PATHOPHYSIOLOGY AND PREVENTION OF ADHESION FORMATION

Maria Mercedes BINDA

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LEUVEN UNIVERSITY PRESS

ACTA BIOMEDICA LOVANIENSIA 417
Katholieke Universiteit te Leuven
Faculteit Geneeskunde
Departement Vrouw en Kind, Afdeling Vrouw
Experimenteel Labo Gynaecologie

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Thesis submitted in partial fulfillment of the requirements for the degree of «Doctor in de Medische Wetenschappen»

ISBN 978 90 5867 667 2
D/2008/1869/4
NUR: 876

"Science moves with the spirit of an adventure characterized both by youthful arrogance and by the belief that the truth, once found, would be simple as well as pretty."

James D. Watson

To Nicolò and Leo

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ACKNOWLEDGEMENTS

I would like to present this thesis to the Katholieke Universiteit Leuven through its rector Prof. Marc Vervenne and to the Faculty of Medicine through its dean Prof. Bernard Himpens.

I am grateful to the members of the jury, Prof. Ignace Vergote (Departement of Obstetric and Gynecology, KUL), Prof. Raf Sciot (Departement of Medical Diagnostic Sciences, KUL), Prof. Marc Miserez (Department of Abdominal Surgery, KUL), Prof. Michel Canis (Polyclinique de l'Hôtel Dieu, Clermont-Ferrand, France), Prof. Hans Jeekel (Department of Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands), for their useful comments and suggestions.

I would like to thank Prof. Philippe Koninckx for all his support and especially for his enthusiasm during this thesis; especially for his patience in reading carefully the manuscripts several times till they were ready to submit, something that helped me a lot to learn how to write a scientific paper. He always encouraged me to think and to work independenly.

I would like also to thank my colleagues from the Laboratory for Experimental Gynaecology. I am thankful to Roger Molinas for teaching me the laparoscopic mouse model and everything related with the experimental work and most of all for his friendship. I thank Silvia Caluwaerts, Salwan Al-Nasiry and Rieta Van Bree for being always smiling and positive while supporting me. I thank Prof Robert Pijnenborg, Lisbeth Vercruysse, Catherine Luyten, Lieve Verbist, Suzan Lambin and Adriana Bastidas for all their help. Thanks also to the gynecology team Kristel Van Calsteren, Isabelle Cadron and Tim Van Mieghem for answering all my questions during Leo's pregnancy. I thank Diane Wolput and Marleen Craessaerts for all the logistic support.

I am grateful to Eugeen Steurs from Storz who helped me – always in a good mood - to solve problems with the laparoscopic stuff.

I would like to thank all my friends for their support and especially Elif for taking me always out, and Hilde for hosting me almost every day when I was feeling lonely.

I would like to thank my family that supported me in my decision to come to Leuven.

Finally, I can't find the words to thank the person who arrived in my life and changed it completly, my husband Nicolò. The last steps of this thesis would not have been possible without his patient and incondicional support. Last but not least, I would like to dedicate this thesis to our son Leo delivered almost togheter with the first draft of the thesis.

February 2008
Bruxelles, Belgium.

Chapter 1

INTRODUCTION

1.1 Definition, aetiology and incidence of intraperitoneal adhesions

Adhesions have been defined as abnormal fibrous connections between surfaces within body cavities. Many different insults, as infections, surgery, chemical irritation, endometriosis, can disrupt the peritoneum, produce inflammation and adhesions development (1).

Adhesions can be classified, according to the aetiology, in congenital or acquired, either post-inflammatory or postoperative (2). Abdominal surgery is the most common cause of adhesions; the incidence ranges from 63% to 97% (2-4). Some 10% of patients without previous surgery have adhesions (92% post inflammatory and 8% congenital). In contrast, adhesions are found in 93% of patients with at least one previous surgery (98% postoperative, 1% post inflammatory and 1% congenital) (3).

Three types of postoperative adhesions should be distinguished: adhesions formed at the surgery site, adhesions formed at non-operative sites and adhesions formed after the lyses of previous adhesions. The terminology used is not uniform and similar processes have received different names by different authors. Diamond *et al* distinguish *adhesions type 1* or *de novo adhesion formation* (adhesions formed at sites that did not have adhesions previously), including *type 1A* and *type 1B*, without or with previous operative procedures at the site of adhesions, respectively, and *adhesions type 2* or *adhesion reformation* (adhesion formed at sites where adhesiolysis was performed), including *type 2A* and *type 2 B*, without or with operative procedures at the adhesion site besides adhesiolysis, respectively (5). We will use terminology used by Pouly *et al*., i.e. *adhesion formation* (adhesions formed at the operative sites), *de novo adhesion formation* (adhesions formed at non-operative sites) and *adhesion reformation* (adhesions formed after the lyses of previous adhesions) (6).

1.2 Clinical significant of intraperitoneal adhesions

Adhesions are the major cause of intestinal obstruction: 30–41% of patients who require abdominal reoperation have adhesion-related intestinal obstruction (7). For small-bowel obstruction, the proportion rises to 65–75% (7; 8). Adhesions are also cause of female infertility (9). They were found in 37% of 733 infertile patients; in 15% of these cases adhesions were the sole factor for infertility and in the rest of the cases, their presence was associated with tubal occlusion, endometriosis, or other infertility factors (10). After adhesiolysis it has been observed both a pregnancy rate of 48% in women with infertility and a decrease in the recurrent loss of pregnancy from 86.5 to 42.8% (11). Moreover, adhesions can cause chronic pain, with a pain relieve of 60-90% of the cases after adhesiolysis (12), and also difficulties at the time of re-operation.

The burden of postoperative adhesions is best illustrated by the study showing that 5.7% of all readmissions of patients undergoing open abdominal or pelvic surgery were classified as being directly related to adhesions, and 3.8% of the patients were managed operatively (2). Moreover, 34.6% of the patients who underwent open abdominal or pelvic surgery were readmitted 2.1 times over 10 years for a disorder directly or possibly related to adhesions and 22.1% of all outcome readmissions occurred in the first year after initial surgery. The same has been shown in women having had open gynaecological surgery; 34.5% of them were readmitted on average 1.9 times in the following ten years for a problem potentially related to adhesions or for further intra-abdominal surgery that could be complicated by adhesions (13). Consistent with this, a cohort study of 12584 patients undergoing in open lower abdominal surgery show that the 32,6% of them were readmitted a mean of 2.2 times in the subsequent ten years for a potencial adhesion-related problem and the 25.4% of readmitions were in the first postoperative year (14). Last but not least, the financial consequences of the adhesions are enormous. Ray *et al* evaluated the direct costs of adhesiolysis in USA during 1988 (15). In total, adhesiolysis hospitalizations during that year accounted for $1180 million in health care expenditures, with $925 million going towards in hospital expenses and $ 255 million in surgeon fees.

1.3 Pathophysiology of intraperitoneal adhesions

Peritoneal injury, due to surgery, infection or irritation, initiates an inflammatory reaction that increases peritoneal fluid, that includes proteins and cells. This fibrinous exudate leads to formation of fibrin (16), by activation of the coagulation cascade, which transforms

prothrombin (Factor II) into thrombin (Factor IIa). Thrombin then triggers the conversion of fibrinogen into monomers of fibrin, which interact and polymerize. The initially soluble polymer becomes insoluble by coagulation factors such as Factor XIIIa and is deposited on the wound surface (17).

Within this fibrinous exudate, polymorphonuclears (PMN), macrophages, fibroblasts and mesothelial cells migrate, proliferate and/or differentiate. During the first two postoperative days, a large number of PMN enter and, in absent of infection, depart within 3-4 days. Macrophages increase in number and become the most important component of the leukocyte population after day 5. They also change functions, e.g. more accurate phagocytosis, greater respiratory burst activity and secretion of a variety of substances, like cytokines and growth factors, that recruit mesothelial cells onto the injured surface. Mesothelial cells form islands throughout the injured area, proliferate and cover the denuded area. This re-epithelialisation process differs from the one of the skin. In the skin, healing occurs at the periphery of the injury and, as result, the duration of the healing directly correlates with the size of the injury, i.e. larger injuries take longer to heal than smaller ones. In contrast, re-epithelialisation of peritoneal injuries occurs by the formation of multiple islands of new mesothelial cells scattered upon the surface of the peritoneum which divide until the surface of the entire site of injury is covered by new mesothelium (18).

All these cells, PMN, macrophages, fibroblasts and mesothelial cells, release a variety of substances such as plasminogen system components, arachidonic acid metabolites, reactive oxygen species (ROS), cytokines and growth factors such as interleukins (IL), tumour necrosis factor (TNF), transforming growth factors α and β (TGF-α and TGF-β). These factors modulate the process of peritoneal healing and adhesion formation at different stages.

The fibrinous exudate and fibrin deposition is an essential part of normal tissue repair, but its complete resolution is required for normal healing. The degradation of fibrin is regulated by the plasminogen system. The inactive proenzyme plasminogen is converted into plasmin by tissue-type plasminogen activator (tPA) and/or urokinase-type plasminogen activator (uPA), which are inhibited by the plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2). Plasmin is a serine protease which degrades fibrin into fibrin degradation products. Plasmin has, in addition, a role in other stages of tissue repair, e.g. extracellular matrix (ECM) degradation, activation of proenzymes of the matrix metalloprotease (MMP) family, and activation of growth factors. Plasmin can be directly inhibited by plasmin inhibitors, i.e. 2-macroglobulin, 2-antiplasmin and 1-antitrypsin, but their role in peritoneal fibrinolysis is not

well defined (16). Fibrinolytic agents as streptokinase, plasmin preparations, urokinase and tPA have been reported to decrease adhesion formation and reformation in several animal models (36). Moreover, the i.p. administration of antibodies against PAI-1 reduce adhesions formation (37).

During peritoneal healing, cell–cell interactions between mesothelial cells, macrophages and also fibroblasts contribute to peritoneum healing. Fibroblasts extracted from adhesions were shown to have a specific phenotype: compared with normal peritoneal fibroblasts, adhesion fibroblasts have increased basal levels of collagen I, fibronectin MMP-1, tissue MMP-1, transforming growth factor (TGF), PAI-1, IL-10 and decreased levels of tPA (19). Consistent with this, it was demonstrated that the use of anti-inflammatories, as corticoids and NSAIDs, reduce adhesion formation in animals models (20-35) since they suppress the inflammatory exudate and fibroblast invasion and proliferation.

The balance between fibrin deposition and degradation is critical in determining normal peritoneal healing or adhesion formation. If fibrin is completely degraded, normal peritoneal healing will occur. In contrast, if fibrin is not completely degraded, it will serve as a scaffold for fibroblasts and capillary ingrowth. Fibroblasts will invade the fibrin matrix and extracelullar matrix (ECM) will be produced and deposited. This ECM is normally completely degraded by MMPs, leading to normal healing. If this process is inhibited by tissue inhibitors of MMPs (TIMPs), peritoneal adhesions will be formed.

In addition, the formation of new blood vessels or angiogenesis has been claimed to be important in adhesion formation (38). Preclinical prevention studies demonstrate that the use of TNP-40, a potent endothelial cell inhibitor (39), neutralizing antiserum against vascular endothelial growth factor (VEGF) (40), antibodies against placental growth factor (PlGF) (41) and against VEGF receptor 1 (VEGFR1) (42) can reduce adhesion formation.

1.4 Postoperative adhesions: laparoscopy vs. laparotomy

It is generally assumed that laparoscopy is less adhesiogenic than laparotomy. In comparison with laparotomy, animal studies indicate that laparoscopy could induce less adhesion formation (43-45); however, this remains controversial (46-48). In humans, laparoscopy could induce less *de novo* adhesions (49; 50) but not for adhesion reformation (6; 49-51). In conclusion, it is unclear whether laparoscopy induces less adhesion formation and this is surprising since laparoscopy produces less surgical trauma, has gentle tissue handling,

meticulous haemostasis and constant irrigation causing less inflammatory response developed (52).

1.5 Laparoscopy and pneumoperitoneum

Laparoscopy requires a pneumoperitoneum. CO_2 is the most common gas used because of safety reasons, i.e. its high solubility in water and its high exchange capacity in lungs. However, CO_2 pneumoperitoneum induces systemic and local effects.

Systemically, CO_2 pneumoperitoneum induces acidosis/hypercarbia (53; 54) and hypothermia (55). Locally, CO_2 pneumoperitoneum decreases the pH (56) and the microcirculation through compression (57). Moreover, the kind of gas used to induce the pneumoperitoneum can have an effect on the inflammatory response. In rats, CO_2 causes less inflammatory reaction with a lower expression of α2-macroglobulin (a hepatic protein of acute phase inflammatory response) than helium (58; 59). Consistent with that, macrophages incubated *in vitro* with CO_2 produced less TNF-α and interleukin-1 in response to LPS compared to macrophages incubated with air or helium (58; 60). Peritoneal macrophages derived from rats exposed to CO_2 pneumoperitoneum release less TNF-α than macrophages derived from animals exposed to air or helium (60). This equal effect of air and helium is quite surprising since helium in comparison with air may induce mesothelium hypoxia.

In addition, humidification and temperature of the gas influence the inflammatory response. Warm and humidified CO_2 in comparison with cold and dry CO_2 reduced the TNF-α concentration in pigs (61) and diminished the increased lymphocytes in rats (62), with overall a reduced duration of inflammation. Pneumoperitoneum, especially when dry gas is used, induces desiccation affecting the morphology of the mesothelial cells, i.e. bulging up, the intercellular clefts increased in size, and the underlying basal lamina became visible (63-65).

1.6 Pneumoperitoneum as a cofactor in adhesion formation

Pneumoperitoneum is co-factor in adhesion formation since adhesions increase with duration of pneumoperitoneum and insufflation pressure and decrease with the addition of oxygen to both CO_2 and helium pneumoperitoneum in rabbits (66; 67) and mice (68; 69). These observations suggest mesothelial hypoxia as a mechanism. Pneumoperitoneum-

enhanced adhesion has been suggested to be mediated by mesothelial hypoxia because similar effects were observed with helium pneumoperitoneum, because the addition of 2-4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation (69; 70). Moreover, pneumoperitoneum-enhanced adhesion was absent in mice deficient for genes encoding for factors regulated by hypoxia, such as hypoxia inducible factor (HIF) (71), for plasminogen activator inhibitor 1 (PAI-1) (72), for vascular endothelial growth factor (VEGF) or for placental growth factor (PlGF) (41). This hypothesis is supported by the observation that during CO_2 or helium pneumoperitoneum the partial pressure of oxygen in the abdominal wall was reduced, whereas insufflation with a 80%CO_2 and 20%O_2 induced no changes (73).

Whereas adhesion formation is decreased with the addition of 3-4% oxygen to the CO_2 pneumoperitoneum, the addition of more than 10% oxygen concentration is deleterious (74). The beneficial effect of the addition of 3-4% oxygen could be explained by the fact that 3-4% oxygen at 770 mmHg (atmospheric pressure of 760 mmHg plus insufflation pressure of 10 mmHg) results in a pO_2 of 23 mmHg, which is remarkably similar to normal intracellular pO_2 (75) and therefore, mesothelial cells would be in a normoxic environment. The addition of 12% oxygen at 770 mmHg results in a pO_2 of 92 mmHg, which is higher than the normal intracellular pO_2 and, therefore, mesothelial cells would be in a hyperoxic environment. During pure CO_2 pneumoperitoneum, mesothelial cell would be in a hypoxic environment.

1.7 Adhesion prevention

A variety of approaches have been developed to prevent adhesions. Historically these approaches can be divided in five categories described in 1942 as the “five fundamental attacks directed towards the prevention of adhesions” (76):

1. Limitation or prevention of initial peritoneal injury.
2. Prevention of coagulation of the serous exudates.
3. Removal or dissolution of deposited fibrin.
4. Prevention of the adherence of surfaces of adjacent structures by keeping them apart.
5. Prevention of the organization of the persisting fibrin by means of inhibiting the fibroblast proliferation.

Although these five categories include the majority of the mechanisms, today new categories can be also considered:

6. Prevention of the angiogenesis
7. Prevention of the oxidative stress
8. Prevention of the hypoxia

1.7.1 Limitation or prevention of initial peritoneal injury

It is universally accepted that the avoidance of the peritoneum damage is an effective approach for adhesion prevention. The first surgeon who recognized the importance of avoiding the damage was William Stewart Halsted (1852-1922) and these principles are known as the "Halstedian principles" (77). The avoidance of the damage can be achieved by careful tissue handling, meticulous haemostasis, and continuous irrigation and by avoiding unnecessary drying, suturing and clamping of tissue. This is to diminish the presence of blood and ischemic tissues which provide a source of fibrin and results in adhesion formation by releasing thromboplastin with subsequent activation of the clotting cascade. Other effective measures include avoiding large abdominal wounds and unnecessary dissection and using micro and atraumatic instruments to reduce serosal injury (78). The use of fine and biocompatible suture material, starch free gloves and non-linting swabs were reported to reduce adhesion formation. In this prevention level, the importance of a good training for the surgeon to improve the surgical techniques is also fundamental (79).

1.7.2 Prevention of coagulation and fibrin deposition

This therapy attempts to prevent fibrin formation and deposition and includes peritoneal lavages to dilute or wash away fibrinous exudates and anticoagulant solutions.
Peritoneal irrigation with saline or Ringer's lactate, with or without anticoagulant agents such us heparin, was often used to reduce adhesion formation, but clinical and animal studies reported contradictory results (80).

1.7.3 Removal or dissolution of deposited fibrin

Although fibrin deposition is an essential component of normal tissue repair, resolution of the fibrin deposit is required to restore the preoperative conditions or conditions before inflammation. A depression in endogenous fibrinolytic activity was observed during surgery and following peritoneal injury (81). Therefore, fibrinolytic agents may supplant this deficiency and they have been applied to prevent adhesion formation. There are several strategies.

First strategy is to apply directly fibrinolytic agents. Plasmin preparations (plasmin, actase, and fibrinolysin) and plasmin activators (streptokinase, urokinase, and tissue-type plasminogen activator) were found to be efficacious in preventing adhesion formation in the animal and clinical studies (36). From the current literature, it can be concluded that postoperative intraperitoneal administration of thrombolytic agents can significantly decrease adhesion formation.

Second strategy is to stimulate the production of fibrinolytic proteins. This can be done by using an antagonist of the proinflammatory peptide substance P (SP). If the binding of SP to the neurokinin 1 receptor (NK-1R) was blocked, adhesion formation was reduced. NK-1R antagonist administration was shown to increase the tPA mRNA levels in peritoneal tissue and the tPA protein in the peritoneal fluid. These data suggested that activation of the NK-1R promotes peritoneal adhesion formation by limiting fibrinolytic activity in the postoperative peritoneum (82). Recently, the same group demonstrated that the NK-1R antagonist may limit adhesions, in part by reducing postoperative oxidative stress through an inhibition of neutrophil recruitment and an increase in peritoneal fluid antioxidant capacity (83). Another way to increase the peritoneal fibrinolytic activity is the use of statins, as lovastatin or atorvastatin (84).

Third strategy is to avoid the prevention of early depression of local fibrinolytic activity using intraperitoneally the protein inhibitor aprotinin (85; 86). Ozogul *et al.* postulated that a depression of fibrinolysis occurs during surgery and the anti-inflammatory effects of the aprotinin may lead to a decrease in adhesion formation in rats.

1.7.4 Prevention of the adherence of surfaces of adjacent structures by keeping them apart

This strategy is based on keeping apart the peritoneal surfaces until mesothelization occurres. An ideal barrier must obviously be biodegradable, safe, non-immunogenic and easily applied (80). The most common components used for barriers are derived from the hyaluronic acid since it is an important extracellular matrix component that is involved in cell movement and tissue repair and it is a naturally component of peritoneal fluid (87).

At this stage, we can consider the use of liquid barriers, semi-solid barriers, mechanical barriers and surfactant-like substances.

1.7.4.1 Mechanical barriers

The mechanical barriers commonly used are: hyaluronic acid-carboxymethylcellulose, oxidized regenerated cellulose and expanded polytetrafluoroethylene.

Hyaluronic acid-carboxymethylcellulose or HA-CMC (Seprafilm, Genzyme, Cambridge, MA, USA),

Since Seprafilm is a film, it can only be used only during laparotomy. It was used in several animal models in which adhesions were induced by abrasion or by colon resection and a reduction in adhesions formation was shown (85; 88; 89). In a prospective clinical trial with patients that were requiring a Hartmann procedure, it was demonstrated that intraperitoneal placement of Seprafilm was effective in reducing the severity of postoperative adhesions; however, their incidence was not diminished (90). In another prospective, blinded, randomized, multicenter clinical study, treatment of patients after myomectomy with Seprafilm significantly reduced the incidence, severity, extent, and area of postoperative uterine adhesions (91). However, wrapping the suture or staple line of a fresh bowel anastomosis with Seprafilm should be avoided, because it was demonstrated in a prospective, randomized, multicenter, controlled study that this practice may increase the risk of sequelae associated with anastomotic leak (92). In conclusion, Seprafilm was proved to be effective in reducing adhesions but its problem is that can only be used during laparotomy and it should be avoid during anastomosis.

Oxidized regenerated cellulose (ORC) (Interceed®, Johnson&Johnson, Gynecare, Unit, NJ, USA)

Interceed has been used in animal models (93) and in human (94; 95) during open surgery showing a significant reduction in the incidence, extent, and severity of postsurgical pelvic adhesions. In addition, Interceed in combination with heparin had a synergist effect in the reduction of adhesions in a rabbit uterine horn model (96). In conclusion, Interceed has good results preventing adhesions but it can be used only during laparotomy.

Expanded polytetrafluoroethylene (ePTPE) (Gore-Tex Surgical Membrane, WL Gore &Associates, Inc, Newark)

This barrier was evaluated in a randomized clinical trial in women with bilateral pelvic sidewall adhesions and its use was associated with fewer postsurgical adhesions (97). The

problem with this barrier is that must be sewn in place and removed during a second surgical procedure.

1.7.4.2 Semisolid barriers

0.5% ferric hyaluronate gel (Intergel, Johnson&Johnson, Gynecare Unit, NJ, USA)

When the hyaluronic acid is cross-linked with carboxilate groups and trivalent iron (Fe^{+3}) produces a significant increase in the solution viscosity (98). The efficacy difference between the Intergel and non-cross-linked hyaluronic acid formulations appears to result from the longer residence time of Intergel in the peritoneal cavity compared to hyaluronic acid (98). In a randomized study, patients treated with Intergel had significantly less adhesions compared to controls, and adhesion extent and severity were also significantly reduced (99). However, Intergel has been removed from the marker due to the reported pelvic pain and allergic reactions (100).

Polyethylenglycol (SprayGel, Confluent Surgical, Waltham, MA, USA)

The SprayGel™ Adhesion Barrier consists of two liquids, that when mixed together, rapidly cross-link to form a biocompatible absorbable hydrogel. This form adheres to the tissue and within one week degrades into water-soluble polyethylene glycol molecules that are absorbed and cleared mainly through the kidneys. A double-blind controlled study with SprayGel was performed in rats and rabbits, with cecum and uterine horn abrasion models, respectively, reducing significantly the postoperative adhesion formation (101). This is confirmed in a porcine model showing that incidence, extent and severity of adhesions were reduced significantly (102).

In a prospective, randomized, clinical trial, patients to whom a bilateral adnexal surgery was performed, frequency, extent and severity of adhesions were statistically reduced (103). Same results were obtained in another prospective, randomized, controlled, multicenter trial in open or laparoscopic myomectomy. Patients randomized to SprayGel were 3.6 times more likely to be adhesion free than controls. SprayGel was effective in reducing severity, frequency, tenacity and extent of adhesions (104; 105). In conclusion, Spraygel is effective to prevent adhesion formation.

Auto-cross linked hyaluronic acid gel (Hyalobarrier Gel, Baxter, Italy)

Hyalobarrier Gel reduces adhesion formation during laparoscopic (106) and open surgery (107-109) models in rabbits and rats. It was also proven to be effective in clinical trials during laparoscopic myomectomy (110; 111) and hysteroscopic surgery (112; 113). In conclusion, from the preclinical and clinical data, HyalobarrierGel is effective at preventing postoperative adhesions.

1.7.4.3 Liquid barriers or hydroflotation

Crystalloids

Ringer's lactate was effective in preventing adhesion formation in a uterine horn abrasion rat model (88). Although, the technique of instillating large volumes of isotonic solutions is the most popular and cheap treatment used in adhesion prevention, a meta-analysis of clinical trials has shown that crystalloids do not reduce postoperative adhesions after laparoscopy or laparotomy (114). This can be explained by the rapid absorption rate of the peritoneum since it was found by ultrasonographic methods that 24 hours after instillation of 250 mL of Ringer's lactate, the volume of fluid found in the pelvis (12 ml) was not different from the volume found in patients in whom no fluid was instilled (7 ml) (115).

Dextran 70 (32% w/v) in dextrose (Hyskon®; Pharmacia, Uppsala, Sweden)

Hyskon was shown to decrease adhesions in a rabbit model (116; 117) whereas no effect was observed in other reports, i.e. in rabbits (118-120), hamsters (121) and rats (122).

In clinical practice, although Hyskon has shown to reduce adhesion formation (123), some undesirable side effects were observed, i.e. abdominal pain, dyspnea, oedema of the vulva and of the thigh, bradycardia, pleural effusions containing dextran (124), therefore its use was abandoned.

Icodextrin (ADEPT, Baxter, USA)

Icodextrin is a biodegradable, biocompatible, α-1-4 glucose polymer of high molecular weight which is metabolized to glucose by the α-amylase, an enzyme that is absent in the human peritoneal cavity but it is present in the systemic circulation. When icodextrin is instilled in the abdominal cavity, it is absorbed slowly, i.e. the volume is maintained for up to 48 hr, and half the instilled volume remained after 72 and 96 hr, this result would allow extensive and prolonged coverage of the peritoneal surface (125). In rabbit, in a uterine horn model, a reduction of adhesion formation was demonstrated with 50 ml of 4% and 20%

icodextrin (126). On the contrary, icodextrin enhanced adhesion and abscess formation in a standardized peritonitis rat model induced by the cecal ligation and puncture model (127).

In clinical trials, safety and tolerability of icodextrin were good and incidence, extent and severity of adhesions were reduced in icodextrin-treated patients (128).

1.7.4.4 Tissue precoating

0.4% hyaluronic acid (Sepracoat, Genzyme, Cambridge, MA, USA)

This solution is applied intraperitoneally to protect peritoneal surfaces from indirect trauma (desiccation, abrasion) rather than to separate surfaces postoperatively after they are traumatized (129). This concept is known as "tissue precoating". Postsurgical adhesions were significantly less in the Sepracoat treated group in comparison with the untreated control in a uterine horn rat model (88). It was also effective in preventing adhesions when was applied before the serosal injury in a rat model (130) whereas was not effective in reducing adhesion reformation after microsurgical adhesiolysis (131).

In humans, Sepracoat was significantly more effective than placebo and was safe in reducing the incidence, extent, and severity of *de novo* adhesions to multiple sites indirectly traumatized by gynaecologic surgery via laparotomy (132). No studies evaluating Sepracoat during laparoscopy has been found in the bibliography.

1.7.4.5 Surfactant-like substances

Phospholipids:

Phospholipids, polar phosphoric acid diesters, are natural constituents of abdominal cavity and cell membranes. They are zwitterions with a positively charged quaternary ammonium ion that is able to bind negative charges of epithelial surfaces. Mesothelial cells of the normal peritoneum secrete surface-active phospholipids (SAPL) for protecting itself and the membrane that forms with its neighbours. It is shown how SAPL, if adsorbed to mesothelium, can impart excellent lubricity, antiwear and release (antistick) properties, while impeding surgical adhesion formation (133).

Adhesion formation was decreased after phospholipids application in a rabbit model after median laparotomy and standardized abdominal wall, cecum, ileum or uterine horns abrasions (134; 135).

Other phospholipids, as phosphatidylcholine, phosphatidylinositol or DL-phosphatidylcholine dilauryl, have shown a reduction in adhesion formation in animal models (136; 137). There are not any clinical trials available about this agent.

1.7.5 Prevention of the organization of the persisting fibrin by means of inhibiting the fibroblastic proliferation.

After fibrin deposition and organization, this will serve as a scaffold for fibroblasts and capillaries ingrowth. Fibroblasts will proliferate and invade the fibrin matrix and they will produce extracellular matrix. Therefore, this prevention step consists in inhibiting its proliferation and also inflammation in general. This has been attempted by using corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), calcium channel blockers, and immunosuppressors.

These products have been studied during both open and laparoscopic surgery in animal models. During open surgery, many of these products have been demonstrated to decrease postoperative adhesion formation i.e. corticosteroids (20-23), non-steroidal anti-inflammatory drugs (22; 24-35), hormones (138), and calcium channel blockers (139-143). Following laparoscopic surgery these products have not been addressed in animal models. In clinical trials, glucocorticoids were tested during laparotomy with no effect in adhesion prevention (144).

1.7.6 Prevention of the angiogenesis

Angiogenesis is the formation of new blood vessels from the pre-existening vessels. After the organization of the extracellular matrix, angiogenesis will happen after some 4 to 5 days if healing has not been completed. Antiangiogenesis therapy with products as TNP-470 and anti-vascular permeability factor/vascular endothelial growth factor were used in mice during laparotomy and they were found to reduce adhesion formation (39; 40). During laparoscopy, other compounds like antiPlGF and antiVEGFR1 antibodies were tried in a mouse model reducing adhesion formation (41; 42). No studies were done in humans.

1.7.7 Prevention of the oxidative stress

The generation of ROS after open and laparoscopic surgery is well reported (145; 146). Laparoscopic surgery increases ROS availability by increasing ROS production (146) or by decreasing ROS scavengers (57; 147). ROS scavengers reduce adhesion formation

following open surgery in different animal models. Indeed, Catalase (CAT), superoxidismutase (SOD) and trimetazidine reduce adhesion formation in rats (148-150); CAT and SOD also reduce adhesion formation in an endometriosis rabbits model (151). In addition, intraperitoneal administration of methylene blue reduces adhesion formation in rats (152); intraperitoneal administration of melatonin also prevents adhesion formation in rats (153). The administration of vitamin E produced contradictory results in rats (154)(155). Moreover, ROS had a direct cytotoxic effect on human mesothelial cells and, in addition, mesothelial cells apoptosis was induced by ROS *in vitro* (150). These mechanisms create a further damage of the mesothelial lining extending beyond the damage created during surgery and enhancing the possibility to create postoperative adhesions.

Moreover, during pneumoperitoneum, there is ischemia at the time of insufflation, and reperfusion at the time of deflation, thus additional ROS can be produced. No data exists about prevention of the oxidative stress by using ROS scavengers during laparoscopy in animal models. In humans, there are no data neither for laparoscopic nor for open surgery.

1.7.8 Prevention of the hypoxia

Mesothelial hypoxia is one of the mechanisms of CO_2 pneumoperitoneum enhanced adhesion formation as explained in paragraph 1.6 of this introduction. The increase in adhesions can be prevented by adding 3% of oxygen to the CO_2 pneumoperitoneum (70). Although this concept has been demonstrated in the mouse model, there is no data available in humans yet. Moreover, during this thesis, the use of hypothermia to prevent the toxic effects of the hypoxia, of the ischemia-reperfusion and of the inflammation processes will be described.

1.7.9 Summary: adhesion prevention today.

In summary, a variety of approaches have been developed to prevent adhesion formation. In human, only barriers are effectively used to reduce adhesions. Seprafilm, Interceed, Preclude, Adept, SprayGel, Hyalobarrier Gel are the most commonly used mechanical and semi solid barriers. Liquid barriers, such as hyaluronic acid, crystalloids and icodextrin, have been used to separate injured surfaces by “hydroflotation” and to reduce adhesions. In animal models, many products have been demonstrated to decrease postoperative adhesions during open surgery i.e. corticosteroids, non-steroidal anti-inflammatory drugs, fibrinolytic agents, surfactants, flotation agents, mechanical barriers,

hormones, calcium channel blockers, ROS scavengers and antiangiogenesis therapy. However, results have been variable and inconsistent for different animal models and a comprehensive understanding is still lacking.

In conclusion, the pathophysiology of adhesion formation is poorly understood. Clinical adhesion prevention is limited to barriers methods. Therefore, we planned to evaluate during this thesis the pathophysiology in order to develop a comprehensive model and hopefully a more appropriated prevention.

1.8 General aims of this thesis:

Aim #1: Standardise and characterise the laparoscopic mouse model for adhesion formation. Adhesion formation varies with the mouse strain used, with the duration and pressure of the pneumoperitoneum and its humidification and oxygen content. Moreover not only desiccation but also ventilation and anaesthesia decrease the body temperature in mice. The understanding and standardisation of each of these parameters is important to understand the pathophysiology and is a prerequisite to have a stable model for adhesion formation. Since the effect of oxygen was already investigated we focused on

a. the effect of different mouse strains upon adhesion formation.
b. the effect of the temperature upon adhesion formation.
c. the effect of desiccation upon adhesion formation.

Aim #2: Screen in our laparoscopic mouse models all the products which have been described to affect adhesion formation in order to compare their relative effectiveness and investigate their mechanisms of action.

Aim #3: Explorate the pathophysiology of adhesion formation and its prevention. Aim#3 clearly will be based upon the results of Aim #1 and #2. Once the pathophysiology will be understood, *combine treatments* will be done to evaluate if the effects upon adhesion

formation are synergistic or additive or not. If effects are additive the pathways are probably different whereas non additive effects probably use similar pathways.

Aim #4: Finally these data will be used to *establish which combination of treatments will give a maximum reduction of adhesions*. This should form the basis for a randomised controlled clinical trial.

Chapter 2

RESULTS

2.1 RESULTS OF AIM #1: FINAL STANDARIZATION OF THE LAPAROSCOPIC MOUSE MODEL.

The laparoscopic mouse model had been standardised as follows: intubation and adequate ventilation, 15 mm Hg insufflation pressure for the pneumoperitoneum, standard bipolar opposing lesions and scoring of adhesions. The effect of mouse strain, temperature and desiccation upon adhesions has been studied during this thesis.

2.1.1. EFFECT OF MOUSE STRAIN UPON ADHESION FORMATION.

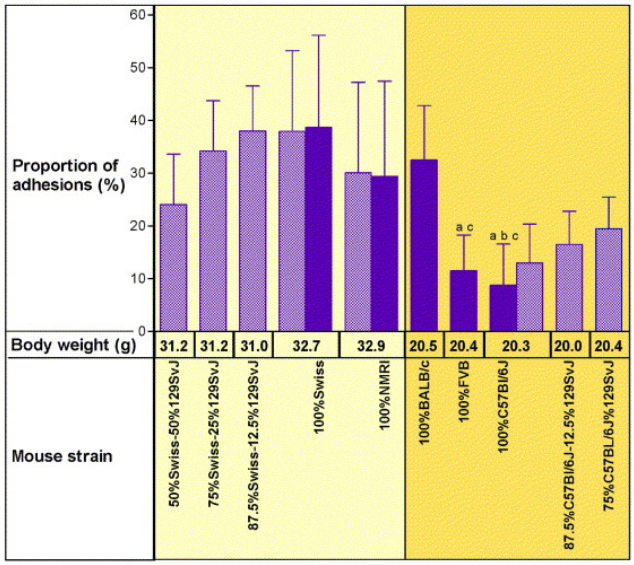
Molinas CR, ***Binda MM****, Campo R, Koninckx PR. Adhesion formation and interanimal variability in a laparoscopic mouse model varies with strains. Fertility & Sterility 83(6):1871-4, 2005 (156)* (Addendum 3)

During this aim pneumoperitoneum-enhanced adhesion formation was evaluated in mice of different wild type strains and/or percentages of strains, e.g., 100%Swiss, 87.5%Swiss-12.5% 129SvJ, 75%Swiss-25% 129SvJ, 50%Swiss-50% 29SvJ, 100%NMRI, 100%C57BL/6J, 87.5%C57BL/6J-12.5%129SvJ and 75%C57BL/6J-25% 29SvJ, 100% BALB/c and 100% FVB mice (n=115).

Swiss, NMRI, and BALB/c mouse strains developed more postoperative adhesions than FVB and C57BL/6J mice (Figure 1).

Figure 1

Postoperative adhesion formation in inbred and outbred mice of different strains.



Postoperative adhesion formation was evaluated in inbred and outbred mice of different strains and with different body weights (means are indicated). Animals, body weight clustered into two groups, one of ~32 g (*yellow background*) and another of ~20 g (*orange background*). Pneumoperitoneum-enhanced adhesions were induced with standardized lesions during laparoscopy (CO_2 pneumoperitoneum for 60 minutes at 20cm H_2O) and scored after 7 days during laparotomy in a retrospective study (*grid bars*) and in a prospective randomized study (*closed bars*). The proportion of adhesions (means ± SD) with differences statistically significant vs. Swiss (a), NMRI (b), and BALB/c (c) mice are indicated.

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The variability of adhesion formation between animals was calculated as the coefficient of variation (CV). The CV was much less for inbred strains than for outbred strains, being 45% for Swiss mice and 61% for NMRI mice (both outbred strains) and 32% for the inbred BALB/c mice. For the inbreed FVB and C57BL/6J mice, the CV were 59% and 90%, respectively, reflecting a very low adhesion formation potential. For this reason, experiments were initially carried out in NMRI and later in BALB/c.

2.1.2. EFFECT OF ENVIRONMENTAL TEMPERATURE, DRY VENTILATION, DESICCATION AND OVERSATURATED INSUFFLATION GAS UPON MOUSE BODY TEMPERATURE.

Binda MM, *Molinas C.R., Mailova K. and Koninckx P.R. Effect of temperature upon adhesion formation in a laparoscopic mouse model. Human Reproduction 19(11):2626-32, 2004) (Experiments 1 and 2) (157) (Addendum 4)*

Binda MM, *Molinas CR, Hansen P, Koninckx PR. Effect of desiccation and temperature during laparoscopy on adhesion formation in mice. Fertil Steril 2006;86:166-175(Experiment 4) (158) (Addendum 5)*

We first observed that mouse body temperature (BT) decreased under anaesthesia at room temperature (RT). Moreover, the variability between experiments was high with apparently more pneumoperitoneum-enhanced adhesions in summer.

A series of four experiments were performed. For clarity we will first describe all the experiments in which the effect of different variables were study upon BT, i.e. (1) the environmental temperature, (2) the use of dry air for ventilation, (3) the use of dry insufflation gas for the pneumoperitoneum and (4) the use of oversaturated CO_2 for the pneumoperitoneum. Subsequently, the effects of temperature upon adhesion formation will be described.

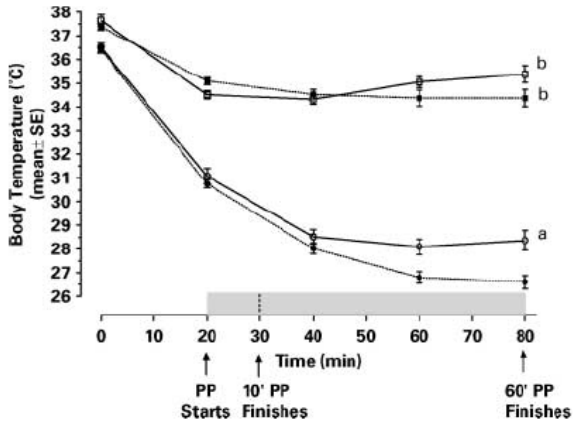
Experiment 1: Effect of environmental temperature upon mouse body temperature.

The effect of environmental temperature upon BT and basal and pneumoperitoneum-enhanced adhesions was evaluated in NMRI mice (n=32). Temperature was modulated by keeping the mice at RT or in a heated chamber at 37°C. Non-humidified pure CO_2 was used for the pneumoperitoneum. To prevent desiccation there was no flow through the abdominal cavity.

At RT, mice BT decreased progressively to 28.5°C and to 26.5°C at T_{80} in mice with 10 and 60 min of pneumoperitoneum, respectively (Figure 2). At a 37°C chamber, BT remained constant at some 35.5°C and 34.5°C up to T_{80} in mice with 10 and 60 min of pneumoperitoneum, respectively. BT were lower after 60 than after 10 min of pneumoperitoneum at both RT ($p<0.0001$) and 37°C (NS). BT also were lower at RT than at 37°C after both 10 ($p<0.0001$) and 60 ($p<0.0001$) min of pneumoperitoneum (Two-way ANOVA).

Figure 2

Effect of environmental temperature upon body temperature in mice.



Basal and pneumoperitoneum (PP)-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure and mice were kept either at room temperature or at 37°C.

Symbols: ○, 10 min PP, room temperature; ● 60 min PP, room temperature; □10 min PP, 37°C; ■60 min PP, 37°C, $P<0.05$: [a]10 versus 60 min at room temperature or at 37°C, [b]room temperature versus 37°C at 10 or at 60 min (two-way ANOVA for temperature).

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These data show that BT during anaesthesia depends on the environmental temperature, and this cooling effect is very pronounced at RT. They also indicate that the cooling increases with the pneumoperitoneum duration.

Experiment 2: Effect of ventilation with non-humidified air upon mouse body temperature

Humidified or non-humidified room air ventilation upon BT was evaluated in NMRI mice (n=6), placed at 37°C during 60 min of humidified CO_2 pneumoperitoneum.

BT was some 1°C lower when non-humidified air was used for ventilation (p=0.003, two-way ANOVA), being 38.1±0.1 (T_0), 36.4±0.1 (T_{10}), 35.9±0.3 (T_{20}), 36.2±0.5 (T_{30}), 36.5±0.6 (T_{40}), 36.5±0.6 (T_{50}), 36.8±0.5 (T_{60}), 37.0±0.5 (T_{70}) and 37.1±0.5 (T_{80})°C for humidified ventilation, and 37.8±0.4 (T_0), 36.1±0.1 (T_{10}), 35.0±0.4 (T_{20}), 35.3±0.5 (T_{30}), 35.4±0.5 (T_{40}), 35.8±0.7 (T_{50}), 35.7±0.6 (T_{60}), 35.6±0.5 (T_{70}) and 36.1±0.5 (T_{80})°C for non-humidified ventilation. This explained why in the previous experiment mice BT decreased

even when placed in a box at 37°C. In conclusion, this experiment demonstrated that non-humidified ventilation decreased BT.

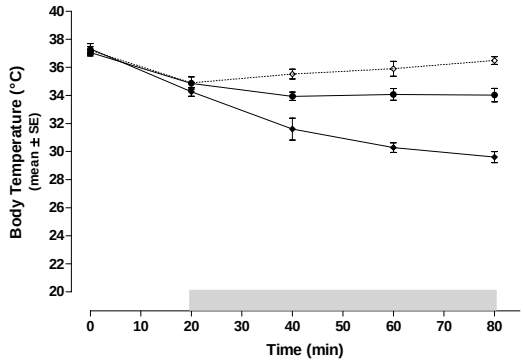
Experiment 3: Effect of desiccation upon mouse body temperature (*non- published data*).

Pneumoperitoneum-enhanced adhesion formation, together with BT, was evaluated in NMRI mice (n=30) placed at 37°C. Pure CO_2 and non-humidified insufflation gas was used. Flows of 23 ml/min and 492 ml/min through the abdominal cavity were used. For the highest flow, humidified insufflation gas was also used.

Desiccation reduced BT (non-humidified gas, low vs high flow, $p<0.0001$) (Figure 3), and this cooling is prevented by using humidified gas (high flow; non-humidified vs humidified; $p<0.0001$; Two-way ANOVA).

Figure 3

Effect of desiccation upon body temperature.



Pneumoperitoneum-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure.

◆ 492 ml/min-flow PP non-humidified gas, ● 23 ml/min-flow PP non-humidified gas.◇ 492 ml/min-flow PP, humidified gas ▒ Pneumoperitoneum

Statistic: 492 vs 23 ml/min-flow (non-hum) $p<0.0001$, Hum vs non-hum (492 ml/min-flow) $p<0.0001$ (Two-way ANOVA).

Experiment 4: Effect of over-saturating the insufflation gas upon body and pneumoperitoneum temperatures.

For this experiment, the humidifier MR860 (Fisher & Paykel Healthcare Ltd, NZ) was used. This humidifier permits "oversaturation" of the CO_2, with some condensation in the peritoneal cavity (61; 158). Discrete levels of humidification, which, expressed relative to body temperature saturated (BTS) conditions (37°C, 100%RH, i.e., 44 mg water/L CO_2),

corresponded to 0%, 75% (33 mg water/L), 100% (44 mg water/L), and 125% (55 mg water/L) BTS. For the dry group (0%BTS), same humidifier was used but the humidification chamber was not filled with water. BT and RH and temperature of the pneumoperitoneum were evaluated using non-humidified (group I), and humidified CO_2 corresponding to 75% (group II), 100% (group III), and 125% (group IV) BTS, respectively. NMRI mice were used (n=52).

Briefly, BT further decreased to 33°C when non-humidified CO_2 was used (group I) (Figure 4A). With humidified CO_2, BT increased progressively to 36, 36.5, and 37°C in mice of groups II, III and IV, respectively. By ANOVA, BT was lower in mice of group I than in mice of groups II ($p<0.0001$), III ($p<0.0001$), and IV ($p<0.0001$). BT was also lower in mice of group II than in mice of groups III ($p=0.02$) and IV ($p=0.04$). Differences between groups III and IV were not significant.

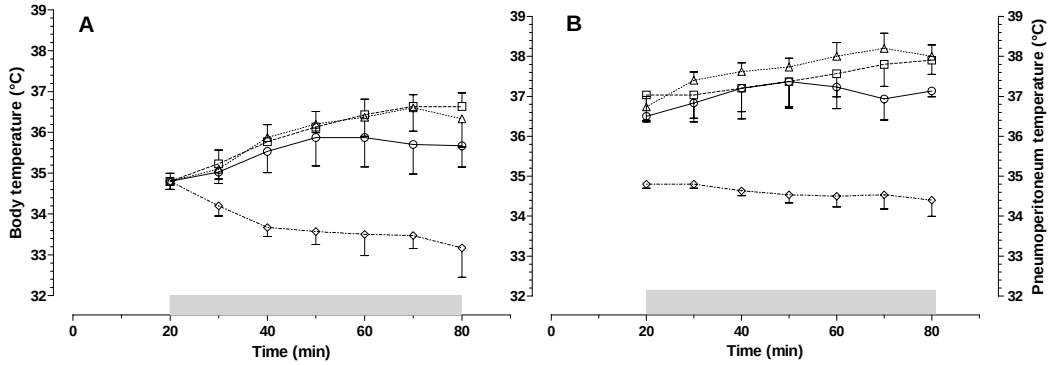
Pneumoperitoneum temperature in mice of group I decreased slowly to 34.5°C (Figure 4B). In mice with humidified CO_2, pneumoperitoneum temperatures were higher around 37°C and increased slowly thereafter to 37.8°C, especially in group IV, reflecting the increase in BT. By ANOVA, pneumoperitoneum temperature was lower in mice of group I than in mice of groups II ($p<0.0001$), III ($p<0.0001$), and IV ($p<0.0001$). It was also lower in mice of group II than in mice of groups III ($p=0.04$) and IV ($p=0.004$) and lower in mice of group III than in mice of group IV ($p<0.0001$).

The RH of the pneumoperitoneum remained 100% in all groups throughout the experiment, except for mice of group I. In this group RH of the pneumoperitoneum was initially (at T_{20}) 82.9% ± 1.9%, and decreased slightly thereafter to 80.8% ± 4.2%, reflecting the slightly lower humidification capacity of the peritoneum at lower temperatures (data not shown).

In conclusion, the insufflation with oversaturated gas, i.e. at higher temperature, increased slightly peritoneal cavity temperature and body temperature.

Figure 4

Effect of over-saturating the insufflation gas upon body (A) and pneumoperitoneum (B) temperature.



Effect of CO_2 pneumoperitoneum with discrete levels of humidification, expressed in relation to BTS conditions (37°C, 100% RH) on body (A) and pneumoperitoneum (B) temperature. Non-humidified gas (group I) and humidified gas at 75% BTS (group II), 100% BTS (group III), and 125% BTS (group IV) conditions and a flow of 23 mL/min through the abdominal cavity were used. *Symbols*: group I (◊), group II (O), group III (□), and group IV (Δ); pneumoperitoneum (shaded bar). Means ± SE are indicated.

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2.1.3. EFFECT OF TEMPERATURE UPON ADHESION FORMATION.

Binda MM, *Molinas C.R., Mailova K. and Koninckx P.R. Effect of temperature upon adhesion formation in a laparoscopic mouse model. Human Reproduction 19(11):2626-32, 2004 (Experiments 1 and 2)(157) (Addendum 4)*

Binda MM, *Molinas CR, Hansen P, Koninckx PR. Effect of desiccation and temperature during laparoscopy on adhesion formation in mice. Fertil Steril 2006;86:166-175(Experiment 3) (158) (Addendum 5)*

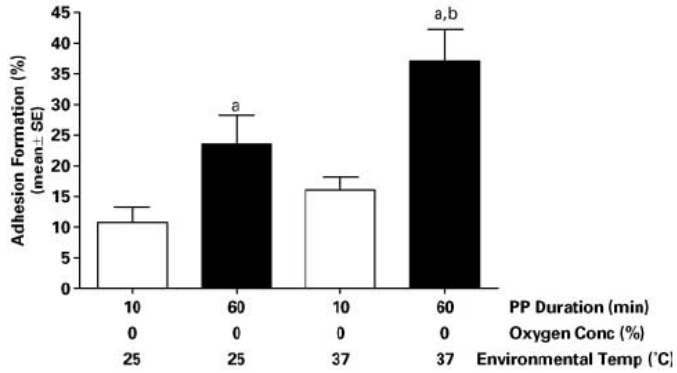
In this section the effects of (1) environmental temperature, (2) BT, (3) the increase in BT by the use of oversaturated CO_2 for the pneumoperitoneum, upon adhesion formation will be described. These are the results in adhesion formation of the experiments explained in the section 2.1.2.

Experiment 1: Effect of environmental temperature upon adhesion formation.

Adhesions were higher at 37°C than at RT, clearly for pneumoperitoneum-enhanced adhesions (p=0.04) and slightly for basal adhesions (NS) (Figure 5; Mann Whitney test). In conclusion, these data suggested that higher BT were associated with more adhesions.

Figure 5

Effect of environmental temperature upon adhesion formation in mice.



Basal and pneumoperitoneum (PP)-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure and mice were kept either at room temperature or at 37°C.
Symbols: $P<0.05$: [a]10 versus 60 min at room temperature or at 37°C, [b]room temperature versus 37°C at 10 or at 60 min (Mann–Whitney test for adhesion formation).

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Experiment 2: Hypothermia reduces adhesions formation

Our aim was first to study the effect of BT (range: 32°C to 37°C) upon adhesion formation. Secondly, our aim was to study at 37°C the effect of pneumoperitoneum duration and of adding 3% and 12% oxygen to the CO_2 pneumoperitoneum upon adhesion formation.

BT was modulated in the following way. To achieve BT with minimal cooling, i.e. around 37°C, mice were placed in an environment of 37°C and ventilated with humidified air. To achieve a slightly lower BT (around 36°C), mice were placed in an environment of 37°C and non-humidified air was used for ventilation. To achieve BT of some 32°C, mice were placed alternatively at RT and 37°C and humidified air was used for ventilation (Figure 6, left). Pneumoperitoneum-enhanced adhesion formation was evaluated at 37°C (group I), 36°C (group II) and 32°C (group III) using humidified and pure CO_2 as insufflation gas.

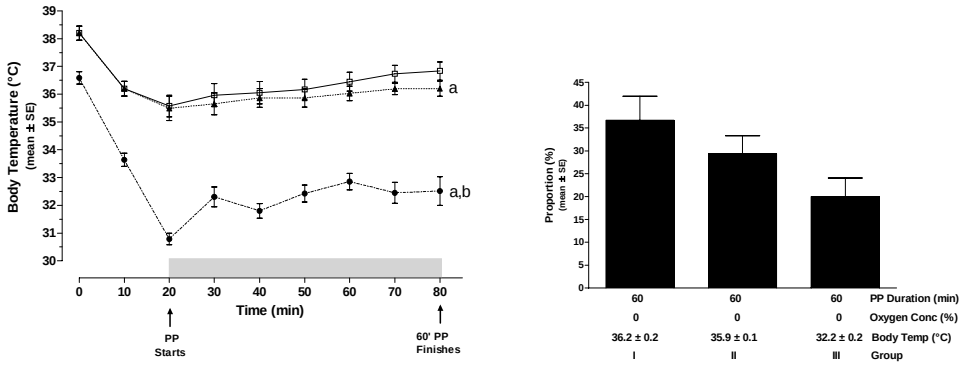
Pneumoperitoneum-enhanced adhesion formation at 37°C was also evaluating using CO_2 with 3% (group IV) and 12% oxygen (group V). Simultaneously, basal adhesion formation was evaluated using pure CO_2 (group VI). For all groups, a 23 ml/min flow through the abdominal cavity was used and BT was registered. NMRI mice were used (n=48).

Pneumoperitoneum-enhanced adhesion decreases with hypothermia (Pearson correlation, p=0.02; Figure 6 right).

Figure 6

Effect of different body temperature upon adhesion formation.

Body Temperature **Adhesion formation**



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Pneumoperitoneum (PP)-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure.
☐ group I, ▲ group II, ● group III.
Pneumoperitoneum

Statistic: [a]p<0.01 vs group I, [b]p<0.0001 vs group II (Two-way ANOVA).

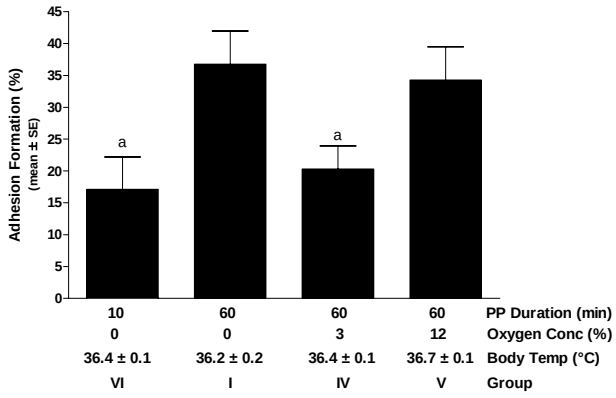
Mean ± SE of BT during T_{20}-T_{80} are indicated in the adhesion formation graph.

Statistic: p=0.02 (Pearson Correlation).

As demonstrated previously, we confirmed that in animals kept at 37°C (Figure 7), adhesion formation increased when pneumoperitoneum is prolonged from 10 to 60 min (p=0.04) (69). In comparison with pure CO_2 (group I), the addition of 3% oxygen to the pneumoperitoneum (group IV) decreased adhesion formation (p=0.03) whereas no differences were found with the addition of 12% of oxygen (group V) (Mann Whitney test).

Figure 7

Effect at 37°C of duration and adding oxygen to the pneumoperitoneum upon adhesion formation in mice.



Basal (group VI) and PP-enhanced adhesions (group I, IV and V) were induced during laparoscopy at 20 cm H_2O with CO_2 containing 0% (group I and VI), 3% (group IV) or 12% of oxygen (group V). Animals were kept at 37°C. Adhesions were quantitatively scored after seven days during laparotomy.

Mean ± SE of body temperature during T_{20}-T_{80} are indicated.
Statistics: [a]$p<0.05$ vs group I, (Mann Whitney test).

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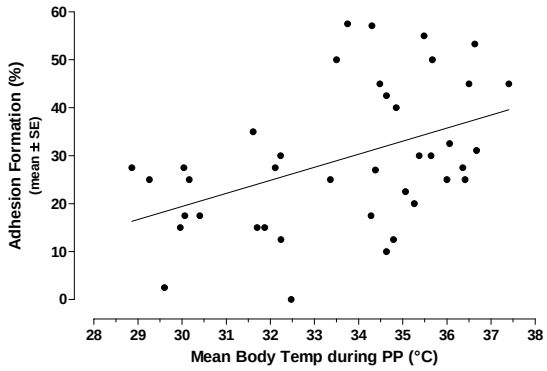
To evaluate the effect of BT upon adhesion formation, data of experiments 1 and 2 were combined (Figure 8). Taken all data together, pneumoperitoneum-enhanced adhesion formation decreased with lower BT ($p=0.004$, Linear regression and Pearson correlation).

Figure 8

Relationship between body temperature and adhesion formation.



Individual values of the mean of body temperature between T_{20} and T_{80} with their respective proportion of adhesions are depictured for pneumoperitoneum (PP)-enhanced adhesion of the data of experiments 1 and 2.

Statistic: $p=0.004$ (Pearson correlation).

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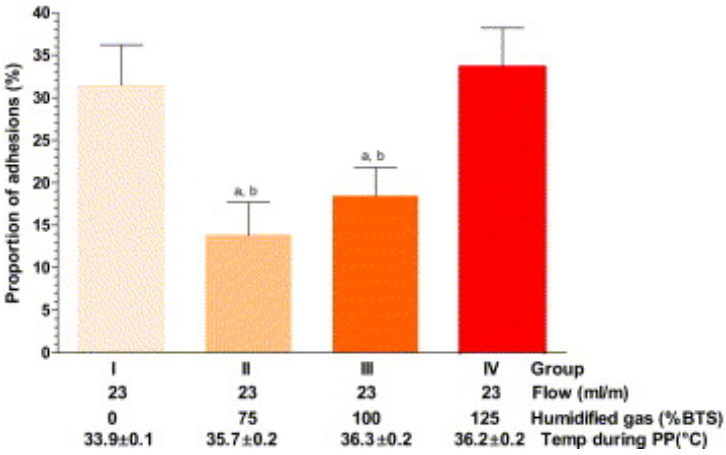
In conclusion, these data clearly demonstrate that hypothermia reduces adhesion formation. In addition, we confirm at 37°C previous results at RT that adhesion formation increases with the duration of the pneumoperitoneum and that the addition of 3% of oxygen to the pneumoperitoneum reduces adhesion formation, whereas the addition of 12% doesn't have any effect.

Experiment 3: Effect of over-saturating the insufflation gas upon adhesion formation.

Adhesion formation in group I (important desiccation and much lower temperatures) was higher than in groups II (p=0.02) and III (p=0.05, Wilcoxon), but not different from group IV (no desiccation and higher intraperitoneal and BTs) (Figure 9). In group III, adhesions were less than in group IV (p=0.03). Adhesion formation in group II (slight desiccation and slightly lower temperatures) was not different from group III but lower than group IV (p<0.01).

Figure 9

Effect of over-saturating the insufflation gas upon adhesion formation.



Effect of CO_2 pneumoperitoneum with discrete levels of humidification, expressed in relation to BTS conditions (37°C, 100% RH) upon adhesion formation.
Non-humidified gas (group I) and humidified gas at 75% BTS (group II), 100% BTS (group III), and 125% BTS (group IV) conditions and a flow of 23 mL/min through the abdominal cavity were used. Pneumoperitoneum-enhanced adhesions were induced during laparoscopy and quantitatively scored after 7 days during laparotomy.

Means ± SE are indicated. [a]p vs. group I <0.05, [b]p vs. group IV <0.03 (Wilcoxon test).

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In conclusion, these results demonstrated that adhesion formation can be reduced by using humidified gas (group III). They also confirmed the effect of temperature upon adhesions. For group IV, although humidified gas was used, the gas had high enthalpy, giving energy and heating the abdominal cavity and increasing pneumoperitoneum and BT and, thus, increasing adhesions. For group I, there is cooling and also desiccation, a topic that will be study in next aim.

2.1.4. EFFECT OF DESICCATION UPON ADHESION FORMATION.

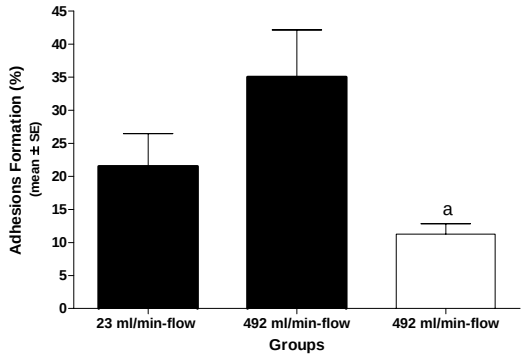
In this section two experiments were done to study the effect of the desiccation upon adhesions.

Experiment 1: (*non- published data*)

Desiccation increases adhesions (Figure 10); however, differences did not reach statistical significance (non-humidified gas, low vs high flow, NS). Humidified gas reduced desiccation-induced adhesions (high flow, non-humidified vs humidified gas, p=0.02; Mann Whitney test).

Figure 10

Effect of desiccation upon adhesion formation.



Pneumoperitoneum-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure.

Filled bar: non-humidified gas, no-filled bar: humidified gas.

Statistic: [a]Non-hum vs hum gas (492 ml/min-flow) p=0.02, Low vs high flow (non-hum) p=NS (Mann Whitney test).

In conclusion, desiccation increases adhesions and, it is associated with a reduction of BT or cooling. Since desiccation is associated with cooling, next step was, to study specifically the effect of desiccation without any associated cooling upon adhesions.

Experiment 2

Binda MM, *Molinas CR, Hansen P, Koninckx PR. Effect of desiccation and temperature during laparoscopy on adhesion formation in mice. Fertil Steril 2006;86:166-175 (158) (Addendum 5).*

The aim of this experiment was to study the effect of desiccation without any associated cooling upon adhesion formation and to confirm the effect of hypothermia upon adhesions.

Non-humidified CO_2 gas was used to induce the pneumoperitoneum at flows of 23 (group II) and 100 (group III) mL/min through the abdominal cavity. Two control groups with minimal desiccation were used: the first with no flow and non-humidified gas (group I) and the second with a flow of 100mL/min and humidified gas (group IV). Because desiccation decreases BT, a homeothermic blanket was used to keep BT strictly at 37°C. Another control, a group of animals was treated with a flow of 100 mL/min and non-humidified gas and without the homeothermic blanket (group V). BT and pneumoperitoneum temperature (calculated as the differences between peritoneum and body temperatures or δT) and RH were measured. Balb/c mice were used (n=71).

The heating blanket kept BT constant at 37.5°C in groups I, II, and III throughout the experiment (between T_{20} and T_{80}) without intergroup differences (data not shown). In group IV, BT increased up to 39°C and was higher than in groups I ($p<0.0001$), II ($p<0.0001$), and III ($p<0.0001$). In group V BT decreased progressively to 31°C and was lower than in groups I ($p<0.0001$), II ($p<0.0001$), III ($p<0.0001$) and IV ($p<0.0001$) (two-way ANOVA).

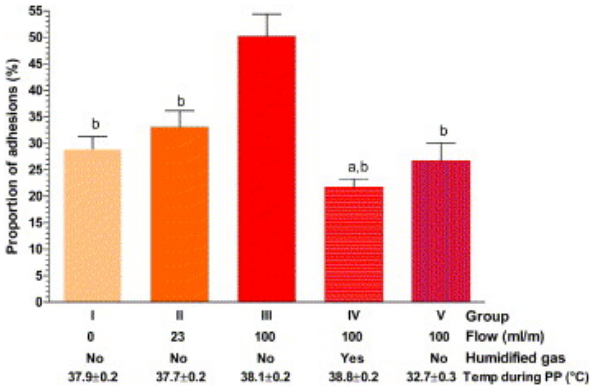
The differences between peritoneum and BT (δT) measured after an equilibration period (T_{40}) were not significant (Proc Univariate) except for group IV ($p=0.03$), being 0.2 ± 0.1°C, −0.5 ± 0.3°C, −0.6 ± 0.4°C, 0.6 ± 0.1°C, and 0.5 ± 0.2°C for groups I, II, III, IV, and V, respectively. The pneumoperitoneum RH remained 100% in all groups throughout the experiment, also when non-humidified CO_2 was used for insufflation, reflecting the high humidification capacity of the peritoneal cavity up to the end of the experiment (data not shown).

Desiccation without affecting BT increased adhesions (Figure 11). In comparison with group I, adhesions increased slightly in group II (NS) and significantly in group III ($p=0.01$; Wilcoxon test). As expected, this increase in adhesions was prevented by using humidified gas (group IV vs III, $p=0.004$). Hypothermia decreased adhesions caused by desiccation

(group V vs III, p=0.01), although not completely up to the level of the no desiccation group (group I), possibly a consequence of the slightly higher temperature.

Figure 11

Effect of desiccation and hypothermia upon adhesion formation.



Adhesions were induced during laparoscopy with 60 minutes of CO_2 pneumoperitoneum at 20 cm of water and quantitatively scored after 7 days during laparotomy. Nonhumidified gas at flows of 0 mL/min (group I), 23 mL/min (group II), 100 mL/min (groups III and V), and humidified gas at a flow of 100 mL/min (group IV) through the abdominal cavity were used. Mice were covered (groups I–IV) or not (group V) with a homeothermic blanket to ascertain body temperature within normal limits.

Mean ± SE of BT during T_{20}–T_{80} is indicated.
^{a}P vs. group I <0.05, ^{b}P vs. group III <0.05 (Wilcoxon test).

Reproduced with permission from *Fertil Steril* (158) (Addendum 5)

In conclusion, desiccation increases adhesions. Normally, two opposite phenomena occurs: desiccation increases adhesions and desiccation produces cooling and this is associated with a reduction in adhesion formation. Hypothermia reduces adhesion formation.

Overall conclusion of Aim #1:

Adhesion formation varies with the *genetic background* (156). In laparoscopy, a decrease or increase in the physiologic *partial oxygen tension* increase adhesion formation. Therefore, in comparison with the physiologic 3% of oxygen, pure CO_2 or CO_2 with the addition of 12% oxygen increases adhesions (74). In addition, the higher the *insufflation pressure* or the longer *duration of the pneumoperitoneum* the more adhesions mice develop (69) (probably due to the hypoxia). Adhesions clearly decrease with low *temperature* probably because cells are, may be, more resistant to hypoxia (157). Finally *desiccation* enhances adhesions (158).

2.2. RESULTS OF AIM #2: SCREENING OF ANTI-ADHESION PRODUCTS IN OUR MODELS

2.2.1. Screening of products which have been described to affect adhesions in a model with 60 min pure CO_2 pneumoperitoneum, i.e. the hypoxia model.

***Binda MM**, Molinas CR, Bastidas A. and Koninckx PR Effect of reactive oxygen species scavengers, antiinflammatory drugs, and calcium-channel blockers on carbon dioxide pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. Surgical Endoscopy 21(10):1826-34. 2007 (159)(Addendum 7).*

***Binda MM**, Molinas CR, Bastidas A, Jansen M and Koninckx PR Efficacy of barriers and hypoxia inducible factor inhibitors to prevent CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. Journal of Minimally Invasive Gynecology 14(5):591-9, 2007 (160)(Addendum 8).*

***Binda MM**, Hellebrekers B, Declerck P, Koninckx PR. Effect of Reteplase and PAI-1 antibodies upon postoperative adhesion formation in a laparoscopic mouse model (submitted; Addendum 10)*

In these screening experiments, adhesions were induced during laparoscopy in BALB/c female mice by performing a bipolar lesion. CO_2 pneumoperitoneum was maintained for 60 minutes using humidified CO_2. BT was strictly maintained at 37°C. Adhesions were scored after 7 days during laparotomy.

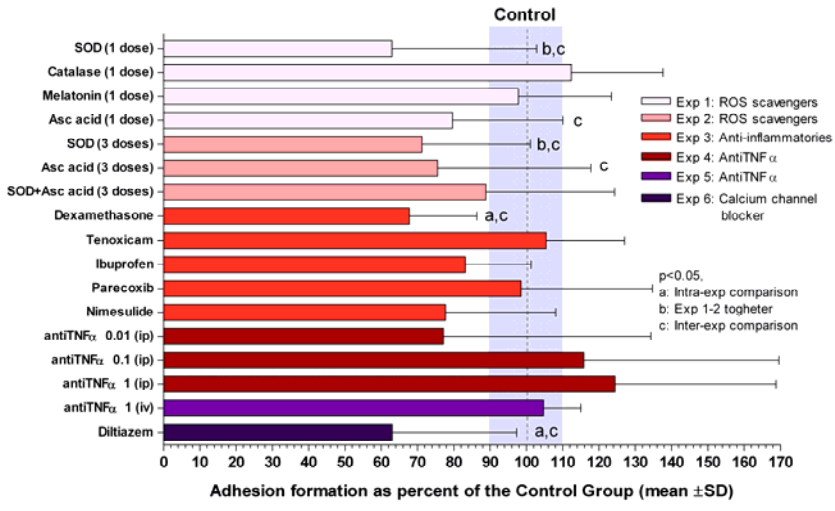
In 12 experiments (n=418), some of the mechanisms affecting the adhesion formation process were analysed. Firstly, the ischemia-reperfusion and the inflammation processes were addressed. During ischemia-reperfusion reactive oxygen species (ROS) are produced, therefore, we analysed the effect of ROS scavengers, molecules which remove ROS, i.e., superoxide dismutase (SOD), melatonin, catalase and ascorbic acid. As anti-inflammatory agents, corticosteroid (dexamethasone), non-steroideal anti-inflammatory drugs (NSAIDs) COX-2 selectives (parecoxib, nimesulide) and COX-2 non-selectives (tenoxicam, ibuprofen)

and neutralising antibodies against TNF-alpha were tried. In addition, diltiazem, a calcium channel blocker, was also used since it works in the inflammation process and also in other pathways. Secondly, the effect of molecules that are lubricating and/or separating two surfaces like barriers (Hyalobarrier Gel, Spraygel), flotation agents (Hyskon and carboximethylcelulose or CMC) and surfactant (phospholipids) were also tried. In addition, since we demonstrated that hypoxia inducible factors (HIF) are involved in the adhesions formation process (71), HIF inhibitors (17-AAG, radicicol, rapamycin and wortmannin) were tried. Finally, fibrinolitic agents were also tested, i.e. Reteplase or r-PA and PAI-1 antibodies.

Adhesion were reduced by SOD ($p<0.01$, Proc GLM of one dose and three doses experiments analysed together), diltiazem ($p=0.05$; Wilcoxon) and dexamethasone ($p<0.03$) but not by NSAIDs, neither by anti-TNF alpha. If all experiments were grouped for analysis, adhesions also decreased with one and three doses of SOD ($p<0.01$; $p<0.01$ respectively) and one and three doses of ascorbic acid ($p<0.02$; $p=0.05$, respectively) showing a border line effect (Figure 12).

Figure 12

Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model



Proportions of adhesions are indicated. In order to visualise the results of all experiments in one graph, the percentage of change in comparison with the controls is given for each treatment. The coefficient of variation of all the controls is indicated as the shadowed area.

Reproduced with permission from *Surgical Endoscopy (159)* (Addendum 7).

Statistics

[a]$p<0.05$ intra-experiment comparisons (each group compared to its own control group) (Wilcoxon test).

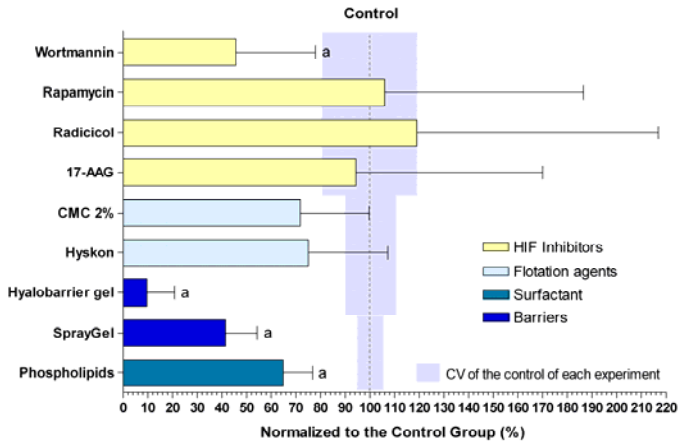
[b]$p<0.05$ Experiment 1 and 2 analysed together (proc GLM, 2 groups, 2 variables, i.e. experiment and treatment); p value of the variable treatment.

[c]$p<0.05$ inter-experiment comparisons (each group compared to a control group grouping all the controls) (Wilcoxon test).

Moreover, adhesion formation decreased with the administration of wortmannin ($p<0.01$), phospholipids ($p<0.01$), Hyalobarrier Gel ($p<0.01$) and SprayGel ($p<0.01$) (Figure 13)

Figure 13

Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model.



Proportions of adhesions are indicated. In order to visualise the results of all experiments in one graph, the percentage of change in comparison with the controls is given for each treatment. The coefficient of variation of the controls of each experiment is indicated as the shadowed area.
To make the table clearer, only the comparisons to the control groups were placed.

[a]$p<0.05$ intra-experiment comparisons (each group compared to its own control group) Wilcoxon test.

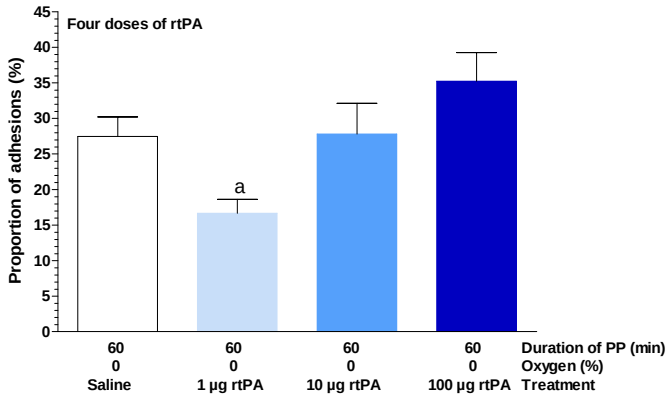
Reproduced with permission from *JMIG* (160) (Addendum 8).

Adhesion formation was reduced with the administration of four doses of 1 µg of Reteplase comparing with the control group ($p<0.04$, Wilcoxon) (Figure 14). There was not any effect of 10 and 100 µg comparing with the control group (NS each comparison). The administration of 1 µg of Reteplase also reduced adhesion formation comparing with animals treated with 10 µg ($p=0.05$) or 100 µg ($p<0.01$). This means that 1 µg of Reteplase produced a 55% of adhesions comparing with the control group (100%).

No effect was observed with the neutralizing anti PAI-1 antibodies comparing with the non-neutralizing antibodies (NS) (data non-shown).

Figure 14

Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model.



Pneumoperitoneum was maintained for 60 minutes using pure CO_2. The gas was humidified and the insufflation pressure was 20 cm H_2O. Adhesions were induced during laparoscopy by performing a bipolar lesion. Four doses of 1, 100 and 100 µg of rtPA were administrated. A control group was done by administrated 4 times saline. Adhesions were scored after 7 days during laparotomy.

$p<0.05$, Wilcoxon test: [a] comparing with the control, with 10 µg and with 100 µg of Reteplase.

Manuscript submitted (Addendum 10)

Other drugs tested: (data non-published)

Adhesions were induced during laparoscopy in BALB/c female mice by performing a bipolar lesion (n= 24). CO_2 pneumoperitoneum was maintained for 60 minutes using humidified CO_2. BT was strictly maintained at 37°C. Resolvin and lipoxin, two molecules with anti-inflammatory activity, were tried i.p. the day of the surgery and the day after (4 doses). We decided to try these two new molecules because they are also associated with several other biological functions including leukocyte activation, chemotaxis promotion, monocyte migration and adhesion and protection during ischemia-reperfusion injury.

Comparing with the control group, there was a small reduction in adhesion formation after applying lipoxin and resolvin, although non-significant (data non-shown).

Conclusions of section 2.2.1:

In our laparoscopic mouse model, adhesion formation was reduced by using dexamethasone, SOD, ascorbic acid and Diltiem. The absence of effect of the other anti-inflammatory drugs, of anti TNF alpha, resolvin and lipoxin is surprising. These experiments also confirm the efficacy of barriers and phospholipids to separate or lubricate damaged surfaces. They may also confirm the role of mesothelial hypoxia in this model, by the efficacy of the HIF inhibitor wortmannin, and the role of the fibrinolysis by the efficacy of recombinant Reteplase.

2.2.2. Screening of selected products which have been described to affect adhesions in a model with 60 min CO_2 pneumoperitoneum +12% oxygen, i.e. the hyperoxia model.

Binda MM *and Koninckx PR. Prevention of hyperoxia enhanced adhesions in a laparoscopic mouse model (in preparation; Addendum 11)*

2.2.2.1. To confirm the hypothesis that hypothermia prevents adhesions by making cells more resistant to the toxic effects of the hyperoxia.

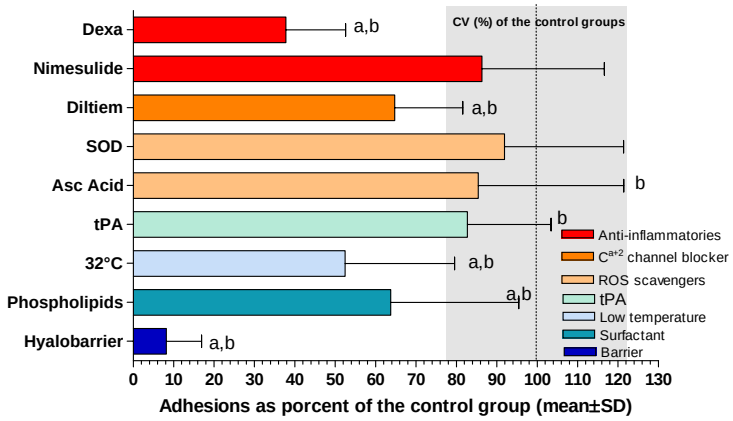
2.2.2.2. To screen some of the products used in 2.2.1 in order to know their relative effectiveness also in the hyperoxia model.

In two experiments, pneumoperitoneum-enhanced adhesion was induced using humidified and CO_2 with the addition of 12% oxygen as insufflation gas. The effect of low temperature was investigated and BT was controlled to 32° or 37°C, as explained in Section 2.1.3, experiment 2. BALB/c mice were used (n=96). In addition, we decided to choose one or two drugs representative of each group of drugs: anti-inflammatories (corticoid: dexamethasone, NSAIDs: nimesulide), calcium channel blockers (diltiazem), ROS scavengers (SOD and ascorbic acid), barriers (hyalobarrier gel), surfactant (phospholipids) and fibrinolytic agent (Reteplase).

Hyperoxia-induced adhesions were reduced by using low temperature ($p<0.02$), phospholipids ($p<0.03$), hyalobarrier gel ($p<0.004$), dexamethasone ($p<0.005$) and diltiazem ($p<0.01$). A low effect of ascorbic acid (NS) and Reteplase (NS) and non effect of the nimesulide, SOD and ascorbic acid were observed (Figure 15). If all experiments were grouped for analysis, adhesions also decreased with ascorbic acid ($p<0.002$) and Reteplase ($p<0.005$) showing a border line effect (Wilcoxon test).

Figure 15

Prevention of pneumoperitoneum-enhanced adhesions in the hyperoxia model.



Proportions of adhesions are indicated. In order to visualise the results of all experiments in one graph, the percentages of change in comparison with the controls are given for each treatment. The coefficient of variation of all the controls is indicated as the shadowed area.

Wilcoxon test
[a] $p<0.05$ Intra-experiment comparisons
[b] $p<0.05$ Inter-experiment comparisons

In conclusion, in the hyperoxia model, adhesion formation decreases with the reduction in BT to 32°C, the use of dexamethasone, a calcium channel blocker, phospholipids and hyalobarrier gel. A border line effect of ascorbic acid and Reteplase was observed. The hypothesis that hypothermia prevents adhesions in the hyperoxia model was also confirmed. We hypothesize that hypothermia is making cells more resistant to the toxic effects of the hyperoxia.

2.3. RESULTS OF AIMS #3 and #4: SCREENING OF COMBINATION OF TREATMENTS

Binda MM *and Koninckx PR. Combination of treatments to prevent adhesion formation in a laparoscopic mouse model (in preparation, Addendum 12).*

2.3.1. To confirm the hypothesis that hypothermia prevents adhesions by making cells more resistant to hypoxia.

2.3.2. To screen some of the products used in the Section 2.2.1 in order to know their relative effectiveness also in the normoxia model and to combine with the low-temperature treatment.

2.3.3. To confirm that the addition of an extra trauma like bleeding increases adhesion formation.

Pneumoperitoneum-enhanced adhesion was induced using humidified and pure CO_2 or CO_2 + 3% of oxygen, hypoxia and normoxia models, respectively. BALB/c mice were used (n=104). BT was controlled to 32° or 37°C, as explained in Section 2.1.3 experiment 2.

Products representative of each group of drugs were choosen and combined with two treatments that previously showed a reduction in adhesion formation (the addition of 3% oxygen and the low temperature). Anti-inflammatories (corticoid: dexamethasone, NSAIDs: nimesulide), a calcium channel blockers (diltiazem), ROS scavengers (SOD and ascorbic acid), a barrier (hyalobarrier gel), a surfactant (phospholipids) and a fibrinolytic agent (Reteplase) were used. In addition, the effect of low temperature was also investigated in both pneumoperitoneum with pure CO_2 and with the addition of 3% oxygen as controls.

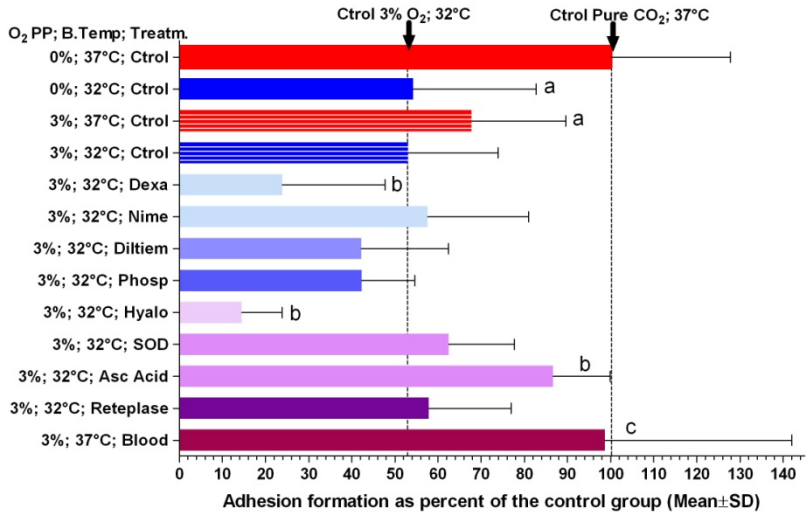
To investigate the effect of an extra trauma (bleeding) 1 ml of blood extracted from another mouse was injected i.p. after performing the lesions.

Comparing to pure CO_2 pneumoperitoneum at 37°C, adhesions were reduced by adding 3% oxygen to the pneumoperitoneum ($p<0.05$), by lowering BT ($p=0.03$, Wilcoxon test), as previously demonstrated (69; 157), and by applying both 3% oxygen and low temperature ($p=0.02$) (Figure 16). Taking into account the groups with 3% of oxygen, lowering BT from 37°C to 32°C did not reduce more the adhesion formation (NS) showing that the effects of both treatments, addition 3% oxygen and lowering BT, do not have additive

effects. Comparing to the control group with 3% oxygen and 32°C BT, adhesion formation were reduced by using dexamethasone (p=0.04), hyalobarrier gel (p=0.0161) whereas a low reduction was observed with diltiazem and phospholipids (NS both comparisons). Non effect was observed neither with Reteplase, nor with SOD and nimesulide (NS for the 3 comparisons) and, surprising, an increase in adhesions formation was observed with ascorbic acid (p=0.0085). Comparing to the group with 3% oxygen and 37°C BT, the addition of an extra trauma like bleeding increases significatively adhesion formation (p=0.01) till the values gotten in the hypoxia model and 37°C BT control group.

Figure 16

Combination of treatments in the laparoscopic mouse model.



Proportions of adhesions are indicated. In order to visualise the results of all experiments in one graph, the percentage of change in comparison with the control pure CO_2 PP; 37°C is given for each treatment.

Wilcoxon test: $p<0.05$

[a] Comparison to pure CO_2 PP, 37°C.

[b] Comparison to 3%O_2 PP, 32°C.

[c] Comparison to 3%O_2 PP, 37°C.

In conclusion, low temperature and addition of 3% oxygen do not have additive effects. Dexamethasone, diltiazem and phospholipids have additive effects to the low temp and 3% oxygen treatments. Nimesulide, SOD, Reteplase do not have any extra effect. Ascorbic acid adds an extra trauma reducing the protective effect caused by the low temperature and normoxia. Hyalobarrier gel gave the same percentage of inhibition than in the hypoxia model and 37°C control group (Figure 13), meaning no additive effects to the low temperature and normoxia. An extra trauma as bleeding increases adhesion formation. A detailed analysis will be done in the discussion of this thesis.

2.3.4. Establishment of which combination of treatments will give a maximum of adhesion reduction.

In order to summarize all the prevention results, a table was done (Table 1). In the figures 12, 13, 15 and 16, data are visualized as percentage of change in comparison to the controls of each experiment, for instances, dexamethasone in Figure 12 has 68% of adhesions in comparison to its control group considered as 100%. In this table, the percentage of inhibition of each agent was calculated, for example for dexamethasone, as 100%- 68%=32%.

The aim of this table was to shown the prevention results all together and to see in each model the percentage of inhibition of the agent used. The comparisons were already done for each model separately as seen in Figures 12, 13, 15 and 16. A new analysis was done to compare the columns “Hypoxia+37°C Model” and “Normoxia+Low temp Model” in order to see if the effect of the treatments (agent and low temperature/adding 3% Oxygen) are additives. Comparing to the dexamethasone application in the hypoxia and normothermia model (32% inhibition), adding low temp and 3%oxygen decreases significantly adhesions formation (76% inhibition) ($p=0.0061$, Mann Whitney test). Comparing to the diltiem application in the hypoxia and normothermia model (36% inhibition), adding low temp and 3%oxygen also decreases significantly adhesions formation (58% inhibition) ($p=0.032$). Comparing to the phospholipids application in the hypoxia and normothermia model (35% inhibition), adding low temp and 3%oxygen decreases significantly adhesion formation (58% inhibition) ($p=0.049$). The other comparisons were not significant or not done since not all the products were tested as explained.

Table 1

Drug	Mechanism	Hypoxia + 37°C Model[a]	Hyperoxia+ 37°C Model[b]	Normoxia + Low temp Model[a]	Comparing to Normoxia + Low temp [C]
#1: Dexamethasone	Steroideal antiinflamatory; inhibitor of fibroblasts proliferation and release of cytokines, immunosuppressive	32% inhibition	62% inhibition	76% inhibition*	54% inhibition
#2: Nimesulide	NSAID: COX-2 selectives	22% inhibition (NS)	14% inhibition (NS)	42% inhibition	No effect
#3: Parecoxib		No effect	*Not done*	*Not done*	*Not done*
#4: Ibuprofeno	NSAID: COX-2 non-selectives	15% inhibition (NS)	*Not done*	*Not done*	*Not done*
#5: Tenoxicam		No effect	*Not done*	*Not done*	*Not done*
#6: Anti-TNF alpha	antiinflammatory	No effect	*Not done*	*Not done*	*Not done*
#7: Diltiem	Calcium channel blocker, protection against the toxic effect of the ischemic-reperfusion cell injury, activation of cellular processes	36% inhibition	34% inhibition	58% inhibition*	19% inhibition
#8: Asc acid	ROS scavengers (1 dose)	20% inhibition (border effect)	14% inhibition (S, interexp)	14% inhibition	Increase in 65%
#9: Melatonine		No effect	*No done*	*No done*	*No done*
#10: SOD		36% inhibition (border effect)	8% inhibition (NS)	38% inhibition	No effect
#11: Catalase		No effect	*Not done*	*Not done*	*Not done*
#8: Asc acid	ROS scavengers (3 doses)	24% inhibition (border effect)	*Not done*	*Not done*	*Not done*
#10: SOD		30% inhibition (border effect)	*Not done*	*Not done*	*Not done*
#12: SOD + Asc Acid		12% inhibition (NS)	*Not done*	*Not done*	*Not done*
#13: Wortmanin	HIF inhibitors	54% inhibition	*Not done*	*Not done*	*Not done*
#14: 17-AAG		No effect	*Not done*	*Not done*	*Not done*
#15: Radicicol		No effect	*Not done*	*Not done*	*Not done*
#16: Rapamicyn		No effect	*Not done*	*Not done*	*Not done*
#17: Spraygel	Barriers	58% inhibition	*Not done*	*Not done*	*Not done*
#18: Hyalobarrier gel		90% inhibition	92% inhibition	85% inhibition	71% inhibition
#19: Hyskon	Flotation agents	24% inhibition (NS)	*Not done*	*Not done*	*Not done*
#20: CMC 2%		28% inhibition (NS)	*Not done*	*Not done*	*Not done*
#21: Phospholipids	Surfactant	35% inhibition	36% inhibition (S)	58% inhibition*	19% inhibition
#22: Resolvin	Anti inflammatories	No effect	*Not done*	*Not done*	*Not done*
#23: Lipoxine			*Not done*	*Not done*	*Not done*
#24:Reteplase	Fibrinolysis	40% inhibition	17% inhibition (S, interexp)	42% inhibition	No effect
#25: anti-PAI-1		No effect	*Not done*	*Not done*	*Not done*
#26: Low temperature	Prevention toxic effects of hypoxia	51% inhibition	48% inhibition (S)	48% inhibition	*Not correspond*
#27: Adding 3% O_2	Prevention toxic effects of hypoxia	32% inhibition	*Not correspond*	*Not correspond*	*Not correspond*

The inhibition values were gotten: [a]Considering as 100% the hypoxia and 37°C control group; [b]Considering as 100% the hyperoxia and 37°C control group; [c]Considering as 100% the normoxia and 32°C control group.

Statistic: * $p<0.05$, Mann Whitney test. Values of column "Hypoxia+37°C Model" vs "Normoxia+Low temp Model".

Chapter 3

DISCUSSION

3.1 Animal models to study adhesion formation

3.1.1 Existing models

Several animal models have been used to investigate the pathogenesis of adhesion formation and to evaluate prevention during open and laparoscopic surgery. Many studies deal with adhesion formation and its prevention in animal models during open surgery; few studies, however, deal with laparoscopy.

Adhesion formation was studied during laparotomy in mouse, rats, rabbits, pigs, etc. In mice, different kind of lesions were used during laparotomy, i.e. cecal abrasion (161; 162), uterine abrasion (163), sharp abrasion of a 2-cm^2 area on the parietal peritoneum leading to microhemorrhage (164), a 2x15 mm defect on the parietal peritoneum created by sharp incision followed by excision with a pair of scissors and closing of the lesion with sutures (165), electrocauterization of an area of 2×4 mm on the uterine wall (166). In rats, different kind of lesions also were induced during laparotomy, i.e. scratching on the cecum and on the abdominal wall (101; 167), liver resection (168), creation of ischemic buttons on the parietal peritoneum (169), cecal ligation and puncture (170), cecum abrasion using a brush until bleeding and denudation of the serosa on the uterine horn with a scalpel until macroscopic punctuate (171). In rabbits, the lesions that were induced during laparotomy were scraping of the large bowel with a scalpel blade and cecum abrasion with a gauze, in both cases until bleeding (172), cecal abrasion with a brush until bleeding (173), denudation of the ascending colon with a scalpel until bleeding and application of 3% povidine-iodine solution on the intestinal wall to stimulate inflammation (174), uterine horn abrasion until punctuate bleeding developed (101; 175), uterine horns incision and reanastomosis (176). In pigs, different kind of lesions were induced during laparotomy, i.e. by performing a cholecystectomy (177), small bowel resection (178), uterine horn transection and re-anastomosis (102), nephrectomy (179), hysterectomy bilateral salpingo-oophorectomy, and resection of pelvic peritoneum (180). In dogs, there are a few studies: the lesions that were performed during laparotomy were cholecystectomy (181), cecal resection and deserosation of the abdominal wall (45), sternotomy and pericardiotomy followed by 2-hour of forced warm air desiccation and

abrasion of the pericardial and epicardial surfaces (182). In horses, there are also a few models to induce lesions during laparotomy, i.e. jejunal abrasion and jejunal resections and end-to-end anastomoses (183). It should be stressed that all these studies were generally reported using different species, different kind of induced lesions, using different scoring systems and the prevention was done by testing only one or two drugs. Therefore, a comprehensive quantitative evaluation of efficacy in one model is still lacking.

Following laparoscopic surgery, tumor growth and implantation have been studied in animal models (184-190). However, prevention of adhesion formation has been poorly addressed. A few studies have been reported during laparoscopy, i.e. in rats (191), rabbits (106; 192; 193) and pigs (194). However, laparoscopy in mice has been reported only for the tumour growth and port site metastasis studies (195; 196), immune response (197) and morphologic alteration of the mesothelium (64) and its use in adhesion formation studies has been poorly addressed. The first study of laparoscopic surgery done in mice has been reported by our group in 1999 (68).

In these models, animals, lesions and circunstances are variable. These models are, moreover, poorly characterised. It is not known neither how critical all the parameters are in the model, nor how strictly they have been controlled. In order to study pathogenesis the first aim of this thesis was to standardize and characterize the laparoscopic mouse model for adhesions. In order to develop a comprehensive model of adhesion prevention, it was necessary to evaluate all known products in one model, something missing today.

3.1.2 The mouse model

Each animal model has advantages and limitations. The main disadvantage of mice is the size. In addition, the thickness of the entire abdominal wall is equivalent to the first muscle layer in rats and the same relationship exists between rats and rabbits. The ratio of peritoneal surface area/ body weight is higher in small than in larger animals, being 0.195 m^2/kg in mice, 0.115 m^2/kg in rats, 0.084 m^2/kg in rabbit and 0.026 m^2/kg in humans. The volume of peritoneal fluid is disproportional higher in small than in larger animals being 18.1 ml/kg in mice, 10.7 ml/kg in rats, 7.7 ml/kg in rabbit and 2.4 ml/kg in humans (198).

The mouse model has advantages: they are inexpensive and easy to handle. Several well characterised types are available, such as inbred strains, genetically manipulated mice, e.g. mice non-expressing or over-expressing specific genes, mice with altered immune system, e.g. *nude* and SCID. Moreover, many specific assays and monoclonal antibodies

exist. In addition, mice and rats, in contrast with rabbit, do not require strict sterile conditions for surgery.

3.1.3 Experimental factors which should be controlled and/or standardised to investigate adhesion formation

We developed a mouse model in which many parameters were modulated and/or monitored: conditions of the pneumoperitoneum (duration, pressure, type of gas, humidification and temperature), body temperature, mouse strain and ventilation. All these parameters can have an influence in adhesion development (Table 2).

We previously demonstrated that adhesion formation increases with the duration of the pneumoperitoneum (69). During this thesis the pneumoperitoneum was maintained for the minimum time to perform the lesion, standardised at 10 min, or for 60 min. We called “basal adhesions” to those adhesions resulting from the electrosurgical trauma and with a minimum effect of the pneumoperitoneum (10 min) and “pneumoperitoneum-enhanced adhesion” to those adhesions resulting from the electrosurgical trauma plus the additional effect of 60 min of pneumoperitoneum, both using pure CO_2 at 20 cm H_2O.

Moreover, the insufflation pressure increased adhesions as demonstrated in rabbits (67) and in mice (69; 74). During this thesis the insufflation pressure used to induce the pneumoperitoneum was 15 mm of Hg. We recognized that this insufflation pressure could be too high for a mouse. Since our working hypothesis was that the pneumoperitoneum induces ischemia and, therefore, hypoxia, and since adhesions are pressure-dependent, we decided to use the maximum pressure in order to observe maximum effect. In addition, a pneumoperitoneum with a pressure of 15 mm Hg could be relevant since it can mimic the conditions that happen in surgery on humans.

The type of gas has also an influence in adhesion formation. Adhesion formation decreases with the addition of 2-4% of oxygen to both CO_2 and helium pneumoperitoneum (69), and increased when higher concentrations than 3% Oxygen were added to the pneumoperitoneum (74). For all these observations, mesothelial hypoxia was postulated as a cofactor in adhesions and this will be explained in detail in next section.

The mouse body temperature has also to be controlled. There are several factors, as demonstrated during this thesis, that affects mouse body temperature, i.e. anaesthesia, ventilation with non-humidified gas, environmental temperature and use of non-humidified

gas to induce the pneumoperitoneum (157; 158). All these factors must be well controlled since it has been demonstrated that hypothermia prevents adhesions.

The humidification of the insufflation gas has also to be controlled. When non-humidified gas is used to induce the pneumoperitoneum, desiccation occurs and adhesions increase (158). In addition, desiccation is associated with cooling.

It was also shown in our model the importance of a proper ventilation (53). CO_2 pneumoperitoneum causes acidosis and hypercarbia and this can be corrected with assisted ventilation. Moreover, it was suggested that pneumoperitoneum-induced acidosis plays a role in the mechanism of CO_2 pneumoperitoneum-enhanced adhesion formation.

Finally, the genetic background has an influence in adhesion formation. Some mouse strains developed more adhesions than others and, in addition, outbred strains have more variability in the results than inbred strains (156).

Table 2:

Model	Model variables	Elements tested	Effect upon adhesions	Interaction with other variables	References
Hypoxia	60 min pure CO_2 PP, humidified gas, 15 mm Hg, 37°C BT, lesion	ventilation	Reduces adhesions	Reduces acidosis-hypercarbia	(53)
		insufflation pressure	The higher the pressure the more adhesions	-	(74)
		desiccation alone	Increases adhesions	Low temperature reduces desiccation-enhanced adhesions	(158)
		cooling alone	Reduces adhesions	-	(157)
		Oversaturation of the insufflation gas	Increases adhesions	Increases PP temp and PP RH.	(158)
Hyperoxia	60 min, CO_2+12% O_2 PP, humidified gas, 15 mm Hg, 37°C BT, lesion	insufflation pressure	The higher the pressure the more adhesions	-	(74)
		cooling alone	Reduces adhesions	-	*In preparation*
Normoxia	60 min, CO_2 + 3-4% O_2 PP, humidified gas, 15 mm Hg, 37°C BT, lesion	cooling alone	No effect	No interaction with low temperature	*In preparation*

3.1.4 New conceps of pathophysiology that have been derived from the mouse model

3.1.4.1 Peritoneal hypoxia, normoxia and hyperoxia

Since adhesion formation increases with the duration and with the insufflation pressure and it decreases with the addition of 3% oxygen to the pneumoperitoneum, mesothelial hypoxia was suggested as a driving mechanism. This mesothelial hypoxia hypothesis is consistent with the fact that adhesions were absent in mice deficient for hypoxia inducible factor (HIF) (71), plasminogen activator inhibitor 1 (PAI-1) (72) and vascular endothelial growth factor (VEGF) (41), all factors up-regulated during hypoxia. Moreover, preliminary results show positive staining of the mesothelial layer for a hypoxia marker after 60 min of CO_2 pneumoperitoneum and a significative reduction of this staining (meaning less hypoxia) after the exposition to a 60 min CO_2 pneumoperitoneum with the addition of 3% oxygen (unpublished observations).

The hypothesis of the mesothelial hypoxia can be explained by postulating that the pneumoperitoneum changes the mesothelial pO_2. According to the oxygen cascade model of mammals, the pO_2 decreases progressively from 159 mm Hg in air to 95 mm Hg in the arterial end of capillaries, 40 mm Hg in the interstitial fluid, and some 23 mm Hg in the peripheral cells (75). This intracellular pO_2 varies from 5–40 mm Hg, depending on the type of cells and on the distance to the capillaries (75; 199-203). Taking these concepts into account, it is clear that intracellular pO_2 lower or higher than 5–40 mm Hg ("cellular normoxia") should be considered "cellular hypoxia" or "cellular hyperoxia," respectively. The pneumoperitoneum, independent of the insufflation gas, will compress the capillary flow in the peritoneal layer, reducing tissue perfusion, inducing ischemia and reducing the pO_2 in the mesothelial cells up to hypoxic levels (204-207). Consistent with that, the higher the insufflation pressure, the higher the capillary compression, therefore more hypoxia and ischemia will occur and more developed of adhesions (67; 74). In addition, the insufflation gas will diffuse to the bloodstream, reducing the mesothelial pO_2 and inducing hypercarbia and acidosis if not corrected by assisted ventilation (53; 54). Although we suggest that the environment during a CO_2 pneumoperitoneum is hypoxic, it was shown, surprising, that a significative elevation in the $PitO_2$ occurs during a CO_2 pneumoperitoneum in a laparoscopic mouse model (208). Although this model is quite similar to our model, the insufflation pressure used is much lower and this could explain the differences with our hypothesis.

During standard laparoscopy, i.e. pure CO_2 pneumoperitoneum, the mesothelial cells would not receive sufficient oxygen supply from the capillaries and, in addition, pure CO_2

will diffuse to the mesothelial cells (mesothelial hypoxia). During laparoscopy with a pneumoperitoneum with 3% oxygen, the mesothelial cells would not receive neither sufficient oxygen supply from the capillaries but the fact that 3% oxygen at 775 mm Hg (atmospheric pressure of 760 mm Hg plus insufflation pressure of 15 mm Hg) results in a pO_2 of around 23 mm Hg, which is remarkably similar to normal intracellular pO_2 and that can be absorbed raising the intracellular pO_2 up to more physiological levels, will make cells feel normoxic (mesothelial normoxia) (75). During laparoscopy with a pneumoperitoneum with 12% oxygen, the 12% oxygen at 775 mm Hg results in a pO_2 of 92 mm Hg, which is higher than the normal intracellular pO_2 (mesothelial hyperoxia). Therefore, we can define three models: the hypoxia, normoxia and hyperoxia models in which pure CO_2 or CO_2 with 3% and 12% oxygen, respectively, are used to induce the pneumoperitoneum. Without taking into account the differences in pressures, the hyperoxia model could be considered similar to the open surgery, which is performed in air that also has a relatively high oxygen concentration.

Because a pneumoperitoneum with 12% oxygen induces mesothelial hyperoxia, the increase in adhesion formation might be caused by ROS (addendum 2, (209)). Indeed, hyperoxia generates ROS (e.g., superoxide anion, hydrogen peroxide, and nitric oxide), which have deleterious effects in cells. Cells protect themselves from the deleterious effects of ROS by producing ROS scavengers. The balance between ROS and ROS scavengers will determinate ROS availability and toxicity. ROS are suggested to be involved in tissue destruction and fibrosis in patients with endometriosis (210) and in adhesion formation (57; 147). Furthermore, this latter effect was shown to be reduced by ROS scavengers, such as catalase and superoxide dismutase (148; 151), vitamin E (154), methylene blue (152), and melatonin (153). In addition, ROS might be involved in the increased adhesion formation after pure CO_2 pneumoperitoneum because ROS can be generated after the reperfusion of an ischemic tissue (205) (i.e., after mesothelial hypoxia during CO_2 pneumoperitoneum). Furthermore, the generation of ROS after open and laparoscopic surgery is well reported (145; 146). Laparoscopic surgery increases ROS availability by increasing ROS production (146) or by decreasing ROS scavengers (57; 147). Therefore, we hypothesize that this increased ROS availability plays a role in adhesion formation (addendum 2, (209)). This is fully consistent with the similar adhesion formation observed with CO_2 pneumoperitoneum with 0% or 12% oxygen and with the reduction of adhesion formation with 3% oxygen. Indeed, with 12% oxygen, mesothelial cells are in a hyperoxic environment that could lead to increased ROS production or decreased ROS scavenger production, whereas pneumoperitoneum with both 0% and 12% oxygen causes an ischemia/reperfusion process,

especially at high insufflation pressure, that could be an additional source of ROS. Although pneumoperitoneum with 3% oxygen also alters microcirculation, cells do not become hypoxic because they receive oxygen from the more physiologic pneumoperitoneum environment (pO_2 around 23 mm Hg).

3.1.4.2 Hypothemia decreases adhesions

Anaesthesia, ventilation and pneumoperitoneum affect body temperature

This study confirmed and extended previous data concerning of the effects of anaesthesia, ventilation and pneumoperitoneum upon body temperature.

We confirmed that anaesthesia decreases mouse body temperature, as demonstrated previously in rats (211), mice (212) and humans (213). As expected, this cooling effect is influenced by the environmental temperature, being more pronounced at RT than at 37°C (T_0-T_{20} in Figures 2, 3, 4 and 6). These observations are consistent with the reported effect of operating room temperature in humans. Indeed, patients remain normothermic after anaesthesia when they are kept in a warmer operating room, whereas they become hypothermic when placed in an operating theatre with colder temperature (214-216). This pure anaesthetic side effect is caused by cutaneous vasodilatation, which abolishes heat conservation. Consequently, anaesthetised subjects become poikilothermic and their body temperature varies with environmental temperatures (215).

We demonstrated that the pneumoperitoneum itself can cause a decrease in body temperature or cooling, especially when dry gas was used (Figures 2, 3 and 4). Since dry gas at RT is commonly used to induce the pneumoperitoneum, this cooling effect can be explained by the heat energy loss required to warm up the cool gas and/or to evaporate body water in order to humidify the dry gas. Our thermodynamic calculations indicates that 577 cal is needed to vaporize 1 mL of water at 37°C (63 cal to heat 1 mL to 100°C + 514 cal to vaporize), whereas only 0.00003 cal is needed to heat 1 mL of CO_2 by 1°C (158). Thermodynamic calculations done by other authors indicates that, with a flow of 10 l/min over 3 hrs, the heat energy required to raise gas temperature from 25˚C to 37˚C is 0.9 W and from 30˚C to 37˚C is 0.48 W, whereas the latent heat of vaporization (amount of energy required to change a unit mass from liquid to gas) required to evaporate body water to saturate the initially dry gas at 37˚C is 18 W in pigs (217). Those calculations indicate that the cooling is caused by the considerably heat energy loss expended to evaporate body water in order to

humidify the dry CO_2 rather than the used to raise the ambient temperature of the gas to body temperature (55; 217; 218).

This water evaporation done in order to humidify the dry gas causes not only a lowering of body temperature but also desiccation in the abdominal cavity. For these reasons we postulate that desiccation and cooling are intimately linked. This desiccation is clearly influenced by the flow rate and by the time (67; 68) being the gas less likely to be saturated at high flows than at low flows (218). This is consistent with our results in which the cooling effect increased with the flow through the abdominal cavity (Figure 3) and with the time of the surgery (Figures 2, 3 and 4). These observations are also consistent with the association found between water evaporation and temperature reduction in the surface of exposed viscera during open surgery (219). In all the experiments, gas temperature was controlled by environment temperature. Moreover, it is also known that desiccation depends on the temperature since the capacity of a gas to hold water depends on its temperature, i.e., 100% relative humidity corresponds to 25 mg of water/litre of gas at 25°C and to 44 mg of water/litre of gas at 37°C. This means that if mice at RT are exposed to RT insufflation gas, the hypothermia would be lower than if mice at RT and exposed to 37˚C insufflation gas, since desiccation would be less pronounced. In addition, we showed that this cooling effect caused by desiccation is prevented using warm and humidified gas (Figures 3, 4 and 11) which is consistent with previous reports in rats (63), pigs (55; 217; 220) and humans (221). As expected, the cooling observed during laparoscopy with cold and dry gas can be fully prevented using warm and humidified gas (55; 63; 220) but not warm and dry gas (63; 217). In summary, pneumoperitoneum-induced desiccation increases with the duration of the pneumoperitoneum, with the flow rate through the abdominal cavity and with the gas temperature. The higher the desiccation, the higher the cooling and both desiccation and cooling can be prevented with humidified gas.

This study demonstrated that ventilation with non-humidified room air can decrease body temperature [Section 2.1.2 experiment 2 (157)]. As explained for pneumoperitoneum, dry air for ventilation will take water by evaporation from a wet surface until reaching the equilibrium, therefore, our data suggest that the water loss or desiccation from the respiratory ways would be cooling the animal down, confirming previous data in humans (222-224).

Body temperature affects adhesion formation

It has been demonstrated for the first time that adhesion formation increases with body temperature or, in other words, that hypothermia reduces adhesion formation (Figures 5, 6, 8,

9 and 11). In Figure 5, no-needle was placed in the mouse abdomen, and then, theoretically, no-flow through the abdominal cavity occured during pneumoperitoneum. This increase in adhesion formation could be explained by the fact that pneumoperitoneum-induced desiccation increases with gas temperature and/or by the fact that body temperature increases. Therefore, a mixed effect of desiccation and temperature is observed in this experiment 1 of section 2.1.4. This is especially cumbersome since the relation between both is complex. As it was said above, desiccation causes cooling and desiccation decreases when gas temperature is lower because the gas can hold less water. Therefore, great care was taken in the Experiment 2 of Section 2.1.3, to prevent pneumoperitoneum-induced desiccation using humidified gas with a little flow through the abdominal cavity and, in addition, mouse body temperature was also carefully manipulated (Figures 6, 7 and 8). In this experiment we clearly demonstrated that hypothermia decreases adhesion formations (Figures 6 and 8). Not only pneumoperitoneum-enhanced adhesions, but also the differences between basal and pneumoperitoneum-enhanced adhesion formation increased with body temperature (Figure 5). Taking into account these results, a positive correlation between pneumoperitoneum-enhanced adhesion and body temperature was found (Figure 8).

Previous reports dealing with the relationship between body temperature and adhesion formation are scanty. In humans, local hypothermia after laparotomy was reported to decrease the inflammatory reaction and to increase intestinal peristalsis, thus decreasing adhesion formation (225). In rats, irrigation with saline at more than 37°C, i.e. between 37°C and 60°C, increases adhesion formation (226). Our results may suggest that hypothermia could modulate the response to CO_2 pneumoperitoneum-induced hypoxia, protecting tissues and cells from the toxic effects of hypoxia. This hypothesis is supported by several facts observed during the ischemia-reperfusion process. Firstly, hypothermia decreases the global cerebral metabolic rate during ischemia, slowing the breakdown of glucose, phosphocreatine and ATP and the formation of lactate and inorganic phosphate (227). Secondly, hypothermia reduces the production of ROS during reperfusion in brain (228), forebrain (229), heart (230), gut (231), endothelium (232) and muscle (233). Thirdly, hypothermia improves recovery of energetic parameters during reperfusion (227). Fourthly, hypothermia also suppresses the inflammatory response after hepatic ischemia-reperfusion, decreasing the infiltration of polymorphonuclears (234), and the production of tumor necrosis factor-alpha, interleukin-1 beta and macrophage inflammatory protein-2 (234; 235).

In these experiments, consistent with our hypoxia hypothesis, we also extended our previous observations at RT demonstrating that at 37°C the addition of 3% of oxygen to the

pneumoperitoneum decreases adhesion formation (69; 70) and that the addition of 12% of oxygen has not effect in reducing adhesions (Figure 7) (74).

All the experiments related with temperature were done at the same insufflation pressure, i.e. 15 mm of Hg. The effect of different insufflation pressures at low temperature upon adhesions has not been addressed yet. Since insufflation pressure increases adhesions, we can estipulate that the use of a lower pressure combined with low temperature would give more reduction of adhesions. Experiments have to be done to confirm this hypothesis.

3.1.4.3 Desiccation increases adhesion formation

Although adhesion formation has been associated with desiccation (236), to the best of our knowledge, this is the first time that the association has been proved. We demonstrated in an *in vivo* model, that pneumoperitoneum-induced desiccation increases adhesions (Figure 10). Using non-humidified gas at a flow through the abdominal cavity of 492 ml/min, more adhesions were developed than at a flow of 23 ml/min, and that was prevented by using humidified insufflation. However, in this experiment, since desiccation and cooling are intimately associated, the higher the flow with non-humidified gas, the more adhesions mice developed but also the more cooling occurred. For this reason, next experiment was designed to study the effect of desiccation without any associated cooling (Figure 11). In this experiment a homeothermic blanket was used to keep mice at 37°C. We demonstrated that using non-humidified insufflation gas and without any cooling, adhesion formation increases with the flow rate through the abdominal cavity this means with desiccation. These desiccation-induced adhesions can be prevented by using humidified gas or by lowering body temperature (no homeothermic blanket used). This can be explained since less desiccation occurs at lower temperatures.

The increase in adhesion formation due to desiccation can also be explained by the alteration of mesothelium morphology produced by the dry gas, i.e. destruction of microvilli, bulging up of cells, and exposure of the basal lamina (63-65; 220).

Studying the effect of desiccation upon body temperature and upon adhesion formation, a new humidifier which produces different levels of humidification was used for our experiments (158). Our hypothesis was that the over-saturation of the insufflation gas would prevent adhesions or, the same, the higher the humidification level of the insufflation gas, the less adhesion mice would develop. However, the results were not the expected (Figure 9). Adhesion formation in group with 0%BTS (important desiccation) was higher than in groups with 75 and 100% BTS, but not different from group of 125%BTS (over saturated

insufflation gas). Surprising, in group with 100%BTS adhesions were less than in group with 125%BTS and in group with 75%BTS adhesion formation was not different from group with 100% BTS but lower than group with 125%BTS. To interpret these data, the opposing effects of desiccation and hypothermia should be considered, knowing that both are intimately linked and that although the former increases adhesion formation, the latter reduces adhesion formation. Pneumoperitoneum and body temperatures have to be also considered to understand these results (Figure 4). Because adhesion formation was much higher in the 0% BTS group, the effect of desiccation on adhesion formation was clearly confirmed. Because in this group body temperature was much lower, the adhesiogenic effect of desiccation must be clearly underestimated. Adhesions were slightly lower in the 75% BTS group than in the 100% BTS group, which can only be interpreted by the slightly lower temperature, as some evaporation must have occurred, considering the 100% RH in the peritoneal cavity. In the 75% BTS group, the effect of temperature is underestimated, as without desiccation adhesions would even have been less. Adhesions were slightly higher in the 125% BTS group than in the 100% BTS group, which can only be explained by the slightly higher temperature, as desiccation can be ruled out. In this experiment we confirmed not only the effect of desiccation upon adhesion formation but also the effect of temperature upon adhesions since the gas in the 125%BTS group (high enthalpy or energy) by condensation increases intraabdominal temperature and, thus, increase adhesion formation. In this experiment, body and pneumoperitoneum temperatures were measured. We would have liked to measure directly peritoneal temperature but it was technically no possible due to the thickness of the mouse peritoneal layer, therefore, pneumoperitoneum temperature was considered closer to peritoneal temperature than body temperature.

Consistent with the hypothesis that pneumoperitoneum induces desiccation, it can be deducted from these experiments that the peritoneal cavity has a high humidifying capacity. All groups in which non-humidified gas (0% RH) was used the RH of the pneumoperitoneum were 100% (Section 2.1.4, Experiment 2) and 80.8% ± 4.2 % (Section 2.1.2, Experiment 4), meaning that water content from the serous fluid was continuously being evaporated to humidify the pneumoperitoneum. We can hypothesised that a water loss from the peritoneum of 1 and 4.4 mg water/min for groups with a flow of 23 and 100 mL/min, respectively, and theoretically, no water loss for the groups with no flow through the abdominal cavity or with humidified gas occur. This high humidifying capacity of the peritoneum was already shown in open surgery in humans; that is, when bowels are exteriorized, the water loss by evaporation

is approximately 32 g/h and this causes their surface temperature to decrease by 3°–5°C (219).

All the experiments related with desiccation were done at the same insufflation pressure, i.e. 15 mm of Hg. The effect of different insufflation pressures together with different levels of desiccation upon adhesions has not been addressed yet. Since both insufflation pressure and desiccation increase adhesions, we can estipulate that the use of a lower desiccation combined with low insufflation pressure would give less adhesions. The best condition would be the use of a humidified gas and low insufflation pressure. High desiccation levels produce a very important trauma in the peritoneum; therefore, we can also estipulate that the effect of the pressure can be masked by the desiccation. Of course, experiments have to be done to confirm this hypothesis.

In clinical trials, the effect of different gas conditions was studied but the results remain controversial. The use of warm and humidified gas was claimed to reduce the postoperative pain (237-240) and the recovery time after the surgery (237) in comparison with cold and dry gas. However, these results are still controversial (240-244). Comparing with cold and dry gas, the use of warm and dry gas produces more intensive shoulder and subcostal pains (245) but without differences in the duration of hospitalization (245; 246). This might be explained by the higher desiccation produced by a warmer gas. The only article analysing dry and warm, dry and cold, humidified and warm and humidified and cold did not find any differences for the body temperature, intraabdominal humidity, postoperative pain, recovery time and length of hospitalization (244). This trial, however, had a small sample size and the patients were very obese (BMI= 50).

3.1.4.4 Adhesion formation depends on the genetic background

Strain differences have been reported for other processes involving fibrosis and healing responses such as hepatic, lung, and colorectal fibrosis (247-250), myocardial, ear (251-253) and skin (254) wound healing, and bone regeneration (255). To the best of our knowledge this is the first study indicating that genetic background also influences adhesion formation, at least after laparoscopic surgery. Among the strains evaluated we found that Swiss, NMRI, and BALB/c mice developed more adhesions compared to FVB and C57BL/6J mice, in which adhesion formation was minimal (Figure 1). About the mechanisms causing these inter-strain differences, at present, we only can speculate. For none of the potential mechanisms, such as cellular interaction (e.g., macrophages, fibroblasts, mesothelial and

endothelial cells) or molecular expression (e.g., plasminogen system, vascular endothelial growth factor, hypoxia-inducible factors, reactive oxygen species, matrix metalloproteases), modulating the processes of inflammation, fibrin deposition/degradation, extracellular matrix deposition/degradation, and angiogenesis (256-258), clear data about strain differences are available.

Our data also demonstrate that interanimal variability is less in the inbred BALB/c mice than in the outbred Swiss and NMRI mice. This is not surprising because inbred strains, maintained by sibling (brother × sister) mating for 20 or more generations, are genetically almost identical, homozygous at virtually all loci, and with high phenotypic uniformity (259). This less interanimal variability in inbred strains has been reported for many processes such as sleeping time under anesthesia (260; 261). The high variability in the inbred FVB and C57BL/6J mice, with very low adhesion formation potential, is also not surprising because the absence of adhesions in many of these animals leads to artificially high coefficient of variation.

These observations on genetic influences contribute to the usefulness of the mouse model for adhesion formation studies. As explained at the beginning of the discussion, the mouse model has many advantages compared to other animal models because it is relatively cheap, easy to handle, and does not require strict sterile conditions for surgery. Furthermore, it is particularly useful for mechanistic studies because of the availability of animals with low genetic variability (i.e., inbred mice), underexpressing/overexpressing specific genes (i.e., transgenic mice), and immunodeficient by spontaneous mutation (i.e., nude mice [T-cell deficient] and SCID mice [T&B cell deficient]). In addition, many specific mouse assays and monoclonal antibodies are available.

Both observations (i.e., strain differences in adhesion formation potential and in interanimal variability) point to genetics effects on adhesion formation, which is not surprising and confirms clinical observations. The importance of these observations is twofold. First, to study the genetic involvement in detail, the use of two strains with high and low adhesion formation potential can be considered as an important experiment. In addition, mating the strain with low and high adhesions and checking the adhesion formation of the offspring may be useful to study the segregation of the genes involved in the adhesions process. Second, to study adhesion formation and its prevention, it is preferable to use a strain with high adhesion formation potential and low interanimal variability, such as BALB/c mice. Furthermore, fewer inbred animals will be needed to achieve a given level of statistical precision than if outbred animals had been used (259). For these reasons after these results,

we decided to change the mouse strain from NMRI (high adhesion and variability) to BALB/c (high adhesion and less variability).

3.2 Adhesion prevention

One of the aims of this thesis was to screen in our laparoscopic mouse models all the products which have been described to affect adhesion formation in order to compare their relative effectiveness and investigate their mechanisms of action. The products chosen were acting in different pathways of the adhesion formation process and they were, first, tested in the hypoxia model, secondly, in the hyperoxia model and, afterwards, in the normoxia model. Once the pathophysiology was understood, combine treatments were tried in order to evaluate if the effects upon adhesion formation were synergistic or additive or not. Finally these data will be used to establish which combination of treatments gave a maximum reduction of adhesions.

In our screening experiments, a 60 min of pneumoperitoneum was induced at 15 mm Hg of insufflation pressure. Pure CO_2 or CO_2 with the addition of 3% or 12% oxygen was used depending on the model analyzed, i.e. hypoxia, normoxia or hyperoxia models, respectively. The insufflation gas was humidified and heated at 37°C in order to avoid desiccation, an extra trauma for the abdominal cavity as demonstrated in this thesis. Although fewer adhesions are produced at 32°C BT (hypothermia), we decided to keep BT at 37°C (normothermia) since we aim to see differences in between treatments. Therefore, a model with higher proportion of adhesions was chosen for the screening experiments in the hypoxia and hyperoxia models. For the combination of treatments, the normoxia model was chosen in combination with the low temperature in order to get a maximum reduction of adhesions.

3.2.1 Prevention in the hypoxic model

Our first screening of drugs was done in the hypoxia model. Some of the mechanisms affecting the adhesion formation process were analysed in this set-up.

As explained in detail in the introduction, the pathogenesis of the adhesions involves several mechanisms. The first process of the pathophysiology analysed was the ischemia-reperfusion. During pneumoperitoneum, there is ischemia at the time of insufflation, and reperfusion at the time of deflation, thus ROS can be produced. Therefore, our hypothesis was that ROS scavengers, molecules which remove ROS, i.e., superoxide dismutase (SOD), melatonin, catalase and ascorbic acid would have an effect on reducing adhesion formation. In

our laparoscopic mouse model we confirmed that ROS scavengers can decrease adhesion formation although the overall effect was small. In the first experiment [Figure 12; (159)], we decided to try one dose of these scavengers and to administrate 5 min before the pneumoperitoneum was ended, this means before the reperfusion starts. Since no effect of melatonin and catalase and a border line effect of SOD and ascorbic acid were observed (significative only when two experiments were grouped), three doses of SOD and ascorbic acid were tested in a second experiment and, in addition, oral administration of ascorbic acid was also given. A border line effect was again observed for these two compounds. That not all products decreased significantly adhesions is not surprising given the small numbers of animals in each group. Increasing the numbers of animals and repeating the experiments would not have changed the message that ROS scavengers can be effective but that the effect is small. It is unlikely but it cannot be excluded that i.p. administration as we used is less effective than i.v. administration. Possibly results differ between mice and rats. In rats the same dose of SOD and catalase, i.e., 15.000 U/kg i.v. decreased intraperitoneal adhesions (148) and in rats also for melatonin, the same i.p. dose of 10 mg/kg body weight was effective (153). Most important, however, is that effectiveness is more pronounced after open surgery since exposure to air with around 21% of oxygen, i.e. a partial pressure of 160 mm Hg, might generate much more ROS, than the ischemia-reperfusion process after laparoscopy. In conclusion, we confirmed in our laparoscopic mouse model that ROS scavengers can reduce adhesion formation but that the effect is small.

During CO_2 pneumoperitoneum, since mesothelial cells do not receive sufficient oxygen supply from the capillaries and, since the CO_2 will diffuse to the mesothelial cells, mesothelial hypoxia can be postulated. Therefore, the second mechanism investigated was the hypoxia. To confirm the role of HIF up-regulation as a mechanism of pneumoperitoneum-enhanced adhesion formation (71), next group of drugs analyzed were HIF inhibitors [Figure 13, (160)]. HIF was blocked through the inhibition of the Hsp-90 (using 17-AAG and radicicol) or of the PI3K signaling pathways (using wortmannin and rapamycin). Comparing with the vehicle-treated control group, wortmannin reduced pneumoperitoneum-enhanced adhesions (54% of inhibition comparing with the control group, Table 1). Surprising, the comparison of wortmannin with the untreated control group was not significant, although there were not differences between both controls groups. This may be explained by the higher SE obtained in this control group. If wortmannin decreases adhesion formation through HIF inhibition, it might be surprising that 17-AAG, rapamycin and radicicol did not. Firstly, these were screening experiments and it cannot be excluded that different doses or way of

administration could become effective. Specifically, radicicol is known to be very unstable (262) and one injection could be insufficient. Secondly, the variability of adhesions formation in this experiment was surprising high, possible related to the use of DMSO as solubilising agent. Thirdly, since 14-AAG and radicicol act through inhibition of Hsp-90 whereas wortmannin and rapamycin through inhibition of the PI3K pathway, the later pathway could be more effective for adhesion reduction. Finally, the PI3K pathway is not only effective in HIF inhibition but also it has other effects. PI3K pathway is involved in cell survival and proliferation and in many aspects of angiogenesis (263) and fibrinolysis, such as VEGF (264), uPA (265) and PAI-1 (266) up-regulations. Each one of these factors is involved in the adhesion formation (41; 72). PI3K signalling is also involved in the inflammatory response since blocking its activity reduces neutrophil influx by diminishing their attachment and migration (267) and reduces the adherence and spreading of macrophages (268). The inhibition of PI3K/Akt contributes to Hsp synthesis in addition to attenuating HIF-1α translation (269). Specifically, wortmannin inhibits the superoxide release by the polymorphonuclear leukocytes (PMNs) (270) and during myocardial ischemia reperfusion injury, it can attenuate PMN infiltration into the myocardium and suppress of superoxide release by PMNs (271). Therefore, a beneficial effect of wortmannin in reducing the toxic effect of ROS produced during ischemia/reperfusion process can also be postulated. In conclusion, the effect of wortmannin could be considered as supporting the hypothesis that HIF is up-regulated during pneumoperitoneum-enhanced adhesions. The absent of effect of the other products do not refute the hypothesis explained. Moreover, it cannot be excluded that wortmannin might be effective through many other mechanisms. To answer this, a detailed experiment should be done.

The peritoneal injury initiates an inflammatory reaction. Therefore, the third mechanism investigated was the inflammation process. Anti-inflammatory agents, corticosteroid (dexamethasone), non-steroideal anti-inflammatory drugs (NSAIDs) COX-2 selectives (parecoxib, nimesulide) and COX-2 non-selectives (tenoxicam, ibuprofen) and neutralising antibodies against TNF-alpha were tried. In addition, diltiazem, a calcium channel blocker, was also used since it can work in the inflammation process and also in other pathways [Figure 12, (159)]. Anti-inflammatory drugs are widely accepted to reduce adhesion formation in mice (26; 161), in rats (20; 21; 23-25; 27-29; 32) and in rabbits (22; 30; 31; 33-35) during open surgery. However, these effects were not always consistent in open surgery models, i.e. ibuprofen failed to reduce adhesion formation in rats (23). Anti-TNF alpha antibodies also failed to reduce adhesions in a rat cecal serosal abrasion model (272). In our

laparoscopic mouse model we confirmed the effectiveness of dexamethasone (32% of inhibition comparing with the control group, Table 1) but we failed to demonstrate any important effects of NSAIDs as COX-1 and COX-2 inhibitors and of neutralizing anti-TNF alpha antibodies. Furthermore, two new generation anti-inflammatories, resolvin and lipoxin, were also tried. Although, resolving and lipoxin are associated with several other biological functions including leukocyte activation, chemotaxis promotion, monocyte migration and adhesion and protection during ischemia-reperfusion injury (273; 274), they did not show any significant effect.

Taken all these observations together, these data suggest that the inflammatory reaction may be less important for adhesion formation after laparoscopy, at least in our model. The absence of effect of anti-TNF alpha antibodies, which it is known to have strong effects on inflammation, supports this suggestion. That dexamethasone, nevertheless, had some effect on adhesion prevention suggests other mechanisms to be involved. Glucocorticoids, indeed, can inhibit fibroblast proliferation and can have immunosuppressive effects affecting production and release of cytokines (275). Another drug tried with the group of anti-inflammatories was the diltiazem, a calcium channel blocker. Diltiazem reduced adhesion formation in our laparoscopic model (36% of inhibition comparing with the control group, Table 1) confirming results in open surgery (139-142). This effect can be explained not only by its effect on the inflammatory response (276), but also in the protection against the toxic effect of the ischemic-reperfusion cell injury (277) and activation of other cellular processes (278).

The inflammatory reaction generates a fibrinous exudate and formation of fibrin. The balance between fibrin deposition and degradation is critical for normal peritoneal healing or adhesion formation. Therefore, another mechanism of the adhesion process evaluated in our hypoxia model was the fibrinolysis (Figure 14; Addendum 10). In this study we tested different doses of Reteplase, and neutralizing antibodies against PAI-1. Although tPA and other fibrinolytic agents have been tried in several animal models (36), this is the first time that Reteplase has been tested. After analyzing the bibliography, we decided to use the doses 0.125, 0.25, 0.5 and 1 mg; however, these doses showed an increase in adhesion formation. This increase in adhesion formation might be explained by the “plasminogen steal” induced by a high concentration of r-PA (279), this means that a high concentration of PA is associated with a depletion of plasminogen and, therefore, no plasmin will be formed and no fibrin degradation will occur. Consistent with that, an *in vitro* model showed that the speed of clot lysis increases with increasing t-PA concentrations up to 2 µg/ml and it decreases above 2

µg/ml, giving a bell-shaped curve of fibrinolysis (280). We, therefore, decided to reduce the doses of Reteplase, resulting in that 1 µg showed a reduction of 40% in adhesion formation (Figure 14). Surprising, non effect of PAI-1 antibodies was observed.

Flotation agents and barriers, the most well known substances to reduce adhesions, were also tested during these studies [Figure 13, (160)]. In this experiment, hyalobarrier gel was the most effective in decreasing adhesions (90% of inhibition comparing with its control group, Table 1). Moreover, 50% of mice (4 of 8 mice) did not develop any adhesion in the Hyalobarrier gel-treated group. It should be emphasized that this is exceptional and that the incidence of adhesion formation in the other groups (control and non-control groups) was 100%. These results are consistent with previous observations in laparoscopic (106) and in open surgery models in rabbits (107; 108) and rats (109). Hyalobarrier gel was also proven to be effective in clinical trials, i.e. in laparoscopic myomectomy (110; 111) and in hysteroscopic surgery (112; 113). The ability of this barrier in preventing adhesion formation may be explained, not only for being a barrier, but also by its inflammatory modulating activity, e.g. by induction of interleukin (IL)-1(281; 282), IL-8 (283), IL-12 (284) and tumor necrosis factor alpha (282) productions. Hyaluronic Acid (HA) also improved wound healing (285). On the other hand, HA can act as a ROS scavenger (286; 287). It was recently demonstrated that HA increases the proliferation rate of human peritoneal mesothelial cells (288) and increases the fibrinolytic response (289).

Although some decrease in adhesion formation was observed with CMC 2% in our laparoscopic model, the differences were not statistically significant (Figure 13, (160)). CMC 2% was shown to reduce intra-abdominal adhesions in rats (290-292) and rabbits (293) but the reports were not consistent, i.e. no effect has been seen in rats (122) and rabbits (118). In conclusion, CMC probably has some effectiveness but the effect is small when used as a single product. We failed to demonstrate effectiveness of Hyskon in our model. Hyskon was shown to decrease adhesions in a rabbit model (116; 117) whereas in other reports no effect upon adhesion formation was observed in rabbits (118-120), hamsters (121) and rats (122). Injection of 0.5 ml Hyskon in the mouse abdominal cavity (25 ml/kg) was, moreover, associated with a mortality of 20%. This was also observed in rats in which 20 ml/kg produced 75% of mortality (292). SprayGel was effective in our model (58% of inhibition comparing to the control group, Table 1) which is consistent with previous observations during open surgery in rats, rabbits (101) and pigs (102) and in humans after a laparoscopic ovarian surgery (103) and laparoscopic and open myomectomy (105). Phospholipids were effective on adhesion prevention in our laparoscopic mouse model (35% of inhibition

comparing to the control group, Table 1), as previously demonstrated during open surgery in rabbits (175; 294). They were, however, not effective in an open mouse model (295). This is the first time that phospholipids are tried during laparoscopic surgery. The lipid more predominant of the peritoneal cavity is the phosphatidylcholin (296) and the composition of the phospholipids solution used in this experiment was phosphatidylcholin 70% by weight, phosphatidylethanolamin, 15% by weight, neutral lipids 8% by weight, sphingomyelin < 3% by weight and lysophosphatidylcholin < 3% by weight (297), therefore, this solution may be helping to prevent adhesions by increasing the local physiologic phospholipids. The ability of the phospholipids in preventing adhesion formation can be explained by its induction of lubricity, antiwear and release or antistick properties (133).

If fibrin is not completely degraded, it will serve as a scaffold for fibroblasts and capillary ingrowth (38). Antiangiogenic compounds as antiPlGF and antiVEGFR1 antibodies were already tested in our laparoscopic mouse model but in another mouse strain, Swiss. Anti-PlGF antibodies gave a 77% of inhibition of adhesions comparing with the control (proportions of adhesions: ctrol vs treated group: 43% vs 10%, respectively) (41). Anti-VEGFR1 gave a 60% of inhibition of adhesions comparing to the control (proportions of adhesions: ctrol vs treated group: 40% vs 17%, respectively) (42).

In summary, we confirmed in our laparoscopic mouse model the effects of ROS scavengers as SOD and ascobic acid, a calcium channel blocker as diltiem, an anti-inflammatory as dexamethasone, a HIF inhibitor as wortmannin, a fibrinolytic agent as Reteplase and barriers as Hyalobarrier gel, Spraygel and phospholipids. The absence of effect of the other anti-inflammatory drugs and of anti TNF alpha antibodies and also of the other ROS scavengers are surprising. It cannot be excluded that different doses or way of administration could become effective.

3.2.2 Prevention in the hyperoxia model

After doing the screening in the hypoxia model, our aim was to study the pathophysiology of the adhesion formation also in the hyperoxia model. We decided to investigate the effect of low temperature upon hyperoxia-enhanced adhesion and also to screen some of the products representative of each mechanism and to check their relative effectiveness in this model.

Hyperoxia-induced adhesions were reduced by around 48% using low temperature (Figure 15, Table 1). This percentage of inhibition is comparable to the effect that low

temperature has in the hypoxia model (51% of inhibition, Table 1), meaning that low temperature also prevents the toxic effects of the hyperoxia. As explained above, ROS might be involved in pneumoperitoneum-enhanced adhesions because ROS can be generated after the reperfusion of an ischemic tissue (205) and this is valid for both the hypoxia and the hyperoxia model. Consistent with this, the generation of ROS after laparoscopic surgery is well reported (146). Laparoscopic surgery increases ROS availability by increasing ROS production (146) or by decreasing ROS scavengers (57; 147). In addition, with 12% oxygen, mesothelial cells are in a hyperoxic environment that could lead to increased ROS production or decreased ROS scavenger production in comparison with a hypoxic or normoxic environment. As explained above, hypothermia can be preventing adhesions since it can reduce the production of ROS during reperfusion in different organs (228-233), it can improve recovery of energetic parameters during reperfusion (227) and it can suppress the inflammatory response after ischaemia–reperfusion (234; 235). Therefore hypothermia can be protecting the toxic effect of the ischemia reperfusion also in this model.

Dexamethasone showed a stronger inhibition in the hyperoxia model in comparison with the hypoxia model (62% vs 32% of inhibition, respectively). It has been demonstrated that the administration of dexamethasone during hyperoxia conditions accelerated the maturation of antioxidant enzyme system in fetal lungs, showing an elevated superoxide dismutase, catalase, and glutathione peroxidase activities (298; 299). We can estipulate that the dexamethasone is, may be, also increasing the antioxidant system in the peritoneum during hyperoxia but not during hypoxia, but it has to be confirmed with further experiments. Dexamethasone also can reduce the hyperoxia-induced IL-8 release and mRNA synthesis by human alveolar macrophages (300). Therefore, it may be possible that the high oxygen tension increases the inflammatory response by increasing cytokines production in the hyperoxia model but not in the hypoxic model, and the dexamethasone acts inhibiting both pathways the inflammation/fibroblast proliferation, in the hypoxic and hyperoxic model, and the production of cytokines, in the hyperoxic model. More experiments have to be done to confirm these expeculations.

Another anti-inflammatory, nimesulide, was tried showing non significant effect. The calcium channel blocker diltiazem showed around the same percentage of inhibition, i.e. 36% (Table 1) in the hyperoxia (Figure 15) than in the hypoxia model (Figure 12), demonstrating that the mechanisms in which the calcium channel blocker acts, i.e. interference with the inflammatory response (276) or protection against the toxic effect of the ischemic-reperfusion cell injury (277) are involved in both models. Although we were expecting a significant effect

of ROS scavengers, SOD and ascorbic acid did not show it. Ascorbic acid showed a border line effect after grouping all the control groups and doing an inter-experiment comparison, demonstrating that the sample size may be too small to detect small differences. Although non significant, a lower effect of SOD was shown in the hyperoxia in comparison to the hypoxia model (8% vs 36%, respectively, Table 1). This may be explained since the hyperoxic environment can induce more ROS production and, may be, the dose, that was the same in both models, was not enough to produce a bigger inhibition.

Reteplase shows a border line effect (only significant when inter-experiments comparisons were done) in the hyperoxia model, a lower inhibition comparing with the hypoxia model (17% vs 40%, respectively, Table 1). In cell culture, it was demonstrated that the fibrinolytic activity was regulated by oxygen tension, i.e uPA and PAI levels were both at a minimum in hypoxic conditions, uPA release peaked at a normoxic media pO_2 whereas PAI release was highest at a hyperoxic pO_2 (301). If we applied that to our model, since PAI inhibits PA and PAI release was higher in hyperoxic conditions, less fibrinolysis would be expected in the hyperoxic conditions in comparison with the hypoxic conditions. Although these are again expeculations, this may explain the lower effect of the Reteplase in the hyperoxia model.

As expected, phospholipids and hyalobarrier gel showed the same percentage of inhibition in the hypoxic and hyperoxic models, i.e. 36% and around 90% (Table 1), since the effect of both compounds are mechanics, lubrication of the peritoneum and separation of 2 damaged layers.

In conclusion, in the hyperoxic model adhesion formation was decreased by using low temperature, dexamethasone, a calcium channel blocker and barriers, as phospholipids and hyalobarrier gel. A border line effect of ascorbic acid and Reteplase was observed. Since dexamethasone and Reteplase showed different percentages of inhibition in the hypoxia model in comparison with the hyperoxia model, we can postulate that these two mechanisms, the inflammatory/immune pathways/fibroblasts proliferation and the fibrinolysis, are involved but, in addition, other pathways are activated. Possible explanations could be that higher oxygen concentration is activating PAI-1 production or is inducing the Reteplase steal resulting in a lower fibrinolytic effect. The higher oxygen concentration might be also enhancing the anti-inflammatory effect of the dexamethasone. These are questions that must be confirmed in future experiments.

3.2.3 Combination of treatments

As previously demonstrated, hypothermia and the addition of 3% oxygen to the pneumoperitoneum, each separately reduced adhesion formation in the hypoxia model (74; 157). Comparing to pure CO_2 pneumoperitoneum and normothermia, there is a 48% inhibition in adhesion formation by lowing down body temperature to 32°C (Figure 16, Table 1) and a 32% of inhibition by adding 3% oxygen (Figure 16, Table 1). Taking into account mice in which pneumoperitoneum + 3% oxygen was used, the decrease in body temperature from 37°C to 32°C reduce a bit adhesion formation (normothermia vs hypothermia: percentage of the control group: 68% vs 52% in Figure 16), although that was not significant. Comparing to pure CO_2 pneumoperitoneum and normothermia, the application of both treatments hypothermia and addition of 3% oxygen to the pneumoperitoneum reduced in a 48% adhesion formation (Figure 16, Table 1). Comparing to pure CO_2 pneumoperitoneum and hypothermia, no additive effects were found when applying both treatments hypothermia and 3% oxygen to the pneumoperitoneum since both groups gave around 50% of inhibition. Since the effect of the treatments hypothermia and adding 3% oxygen to the pneumoperitoneum are not additive, we can say that both are acting in the same pathway. This means that the addition of 3% oxygen to the pneumoperitoneum, as well the hypothermia, are both preventing the toxic effect of the hypoxia. As explained in the section 3.1.4.1 of the discussion, our hypothesis is that the addition of 3% oxygen to the pneumoperitoneum would make mesothelial cells to feel “normoxic” since the fact that 3% oxygen is added at 775 mm Hg results in a pO_2 of around 23 mm Hg, which is similar to normal intracellular pO_2. Hypothermia would be also protecting tissues and cells from the toxic effects of hypoxia. As explained in section 3.1.4.2 of this discussion, hypothermia would be reducing the production of ROS during reperfusion and improving the recovery of energetic parameters during reperfusion (234; 235). These are all hypothesis and they have to be confirmed in specific experiments.

Comparing to the hypoxia model and normothermic control group, the administration of dexamethasone produces an inhibition of 32% (Figure 12, Table 1). When dexamethasone is combined with low body temperature and normoxia, the inhibition increased to 76% comparing to the hypoxia model and normothermic control group (Figure 16, Table 1), and around 54% comparing to the normoxia model and hypothermic control group (Figure 16, Table 1). Therefore, we can conclude that dexamethasone has additive effect together with low temperature and adding 3% oxygen to the pneumoperitoneum.

Nimesulide gave an inhibition of 22% in the hypoxia model (Figure 12, Table 1) although not significant. When low temperature and 3% oxygen were combined with nimesulide, no additive effects are shown since the inhibition is comparable to the normoxia model and low temperature control group: around 50% inhibition (Figure 16, Table 1). Diltiazem, a calcium channel blocker, gave a 36% of inhibition of adhesions in the hypoxia model (Figure 12, Table 1). When low temperature and 3%oxygen were added to the Diltiazem a 58% of inhibition was observed showing a higher reduction in comparison with the control low temperature and 3% oxygen (48% of inhibition) (Figure 16, Table 1). Same observations were obtained with the application of phospholipids (Figure 13, Table 1). This means that there is an additive effect of the diltiazem and of the phospholipids to the low temperature and the addition of 3% oxygen. The function of the phospholipids is to lubricate the peritoneum and it is not surprising that has an additive effect to the low temperature/normoxia since these are two different pathways. Same explanation for diltiazem that acts in the inflammatory response, protects against the toxic effect of the ischemia-reperfusion injury and activates cellular pathways.

Hyalobarrier gel together with hypothermia and adding 3% oxygen showed approximately the same adhesion reduction as in the hypoxia model: around 90% of inhibition (Figures 13 and 16, Table 1) indicating that the effect of the barrier separating 2 surfaces was more important than the effect of the hypothermia and addition 3% oxygen and, of course, there is not additives effects between the application of the barrier and the low temperature.

SOD in the hypoxia model and normothermia showed a 36% of inhibition although significant when sample size increased showing a border line effect (Figure 12, Table 1). When 3% oxygen and low body temperature were combined with SOD, a 38% of inhibition was obtained, showing no additive effect in comparison with the normoxia model and low body temperature control group: 48% inhibition (Figure 16, Table 1). Ascorbic acid (AA) in the hypoxia model and normothermia showed a 20% of inhibition although significant when sample size increased showing also a border line effect (Figure 12, Table 1). Unexpectively, when 3% oxygen and hypothermia were added to the AA, an increase of adhesion formation was observed (only 14% of inhibition vs 48% of inhibition in the normoxia model and low body temperature control group) may be possibly due to an irritative effect (Figure 16, Table 1).

Reteplase in the hypoxia model and normothermia showed a 40% of inhibition (Figure 12, Table 1). When 3% oxygen and hypothermia were added, a 42% of inhibition was

obtained, showing no additive effect to the normoxia model and low body temperature control group (48% inhibition) (Figure 16, Table 1).

Although the side effects of the low temperature are well known, induced hypothermia is one of the most promising neuroprotective therapies (302). In addition, combined therapies with mild hypothermia (33°C) were demonstrated to be efficient during cerebral ischemia for neuroprotection in cerebrovascular surgery (303). Moreover, hypothermia-cerebroprotection effect can be improved by additional pharmacotherapy in rats subjected to ischemia reperfusion by a transient middle cerebral artery occlusion (304). Application of therapeutic hypothermia (32-34°C for 12-24 h) after cardiac arrest could help to improve the neurological recovery (305). In adult patients, hypothermia improves neurological outcome in survivors of cardiac arrest (306; 307) and its use after cardiac arrest is recommended by the International Liaison Committee on Resuscitation (ILCOR) (308). In infants with hypoxic-ischemic encephalopathy, hypothermia of 33.5°C for 72 hours was safe, reduced fatality, and improved neurodevelopmental outcome (309). These examples are different from our model, however, they both have the common pathway of the ischemia-reperfusion and the protection of its toxic effects. Since hypothermia was induced for longer time and in the whole body, those would be extreme examples showing that hypothermia can also be used in humans. We propose to apply hypothermia locally in the abdominal cavity and only during the surgery, therefore, the exposition to the low temperature will be also shorter and local (not the whole body) and, of course, less traumatic than in the cases of neuroprotective therapies. A recent article of Ozgonul et al (310) will be supporting our theory of using low temperature locally. In this study hypothermic CO_2 (21°C) used for pneumoperitoneum was compared with isothermic gas (37°C) during laparoscopic cholecystectomy in a prospective randomized study (n=62). Measurements were done before insufflation, at 30 minutes of pneumoperitoneum, and 30 minutes after desufflation. No significant difference was observed in core body temperature and blood arterial pH, arterial carbon dioxide pressure, arterial oxygen pressure, and bicarbonate values, only the mean skin body temperature was significantly higher in the isothermic group than the hypothermic group. They concluded that warm insufflated CO_2 does not affect blood gases.

In summary, we can conclude from these experiments that using a *less traumatic pneumoperitoneum*, this means using humidified CO_2 with the addition of 3% oxygen, reducing body temperature to 32°C and with the application of, first, an anti-inflammatory as dexamethasone or a calcium channel blocker as diltiazem during and after the surgery, and, second, applying a barrier as hyalobarrier gel or a surfactant as phospholipids after the

surgery, adhesions can be reduced in between 48% (only low temp+3%O_2+humidified gas) and 90% [76% (low temp+3% O_2+humidified gas+ dexa), 58% (low temp+3% O_2+humidified gas+ diltiazem or phospholipids), around 90% (low temp+3%oxygen+humidified gas+ hyalobarrier gel)]. Of course, new therapies have to be tested in combination with low temperature, no desiccation and 3%O_2 pneumoperitoneum.

3.3 Pathophysiology of adhesion formation

Several mechanisms are involved in the pathogenesis of adhesions. During surgery the peritoneum suffers a trauma. Generally, trauma may include exposure to infection or to intestinal contents, ischemia, irritation from foreign materials such as sutures, gauze particles, or glove dusting powder, abrasion, overheating by lamps or irrigation fluid and many others (311).

Although laparoscopy induces less direct trauma than laparotomy, we postulate that the pneumoperitoneum could also be detrimental. We previously demonstrated that CO_2 pneumoperitoneum increases adhesion in time- and pressure-dependent (69). Those observations suggest that mesothelial hypoxia could be the driving mechanism. Consistent with this hypothesis, similar effects in adhesion formation were observed with helium and CO_2 pneumoperitoneum and the addition of 2-4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation (69; 70). Moreover, this effect was absent in mice deficient for genes encoding for factors up regulated by hypoxia, such as HIF (71), PAI-1 (72), VEGF or PlGF (41). We demonstrate during this thesis that HIF inhibitors as wortmannin reduced pneumoperitoneum-enhanced adhesion (160). Moreover, it is well know that during surgery, there is an oxidative stress and ROS are produced (57; 146; 147) adding an additional trauma. In addition to the surgical trauma, ROS can also be produced during the ischemia and reperfusion process that occurs during insufflation and deflation (205). Consistent with that, it has been demonstrated during laparotomy that ROS scavengers reduced adhesion formation (148-154). SOD and ascorbic acid, two ROS scavengers, have been shown to reduce adhesion formation during this thesis (159) and, to the best of our knowledge, for the first time during laparoscopy.

During pneumoperitoneum mesothelial cells are also in contact with dry CO_2. When CO_2 is not humidified, water will be evaporated from the tissue to the insufflation gas and desiccation will occur. We demonstrate during this thesis that desiccation increases adhesion formation and this can be reduced by using humidified gas (158). In order to evaporate water

to humidify the dry insufflation gas, body will loose energy causing a reduction in body temperature or hypothermia and, moreover, we demonstrated that hypothermia reduces adhesions (157). Furthermore, we demonstrate that the trauma produced by the pure CO_2 pneumoperitoneum can be reduced by adding 3% of oxygen to pneumoperitoneum, by using humidified gas and by reducing body temperature; combining all these treatments the final effect is a reduction in adhesion formation of around 50%.

The peritoneal injury that occurs during the surgery initiates an inflammatory reaction: polymorphonuclears, macrophages and fibroblasts cells migrate, proliferate and/or differentiate. This inflammatory reaction can be reduced by using anti-inflammatory drugs as corticoids and NSAIDs preventing adhesion formation as previously demonstrated in many animal studies during laparotomy (20-35) and confirmed for dexamethasone during laparoscopy during this thesis (159). Calcium channel blocker can also be used to reduce the inflammation process reducing adhesions as previously demonstrated (139-142) and confirmed during this thesis for diltiem (159).

After inflammation, the healing attempt begins with the formation, through coagulation, of a fibrin gel matrix. Fibrin can be reduced by using fibrinolitic agents as plasmin, streptokinase, uPA, and tPA (36). We previously demonstrated that tPA and uPA are involved in adhesion formation since pneumoperitoneum-enhanced adhesions were absence in tPA(-/-), uPA(-/-) knock out mice (72). A reduction in adhesion formation has been shown applying fibrinolytic agents in several animal models during open surgery (36). Particularly during laparoscopy, Reteplase has been shown during this thesis to reduce adhesions (Addendum 10). The tPA and uPA are inhibited by plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2) and, in addition, we previously demonstrated that PAI-1 is involved in adhesion formation since pneumoperitoneum-enhanced adhesion was absence in PAI-1(-/-) knock out mice (72). The use of antibodies against PAI-1 showed a reduction upon adhesion formation during laparotomy (37). However, no effect has been found during laparoscopy as demonstrated during this thesis (159). Furthermore, it is known that surgery dramatically reduces fibrinolytic activity, by both increasing plasminogen activator inhibitors levels (312) and by decreasing tPA levels (81).

The fibrin matrix is the ‘‘ground’’ through which mesothelial cells can migrate and accomplish reepithelialization. When two injured peritoneal surfaces covered with this sticky fibrin matrix come into apposition, sticky bands and bridges of fibrin can be formed. In this step, the use of flotation agents and barriers has been shown to be effective in reducing adhesion formation during laparotomy (101; 102; 107-109; 116; 117; 175; 290-294) and, as

shown within this thesis, during laparoscopy for Hyalobarrier gel, phospholipids and spraygel (160). Flotation agents as CMC and Hyskon show an small and not significant effect (160), consistent with the results of other groups (118-122).

If the fibrinous matrix persists, it will be infiltrated by proliferating fibroblasts which subsequently depose collagen. Mesothelial cells also migrate and form an uninterrupted layer on the surface of the already constituted adhesion. In this step anti-inflammatories and calcium channel blockers have an influence upon adhesions formation inhibiting the growth of fibroblast and mesothelial as demonstrated previously (20-25; 27-34; 101; 139; 140; 142; 143) and during this thesis for dexamethasone and diltiazem (159).

As the tissue underlying the adhesion is usually relatively hypoxic, signals initiating angiogenesis will be elaborated, resulting in a vascularized adhesion. Preclinical prevention studies demonstrate that the TNP-40 use, a potent endothelial cell inhibitor (39), neutralizing antiserum to VEGF (40), antibodies against PlGF (41) and against VEGFR1 (42) can reduce adhesion formation.

Taking into account the pathophysiology of the adhesions and the results of this thesis, we can conclude that using a *less traumatic pneumoperitoneum* to avoid the surgical trauma, this means adding 3% oxygen to avoid hypoxia, using humidified gas to avoid desiccation and decreasing body temperature to reduce inflammation, would have an effect in reducing adhesion formation. In addition, the combination of the *less traumatic pneumoperitoneum* with *the use of agents that act in different step of the adhesion process,* i.e. anti-inflammatories, calcium channel blockers, ROS scavengers, HIF inhibitors, flotation agents, barriers, fibrinolytic agents reduce adhesion formation. Last but not least, the training of the surgeon has an important role avoiding the initial surgical trauma (79).

3.4 Future Aspects

Important results have been found during this thesis. Firstly, since the characterization of the laparoscopic mouse model for adhesions is finally understood, new products for adhesion prevention have to be developed and tested. In addition, it would be useful to test new products also in combination with the treatments that decreased adhesions, i.e. low temperature, adding 3% oxygen, dexamethasone, Hyalobarrier gel, Spraygel, phospholipids, ROS scavengers, calcium channel blockers, etc. Moreover, it would be interesting to investigate in the mouse model why low temperature reduces adhesion formation and to learn which are the pathways, molecules and genes that are activated or down regulated with the

hypothermia. Something to be done in the future is to study the effect of low temperature in combination with low pressure of insufflation gas and to see if the effects are additives or not.

Secondly, our data clearly showed that adhesion formation decreases with body temperature. If this observation is confirmed in humans, this will become inmediately clinically relevant for laparoscopic surgery.

Thirdly, since the use of humidified gas prevents mesothelial layer desiccation and it reduces adhesion formation, it would be important to introduce the use of humidified gas in the clinical practice. The importance of using warm and humidified gas during laparoscopy has already been addressed in clinical trials showing a reduction in hypothermia, postoperative pain and recovery time (237-240; 243; 313). However, these results are not consistent (239-244; 313). Since the sample size used in these studies was small, it would be important to do a clinical trial with a bigger sample size and, in addition, to test the cold and humidified gas. A new humidifier which produces cold and humidified gas is being developed and we hope to be able to test it in humans soon. As it was said above, the aim is to decrease some degrees abdominal temperature without affecting body temperature.

Finally, after testing the use of cold and humidified gas during laparoscopy, our prevention results, specially the combination of treatments, have to be tested in the clinical practice. If adhesions can be reduced up to 90% as demonstrated during this thesis, the clinical relevance will be inmediate.

3.5 Summary

Intraperitoneal adhesions are abnormal fibrous connections between surfaces within the abdominal cavity which result mainly from a previous surgery. They are a major clinical problem because they cause chronic pain, intestinal obstruction, female infertility and difficulties at the time of reoperation.

Surgery generates a trauma in the peritoneum and this initiates an inflammatory reaction leading to fibrin deposition. Within this fibrinous exudate, various types of cells migrate, proliferate and/or differentiate, producing molecules that determine either normal healing or adhesion formation. The balance between fibrin deposition and degradation is critical in determining normal peritoneal healing or adhesion formation. If fibrin is completely degraded, normal peritoneal healing will occur. In contrast, if fibrin is not completely degraded, it will serve as a scaffold for fibroblasts and capillary ingrowth. Fibroblast will

invade the fibrin matrix and ECM will be produced and deposited. This ECM is normally completely degraded by MMPs, leading to normal healing. If this process is inhibited by TIMPs, peritoneal adhesions will be formed.

The severity of the trauma is important in developing adhesions. Although laparoscopy induces less direct trauma than laparotomy, we postulate that the pneumoperitoneum during laparoscopy could also be detrimental. We previously demonstrated that CO_2 pneumoperitoneum increases adhesion in time and pressure-dependent manner and this increase is reduced by adding 3-4% oxygen to the pneumoperitoneum, suggesting peritoneal hypoxia as the driving mechanism. During this thesis, we demonstrate that pneumoperitoneum can produce another trauma, desiccation and, in addition, the complex relationship between cooling and desiccation. Desiccation increases adhesion formation, and this effect is generally underestimated as the associated cooling decreases adhesion formation. We confirm the effect of hypothermia in reducing adhesion prevention, an effect that at 32°C is quantitatively as pronounced as humidification. The initial hypothesis that oversaturation of the insufflated gas would be beneficial for adhesion formation, as all desiccation would be prevented, thus proved wrong because of the associated increase in peritoneal temperature and enthalpy of the gas. From these data we anticipate that insufflators, which provide only a heating option that will warm the gas to body temperature without humidification, could be more deleterious for adhesion formation than using an insufflator without a heating option, because of higher temperature and higher desiccation. These data obviously still need to be confirmed in human, in whom a decrease in pneumoperitoneum temperature is not necessarily associated with a decrease in body temperature. If confirmed in human, these results would have very important clinical implications for the design of insufflators and humidifiers, which would minimize the trauma produced by the pneumoperitoneum diminishing postoperative adhesions.

We, therefore, suggest the use of a *less traumatic pneumoperitoneum* to prevent adhesion formation: using humidified gas to avoid desiccation, adding 3% oxygen to the CO_2 insufflation gas to prevent hypoxia and, locally, low temperature to reduce the trauma. Combined with this *less traumatic pneumoperitoneum,* we suggest the application of agents that can act in different pathways of the adhesion formation process as inflammation (anti-inflammatories as dexamethasone, calcium channel blocker as diltiem), ischemia-reperfusion (ROS scavengers as SOD and ascorbic acid), fibrinolysis (Reteplase) and the separation of the two injured surfaces (barriers: Hyalobarrier Gel, Spraygel, phospholipids). The results of this thesis are promising since we demonstrated that adhesion formation can be reduced by around

48% only using a less traumatic pneumoperitoneum and this reduction can be increased to 76 to 90% by applying different products. Of course, this is just the beginning of an important topic. New molecules and combinations of treatments must be tested and, afterwards, confirmed in clinical trials.

3.6 Summary in Dutch

Intraperitoneale adhesies zijn abnormale fibreuze strengen tussen oppervlakten in de abdominale caviteit ten gevolge van voorgaande chirurgische ingrepen. Klinisch vormen ze een groot probleem daar ze aanleiding kunnen geven tot chronische pijn, intestinale obstructie, vrouwelijke infertiliteit en complicaties bij re-operaties.

Ten gevolge van een chirurgische ingreep ontstaat een trauma in het peritoneum, wat aanleiding geeft tot een inflammatoire reactie met vorming van een fibrine beslag. In dit fibreus exudaat, migreren verschillende types cellen, ze prolifereren en/of differentiëren en geven aanleiding tot moleculen die leiden tot ofwel een normale heling of adhesie formatie. De balans tussen fibrine depositie en degradatie is hierin kritisch. Wanneer fibrine volledig gedegradeerd wordt, zal een normale peritoneale heling ontstaan, daarentegen, als fibrine niet volledig degradeert, zal dit aanleiding geven tot een platform voor fibroblasten en capillaire ingroei. Deze fibroblasten zullen invaderen in deze matrix and extracellulaire matrix (ECM) wordt geproduceerd en neergeslagen. ECM wordt normaal volledig gedegradeerd door matrix metalloproteïnasen (MMPS), wat aanleiding geeft tot een normale heling. Als dit proces echter wordt geïnhibeerd door TIMP's, zullen peritoneale adhesies worden gevormd.

Hierbij is ook de ernst van het trauma van belang in het ontwikkelen van adhesies. Ondanks dat een laparoscopische procedure minder trauma veroorzaakt dan een laparotomie, veronderstelden we dat het pneumoperitoneum gebruikt tijdens een laparoscopie eveneens een nadelig effect heeft. In voorgaande studies toonden we aan dat een CO2 pneumoperitoneum aanleiding geeft tot meer adhesies op een tijds- en druk dependente manier en dat deze toename kan gereduceerd worden door de additie van 3-4% zuurstof aan het pneumoperitoneum. Dit suggereert een peritoneale hypoxie als drijvende kracht in dit mechanisme.

Tijdens deze thesis, toonden we aan dat het pneumoperitoneum kan aanleiding geven tot andere trauma nl desiccatie en we verduidelijken de complexe relatie tussen afkoeling en desiccatie. Desiccatie leidt tot een toename van adhesies en dit wordt meestal onderschat doordat de geassocieerde koeling leidt tot een vermindering in adhesie vorming. In deze thesis

bevestigen we het effect van hypothermie op het verminderen van adhesies, een effect dat bij 32°C kwantitatief te vergelijken is met humidificatie. De initiële hypothese dat oversaturatie van het geïnsuffleerde gas een voordelig effect zou hebben op adhesie vorming, doordat alle desiccatie zou vermeden worden, blijkt dus fout te zijn. Dit is het gevolg van de geassocieerde stijging in peritoneale temperatuur en enthalpie van het gas. Uit deze data vermoeden we dan ook dat insufflators die het gas opwarmen tot lichaamstemperatuur zonder bevochtiging een meer nadelig effect zouden hebben op adhesie vorming dan insufflators zonder opwarming ten gevolge van hogere temperaturen en desiccatie. Deze data moeten echter geconfirmeerd worden in humane studies, bij wie een daling in de temperatuur van het pneumoperitoneum niet noodzakelijk geassocieerd is met een daling van de lichaamstemperatuur. Doch indien deze gegevens bevestigd worden in humane studies, heeft dit een belangrijk klinisch impact. Dit betekent immers dat insufflators en bevochtigers zodanig moeten ontwikkeld worden dat trauma ten gevolge van het pneumoperitoneum geminimaliseerd wordt waardoor er minder postoperatieve adhesies zullen ontstaan.

We suggereren dan ook het gebruik van een ‘minder traumatisch pneumoperitoneum’ om adhesies te voorkomen: het gebruik van bevochtigd gas om desiccatie te voorkomen, toevoegen van 3% zuurstof aan het CO2 insufflatie gas om hypoxie te vermijden en, lokaal, lage temperaturen om trauma te voorkomen. In combinatie hiermee stellen we voor agentia te gebruiken die inwerken op de verschillende mechanismen van adhesie vorming zijnde inflammatie (anti-inflammatoire medicatie als dexamethasone, calcium kanaal blokkers als diltiem), ischemie-reperfusie (ROS scavengers zoals SOD en ascorbine zuur), fibrinolyse (Reteplase) en barriers (Hyalobarrier Gel, Spraygel, phospholipiden).

Onze resultaten zijn veelbelovend daar we konden aantonen dat adhesie formatie kan gereduceerd worden met 48% door het gebruik van een ‘minder traumatisch pneumoperitoneum’ en deze reductie kan evenwel nog verminderd worden naar 76-90% door gebruik te maken van verschillende lokale agentia. Verder onderzoek is echter noodzakelijk waarbij nieuwe molecules en combinaties van behandelingen moeten getest worden en gevalideerd in klinische trials.

Chapter 4

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ADDENDUM

Addendum 1: GENERAL METHODS AND PROCEDURES

1.1 The laparoscopic mouse model

Experimental setup, i.e. animals, anaesthesia and ventilation, laparoscopic surgery, induction and scoring of intraperitoneal adhesions, has been described in detail (41; 42; 53; 69; 71; 72; 74; 156-158; 314) (Figure 1, Figure 4).

1.1.1 Animals

Naval Medical Research Institute (NMRI) mice were used initially. During the experiment in which strain influence was evaluated, wild-type mice of the following strains were evaluated: 100% Swiss, 87.5% Swiss-12.5% 129SvJ, 75% Swiss-25% 129SvJ, 50% Swiss-50% 129SvJ, 100% NMRI, 100% C57BL/6J, 87.5% C57BL/6J-12.5% 129SvJ and 75% C57BL/6J-25% 129SvJ, 100% BALB/c and 100% FVB. After it had become clear that the interanimal variability was much less in the inbred strain BALB/c, whereas the adhesion formation was similar than in NMRI mice (156), we decided to use BALB/c for further experiments.

Female mice around 8-10 weeks old were used. They were kept under standard laboratory conditions and they were fed with a standard laboratory diet with free access to food and water. The study was approved by the Institutional Review Animal Care Committee.

1.1.2 Anaesthesia and ventilation

Mice were anesthetized with intraperitoneal (IP) 0.08 mg/g pentobarbital, considered as time 0 (T_0), and after 10 min (T_{10}) intubated with a 20-gauge catheter and mechanically ventilated (Mouse Ventilator MiniVent, type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using humidified or non-humidified room air. Since it was previously demonstrated that CO_2 pneumoperitoneum increased the pCO_2 and decreased the pH and that an assisted ventilation can correct these two parameters, a tidal volume of 250 μL at 160 strokes/min was used during this thesis in order to avoid acidosis and hypercarbia (53).

1.1.3 Laparoscopic surgery

At T_{20} a midline incision was performed caudal to the xyphoides, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity, and the incision was closed gas tight around the endoscope to avoid leakage. Pneumoperitoneum was created with the Thermoflator Plus (Karl Storz, Tuttlingen, Germany) using humidified or non-humidified insufflation gas. The insufflation gas was pure CO_2 or CO_2 mixed with up to 12% oxygen at a pressure of 15 mm of Hg (or 20 cm of H_2O). To maintain accurately the insufflation pressure with minimal fluctuation, a water valve with a free escape of gas was used. The water valve was found to be necessary to adapt the flow rate to a mouse and to dampen pressure changes during insufflation.

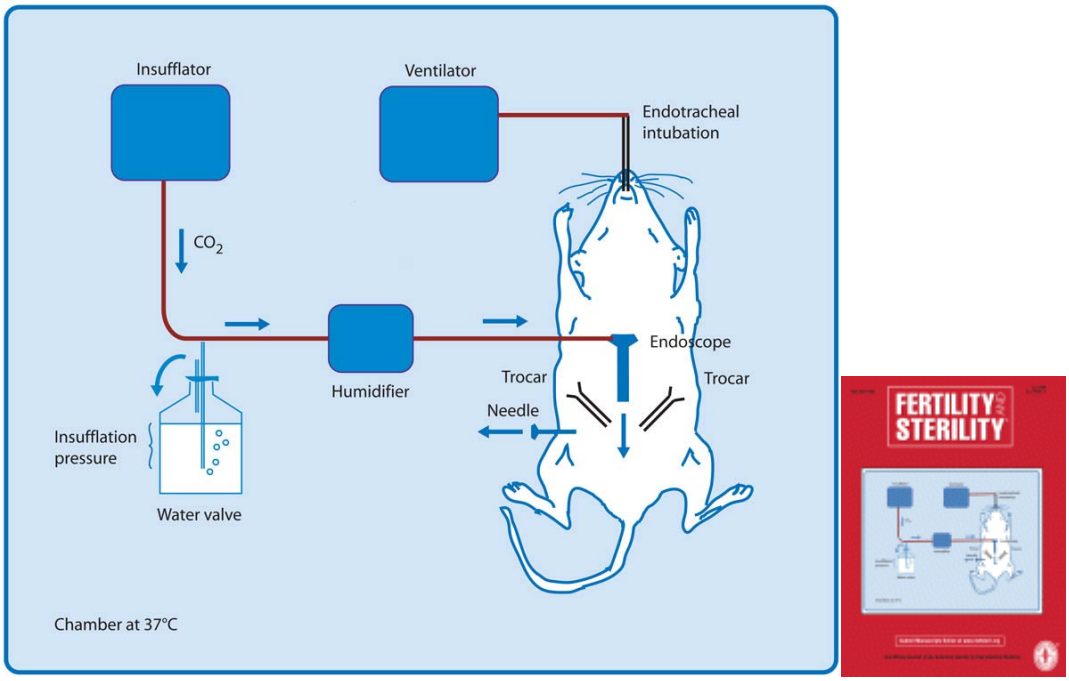
1.1.4 Induction of intraperitoneal adhesions

After the establishment of the pneumoperitoneum, two 14-gauge catheters were inserted under laparoscopic vision. Standardized 10x1.6-mm lesions were performed in the antimesenteric border of both right and left uterine horns and pelvic sidewalls with bipolar coagulation (BICAP, bipolar hemostasis probe, BP-5200A, 5 Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (standard coagulation mode, Autocon 200, Karl Storz).

1.1.5 Scoring of adhesions

Adhesions were qualitatively and quantitatively scored, blindly (the investigator was not informed of the group being evaluated) under microscopic vision during laparotomy 7 days after their induction. The qualitative scoring system assessed as follows: extent (0: no adhesions; 1: 1%–25%; 2: 26%–50%; 3: 51%–75%; 4: 76%–100% of the injured surface involved), type (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), tenacity (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection), and total (extent + type + tenacity). The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: adhesion (%) = (sum of the length of the individual attachments/length of the lesion) × 100. The results are presented as the average of the adhesions formed at the four sites (right and left visceral and parietal peritoneum), which were individually scored.

Figure 1



Reproduced with permission from *Fertil Steril 2006* (158) (Addendum 4)

1.1.6 Our triple model

Our laparoscopic mouse model for adhesion formation with pure CO_2 thus is a model for laparoscopic surgery with the mesothelial cells in hypoxic conditions. When CO_2 is used with 12% of oxygen the partial pressure of oxygen is higher than the physiologic mesothelial oxygen tension: this hyperoxia would mimic the laparotomy conditions in which the abdomen is exposed to 21% oxygen. When CO_2 is used with 3-4% of oxygen, the mesothelial cells are in normoxic conditions and adhesion formation is minimal (normoxia model). In comparison with CO_2 with 3-4% of oxygen the increase in adhesion formation with pure CO_2 is, therefore, called 'pneumoperitoneum- or hypoxia-enhanced adhesion formation or hypoxia model. The increase in adhesion formation caused by CO_2 + 12% of oxygen therefore is called hyperoxia-enhanced adhesion formation or hyperoxia model.

1.2 Set up and general design of the experiments

A strict standardization and control of all parameters was found essential.

1.2.1 Environmental temperature

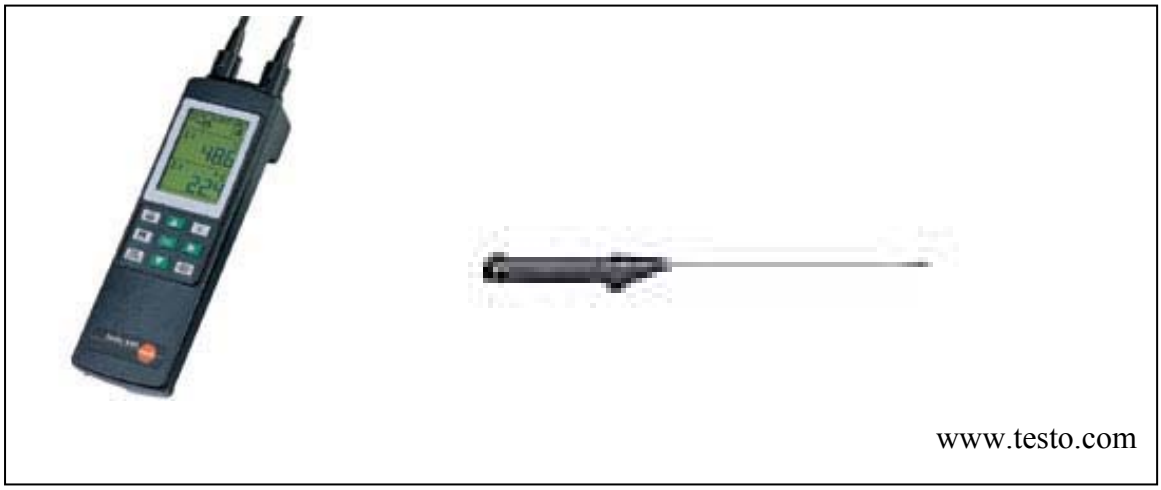
To control animal and gas temperature, animals and equipment (i.e., insufflator, humidifier, water valve, ventilator, and tubing) were placed in a closed chamber maintained at 37°C with heated air (WarmTouch, Patient Warming System, model 5700, Mallinckrodt Medical, Hazelwood, MO) (Figure 2).

1.2.2 Body and pneumoperitoneum temperatures and pneumoperitoneum relative humidity

Animal body temperature (BT) was continuously monitored in the rectum (Hewlett Packard 78353A, Hewlett Packard, Böblingen, Germany) and registered every 10 minutes. Pneumoperitoneum temperature and relative humidity (RH) were measured with the Testo 645 device and a 4-mm probe (Testo N.V./S.A., Lenzkirch, Germany) introduced in the abdomen (Figure 3). Due to the size of this probe, measurements were not done systematically in the same experiments performed to induce adhesions.

Because desiccation or vaporization requires 577 cal/mL of water and thus produces cooling, the mice could not maintain their BT at 37°C during desiccation experiments, notwithstanding the box heated to 37°C. Therefore, to evaluate the pure effect of desiccation without cooling, keeping mouse BT at 37°C, an additional heating system had to be used (the homeothermic Blanket System; Harvard Apparatus LTD, Edenbridge, UK). This system includes a small rectal probe for continuous temperature monitoring and a heating blanket to provide sufficient heat for accurate control of mouse BT, both connected to a control unit. The control unit varies the current flowing through the heating blanket in an inversely proportional manner to the temperature monitored by the temperature probe.

Figure 3: Device and probe to measure temperature and humidity.



1.2.3 Pneumoperitoneum gas conditions: desiccation and humidification.

Usually, since some gas can diffuse from the circulation to the pneumoperitoneum, a 26-gauze needle with free escape of gas from the peritoneal cavity was placed in order to ascertain a constant composition of the pneumoperitoneum.

To induce desiccation, a controlled flow of non-humidified CO_2 was obtained using 26-, 22- and 16-gauge needles, which at 15 mm Hg insufflation pressure induced a 23-, 100- or 492- mL/min flow of CO_2 gas through the abdominal cavity, respectively. Without a needle, in the absence of any leak, no flow through the abdominal cavity occurred.

To humidify the insufflation gas two types of humidifiers were used. For all the experiments in which humidified gas was evaluated, the Storz Humidifier (204320 33, Karl Storz) and the 37°C chamber were used, in which CO_2 at 37°C and nearly 100% RH can be obtained. Only for the experiment 4 Aim#1b, the insufflation humidifier MR860 (Fisher & Paykel Healthcare Ltd, Auckland, New Zealand) was used to avoid any desiccation by oversaturation. To see details about this humidifier please see the references (61; 158).

1.3 Statistics

Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC) and/or the GraphPad Prism (GraphPad Software Inc., San Diego, CA). Individual comparisons were performed with Wilcoxon rank-sum test (Mann Whitney U test), multiples comparisons with ANOVA or logistic regression (proc logistic) and correlation analysis with Person or Spearman for parametric or non-parametric variables, respectively. P values (two tailed) lower than 0.05 were considered significant.

Many expereiments has a factorial design. The advantage of the factorial design is the increase in statistical power for the same total number of animals. A 2x2 factorial design evaluating two effects A and B with *n* animals in each group achieves for a total number of 4*n* animals almost the same statistical power as would be achieved by doing a 4*n* experiment evaluating A and another 4*n* experiment evaluating B, thus requiring almost 50% fewer animals in total (315).

In most studies, at least 6 mice per group were used. Less than 6 mice per group were only used for the temperature experiments in which a very low interanimal variability was shown.

Figure 2: Pictures of the 37°C Chamber

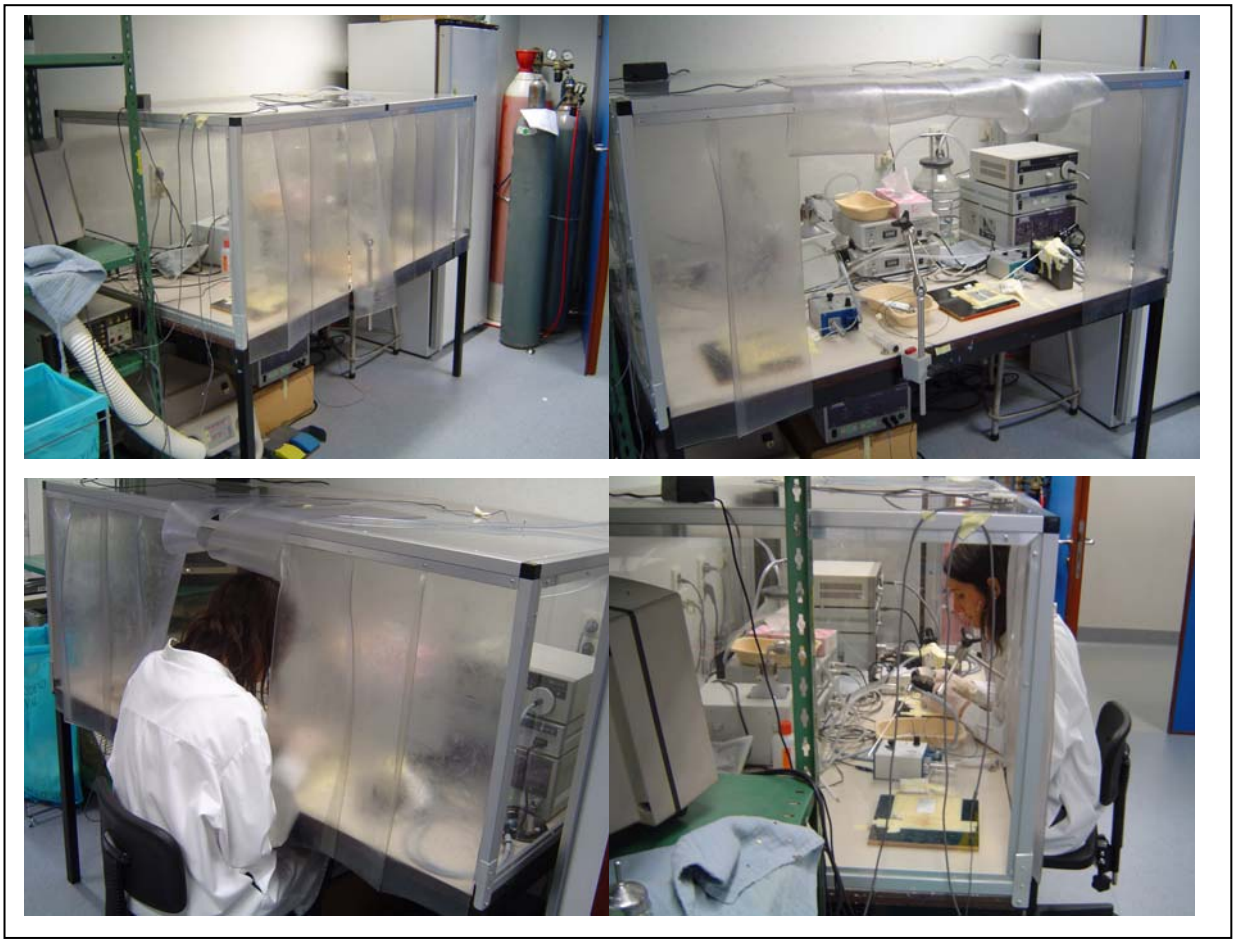
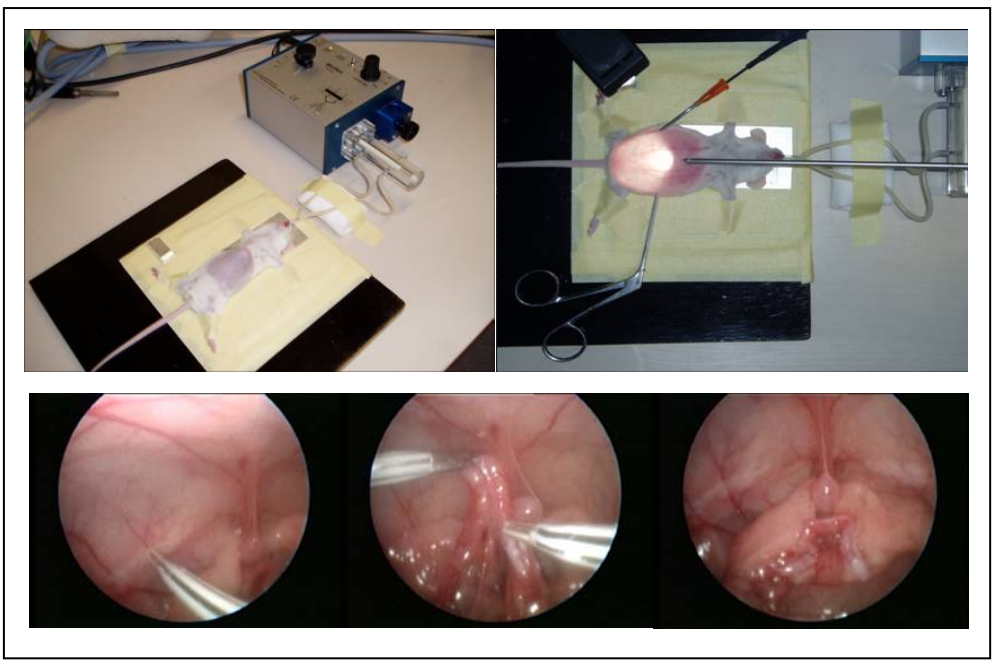


Figure 4: Pictures of the laparoscopic mouse model



Addendum 2

Binda MM, Molinas CR, Koninckx PR. Reactive oxygen species and adhesion formation: Clinical implications in adhesion prevention. *Human Reproduction* 18 (12): 2503-2507, 2003.

Human Reproduction Vol.18, No.12 pp. 2503–2507, 2003 DOI: 10.1093/humrep/deg481

DEBATE—continued

Reactive oxygen species and adhesion formation

Clinical implications in adhesion prevention

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Postoperative adhesion formation is a major clinical problem. It has been demonstrated that the pneumoperitoneum used during laparoscopy is a cofactor in adhesion formation. Reactive oxygen species (ROS) are produced in a hyperoxic environment and during the ischaemia/reperfusion process. ROS activity is deleterious for cells, which protect themselves by an antioxidant system known as ROS scavengers. ROS activity can increase by up-regulation of ROS themselves or by down-regulation of ROS scavengers. Recent data also point to a role for ROS in adhesion formation since the administration of ROS scavengers decreases adhesion formation in several animal models. ROS activity increases during both laparotomy and laparoscopy. During laparoscopy, the pneumoperitoneum determines ischaemia at the time of insuflation and reperfusion at the time of deflation. During laparotomy, the environment has a 150 mmHg partial pressure of oxygen (pO_2), which is much higher than the intracellular pO_2 (5–40 mmHg). This can explain the increase in ROS activity. The aim of this debate is to open a discussion about the importance of ROS activity, besides the known players and mechanisms involved, in adhesion formation and in adhesion prevention.

Key words: adhesion formation/antioxidants/free radical scavengers/pneumoperitoneum/reactive oxygen species

Introduction

Following surgery, adhesions form in >80% of women and can cause female infertility (Drake and Grunert, 1980), intestinal obstruction (Ellis, 1997), chronic pelvic pain (Duffy and diZerega, 1996) and difficulties at the time of reoperation. The burden of postoperative adhesions is best illustrated by the study showing that 35% of women having open gynaecological surgery will be readmitted on average 1.9 times in the following 10 years for reoperation due to adhesions (Ellis, 2000).

Pathophysiology of intraperitoneal adhesions

Peritoneal injury, due to surgery, infection or irritation, initiates an inflammatory reaction that increases peritoneal fluid, including proteins and cells. This fibrinous exudate leads to formation of fibrin (Holmdahl, 2000), by activation of the coagulation cascade, which transforms prothrombin (Factor II) into thrombin (Factor IIa). Thrombin then triggers the conversion of fibrinogen into monomers of fibrin, which interact and polymerize. The initially soluble polymer becomes insoluble by coagulation factors such as Factor XIIIa and is deposited on the wound surface (diZerega, 2000). Within this fibrinous exudate, polymorphonuclears (PMN), macrophages, fibroblasts and mesothelial cells migrate, proliferate and/or differentiate. Macrophages increase in number and change functions, e.g. more accurate phagocytosis, greater respiratory burst activity and secretion of a variety of substances that recruit mesothelial cells onto the injured surface. Mesothelial cells form islands throughout the injured area, proliferate and cover the denuded area. All these cells release a variety of substances such as plasminogen system components, arachidonic acid metabolites, reactive oxygen species (ROS), cytokines and growth factors such as interleukins (IL), tumour necrosis factor α (TNFα), transforming growth factors α and β (TGFα and TGFβ). These factors modulate the process of peritoneal healing and adhesion formation at different stages.

The fibrinous exudate and fibrin deposition is an essential part of normal tissue repair, but its complete resolution is required for normal healing. The degradation of fibrin is regulated by the plasminogen system. The inactive proenzyme plasminogen is converted into plasmin by tissue-type plasminogen activator (tPA) and/or urokinase-type plasminogen activator (uPA), which are inhibited by the plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2). Plasmin is a serine protease which degrades fibrin into fibrin degradation products. Plasmin has, in addition, a role in other stages of tissue repair, e.g. extracellular matrix (ECM) degradation, activation of

proenzymes of the matrix metalloprotease (MMP) family, and activation of growth factors. Plasmin can be directly inhibited by plasmin inhibitors, i.e. α_2-macroglobulin, α_2-antiplasmin and α_1-antitrypsin, but their role in peritoneal fibrinolysis is not well defined (Holmdahl, 2000).

The balance between fibrin deposition and degradation is critical in determining normal peritoneal healing or adhesion formation. If fibrin is completely degraded, normal peritoneal healing will occur. In contrast, if fibrin is not completely degraded, it will serve as a scaffold for fibroblasts and capillary ingrowth. Fibroblasts will invade the fibrin matrix and ECM will be produced and deposited. This ECM is normally completely degraded by MMPs, leading to normal healing. If this process is inhibited by tissue inhibitors of MMPs (TIMPs), peritoneal adhesions will be formed. In addition to fibroblast invasion and ECM deposition, the formation of new blood vessels has been universally claimed to be important in adhesion formation.

During peritoneal healing, cell–cell interactions between mesothelial cells, macrophages and also fibroblasts contribute to the healing of the peritoneum. Adhesion fibroblasts have developed a specific phenotype. Compared with normal peritoneal fibroblasts, adhesion fibroblasts have increased basal levels of collagen I, fibronectin, MMP-1, tissue MMP-1, TGFβ, PA-1, IL-10 and decreased levels of tPA (Saed *et al*., 2001).

Pneumoperitoneum-enhanced adhesion formation

Laparoscopy, in comparison with laparotomy, was claimed to be less adhesiogenic, but the data are not conclusive (Pouly and Seak-San, 2000). In recent years the effects of CO_2 pneumoperitoneum have become increasingly scrutinized. CO_2 pneumoperitoneum induces adverse effects such as hypercarbia, acidosis (West *et al*., 1997), hypothermia and desiccation (Gray *et al*., 1999). It alters peritoneal fluid (Ott, 2001) and the morphology of the mesothelial cells (Volz *et al*., 1999; Hazebroek *et al*., 2002). Pneumoperitoneum is a cofactor in adhesion formation since adhesions increase with the duration of the pneumoperitoneum and with the insufflation pressure in rabbits (Ordonez *et al*., 1997; Yesildaglar and Koninckx, 2000) and mice (Yesildaglar *et al*., 1999; Molinas *et al*., 2001). This pneumoperitoneum-enhanced adhesion formation has been suggested to be mediated by mesothelial hypoxia because similar effects were observed with helium pneumoperitoneum because the addition of 2–4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation (Molinas and Koninckx, 2000; Molinas *et al*., 2001) and because this effect was absent in mice deficient for hypoxia inducible factor (HIF) (Molinas *et al*., 2003a), for PAI-1 (Molinas *et al*., 2003b), for vascular endothelial growth factor (VEGF) or for placental growth factor (PlGF) (Molinas *et al*., 2003c).

Reactive oxygen species

Reactive oxygen species are produced in a series of conditions such as cells maintained under hyperoxic conditions (Bostek, 1989) and during reperfusion following ischaemia (Eleftheriadis *et al*., 1996; Glantzounis *et al*., 2001). They are also produced during a bactericidal immune response (Babior *et al*., 1973). ROS comprises free radicals and non-free radicals. Free radicals, i.e. superoxide anion (O_2^-), hydroxyl radical (·OH) and nitric oxide (NO), are unstable atoms or molecules with an unpaired electron: they take an electron from other stable molecules to stabilize themselves, thus causing a chain reaction by destabilizing other molecules. Non-free radicals, i.e. hydrogen peroxide (H_2O_2), have paired electrons but by their natural instability they can easily become free radicals. Since ROS are deleterious for cells, they protect themselves by an antioxidant system known as ROS scavengers. These comprise antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Forman and Torres, 2002) and antioxidant molecules such as glutathione, carotenoids, retinoic acid, ascorbic acid and vitamin E. The balance between ROS and ROS scavengers will determine ROS activity and toxicity.

ROS inhibit cellular proliferation and produce cellular senescence (Honda *et al*., 2001, 2002), induce molecular damage of molecules such as DNA, proteins and lipids (Wei and Lee, 2002), and cause ageing (Wei and Lee, 2002) and apoptosis (Taglialatela *et al*., 1998). ROS are also involved in a variety of diseases such as in the inflammatory reaction associated with endometriosis (Ota *et al*., 1999; Van Langendonckt *et al*., 2002), in neurodegenerative diseases as Alzheimer's (Nourooz-Zadeh *et al*., 1999), in autoimmune diseases as systemic lupus erythematosus (Ahsan *et al*., 2003) and in the pathogenesis of diabetic nephropathy (Ha and Lee, 2001). ROS have also been associated with surgery and postoperative adhesion formation (Tsimoyiannis *et al*., 1989; Portz *et al*., 1991; Hemadeh *et al*., 1993; Galili *et al*., 1998; Taskin *et al*., 1999).

Surgery and reactive oxygen species

During open surgery, an increase in ROS production has been reported, i.e. an increase of superoxide anions (Elkins *et al*., 1991), of xanthine oxidase, an enzyme involved in ROS formation (Anup *et al*., 1999), and of malondialdehyde, a marker of ROS production (Souza *et al*., 2003). Also during laparoscopic surgery, ROS increase. Indeed, recent findings showed an increase of markers of ROS production such as 8-isoprostaglandin $F_{2\alpha}$ and hydroxyeicosatetranoic acid in human peritoneum in a time- and CO_2 volume-dependent manner (Souza *et al*., 2003) and of malondialdehyde in the intestine, liver and lung in rats (Eleftheriadis *et al*., 1996). In addition, a negative correlation between ROS scavengers such as GSH-Px, SOD, CAT and GSH, and duration/amount of CO_2 exposure was observed (Taskin *et al*., 1998, 1999). Since abdominal insufflation/deflation causes ischaemia/reperfusion (Caldwell and Ricotta, 1987; Kotzampassi *et al*., 1993; Shuto *et al*., 1995; Eleftheriadis *et al*., 1996; Gutt and Schmandra, 1999; Kotzampassi *et al*., 2000; Schmandra *et al*., 2001), which is well known to generate ROS (Glantzounis *et al*., 2001), a causal role of the pneumoperitoneum, especially at high

insufflation pressure in increased ROS production, can be postulated.

Adhesion formation and reactive oxygen species

It has been suggested that ROS can be involved in postoperative adhesion formation but direct data to support this are scant. It has been demonstrated *in vitro* that free radicals contribute to the formation of cross-linked proteins that may serve as an initial scaffolding for the development of adhesions frequently seen in joints (Dijkgraaf *et al*., 2003). In humans, in comparison with microlaparoscopy (2 mm endoscope, local anaesthesia and 10 mmHg insufflation pressure), standard laparoscopy (10 mm endoscope, general anaesthesia and 15 mmHg insufflation pressure) was reported to be associated with a higher amount of CO_2 used, with decreased levels of ROS scavengers, i.e. GSH-Px, SOD, CAT and GSH, and with increased adhesion formation (Taskin *et al*., 1999).

Indirectly, a role for ROS in adhesion formation is derived from the observation that ROS scavengers reduce adhesion formation following open surgery in different animal models. Indeed, CAT, SOD and trimetazidine reduce adhesion formation induced by vascular obstruction/reperfusion of an ileal segment in rats (Tsimoyiannis *et al*., 1989, 1990); CAT and SOD also reduce adhesion formation in an endometriosis model in rabbits (Portz *et al*., 1991). In addition, intraperitoneal administration of methylene blue reduces adhesion formation induced by scraping the uterus in rats (Galili *et al*., 1998); intraperitoneal administration of melatonin also prevents adhesion formation induced by monopolar cautery in rats (Özçelik *et al*., 2003). Similarly, oral supplements of vitamin E reduce adhesion formation created by scraping the caecum with mesh gauze in rats (Hemadeh *et al*., 1993). This effect of vitamin E, however, was not confirmed by denuding the serous surface of the uterus in rats (Sanfilippo *et al*., 1995).

Finally, the observation that adhesion formation decreases by adding 2–4% of oxygen to the CO_2 pneumoperitoneum (Molinas and Koninckx, 2000; Molinas *et al*., 2001) could be explained by the fact that this addition of oxygen prevents the decrease of ROS scavengers, and thus the increase of ROS activity.

Discussion

Research in adhesion formation and prevention has been performed with the dogma that laparotomy is the standard and with the assumption that the mechanisms involved in adhesion formation following laparotomy and laparoscopy are comparable. Recent data unequivocally demonstrated a role of the pneumoperitoneum through mechanisms involving HIF, the plasminogen system, i.e. PAI-1, and the VEGF family, i.e. VEGF-A, VEGF-B and PlGF. Simultaneously, accumulating data point to a role for ROS in adhesion formation. Interestingly, an increase in ROS activity has been shown following both laparotomy (Elkins *et al*., 1991; Anup *et al*., 1999; Souza *et al*., 2003) and laparoscopy (Eleftheriadis *et al*., 1996; Taskin *et al*., 1998, 1999; Glantzounis *et al*., 2001; Souza *et al*., 2003). The mechanisms underlying the increased ROS activity could, however, be different. During laparoscopy, ROS scavengers can decrease by hypoxia, whereas after laparoscopy ROS can increase by the ischaemia–reperfusion process. During laparotomy, ROS activity increases, and we suggest that this is due to a hyperoxic environment of the peritoneum. Indeed, during laparotomy the peritoneum is exposed to air with a pO_2 of 150 mmHg whereas the normal pO_2 for peripheral cells is estimated between 5 and 40 mmHg (Guyton and Hall, 2000).

The importance of these observations on ROS activity is that the traditional concepts of adhesion formation, involving tissue trauma, fibrin deposition and fibrinolysis, fibroblast invasion, ECM deposition and angiogenesis, have to incorporate the effect of the environment upon the peritoneal cells, e.g. mesothelial cells, macrophages, fibroblasts in order to understand the differences between laparotomy and laparoscopy. During laparotomy, the pO_2 of the environment is clearly hyperoxic for the peritoneal cells. During laparoscopy, the CO_2 pneumoperitoneum creates a hypoxic environment. Specifically relevant for adhesion formation is that a hypoxic environment induces irreversible molecular changes in peritoneal fibroblast, such as increases in cyclooxygenase 2 (COX-2) (Saed *et al*., 2003), ECM (Saed and Diamond, 2002), and PAI-1 (Saed and Diamond, 2003); moreover, hypoxia modulates the expression of TGFβ1, -2 and -3 and their receptors (Saed *et al*., 2002) and decreases tPA (Saed and Diamond, 2003).

These concepts are fundamental for the clinically important problem of adhesion prevention. Until now, prevention has focused on good surgical techniques minimizing tissue trauma and fibrin deposition and on mechanical separation of surfaces and upon fibrinolysis. The understanding of the role of the pO_2 in adhesion formation and of the mechanisms involving ROS, hypoxia during CO_2 pneumoperitoneum and hyperoxia during laparotomy, and angiogenesis could open new possibilities in adhesion prevention. Recent new approaches in animals, such as the addition of 3% of oxygen to the pneumoperitoneum (Molinas and Koninckx, 2000; Molinas *et al*., 2001), the neutralization of PlGF by monoclonal antibodies (Molinas *et al*., 2003c) and the administration of ROS scavengers (Tsimoyiannis *et al*., 1989, Portz *et al*., 1991; Hemadeh *et al*., 1993; Galili *et al*., 1998; Özçelik *et al*., 2003), open unexpected possibilities for postoperative adhesion prevention.

We fully realize that these concepts are provocative. Yet to stimulate thinking and discussion—'*du choc des idées jaillit la lumière*'—we decided to write this introduction to a Debate.

Indeed, the relative importance of fundamental mechanisms as the fibrinolytic system, angiogenic factors and ROS in adhesion formation is still unclear. Moreover, the role of a fundamental process such as ROS activity is important for many other aspects in medicine e.g. the ischaemia–reperfusion process and transplant surgery, embryo implantation, cell culture, and possibly IVF and embryo culture.

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Addendum 3

Molinas CR, **Binda MM**, Campo R, Koninckx PR. Adhesion formation and interanimal variability in a laparoscopic mouse model varies with strains. *Fertility & Sterility* 83(6):1871-4, 2005.

Adhesion formation and interanimal variability in a laparoscopic mouse model varies with strains

Adhesion formation after laparoscopic surgery was evaluated in mice of different strains. More adhesions were observed in Swiss, NMRI, and BALB/c mice, with less interanimal variability in BALB/c mice. These data point to genetics effects on adhesion formation, which open new insights in its pathogenesis and indicate the importance of a careful strain selection for animal studies. (Fertil Steril® 2005;83:1871–4. ©2005 by American Society for Reproductive Medicine.)

During the past years we developed a laparoscopic mouse model for the study of postoperative adhesion formation and reported that the CO_2 pneumoperitoneum is a cofactor in adhesion formation (1). Because adhesion formation increases with the duration of the pneumoperitoneum and with the insufflation pressure, without differences between CO_2 and helium pneumoperitoneum, our data indicate that this pneumoperitoneum-enhanced adhesion formation is mediated to a large extent by peritoneal hypoxia (1) and to a lesser extent by acidosis (2). This hypothesis of pneumoperitoneum-induced peritoneal hypoxia as a driving mechanism in pneumoperitoneum-enhanced adhesion formation was confirmed in transgenic mice that underexpress/overexpress genes encoding for factors regulated by hypoxia, such as hypoxia-inducible factors (3), members of the vascular endothelial growth factor family (4, 5), and of the plasminogen system (6). These transgenic mice and their controls were from different strains than the previously used NMRI mice (1, 7). The results from these consecutive experiments, evaluated retrospectively for strain differences, strongly suggested important strain effects on postoperative adhesion formation. Therefore, a prospective randomized study was performed to ascertain and document these strain-related differences in adhesion formation.

The studies were approved by the Institutional Review Animal Care Committee and performed in 10- to 12-week-old female mice. Animals were anesthetized with pentobarbital (IM, 0.07 mg/g), intubated and ventilated with room air with a tidal volume of 500 μL at 80 strokes/min (Small Animal Ventilator, model 683; Harvard Apparatus Inc., Holliston, MA) as described (1–6).

Experiments were carried out at room temperature and laparoscopic surgery for induction of intraperitoneal adhesions was performed as described (1–6). Briefly, a 2-mm endoscope with a 3.3-mm sheath was introduced into the abdominal cavity caudal to the xyphoides. Heated (37°C, Optitherm; Karl Storz, Tüttlingen, Germany) and humidified (100% relative humidity, Aquapor; Dräger, Lübeck, Germany) CO_2 at 20 cm H_2O (~14 mm Hg) of insufflation pressure was used for the pneumoperitoneum.

Standardized lesions in uterine horns and pelvic sidewalls were performed with monopolar and bipolar coagulation. The time required to establish the pneumoperitoneum and to perform the lesions was 5–6 minutes but the pneumoperitoneum was maintained for a longer period (60 minutes) to evaluate pneumoperitoneum-enhanced adhesion formation (2–6). After 7 days, adhesions were quantitatively (proportion) and qualitatively (extent, type, tenacity, and total) scored during laparotomy, as described (1–6).

Statistical analyses were performed with the GraphPad Prism 4 (GraphPad Prism Software Inc., San Diego, CA) using Kruskal-Wallis with Dunn's multiple comparisons tests for comparisons of nonparametric variables (adhesion scores) and one-way ANOVA with Bonferroni test for comparisons of parametric variables (body weights). Spearman test was used for correlation of strain and adhesion formation and of body weight and adhesion formation. Two-tailed P values $<.05$ were considered significant. Because the same trend was observed with both scoring systems, only the mean of the proportion of adhesions formed at the four individual sites are presented (means ± SD).

In the retrospective study (Fig. 1), wild-type mice of the following strains were evaluated: 100% Swiss (n = 20; 32.8 ± 1.9 g) (4, 5), 87.5% Swiss-12.5% 129SvJ (n = 5; 31.0 ± 1.0 g) (3), 75% Swiss-25% 129SvJ (n = 5; 31.2 ± 1.3 g) (4), 50% Swiss-50% 129SvJ (n = 10; 31.2 ± 1.2 g) (3, 4), 100% NMRI (n = 10; 32.8 ± 1.8 g) (2), 100% C57BL/6J (n = 5; 20.2 ± 0.8 g) (4), 87.5% C57BL/6J-12.5% 129SvJ (n = 5; 20.0 ± 0.7 g) (6), and 75% C57BL/6J-25% 129SvJ (n = 5; 20.4 ± 1.1 g) (6). In mice with a mixture of Swiss and 129SvJ background, more adhesions were observed with more Swiss background (r = 0.5,

Received June 20, 2004; revised and accepted November 15, 2004.
Supported in part by Karl Storz Endoscopy (Tuttlingen, Germany) and by the Fonds Wetenschappelijk Onderzoek, Brussels, Belgium, grant G.0324.01 and Onderzoeks Toelagen Katholieke Universiteit Leuven, Leuven, Belgium, grant Nr. TBA/00/27.
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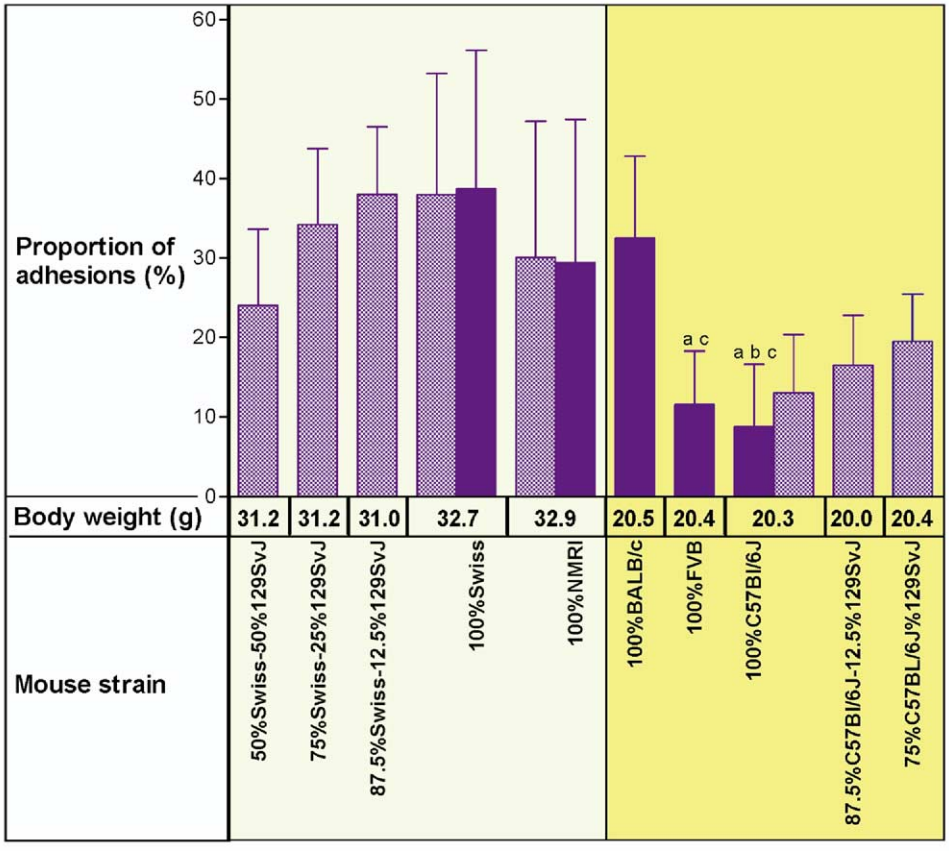
FIGURE 1

Postoperative adhesion formation was evaluated in inbred and outbred mice of different strains and with different body weights (means are indicated). Animals, body weight clustered into two groups, one of ~32 g (*yellow background*) and another of ~20 g (*orange background*). Pneumoperitoneum-enhanced adhesions were induced with standardized lesions during laparoscopy (CO_2 pneumoperitoneum for 60 minutes at 20 cm H_2O) and scored after 7 days during laparotomy in a retrospective study (*grid bars*) and in a prospective randomized study (*closed bars*). The proportion of adhesions (means ± SD) with differences statistically significant vs. Swiss (a), NMRI (b), and BALB/c (c) mice are indicated.



Molinas. Mouse strain and adhesion formation. Fertil Steril 2005.

P=.0002, Spearman correlation). In mice with a mixture of C57BL/6J and 129SvJ background, more adhesions were observed with less C57BL/6J background ($r = -0.5$, P=.01, Spearman correlation).

In the prospective randomized study (Fig. 1), wild-type mice of the following strains were used: 100% Swiss (n = 10; 32.6 ± 1.7 g) a 100% NMRI (n = 10; 33.1 ± 1.5 g), 100% BALB/c (n = 10; 20.6 ± 1.3 g), 100% FVB (n = 10; 20.4 ± 1.3 g), and 100% C57BL/6J (n = 10; 20.4 ± 0.8 g). The choice of these strains was determined by the availability at the Katholieke Universiteit Leuven. The study was carried out using block randomization by days (i.e., one block comprising one animal of each strain was operated on the same day), and within a block animals were operated on in a random order. In this study intergroup differences in adhesion formation were evaluated (Kruskal-Wallis with Dunn's multiple comparisons tests). No differences in adhesion formation was observed between Swiss, NMRI, and BALB/c mice (P = not significant [NS] for all comparisons), but in all these strains adhesion formation was higher than in FVB (P<.01, P=NS, and P<.01) and in C57BL/6J (P<.001, P<.05, and P<.01) mice. No differences in adhesion formation were observed between FVB and C57BL/6J mice (P=NS).

In addition to the amount of adhesion formation, the variability of adhesion formation between animals was calculated. The coefficient of variation (SD/mean × 100) was 45% for Swiss mice and 61% for NMRI mice, both outbred strains, but only 32% for the inbred BALB/c mice. For the inbreed FVB and C57BL/6J mice, the coefficient of

variation were 59% and 90%, respectively, reflecting the very low adhesion formation potential.

Differences in body weight (one-way ANOVA with Bonferroni test) and the relationship between body weight and adhesion formation (Spearman correlation) were evaluated in detail because it seemed that most strains with low weight developed less adhesions than strains with high weight. Two clusters of body weight (yellow and orange background in Fig. 1) without overlap were found, that is, one cluster of mice weighing ~32 g (Swiss and NMRI mice) and another cluster of mice weighing ~20 g (BALB/c, FVB and C57BL/6J mice). Within each cluster no significant differences in body weight were observed (P=NS for all comparisons), whereas both Swiss and NMRI mice weighed more than BALB/c ($P<.001$, $P<.001$), FVB ($P<.001$, $P<.001$), and C57BL/6J ($P<.001$, $P<.001$) mice. Within each cluster no correlation between body weight and adhesion formation was found. An overall correlation could, obviously, not be done because body weight clustered in two groups only. Moreover, BALB/c mice with a low body weight had more adhesions than all other strains with low body weight, whereas compared to mice with high body weight adhesion formation was not statistically different.

Strain differences have been reported for other processes involving fibrosis and healing responses such as hepatic, lung, and colorectal fibrosis (8–11), myocardial and ear wound healing (12, 13), and bone regeneration (14). To the best of our knowledge this is the first study indicating that genetic background also influences adhesion formation, at least after laparoscopic surgery. Among the strains evaluated we found that Swiss, NMRI, and BALB/c mice developed more adhesions compared to FVB and C57BL/6J mice, in which adhesion formation was minimal. About the mechanisms causing these interstrain differences, at present, we only can speculate. For none of the potential mechanisms, such as cellular interaction (e.g., macrophages, fibroblasts, mesothelial and endothelial cells) or molecular expression (e.g., plasminogen system, vascular endothelial growth factor, hypoxia-inducible factors, reactive oxygen species, matrix metalloproteases), modulating the processes of inflammation, fibrin deposition/degradation, extracellular matrix deposition/degradation, and angiogenesis (15–17), clear data about strain differences are available.

Our data also demonstrate that interanimal variability is less in the inbred BALB/c mice than in the outbred Swiss and NMRI mice. This is not surprising because inbred strains, maintained by sibling (brother × sister) mating for 20 or more generations, are genetically almost identical, homozygous at virtually all loci, and with high phenotypic uniformity (18). This less interanimal variability in inbred strains has been reported for many processes such as sleeping time under anesthesia (19). The high variability in the inbred FVB and C57BL/6J mice, with very low adhesion formation potential, is also not surprising because the absence of adhesions in many of these animals leads to artificially high coefficient of variation.

These observations on genetic influences contribute to the usefulness of the mouse model for adhesion formation studies. The mouse model has many advantages compared to other animal models because it is relatively cheap, easy to handle, and does not require strict sterile conditions for surgery. Furthermore, it is particularly useful for mechanistic studies because of the availability of animals with low genetic variability (i.e., inbred mice), underexpressing/overexpressing specific genes (i.e., transgenic mice), and immunodeficient by spontaneous mutation (i.e., nude mice [T-cell deficient] and SCID mice [T&B cell deficient]). In addition, many specific mouse assays and monoclonal antibodies are available.

Both observations (i.e., strain differences in adhesion formation potential and in interanimal variability) point to genetics effects on adhesion formation, which is not surprising and confirms clinical observations. The importance of these observations is twofold. First, to study the genetic involvement in detail, the use of two strains with high and low adhesion formation potential can be considered as an experiment of nature. Second, to study adhesion formation and prevention, it is preferable to use a strain with high adhesion formation potential and low interanimal variability, such as BALB/c mice. Furthermore, fewer inbred animals will be needed to achieve a given level of statistical precision than if outbred animals had been used (18). We, however, want to point out that inbred strains in general weigh less than outbred strains (average of 20 g vs. 32 g), which increases the technical skills required to do the experiments, especially those involving laparoscopic surgery.

In conclusion, this study demonstrates that some mouse strains develop more postoperative adhesions than others and that the interanimal variability in inbred strains is less. These data should not be underestimated for adhesion formation studies in animal models and, although very preliminary, can open new insights in the pathogenesis of adhesion formation in humans.

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Addendum 4

Binda MM, Molinas C.R., Mailova K. and Koninckx P.R. Effect of temperature upon adhesion formation in a laparoscopic mouse model. *Human Reproduction* 19(11):2626-32, 2004.

Human Reproduction Vol.19, No.11 pp. 2626–2632, 2004 doi:10.1093/humrep/deh495
Advance Access publication August 27, 2004

Effect of temperature upon adhesion formation in a laparoscopic mouse model

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BACKGROUND: Pneumoperitoneum can be a cofactor in adhesion formation. Pneumoperitoneum with non-humidified gas causes desiccation in the peritoneal cavity which decreases temperature. The effect of desiccation upon adhesion formation is widely accepted. The specific effect of the associated cooling upon adhesion formation remains unexplored, and was addressed specifically in our laparoscopic mouse model. METHODS: Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Pneumoperitoneum was performed using CO_2 or CO_2 with oxygen with or without humidification. Animals were placed at different environmental temperatures to modulate body and intraperitoneal temperature. RESULTS: Anaesthesia, environment with a lower temperature and pneumoperitoneum all independently decrease body temperature. A decrease in body temperature decreases adhesion formation ($P = 0.004$). Therefore, at 37°C, pneumoperitoneum-enhanced adhesion formation is more important than at room temperature ($P = 0.04$). As was observed at room temperature, adhesion formation at 37°C increases with the duration ($P = 0.01$) of pneumoperitoneum and decreases with the addition of 3% of oxygen ($P = 0.03$). CONCLUSIONS: Hypothermia reduces pneumoperitoneum-enhanced adhesion formation, which supports hypoxia as a driving mechanism, since hypothermia decreases the toxic effects of hypoxia and of the ischaemia–reperfusion process. These data could open up new possibilities for adhesion prevention in laparoscopic surgery.

Key words: body temperature/desiccation/hypoxia/intraperitoneal adhesion formation/laparoscopy/pneumoperitoneum

Introduction

CO_2 pneumoperitoneum during laparoscopy can decrease body temperature, especially when cold and dry CO_2 gas at high flow rates is used (Bessell *et al.*, 1999). As can be expected from thermodynamics, this cooling effect is caused less by the gas temperature but mainly by the energy necessary to evaporate body water in order to humidify the dry CO_2 (Bessell *et al.*, 1995). Indeed, cooling cannot be prevented with warm and dry gas (Bessell *et al.*, 1995; Hazebroek *et al.*, 2002), whereas cooling can be prevented to a large extent by cold and humidified gas (Hazebroek *et al.*, 2002). Cooling can be fully prevented using warm and humidified gas, as shown in rats (Hazebroek *et al.*, 2002) and pigs (Bessell *et al.*, 1995, 1999; Mouton *et al.*, 1999).

Pneumoperitoneum with dry and cold CO_2 alters the morphology of the mesothelium, i.e. destroys the hexagonal pattern, reduces the microvilli (Hazebroek *et al.*, 2002) and bulges up the cells (Volz *et al.*, 1999; Suematsu *et al.*, 2001). Whether this can be prevented with warm and humidified gas is controversial (Mouton *et al.*, 1999; Hazebroek *et al.*, 2002). Anyway, since the introduction of high flow insufflators for endoscopic surgery in the human (Koninckx and Vandermeersch, 1991), the CO_2 used became progressively warmed and humidified. The use of warm and humidified gas was claimed to reduce post-operative pain and duration of hospitalization (Demco, 2001) and to reduce intraperitoneal cytokine response (Puttick *et al.*, 1999) and tumour growth (Nduka *et al.*, 2002).

Over the last years, CO_2 pneumoperitoneum became known as a cofactor in post-operative adhesion formation (Ordonez *et al.*, 1997), and several mechanisms seem to be involved. First, peritoneal hypoxia was suggested as a mechanism since the effect increased with duration of pneumoperitoneum and with insufflation pressure, since similar effects were observed with helium pneumoperitoneum and since the addition of 2–4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation (Molinas and Koninckx, 2000; Molinas *et al.*, 2001). This was supported recently by the observations that the partial pressure of oxygen in the abdominal wall was reduced during a pneumoperitoneum with CO_2 and with helium, whereas insufflation with a non-hypoxic gas mixture (80% CO_2 and 20% O_2) induced no changes (Wildbrett *et al.*, 2003). This pneumoperitoneum-induced hypoxia was also supported by the fact that this effect was absent in mice deficient for hypoxia-inducible

factor (HIF) (Molinas *et al.*, 2003b), plasminogen activator 1 (PAI-1) (Molinas *et al.*, 2003a), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Molinas *et al.*, 2003c). Secondly, a role for reactive oxygen species (ROS) in adhesion formation has been suggested (Binda *et al.*, 2003) since ROS activity increases during both laparotomy and laparoscopy, since they are produced during the ischaemia–reperfusion process and since the administration of ROS scavengers decreases adhesion formation in several animal models. Thirdly, other mechanisms could be involved such as cooling and desiccation.

During pneumoperitoneum, desiccation and cooling are intimately linked. First, desiccation is a key factor in cooling since at 37°C (body temperature), 577 cal are needed to vaporize 1 g of water, in contrast to only 1 cal needed to cool 1 g of water by 1°C and 0.2 cal to cool 1 g of CO_2 by 1°C. Secondly, desiccation will be more important at higher gas temperature since absolute humidity increases with temperature, e.g. relative humidity of 100% corresponds to 25 mg of water/l of gas at 25°C and to 44 mg of water/l of gas at 37°C. Therefore, in desiccation experiments to study adhesion formation, some cooling will always be evident unless temperature is strictly controlled.

Since the exact roles of desiccation and cooling in peritoneal damage and in adhesion formation have not yet been studied in detail, we therefore planned to evaluate in our laparoscopic mouse model the specific effect of cooling during CO_2 pneumoperitoneum upon adhesion formation.

Material and methods

The experimental set-up, i.e. animals, anaesthesia and ventilation, laparoscopic surgery, induction of intraperitoneal adhesions and scoring of adhesions, has been described in detail previously (Molinas *et al.*, 2001, 2003a,b,c; Elkelani *et al.*, 2002).

Animals

The study was performed in 86 female, Naval Medical Research Institute (NMRI), 9- to 10-week-old mice weighing 30–40 g. The animals were kept under standard laboratory conditions and they were fed with a standard laboratory diet with free access to food and water at any time. The study was approved by the Institutional Review Animal Care Committee.

Anaesthesia and ventilation

Animals were anaesthetized with i.p. pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium) with a dose of 0.08 mg/g. The abdomen was shaved and the animal was secured to the table in the supine position. Animals were intubated with a 20-gauge catheter and ventilated with a mechanical ventilator (Mouse Ventilator MiniVent, Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using non-humidified or humidified room air (according to the experiment) with a tidal volume of 250 µl at 160 strokes/min.

Laparoscopic surgery

A midline incision was performed caudal to the xyphoides appendix, a 2 mm endoscope with a 3.3 mm external sheath for insufflation (Karl Storz, Tüttlingen, Germany) was introduced into the abdominal cavity and the incision was closed gas tight around the endoscope in order to avoid leakage.

The pneumoperitoneum was created using the Thermoflator Plus (Karl Storz, Tüttlingen, Germany), which permits addition of a variable concentration of O_2 to the CO_2. Insufflation gas, humidification and temperature varied with the experimental design. For humidification, the Storz Humidifier 204320 33 (Karl Storz, Tüttlingen, Germany) was used.

Induction of intraperitoneal adhesions

After the establishment of the pneumoperitoneum, two 14-gauge catheters (Insyte-W, Vialon, Becton Dickinson, Madrid, Spain) were inserted under laparoscopic vision. Standardized 10 mm × 1.6 mm lesions were performed in the antimesenteric border of both right and left uterine horns and in both the right and left pelvic side walls with monopolar coagulation (10 W, standard coagulation mode, Autocon 350, Karl Storz, Tüttlingen, Germany).

The pneumoperitoneum was maintained for the minimum time needed to perform the surgical lesions, standardized at 10 min or for 60 min (basal and pneumoperitoneum-enhanced adhesion, respectively).

Scoring of adhesions

During laparotomy 7 days after the induction of adhesions, the adhesions were scored blind (of the group being evaluated) under microscopic vision using a qualitative and a quantitative scoring system. The qualitative scoring system assessed: extent (0, no adhesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of the injured surface involved, respectively), type (0, no adhesions; 1, filmy; 2, dense; 3, capillaries present), tenacity (0, no adhesions; 1, easily fall apart; 2, require traction; 3, require sharp dissection) and total (extent + type + tenacity). The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: (sum of the length of the individual attachments/length of the lesion) × 100. The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were scored individually.

Environmental and animal temperatures

To control temperature, animals and equipment, i.e. insufflator, humidifier, water valve, ventilator and tubing, were placed either at 23–25°C (room temperature) or in a closed chamber maintained at 37°C (heated air, WarmTouch, Patient Warming System, model 5700, Mallinckrodt Medical, Hazelwood, MO). The insufflation gas temperature was determined by the environmental temperature, i.e. either at room temperature or at 37°C. Indeed, previous experiments showed equilibration of the gas temperature with environmental temperature after some 50 cm of tubing with 7 mm inner diameter and a flow rate of 2.5 l/min.

The temperature of the environment was measured with Testo 645 (Testo N.V./S.A., Lenzkirch, Germany), whereas the temperature of the animal was measured via the rectum with the Hewlett Packard 78353A device (Hewlett Packard, Böblingen, Germany) and recorded every 10–20 min.

Experimental design

The time of anaesthesia injection was considered time 0 (T_0). The animal preparation and ventilation started after 10 min (T_{10}). The pneumoperitoneum started at 20 min (T_{20}) and was maintained for 10 min (T_{20}–T_{30}) or 60 min (T_{20}–T_{80}). Ventilation always finished at T_{80}.

In experiment I ($n = 32$), basal and pneumoperitoneum-enhanced adhesion formation, together with body temperature, were evaluated in mice placed either at room temperature or at 37°C. Non-humidified CO_2 was used for the pneumoperitoneum and special care was taken to have a gas tight seal around the trocar in order to avoid any flow through the abdominal cavity and thus minimize desiccation. Ventilation was performed with non-humidified air (four groups, $n = 8$ per group).

In experiment II ($n = 6$), the effect of ventilation with or without humidified air upon body temperature was evaluated in mice placed at 37°C during 60 min of humidified CO_2 pneumoperitoneum (two groups, $n = 3$ per group).

In experiment III ($n = 48$), the effect of body temperature during humidified pneumoperitoneum upon adhesion formation was evaluated. To achieve a body temperature with minimal cooling, i.e. ~37°C, mice were placed at 37°C and ventilated with humidified air. To achieve a slightly lower body temperature, i.e. ~36°C, mice were placed at 37°C and ventilated with non-humidified air. To achieve a body temperature of some 32°C, mice were placed alternately at room temperature (T_0–T_{20}, T_{30}–T_{40}, T_{50}–T_{60} and T_{70}–T_{80}) and at 37°C (T_{20}–T_{30}, T_{40}–T_{50} and T_{60}–T_{70}) and ventilated with humidified air. These settings were determined based on previous experiments. Pneumoperitoneum-enhanced adhesion formation was evaluated using pure CO_2 in mice at 37°C (group I), 36°C (group II) and 32°C (group III). Pneumoperitoneum-enhanced adhesion formation at 37°C was also evaluated using CO_2 with 3% oxygen (group IV) and 12% oxygen (group V). Simultaneously, basal adhesion formation was evaluated using pure CO_2 (group VI). A flow of 23 ml/min through the abdominal cavity was used in all the groups (six groups, $n = 8$ per group).

Statistics

Statistical analyses were performed with GraphPad Prism version 4 (GraphPad Software Inc., San Diego CA). The Mann–Whitney test was used to compare adhesion formation between individual groups. Intergroup differences in body temperature were evaluated with two-way ANOVA. Linear regression and Pearson correlation were used to analyse adhesions and body temperature data. All data are presented as the mean ± SEM.

Results

In experiment I (Figure 1, Table I), during anaesthesia and ventilation only (T_0–T_{20}), body temperature decreased from some 36.5 to 31°C and from 37.5 to 35°C at room temperature and 37°C, respectively. At room temperature, body temperature further decreased to 28.5 and to 26.5°C at T_{80} in mice with 10 and 60 min of pneumoperitoneum, respectively. At 37°C, body temperature remained constant at some 35.5 and 34.5°C up to T_{80} for 10 and 60 min of pneumoperitoneum, respectively. Overall, body temperatures were always lower after 60 min of pneumoperitoneum than after 10 min, ie. both at room temperature ($P < 0.0001$) and at 37°C (P = NS); body temperatures also were always lower at room temperature than at 37°C, i.e. after both 10 min ($P < 0.0001$) and 60 min ($P < 0.0001$) of pneumoperitoneum (two-way ANOVA). At room temperature, adhesion formation increased with the duration of pneumoperitoneum (proportion, $P < 0.05$). At 37°C, this effect of duration of pneumoperitoneum was more pronounced (proportion, $P = 0.01$; total, $P = 0.04$; extent, $P = 0.02$; type, $P = 0.03$). In addition, at 37°C, adhesion formation was higher than at room temperature, clearly for pneumoperitoneum-enhanced adhesions (proportion, $P = 0.04$; total, $P < 0.05$; extent, $P = 0.03$) and slightly for basal adhesions (P = NS) (Mann–Whitney test).

In experiment II, body temperatures were some 1°C higher when humidified air was used for ventilation ($P = 0.003$, two-way ANOVA), being 38.1 ± 0.1 (T_0), 36.4 ± 0.1 (T_{10}), 35.9 ± 0.3 (T_{20}), 36.2 ± 0.5 (T_{30}), 36.5 ± 0.6 (T_{40}), 36.5 ± 0.6 (T_{50}), 36.8 ± 0.5 (T_{60}), 37.0 ± 0.5 (T_{70}) and 37.1 ± 0.5 (T_{80})°C for humidified ventilation, and 37.8 ± 0.4 (T_0), 36.1 ± 0.1 (T_{10}), 35.0 ± 0.4 (T_{20}), 35.3 ± 0.5 (T_{30}), 35.4 ± 0.5 (T_{40}), 35.8 ± 0.7 (T_{50}), 35.7 ± 0.6 (T_{60}), 35.6 ± 0.5 (T_{70}) and 36.1 ± 0.5 (T_{80})°C for non-humidified ventilation.

In experiment III (Figure 2), during anaesthesia and ventilation only, body temperature decreased to 35.5°C for groups I and II and to 31°C for group III at T_{20}. Afterwards, body temperature remained constant till T_{80} at some 37°C for

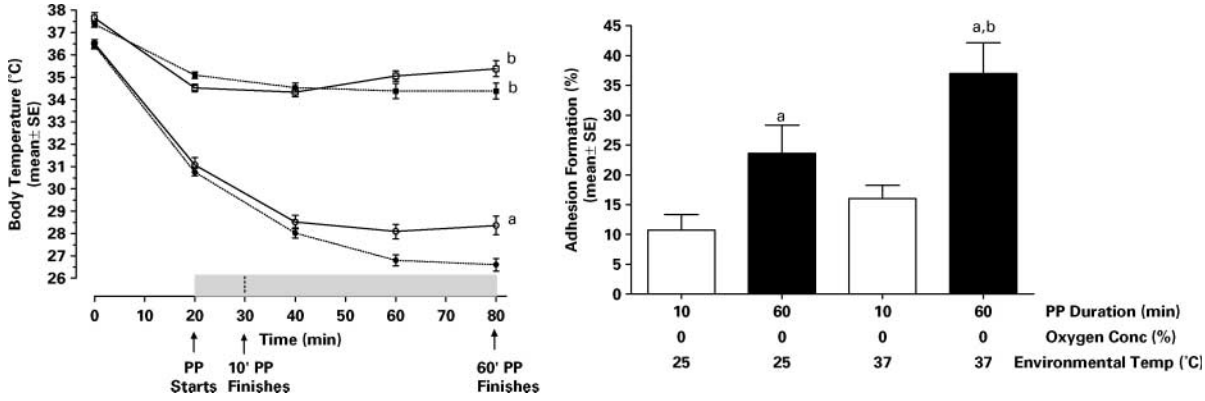

Figure 1. Effect of environmental temperature upon body temperature (left) and adhesion formation (right) in mice. Basal and pneumoperitoneum (PP)-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure and mice were kept either at room temperature or at 37°C. Symbols: ○, 10 min PP, room temperature; • 60 min PP, room temperature; □ 10 min PP, 37°C; ■ 60 min PP, 37°C, $P < 0.05$: [a]10 versus 60 min at room temperature or at 37°C, [b]room temperature versus 37°C at 10 or at 60 min (two-way ANOVA for temperature and Mann–Whitney test for adhesion formation).

Table I. Effect of environmental temperature upon adhesion formation in mice

Environmental temperature	Pneumoperitoneum		Adhesion scores (mean ± SE)			
	Oxygen	Duration	Extent	Type	Tenacity	Total
25 °C	0%	10 min	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.9 ± 0.4
	0%	60 min	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	2.9 ± 0.4
37 °C	0%	10 min	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	2.8 ± 0.3
	0%	60 min	1.8 ± 0.2[a,b]	1.5 ± 0.2[a]	1.3 ± 0.2	4.5 ± 0.6[a,b]

Adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure.
[a]10 versus 60 min at room temperature (25°C) or 37°C, $P < 0.05$.
[b]Room temperature versus 37°C, 10 or 60 min $P < 0.05$, Mann–Whitney test.

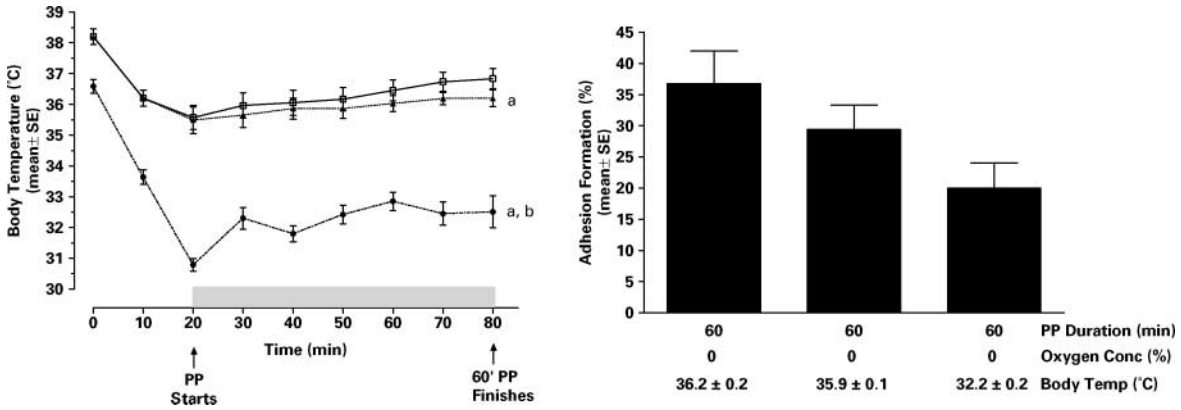

Figure 2. Effect of body temperature (left) upon adhesion formation (right) in mice. Pneumoperitoneum (PP)-enhanced adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure. Symbols: □ group I, ▲ group II, • group III. The mean ± SE of body temperature during T_{20}–T_{80} is indicated on the adhesion graph. $P < 0.05$: [a]versus group I, [b]versus group II (two-way ANOVA for temperature), $P = 0.02$ (Pearson correlation, for adhesions).

group I, 36°C for group II and 32.5°C for group III (group I versus III, $P < 0.0001$; II versus III, $P < 0.0001$; and I versus II, $P = 0.02$). Body temperatures of groups IV, V and VI were similar to those of group I (P = NS, data not shown, two-way ANOVA). Adhesion formation after 60 min of pure CO_2 pneumoperitoneum (groups I, II and III) decreases with body temperature (proportion, $P = 0.02$ Pearson correlation, Figure 2, Table II). In mice with body temperature of 37°C (Figure 3, Table II), adhesion formation increased with the duration of pneumoperitoneum (proportion, $P = 0.04$; total, $P = 0.02$; extent, $P = 0.04$; type, $P =$ NS; tenacity, $P = 0.04$). In comparison with pure CO_2 (group I), the addition of 3% oxygen to the pneumoperitoneum (group IV) decreased adhesion formation (proportion, $P = 0.03$; total, $P = 0.04$; extent, $P < 0.05$; type, $P =$ NS; tenacity, $P =$ NS), whereas no differences were observed with the addition of 12% oxygen (group V).

To evaluate the effect of body temperature upon adhesion formation, data of experiments I and III were combined (Figure 4). Taking all data together, pneumoperitoneum-enhanced adhesion formation decreased with lower body temperatures (proportion, $P = 0.004$; total, $P = 0.02$, linear regression and Pearson correlation).

Discussion

This study confirmed and extended previous data concerning the effects of anaesthesia, ventilation and pneumoperitoneum upon body temperature.

We confirmed that anaesthesia decreases the body temperature of mice, as demonstrated previously in rats (Torbati *et al.*, 2000), mice (Gardner *et al.*, 1995) and humans (Buhre and Rossaint, 2003). As expected, this cooling effect is more pronounced at room temperature than at 37°C. Patients remain normothermic after anaesthesia when they are kept in a warmer operating room, whereas they become hypothermic in a colder operating theatre (Morris and Wilkey, 1970; Morris, 1971a,b). This pure anaesthetic side effect is caused by cutaneous vasodilatation, which abolishes heat conservation. Consequently, anaesthetized subjects become poikilothermic and their body temperature varies with environmental temperatures (Morris, 1971b).

This study demonstrated in mice that non-humidified ventilation can decrease body temperature, confirming previous data in humans (Dery, 1973; Fonkalsrud *et al.*, 1980; Bissonnette and Sessler, 1989). Since unsaturated air will absorb water by evaporation from a wet surface (Williams *et al.*, 1996), water loss from the respiratory airways is the most plausible explanation.

We confirmed that pneumoperitoneum causes cooling. Since non-humidified gas at higher flow rates causes important cooling due to desiccation, we took great care to prevent desiccation as much as possible by avoiding any flow through the abdomen (experiment I) and by humidifying the gas (experiments II and III). It is difficult, however, to rule out completely at least some desiccation by the pneumoperitoneum since gas-tight seals of the trocar insertions are

Table II. Effect of body temperature, duration of the pneumoperitoneum and addition of oxygen upon adhesion formation in mice

Group	Body temperature[b] (mean ± SE)	Pneumoperitoneum		Adhesion scores (mean ± SE)			
		Oxygen	Duration	Extent	Type	Tenacity	Total
I	36.2 ± 0.2°C	0%	60 min	1.7 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	4.4 ± 0.4
II	35.9 ± 0.1°C	0%	60 min	1.4 ± 0.2	1.2 ± 0.3	1.2 ± 0.2	3.7 ± 0.6
III	32.2 ± 0.2°C	0%	60 min	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.2	3.1 ± 0.5
IV	36.4 ± 0.1°C	3%	60 min	1.1 ± 0.2[a]	1.1 ± 0.2	1.1 ± 0.1	3.2 ± 0.4[a]
V	36.7 ± 0.1°C	12%	60 min	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	4.1 ± 0.5
VI	36.4 ± 0.1°C	0%	10 min	0.9 ± 0.2[a]	0.7 ± 0.2	0.8 ± 0.2[a]	2.4 ± 0.6[a]

Adhesions were induced during laparoscopy at 20 cm H_2O insufflation pressure.
[a]$P < 0.05$ versus group I, Mann–Whitney test.
[b]Body temperature during T_{20}–T_{80} is indicated.

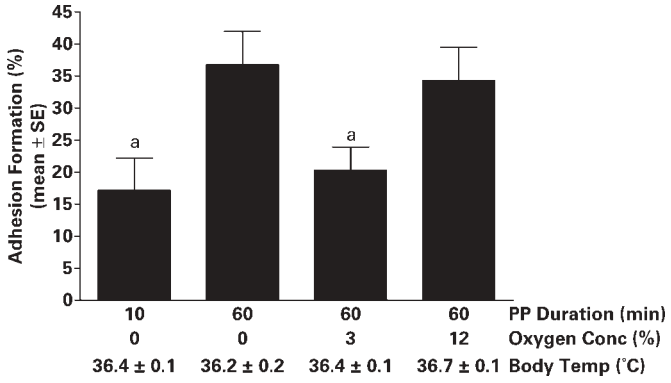

Figure 3. Effect at 37°C of duration of pneumoperitoneum (PP) and adding oxygen upon adhesion formation in mice. Basal (group VI) and PP-enhanced adhesions (groups I, IV and V) were induced during laparoscopy at 20 cm H_2O insufflation pressure with CO_2 containing 0% (group I and VI), 3% (group IV) or 12% oxygen (group V). The mean ± SE of body temperature during T_{20}–T_{80} is indicated. $P < 0.05$: [a]versus group I (Mann–Whitney test).

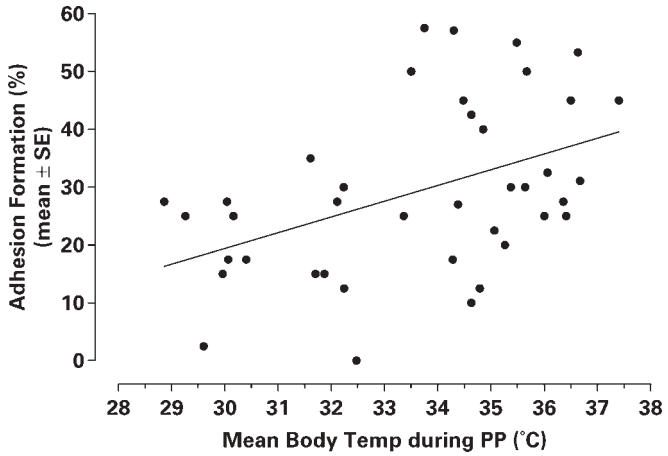

Figure 4. Relationship between body temperature and adhesion formation. Individual values of the mean of body temperature between T_{20} and T_{80} with their respective proportion of adhesions are depictured for pneumoperitoneum-enhanced adhesion for experiments I and III. $P = 0.004$ (Pearson correlation).

extremely difficult to obtain and since any relative humidity $<100\%$ will cause some desiccation. This might explain why 60 min of pneumoperitoneum causes slightly more cooling than 10 min. These data obviously do not yet permit us to rule out other simultaneous effects such as vascular compression and reduced circulation.

These experiments clearly demonstrated that hypothermia reduces adhesion formation. Not only do pneumoperitoneum-enhanced adhesions increase with the body temperature, but also the differences between basal and pneumoperitoneum-enhanced adhesion formation increase with body temperature. Previous reports dealing with the relationship between body temperature and adhesion formation are scanty. In humans, local hypothermia after laparotomy was reported to decrease the inflammatory reaction and to increase intestinal peristalsis, thus decreasing adhesion formation (Gataullin *et al.*, 1971). In rats, irrigation with saline at >37°C, i.e. between 37 and 60°C, increases adhesion formation (Kappas *et al.*, 1988). These observations support our hypoxia hypothesis as the driving mechanism. First, hypothermia could directly protect tissues and cells from the pneumoperitoneum-induced hypoxia, since oxygen consumption by cells decreases with temperature. Hypothermia decreases the global cerebral metabolic rate during ischaemia, slowing the breakdown of glucose, phosphocreatine and ATP and the formation of lactate and inorganic phosphate (Erecinska *et al.*, 2003). Secondly, pneumoperitoneum-enhanced adhesion formation can be considered as an ischaemia–reperfusion process. Hypothermia reduces the production of ROS during reperfusion in brain (Zhao *et al.*, 1996), forebrain (Horiguchi *et al.*, 2003), heart (Prasad *et al.*, 1992), gut (Attuwaybi *et al.*, 2003), endothelium (Zar and Lancaster, 2000) and muscle (Yoshioka *et al.*, 1992). Hypothermia improves recovery of energetic parameters during reperfusion (Erecinska *et al.*, 2003). Hypothermia also suppresses the inflammatory response after hepatic ischaemia–reperfusion, decreasing the infiltration of polymorphonuclear cells (Patel *et al.*, 2000), and the production of tumour necrosis factor-α, interleukin-1β and macrophage inflammatory protein-2 also decreases (Patel *et al.*, 2000; Kato *et al.*, 2002).

The relationship between desiccation and cooling is complex, since desiccation causes cooling whereas desiccation is more important at higher gas temperature. This might explain why the effect upon adhesion formation can be variable if both desiccation and cooling are not strictly controlled. Therefore, experiments evaluating the effect of desiccation

might underestimate the effect since desiccation decreases temperature which itself reduces desiccation.

We confirmed and extended to 37°C our previous observations at room temperature which showed that the addition of 3% oxygen to the pneumoperitoneum decreased pneumoperitoneum-enhanced adhesion formation, and that the addition of 12% oxygen in comparison with 3% oxygen increases adhesion formation to a similar level as with pure CO_2 (Molinas and Koninckx, 2000; Molinas *et al.*, 2001).

In summary, this study confirms that environmental temperature, anaesthesia, ventilation and pneumoperitoneum all influence the body temperature of mice. The most important observation is that hypothermia decreases pneumoperitoneum-enhanced adhesion formation. This could be due to prevention of the toxic effects caused by hypoxia and/or the ischaemia–reperfusion process. Other effects, i.e. reduction of the inflammatory response and an increase of intestinal peristaltic movements, cannot be excluded. Whether basal adhesions, i.e. without a pneumoperitoneum, are also decreased by lower temperatures is still uncertain since, in our model, the group of 'basal adhesions' still had 10 min of pneumoperitoneum. Before being extrapolated to the human, the data obviously need to be confirmed in larger animals. The concept that the decrease of the intraperitoneal temperature by a few degrees could prevent adhesion formation remains very attractive.

Acknowledgements

We thank Professor R.Pijnenborg, S.Caluwaerts, S.Lambin, S.Al-Nasiry, R.Van Bree, M.Craessaerts (Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg) and Paul Hanson (Fisher & Paykel Healthcare Ltd, Auckland, New Zealand) for their help. The technical support department of the University Hospital Gasthuisberg is acknowledged for the design of the 37°C chamber and Karl Storz Endoscopy for generous supply of the laparoscopic equipments. This study was partially supported by Fonds Wetenschappelijk Onderzoek, Brussels, Belgium grant No. G.0324.01 and Onderzoeks Toelagen Katholieke Universiteit Leuven, Leuven, Belgium grants Nos TBA/00/27 and OT/03/51.

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Submitted on May 13, 2004; accepted on August 2, 2004

Addendum 5

Binda MM, Molinas CR, Hansen P and Koninckx PR. Effect of desiccation and temperature during laparoscopy on adhesion formation in mice *Fertility & Sterility* 86(1):166-175, 2006.

Effect of desiccation and temperature during laparoscopy on adhesion formation in mice

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Objective: To investigate the effects of desiccation (without cooling) and of oversaturation of the pneumoperitoneum on adhesion formation.
Design: Prospective randomized trial.
Setting: Academic research center.
Animal(s): BALB/c and NMRI female mice.
Intervention(s): The effect of desiccation using nonhumidified CO_2 on adhesion formation was evaluated in a laparoscopic mouse model. Body temperature (BT) was maintained at 37°C using a homeothermic blanket. In addition to controls without desiccation, the effect of both hypothermia and desiccation on adhesion formation was evaluated. Subsequently the effect of oversaturating the pneumoperitoneum using a high energy gas to avoid any desiccation was studied.
Main Outcome Measure(s): During surgery BT, pneumoperitoneum temperature, and relative humidity were monitored. Adhesions were scored after 7 days.
Result(s): Adhesions increased with increasing levels of desiccation when BT was kept at 37°C. This was prevented with humidified gas. If BT decreased, adhesions were fewer. Oversaturating the pneumoperitoneum increased adhesions due to high energy gas causing an increase in both BT and pneumoperitoneum temperature.
Conclusion(s): Adhesions increase with desiccation and decrease when BT is reduced. Adhesions are minimized when humidified gas is used. Since desiccation is associated with cooling, its effect is generally underestimated because of the counterbalance with cooling. The concept of combining controlled intraperitoneal cooling with a rigorous prevention of desiccation might be important for clinical adhesion prevention. (Fertil Steril® 2006;86: 166–75. ©2006 by American Society for Reproductive Medicine.)

Key Words: Body temperature, desiccation, hypothermia, hypoxia, intraperitoneal adhesion formation, laparoscopy, pneumoperitoneum, humidification

The CO_2 pneumoperitoneum has become known as a cofactor in postoperative adhesion formation (1) and several mechanisms seem to be involved. First, peritoneal hypoxia was suggested as a mechanism, as adhesion formation increased with insufflation pressure and with duration of pneumoperitoneum, as similar effects were observed with CO_2 and helium pneumoperitoneum, and as the addition of 2%–4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation (2–4). This hypothesis was supported by the observation that the partial pressure of oxygen in the abdominal wall is reduced during CO_2 or helium pneumoperitoneum (5). In addition, pneumoperitoneum-enhanced adhesion formation was absent in mice deficient for genes encoding for factors upregulated by hypoxia, such as hypoxia inducible factors (6), vascular endothelial growth factor and placental growth factor (7), and plasminogen activator 1 (8).

Second, the pneumoperitoneum induces ischemia at the time of insufflation and reperfusion at the time of deflation. Pneumoperitoneum-enhanced adhesion formation thus could be the consequence of an ischemia–reperfusion process with a role of reactive oxygen species (ROS) (9). This ischemia–reperfusion hypothesis is supported by a reduced adhesion formation after the administration of ROS scavengers in several animal models (10–15).

A third mechanism is peritoneal temperature. We recently demonstrated that adhesion formation is less when body temperature is lower (16). This indirectly supports the previous hypotheses—hypothermia decreases the toxic effects of hypoxia and of the ischemia–reperfusion process, suppressing the inflammatory response (17–25).

Finally, desiccation has been claimed to enhance adhesion formation, although clear experimental evidence is lacking. Dry and cold gas for the pneumoperitoneum not only induces desiccation (26), but also is deleterious for the peritoneum,

Received January 18, 2005; revised and accepted November 29, 2005.
Supported by Onderzoeks Toelagen Katholieke Universiteit Leuven (Leuven, Belgium grant TBA/00/27 and in part by Karl Storz Endoscopy (Tuttlingen, Germany).
Part of these results were presented at the 13th Annual Congress of the European Society for Gynecological Endoscopy, which was held in Cagliari, Sardinia, Italy, on October 14–17, 2004, and was awarded with the "R. Palmer Prize" for the best oral presentation.
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0015-0282/06/$32.00
doi:10.1016/j.fertnstert.2005.11.079

altering the morphology of the mesothelium, destroying the microvilli, and bulging up the cells with exposure of the basal lamina (27–30).

Desiccation in the abdominal cavity will inevitably occur whenever the gas entering the peritoneal cavity is not fully saturated at the intraperitoneal temperature, normally 37°C. The peritoneum has a large surface with a thin serous fluid layer facilitating humidification of the pneumoperitoneum gas. Desiccation can be locally aggravated by a jet stream of CO_2 forcing tissue surfaces apart and exposing directly the tissue surfaces to this stream of gas (26).

Desiccation requires high amounts of energy and thus is associated with cooling. Quantitatively, 577 cal is needed to vaporize 1 mL of water at 37°C, whereas only 0.00003 cal is needed to heat 1 mL of CO_2 by 1°C (31). The caloric equivalent of heating cold dry gas is thus very small in comparison with the effect of vaporization. This cooling effect of desiccation in the airways during ventilation (16, 32–34) and in the abdomen during both open (35) and laparoscopic (36) surgery has been well documented. As expected, the cooling observed during laparoscopy with cold and dry gas can be fully prevented using warm and humidified gas (27, 28, 36) but not warm and dry gas (27, 37). Desiccation quantitatively depends on the volume of gas to be humidified, and thus increases tremendously when a continual supply of gas through the abdominal cavity occurs (e.g., due to leaks).

The loss of water content from the serous fluid, moreover, increases the osmolarity of the fluid, causing an osmotic imbalance between the intracellular and the extracellular space of the mesothelial cells. This then causes fluid of the intracellular space to diffuse through the cell membrane to equalize the osmotic imbalance. This mechanism then dehydrates the cell, leading to desiccation and trauma of the cell (27–30), resulting in a peak inflammatory response (38, 39).

Desiccation and cooling, two intimately linked processes, have opposite effects on adhesion formation, the former increasing (widely accepted but not proven) and the latter decreasing (16) adhesions. Therefore, the aim of this study was, first, to confirm that desiccation increases adhesion formation and to quantify this effect when the associated cooling was prevented. Second, the effect of avoiding completely desiccation by insufflating oversaturated gas turned out to be predominantly an experiment of increasing the intra-abdominal temperature due to the condensation.

MATERIALS AND METHODS

The Laparoscopic Mouse Model for Adhesion Formation

Experimental setup, that is, animals, anesthesia and ventilation, laparoscopic surgery, induction, and scoring of intraperitoneal adhesions (Fig. 1), has been described in detail previously (3, 4, 6–8, 16, 40).

Animals In the oversaturation experiment, 10-week-old female Naval Medical Research Institute (NMRI) mice weighing 25–35 g were used as in previous experiments. In the desiccation experiment, 10-week-old female BALB/c mice weighing 19–21 g were used. After it had become clear that the interanimal variability was much less in this inbred strain, whereas the adhesion formation was similar than in NMRI mice (41), we decided to use this strain for further experiments.

Animals were kept under standard laboratory conditions and they were fed with a standard laboratory diet with free access to food and water. The study was approved by the Institutional Review Animal Care Committee.

Anesthesia and Ventilation Mice were anesthetized with intraperitoneal (IP) 0.08 mg/g pentobarbital, intubated with a 20-gauge catheter and mechanically ventilated (Mouse Ventilator MiniVent, type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using humidified room air with a tidal volume of 250 μL at 160 strokes/min. Humidified air for ventilation was used to prevent cooling, as occurs during ventilation with nonhumidified air (16).

Laparoscopic Surgery A midline incision was performed caudal to the xyphoides, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tüttlingen, Germany) was introduced into the abdominal cavity, and the incision was closed gas tight around the endoscope to avoid leakage.

The pneumoperitoneum was created with the Thermoflator Plus (Karl Storz) using humidified or nonhumidified insufflation gas.

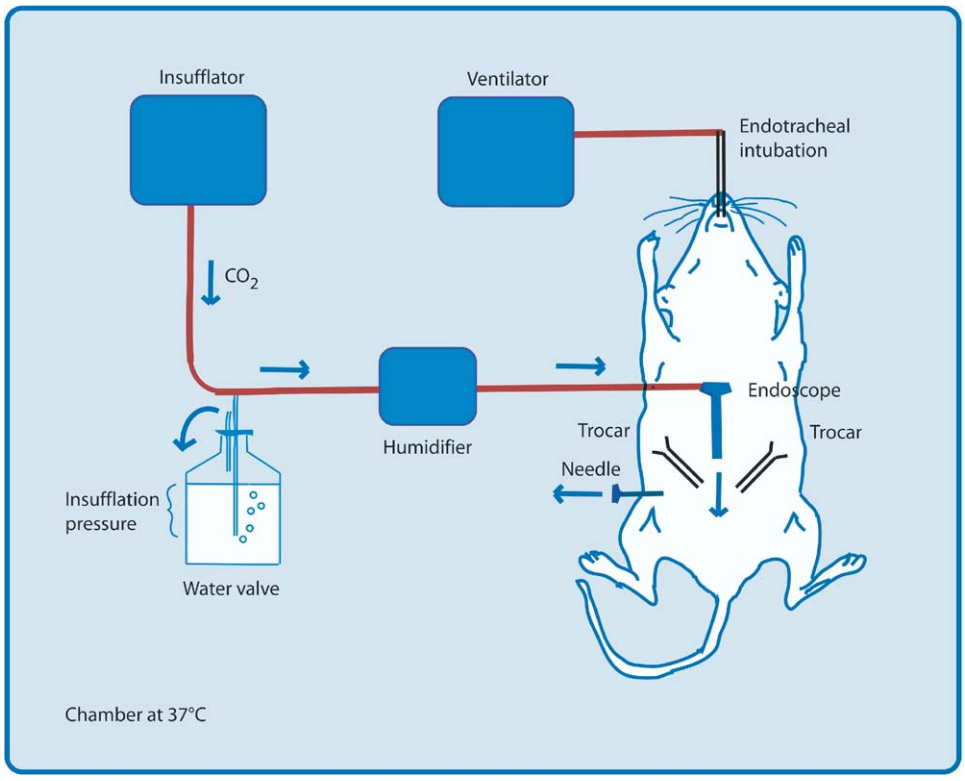
Induction of Intraperitoneal Adhesions After the establishment of the pneumoperitoneum, two 14-gauge catheters were inserted under laparoscopic vision. Standardized 10- by 1.6-mm lesions were performed in the antimesenteric border of both right and left uterine horns and pelvic sidewalls with bipolar coagulation (BICAP, bipolar hemostasis probe, BP-5200A, 5 Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (standard coagulation mode, Autocon 200, Karl Storz).

Because previous data indicate that adhesion formation increases with the duration of the pneumoperitoneum (3), pneumoperitoneum-enhanced adhesion formation was evaluated by maintaining the pneumoperitoneum for 60 minutes.

Scoring of Adhesions Adhesions were qualitatively and quantitatively scored, blindly (the investigator was not informed of the group being evaluated) under microscopic vision during laparotomy 7 days after their induction. The qualitative scoring system assessed as follows: extent (0: no adhesions; 1: 1%–25%; 2: 26%–50%; 3: 51%–75%; 4: 76%–100% of the injured surface involved), type (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), tenacity (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection), and total (extent + type + tenacity). The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: adhesion (%) =

FIGURE 1

Laparoscopic mouse model.



Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

(sum of the length of the individual attachments/length of the lesion) × 100. The results are presented as the average of the adhesions formed at the four sites (right and left visceral and parietal peritoneum), which were individually scored.

Setup and Design of the Experiments

Environmental Temperature To control animal and gas temperature, animals and equipment (i.e., insufflator, humidifier, water valve, ventilator, and tubing) were placed in a closed chamber maintained at 37°C with heated air (WarmTouch, Patient Warming System, model 5700, Mallinckrodt Medical, Hazelwood, MO).

Body and Pneumoperitoneum Temperature and Pneumoperitoneum Relative Humidity Animal body temperature was continuously monitored in the rectum (Hewlett Packard 78353A, Hewlett Packard, Böblingen, Germany) and registered every 10 minutes. Pneumoperitoneum temperature and relative humidity (RH) were measured with the Testo 645 device and a 4-mm probe (Testo N.V./S.A., Lenzkirch, Germany) introduced in the abdomen. Due to the size of this probe, measurements were not done systematically in the same experiments performed to induce adhesions.

Because desiccation or vaporization requires 577 cal/mL of water and thus produces cooling, the mice could not maintain their body temperature at 37°C during desiccation experiments, notwithstanding the box heated to 37°C. Therefore, to evaluate the pure effect of desiccation without cooling, keeping mouse body temperature at 37°C, an additional heating system had to be used (i.e., the homeothermic Blanket System; Harvard Apparatus LTD, Edenbridge, UK). This system includes a small rectal probe for continuous temperature monitoring and a heating blanket to provide sufficient heat for accurate control of mouse body temperature, both connected to a control unit. The control unit varies the current flowing through the heating blanket in an inversely proportional manner to the temperature monitored by the temperature probe.

Desiccation and Humidification of Pneumoperitoneum To induce desiccation, a controlled flow of nonhumidified CO_2 was obtained using 26- and 22-gauge needles, which at 15 mm Hg insufflation pressure induced a 23- or 100-mL/min flow of CO_2 gas through the abdominal cavity, respectively. Without a needle, in the absence of any leak, no flow through the abdominal cavity occurred.

To humidify the insufflated gas two types of humidifiers were used. For the desiccation experiment, the Storz Humidifier (204320 33, Karl Storz) and the 37°C chamber were used, in which CO_2 at 37°C and nearly 100% RH can be

obtained (this was measured in pilot studies). For the oversaturation experiment, the insufflation humidifier MR860 (Fisher & Paykel Healthcare Ltd, Auckland, New Zealand) was used to avoid any desiccation by oversaturation. This newly developed humidifier permits "oversaturation" of the CO_2, with some condensation in the peritoneal cavity. By varying the temperature in the humidification chamber, discrete levels of absolute humidity can be obtained (42). To prevent condensation between the humidifier and the animal or trocar, the tubing heats the CO_2 gas temperature above the dew point of the gas, using an internal heating wire. With entrance into the peritoneal cavity, the CO_2 will cool to 37°C, and if the absolute humidity is above 44 mg/L condensation will occur. In the oversaturation experiment, the humidifier was used at discrete levels of humidification, which, expressed relative to body temperature saturated (BTS) conditions (37°C, 100% RH, i.e., 44 mg water/L CO_2), corresponded to 0%, 75% (33 mg water/L), 100% (44 mg water/L), and 125% (55 mg water/L) BTS. For the dry group or 0% BTS, the same humidifier was used but the humidification chamber was not filled with water.

Experimental Design Because anesthesia and ventilation can influence body temperature, the timing was strictly controlled. The time of the anesthesia injection was considered time 0 (T_0). The animal preparation and ventilation started after exactly 10 minutes (T_{10}). The pneumoperitoneum started at 20 minutes (T_{20}) and was maintained for 60 minutes until T_{80}.

Two sets of experiments were performed. Historically, the oversaturation experiment was done first and later the desiccation experiment. Because it is easier and more logical to present the desiccation experiment first and subsequently the oversaturation experiment, we deliberately chose to describe throughout the article, first, the effect of desiccation without cooling and subsequently the effect of oversaturating the insufflation gas. In each experiment the measurement of temperature and humidification and the evaluation of adhesion formation were done in different mice to avoid any influence of the temperature and humidification measurements on adhesion formation.

In the desiccation experiment, desiccation was induced using nonhumidified CO_2 for the pneumoperitoneum at flows of 23 mL/min (group II) and 100 mL/min (group III) through the abdominal cavity. Two control groups with minimal desiccation were used: the first with no flow of nonhumidified gas (group I) and the second with a flow of 100 mL/min of humidified gas (group IV). Because desiccation decreases body temperature, a homeothermic blanket was used to keep body temperature strictly at 37°C. As a control for the effect of the homeothermic blanket on temperature and adhesion formation, a group of animals was treated with a flow of 100 mL/min of nonhumidified gas and without the homeothermic blanket (group V).

In the desiccation experiment, first body temperature, pneumoperitoneum temperature, and RH were measured, and the difference between peritoneum and body temperatures (δT = peritoneum − body temperature) was calculated (5 groups, n = 3/group). Subsequently, the effect of desiccation, without the associated decrease in body temperature, was evaluated on adhesion formation (5 groups, n = 56). A total of nine animals per group was planned. In group I, however, intended to have no flow through the abdominal cavity, an important leakage around the port sites occurred in four animals and this resulted in a dry abdominal wall and hypothermia, despite of the use of the homeothermic blanket. Because the degree of desiccation could not be estimated, these mice were immediately replaced during the experiments without changing the randomization order to have the required number of animals with temperature at 37°C. Also in groups II and III, a leakage occurred in two and five mice, respectively, and these mice could not maintain their body temperature at 37°C notwithstanding the homeostatic blanket. These mice also were replaced during the experiment without changing the randomization order, as the aim of this study was to maintain body temperature.

In the oversaturation experiment, the effect of oversaturating the CO_2 with some condensation (to avoid any desiccation) was analyzed. First, body temperature, pneumoperitoneum temperature, and RH were evaluated using nonhumidified CO_2 (group I), and humidified CO_2 corresponding to 75% (group II), 100% (group III), and 125% BTS (group IV), respectively (4 groups, n = 3 per group). Subsequently, the effect of oversaturating the CO_2 on adhesion formation was evaluated using the same discrete levels of humidification (4 groups, n = 10 per group).

Statistics

Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC) and the GraphPad Prism (GraphPad Software Inc., San Diego, CA). Differences in body temperature were evaluated with two-way ANOVA. Differences between pneumoperitoneum and body temperatures were evaluated with Proc Univariate. Differences in adhesion formation were evaluated with Wilcoxon test for the univariate analysis and with General Linear Methods (proc GLM) for the multivariate analysis to evaluate simultaneously the effect of flow and body temperature. All data are presented as the mean ± standard error of the mean (SE).

RESULTS

In the desiccation experiment, the heating blanket kept body temperature constant at 37.5°C in groups I, II, and III throughout the experiment (between T_{20} and T_{80}) without intergroup differences (data not shown). In group IV body temperature increased up to 39°C and was higher than in groups I ($P<.0001$), II ($P<.0001$), and III ($P<.0001$). In group V body temperature decreased progressively to 31°C and was lower than in groups I ($P<.0001$), II ($P<.0001$), III ($P<.0001$), and IV ($P<.0001$) (two-way ANOVA).

The differences between peritoneum and body temperatures (δT) measured after an equilibration period (T_{40}) were not significant (Proc Univariate) except for group IV ($P=.03$), being 0.2; ± 0.1°C, −0.5 ± 0.3°C, −0.6 ± 0.4°C, 0.6 ± 0.1°C, and 0.5 ± 0.2°C for groups I, II, III, IV, and V, respectively. The RH of the pneumoperitoneum remained 100% in all groups throughout the experiment, also when nonhumidified CO_2 was used for insufflation reflecting the high humidification capacity of the peritoneal cavity up to the end of the experiment (data not shown).

In the desiccation experiment, adhesion formation was first evaluated in the mice that maintained their body temperature at 37°C (n = 9 per group). Desiccation without affecting body temperature increased adhesion formation (Fig. 2, Table 1). In comparison with group I, adhesion formation increased slightly in group II (P = not significant [NS]) and significantly in group III (proportion: $P=.01$, total: $P=.01$, extent: $P=.02$, type: $P=.04$, tenacity: $P=.05$, Wilcoxon test). As expected, this increase in adhesion formation was prevented by using humidified gas (group IV vs. III, proportion: $P=.004$, total: $P=.01$, extent: $P=.01$, type: $P=.01$, tenacity: $P=.01$). Hypothermia decreased adhesion formation caused by desiccation (group V vs. group III, proportion: $P=.01$, total: $P=.01$, extent: $P=.01$, type: $P=.02$, tenacity: $P=.04$), although not completely up to the level of the group with no desiccation (group I), possibly a consequence of the slightly higher temperature. Unexpectedly, comparing with group IV adhesion formation was lower than group I (proportion: $P=.04$, total: $P=.02$, extent: $P=.03$, tenacity: $P=.02$) notwithstanding the higher peritoneal temperature, suggesting that also in group I desiccation occurred in some animals due to leaks around the ports. No adhesions were found in the animals either in laparoscopic ports or in the nonoperative sites.

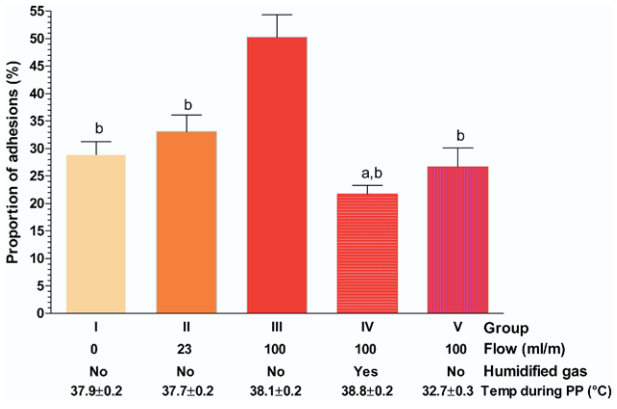
If all animals treated with nonhumidified CO_2, including those that were unable to maintain their body temperature at 37°C, were analyzed together (proc GLM; four groups, i.e., I, II, III, and V; two variables, i.e., desiccation [reflected by flow through the peritoneal cavity] and mean of body temperature), adhesions increased with desiccation (proportion: $P<.0001$; total: $P=.005$; extent: $P=.001$) and decreased with lower body temperature (proportion: $P<.0001$; total: $P=.0005$; extent: $P<.0001$; type: $P=.02$; tenacity: $P=.03$; Fig. 3). If only mice with body temperature close to 37°C were analyzed simultaneously (proc GLM; three groups; two variables, i.e., desiccation and temperature), adhesions increased with desiccation (proportion: $P<.0001$; total: $P=.001$; extent: $P=.001$; type: $P=.01$; tenacity: $P=.03$; Fig. 2) and, obviously, the effect of the minor differences of temperature around 37°C was not significant.

In the oversaturation experiment, as observed previously, that is, without the heating blanket (16), body temperature decreased from 37.5°C at T_0 to 35°C at T_{20}—the period before pneumoperitoneum was started. After this, body temperature further decreased to 33°C when nonhumidified CO_2 was used (group I). When humidified CO_2 was used temperature increased progressively to 36, 36.5, and 37°C in mice of group II (75% BTS), III (100% BTS), and IV (125% BTS), respectively (Fig. 4A). By ANOVA, body temperature between T_{20} and T_{80} was lower in mice of group I than in mice of groups II ($P<.0001$), III ($P<.0001$), and IV ($P<.0001$). Body temperature was also lower in mice of group II than in mice of groups III ($P=.02$) and IV ($P=.04$). Differences between groups III and IV were not significant (P=NS).

The pneumoperitoneum temperature in mice of group I was initially (T_{20}) almost identical to the body temperature at 35°C (Fig. 4B). Thereafter, the pneumoperitoneum temperature decreased slowly to 34.5°C, corresponding to the progressively decreasing body temperature. In the mice with humidified CO_2, pneumoperitoneum temperatures were higher around 37°C and increased slowly thereafter to 37.8°C, especially in group IV, reflecting the increase in body temperature (Fig. 4A). By ANOVA, pneumoperitoneum temperature was lower in mice of group I than in mice of groups II ($P<.0001$), III ($P<.0001$), and IV ($P<.0001$). It was also lower in mice of group II than in mice of groups III

FIGURE 2

Effect of desiccation and hypothermia during pneumoperitoneum on adhesion formation. Adhesions were induced during laparoscopy with 60 minutes of CO_2 pneumoperitoneum at 20 cm of water and quantitatively scored after 7 days during laparotomy. Nonhumidified gas at flows of 0 mL/min (group I), 23 mL/min (group II), 100 mL/min (groups III and V), and humidified gas at a flow of 100 mL/min (group IV) through the abdominal cavity were used. Mice were covered (groups I–IV) or not (group V) with a homeothermic blanket to ascertain body temperature within normal limits. Mean ± SE of body temperature during T_{20}–T_{80} is indicated. [a]P vs. group I <.05, [b]P vs. group III <.05 (Wilcoxon test).



Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

TABLE 1

Effect of desiccation and hypothermia during pneumoperitoneum on adhesion formation.

Group	Pneumoperitoneum: Flow (mL/min)	Pneumoperitoneum: Humidified gas	Body Temp[c] (°C)	Adhesion scores (mean ± SE): Extent	Type	Tenacity	Total
I	0	No	37.9 ± 0.2	1.5 ± 0.1[b]	1.3 ± 0.1[b]	1.6 ± 0.1[b]	4.4 ± 0.2[b]
II	23	No	37.7 ± 0.2	1.7 ± 0.1[b]	1.3 ± 0.1	1.5 ± 0.1[b]	4.5 ± 0.3[b]
III	100	No	38.1 ± 0.2	2.3 ± 0.2	1.6 ± 0.1	1.9 ± 0.1	5.8 ± 0.4
IV	100	Yes	38.8 ± 0.2	1.1 ± 0.1[a,b]	0.9 ± 0.1[b]	1.3 ± 0.1[a,b]	3.3 ± 0.3[a,b]
V	100	No	32.7 ± 0.3	1.4 ± 0.1[b]	1.1 ± 0.1[b]	1.5 ± 0.1[b]	4.0 ± 0.3[b]

Note: Adhesions were induced during laparoscopy with 60 minutes of CO_2 pneumoperitoneum at 20 cm of H_2O and qualitatively scored after 7 days during laparotomy.

[a] *P* vs. group I <.05; [b] *P* vs. group III <.05.

[c] Mean of body temperature during T_{20}–T_{80} is indicated.

Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

(*P*=.04) and IV (*P*=.004) and lower in mice of group III than in mice of group IV (*P*<.0001).

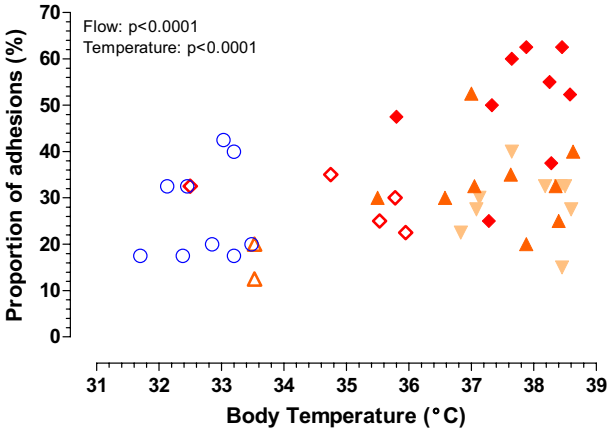
Peritoneum temperature was higher than body temperature (δT) after an equilibration period (T_{40}) (*P*<.05 for each group, Proc Univariate), being 1.4 ± 0.1°C, 1.2 ± 0.1°C, 1.4 ± 0.1°C, and 0.7 ± 0.1°C for groups II, III, IV, and I, respectively. The δTs remained constant up to T_{80}, being 1.3 ± 0.1°C, 1.0 ±0.1°C, 1.3 ±0.2°C, and 1.0 ± 0.2°C for groups II, III, IV, and I, respectively (*P*<.05 for each group, Proc Univariate).

The RH of the pneumoperitoneum remained 100% in all groups throughout the experiment, except for mice of group I. In this group RH of the pneumoperitoneum was initially (at T_{20}) 82.9% ± 1.9%, and decreased slightly thereafter to 80.8% ± 4.2%, reflecting the slightly lower humidification capacity of the peritoneum at lower temperatures (data not shown).

In the oversaturation experiment (Fig. 5, Table 2), adhesion formation in group I (important desiccation and much lower temperatures) was higher than in groups II (proportion: *P*=.02, total: *P*<.01, extent: *P*=.02, type: *P*<.01, tenacity: *P*<.01) and III (proportion: *P*=.05, total: *P*=.05), but not different from group IV (Wilcoxon). In group III, adhesion formation was lower than in group IV (proportion: *P*=.03, extent: *P*=.02). Adhesion formation in group II (slight desiccation and slightly lower temperatures) was not different from group III but lower than group IV (proportion: *P*<.01, total: *P*<.01, extent: *P*<.01, type: *P*<.01, tenacity: *P*=.03).

FIGURE 3

Relationship between adhesion formation and body temperature with different levels of desiccation. Individual values of mean of body temperature between T_{20} and T_{80} with their respective proportion of adhesions are depictured for pneumoperitoneum-enhanced adhesion for groups I (▼), II (▲), II with low temperature (△), III (♦), III with low temperature (◇), and V (○). Effect of flow: *P*<.0001, effect of temperature: *P*<.0001 (ProcGLM).



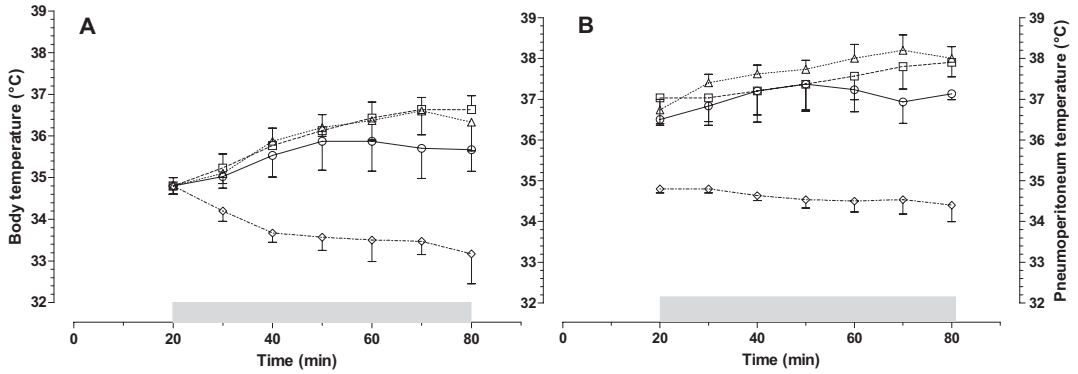
Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

DISCUSSION

The peritoneal cavity has a high humidifying capacity, as in this study in all groups with nonhumidified gas (0% RH) the RH of the pneumoperitoneum was 100% (desiccation experiment) and 80.8% ± 4.2 % (oversaturation experiment), meaning that water content from the serous fluid was continuously being evaporated to humidify the pneumoperitoneum. This then leads to tissue dehydration and desiccation. This corresponds to a water loss from the peritoneum of 1 and 4.4 mg water/min for groups with a flow of 23 and 100 mL/min, respectively, and theoretically, no water loss for the

FIGURE 4

Effect of CO_2 pneumoperitoneum with discrete levels of humidification, expressed in relation to BTS conditions (37°C, 100% RH) on body (**A**) and pneumoperitoneum (**B**) temperature. Nonhumidified gas (group I) and humidified gas at 75% BTS (group II), 100% BTS (group III), and 125% BTS (group IV) conditions and a flow of 23 mL/min through the abdominal cavity were used. Symbols: group I (◇), group II (○), group III (□), and group IV (△); pneumoperitoneum (*shaded bar*). Means ± SE are indicated.

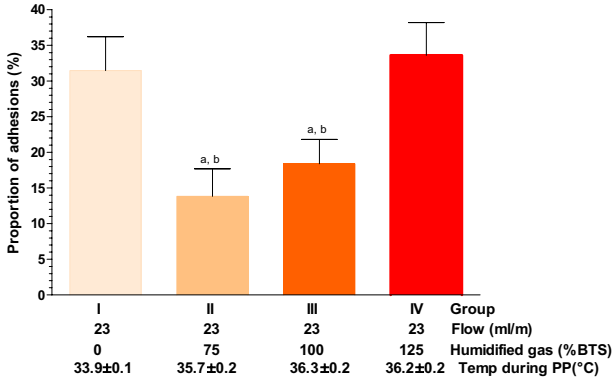


Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

groups with no flow through the abdominal cavity or with humidified gas. This high humidifying capacity of the peritoneum was already shown in open surgery in humans; that is, when bowels are exteriorized, the water loss by evaporation is approximately 32 g/h and this causes their surface temperature to decrease by 3°–5°C (35).

FIGURE 5

Effect of CO_2 pneumoperitoneum with discrete levels of humidification, expressed in relation to BTS conditions (37°C, 100% RH) on adhesion formation. Nonhumidified gas (group I) and humidified gas at 75% BTS (group II), 100% BTS (group III), and 125% BTS (group IV) conditions and a flow of 23 mL/min through the abdominal cavity were used. Pneumoperitoneum-enhanced adhesions were induced during laparoscopy and quantitatively scored after 7 days during laparotomy. Means ± SE are indicated. [a]*P* vs. group I <.05, [b]*P* vs. group IV <.03 (Wilcoxon test).



Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

As explained in the introduction, desiccation requires a high amount of energy. Taking into consideration energy calculations, whereas 1 cal is needed to heat 1 mL of water by exactly 1°C and 0.00003 cal is needed to heat 1 mL of CO_2 by 1°C, the energy to vaporize 1 mL of water at 37°C is 577 cal (63 cal to heat 1 mL to 100°C + 514 cal to vaporize) (31). This means that much more energy is needed to evaporate water than to heat water or CO_2 by 1°C. Applied to the desiccation experiment, using nonhumidified gas and assuming 100% RH in the pneumoperitoneum by evaporation of body water, body temperature of 37°C, and gas temperature of 37°C before entering the abdominal cavity, mice with a flow rate of 23 and 100 mL/min through the abdomen would lose 0.6 and 2.5 cal/min, respectively, whereas mice with no flow or with humidified gas (100% RH) would not require extra energy. The same calculations can be applied to the oversaturation experiment; the 0% BTS condition would require 0.6 cal/min, the 75% BTS 0.14 cal/min, and the 100% BTS 0 cal/min, whereas the 125% BTS would add 0.14 cal/min by condensation.

Animal body temperature changes in this study can, therefore, be explained by the energy required for evaporation or released at condensation. This decrease in body temperature was, however, masked by the homeothermic blanket in the desiccation experiment (groups I, II, and III), but fully evident when the homeothermic blanket was not used (group V). In that case body temperature decreased to 31°C, confirming observations in rats (27) and pigs (37). This cooling can be prevented by using warm and humidified gas, demonstrated in previous studies (27, 28, 36) and confirmed in this study (group IV). In the oversaturation experiment, we confirm that with warm and

TABLE 2

Effect of humidification and temperature during pneumoperitoneum on adhesion formation.

Group	Pneumoperitoneum: Flow (mL/min)	Pneumoperitoneum: Humidified gas (%BTS)	Body Temp[c] (°C)	Adhesion scores (mean ± SE): Extent	Type	Tenacity	Total
I	23	0	33.9 ± 0.2	1.5 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	4.2 ± 0.4
II	23	75	35.7 ± 0.2	0.7 ± 0.2[a,b]	0.7 ± .01[a,b]	0.9 ± 0.1[a,b]	2.3 ± 0.3[a,b]
III	23	100	36.3 ± 0.2	0.9 ± 0.1[b]	1.0 ± 0.1	1.0 ± 0.2	2.9 ± 0.4[a]
IV	23	125	36.2 ± 0.2	1.6 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	4.3 ± 0.5

Note: Discrete levels of humidification, expressed in relation to body temperature saturated (BTS) conditions (37°C, 100% RH, 44 mg of water/liter) were used. Adhesions were induced during laparoscopy with 60 minutes of CO_2 pneumoperitoneum at 20 cm of H_2O and qualitatively scored after 7 days during laparotomy.

[a] *P* vs. group I <.05; [b] *P* vs. group IV <.05.

[c] Mean of body temperature during T_{20}–T_{80} is indicated.

Binda. Adhesions, temperature, and desiccation. Fertil Steril 2006.

nonhumidified CO_2, animals cool down to 33°C, which can be explained solely by the evaporation of water. In the 100% BTS group, body temperature slightly increased, that is, returned to the initial 37°C before anesthesia, in the absence of any cooling. Moreover, some additional energy could have been provided if the gas was not completely cooled down in the trocar. As expected, this increase in temperature is more pronounced in the 100% and 125% BTS groups, particularly the 125% BTS group, where energy is released by the condensation due to the higher enthalpy of the gas.

The pneumoperitoneum temperature will be a function of the temperature of the insufflated gas, the flow through the abdominal cavity, the energy released or required by condensation or evaporation, the animal body temperature, and the surrounding environment. This explains why, in the desiccation experiment, in comparison with body temperature, pneumoperitoneum temperature was comparable in the group with no flow, slightly lower (NS probably because n = 3 only) in the groups with flows of 23 mL/min and 100 mL/min and nonhumidified gas, and significantly higher for the group with humidified gas. This shows that as the cold, nonhumidified gas flow increases, the temperature in the pneumoperitoneum decreases, whereas even at high flows the temperature remains close to body temperature when humidified gas is used. Also in the group with hypothermia the pneumoperitoneum temperatures were slightly higher than body temperatures. This is logical for the group with hypothermia because body temperature was approximately 31°C and insufflated gas temperature was approximately 37°C. The same holds true for the oversaturation experiment, explaining why in all the groups the pneumoperitoneum temperature remained higher than body temperatures, especially in the 75%, 100%, and 125% BTS groups.

This is to our knowledge the first direct demonstration that desiccation enhances adhesion formation. Unless great effort is taken to prevent the associated cooling, the effect will be underestimated, as the associated cooling will decrease adhesion formation (16). Even in the desiccation experiment, in which cooling was prevented with the homeothermic blanket, some underestimation by cooling cannot be ruled out. Pneumoperitoneum temperatures were, as expected, slightly lower when desiccation occurred; moreover, we can speculate that in the peritoneum, where desiccation occurred, the temperature was probably even lower. In all previous published experiments, desiccation was always associated with cooling. Also for effects such as alteration of mesothelium morphology, destruction of microvilli, and bulging up of cells with exposure of the basal lamina (27–30), it is difficult to judge the independent effects of desiccation and cooling.

Desiccation-enhanced adhesions are clearly prevented by using humidified gas. Adhesions were even slightly lower in the group with high flow and humidified gas than in the group with no flow and nonhumidified gas. This can be explained by the gas leakage during the surgical procedure to induce adhesions, a problem we were not aware of during the experiments. Leakage occurred from the 14-gauge catheters between their insertion and the insertion of the surgical instruments; slight leakage occurred during the surgery; more important leakage occurred after removal of the catheters until suturing was finished. The difficulty of avoiding leakage varies with the expertise of the surgeon. Considering the diameter of the 14-gauge catheter, leakage for 1 minute only can easily amount to more than 500 mL of CO_2, which accounts for nonnegligible desiccation. The relative importance of this leakage is huge in group I, considered as without desiccation; still important in group II, with total leakage of 1,380 mL; and less important in group III, with leakage of 6,000 mL. Thus, groups I, II, and III had desiccation of 500, 1,880, and 6,500 mL instead of 0, 1,380, and 6,000 mL. Without this leakage during surgery, we can speculate that adhesions in group I would have been consid-

erably less and in group II slightly less. In future experiments this leakage during surgery must be controlled.

These experiments confirm and extend previous observations that adhesions decrease with hypothermia (16). It remains surprising, however, that quantitatively this effect, at least under these experimental conditions, seems as important as using humidified gas. Also mice of groups II and III, which could not maintain their body temperature, had fewer adhesions (Fig. 3). It is unclear whether this decrease in body temperature was a consequence of a leakage and thus enhanced desiccation or of an insufficient metabolic capacity to maintain the body temperature at 37°C. In the former hypothesis, the decrease in body temperature would have a more important effect on adhesions than the increased desiccation. We can only speculate today that cooling might to some extent prevent the deleterious effect of desiccation as it does for the hypoxia. This also might explain why the effects of warm and humidified gas on mesothelium morphology are still controversial (27, 28).

To interpret the adhesion formation data in the oversaturation experiment, the opposing effects of desiccation and hypothermia should also be considered, knowing that both are intimately linked and that although the former increases adhesion formation (desiccation experiment), the latter reduces adhesion formation (16; desiccation experiment). Because adhesion formation was much higher in the 0% BTS group (oversaturation experiment), the effect of desiccation on adhesion formation was clearly confirmed. Because in this group body temperature was much lower, the adhesiogenic effect of desiccation must be clearly underestimated. Adhesions were slightly lower in the 75% BTS group than in the 100% BTS group, which can only be interpreted by the slightly lower temperature, as some evaporation must have occurred, considering the 100% RH in the peritoneal cavity. In the 75% BTS group, the effect of temperature is underestimated, as without desiccation adhesions would even have been less. Adhesions were slightly higher in the 125% BTS group than in the 100% BTS group, which can only be explained by the slightly higher temperature, as desiccation can be ruled out. It is unlikely that excess condensed water poses a hypotonicity challenge, causing cellular damage in the 125% BTS group (43), because of the limited amount of condensation produced.

The effect of heating and humidifying the gas during laparoscopy has been studied in clinical trials. Compared with cold and nonhumidified gas, warm and humidified CO_2 is claimed to reduce postoperative pain after laparoscopy (28, 44, 45), but this observation is still controversial (46). It should be stressed that in all these evaluations the effect of warm and humidified gas was always compared with that of cold and nonhumidified gas. The effect in reducing the pain therefore might be due to prevention of desiccation rather than to the heating of the gas.

In summary, we demonstrate the complex relationship between cooling and desiccation on adhesion formation. Desiccation clearly increases adhesion formation, and the effect is generally underestimated as the associated cooling decreases adhesion formation. We confirm the effect of hypothermia in reducing adhesion formation, an effect that at 32°C is quantitatively as pronounced as humidification. Slight cooling together with slight desiccation (oversaturation experiment) decrease adhesion formation, but this effect of cooling is overruled when desiccation becomes important. These data moreover extend the previous data demonstrating that increased pneumoperitoneum temperatures (above 37°C) increase adhesion formation even further. The initial hypothesis that oversaturation of the insufflated gas would be beneficial for adhesion formation, as all desiccation would be prevented, thus proved wrong because of the associated increase in peritoneal temperature and enthalpy of the gas. This in effect is consistent with the physiologic map (43) in that nonphysiologic gas conditions affect the normal physiologic state (lower than BTS or above BTS). From these data we anticipate that insufflators, which provide only a heating option that will warm the gas to body temperature without humidification, could be more deleterious for adhesion formation than using an insufflator without a heating option, because of higher temperature and higher desiccation. These data will have to be confirmed in larger animals. Moreover, in larger animals a decrease in pneumoperitoneum temperature is not necessarily associated with a decrease in body temperature. If confirmed in larger animals, these results may have very important clinical implications for the design of insufflators and humidifiers, which would minimize adhesion formation. The potential clinical implications of preventing adhesion formations in human surgery are important, especially if the prevention of hypoxia by adding a few percent of oxygen, preventing desiccation, and cooling the pneumoperitoneum to approximately some 32°C would have additive effects.

Acknowledgments: The authors thank Lisbeth Vercruysse, M.Sc., Silvia Caluwaerts, Ph.D., Salwan Al-Nasiry, M.D., Ms. Rita Van Bree, Adriana Bastidas, M.D., Jasper Verguts, M.D., Robert Pijnenborg, Ph.D. (Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg), and Michael Blackhurst, B. Eng (Hons.) (Fisher & Paykel Healthcare Ltd.) for their help. The authors also thank Fisher & Paykel Healthcare Ltd. for the development and supply of the humidifier.

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Addendum 6

Molinas CR, **Binda MM** and Koninckx PR. Angiogenic factors in peritoneal adhesion formation. *Gynecological Surgery* 3: 157-167, 2006.

Gynecol Surg (2006) 3: 157–167
DOI 10.1007/s10397-006-0236-7

REVIEW ARTICLE

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Angiogenic factors in peritoneal adhesion formation

Received: 27 June 2006 / Accepted: 23 July 2006 / Published online: 17 August 2006

Abstract Abdominal surgery is considered as the leading cause of peritoneal adhesions and almost universally as adhesiogenic. Peritoneal injury at the time of surgery initiates an inflammatory reaction determining fibrin deposition on the wound surface. Depending on the balance between the different components of the plasminogen system, this fibrin can be either lysed, leading to normal peritoneal healing, or organised, serving as a scaffold for fibroblast ingrowth, extracellular matrix deposition and angiogenesis, leading to adhesion formation. The mechanism underlying the predisposition to form adhesions in some patients and in some specific anatomic sites and not in others after similar surgical procedures remains unknown. In spite of the many attempts proposed over the years for reducing the incidence of adhesion formation, peritoneal adhesions remain a major clinical problem, inducing intestinal obstruction, pelvic pain, female infertility and difficulties at the time of re-operation. The available evidence indicates that understanding the adhesion formation process at the molecular level is essential for developing successful strategies for preventing adhesions. Fortunately, the advancement in molecular biology during the last years has led to the identification of many molecules with the potential of regulating inflammatory and immune responses, tissue remodelling and angiogenesis, key events in peritoneal healing and adhesion formation. This review focuses on the role of angiogenesis and angiogenic factors in peritoneal adhesion formation.

Keywords Peritoneum · Adhesions · Angiogenesis · Vascular endothelial growth factor · Hypoxia inducible factors · Laparoscopy · CO2

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Definition and aetiology of peritoneal adhesions

Adhesions are pathological bonds between surfaces within body cavities. These bonds can be a thin film of connective tissue, a thick fibrous bridge containing blood vessels and nerve tissue, or a direct contact between two organ surfaces [1]. Adhesions can be found in abdominal, pericardial, pleural, uterine and joint cavities, and in the chamber of the eyes. Adhesions in the abdominal cavity are also known as peritoneal adhesions because the peritoneum is always involved.

Peritoneal adhesions may be classified, according to the aetiology, as congenital or acquired, which in turn can be classified as postinflammatory or postoperative [2]. Abdominal surgery is the most common cause of adhesions, 70–85% of all adhesions being attributed to previous surgery. On the other hand, surgery has been documented as almost universally adhesiogenic, the reported incidence of adhesions in patients undergoing surgery being between 55 and 100% [3].

Among postoperative adhesions, different processes can be distinguished [4]:

- Adhesions type 1 or de novo adhesion formation: adhesions formed at sites that did not have adhesions previously.
  Type 1A: no previous operative procedures at the site of adhesions.
  Type 1B: previous operative procedures at the site of adhesions.
- Adhesions type 2 or adhesion reformation: adhesions formed at sites where adhesiolysis was performed.

Type 2A: no operative procedures at the site of adhesions besides adhesiolysis.
Type 2B: other operative procedures at the site of adhesions besides adhesiolysis.

Clinical significance of peritoneal adhesions

Depending on their location and structure, adhesions may remain silent or cause clinically important complications such as intestinal obstruction, chronic pelvic pain, female infertility and difficulties at the time of re-operation.

Intestinal obstruction is the most serious complication of peritoneal adhesions as it can be life threatening due to strangulation. Adhesions are the leading cause of intestinal obstruction in the Western world, accounting for more than 40% of all cases of intestinal obstruction and for 60–70% of those involving the small bowel [2].

Adhesion formation is a major cause of chronic pelvic pain and it has been reported as the primary cause in some 25% of patients with chronic pelvic pain. It was suggested that pelvic pain is a consequence of the restricted organ mobility imposed by adhesions. After adhesiolysis, a relief of symptoms has been consistently reported. From a clinical point of view, however, the relation between adhesions and chronic pelvic pain is unclear since their association does not necessarily imply a causal relationship. Indeed, it was demonstrated that a large number of infertility patients with adhesions do not experience pelvic pain [5].

Peritoneal adhesions are well recognised as a cause of female infertility. The proposed mechanism of infertility is that adhesions restrict the sweeping of the fimbria over the ovary. Periadnexal adhesions were found in some 20–30% of infertile women and marked increases in pregnancy rates were reported after adhesiolysis [6].

Adhesions increase the technical difficulty for surgeries, increasing the difficulty of accessing the abdomen and/or the operation site, the complication rates, the anaesthesia, operating and recovery time, the use of surgical materials and the need for blood transfusion. Therefore, the magnitude of adhesions related disorders (ARD) is larger than could be anticipated and is better illustrated by the reports showing that hospital readmission for ARD rival the number of hip replacements, heart bypass or appendix surgeries, that 35% of women having open gynaecologic surgery are readmitted 1.9 times in 10 years for operation due to adhesions or complicated by adhesions, and that the estimated annual cost for ARD in the USA is 1.3 billion US$ [7].

Pathogenesis of peritoneal adhesions

The peritoneum is one of the largest organs in humans with a surface of some 10.000 cm^2. It serves to minimise friction and facilitate free movement of abdominal viscera, to resist and localise infections and to store fat, especially in the greater omentum. It forms a closed sac in males and an open sac in females, lining the abdominal walls (parietal peritoneum) and the viscera (visceral peritoneum). It is composed of a continuous layer of mesothelial cells and a layer of loose connective tissue [8].

Peritoneal mesothelial cells are highly differentiated, as are pleural and pericardial mesothelial cells, and their apical surface contain abundant long microvilli that increase the functional surface of the peritoneum for absorption and secretion. Mesothelial cells are connected to one another by desmosomes and very loosely attached to the underlining basement membrane. The connective tissue is composed of bundles of collagenous and elastic fibres oriented in different directions and a rich network of blood and lymphatic vessels. Interspersed among these fibres and vessels, there are poorly differentiated epithelioid-like cells, similar to fibroblasts, macrophages, mast cells and fat cells [8].

The intact peritoneal cavity contains 3–50 ml of fluid with a pH of 7.5–8.0 and with a significant buffering capacity. The peritoneal fluid (PF) contains plasma proteins, including a large amount of fibrinogen, and a variety of free-floating cells, including macrophages, lymphocytes, eosinophils, mast cells and desquamated mesothelial cells [8].

Peritoneal injury, due to surgery, infection or irritation, initiates an inflammatory reaction that increases all components of the PF, i.e. proteins and cells, generating a fibrinous exudate and the formation of fibrin [9]. Fibrin formation is the result of the activation of the coagulation cascade, which includes two pathways, i.e. the contact factor or intrinsic pathway and the tissue factor or extrinsic pathway. Activation of these pathways transforms prothrombin (Factor II) into thrombin (Factor IIa) via the common pathway. Thrombin then triggers the conversion of fibrinogen into monomers of fibrin, which interact with each other and polymerise. The initially soluble polymer becomes insoluble by some coagulation factors such as Factor XIIIa and is deposited on the wound surface [9]. Within this fibrinous exudate, polymorphonuclears (PMN), macrophages, fibroblasts and mesothelial cells migrate, proliferate and/or differentiate. During the first two postoperative days, a large number of PMN enter and, in the absence of infection, depart within 3–4 days. Macrophages increase in number and change their functions, becoming the most important component of the leukocyte population after day 5. They phagocyte more accurately, have greater respiratory burst activity and secrete a variety of substances including cytokines and growth factors that recruit new mesothelial cells onto the injury surface. Mesothelial cells migrate, form islands throughout the injured area and proliferate in order to cover the denuded area. This healing process is different from that occurring in the skin because the entire surface becomes epithelialised simultaneously from the islands of mesothelial cells and not gradually from the borders. Therefore, it is irrespective of the size of the injury and is complete in 5–7 days [8].

These cells release a variety of substances including plasminogen system components, arachidonic acid metab-

olites, reactive oxygen species (ROS), cytokines and growth factors such as interleukins (IL), tumour necrosis factor α (TNF-α), transforming growth factors α and β (TGF-α and TGF-ß), which modulate the process of peritoneal healing and adhesion formation at different stages [9, 10].

This fibrinous exudate and fibrin deposition is an essential part of normal tissue repair, but its complete resolution is required to restore the preoperative conditions. The degradation of fibrin is regulated by the plasminogen system. In this system, the inactive proenzyme plasminogen is converted into active plasmin by plasminogen activators (PAs), which are inhibited by plasminogen activator inhibitors (PAIs) [11]. Plasminogen is a glycoprotein synthesised in the liver that is abundant in almost all tissues. It is the inactive precursor of plasmin, a serine protease that is highly effective in the degradation of fibrin into fibrin degradation products (FDP) and that has a role in other stages of tissue repair such as extracellular matrix (ECM) degradation, [12] activation of proenzymes of the matrix metalloprotease (MMP) family [13] and activation of growth factors [14]. The principal activator of plasminogen is the serine protease tissue-type PA (tPA), which is expressed in endothelial cells, mesothelial cells and macrophages. tPA has a high affinity for fibrin and binds to a specific receptor, which exposes a strong plasminogen-binding site on the surface of the fibrin molecule. Therefore, in the presence of fibrin the activation rate of plasminogen is strikingly enhanced, whereas in the absence of fibrin, tPA is a poor activator of plasminogen [15, 16]. This results in higher plasminogen activation on the sites where it is required, whereas systemic activation is prevented. The other activator of plasminogen is the serine protease urokinase-type PA (uPA). The properties of uPA differ from those of tPA as it lacks high-affinity binding for fibrin and thus the increased activity in the presence of fibrin. Therefore, uPA is limited in its capacity to activate plasminogen [17].

The action of the PAs is counteracted by PAI-1 and PAI-2 through the formation of inactive complexes. The most potent inhibitor of tPA and uPA is the glycoprotein PAI-1, which is expressed in endothelial cells, mesothelial cells, macrophages, platelets and fibroblasts. The glycoprotein PAI-2 is a relatively poor inhibitor of tPA and uPA and is expressed in mesothelial cells, macrophages and epithelial cells. The role of other PAIs, i.e. PAI-3 and protease nexin 1, and plasmin inhibitors, i.e. α2-macroglobulin, α2-antiplasmin and α1-antitrypsin, in peritoneal fibrinolysis remains unknown.

The balance between fibrin deposition and degradation is critical in determining normal peritoneal healing or adhesion formation. If fibrin is completely degraded, normal peritoneal healing will occur. In contrast, if fibrin is not completely degraded, it will serve as a scaffold for fibroblasts and capillary ingrowth. Indeed, fibroblast will invade the fibrin matrix and ECM will be produced and deposited. The ECM can be completely degraded by MMPs, leading to normal healing. However, if this process is inhibited by tissue inhibitors of MMPs (TIMPs), peritoneal adhesions will be formed.

Angiogenesis, angiogenic factors and adhesion formation

The formation of new blood vessels on peritoneal adhesions has been universally claimed to be important and supported by animal data demonstrating increasing vascularisation over days [18]. The details of the angiogenesis process in peritoneal adhesion formation remains, however, largely unexplored.

Angiogenesis, the formation of new blood vessels extending from existing vessels, occurs when the distance between cells and the nearest capillary exceeds an efficient diffusion range for maintaining an adequate supply of oxygen and nutrients to cells. This process is regulated by cellular hypoxia through the modulation of angiogenic factors and their inhibitors.

Angiogenesis is a self-limited and strictly controlled process that occurs in a sequential manner, involving degradation of the vascular basement membrane and interstitial matrix, migration and proliferation of endothelial cells and finally tubologenesis and formation of capillary loops. The proteolytic enzymes production such as MMPs and PAs, in response to angiogenic factors is fundamental for all stages of angiogenesis, i.e. degradation of perivascular matrix and tissue stroma, migration and proliferation of endothelial cells. Since these proteases are produced in inactive forms and must become activated to initiate their actions, their activities are regulated by naturally occurring physiological inhibitors, i.e. TIMPs and PAIs. Cytokines and growth factors such as IL 1, IL 8, TNF-α, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), TGF-α, TGF-β, plateled-derived growth factor (PDGF) are considered as angiogenic factors due to their ability to regulate the expression of MMPs, PAs and their inhibitors and to modulate endothelial cell migration and proliferation [19].

VEGF, the most potent known angiogenic factor, is a family that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). These factors are transcribed from single genes and processed by alternative splicing into different isoforms. VEGF-A, also known as VEGF, is processed into four isoforms in humans (VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}) and three in mice (VEGF-A_{120}, VEGF-A_{164} and VEGF-A_{188}). VEGF-B is processed into two isoforms in humans and two in mice (VEGF-B_{167} and VEGF-B_{186}), whereas PlGF is processed into three isoforms in humans (PlGF-1, PlGF-2 and PlGF-3) and one in mice (PlGF-2). These factors bind to two high-affinity transmembrane tyrosine kinase receptors with 7 immunoglobulin-like extracellular domains and a kinase intracellular domain, i.e. VEGFR-1/Flt-1 (for VEGF-A, VEGF-B and PlGF) and VEGFR-2/Flk-1 (for VEGF-A). These receptors are selectively but not exclusively expressed on endothelial cells. A truncated

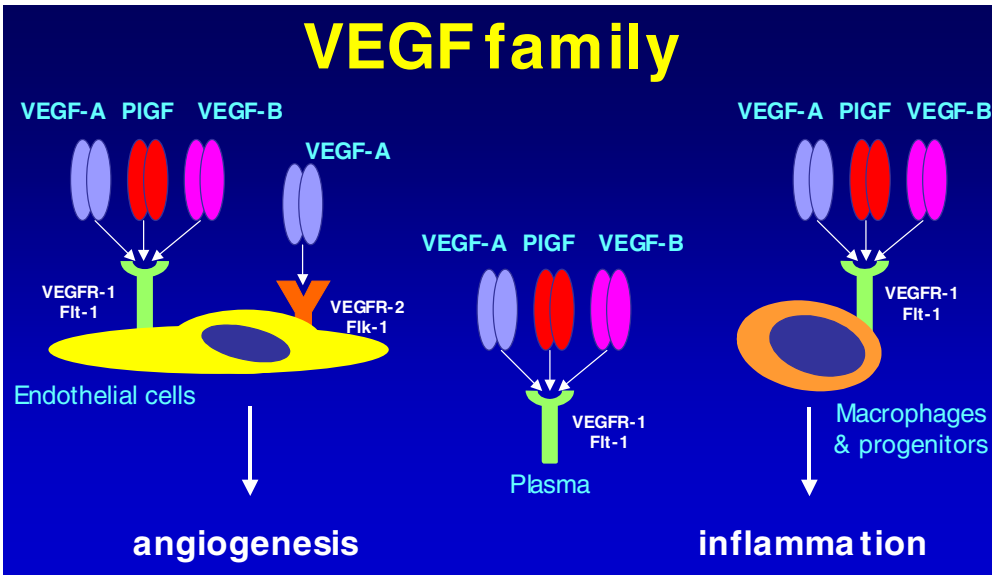

Fig. 1 Vascular endothelial growth factor family

soluble form of VEGFR-1, resulting from alternative splicing and retaining its binding activity, is present in serum. VEGFR-1 is, unlike VEGFR-2, also expressed on inflammatory cells. Therefore, VEGF-A, VEGF-B and PlGF can stimulate inflammation in addition to angiogenesis (Fig. 1) [20–23].

Because of the presence of VEGF in endothelial cells of blood vessels supplying pelvic adhesions, a key role for VEGF in angiogenesis during adhesion formation has been suggested [24]. This observation was supported by studies in rats demonstrating up-regulation of $VEGF_{188}$ and $VEGF_{120}$ during early stages of peritoneal healing and down-regulation of $VEGF_{164}$ 24–48 h following open surgery [25], suggesting a compensatory mechanism to regulated angiogenesis in order to provide nutrients and oxygen to the injured tissues. The role of VEGF is also supported by the reduction of adhesion formation after treatment with antibodies against VEGF in an open surgery mouse model [26].

The role of VEGF-A, VEGF-B and PlGF in adhesion formation after laparoscopic surgery has been addressed in studies using wild type mice (i.e. $VEGF\text{-}A^{+/+}$, $VEGF\text{-}B^{+/+}$, $PlGF^{+/+}$), transgenic mice and monoclonal antibodies. Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Since adhesions increase with the duration of the pneumoperitoneum and the insufflation pressure [27, 28], the CO_2 pneumoperitoneum was maintained at 14 mmHg for the minimum time needed to induce the lesions (10 min) or for a longer period (60 min) to evaluate "basal adhesions" and "pneumoperitoneum-enhanced adhesions", respectively [29]. In all control groups, 60 min of pneumoperitoneum increased adhesion formation. In transgenic mice for VEGF-A, (i.e. deficient for $VEGF\text{-}A_{120}$ and $VEGF\text{-}A_{188}$ and expressing

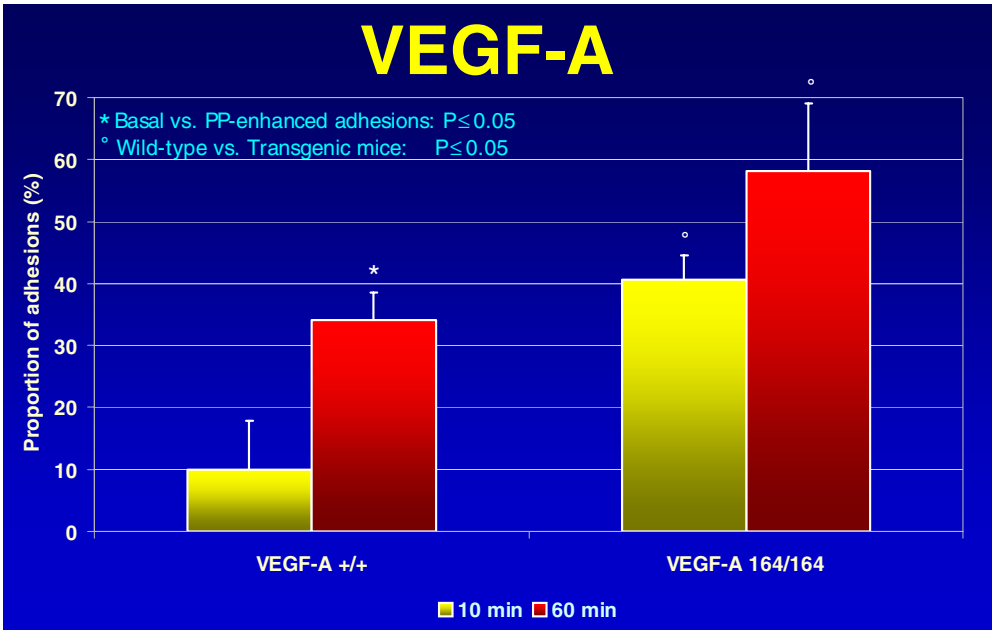

Fig. 2 Role of VEGF-A in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice ($VEGF\text{-}A^{+/+}$) and transgenic mice deficient for $VEGF\text{-}A_{120}$ and for $VEGF\text{-}A_{188}$ isoforms and expressing exclusively $VEGF\text{-}A_{164}$ isoform ($VEGF\text{-}A^{164/164}$). Means±SE are indicated

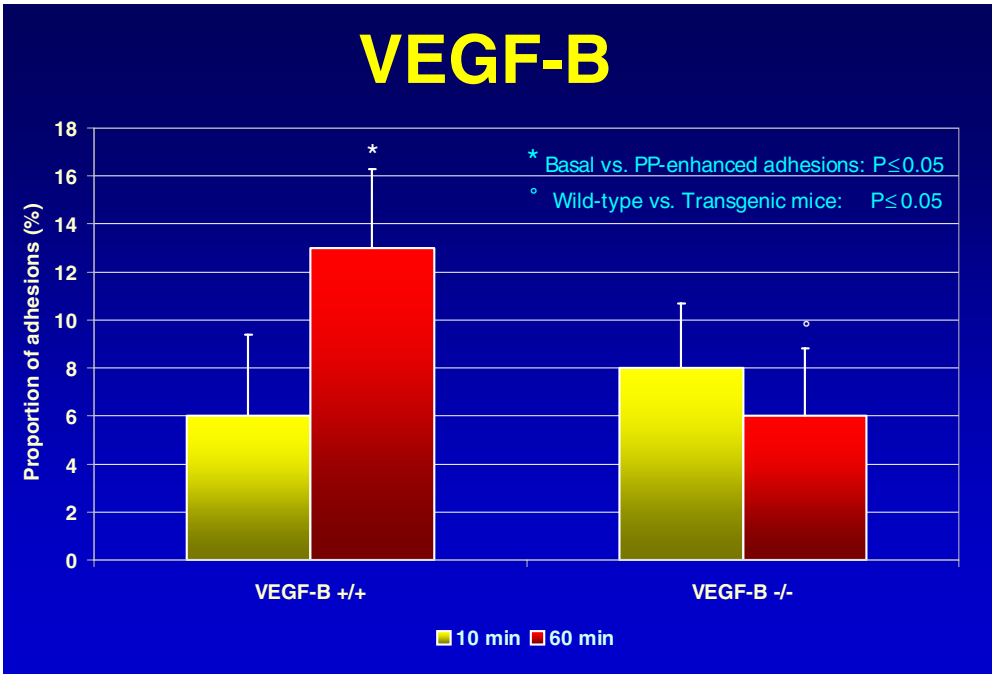

Fig. 3 Role of VEGF-B in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice (VEGF-B$^{+/+}$) and transgenic mice deficient for VEGF-B (VEGF-B$^{-/-}$). Means±SE are indicated

exclusively VEGF-A_{164}: VEGF-A$^{164/164}$), basal adhesions were higher than in VEGF-A$^{+/+}$ mice, the pneumoperitoneum slightly increased adhesions, and "pneumoperitoneum-enhanced adhesions" were higher than in VEGF-A$^{+/+}$ mice (Fig. 2) [30]. In mice deficient for VEGF-B (VEGF-B$^{-/-}$), "basal adhesions" were similar than in VEGF-B$^{+/+}$ mice and the pneumoperitoneum did not increase adhesions, "pneumoperitoneum-enhanced adhesions" being therefore lower than in VEGF-B$^{+/+}$ mice (Fig. 3) [30]. In mice deficient for PlGF (PlGF$^{-/-}$), basal adhesions were slightly lower than in PlGF$^{+/+}$ mice and the pneumoperitoneum did not increase adhesions, pneumoperitoneum-enhanced adhesions being therefore lower than in PlGF$^{+/+}$ (Fig. 4) [30]. The role of PlGF was confirmed by using monoclonal antibodies with different neutralising capacities of the binding of PlGF to its receptor. In mice treated with neutralising antibodies, basal adhesions were lower than in the control groups and the pneumoperitoneum did not increase adhesions, pneumoperitoneum-enhanced adhesions being therefore lower than in the control groups [30] (Fig. 5).

The role of the common receptor of VEGF-A, VEGF-B and PlGF, i.e. VEGF-R1, was evaluated by using mono-

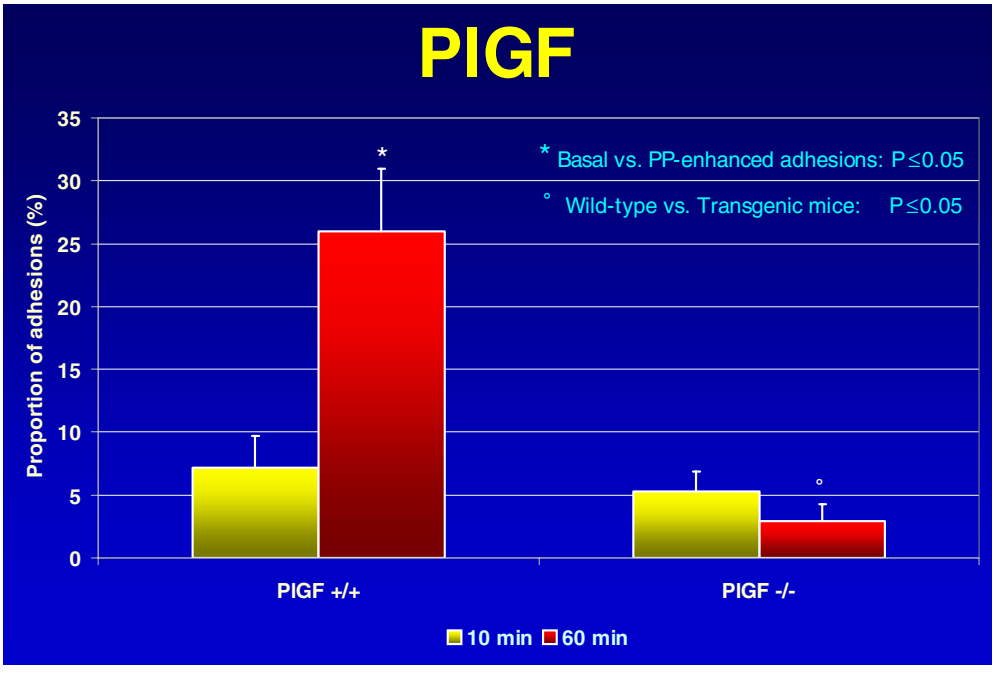

Fig. 4 Role of PlGF in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice (PlGF$^{+/+}$) and transgenic mice deficient for PlGF (PlGF$^{-/-}$). Means±SE are indicated

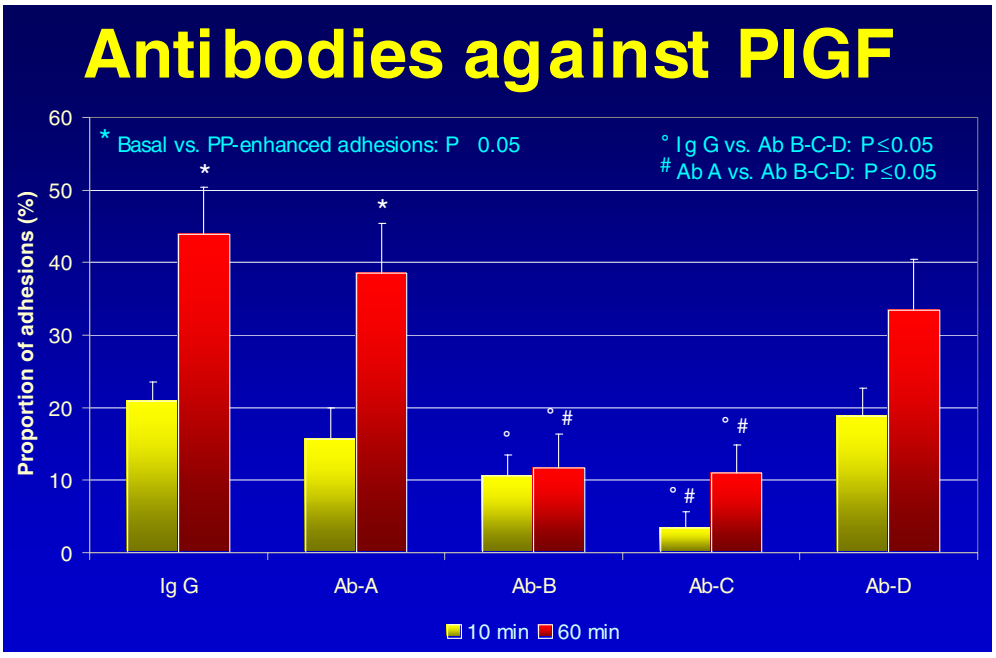

Fig. 5 Role of antibodies against PlGF in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice treated with IgG or with PlGF antibodies with different neutralising capacity according to their ability to inhibit the binding of PlGF to VEGFR-1 (Ab A: no neutralising, Ab B: neutralising, Ab C: neutralising, Ab D: semi-neutralising). Means±SE are indicated

clonal antibodies against VEGFR-1. In the control group, i.e. IgG-treated mice, pneumoperitoneum increased adhesions. In VEGFR-1 antibodies-treated mice, basal adhesions were similar than in IgG-treated mice and the pneumoperitoneum did not increase adhesions, pneumoperitoneum-enhanced adhesions being therefore lower than in IgG-treated mice (Fig. 6) [31].

For basal adhesions the data clearly demonstrate a role for VEGF-A_{164}, whereas for pneumoperitoneum-enhanced adhesions, the data indicate that the pneumoperitoneum increases adhesions through VEGF-B and PlGF up-regulation and probably also through VEGF-A_{164} up-regulation. Indeed, pneumoperitoneum-enhanced adhesions is absent in VEGF-$B^{-/-}$ and $PlGF^{-/-}$ mice because the pneumoperitoneum cannot up-regulate these nonexistent factors. This is fully consistent with the observations in mice treated with PlGF antibodies. The only slight increase in adhesions following 60 min of pneumoperitoneum in VEGF-$A^{164/164}$ mice does not rule out VEGF-A_{164} up-regulation because adhesion formation could already be near maximal due to the over-expression of this factor.

Since PlGF, VEGF-A and VEGF-B have a common receptor, i.e. VEGFR-1, and since antibodies against VEGFR-1 prevent pneumoperitoneum-enhanced adhesions, the data indicate that the effects of the VEGF family are mediated to a large extent by this receptor. This is

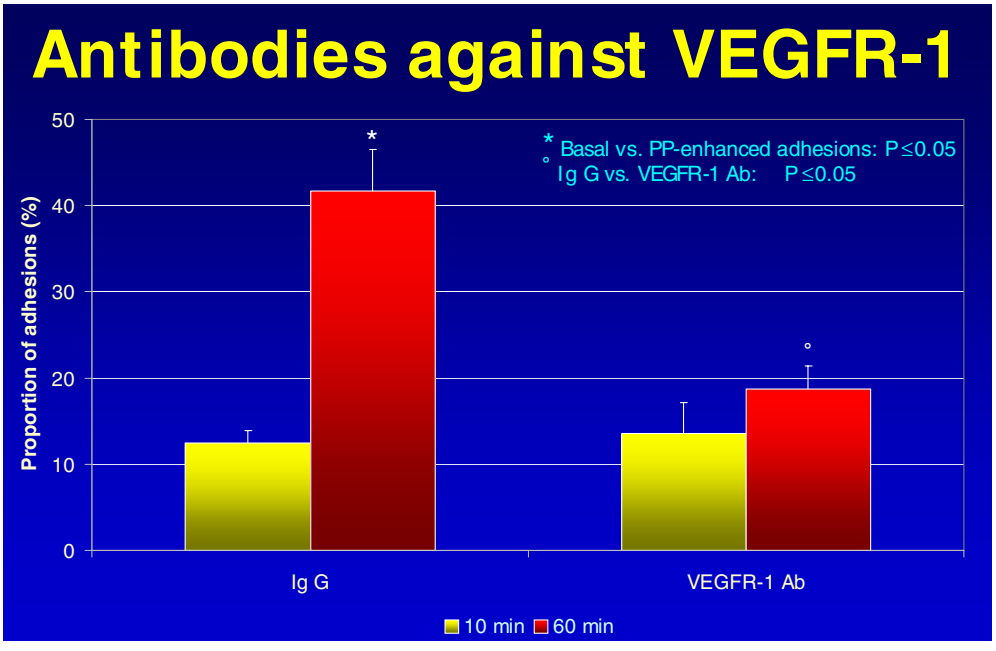

Fig. 6 Role of antibodies against VEGFR-1 in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice treated with IgG or with VEGFR-1 antibodies. Means±SE are indicated

supported by the recently reported reduction of peritoneal fibrosis after soluble VEGFR-1 gene transfer in mice [32], since this isoform, by retaining its binding capacity, reduces the binding of the ligands to the functional cellular receptors. As VEGFR-1 is expressed on endothelial cells and on inflammatory cells, it remains unclear whether these effects are mainly related to stimulation of angiogenesis and/or inflammation.

Several mechanisms have been proposed for VEGF-driven angiogenesis [21, 33]. VEGF-A induces angiogenesis by activating VEGFR-2, while VEGFR-1 might function as an inert "decoy" regulating the availability of VEGF-A to activate VEGFR-2. PlGF stimulates angiogenesis by several mechanisms. First, PlGF displaces VEGF-A from VEGFR-1, increasing the fraction of VEGF-A available to activate VEGFR-2. Second, PlGF up-regulates the expression of VEGF-A. Third, PlGF transmits its own intracellular angiogenic signals through VEGFR-1. Fourth, PlGF activates receptor cross-talk between VEGFR-1 and VEGFR-2, enhancing VEGFR-2-driven angiogenesis. Fifth, PlGF forms heterodimers with VEGF-A. On the other hand, VEGF-driven inflammation is mediated by VEGFR-1 by increasing mobilisation of bone marrow-derived myeloid progenitors into peripheral blood, by increasing myeloid cell differentiation, mobilisation and activation, and by increasing cytokines production by macrophages [21, 36].

Regardless of the main mechanism of action of VEGF, the available data point to peritoneal hypoxia as the trigger factor. The hypoxic response is not restricted to specific specialised cell types and a general similar mechanism might act in a variety of cell types. Most mammalian cells can respond to alterations in oxygen levels by increasing or decreasing the expression of specific genes [34, 35]. The hypoxic regulation of many of these genes takes place at both transcriptional and post-transcriptional levels. The transcriptional regulation is mediated by transcription factors known as hypoxia inducible factors (HIFs) [36–38]. Since VEGF is up-regulated by hypoxia through HIFs and since HIFs have a well-known role in angiogenesis [39], a role for these factors in adhesion formation can be postulated.

HIFs are nuclear proteins that bind to hypoxia response elements (HRE) in the promoter or enhancer regions of hypoxia inducible genes, activating gene transcription in response to hypoxia [43]. HIFs are members of the basic helix-loop-helix (bHLH) periodic (Per) aryl hydrocarbon receptor nuclear translocator (ARNT) single-minded (Sim) (PAS) domain protein family. Several proteins have been identified in this bHLH-PAS family that belong to the α or β classes. Each member of the α class form a stable heterodimer with a member of the β class. Whereas β class members are constitutively expressed in a ubiquitous or a tissue-specific way, α class members are often inducible by environmental stimuli such as light or hypoxia [40]. HIF-1 is composed of HIF-1α and HIF-1β subunits [41–43], whereas HIF-2 is composed of HIF-2α and HIF-1β subunits [44]. HIF-1α and HIF-2α, the specific hypoxia-regulated subunits, are structurally very similar and share the same heterodimerisation partner. Therefore, both HIF-1 and HIF-2 have a high similarity in structure and regulatory domains and are able to bind to the same HRE of target genes (Fig. 7).

The specific role of HIFs in adhesion formation was evaluated in mice partially deficient for HIF-1α or HIF-2α using the model previously described. While, in the control groups, 60 min of pneumoperitoneum increased adhesions, in the transgenic mice this effect was not observed, pneumoperitoneum-enhanced adhesions being therefore nonexistent (Figs. 8 and 9) [45]. These observations are consistent with the effects of the VEGF family [30, 34] and are also supported by the absence of pneumoperitoneum-enhanced adhesions in mice deficient for PAI-1 [29], since PAI-1 is up-regulated by hypoxia through HIF-1α [46].

Angiogenesis not only depends on the angiogenic factors but also on the availability of their inhibitors. Among the angiogenic suppressors are TGF-β, TNF-α, interferons, collagen synthesis modifiers, protamine, cyclosporine, hyaluronic acid, thrombospondin, angiostatin and endogenous oestrogen metabolites [19]. Since peritoneal vascular endothelial cells contain receptors for ovarian steroids [60], these steroids can potentially regulate peritoneal healing and adhesion formation related angiogenesis.

Antiangiogenic therapy for peritoneal adhesion prevention

Although antiangiogenic therapy is widely used in other fields such as cancer, to the best of our knowledge, only recently have antiangiogenic agents been used for evaluating their efficacy for peritoneal adhesion prevention. The angiogenesis inhibitor TNP-4 [34], an analogue of fumagillin secreted by the fungus *Aspergillus fumigatus*, has been shown to reduce peritoneal adhesions and to delay vascular ingrowth in a laparotomy mouse model [48]. However, side effects of the drug such as neurotoxicity and delayed wound healing, precluded further investigations of TNP-470.

Enzymes involved in the transformation of the arachidonic acid as the first step in the prostaglandin synthesis

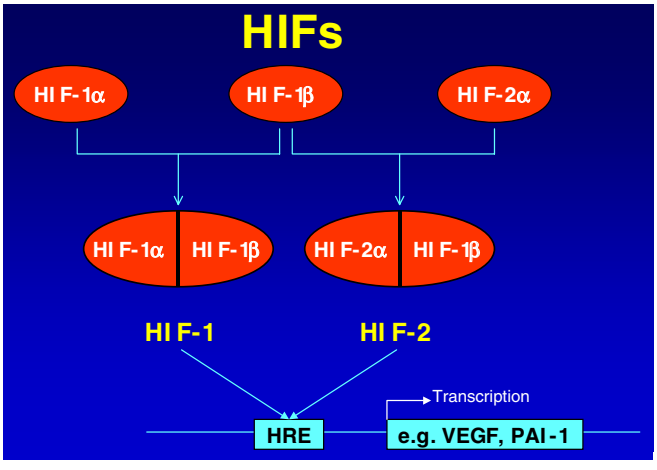

Fig. 7 Hypoxia inducible factors

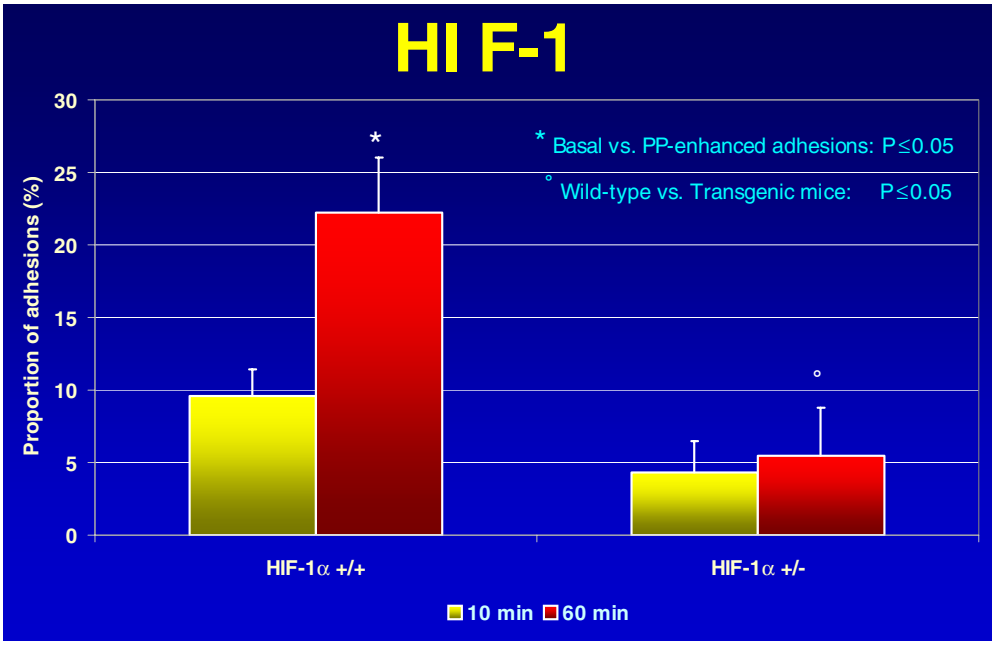

Fig. 8 Role of HIF-1 in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice (HIF-1$\alpha^{+/+}$) and transgenic mice partially deficient for HIF-1α (HIF-1$\alpha^{+/-}$). Means±SE are indicated

pathway, cyclooxygenase-1 and 2 (COX-1 and COX-2), were also evaluated for adhesion prevention. In contrast to COX-1, which is expressed on endothelial cells of normal blood vessels, COX-2 is present on new angiogenic endothelial cells [49], as well as in fibroblasts associated with surgical adhesions [50]. Therefore, COX-2 inhibitors have the potential of reducing angiogenesis and adhesion formation. In a laparotomy mouse model, in which adhesions were created by rubbing the cecum and by a silicone patch attached to the abdominal wall, animals were treated with the selective COX-2 agents, celecoxib or rofecoxib, and the nonspecific COX inhibitors, aspirin, naproxen, ibuprofen or indomethacin. Animals treated with selective and nonselective COX-2 inhibitors, except aspirin, had significantly fewer adhesions than control animals. Celecoxib produced a maximal reduction in adhesion formation compared with rofecoxib and the nonselective COX-2 inhibitors. Adhesions from mice treated with celecoxib had reduced microvessel density, suggesting inhibition of peritoneal adhesions through an antiangiogenic mechanism [51]. These observations were further supported by the reduced human fibroblast expression of VEGF after in vitro treatment with another COX-2 inhibitor, i.e. NS-358, and by stimulation of aerobic metabolism with dichloroacetic acid [52].

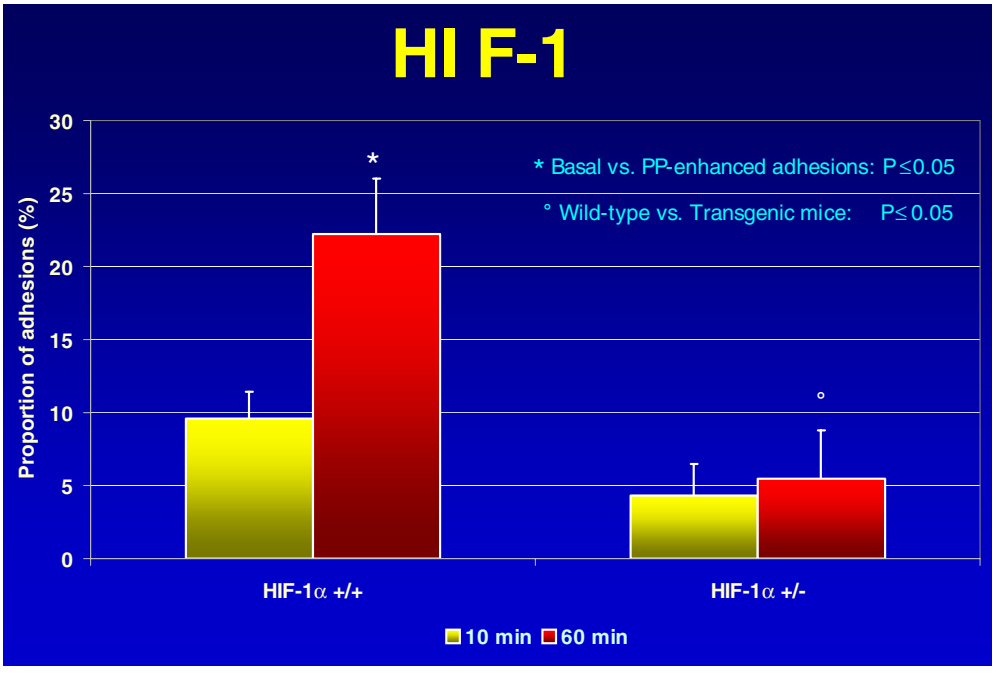

Fig. 9 Role of HIF-2 in adhesion formation. Proportion of basal adhesions (10 min of pneumoperitoneum) and pneumoperitoneum-enhanced adhesions (60 min of pneumoperitoneum) in wild-type mice (HIF-2$\alpha^{+/+}$) and transgenic mice partially deficient for HIF-2α (HIF-2$\alpha^{+/-}$). Means±SE are indicated

Although it was withdrawn from the market for the teratogenic side effects, the antiangiogenesis inhibitor thalidomide was shown to reduce adhesions formation after colonic anastomosis in rabbits [53]. The antiangiogenenic agent tamoxifen, however, did not show any beneficial effect for reducing adhesion formation in an ileo-ileal anastomosis rat model, although no adverse effects on wound or anastomotic healing were reported [54]. As mentioned before, mice data clearly demonstrate a reduction in adhesion formation after treatment with antibodies again VEGF [55], PlGF [30] or VEGFR-1 [31] opening new alternatives for adhesion prevention in humans.

Conclusions

Peritoneal adhesions, induced by infection, inflammation or surgery, are a leading cause of pelvic pain, intestinal obstruction and female infertility and cause increasing difficulties at the time of re-operation as well as increasing medical costs. It remains unknown why peritoneal wounds heal without adhesions in some patients, whereas in others, severe adhesions are formed from seemingly equal procedures, and why adhesions can develop in one surgical site and not in others in the same patient.

These observations, together with the failure of the many strategies developed over the years to prevent or at least to reduce peritoneal adhesions, clearly highlight the importance of understanding adhesion formation at the molecular level. Fortunately, we have witnessed, during the past decade, extraordinary advances in molecular biology that have led to the identification of many molecules, e.g. cytokines, growth factors, chemokines and proteases, with the potential of regulating inflammatory response, tissue remodelling and angiogenesis—events that are central to normal wound healing and to tissue fibrosis. However, the roles of all these molecules, specifically in the peritoneal biology and in the adhesion formation process, remain speculative to a large extent. Recently a specific adhesion phenotype has been reported, describing the substantial differences between the adhesion peritoneum and the apparently normal adjacent peritoneum [57], an observation that may be crucial for prevention of adhesion reformation (type 2 adhesions).

In addition to the cellular players, molecules and processes involved in postoperative adhesion formation, we have reported the importance of taking into account the potential effect of the local environment, i.e. CO_2 pneumoperitoneum for laparoscopy and air for laparotomy, to fully understand the intrinsic mechanisms involved. These observations should not be underestimated since environment-related factors such as hypoxia [28–57], hyperoxia [58, 59], desiccation and hypothermia [60, 61] could modulate every stage of the adhesion formation process in different ways. Indeed, we have initially postulated that the CO_2 pneumoperitoneum induces peritoneal hypoxia by compressing the capillary flow at the time of insufflation, which could enhance the formation of adhesions. This hypothesis was supported by the increase in adhesion formation with the duration of the pneumoperitoneum and the insufflation pressure using both CO_2 and helium pneumoperitoneum and by the decrease in adhesion formation observed after adding 2–4% of oxygen to any of the two insufflation gases [27, 28]. It was also confirmed by the absence of pneumoperitoneum-enhanced adhesions in mice deficient for the genes encoding for factors regulated by hypoxia such as HIFs [54], VEGF [30] and PAI-1 [29]. The important role of hypoxia in adhesion formation was further supported by a series of in vitro studies demonstrating increased expression of many adhesiogenic factors produced by fibroblasts cultured under hypoxic conditions [62–79].

Since angiogenesis is one of the essential steps in adhesion formation, and since it is mainly regulated by hypoxia, all these data together indicate that antiangiogenic measurements, either by preventing hypoxia and thus angiogenesis or by using antiangiogenic drugs, could be an alternative for prevention of peritoneal adhesions.

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Addendum 7

Binda MM, Molinas CR, Bastidas A. and Koninckx PR. Effect of reactive oxygen species scavengers and anti-inflammatory drugs upon CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. *Surgical Endoscopy 21(10):1826-34*, 2007.

Surg Endosc (2007) 21: 1826–1834
DOI: 10.1007/s00464-007-9296-7

SURGICAL ENDOSCOPY
and Other Interventional Techniques

Effect of reactive oxygen species scavengers, antiinflammatory drugs, and calcium-channel blockers on carbon dioxide pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model

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Received: 7 November 2006/Accepted: 17 January 2007/Online publication: 4 May 2007

Abstract
Background: Postoperative adhesions are a clinical problem. They can cause female infertility, intestinal obstruction, chronic pelvic pain, and difficulties at the time of reoperation. A variety of approaches described to prevent adhesions have shown variable and inconsistent results. Therefore, this study aimed to evaluate most known substances in a laparoscopic mouse model to obtain quantitative and comprehensive information on adhesion prevention. Specifically, this first study aimed to investigate the effects of reactive oxygen species (ROS) scavengers, antiinflammatory agents, and a calcium-channel blocker on pneumoperitoneum-enhanced adhesions.
Methods: Adhesions were induced during laparoscopy in BALB/c female mice by creation of a bipolar lesion. Carbon dioxide (CO_2) pneumoperitoneum was maintained for 60 min using humidified CO_2. Six experiments were conducted to evaluate the effects of ROS scavengers (superoxide dismutase [SOD], catalase, melatonin, and ascorbic acid), antiinflammatory agents (dexamethasone, tenoxicam, ibuprofen, parecoxib, nimesulide, anti–tumor necrosis factor [TNF]-alpha), and a calcium-channel blocker (diltiazem). Adhesions were scored after 7 days during laparotomy.
Results: Adhesions were reduced by SOD ($p < 0.01$, proc general linear methods (GLM) of experiments 1 and 2), diltiazem ($p = 0.05$, Wilcoxon), and dexamethasone ($p < 0.03$), but not by nonsteroidal antiinflammatory drugs (NSAIDs) nor by anti–TNF-alpha. When all the experiments were grouped for analysis, adhesions also decreased with one and three doses of SOD ($p < 0.01$ and $p < 0.01$, respectively) and with one and three doses of ascorbic acid ($p < 0.02$ and $p = 0.05$, respectively).
Conclusions: These experiments confirm that SOD, diltiazem, and dexamethasone can decrease adhesion formation. The absence of effect from the other antiinflammatory drugs and anti-TNF-alpha is surprising.

Key words: Antiinflammatory agents — Calcium-channel blockers — Laparoscopy — Pneumoperitoneum-enhanced adhesions — Prevention — ROS scavengers

Postoperative adhesion formation remains an important clinical problem because it causes intestinal obstruction [1], chronic pelvic pain [2], female infertility [3], and difficulties at the time of reoperation. However, adhesion prevention still is inadequate and poorly understood overall.

In animal models, adhesion prevention has been studied during both open and laparoscopic surgery. During open surgery, many products have been demonstrated to decrease postoperative adhesion formation, including corticosteroids, [4–7], nonsteroidal antiinflammatory drugs (NSAIDs) [6, 8–19], fibrinolytic agents [20], surfactants [21–25], flotation agents and semisolid barriers [23, 26–28], mechanical barriers [26, 29–32], hormones [33], calcium-channel blockers [34–38], reactive oxygen species (ROS) scavengers [39–45], and antiangiogenesis therapy [46, 47]. It should be stressed that these observations generally were reported with the use of only one drug in different models with different species and using different scoring systems. Therefore, a comprehensive quantitative evaluation of efficacy in one model still is lacking.

Presented at the XV Annual Congress of the International Society for Gynecological Endoscopy (ISGE), 29 March to 1 April, 2006, Buenos Aires, Argentina

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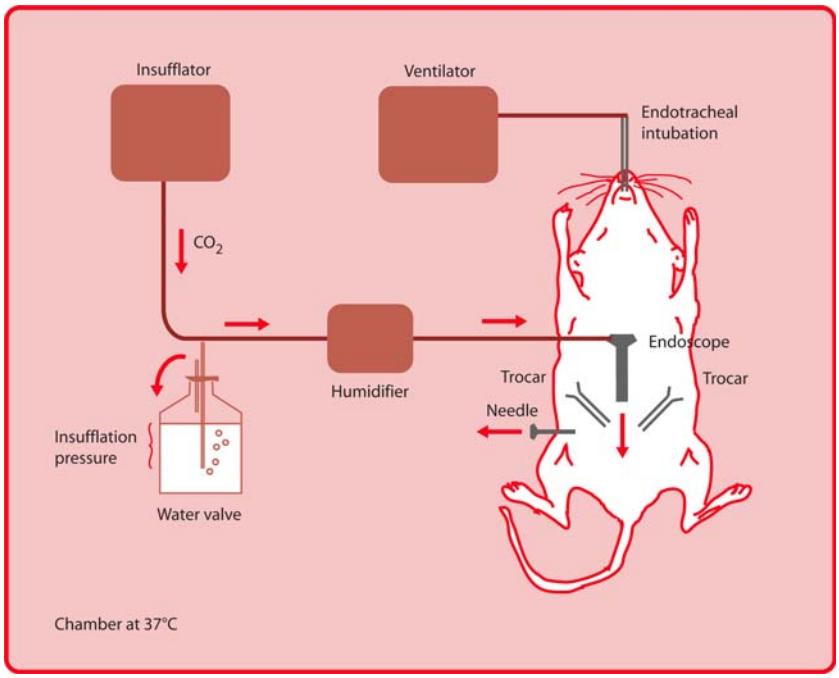

Fig. 1. Laparoscopic mouse model.

Prevention of adhesion formation after laparoscopic surgery has been poorly addressed. Some products have been demonstrated to decrease postoperative adhesions during laparoscopy including antibodies against vascular endothelial growth factor receptor 1 (VEGF-R1) [48], crystalloids [49, 50], 4% icodextrin [50], ferric hyaluronate gel [50–52], Sepracoat [53], a crosslinked hyaluronan solution [54], and hyaluronate membrane [55].

Effectiveness after open surgery for humans was demonstrated in clinical trials for the SprayGel adhesion barrier system [56], Seprafilm [57], Intergel solution [58], 0.5% ferric hyaluronate [59, 60], Sepracoat (HAL-C) solution [61], Interceed [62–64], and glycerol hyaluronate/carboxymethylcellulose [65]. In addition, the following products also were effective in preventing adhesions after laparoscopic surgery in clinical trials: SprayGel adhesion barrier system [56], Interceed [66], Viscoelastic gel [67] and Hyalobarrier Gel [68].

During laparoscopy, a pneumoperitoneum is necessary. This carbon dioxide (CO_2) pneumoperitoneum has been identified as a cofactor in adhesion formation. Mesothelial hypoxia is suggested as the driving mechanism because adhesion formation increased with insufflation pressure and with duration of pneumoperitoneum, because similar effects were observed with CO_2 and helium pneumoperitoneum, and because the addition of 2% to 4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation [69, 70]. This hypothesis also was supported by the observation that pneumoperitoneum-enhanced adhesion formation was absent in mice deficient in genes encoding for factors upregulated by hypoxia, such as hypoxia-inducible factors [71], vascular endothelial growth factor (VEGF) and placental growth factor (PiGF) [72], and plasminogen activator 1 [73].

In addition, we demonstrated that both temperature and desiccation increased adhesion formation [74, 75]. These observations led to the conclusion that the mechanisms involved in adhesion formation after CO_2 pneumoperitoneum may be different from those observed after laparotomy. This also led to new concepts of adhesion prevention such as the addition of 3% oxygen to the pneumoperitoneum [76, 77], the use of anti–VEGF-R1 antibodies [48], using anti-PiGF antibodies [72], and lowering of body temperature [74]. In this model, we demonstrated that avoidance of desiccation prevented adhesion formation [75], whereas the addition of more than 3% oxygen to the pneumoperitoneum increased adhesions [77]. This led to the hypothesis that ROS could be another cofactor in adhesion formation [78].

Because ROS scavengers, antiinflammatory drugs, and calcium-channel blockers have not been investigated for the prevention of pneumoperitoneum-enhanced adhesions, this study aimed to evaluate these drugs in our laparoscopic mouse model. These experiments are part of a series intended to evaluate most known substances in one model to obtain quantitative and comprehensive information on adhesion prevention.

Materials and methods

The laparoscopic mouse model for adhesion formation

The experimental setup (i.e., animals, anesthesia and ventilation, laparoscopic surgery, and induction and scoring of intraperitoneal adhesions) has been described in detail previously [49, 71–77]. Briefly, the model consisted of pneumoperitoneum-enhanced adhesions induced during laparoscopy by creation of a mechanical lesion. The pneumoperitoneum was kept for 60 min using pure and humidified CO_2 at 15 mmHg of insufflation pressure. Gas and body temperatures were kept strictly at 37°C using a heated chamber (Fig. 1).

Animals

This study used 192 female 9- to 10-week-old BALB/c mice weighing 20 g. The animals were kept under standard laboratory conditions. They were fed using a standard laboratory diet with free access to food and water any time. The study was approved by the Institutional Review Animal Care Committee.

Anesthesia and ventilation

The mice were anesthetized with intraperitoneal pentobarbital 0.08 mg/g, intubated with a 20-gauge catheter, and mechanically ventilated (Mouse Ventilator MiniVent, Type 845; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using humidified room air with a tidal volume of 250 μl at 160 strokes/min. Humidified air for ventilation was used to prevent cooling as occurs during ventilation with nonhumidified air [74].

Laparoscopic surgery

A midline incision was performed caudal to the xyphoides. A 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity, and the incision was closed gas tight around the endoscope to avoid leakage.

Pneumoperitoneum was created with pure CO_2 at 15 mmHg insufflation pressure using the Thermoflator Plus (Karl Storz) and a water valve to damper pressure changes. The gas was humidified (Storz Humidifier 204320 33; Karl Storz), and the whole setup was kept in a 37°C chamber to obtain CO_2 at 37°C and with 100% relative humidity. As described previously, we maintained a controlled 23-ml/min flow of CO_2 through the abdominal cavity using a 26-gauge needle to ascertain a continuous 100% CO_2 environment by constant removal of any oxygen that might have diffused from the capillaries.

Induction of intraperitoneal adhesions

Pneumoperitoneum-enhanced adhesion formation was induced by maintaining the pneumoperitoneum for 60 min and creating standardized 10 × 1.6-mm lesions in the antimesenteric border of both the right and left uterine horns and the pelvic sidewalls with bipolar coagulation (BICAP bipolar hemostasis probe, BP-5200A, 5 Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (Autocon 200; Karl Storz, standard coagulation mode).

Scoring of adhesions

Adhesions were qualitatively and quantitatively scored. Scoring was performed blindly, with the investigator not informed of the group being evaluated, after 7 days during laparotomy under microscopic vision. The qualitative scoring system assessed extent (0 [no adhesions], 1 [1–25% of the injured surface involved], 2 [26–50%], 3 [51–75%], 4 [76–100%]), type (0 [no adhesions], 1 [filmy], 2 [dense], 3 [capillaries present]), tenacity (0 [no adhesions], 1 [easily falls apart], 2 [requires traction], 3 [requires sharp dissection]), and total (extent + type + tenacity).

The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: adhesion (%) = (sum of the lengths of the individual attachments/length of the lesion) × 100. The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were individually scored.

Products

ROS scavengers

Superoxide dismutase from bovine erythrocytes and catalase from murine liver (Sigma, Bornem, Belgium) were dissolved in saline (NaCl 0.9%) to 3,000 U/ml and kept at −20°C and 4°C until used, respectively. Melatonin (Sigma) was dissolved in ethanol (60 mg/ml) and kept protected from the light at −20°C. The day of the experiment, it was diluted in saline to 2 mg/ml immediately before use. Ascorbic acid (AA) (Sigma) was dissolved to 20 mg/ml in saline before use.

Antiinflammatory drugs

Dexamethasone (Aacidexam 5 mg for injection; Organon, Bruxelles, Belgium) was prepared the day of the experiment as indicated in the product data sheet, diluted to 80 μg/ml in phosphate-buffered saline (PBS), and kept at 4°C. Nimesulide (Sigma) was dissolved in dimethyl sulphoxide (30 mg/ml), kept at −20°C, and diluted to 0.2 mg/ml in PBS the day of the experiment. Parecoxib (Dynastat injectable; Pfizer, Bruxelles, Belgium) was prepared the day of the experiment as indicated in the product data sheet, diluted to 0.2 mg/ml in PBS, and kept at 4°C. Ibuprofen 10 mg/ml (Office Chimique-Certa S.P.R.L., Braine l'alleud, Belgium) was diluted to 2.8 mg/ml in PBS and kept at 4°C until used. Intravenous (IV) tenoxicam (Tilcotil; Roche, Bruxelles, Belgium) was prepared the day of the experiment as indicated in the product data sheet, diluted to 0.4 mg/ml in PBS, and kept at 4°C.

Anti–TNF-alpha antibody

A neutralizing antibody against mouse tumor necrosis factor (TNF)-alpha and a nonneutralizing antibody (used as a control condition) were kindly provided by Centocor B.V., Leiden, The Netherlands. The concentration of both antibodies was 10 mg/ml. Neutralizing antibodies were diluted to 1 mg/ml and to 0.1 mg/ml in saline and kept at −20°C.

Calcium-channel blockers

Diltiazem hydrochloridum (Tildiem 25 mg IV; Sanofi-Synthelabo S.A.N.V., Bruxelles, Belgium) was prepared the day of the experiment as indicated in the product data sheet, diluted to 0.2 mg/ml in saline, and kept at 4°C.

All the drugs were diluted in saline except the drugs of the experiment, which were diluted with nimesulide. In this experiment, the dilutions were made in PBS because nimesulide precipitated in saline (pH of 5.5) and was completely dissolved in PBS (pH of 7.4).

At least minimally effective doses, according to the literature, were chosen. For superoxide dismutase (SOD) and catalase,15,000 U/kg, administered intraperitonealy (IP) was chosen because this dose given intravenously (IV) was shown to be effective in rats [39]. For melatonin, 10 mg/kg IP was used because it was shown to be effective in rats [42]. For AA, 100 mg/kg IP was used because it was shown to be effective in guinea pigs [79]. In addition, AA 80 and 250 mg/kg IP also were effective in mice [80, 81]. For the diet supplement, AA 8% was used because this concentration was shown to increase the dehydroascobate plasma concentration [82]. For dexamethasone, 2 mg/kg IP was used because 1 mg/kg was effective in rabbits [6]. For nimesulide, 5 mg/kg IP was used because 2.5 mg/kg was effective in rats [9]. For parecoxib, the same dose as with nimesulide was used because they both are a selective NSAID. For Ibuprofen, 70 mg/kg IP was used because this dose was shown to be effective in rabbits [14]. For tenoxicam, 10 mg/kg IP was used because 5 mg/kg IP was shown to be effective in mice [10]. For anti–TNF-alpha, 0.01, 0.1, and 1 mg IP were used because 1 mg IP was shown to be effective in mice [83]. For diltiazem, two daily doses 5 mg/kg IP were used because one daily dose of 10 mg/kg IP was shown to be effective in rats [38]. This drug was strictly controlled because overdoses can produce hypotension and bradycardia [84].

Experimental design

The time of anesthesia injection was considered time 0 (T_0). The animal preparation and ventilation started after exactly 10 min (T_{10}). The pneumoperitoneum was started at 20 min (T_{20}) and maintained for 60 min, until T_{80}. For all the experiments, eight animals per group were used.

Table 1. Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model[a]

Experiment	Group	No. of doses	Route of administration	Dose	Quantitative scoring (Proportion, %)	Qualitative scoring (Total)
1	Control (saline)	1	IP	—	27.8 ± 2.8	3.5 ± 0.4
	SOD	1	IP	300 U	17.5 ± 4.2[b,c]	2.6 ± 0.5[c]
	Catalase	1	IP	300 U	31.3 ± 5.0	3.7 ± 0.5
	Melatonin	1	IP	200 μg	27.2 ± 2.5	3.6 ± 0.3
	AA	1	IP	2 mg	22.1 ± 3.2[c]	3.5 ± 0.4
2	Control (saline)	3	IP	—	30.6 ± 3.1	3.4 ± 0.3
	SOD	3	IP	300 U	21.8 ± 3.1[b,c]	3.1 ± 0.1[c]
	AA	3	IP	2 mg	23.1 ± 4.6[c]	3.3 ± 0.5
	SOD + AA	3	IP	300 U; 2 mg	27.2 ± 3.8	3.5 ± 0.3
3	Control (PBS)	2	IP	—	34.6 ± 2.0	5.0 ± 0.3
	Control (PBS)	4	IP	—	33.7 ± 4.1	4.2 ± 0.4
	Dexamethasone	2	IP	40 μg	23.4 ± 2.3[c,d]	3.2 ± 0.1[c,d]
	Nimesulide	4	IP	100 μg	26.8 ± 3.7	3.8 ± 0.2
	Parecoxib	4	IP	100 μg	34.1 ± 4.4	4.1 ± 0.4
	Ibuprofen	4	IP	1.4 mg	28.8 ± 2.2	4.0 ± 0.4
	Tenoxicam	2	IP	200 μg	36.4 ± 2.8	3.9 ± 0.7
4	Control (Ab)	1	IP	1 mg	29.9 ± 3.8	3.3 ± 0.3
	Anti-TNFα Ab, 0.01	1	IP	0.01 mg	23.0 ± 7.6	3.2 ± 0.6
	Anti-TNFα Ab, 0.1	1	IP	0.1 mg	34.6 ± 6.6	3.4 ± 0.5
	Anti-TNFα Ab, 1	1	IP	1 mg	37.1 ± 5.4	3.6 ± 0.4
5	Control (Ab)	1	IV	1 mg	26.9 ± 3.4	3.6 ± 0.8
	Anti-TNFα Ab, 1	1	IV.	1 mg	28.1 ± 2.8	2.5 ± 0.8
6	Control (saline)	4	IP	—	35.7 ± 3.4	3.9 ± 0.3
	Diltiazem	4	IP	100 μg	22.5 ± 4.6[c,d]	3.0 ± 0.5[c]

IP, intraperitoneal; SOD, superoxide dismutase; AA, ascorbic acid; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IV, intravenous; Ab, antibody

[a] Carbon dioxide (CO_2) pneumoperitoneum using humidified gas at 15 mmHg was maintained for 60 min. Adhesions were induced during laparoscopy by performing a bipolar lesion. Six experiments were performed evaluating the effects of reactive oxygen species (ROS) scavengers (SOD, catalase, melatonin and ascorbic acid), antiinflammatories (dexamethasone, tenoxicam, ibuprofen, parecoxib, nimesulide and anti–TNF-alpha antibodies) and a calcium channel blocker (diltiazem). In this table, quantitative (proportion) and qualitative (total) scores are presented (mean ± standard error of the mean). The volumes administrated per dose were 100 μl for experiments 1, 4, and 5; 200 μl for experiment 2; and 500 μl for experiments 3 and 6

[b] $p < 0.05$: Experiments 1 and 2 analyzed together (proc general linear methods [GLM], 2 groups, 2 variables [i.e., experiment and treatment])

[c] $p < 0.05$: Interexperiment comparisons (each group compared with a control group grouping all the control subjects) (Wilcoxon test)

[d] $p < 0.05$: Intraexperiment comparisons (each group compared with its own control group) (Wilcoxon test)

To make the table clearer, only the comparisons to the control groups were placed

Experiment 1 (5 groups) was designed to evaluate the effect of ROS scavengers on adhesion formation. Because it is well known that ROS scavengers are produced during reperfusion, 100 μl of SOD, catalase, melatonin, and AA were injected IP at T_{75} (i.e., 5 min before the pneumoperitoneum was ended). Similarly, 100 μl of saline was injected in the control group.

Because some inhibitory effect was shown with SOD and AA, experiment 2 (4 groups) was designed for a more detailed study on the effect from higher doses of SOD, AA, and the combination of both on adhesion formation. In this experiment, 100 μl of SOD + 100 μl of saline (SOD group), 100 μl of AA + 100 μl of saline (AA group), 100 μl of SOD + 100 μl of AA (SOD + AA group), or 200 μl of saline (control group) were administrated IP at the beginning (T_{20}), before completion (T_{75}), and 30 min after completion of the pneumoperitoneum (T_{110}). In addition, the mice of groups AA and SOD + AA received AA orally (PO) in their food (Harlan Special Diet 2018 containing 8% ascorbic acid; Horst, The Nederland) 1 week before and after the surgery.

Experiment 3 (7 groups) was designed to evaluate the effect of steroidal (dexamethasone) and cyclooxygenase-2 (COX-2) nonselective (ibuprofen and tenoxicam) and COX-2 selective (nimesulide, parecoxib) NSAIDs on adhesion formation. For the dexamethasone and tenoxicam groups, the mice received two IP doses of 500 μl (immediately after creation of the lesion and on the day after the surgery) because these drugs have a very long half-life (36–72 h and 67 h, respectively) [85, 86]. The mice in the nimesulide, parecoxib, and ibuprofen groups received four IP doses of 500 μl (immediately after creation of the lesion, 6 h later, the day after the surgery in the morning, and 6 h later) because those drugs have a shorter half-life (1.80–4.73 h, 7 h, and 2–4 h, respectively) [85, 87, 88]. Two control groups were used, receiving two and four saline doses of 500 μl, respectively, in the same way as the treated groups.

Experiment 4 (4 groups) was designed to evaluate the effect of anti–TNF-alpha antibodies administered IP on adhesion formation. Immediately after induction of the pneumoperitoneum, 100 μl of either nonneutralizing antibodies 1 mg (control group) or anti–TNF-alpha antibodies 0.01 mg, 0.1 mg, or 1 mg were injected IP under laparoscopic vision.

Experiment 5 (2 groups) was designed to evaluate the effect of anti–TNF-alpha antibodies administered IV on adhesion formation. The day before the surgery, either nonneutralizing antibodies 1 mg/100 μl (control group) or anti–TNF-alpha antibodies 1 mg/100 μl were injected IV in the vein of the animal tail.

Experiment 6 (2 groups) was designed to evaluate the effect of a calcium-channel blocker, diltiazem, on adhesion formation. Because diltiazem has a short half-life (1.5–7 h) [89], the mice received four IP doses of 500 μl (immediately after creation of the lesion, 6 h later, on the day after the surgery in the morning, and 6 h later). Similarly, four saline doses of 500 μl were administered in the control group.

Statistical analysis

Statistical analyses were performed using the SAS System (SAS Institute, Cary, NC, USA). Differences in adhesion formation were evaluated with the Wilcoxon test and with procedure general linear methods (proc GLM) for a two-way analysis of variance (ANOVA) of the data from experiments 1 and 2. In Table 1, all the data are presented as the mean ± standard error of the mean. To represent all data in one figure, proportion data (mean ± standard deviation) were divided by the mean of the their control group and multiplied by 100 to be expressed as a percentage of the control subjects.

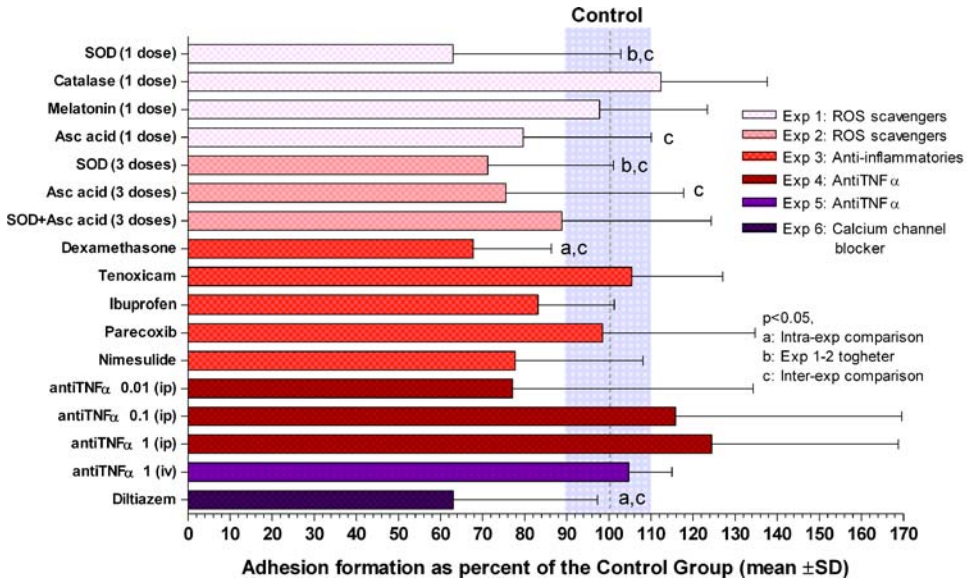

Fig. 2. Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. Carbon dioxide pneumoperitoneum (humidified gas at 15 mmHg) was maintained for 60 min. Adhesions were induced during laparoscopy by performing a bipolar lesion. Six experiments were performed evaluating the effects of reactive oxygen species (ROS) scavengers (superoxide dismutase, catalase, melatonin, and ascorbic acid), antiinflammatories (dexamethasone, tenoxicam, ibuprofen, parecoxib, nimesulide, and anti–tumor necrosis factor [TNF]-alpha antibodies), and a calcium-channel blocker (diltiazem). Proportions of adhesions are indicated. For visualization of the results for all the experiments in one graph, the percentage of change in comparison with the controls is given for each treatment. The coefficient of variation for all the controls is indicated as the shadowed area. $^{a}p < 0.05$: Intraexperiment comparisons (each group compared with its own control group) (Wilcoxon test). $^{b}p < 0.05$: Experiments 1 and 2 analyzed together (proc general linear methods [GLM], 2 groups, 2 variables [i.e., experiment and treatment]); p value of the variable treatment. $^{c}p < 0.05$: Interexperiment comparisons (each group compared with a control group grouping all the controls) (Wilcoxon test).

Results

The results of all six experiments are presented in Table 1. In experiments 1 and 2, the effect of the ROS scavengers, catalase, SOD, melatonin, and AA were evaluated. When both experiments were analyzed together (proc GLM, 2 groups, 2 variables [i.e., experiment and treatment]), SOD reduced adhesion formation: effect of treatment (proportion: $p < 0.01$; extent: $p < 0.01$); effect of experiment (not significant). None of the other drugs showed any important effect on adhesion formation, neither when the two experiments were analyzed together nor when they were analyzed as single experiments (Wilcoxon).

In experiment 3, the effect of antiinflammatory drugs was evaluated. First, no differences were found between two and four doses of saline injected IP in the control groups (Wilcoxon). Dexamethasone reduced adhesion formation (proportion: $p < 0.03$, total: $p = 0.01$; extent: $p < 0.02$, type: $p < 0.01$, tenacity: $p < 0.01$), but no significant effect was observed when ibuprofen, tenoxicam, nimesulide, and parecoxib were used. The mice treated with dexamethasone had less adhesions than the mice treated with tenoxicam (proportion: $p = 0.02$, total: $p < 0.04$, extent: $p < 0.04$), but not less adhesions than the mice treated with nimesulide, parecoxib, and ibuprofen.

In experiments 4 and 5, the effect of neutralizing anti–TNF-alpha antibodies was analyzed. In comparison with the control group, the IP administration of 0.01, 0.1, and 1 mg and the IV administration of 1 mg of the neutralizing antibodies did not have any effect on adhesion formation. In experiment 6, the effect of a calcium-channel blocker was analyzed. Diltiazem reduced adhesion formation in comparison with the control group (proportion: $p = 0.05$).

The results of all the experiments can be visualized in Fig. 2. Adhesion formation in the control groups during the six experiments was comparable (proc GLM, 2 variables [i.e., experiment and block, nonsignificant effect for both]), with a coefficient of variation of 10% only. Given this low variability of adhesion formation between the experiments, a reanalysis was performed grouping the control groups in all the experiments (total, 56) as the comparator. As expected, this confirmed the effects of SOD, dexamethasone, and diltiazem. In addition, adhesions decreased with one IP dose of SOD (proportion: $p < 0.01$, total: $p < 0.01$, extent: $p < 0.01$, type: $p < 0.01$) and with three IP doses of SOD (proportion: $p < 0.01$, total: $p < 0.01$, extent: $p < 0.01$). Moreover, adhesions decreased with one IP dose of AA (proportion: $p < 0.02$, extent: $p < 0.02$) and with the combined use of three IP ascorbic acid doses together with PO administration (proportion: $p = 0.05$).

Discussion

In open surgery models for adhesion formation effectiveness of ROS scavengers, antiinflammatory drugs, and calcium-channel blockers have been shown, albeit in isolated experiments with mice [10, 45], rats [4, 5, 7–9, 11–13, 16, 38–40, 42–44], hamsters [36], and rabbits [6, 14, 15, 17, 18, 31, 34, 37, 41]. In our pneumoperitoneum-

enhanced adhesion mouse model, ROS scavengers (only SOD was significant), dexamethasone and a calcium-channel blocker (diltiazem) decreased adhesion formation, whereas no effect was found for NSAIDs, even for specific COX-2 inhibitors or for anti–TNF-alpha antibodies. These data should be interpreted cautiously because results from one species do not necessarily apply to other species.

In open surgery, ROS scavengers have been described as reducing adhesion formation in mice [45], rats [39, 40, 42–44], and rabbits [41], but the effect is not consistent (e.g., vitamin E failed to decrease adhesion formation in rats) [90]. In our laparoscopic mouse model, we confirmed that ROS scavengers can decrease adhesion formation, although the overall effect was small. It is not surprising that not all products decreased adhesions significantly given the small number of animals in each group. Increasing the number of animals and repeating the experiments would not have changed the message that ROS scavengers can be effective but the effect is small. It is unlikely but cannot be excluded that IP administration such as we used is less effective than IV administration. Possibly, results differ between mice and rats. In rats the same dose of SOD and catalase (i.e., 15,000 U/kg IV) decreased intraperitoneal adhesions [39]. Also for melatonin, the same IP dose of 10 mg/kg body weight was effective in rats [42].

Most important, however, is that effectiveness is more pronounced after open surgery because exposure to air with approximately 21% of oxygen (i.e., a partial pressure of 160 mmHg) may generate much more ROS than the ischemia–reperfusion process after laparoscopy. In conclusion, we confirmed in our laparoscopic mouse model that ROS scavengers can reduce adhesion formation but the effect is small.

Antiinflammatory drugs are widely accepted for reducing adhesion formation in mice [10, 91], rats [4, 5, 7–9, 11–13, 16], and rabbits [6, 14, 15, 17–19]. However, these effects have not always been consistent in open surgery models. Ibuprofen failed to reduce adhesion formation in rats [7]. Anti–TNF-alpha antibodies also failed to reduce adhesions in a rat cecal serosal abrasion model [92]. In our laparoscopic mouse model, we confirmed the effectiveness of dexamethasone, but failed to demonstrate any important effects of NSAIDs as COX-1 and COX-2 inhibitors or of neutralizing anti–TNF-alpha antibodies.

Taken together, these data after both open surgery and laparoscopy suggest that this inflammatory reaction may be less important for adhesion formation. The absence of effect from anti–TNF-alpha antibodies with their strong effects on inflammation supports this suggestion. The fact that dexamethasone, nevertheless, had some effect on adhesion prevention suggests the involvement of other mechanisms. Glucocorticoids indeed can inhibit fibroblast proliferation and can have immunosuppressive effects on the production and release of cytokines [85].

Diltiazem, a calcium-channel blocker, reduced adhesion formation in our laparoscopic model, confirming the results for open surgery [34–38]. The suggested cause of this effect involves mechanisms such as interference with the inflammatory response [93], protection against the toxic effect of the ischemic–reperfusion cell injury [94], and activation of cellular processes [95].

The current understanding of adhesion formation comprises several mechanisms including the initial inflammatory reaction, with exudation and fibrin deposition, and fibroblast migration or differentiation, as well as a role for macrophages and later angiogenesis and collagen deposition. These experiments question the importance of the inflammatory reaction while confirming the effectiveness of ROS scavengers (SOD and AA) and calcium-channel blockers (diltiazem), at least in our model.

These obviously are screening experiments. It remains to be confirmed that the conclusion is valid for other species, especially primates, and for other adhesion formation models. Unfortunately the cost and effort associated with experiments of sufficient size with larger animals is prohibitive for screening experiments. Our screening data nevertheless warrant evaluating at least ROS scavengers such as SOD and AA, antiinflammatory drugs such as dexamethasone, and calcium-channel blockers such as diltiazem.

In conclusion, we confirmed with our laparoscopic mouse model the effects of ROS scavengers, calcium-channel blockers, and dexamethasone. The absence of effect from the other antiinflammatory drugs and anti–TNF-alpha antibodies is surprising. It is clear that we currently can begin to identify mechanisms and players, but we are far from a comprehensive understanding of the adhesion formation process and its prevention.

Acknowledgments. We thank R. Pijnenborg, PhD, Ms. R. Van Bree, J. Verguts, MD, L. Ret, MD, C. De Cicco, MD, and L. Vercruysse, MSc (Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg) for their help. This study was supported by Onderzoeks Toelagen Katholieke Universiteit Leuven (Leuven, Belgium grant TBA/00/27) and in part by Karl Storz Endoscopy (Tuttlingen, Germany).

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Addendum 8

Binda MM, Molinas CR, Bastidas A, Jansen M and Koninckx PR. Efficacy of barriers and hypoxia inducible factor inhibitors to prevent CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. *Journal of Minimally Invasive Gynecology 14(5):591-9, 2007*.

Journal of Minimally Invasive Gynecology (2007) 14, 591–599

THE JOURNAL OF
MINIMALLY INVASIVE
GYNECOLOGY

Efficacy of barriers and hypoxia-inducible factor inhibitors to prevent CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model

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KEYWORDS:
Barriers;
Flotation;
HIF;
Hypoxia;
Intraperitoneal adhesion formation;
Laparoscopy;
Pneumoperitoneum;
Prevention;
Surfactant

Abstract

STUDY OBJECTIVE: To investigate the effects of hypoxia-inducible factor (HIF) inhibitors, flotation agents, barriers, and a surfactant on pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model.

DESIGN: Prospective randomized trial (Canadian Task Force classification I).

SETTING: Department of Obstetrics and Gynecology, University Hospital Gasthuisberg, Catholic University of Leuven.

SUBJECTS: One hundred fourteen female BALB/c mice.

INTERVENTIONS: Adhesions were induced during laparoscopy in BALB/c female mice. Pneumoperitoneum was maintained for 60 minutes with humidified CO_2. In 3 experiments the effects of HIF inhibitors such as 17-allylamino 17-demethoxygeldanamycin, radicicol, rapamycin, and wortmanin, flotation agents such as Hyskon and carboxymethylcellulose, barriers such as Hyalobarrier gel and SprayGel, and surfactant such as phospholipids were evaluated.

MEASUREMENTS AND MAIN RESULTS: Adhesions were scored after 7 days during laparotomy. Adhesion formation decreased with the administration of wortmannin ($p < .01$), phospholipids ($p < .01$), Hyalobarrier Gel ($p < .01$), and SprayGel ($p < .01$).

CONCLUSIONS: These experiments confirm the efficacy of barriers and phospholipids to separate or lubricate damaged surfaces. They also confirm the role of mesothelial hypoxia in this model by the efficacy of the HIF inhibitor wortmannin.

Supported by Onderzoeks Toelagen Katholieke Universiteit Leuven (Leuven, Belgium grant TBA/00/27) and in part by Karl Storz Endoscopy (Tuttlingen, Germany).

Presented, in part, at the 21st annual meeting of European Society of Human Reproduction and Embryology (ESHRE), June 19th–22nd, 2005, Copenhagen, Denmark, and at the XV annual congress of the International Society for Gynecological Endoscopy (ISGE), March 29th–April 1st, 2006, Buenos Aires, Argentina.

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Submitted January 8, 2007. Accepted for publication April 2, 2007.

1553-4650/$ -see front matter © 2007 AAGL. All rights reserved.
doi:10.1016/j.jmig.2007.04.002

One of the complications of abdominal surgery is intra-abdominal adhesion formation. Adhesions can cause intestinal obstruction, chronic pain, and infertility. Although postoperative adhesion formation remains an important clinical problem, its prevention is still inadequate and overall poorly understood.

Over recent years, CO_2 pneumoperitoneum became known as a cofactor in postoperative adhesion formation.[1] Mesothelial hypoxia was suggested as a mechanism, because adhesions increased with duration of CO_2 pneumoperitoneum and with insufflation pressure, because similar effects were observed with helium pneumoperitoneum and because the addition of 2% to 4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation.[1] This hypothesis was supported by the absence of pneumoperitoneum-enhanced adhesions in mice deficient for factors which are up-regulated during hypoxia, such as plasminogen activator inhibitor 1 (PAI-1),[2] vascular endothelial growth factor (VEGF), placental growth factor,[3] and hypoxia-inducible factor 1α and 2α (HIF-1α and HIF-2α).[4]

HIF is an α/β heterodimeric DNA-binding complex that directs an extensive transcriptional response to hypoxia. HIF activity is induced during hypoxia through the stabilization and activation of its subunit HIF-1α whereas during normoxia subunit HIF-1α is rapidly degraded by the ubiquitin-proteosome system.[5] Inhibition of HIF activity can be achieved by decreasing heat shock protein 90 (Hsp-90) or by blocking the phosphatidylinositol 3-kinase (PI3K) pathway. The molecular chaperone Hsp-90 is important to maintain the appropriate folding and conformation and to regulate the balance of synthesis and degradation of its clients such as HIF-1α.[6] Therefore Hsp-90 inhibitors, such as radicicol, geldanamycin, and its derivated 7-allylaminogeldanamycin (17-AAG), decrease HIF-1α activity.[6] In addition, 17-AAG and radicicol bind to the intrinsic ATPasa activity in the N-terminal site of Hsp-90, resulting in degradation of Hsp-90 client proteins by the ubiquitin proteosome pathway. Another approach to decrease HIF activity is the inhibition of the PI3K/Akt pathway with inhibitors such as wortmannin and rapamycin[7] because phosphorylation is involved in the HIF-1α subunit stabilization, as well as in the regulation of HIF-1 transcriptional activity.[8]

Prevention of adhesion formation after laparoscopic surgery has been poorly addressed. Several agents have been tested, such as antibodies against VEGFR1,[9] crystalloids,[10,11] 4% icodextrin,[11] ferric hyaluronate gel,[11–13] Sepracoat,[14] a cross-linked hyaluronan solution,[15] and hyaluronate membrane,[16] which were effective in different animal models. These observations, however, were generally reported with only 1 drug in different models, different species, and with different scoring systems. A comprehensive quantitative evaluation of efficacy in 1 model is still lacking.

These experiments are the second part of a series of experiments to evaluate most known substances in 1 model to obtain quantitative and comprehensive information on adhesion prevention. In this article, the effects of HIF inhibitors, flotation agents, barriers, and a surfactant were investigated.

Materials and methods

The laparoscopic mouse model for adhesion formation

Experimental setup, that is, animals, anesthesia, and ventilation, laparoscopic surgery, induction, and scoring of intraperitoneal adhesions, has previously been described in detail.[1–4,9,10,17–19,27] Briefly, in the pneumoperitoneum-enhanced adhesions model, adhesions were induced during laparoscopy by creating a mechanical lesion. Pneumoperitoneum was maintained for 60 minutes with pure and humidified CO_2 at 15 mm Hg insufflation pressure. Gas and body temperatures were kept strictly at 37°C with a heated chamber (Figure 1).

Animals

One hundred fourteen 9- to 10-week-old female BALB/c mice weighing 20 g were used. Animals were kept under standard laboratory conditions, and they were fed a standard laboratory diet with free access to food and water at any time. The study was approved by the Institutional Review Animal Care Committee.

Anesthesia and ventilation

Mice were anesthetized with intraperitoneal 0.08 mg/g pentobarbital, intubated with a 20-gauge catheter, and mechanically ventilated (Mouse Ventilator MiniVent, Type 845; Hugo Sachs Elektronik-Harvard Apparatus GmbH,

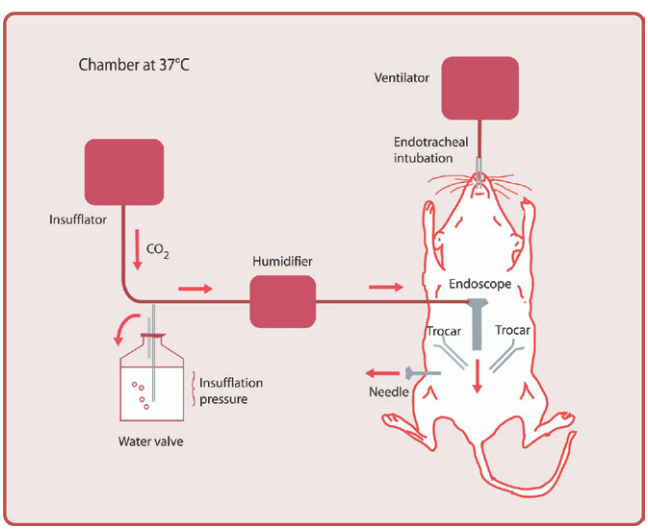

Figure 1 Laparoscopic mouse model.

March-Hugstetten, Germany) by use of humidified room air with a tidal volume of 250 μL at 160 strokes/min. Humidified air for ventilation was used to prevent cooling, as occurs during ventilation with non-humidified air.[17]

Laparoscopic surgery

A midline incision was performed caudal to the xiphoid process, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity, and the incision was closed gas tight around the endoscope to avoid leakage.

Pneumoperitoneum was created with pure CO_2 at 15 mm Hg insufflation pressure using the Thermoflator Plus (Karl Storz) and a water valve to damper pressure changes. The gas was humidified (Storz Humidifier 204320 33; Karl Storz), and the whole setup was kept in a chamber at 37°C to obtain CO_2 at 37°C and with 100% relative humidity. We used, as described previously, a controlled flow of CO_2 through the abdominal cavity of 23 mL/min with a 26-gauge needle, to ascertain a continuously 100% CO_2 environment by removing constantly any oxygen that might have diffused from the capillaries.

Induction of intraperitoneal adhesions

Pneumoperitoneum-enhanced adhesion formation was induced by maintaining the pneumoperitoneum for 10 or 60 minutes and by performing standardized 10-mm × 1.6-mm lesions in the antimesenteric border of both right and left uterine horns and pelvic sidewalls with bipolar coagulation (BICAP, bipolar hemostasis probe, BP-5200A, 5F, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (Autocon 200; Karl Storz; standard coagulation mode).

Scoring of adhesions

Adhesions were qualitatively and quantitatively scored. Scoring was done blindly (the investigator was not informed of the group being evaluated) after 7 days during laparotomy under microscopic vision. The qualitative scoring system assessed the following: extent (0: no adhesions; 1: 1%–25%; 2: 26%–50%; 3: 51%–75%; 4: 76%–100% of the injured surface involved, respectively), type (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), tenacity (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection), and total (extent + type + tenacity).

The quantitative scoring system assessed the proportion of the lesions covered by adhesions with the following formula: adhesion (%) = (sum of the length of the individual attachments/length of the lesion) × 100. The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were individually scored. Because the initial measurements are in millimeters (thus with an error of 0.5 mm), the precision of the division will be around 1%. According to the law of error propagation (where $\Delta Z/Z = \Delta X/X + \Delta Y/Y$) the accuracy yields some 4% CV or 1% for the sum of 4 estimates. Therefore only 1 digit becomes significant.[20]

Products

HIF inhibitors

The 17-allylamino 17-demethoxygeldanamycin (17-AAG) was donated by Kosan Biosciences (Hayward, CA). Radicicol, wortmannin, and rapamycin were bought from A.G. Scientific, Inc., (San Diego, CA). The doses administered were 17-AAG 20 mg/kg, radicicol 25 mg/kg, wortmannin 0.31 mg/kg, and rapamycin 3 mg/kg. Radicicol, rapamycin, 17-AAG, and wortmannin were dissolved in pure dimethylsulphoxide (DMSO) at final concentrations of 7.5 mg/mL, 0.9 mg/mL, 6 mg/mL, and 5 mg/mL, respectively. Afterward, wortmannin stock was diluted to 0.093 mg/mL in saline solution. Stocks were kept at −20°C until they were used.

The doses used in this experiment were those proven effective or nontoxic in the in vivo models. Rapamycin at the dose of 3 mg/kg showed an immunosuppressive effect in mice and rats.[21,22] Wortmannin at the dose of 0.31 mg/kg (MTD/2) showed an antitumor effect in mice.[23] Different MTD doses of 17-AAG were found in the literature, that is, 50 mg/kg and 80 mg/kg, and they both showed an antitumor effect in mice.[24,25] We tried 40 mg/kg, but it was toxic in our model; therefore the dose used was 20 mg/kg. Although the MTD of radicicol did not show any antitumor effect in mice,[26] the dose of 25 mg/kg (MTD/4) was used because it was nontoxic.

Flotation agents

Carboxymethylcellulose 2% was prepared and sterilized by the pharmacists of the Hospital Gasthuisberg. Hyskon (32% dextran 70) was donated by Gynotec (Malden, The Netherlands).

Mechanical barriers

Hyalobarrier Gel is a sterile, transparent, and highly viscous gel obtained by condensation of hyaluronic acid (HA) through an auto-cross-linking process and is indicated for laparoscopic and hysteroscopic or open surgical procedures. It was kindly provided by Fidia Advanced Biopolymers SRL (Abano Terme, Padova, Italy). SprayGel Adhesion Barrier System consists of 2 liquids which cross-link to form a biocompatible absorbable hydrogel; it can be used for laparoscopic and laparotomy procedures. SprayGel (Confluent Surgical, Inc., Waltham, MA) was donated by Medical International AG (Kaltenbach, Switzerland).

Surfactant

Phospholipids solution (9%), donated by Dr. Marc Jansen (Department of Surgery, University Clinic, RWTH Aachen, Germany), was diluted to 3% in saline solution before use.

Experimental design

Because anesthesia and ventilation can influence body temperature, the timing was strictly controlled. The time of anesthesia injection was considered time 0 (T_0). The animal preparation and ventilation started after exactly 10 minutes (T_{10}). The pneumoperitoneum started at 20 minutes (T_{20}) and was maintained for 10 or 60 minutes, until T_{30} or $T_{80,}$ respectively.

We used a sample size of 8 mice because, taking into account the coefficient of variability of 30% for adhesion formation in our experiments in Balb/c mice[27] and the power of the experiment of 70%, a decrease of 40% in adhesions formation can be detected. A decrease of less than 40% in adhesion is not clinically relevant.

Experiment 1 was designed to evaluate the effect of radicicol, rapamycin, 17-AAG, and wortmannin on adhesion formation. Pneumoperitoneum-enhanced adhesions were induced, and 0.1 mL of the HIF pathway inhibitors (17-AAG, rapamycin, radicicol, and wortmannin) was injected immediately before performing the lesions under laparoscopic vision (17-AAG, rapamycin, radicicol and wortmannin groups, respectively). Two control groups for pneumoperitoneum-enhanced adhesions were used, the first without any treatment, the second with injection of 0.1 mL of the vehicle used to dissolve the drugs (control 60 minutes pneumoperitoneum and control vehicle, respectively). Another control group was performed maintaining the pneumoperitoneum for 10 minutes (control 10 minutes pneumoperitoneum or basal adhesion), and no treatment was administered (7 groups, 8 mice per group, n = 56).

Experiment 2 was designed to evaluate the effect of flotation agents, Hyskon and carboxymethylcellulose 2% (CMC 2%) and a barrier Hyalobarrier gel, on adhesion formation. After performing the lesion, a volume of 0.5 mL of Hyskon or CMC 2% were injected intraperitoneally (Hyskon and CMC 2% groups, respectively) and approximately 1 mL of Hyalobarrier gel was applied on the lesions (Hyalobarrier gel group) under laparoscopic vision. The control group received intraperitoneally saline solution 0.5 mL. Injection of Hyskon in the abdominal cavity was associated with 20% of deaths. Because 2 of 8 mice died within the 24 hours after the surgery, they were replaced (2 of 10 mice = 20% mortality rates) (4 groups, 8 mice per group, 10 mice for Hyskon group; n = 32 mice).

Experiment 3 was designed to evaluate the effect of a mechanical barrier (SprayGel) and a surfactant (phospholipids) on adhesion formation. After performing the lesions, a small incision was made, and the 5-mm SprayGel applicator was introduced in the abdominal cavity. SprayGel was applied immediately following the instructions for use, and 2 stitches were made to close the incision. After application of the product, SprayGel-coated tissues were rinsed with 0.5 mL of saline solution (SprayGel group). Phospholipids 3% solution 0.5 mL was applied intraperitoneally after performing the lesion (Phospholipids group). The control group received saline solution 0.5 mL (3 groups, 8 mice per group, n = 24).

Each experiment was performed with block randomization by day to avoid day-to-day variability. Therefore, 1 block of mice comprising 1 animal of each group was operated on the same day, and within a block the animals were operated in a random order.

Statistics

Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC). Since adhesions scores were not normally distributed (Kurtosis test), medians and ranges are shown and differences between groups were evaluated by the nonparametric Wilcoxon rank-sum test. Because several products were used to test the same hypothesis, a Bonferroni correction[28] was used to exclude spurious significances, that is, the alpha value for significance ($\alpha = 0.05$) was divided by the number of products tested.

The p values for all the comparisons were included in the Results section. To make Table 1 and Figure 2 clearer, only the significant values were included.

Results

The results of all 3 experiments are listed in Table 1 and Figure 2. In experiment 1, the effect of HIF inhibitors was evaluated. We confirmed as shown previously that adhesion formation was higher after 60 minutes than after 10 minutes of pneumoperitoneum (proportion: p = .02, total: p = .08, extent: p = .01, type: p = .19, tenacity: p = .15, Wilcoxon rank-sum test). The administration of the vehicle DMSO did not have any effect on adhesion formation (proportion: p = .93; total: p = .96; extent: p = 1.0; type: p = .64; tenacity: p = .85). Wortmannin reduced pneumoperitoneum-enhanced adhesion formation in comparison with both controls groups, that is, the untreated (proportion: p = .04; total: p = .08; extent: p = .04; type: p = .02; tenacity: p = .19) or vehicle-treated (proportion: p = .01; total: p = .03; extent: p = .01; type: p = .07; tenacity: p = .22) control groups. After wortmannin treatment in the 60-minute pneumoperitoneum group, adhesions were no longer different from basal adhesions, that is, 10 minutes of pneumoperitoneum (proportion: p = .43, total: p = .75, extent: p = .41, type: p = .83, tenacity: p = .70). The 17-AAG, rapamycin, and radicicol did not reduce adhesions neither in comparison with the untreated-control group (proportion: p = .49; total: p = .89; extent: p = .72; type: p = .89; tenacity: p = 1.0; proportion: p = .48; total: p = .54; extent: p = .48;

Table 1 Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model

Experiment	Group	Concentration; volume of the dose	Qualitative scoring (total)
1	Control 10 min PP: basal	—	1.5 (0–2.5)
	Control 60 min PP: untreated	—	2.3 (1.3–7.0)
	Control: 60 min PP: DMSO	0.1 mL	2.3 (1.8–3.0)
	17-AAG	7.5 mg/mL; 0.1 mL	2.3 (1.0–5.5)
	Radicicol	9 mg/mL; 0.1 mL	3.0 (0.0–6.0)
	Rapamycin	5 mg/mL; 0.1 mL	2.8 (0.0–6.3)
	Wortmannin	93 μg/mL; 0.1 mL	1.3 (0.0–3.0)†
2	Control (saline solution)	0.5 mL	3.5 (3.3–5.3)
	Hyskon	0.5 mL	3.3 (2.5–5.3)
	CMC	2%; 0.5 mL	3.1 (1.8–3.8)
	Hyalobarrier Gel	Around 1 mL	0.5 (0.0–2.0)*
3	Control (saline solution)	0.5 mL	3.8 (3.0–4.8)
	SprayGel	Quantity necessary to cover the lesions	2.8 (1.0–2.8)*
	Phospholipids	3%; 0.5 mL	3.0 (3.0–4.5)

17-AAG = 7-allylaminogeldanamycin; CMC = carboxymethylcellulose; DMSO = dimethylsulphoxide; PP = CO_2 pneumoperitoneum.

CO_2 pneumoperitoneum was maintained for 60 minutes (humidified gas, 15 mm Hg insufflation pressure). Adhesions were induced during laparoscopy by performing a bipolar lesion. Three experiments were performed evaluating the effects of HIF inhibitors (17-AAG, radicicol, rapamycin, and wortmannin), surfactants (phospholipids 3%), flotation agents (Hyskon and CMC), and barriers (Hyalobarrier gel and SprayGel). Adhesions were scored after 7 days during laparotomy.

The qualitative scoring system (total) is represented (median, range). To make the table clearer, only the significant comparisons to the control groups were placed.

*p <.05 intraexperiment comparisons (each group compared to its own control group).

†p <.05 intraexperiment comparisons (each group compared to the vehicle control group for experiment 1).

type: p = .53; tenacity: p = .41; proportion: p = .79; total: p = .83; extent: p = .79; type: p = .71; tenacity: p = .65; respectively) nor with the vehicle treated-control group (proportion: p = .86; total: p = .96; extent: p = .93; type: p = .85; tenacity: p = .85; proportion: p = .36; total: p = .38; extent: p = .25; type: p = .55; tenacity: p = .21; proportion: p = .46; total: p = .40; extent: p = .54; type: p = .70; tenacity: p = .42; respectively).

In experiment 2, the effect of Hyskon, CMC 2%, and Hyalobarrier gel were evaluated on 60 minutes of pneumoperitoneum-enhanced adhesions. In comparison with the control group, adhesion formation decreased strongly with Hyalobarrier gel (proportion: p <.01; total: p <.01; extent: p <.01; type: p <.01; tenacity: p <.01), but not significantly with Hyskon (proportion: p = .18; total: p = .23; extent: p = .17; type: p = .19; tenacity: p = .26) or CMC 2% (proportion: p = .08; total: p = .07; extent: p = .09; type: p = .06; tenacity: p = .12). Hence, it is not surprising that adhesion formation scores were lower in the Hyalobarrier gel group than in the Hyskon (proportion: p <.01; total: p <.01; extent: p <.01; type: p <.01; tenacity: p <.01) or CMC 2% (proportion: p <.01; total: p <.01; extent: p <.01; type: p <.01; tenacity: p <.01) groups.

In experiment 3, the effect of a mechanical barrier (SprayGel) and a surfactant (phospholipids 3%) were analyzed. Adhesion formation decreased with SprayGel (proportion: p <.01; total: p <.01; extent: p <.01; type: p = .08; tenacity: p <.04) and with phospholipids 3% (proportion: p <.01; total: p = .12; extent: p <.02; type: p = .78; tenacity: p = .65). SprayGel was more effective in reducing

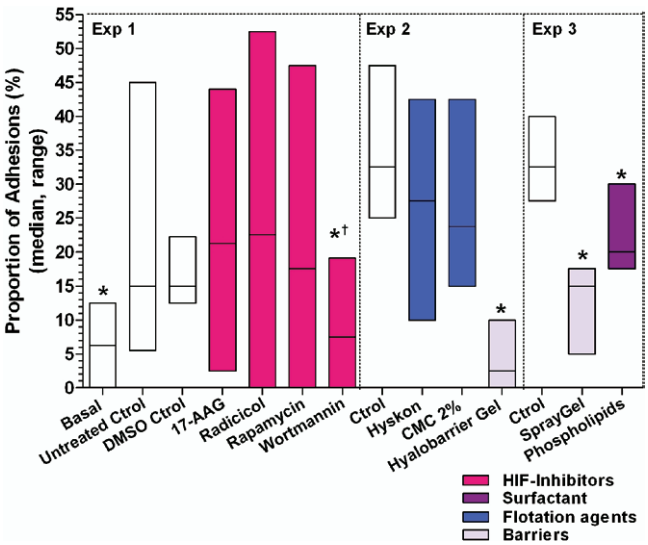

Figure 2 Prevention of pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. CO_2 pneumoperitoneum was maintained for 60 minutes (humidified gas, 15 mm Hg insufflation pressure). Adhesions were induced during laparoscopy by performing a bipolar lesion. Three experiments were performed evaluating the effects of HIF inhibitors (17-AAG, radicicol, rapamycin, and wortmannin), surfactants (phospholipids 3%), flotation agents (Hyskon and CMC), and barriers (Hyalobarrier gel and SprayGel). Adhesions were scored after 7 days during laparotomy. The quantitative scoring (proportions of adhesions) is indicated (median and range). To make the figure clearer, only the significant comparisons to the control groups were placed. *p <.05 intraexperiment comparisons (each group compared with its own control group). †p<.05 intraexperiment comparisons (each group compared to the vehicle control group for experiment 1).

adhesions than phospholipids 3% (proportion: $p < .01$; total: $p < .01$; extent: $p < .01$; type: $p = .05$; tenacity: $p = .05$).

Discussion

These experiments are part of a series of experiments designed to evaluate most known and new substances in 1 model to obtain quantitative and comprehensive information on adhesion prevention. These experiments aimed to confirm the role of HIF up-regulation as a mechanism of pneumoperitoneum-enhanced adhesion formation by blocking HIF through the inhibition of the Hsp-90 (17-AAG and radicicol) or of the PI3K signaling pathway (wortmannin and rapamycin). Taking into account these 2 mechanisms involved in HIF inhibition and also the Bonferroni correction, we can define 2 hypotheses. If the hypothesis was that HIF inhibitors decrease pneumoperitoneum-enhanced adhesion formation, 4 products can be considered leading to an α of 0.0125. If, however, the hypothesis was that inhibition of PI3K decreases adhesions through HIF, only 2 similar products (wortmannin and rapamycin) can be considered leading to an α of 0.025. Comparing with the vehicle-treated control group, wortmannin reduced pneumoperitoneum-enhanced adhesions either considering α of 0.0125 or of 0.025. Surprising, the comparison of wortmannin with the untreated control group was not significant considering both α values, although there were no differences between both control groups. This may be explained by the higher SE obtained in this control group.

If wortmannin decreases adhesion formation through HIF inhibition, it might be surprising that 17-AAG, rapamycin, and radicicol did not. First, these were screening experiments, and it cannot be excluded that different doses or way of administration could become effective. Specifically, radicicol is known to be very unstable,[26] and 1 injection could be insufficient. Second, the variability of adhesion formation in this experiment was surprisingly highly possible related to the use of DMSO as a solubilizing agent. Third, because 14-AAG and radicicol act through inhibition of Hsp-90 whereas wortmannin and rapamycin act through inhibition of the PI3K pathway, the later pathway could be more effective for adhesion reduction. Finally, the PI3K pathway is not only effective in HIF inhibition but also has other effects. PI3K pathway is involved in cell survival and proliferation and in many aspects of angiogenesis[29] and fibrinolysis, such as VEGF,[30] uPA,[31] and PAI-1[32] up-regulations. Each one of these factors is involved in the adhesion formation.[2,3] PI3K signaling is involved in the inflammatory response because blocking its activity reduces neutrophil influx by diminishing their attachment and migration[33] and reduces the adherence and spreading of the macrophages.[34] The inhibition of PI3K/Akt contributes to Hsp synthesis in addition to attenuating HIF-1α translation.[35] Specifically, wortmannin inhibits the superoxide release by the polymorphonuclear leukocytes (PMNs)[36] and during myocardial ischemia reperfusion injury, it can attenuate PMN infiltration into the myocardium and suppress superoxide release by PMNs.[37] Therefore a beneficial effect of wortmannin in reducing the toxic effect of reactive oxygen species produced during ischemia/reperfusion process can also be postulated.

In conclusion, the effect of wortmannin could be considered as supporting the hypothesis that HIF is up-regulated during pneumoperitoneum-enhanced adhesions. The absence of effect of the other products does not refute the hypothesis explained. Moreover, it cannot be excluded that wortmannin might be effective through many other mechanisms. To answer this, a detailed experiment should be done.

Flotation agents and barriers are the most well-known substances to reduce adhesions. For the barriers, we can consider that 4 barriers (flotation agents are barriers) were tested correcting the α value to 0.0125, or that 2 flotation agents and 2 mechanical barriers were tested leading an α of 0.025 for significance. In this experiment, Hyalobarrier gel was the most effective in decreasing adhesion, even significant compared with the smaller α. Moreover, in the Hyalobarrier gel–treated group, 50% (4 of 8 mice) of mice did not develop any adhesion. It should be emphasized that this is exceptional and that the incidence of adhesion formation in the other groups (control and noncontrol groups) was 100%. These results are consistent with previous observations in a laparoscopic model[13] and in an open surgery model[38,39] in rabbits and in rats.[40] Hyalobarrier gel was also proven to be effective in clinical trials, that is, in laparoscopic myomectomy[41,42] and in hysteroscopic surgery.[43,44] The ability of the Hyalobarrier gel in preventing adhesion formation may be explained, in addition to being a barrier, by its inflammatory modulating activity, for example, by induction of interleukin-1,[45,46] interleukin-8,[47] interleukin-12,[48] and tumor necrosis factor alpha[46] production. HA also improved wound healing.[49] On the other hand, HA can act as a reactive oxygen species scavenger.[50,51] It was recently demonstrated that HA increases the proliferation rate of human peritoneal mesothelial cells[52] and increases the fibrinolytic response.[53]

Although some decrease in adhesion formation was observed with CMC 2% in our laparoscopic model, the differences were not statistically significant. CMC 2% was shown to reduce intraabdominal adhesions in rats[54–56] and in rabbits,[57] but the reports were not consistent. No effect of CMC was seen in rats[58] and in rabbits.[59] In conclusion, CMC probably has some effectiveness, but the effect is small when used as a single product.

We failed to demonstrate effectiveness of Hyskon in our model. Hyskon was shown to decrease adhesions in a rabbit model,[60,61] whereas in other reports no effect on adhesion formation was observed. No effects were seen in rabbits,[59,62,63] in hamsters,[64] and in rats.[58] Injection of Hyskon 0.5 mL in the mouse abdominal cavity (25 mL/kg) was, moreover, associated with a mortality rate of 20%. This was

also observed in rats in which 20 mL/kg produced a mortality rate of 75%.[55]

SprayGel was effective in our model, even also comparing the $\alpha = 0.0125$, which is consistent with previous observations during open surgery in rats, rabbits,[65] and pigs[66] and in the human being after a laparoscopic ovarian surgery[67] and laparoscopic and open myomectomy.[68]

Phospholipids were effective in adhesion prevention in our laparoscopic mouse model, as previously demonstrated in a rabbit during open surgery.[69,70] They were, however, not effective in an open mouse model.[71] This is the first time that phospholipids were tried during laparoscopic surgery. The composition of the phospholipids solution used in this experiment was phosphatidylcholine 70% by weight, phosphatidylethanolamine 15% by weight, neutral lipids 8% by weight, sphingomyelin <3% by weight and lysophosphatidylcholin <3% by weight[72]; the large concentration of phosphatidylcholine, the lipid more predominant of the peritoneal cavity[73] may be helping to prevent adhesions. The ability of the phospholipids in preventing adhesion formation can be explained by its induction of lubricity, antiwear, and release or antistick properties.[74]

Conclusion

In conclusion, wortmannin at a dose of 0.31 mg/kg body weight clearly prevents pneumoperitoneum-enhanced adhesion. The mechanism involved probably is the prevention of HIF up-regulation, but other mechanisms as inhibition of angiogenesis, inflammation, oxidative stress, and fibrinolysis inhibition, cannot be ruled out. It is premature to exclude effectiveness of the other HIF inhibitors on pneumoperitoneum-enhanced adhesion. Barriers such as Hyalobarrier gel and SprayGel were confirmed to be highly effective, and phospholipids 3% were also shown to be effective. These results should not be viewed as stand-alone observations but could help to develop an overall strategy to reduce adhesions by combining treatments aiming at the different pathophysiological mechanisms of adhesion formation.

Acknowledgments

We would like to thank Prof. R. Pijnenborg, R. Van Bree, and L. Vercruysse (Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg) for their help. F. Torasso and A. Frasson (Fidia Advanced Biopolymers s.r.l.), A. van der Veek (Q Medical International), and T. Koninckx are also acknowledged. The pharmacists of the Hospital Gasthuisberg are acknowledged for the preparation of the carboxymethylcellulose solution.

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Addendum 9

Binda MM and Koninckx PR. The role of the pneumoperitoneum in adhesion formation *Adhesions News & Views* 10: 11-13, 2007.

MERCEDES BINDA AND PHILIPPE KONINCKX EXPLAIN THE PNEUMOPERITONEUM'S ROLE IN ADHESION FORMATION

The role of the pneumoperitoneum in adhesion formation

Adhesions are a major cause of female infertility,[1] bowel obstruction,[2] chronic pain[3] and difficulties at the time of re-operation. The burden of postoperative adhesions is best illustrated by a study by Lower *et al*, which showed that 34.5% of women having open gynaecological surgery will be readmitted on average 1.9 times in the following 10 years for reoperation due to adhesions.[4] The aim of this article is to review the effect of the pneumoperitoneum as a cofactor in adhesion formation in laparoscopic surgery.

Pathophysiology of intraperitoneal adhesions

The pathogenesis of adhesions involves several mechanisms. Briefly, the peritoneal injury initiates an inflammatory reaction, generating a fibrinous exudate and formation of fibrin. The balance between fibrin deposition and degradation is critical for normal peritoneal healing or adhesion formation. If fibrin is degraded completely, normal peritoneal healing will occur. Fibrin degradation is regulated by the plasminogen system. The inactive proenzyme plasminogen is converted into plasmin by tissue-type plasminogen activator (tPA) and/or urokinase-type plasminogen activator (uPA), which are inhibited by the plasminogen activator inhibitors 1 (PAI–1) and 2 (PAI–2). Plasmin degrades fibrin into fibrin degradation products. In contrast, if fibrin is not degraded completely, it will serve as a scaffold for fibroblasts and capillary ingrowth. This process of healing or adhesion formation has specific time courses: if healing is not achieved within 3–4 days, adhesion formation starts with capillary ingrowth from day 6 onwards.[5]

Postoperative adhesion formation: laparoscopy versus laparotomy

To understand fully the differences in adhesion formation between laparoscopy and laparotomy, it is necessary to differentiate between adhesion formation (adhesions formed at operative sites), *de novo* adhesion formation (adhesions formed at non-operative sites) and adhesion reformation (adhesions formed after lyses of previous adhesions).

In comparison with laparotomy, animal studies indicated that laparoscopy could induce less adhesion formation,[6] but this remains controversial.[7] Laparoscopy could induce less *de novo* adhesion formation[8] but, for adhesion reformation, no differences were found.[9] In addition, the SCAR–2 epidemiological study indicated no differences in adhesion-related readmissions between laparoscopy and laparotomy.[10] In conclusion, it is unclear whether laparoscopy induces less adhesion formation. This is surprising since laparoscopy produces less surgical trauma, involves gentle tissue handling, meticulous haemostasis and constant irrigation, resulting in less of an inflammatory response.[11]

Laparoscopy and pneumoperitoneum

Effects of the pneumoperitoneum

Laparoscopy requires a pneumoperitoneum. CO_2 is the most common gas used for pneumoperitoneum because of safety reasons, i.e. its high solubility in water and high exchange capacity in lungs. However, CO_2 pneumoperitoneum induces systemic and local effects.

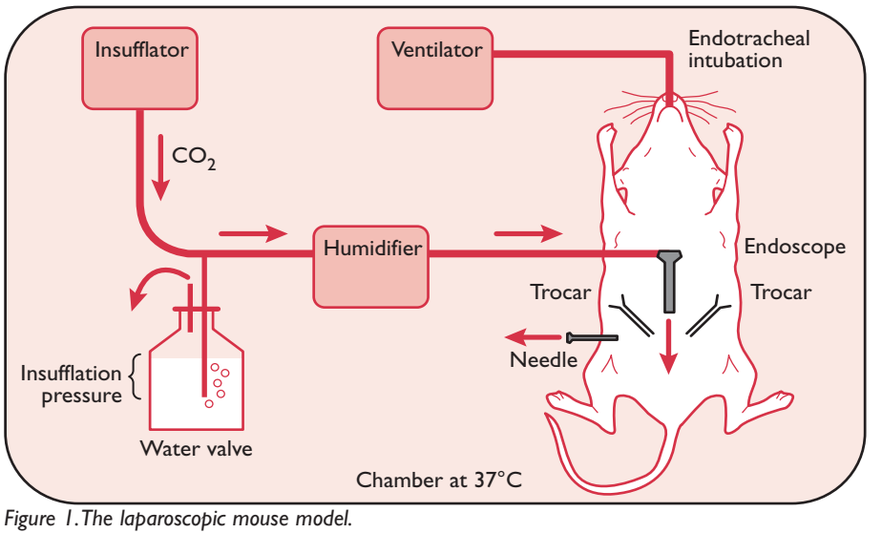

Figure 1. The laparoscopic mouse model.

Locally, CO_2 pneumoperitoneum decreases the pH[12] and the microcirculation through compression.[13] Moreover, the type of gas used to induce the pneumoperitoneum can have an effect on the inflammatory response. In rats, CO_2 causes less inflammatory reaction with a lower expression of α_2-macroglobulin (an hepatic gene of acute phase inflammatory response) compared with helium pneumoperitoneum.[14] Consistent with this, macrophages incubated *in vitro* with CO_2 produced less TNF–α and interleukin–1 in response to LPS compared to the macrophages incubated with air or helium.[15] Peritoneal macrophages derived from rats exposed to CO_2 pneumoperitoneum released less TNF–α than macrophages derived from animals exposed to air or helium.[15] In addition, the humidification and temperature of the gas also influence the inflammatory response. Warm, humidified CO_2 has been shown to reduce the TNF-α concentration in pigs[16] and diminish the increased number of lymphocytes in rats,[17] with an overall reduced duration of inflammation. Pneumoperitoneum, in particular when dry gas is used, induces desiccation: this affects the morphology of the mesothelial cells (i.e. the cells bulged), increasing the size of the intercellular clefts and exposing the underlying basal lamina.[18,19] Systemically, CO_2 pneumoperitoneum induces acidosis/hypercarbia[20] and hypothermia.[21]

Mercedes Binda PhD, Post-doctoral Researcher and Philippe Koninckx MD, PhD, Head, Division of Endoscopic Surgery, Department of Gynecology and Obstetrics, Catholic University of Leuven, Leuven, Belgium

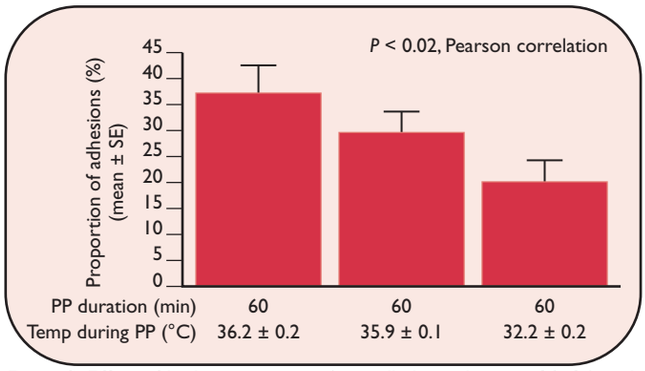

Figure 2. Effect of body temperature (controlled strictly up to 32, 36 and 37°C) upon adhesion formation in mice.

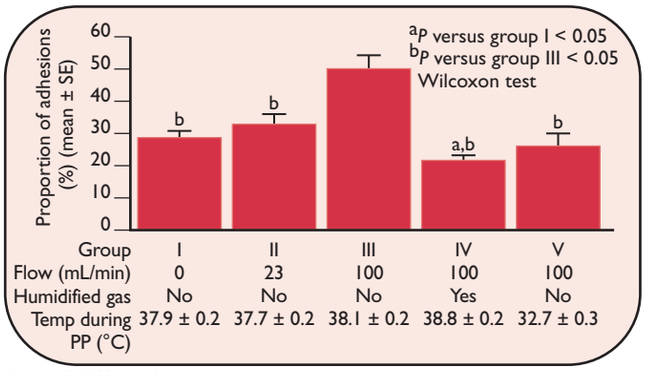

Figure 3. Effect of desiccation and hypothermia during pneumoperitoneum upon adhesion formation in mice.

In clinical trials, the effects of different gas conditions have been studied, but the results remain controversial. The use of warm, humidified gas was claimed to reduce postoperative pain and recovery time after surgery[22] in comparison with cold, dry gas. However, these results remain controversial.[23,24] Compared to cold, dry gas, the use of warm, dry gas produces more intensive shoulder and subcostal pain, but without differences in the duration of hospitalisation.[25] This might be explained by the higher desiccation produced by a warmer gas. The only study that has analysed dry and warm, dry and cold, humidified and warm, and humidified and cold gas did not find any differences in terms of body temperature, intra–abdominal humidity, postoperative pain, recovery time and length of hospitalisation.[24] This trial, however, had a small sample size and the patients were very obese (BMI = 50).

Pneumoperitoneum and postoperative adhesions

The effect of the pneumoperitoneum on adhesion formation has been well addressed in our rabbit and mouse laparoscopic models. In the rabbit model, animals were anaesthetised and intubated. A 10 mm endoscope was used together with a pneumoperitoneum and two 5 mm trocars were introduced under direct vision in the left and right flank to allow the introduction of the necessary instruments.[26] Standardised opposing lesions of 2 cm^2 were performed randomly on the uterine horns and in the pelvic side walls by bipolar coagulation on one side and by bipolar coagulation or CO_2 laser on the other side. Adhesion formation was scored after 7 days by second-look laparoscopy.

In our mouse model (Figure 1), animals were anaesthetised with pentobarbital, intubated with a 20-gauge catheter and ventilated with a mechanical ventilator, using humidified room air to avoid desiccation of the airways.[27,28] Through a midline incision, a 2 mm endoscope with a 3.3 mm external sheath for insufflation was introduced and the incision was closed gas-tight in order to avoid leakage. Two 14-gauge catheters were used as secondary trocars and standardised 10 mm x 1.6 mm lesions were created in the anti-mesenteric border of both right and left uterine horns and in both the right and left pelvic side walls using a bipolar coagulator. After 7 days, adhesions were scored blindly during laparotomy under microscopic vision using both a qualitative and a quantitative scoring system.

The pneumoperitoneum variables that we investigated were gas (pure CO_2, helium, with or without the addition of 1–12% of oxygen), duration of the pneumoperitoneum, humidification and temperature. To achieve this, the animals and equipment were placed in a closed chamber, which was maintained at 37°C. In the experiments in which high desiccation was induced, a homeothermic blanket was also used.[29]

In both models, CO_2 pneumoperitoneum was a co-factor in postoperative adhesion formation.[26,27] We suggested that mesothelial hypoxia was a driving mechanism, since adhesion formation increased with duration and insufflation pressure[27,30] and similar effects were observed with helium pneumoperitoneum. Furthermore, the addition of 2–4% of oxygen to both CO_2 and helium pneumoperitoneum decreased adhesion formation.[26,31] This effect was absent in mice deficient for hypoxia-inducible factor (HIF),[32] plasminogen activator inhibitor–1 (PAI–1)[33] and vascular endothelial growth factor (VEGF)[34] — factors that are all up-regulated during hypoxia.

Some observations have suggested that reactive oxygen species (ROS) scavengers decrease adhesion formation in several animal models.[35,36] In addition, it is known that during laparoscopy the pneumoperitoneum determines ischaemia at the time of insufflation and reperfusion at the time of deflation. For these reasons, a role for ROS in adhesion formation has been suggested.[37]

Moreover, CO_2 pneumoperitoneum produces hypothermia, particularly when cold, dry gas is used.[21] This hypothermia can be explained by the loss of energy as a result of two events. One is that the dry gas has to become humidified; therefore, body water will be vaporised from the abdominal cavity to humidify the dry gas, resulting in the mesothelial layer becoming desiccated. The other event is that the cold gas has to be heated to body temperature. For both processes, energy is expended: quantitatively, 577 cal is needed to vaporise 1 mL of water at 37°C, whereas only 0.00003 cal is needed to heat 1 mL of CO_2 by 1°C.[28] In conclusion, much more energy is needed to vaporise body water than to heat the gas and this loss of energy will produce a decrease in body temperature or cooling. Therefore, desiccation and cooling are intimately linked. In addition, the capacity of a gas to hold water increases with temperature: for this reason, desiccation and the associated cooling will be higher at higher temperatures. Indeed, desiccation-associated cooling cannot be prevented with warm, dry gas because of the higher desiccation,[18,38] whereas it can be prevented to a large extent by cold, humidified gas[18] and fully prevented using warm, humidified gas, as shown in rats,[18] pigs[21] and humans.[39]

We recently demonstrated that hypothermia reduces adhesion formation and, to the best of our knowledge, this is the first time that it has been proven.[28] In our laparoscopic mouse model, when adhesions were induced during 60 minutes of pneumoperitoneum with pure CO_2 and body temperature was controlled strictly up to 32, 36 and 37°C, adhesion formation was lower than when body temperature was lower (Figure 2). Supporting these results, it was reported that irrigation with saline at > 37°C, i.e. between 37°C and 60°C, increases adhesion formation in rats.[40] Furthermore, in humans, it was found that local hypothermia after laparotomy decreases the inflammatory reaction and increases intestinal peristalsis, thus possibly decreasing adhesion formation.[41]

Hypothermia could protect tissues and cells directly from the pneumoperitoneum-induced hypoxia, since oxygen consumption by cells decreases with temperature.

Hypothermia decreases the global cerebral metabolic rate during ischaemia, slowing the breakdown of glucose, phosphocreatine and ATP and the formation of lactate and inorganic phosphate.[42] Pneumoperitoneum-enhanced adhesion formation can be considered to be an ischaemia–reperfusion process and hypothermia is known to reduce the production of ROS during reperfusion in several tissues.[43,44] Hypothermia also suppresses the inflammatory response after hepatic ischaemia–reperfusion, decreasing the infiltration of polymorphonuclear cells[45] and the production of tumour necrosis factor–α, interleukin–1 and macrophage inflammatory protein–2.[45]

Finally, it has always been claimed that desiccation increases adhesion formation,[46] but this has not, until now, been demonstrated directly. We recently proved that desiccation increases adhesion formation.[29] In our experiment (Figure 3), different levels of desiccation were induced by using dry CO_2 and by placing in the abdomen a needle of different gauge size in order to generate various gas flow rates through the abdominal cavity. Body temperature was controlled strictly at 37°C in order to avoid the desiccation-associated effect of cooling. An increase in adhesions was observed when desiccation increased (groups I to III). Desiccation-enhanced adhesion was prevented when humidified gas (group III versus IV) or low temperature were used (group III versus V).

Conclusion

In summary, pneumoperitoneum has a role in adhesion formation, affecting the entire abdominal cavity. Several pathophysiological factors have been identified to date. First, pneumoperitoneum causes trauma in the abdominal cavity because it induces hypoxia. Second, ischaemia/reperfusion occurs and ROS can be produced during inflation of the gas into the abdominal cavity and on deflation. Third, pneumoperitoneum may induce desiccation and cooling, particularly when a dry gas is used. Desiccation increases and cooling decreases adhesions. The higher the gas temperature, the more desiccation produced. We therefore suggest the use of a less traumatic pneumoperitoneum to prevent adhesion formation: using humidified gas to avoid desiccation, adding 3% oxygen to the CO_2 insufflation gas to prevent hypoxia and, locally, using low temperature to reduce the trauma.

These data obviously still need to be confirmed in humans, in whom a decrease in pneumoperitoneum temperature is not necessarily associated with a decrease in body temperature. If confirmed in humans, these results may have very important clinical implications for the design of insufflators and humidifiers, which would minimise the trauma produced by the pneumoperitoneum, thus diminishing postoperative adhesions.

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SUMMARY

- *Pneumoperitoneum has a role in adhesion formation, affecting the entire abdominal cavity*
- *Contributing factors include trauma in the abdominal cavity and desiccation and cooling, particularly when a dry gas is used*
- *Recommendations are to use humidified gas, adding 3% oxygen to the CO_2 insufflation gas and maintaining a low temperature to reduce trauma*

Addendum 10

Binda MM, Hellebrekers B, Declerck P, Koninckx PR. Effect of Reteplase and PAI-1 antibodies upon postoperative adhesion formation in a laparoscopic mouse model (*submitted*)

EFFECT OF RETEPLASE™ AND PAI-1 ANTIBODIES UPON POSTOPERATIVE ADHESION FORMATION IN A LAPAROSCOPIC MOUSE MODEL

Running title: Postoperative adhesion formation and fibrinolysis.

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ABSTRACT

Background: Postoperative adhesions remain an important clinical problem accounting for infertility, chronic pain and bowel obstruction. Its prevention still is inadequate and overall poorly understood. The aim of this study was to investigate the effect of Reteplase (a recombinant plasminogen activator or r-PA) and of PAI-1 antibodies upon adhesion formation in a laparoscopic model. **Methods:** Pneumoperitoneum-enhanced adhesions were induced by performing a bipolar lesion in female BALB/c mice and by using pure and humidified CO_2 as insufflation gas for 60 min. In experiment 1, four doses of 0.125, 0.25, 0.5 and 1 mg/0.5 ml of r-PA and 1 and 2 doses of 1 mg r-PA were i.p. administrated. Two control groups were done, one without any treatment and the second one receiving 4 times 0.5 ml of saline. In experiment 2, four doses of 0, 1, 10 and 100 µg/0.5 ml of r-PA were i.p. administrated. In experiment 3, PAI-1 neutralising and non-neutralising antibodies were injected i.p. after performing the lesion on day 0 and days 2 and 4. Adhesions were scored after 7 days. **Results:** Adhesion formation was less with the administration of four doses of 1 µg of r-PA (proportion, $p<0.05$, Wilcoxon). An increase in adhesion formation was observed when higher number of doses and amounts of r-PA were used (Proc GLM, 8 groups, 2 variables, $p=0.05$ for the amount of r-PA and $p<0.02$ for the number of doses administrated). Non effect was observed with the PAI-1 antibodies. **Conclusions:** These experiments demonstrated that low doses of r-PA can be used during laparoscopy to reduce adhesions.

Key Words: adhesion formation, laparoscopy, mouse model, PAI-1 antibodies, Reteplase, r-PA.

INTRODUCTION

Peritoneal adhesions are pathological bonds between surfaces within body cavities. They are formed when the parietal or visceral peritoneum is damaged and the basal membrane of the mesothelial layer is exposed to the surrounding tissues. This injury to the peritoneum, due to either surgery or infection, causes a local inflammatory reaction which leads to the formation of a serosanguineous exudate that is rich in fibrin. The fibrinous exudate is part of the hemostatic process and facilitates tissue repair by providing a matrix for invading fibroblasts and new bloodvessels. On the one hand, this deposition of fibrin is an essential component of normal tissue repair, but on the other hand, resolution of this fibrin deposit is required to restore the preoperative conditions or conditions before inflammation. The degradation of fibrin is regulated by the plasminogen system. The inactive proenzyme plasminogen is converted into plasmin by tissue-type plasminogen activator (t-PA) and/or urokinase-type plasminogen activator (u-PA), which are inhibited by the plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2). Plasmin degrades fibrin, the matrix structure of fibrinous adhesions. When the fibrinolytic capacity is insufficient, persistence of deposited fibrin may occur and the fibrinous adhesions may develop. The fibrinous adhesions become organized, characterized by deposition of collagen and concomitant vascular ingrowth, as a consequence of which the adhesions are changed into fibrous, permanent adhesions. Thus, a disbalance between fibrin deposition and fibrin dissolution is the key event in the development of adhesion formation (1).

We previously demonstrated that adhesion formation increases with the duration of the pneumoperitoneum (2). This effect of pneumoperitoneum-enhanced adhesions was not observed in PAI-1(-/-), u-PA (-/-), and t-PA (-/-) knock out mice (3). Compared with wild-type mice, PAI-1 knock out mice developed fewer adhesions, whereas both u-PA and t-PA knock out mice developed more adhesions. This effect was expected since the lack of u-PA and tPA reduces plasmin activation and fibrin degradation, thus leading to adhesion formation, whereas the lack of PAI-1 reduces the inactivation of uPA and tPA, increasing plasmin levels and fibrin degradation, thus reducing adhesion formation (3). In addition, an increase in PAI-1 expression in the abdominal wall indicates that PAI-1 up-regulation by CO_2 pneumoperitoneum is a mechanism of pneumoperitoneum-enhanced adhesion formation (3). Same susceptibility to develop more adhesion was observed also in tPA and uPA knock out mice during open surgery (4).

After the initial use of streptokinase, continuing research has given rise to the development of second-generation (recombinant human tissue plasminogen activator, rt-PA, Alteplase™) and third-generation (mutants of rt-PA such as recombinant plasminogen activator or r-PA or Reteplase™) plasminogen activators (5). Reteplase™ lacks the finger, epidermal growth factor, and kringle-1 domain. The slower clearence resulting from these changes in the molecule allows Reteplase to be given as a bolus. Fibrinolytic agents as streptokinase, plasmin preparations, urokinase and rt-PA have been reported to decrease adhesion formation and reformation in several animal models (5). In these experiments, different numbers of dosages, concentrations, vehicles and animal models have been used, and, adhesions were induced during open surgery or laparotomy. However, nothing is known about the effect of fibrinolytic agents during laparoscopy. Specifically, Reteplase and PAI-1 antibodies have ever been tested neither during laparotomy nor during laparoscopy for their

anti-adhesive properties. Therefore, the aim of the present study was to investigate the effect of Reteplase and PAI-1 antibodies upon adhesion formation in a laparoscopic mouse model.

MATERIALS AND METHODS

The laparoscopic mouse model for adhesion formation

Experimental setup, i.e. animals, anaesthesia and ventilation, laparoscopic surgery, induction and scoring of intraperitoneal adhesions, has been described in detail previously (2; 3; 6-15).

Animals

One hundred twelve 9-10 weeks old female BALB/c mice weighing 20 g were used. Animals were kept under standard laboratory conditions and they were fed with a standard laboratory diet with free access to food and water at anytime. The study was approved by the Institutional Review Animal Care Committee.

Anesthesia and ventilation

Mice were anaesthetised with intraperitoneal 0.08 mg/g pentobarbital, intubated with a 20-gauge catheter and mechanically ventilated (Mouse Ventilator MiniVent, Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using humidified room air with a tidal volume of 250 µL at 160 strokes/min. Humidified air for ventilation was used in order to prevent cooling, as occurs during ventilation with non-humidified air (8).

Laparoscopic surgery

A midline incision was performed caudal to the xyphoides, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity and the incision was closed gas tight around the endoscope in order to avoid leakage.

The CO_2 pneumoperitoneum was created with the Thermoflator Plus (Karl Storz, Tuttlingen, Germany). Using humidified gas (Storz Humidifier 204320 33, Karl Storz, Tuttlingen, Germany) and the 37°C chamber, gas at 37°C and 100% relative humidity is obtained. A controlled flow of the gas was obtained using a 26 gauge needle placed in the abdomen, which at 15 mm Hg insufflation pressure, induced a 23 ml/min flow of gas through the abdominal cavity.

Induction of intraperitoneal adhesions

After the establishment of the pneumoperitoneum, two 14-gauge catheters were inserted under laparoscopic vision. Standardized 10-mm x 1.6-mm lesions were performed in the antimesenteric border of both right and left uterine horns and pelvic sidewalls with bipolar coagulation (BICAP^{TM}, bipolar hemostasis probe, BP-5200A, 5 Fr, 200 cm; IMMED

Benelux, Linkebeek, Belgium) at 20 watts (Autocon 200, Karl Storz, Tuttlingen, Germany, standard coagulation mode).

Scoring of adhesions

Adhesions were qualitatively and quantitatively scored, blindly (the investigator was not informed of the group being evaluated) under microscopic vision during laparotomy seven days after their induction. The qualitative scoring system assessed: extent (0: no adhesions; 1: 1-25%; 2: 26-50%; 3: 51-75%; 4: 76-100% of the injured surface involved, respectively), type (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), tenacity (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection) and total (extent + type + tenacity). The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: adhesion (%) = (sum of the length of the individual attachments/length of the lesion) x100. The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were individually scored.

Products

Recombinant human PA: Reteplase (Rapilysin® 10 U, Roche) was prepared as indicated in the product data sheet and diluted to 0.25, 0.5, 1 and 2 mg/ml in saline (Experiment 1) and to 2, 20 and 200 µg/ ml (Experiment 2) and kept at -20°C.

PAI-1 neutralizing antibodies: PAI-1 monoclonal antibody MA33H1F7 (2 mg/ml, 0.49U/mg endotoxin) and PAI-1 non-neutralizing antibody MA32K3 (2 mg/ml, 2.88 U/mg endotoxin) were kindly given by Professor Paul Declerck (Laboratorium voor Farmaceutische biologie en fytofarmacologie, Catholic University of Leuven). This antibody was chosen because it was proved its cross-reactivity with murine PAI-1 (16).The antibodies were diluted to 0.06 mg/ml with saline and keep at -20°C.

Dosages

Recombinant human PA: From existing literature it was difficult to assess which minimal dosages are required to prevent adhesions. The treatment programs are hard to compare because different animal models, different methods of inducing adhesions, and different routes of administration and dosages were used (5). In addition, no studies in mouse models have been found. For this reason, we decided to choose for the experiment 1 the higher concentration found in the bibliography in a rat model, i.e. 5 mg rt-PA/rat (or 25 mg rt-PA /kg b.w.) (17), and to duplicate it in order to be sure that enough PA was available. Therefore, the higher dose used in the experiment 1 was 1 mg r-PA /mouse (or 50 mg r-PA /kg b.w.). Moreover, the r-PA was injected i.p. since greater availability was demonstrated when it is given i.p. (18). With regard to the length of treatment, we decided to administrate the r-PA for 2 days since a reduction of adhesions formation from 35-40% to 6.3% +/- 1.57 (control vs rt-PA treated groups) was observed after 2 days treatment (19). Consistent with this, Orita *et al* concluded that effective adhesion prevention occurred with 2 days of rt-PA treatment, beginning on the day of operation in a study in rabbits (20).

From the results of experiment 1, we realized that the doses used were too high for our experimental model and then experiment 2 was performed in which lower r-PA doses were used.

PAI-1 neutralizing antibodies:
The quantity of anti-PAI-1 antibody was 1.5 mg/kg b.w. based on Schoots *et al* studies done in rats (21). This corresponds to a dose of 30 µg/mouse.

Experimental design

Since anaesthesia and ventilation can influence body temperature, the timing was strictly controlled. The time of anaesthesia injection was considered time 0 (T_0). The animal preparation and ventilation started after 10 min (T_{10}). The pneumoperitoneum started at 20 min (T_{20}) and was maintained for 60 min (T_{20} to T_{80}) to induce pneumoperitoneum enhanced adhesions.

The experiment 1 was designed to evaluate the effect of different doses of r-PA and the timing of the r-PA administration upon adhesion formation. Pneumoperitoneum- enhanced adhesions were induced and r-PA or saline were administrated i.p. depending on the groups: 2 doses the day of the surgery (immediately after performing the lesion and 6 hrs after that) and 2 doses the day after (in the morning and after 6 hrs). Four groups receiving four doses of 0.125, 0.25, 0.5 and 1 mg/0.5 ml of r-PA were performed. Another two groups in which one dose of 1 mg r-PA (and 3 times of saline) or 2 times 1 mg r-PA (and 2 times of saline) were administrated. Two control groups were done, one without any treatment and the second one receiving 4 times 0.5 ml of saline (untreated control and saline control, respectively) (8 groups, 8 mice per group, n= 64).

After completing experiment 1, it was hypothesized that the concentrations used might be too high and experiment 2 was designed in order to evaluate the effect of lower concentrations of r-PA upon adhesion formation. Pure CO_2 pneumoperitoneum was induced and four doses of 1, 10 and 100 µg/0.5 ml of r-PA were i.p. administrated: 2 doses the day of the surgery (immediately after performing the lesions and 6 hrs after that) and 2 doses the day after the surgery (in the morning and after 6 hrs). A control group receiving 4 times of saline was also done (4 groups, 8 mice per group, n=32).

The experiment 3 was designed to evaluate the effect of PAI neutralizing antibodies in pneumoperitoneum-enhanced adhesions. PAI neutralizing and non-neutralizing antibodies were injected i.p. day 0 (after performing the lesion) and days 2 and 4 (2 groups, 8 mice per group, n=16).

Each experiment was performed using block randomisation by days. Therefore, one block of mice, comprising one animal of each group, was operated during the same day, and within a block the animals were operated in a random order.

Statistics

Statistical analyses were performed with the SAS System (SAS Institute, Cary, NC, USA). Differences in adhesion formation were evaluated with Wilcoxon test for the univariate analysis and with General Linear Methods (proc GLM) for the multivariate analysis to

evaluate simultaneously the effect of number of doses and amount of r-PA. All data are presented as the mean ± standard error of the mean (SE).

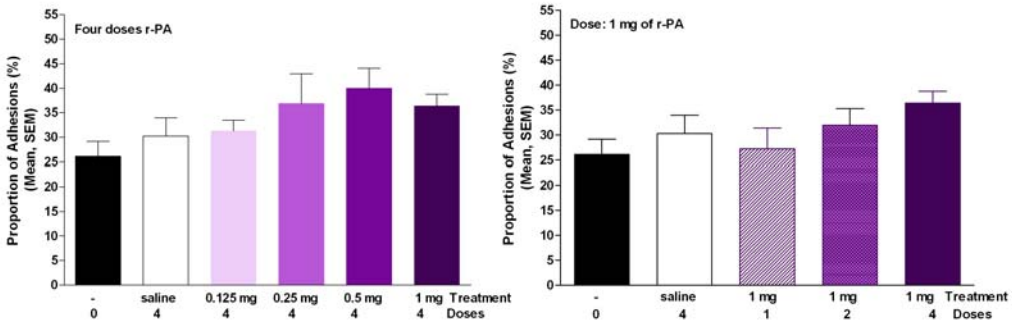
RESULTS

In experiment 1, the effect of different amounts and numbers of doses of r-PA were evaluated upon pneumoperitoneum-enhanced adhesions (Figure 1). When 4 times of r-PA were administrated, adhesion formation increased with the amount of r-PA (proportion: $p=0.05$; total: $p<0.0004$; extent: $p<0.02$; type: $p<0.0001$; tenacity: $p<0.002$; Figure 1 left). When the amount of 1 mg was administrated, adhesion formation increases with the number of doses administrated (proportion: $p<0.02$; total: $p<0.01$; extent: $p<0.01$; type: $p<0.01$; tenacity: $p<0.02$; Figure 1 right) (proc GLM, 8 groups, 2 variables, i.e. amount and number of doses of r-PA).

In experiment 2 (Figure 2), adhesion formation was reduced with the administration of 1 µg of r-PA comparing with the control group (proportion: $p<0.04$, Wilcoxon). There was not any effect of 10 and 100 µg comparing with the control group (NS each comparison). The administration of 1 µg of r-PA also reduced adhesion formation comparing with animals treated with 10 µg (proportion: $p=0.05$) or 100 µg (proportion: $p<0.01$, extent: $p<0.02$).

In experiment 3, the effect of PAI neutralizing antibodies were evaluated (Table 1). No effect was observed with the PAI-1 neutralizing antibodies comparing with the non-neutralizing antibodies (NS).

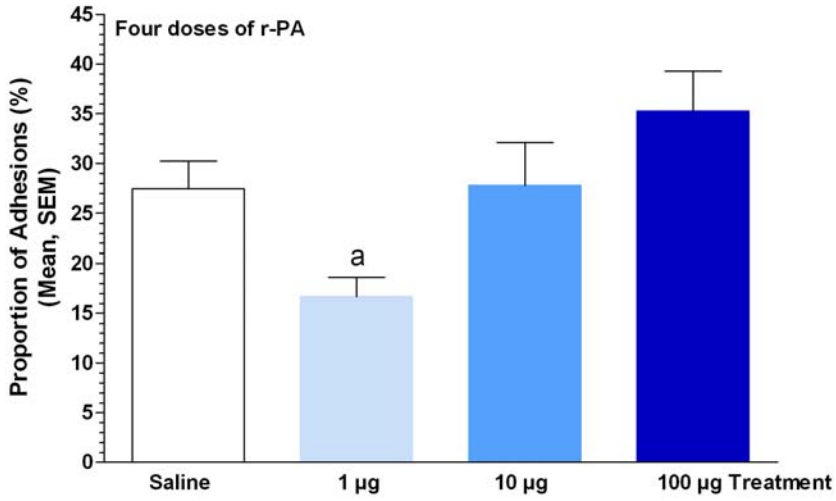
FIGURE 1



Statistics:

Proc GLM, 8 groups, 2 variables, i.e. amount and number of doses of r-PA, for the amount: proportion: $p=0.05$, for the number of doses: proportion: $p<0.02$.

FIGURE 2



Statistics:
[a] $p<0.05$, comparing to the control, with 10 µg and with 100 µg of r-PA (Wilcoxon test).

FIGURE LEGENDS

FIGURE 1

Effect of different amounts and number of doses of r-PA upon pneumoperitoneum-enhanced adhesions.
Pneumoperitoneum was maintained for 60 minutes and humidified pure CO_2 at 20 cm H_2O insufflation pressure was used. Adhesions were induced during laparoscopy by performing a bipolar lesion. Different amounts and doses of Reteplase (r-PA) upon adhesion formation were evaluated. Adhesions were scored after 7 days during laparotomy. Quantitative scoring system (proportions) is represented on this figure.

Statistics: Proc GLM, 8 groups, 2 variables, i.e. amount and number of doses of r-PA, for the amount: proportion: $p=0.05$, for the number of doses: proportion: $p<0.02$.

FIGURE 2

Effect of the administration of lower doses of r-PA upon pneumoperitoneum-enhanced adhesions.
Pneumoperitoneum was maintained for 60 minutes using pure CO_2. The gas was humidified and the insufflation pressure was 20 cm H_2O. Adhesions were induced during laparoscopy by performing a bipolar lesion. Four doses of 1, 100 and 100 µg of r-PA were administrated. A control group was done by administrated 4 times saline. Adhesions were scored after 7 days during laparotomy. Quantitative scoring system (proportions) is represented on this figure.

Statistics: [a] $p<0.05$, comparing to the control, with 10 µg and with 100 µg of tPA (Wilcoxon test).

TABLE 1

Experiment	Time of administration	Concentration r-PA or PAI-1 antibodies	Qualitative Scoring (mean±SE)			
			Extent	Type	Tenacity	Total
1 (r-PA)	0	-	1.3±0.1[a]	1.0±0.1[a]	1.1±0.1[a]	3.4±0.3[a]
	4	500 µl saline	1.4±0.2[a]	1.0±0.1[a]	1.1±0.1[a]	3.5±0.4[a]
	4	0.125 mg/500 µl	1.5±0.1[a]	1.2±0.1[a]	1.3±0.1[a]	4.0±1.1[a]
	4	0.250 mg/500 µl	1.8±0.2[a]	1.4±0.1[a]	1.4±0.1[a]	4.6±0.5[a]
	4	0.500 mg/500 µl	1.8±0.2[a]	1.6±0.1[a]	1.6±0.1[a]	5.0±0.4[a]
	4	1 mg/500 µl	1.8±0.1[a]	1.6±0.1[a]	1.6±0.1[a]	5.0±0.2[a]
	2	1 mg/500 µl	1.5±0.1[a]	1.2±0.1[a]	1.3±0.2[a]	4.0±0.4[a]
	1	1 mg/500 µl	1.3±0.2[a]	1.1±0.1[a]	1.0±0.1[a]	3.4±0.2[a]
2 (r-PA)	4	500 µl saline	1.3±0.1	1.0±0.1	1.0±0.2	3.3±0.4
		1 µg/500 µl	0.9±0.1[b]	1.1±0.1	1.2±0.1	3.2±0.4
		10 µg/500 µl	1.3±0.2	1.2±0.1	1.1±0.1	3.6±0.4
		100 µg/500 µl	1.6±0.2	1.3±0.1	1.3±0.1	4.2±0.4
3 (PAI-1 antibodies)	3 (days 0, 2, 4)	30 µg/500 µl non-neutralizing Ab	1.2±0.2	1.1±0.1	1.2±0.2	3.5±0.5
		30 µg/500 µl neutralizing Ab	1.3±0.2	1.0±0.1	1.0±0.1	3.3±0.4

TABLE 1 LEGEND

Pneumoperitoneum was maintained for 60 minutes and humidified pure CO_2 at 20 cm H_2O insufflation pressure was used. Adhesions were induced during laparoscopy by performing a bipolar lesion. Different amounts and doses of Reteplase (r-PA) were evaluated upon adhesion formation in experiment 1 and 2. PAI-1 antibodies upon adhesion formation were evaluated in experiment 3. Adhesions were scored after 7 days during laparotomy. Qualitative scoring system is represented on this table.

Statistics: [a] $p<0.05$. Proc GLM, 8 groups, 2 variables, i.e. amount and number of doses of r-PA. Adhesion formation increased with the dose of r-PA when 4 times of r-PA were administrated, also with the number of doses administrated when the dose of 1 mg was administrated. [b] $p<0.05$ vs 100 µg r-PA (Wilcoxon test)

DISCUSSION

Until some years ago, streptokinase and urokinase were used in thrombolytic therapy. Afterwards, the second-generation of plasminogen activators (recombinant human tissue plaminogen activator [rt-PA] or Alteplase) and third-generation of plasminogen activators (mutants of rt-PA, e.g. recombinant human PA [r-PA] or Reteplase) were developed (5). These PA's have several advantages over the streptokinase and urokinase, i.e., they are not antigenic, they do no cause immunogenic reactions, they have little or no general side effects. These fibrinolytic agents have already been tested to prevent adhesions. In these experiments, different numbers of dosages, concentrations, vehicles and animal models have been used, and, adhesions were induced during open surgery or laparotomy (5). However, nothing is known about the effect of these fibrinolytic agents during laparoscopy. Specifically, Reteplase and PAI-1 antibodies have ever been tested neither during laparotomy nor during laparoscopy for their anti-adhesive properties. Therefore, the aim of the present study was to evaluate the effect of both Reteplase and PAI-1 antibodies upon adhesion formation in a laparoscopic mouse model.

From our results, we can confirm that r-PA is able to prevent adhesion to some extent, i.e. four i.p. doses of 1µg of r-PA reduced adhesion formation during laparoscopy. These experiments also show the importance of using the right dose. As explained in Material & Methods, we initially decided to choose the higher concentration found in the bibliography in a rat model, 5 mg rt-PA/rat (or 25 mg rt-PA/kg b.w.) (17) and to duplicate it in order to be sure there was enough PA available. Therefore, the doses 0.125, 0.25, 0.5 and 1 mg r-PA /mouse were used at experiment 1 (1 mg r-PA/mouse = 50 mg r-PA/kg b.w.). However, these doses showed an increase in adhesion formation. From the experiment 1, we can conclude that the doses were too high for our experimental model since adhesion formation increased significatively with both the number of doses and the concentration of r-PA used. This increase in adhesion formation can be explained by the "plasminogen steal" induced by a high concentration of r-PA, this means that a high concentration of PA is associated with a depletion of plasminogen and, therefore, no plasmin will be formed and no fibrin degradation will occur (22). Consistent with that, it was showed in an *in vitro* model that the speed of clot lysis increases with increasing t-PA concentrations up to 2 µg/ml and it decreases above 2 µg/ml, giving a bell-shaped curve of fibrinolysis (23). We, therefore, decided to reduce the doses of Reteplase in experiment 2, resulting in that 1 µg showed a reduction of 40% in adhesion formation confirming the "plasminogen stealing" hypothesis.

In experiment 3, PAI-1 antibodies were tested in our model. Surprising, non effect of these antibodies was observed. Although the efficiency of PAI-1 inhibition *in vivo* by monoclonal antibodies has been demonstrated in a number of studies (24; 25), they were all tested in thrombosis models. Specifically, the antibody used in this experiment, the MA-33H1F7, were also having an effect in reducing the thrombus formation in rat models (26; 27). However, this is the first time that these antibodies were tried in a laparoscopic mouse model and, may be, this absent of effect could explain because the dose was not the right one. In addition, the antibodies were injected i.v. in thrombosis models, and in this experiment, it was done i.p. that may explain also the absent of effect.

It was demonstrated that pneumoperitoneum can have an influence on the fibrinolytic system. First, *in vitro* experiments show that mesothelial cells increased the synthesis and release of PAI-1 when exposed to CO_2 (28). Second, mesothelial cells seem to respond to

acidification by an increased release and production of PAI-1 *in vitro* (29). On the contrary, mesothelial cell *in vitro* exposed to CO_2 and helium showed an enhanced PA activity associated with a decrease in PAI-1 concentrations compared with the control (CO_2 with oxygen) concluding that these changes may participate in the observed reduction in adhesions after laparoscopic surgery relative to open surgery (30). It was shown in *in vivo* experiments, that pneumoperitoneum enhanced adhesions in PAI-1, uPA, and tPA wild-type mice, and that compared with those wild-type mice, pneumoperitoneum did not enhance adhesions in knock-out mice for each one of those factors (3). In addition, PAI-1 concentration increased after 60 minutes of pneumoperitoneum whereas tPA concentration did not change in wild type mice. These two observations indicate that PAI-1 up-regulation by carbon dioxide pneumoperitoneum is a mechanism of pneumoperitoneum-enhanced adhesion formation.

CO_2 pneumoperitoneum was postulated to be a cofactor in adhesion formation since adhesions increase with the duration of the pneumoperitoneum and with the insufflation pressure, and since the addition to 3% oxygen to both CO_2 and helium pneumoperitoneum reduce adhesions (2). Moreover, it was demonstrated that avoiding desiccation by using humidified gas and that reducing body temperature, adhesion formation were decreased (8; 9). Therefore, we postulate the use of both a *less traumatic pneumoperitoneum*, for instance, using as insufflation gas humidified CO_2 with the addition of 3% of oxygen, lowering body temperature, and the *application of products which decreases adhesions*, i.e., anti-inflammatory agents, calcium channel blockers, barriers, surfactants (10; 11) and r-PA, as demonstrated in this study, may be a way to reduce adhesions. This is the third part of our screening experiments in which different agents are being evaluated during laparoscopy. We hope in a near future to be able to define which is the best combination of treatments to reduce adhesions to the minimum.

ACKNOWLEDGEMENTS

We thank Prof. R. Pijnenborg, R. Van Bree and A. Bastidas (Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg) for their help. Karl Storz Endoscopy is acknowledged for the generous supply of the laparoscopic equipments. This study was partially supported by Onderzoeks Toelagen Katholieke Universiteit Leuven, Leuven, Belgium grants Nr. TBA/00/27

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Addendum 11

Binda MM and Koninckx PR. Prevention of hyperoxia enhanced adhesions in a laparoscopic mouse model (in preparation).

ABSTRACT

Background: We previously demonstrated that the addition of 3% of oxygen to the pneumoperitoneum reduced adhesions formation and when more than 4% oxygen was used an increase was observed. The aim of this study was to investigate the effect of different treatments upon adhesion formation in the hyperoxia model in order to know their relative effectiveness and to understand the pathophysiology of the hyperoxia enhanced adhesions. **Methods:** Pneumoperitoneum-enhanced adhesion was induced using humidified and CO_2 with the addition of 12% oxygen as insufflation gas (hyperoxia model). The effect of low temperature was investigated and BT was controlled to 32° or 37°C. In addition, we choosed different drugs representative of different pathways of the adhesions process: anti-inflammatories (corticoid: dexamethasone, NSAIDs: nimesulide), calcium channel blockers (diltiazem), ROS scavengers (SOD and ascorbic acid), barriers (hyalobarrier gel), surfactant (phospholipids) and fibrinolytic agent (Reteplase). Adhesions were scored after 7 days. **Results:** Hyperoxia-induced adhesions were reduced using low temperature ($p<0.02$), phospholipids ($p<0.03$), hyalobarrier gel ($p<0.004$), dexamethasone ($p<0.005$) and diltiazem ($p<0.01$). A low effect of the ascorbic acid (NS) and Reteplase (NS) and non effect of the nimesulide, SOD and ascorbic acid was observed. If all experiments were grouped for analysis, adhesions also decreased with ascorbic acid ($p<0.002$) and Reteplase ($p<0.005$) showing a border line effect (Wilcoxon). **Conclusions:** In the hyperoxia model, adhesion formation decreases with the reduction in BT to 32°C, the use of dexamethasone, a calcium channel blocker, phospholipids and hyalobarrier gel. A border line effect of the ascorbic acid and Reteplase was observed. The hypothesis that hypothermia prevents also adhesions was confirmed, may be, by making cells more resistant to the toxic effects of the hyperoxia.

Key Words: adhesion formation, laparoscopy, mouse model, hyperoxia, prevention.

Addendum 12

Binda MM and Koninckx PR. Combination of treatments to prevent pneumoperitoneum enhanced adhesions in a laparoscopic mouse model (in preparation).

ABSTRACT

Background: Since we demonstrated that adhesion formation was reduced by adding 3% oxygen to the pneumoperitoneum (PP), by lowering body temperature (BT) and by applying different products during surgery, the aim of this study was to investigate the effect of the combination of these treatments in order to reduce adhesions. **Methods:** BALB/c mice were used (n=104). PP-enhanced adhesion was induced using humidified and pure CO_2 or CO_2 + 3% of oxygen, hypoxia and normoxia models, respectively. BT was controlled, to 32° or 37°C. Products representative of each mechanism were used and they were combined with two treatments: the addition of 3% oxygen and the low BT. Anti-inflammatories (dexamethasone, nimesulide), calcium channel blockers (diltiazem), ROS scavengers (SOD and ascorbic acid), barriers (hyalobarrier gel), surfactant (phospholipids) and fibrinolytic agent (Reteplase) were used. In addition, the effect of low temperature was also investigated in both pneumoperitoneum with pure CO_2 and with CO_2 + 3% oxygen. Adhesions were scored after 7 days. **Results:** Comparing to pure CO_2 PP at 37°C, adhesions were reduced by adding 3% oxygen to the PP ($p<0.05$), by lowering BT ($p=0.03$, Wilcoxon), as previously demonstrated, and by applying both 3% oxygen and low temperature ($p=0.02$). Taking into account the groups with 3% of oxygen, lowering BT from 37°C to 32°C did not reduce more the adhesion formation (NS) showing that both treatments, addition 3% oxygen and lowering BT, do not have additive effects. Comparing to the group with 3% oxygen and 32°C BT, adhesions were reduced by using dexamethasone ($p=0.04$), hyalobarrier gel ($p=0.0161$) whereas a low reduction was observed with diltiazem and phospholipids. Non effect was observed neither with Reteplase, nor with SOD and nimesulide and, surprising, an increase in adhesions was observed with ascorbic acid ($p=0.0085$). **Conclusions:** The use of a less traumatic pneumoperitoneum (adding 3% oxygen, humidified gas and low temperature) in combination with dexamethasone, diltiazem or phospholipids can be a way to reduce adhesions. New treatments and their combinations have still to be tested to get a maximum reduction of postoperative adhesions.

Key Words: adhesion formation, laparoscopy, mouse model, prevention.

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