Providing Direction Improves Function: Comparison of a Radial Pore Orientated Acellular Collagen Scaffold to Clinical Alternatives in a Surgically Induced Rabbit Diaphragmatic Tissue Defect Model

SHORT TITLE: Testing Novel Scaffolds for Diaphragmatic Hernia Repair

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CONFLICTS OF INTEREST

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ABSTRACT

Gore-Tex® is a widely used durable patch for repair of congenital diaphragmatic defects yet may result in complications. We compared Gore-Tex to a composite of a radial pore orientated collagen scaffold (RP-Composite) and clinically used porcine small intestinal submucosa (SIS; Surgisis®) in a rabbit model for diaphragmatic hernia. The growing rabbit mimics the rapid rib cage growth and re-herniation rates seen in children. We created and immediately repaired left hemi-diaphragmatic defects in 6-week-old rabbits with Gore-Tex, SIS and a RP-Composite scaffold. An additional group of rabbits had a sham operation. At 90 days, survivors more than doubled in weight. We observed few re-herniations or eventrations in Gore-Tex (17%) and RP-Composite (22%) implanted animals. However, SIS failed in all rabbits. Maximum transdiaphragmatic pressure was lower in Gore-Tex (71%) than RP-Composite implanted animals (112%) or sham (134%). Gore-Tex repairs were less compliant than RP-Composite, which behaved as sham diaphragm ($p < 0.01$). RP-Composite induced less foreign body giant cell reaction than Gore-Tex ($p < 0.05$) with more collagen deposition ($p < 0.001$), although there was a tendency for the scaffold to calcify. Unlike Gore-Tex, the compliance of diaphragms reconstructed with RP-Composite scaffolds were comparable to native diaphragm, whereas re-herniation rates and transdiaphragmatic pressure measurements were similar.

KEY WORDS: diaphragmatic hernia, defect repair, collagen scaffold, pore orientation

1. INTRODUCTION

Congenital diaphragmatic hernia (CDH) occurs in approximately 1/3,000 live births (McGivern et al., 2015). Patch repairs are necessary in around 25%, however this number is likely to rise as increasingly efficacious fetal intervention results in a new population of survivors (Cruz-Martínez et al., 2011; Jani et al., 2009). The diaphragm is a unique skeletal muscle, not only does it separate the peritoneal and thoracic cavity, it has a vital role in both respiration and as an anatomic contribution to the lower esophageal sphincter (Lessa et al., 2016). Its complex structure is not widely studied in the setting of CDH (Ackerman et al., 2012; Alaggio et al., 2015). Diaphragmatic repair in neonates with CDH will face a lifetime of demands, in normal individuals compensated by changing cell density and tensile strength throughout childhood (Steigman SA et al., 2010). Not only does the patch repair have to allow for these demands, it also independently affects the function of the diaphragm as a respiratory muscle (Eastwood et al., 2017). The most commonly used patch is Gore-Tex® (expanded polytetrafluoroethylene), although absorbable alternatives are increasingly used, such as Surgisis® (porcine derived small intestinal submucosa (SIS)) and Alloderm® (cadaveric human skin) (Grethel et al., 2006; Laituri et al., 2010). Although conflicting outcomes have been published, complications such as scoliosis, chest wall deformities, re-herniation, bowel obstruction and poorer long term pulmonary function are more common in children requiring patch repair (Jancelewicz et al., 2013; Jawaid et al., 2013; Panitch et al., 2015; Tsai et al., 2012).

Alternative patches such as decellularized diaphragm and stem cell engineered tissues have been tested, however as of yet they have not translated to clinical practice (Davari et al., 2016; Gubareva et al., 2016; Turner et al., 2011). It could be argued that availability and

cost of the latter may prohibit widespread use. Porous collagen-based tissue engineered solutions may offer a biocompatible alternative, with complete absorption, and minimal risk of late host rejection. Constructed collagen scaffolds can be modified by the addition of growth factors to encourage tissue integration (Brouwer et al., 2013, n.d.) and crosslinking may provide biomechanical strength for long term *in vivo* stability. Furthermore, modifications in pore size and orientation can further enhance tissue ingrowth (Brouwer et al., 2013). Previously, radial pore-orientated collagen scaffolds implanted into the diaphragm of rats showed promising results (Brouwer et al., 2014a), although a slower degrading material was suggested to be necessary for larger animal models (Brouwer et al., 2014b). Few of these materials are extensively tested pre-clinically and available models are often only partly representative. Moreover, long-term functional testing of scaffolds in these animal models is limited. However, we recently developed a comprehensive portfolio of read-outs in our growing rabbit model of diaphragmatic hernia, which reaches full size by approximately six months, closely mimicking the rapidly expanding infant rib-cage (Eastwood et al., 2017).

Herein, we investigate the properties and efficacy of radial pore orientated collagen scaffolds combined with clinically used SIS and crosslinked to provide additional biomechanical strength *in vivo*. We test whether it provides superior functional outcomes to the clinically available alternatives, Gore-Tex and SIS, namely on Pdi measurements whilst preventing re-herniations.

2. MATERIALS AND METHODS

Three scaffolds were investigated in this study: i) 1 mm single layer Gore-Tex (GORE-TEX®, Arizona, USA), ii) four layer (4 ply) non-crosslinked porcine small intestinal submucosa graft (SIS, Cook Biotech, IN, USA), iii) composite crosslinked scaffolds consisting of 4 ply SIS and radial pore orientated type I collagen henceforth known as “RP-Composite”.

2.1 Production and characterization of composite scaffolds with radially oriented pores

Type I collagen with radially oriented pores were prepared according to a method adjusted from Brouwer *et al.* (Brouwer et al., 2013, 2011). It was prepared as a composite scaffold with SIS scaffold in circular shapes (35 mm diameter) (S1). Lyophilised scaffolds were crosslinked in formaldehyde and after crosslinking reaction was quenched (S1). Scaffolds were then γ -irradiation for sterilisation and their structure was visualised using a scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany). The distance between the lamellae in the radial part of the composite scaffold was measured from SEM images using Fiji (Schindelin et al., 2012). The degree of crosslinking was estimated (S1).

2.2 *In vitro* biomechanical scaffold properties

As excessive rehydration has been suggested to cause delamination in SIS (COOK BIOTECH, 2010), we studied the biomechanical properties of sterile SIS and RP-Composite following longer term immersion (60 days) in PBS at room temperature. Data were compared to the characteristics at baseline. For SIS this was following rehydration for 3-5 min in PBS as per the manufacturer guidelines. The investigational RP-Composites scaffolds were shipped under sterile conditions while hydrated in PBS and stored for either seven or 60 days.

Scaffolds were cut into standardized strips using a precut rectangular shape (10x30 mm) and the thickness was measured using a digital micrometer (Mitutoyo, Japan, accuracy 0.01mm).

Uniaxial testing (S2) was performed using a Zwick tensiometer (Zwick GmbH & Co. KG, Ulm, Germany). The load and elongation were recorded throughout the testing period. The strain was calculated by dividing the elongation by the clamp-to-clamp length. The corresponding stress-strain curve was computed (S3).

2.3 *In vivo* evaluation of scaffolds in a growing rabbit model

2.3.1 Ethical approval

The experimental protocol was approved by the institutional animal ethical committee (P112/2014) and performed according to the European guidelines for the care and use of laboratory animals. All animals were group housed in a controlled environment. This study follows the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidance.

2.3.2 Study design

Forty-one six-weeks old (~1.5kg) male rabbits (New Zealand White, Zootechnical Institute KU Leuven) underwent immediate repair of a surgically induced left diaphragmatic hernia. Power calculation based on our previous study (tensiometry results) suggested a repair group size of n=6 would give 80% power (independent t-test) (Mayer et al., 2015). A previously described standardized anesthetic and surgical protocol (S4) was used (Eastwood et al., 2017). Animals were randomly allocated to receive either a sham operation or defect creation and repair.

In total 41 animals entered this experiment. Thirty-one (76%) survived beyond the early post-operative period and are included in the later analysis. Ten died from complications

unrelated to the scaffold; pneumothorax (n=4), unable to maintain airway post-extubation (n=3), died in recovery (<2h; n=1), aspiration (n=1), early post-operative death without identified cause (day 3; n=1).

In sham operated animals (n=6) the diaphragm was left untouched with the abdomen packed for 20min. In all other animals, a standardized 3x3cm left posterior defect was created and immediately repaired with a 3.5x3.5cm i) Gore-Tex (n=9), ii) SIS (n=7) and iii) RP-Composite (n=9), sutured in place with non-absorbable interrupted suture (Prolene 4-0, Ethicon, Zaventem, Belgium). The Gore-Tex and sham animals were also included in a previous publication (Eastwood et al., 2017). The abdominal wall was closed in three layers by running sutures (4-0 vicryl) for the linea alba and SC fascia and 4-0 Monocryl (Ethicon) was used for subcuticular skin closure. The ketamine/propofol infusion was slowed and stopped, with removal of the LMA when the animal gained consciousness. In recovery 4 L/min O₂ was given via a nose cone. Post-operatively, rabbits received once daily (SC) postoperative pain relief (metacam) and antibiotics (enrofloxacin) for three days. All animal caretakers were blinded to treatment group.

2.3.3 Experimental outcomes

In vivo outcome measures were assessed at 90 days following implantation. These are divided into gross functional outcomes (chest X-rays, breathing patterns, transdiaphragmatic pressure measurements, tensiometry) and histopathological outcomes (herniation, adhesion, immunohistochemistry and scaffold integration on nanoCT).

2.3.3.1 Functional outcomes

Chest X-rays

24-48 h prior to euthanasia, animals were sedated (IM ketamine 35mg/kg with xylazine 5 mg/kg as previously) to have posterior-anterior and left lateral chest X-rays (Embrace DM

1000 Mammography System; Agfa-Gevaert, Mortsel, Belgium: collimation size 24x29cm, thickness 280 mm, voltage 28 kVp, engine load 65 and 80mAs). Chest X-rays were assessed for re-herniation by a single observer blinded to the experimental surgery. To determine the degree of scoliosis the Cobb angle was measured between the 4th and 10th vertebrae in animals with no obvious curvature or at the extremes of the spinal curvature (RadiAnt DICOM Viewer 3.2.2, Medixant, Poznan, Poland) (Figure 4a). Measurements were repeated three times by an observer blinded to treatment group and averaged.

Trans-diaphragmatic pressure measurement and pulmonary function testing

Animals were sedated (IM ketamine/xylazine) and commenced on an intravenous (IV) infusion of ketamine/propofol (as above). In brief, a tracheostomy was performed with placement of a pneumotachograph (Hans Rudolph inc, Shawnee, KS, USA) to allow airflow measurements that were integrated to obtain tidal volume (Vt). Then oesophageal and abdominal pressure changes were measured by using balloon catheters (Cooper Surgical, Trumbull, CT, USA) introduced through the mouth and placed in the esophagus and stomach respectively (Eastwood et al., 2017). Transdiaphragmatic pressure (Pdi) was calculated as the difference between abdominal and esophageal pressures. Breathing pattern was recorded at rest, and Pdi and corresponding tidal volume were averaged from five consecutive breaths. Maximal Pdi was recorded as the maximal Pdi value obtained during occluded breaths induced by blocking the tracheostomy tube. The animal was then euthanized with an IV injection of T61 (MSD, Brussels, Belgium).

2.3.3.2 Biomechanical and histopathological outcomes

Following euthanasia animals were shaved and assessed for any signs of incision site breakdown (infection/ dehiscence) before a midline laparotomy was performed as previously described (Eastwood et al., 2017). The abdominal cavity was opened and inspected for fluid

collections, infection, adhesions, re-herniation or eventration of the diaphragm. A left thoracic window was created with removal of the anterior section of the 4th to 6th ribs. The severity of adhesions was graded at the a) original sub-costal incision site (scar) b) scaffold on the abdominal side (mesh) and c) scaffold on thoracic side (left lung). Adhesions were broadly classified into three domains; tenacity (1-3), area (1-4) and type of adhesion (1-4)(Zühlke et al., 1990).

The diaphragm was then explanted *en bloc* and specimens were taken from the left hemidiaphragm as shown (Figure 1). Specimens from the scaffold interface and center were either snap frozen or fixed overnight in 4% paraformaldehyde (buffered formaldehyde, pH 6.8-7.2, Klinipath BV, Duiven, Netherlands), washed in PBS and then placed in 70% ethanol prior to embedding in paraffin. For tensiometry, standardized samples using a dog bone shape cutting form (2 x 25mm) were punched from the anterior scaffold-tissue interface and right hemidiaphragmatic musculo-tendinous junction and mid tendinous area. Testing was performed within 2 h after prelevation; prior to measurements tissues were kept in a wet saline soaked gauze at room temperature (~20°C). Biomechanical testing was described in 2.2.

Following embedding specimens from the scaffold center and interface were cut into 4 µm slides. A hematoxylin and eosin was performed to quantify the presence of polymorphonuclear cells (PMN), foreign body giant cells (FBGC) and angiogenesis. Five randomly selected fields at 400x magnification (Carl Zeiss, Oberkochen, Germany) were scored by an expert observer (EVB) using a semi-quantitative scale; 0 (0), 1-5 (1), 6-9 (2) and ≥10 cells (3) or 0 (0), 1-3 (1), 4-9 (2) and ≥10 (3) vessels. Macrophages were stained using a Mouse anti-Rabbit RAM-11 antibody (MO63; Dako Corp, Carpinteria, CA, USA). After incubation with 0.2M HCl for 10 min followed by blocking 1 h at ambient temperature in 2%

bovine serum albumin, 1% non-fat dry milk, 0.1% Tween 80 in PBS). Slides were incubated with RAM-11 1:50 in PBS overnight at 4°C followed by the secondary antibody (goat anti-mouse 1:50 in PBS; Dako Corp.) and normal rabbit serum (1:25; Dako Corp.) for 30 min at ambient temperature. Final counterstaining was with 2% methyl green solution in PBS for 30 min. Numbers of RAM-11 cells were counted as described above. Collagen maturity and amount was assessed semi-quantitatively using a Movat stain (MOVAT, 1955). Maturity was scored semi-quantitatively from a totally disorganized cellular to a well-organized acellular scar (0-3), amount from absent (0), minimal (1), moderate (2) to abundant (3). Finally, slides were stained (2mins) with Alizarin Red (2g in 100ml of distilled H₂O (pH 4.1-4.3); Sigma-Aldrich) to confirm the presence of scaffold calcification.

2.3.3.3 Scaffold-tissue Interface assessed by nanoCT

A snap frozen specimen from the scaffold-tissue diaphragmatic interface of one Gore-Tex and one RP Composite implanted animal were fixed at room temperature in 4% paraformaldehyde for 24 h, washed in PBS and then placed in iodine 0.1M (KI 40g (Merck, Overise, Belgium), resublimed I₂ crystals 12.7g (Aldrich, Milwaukee, USA) in 1L dH₂O) overnight. They were scanned using standardized settings in nanoCT scanner (Phoenix nanotom m, General Electric Company, Pennsylvania, USA) to enable 3D rendering of the scaffold tissue interface. Standardized settings of 50kV, 210 mA, diamond target and no filter were used, allowing a scan resolution of 4 µm.

2. 4 Statistical analysis

Data were analyzed using Prism for Windows version 5.0 (Graphpad software, San Diego, CA, USA). Normality of the data was checked prior to statistical testing (Kolmogorov-Smirnov test). Quantitative data are represented as mean±standard deviation (SD) or median (IQR).

Between groups *t*-testing or fisher exact testing was used. Paired data were tested using a *t*-test or Wilcoxon signed rank test. One way ANOVA was used to test differences between more than two groups of animals, with *post hoc* testing between groups using Tukey's multiple comparison test or Dunn's multiple comparison test. $P < 0.05$ was considered a statistically significant result.

3. RESULTS

3.1 Production and characterization of composite scaffolds with radially oriented pores

During the freezing process, it was macroscopically observed that freezing of the collagen suspension onto the SIS started from the centrally placed freezing tube going outward in a radial manner. After lyophilisation, the SIS and radial collagen layer were macroscopically well attached, even after prolonged immersion in PBS.

Crosslinking was $47 \pm 8\%$ for sterilized scaffolds and $44 \pm 11\%$ for non-sterilized scaffolds. Please note that the SIS membrane did not completely dissolve during the analysis, resulting in an underestimation of the number of amine groups, both before and after crosslinking, and for sterilized and non-sterilized scaffolds.

Scanning electron microscopy showed that the SIS layer and the radial pore-orientated collagen scaffold were well connected in the RP-Composite (Figure 2a). The lamellae showed clear anisotropic radial pores and were connected by small struts of collagen (Figure 2b, c). The distance between the lamellae in the radial part of the composite scaffold was $47 \pm 20 \mu\text{m}$. No differences in ultrastructure could be observed when comparing the scaffolds before and after γ -irradiation.

3.2 *In vitro* biomechanical implant properties

We studied the biomechanical properties of our biodegradable scaffolds ($n=4$) at the time of implantation or 60 days following immersion in PBS (Figure 3a). Following 3-5 min (0 days) or 60 days immersion in PBS 4-ply SIS has similar biomechanical properties. Our RP-Composite did also not behave differently at the time of implant or following 60 days in PBS. Although

maximal stress withstood was higher in the SIS group, there were no statistical differences between groups.

3.3 *In vivo* experiments

Survival, herniation, weight and adhesion score

Thirty-one survived the initial post-operative period. Three (9%) animals died at a later time-point, all in the Gore-Tex group, with significant diarrhea, pneumonia, and for one no post mortem was undertaken.

Average weight pre-operatively was 1.4 (1.3-1.7) kg and this was similar in all implant groups ($p=0.0725$) (Table 1). At 90 days, sham, Gore-Tex and RP-Composite animals had a similar weight, however the SIS group weighed less ($p<0.05$). Animals more than doubled in size (+154%) with the largest weight gain being in the Gore-Tex group (+220%) and the smallest in the SIS group (+108%). The majority of animals in the SIS group (5/7) did not double in weight.

All SIS patches showed herniation or eventration (100%). There was massive herniation of abdominal contents in the majority of animals implanted with SIS ($n=5$; 71%; Figure 4e/f), while in the remaining SIS implanted animals, one had herniation of a segment of liver at the anterior medial edge of the repair and the second had an eventration with the liver plugging the defect below a flimsy scaffold (Figure 4g). In the SIS group, the scaffold formed a sac-like projection over the herniating organs with an intact suture line (Figure 4e-f). Herniation was significantly more likely in SIS than in the Gore-Tex ($p<0.01$) and RP-Composite groups ($p<0.01$). Gore-Tex (17%) and composite scaffolds (22%) had similar herniation rates; all demonstrating herniation of a segment of liver close to or at the antero-

medial suture line (Figure 4a-c). The two layers in RP-Composite were noted to have separated at the time of harvest (Figure 4d, 6m).

At harvest one animal in the RP-Composite group had a subcutaneous abscess at the scar site, with well healed scars in all other animals. Adhesion scores at the site of the scar were similar in all groups. Sham operated animals had no thoracic adhesions (table 1: left lung), with similar thoracic adhesions present in the three groups ($p=0.6290$) (Figure 4h). Adhesions scores at the scaffolds were only scored in the two SIS animals without major scaffold failure and were similar to the other implanted groups ($p=0.8169$). It was noted however that the dissection of Gore-Tex presented the greatest challenge requiring dissection through the liver on the abdominal side. The liver was particularly adherent to the suture lines and in 10-50% of medial part of the implant.

3.3.3.1 Functional outcomes

Chest X-ray demonstrated easily identifiable herniation of abdominal contents in five SIS cases (Figure 5a). Cobb angles were similar ($p=0.3388$) and in the normal range in all groups: sham $3.817 \pm 1.14^\circ$, Gore-Tex $3.192 \pm 1.59^\circ$, SIS $2.647 \pm 0.98^\circ$, RP-Composite $4.276 \pm 1.77^\circ$.

Functional measurements results are given in at least five animals per group. Not included are two animals in the SIS group and one in the RP-Composite group as they died following sedation for chest X-ray. Furthermore, one sham animal and two RP-Composite animals were excluded as resting intra-abdominal pressures exceeded three times the expected value (>22.8 mmHg) suggesting inadequate relaxation. The equipment was not available for two animals. Tidal volume and airflow were similar between groups (Figure 5b-c). Pdi measurements were possible in five SIS animals; in three the abdominal signal was negative in keeping with the thoracic position of the stomach at harvest. The other two animals had small herniations and positive abdominal pressure signals; maxPdi of -3.4% and 126%. Pdi

(cmH₂O) was similar ($p=0.8854$) at rest in sham (6.63 ± 2.5), Gore-Tex (7.39 ± 1.2) and RP-Composite (7.89 ± 3.2) groups. Following airway occlusion there was a significant increase in Pdi in Sham and RP-Composite implanted animals ($p < 0.05$) (Figure 5d). The increase in maximal Pdi during occluded breaths was greatest in sham operated animal (134%), as compared to RP-Composite (112%), and a 71% increase in Gore-Tex implanted animals (Figure 5e).

3.3.3.2 Biomechanical implant properties at 90 days

In sham animals we compared three samples; left and right hemidiaphragmatic mid tendon and right muscle-tendon interface (Figure 2b-f). The right and left tendinous strips were found to be similar in both comfort zone modulus and elongation to failure whereas the muscle-tendon interface was different in both measurements ($p < 0.01$) (Figure 3b). All samples were tested across the scaffold-tissue interface. The two SIS samples (without massive herniation) failed during pre-conditioning and biomechanical testing was not possible. The left hemidiaphragmatic tendon (sham) was compared with Gore-Tex and RP-Composite explants (Figure 3c-f). In left hemi-diaphragmatic specimens, the comfort zone modulus showed greatest variability in the RP-Composite group (Figure 3d). Gore-Tex had a much lower comfort zone modulus ($p < 0.01$), whereas RP-Composite was not different to sham. Ultimate tensile strength was significantly lower in Gore-Tex implanted animals and not significantly improved in the RP-Composite group (Figure 3e). Elongation to failure was similar in tested groups (Figure 3f). The scaffold-tissue interface was compared to the right hemidiaphragm tendon and muscle-tendon interface in each animal. Gore-Tex at its interface was noted to behave more similarly to the muscle-tendon interface, whereas the RP-Composite – tissue interface behaved more like the diaphragmatic tendon.

Histological outcomes at 90 days

At 90 days, there were no visible scaffolds in SIS implanted animals; they were replaced by fibrous tissue staining for immature collagen (Figure 6c), whereas in RP-Composite implanted animals the layers of SIS were visible but it was difficult to identify the radial pore layer (Figure 6d, 6m, 6p). All RP-Composite implanted scaffolds demonstrated encapsulation compared to 20% in the SIS group and 67% of the Gore-Tex group (Figure 6m, 6n). Furthermore, 44% of RP-Composite explants demonstrated a degree of calcification within the layers of the scaffold (Figure 6c, 6d) compared to 60% of the SIS group. Calcification of both sides of the RP-Composite was confirmed on alizarin red staining (Figure 6p). Virtually no polymononuclear leukocytes were identified at the tissue interface or center of any operated group (Table 2). Overall, macrophages were the predominant cell type (Figure 6q). Gore-Tex displayed the highest cellular reaction with greater numbers of macrophages and FBGC at the scaffold center than the interface (Figure 6b/f/j/q). This pattern was reversed in the biologically degradable groups with increased cell numbers at the scaffold interface (Figure 6q). Compared to sham, Gore-Tex implanted animals had significantly elevated FBGC at both scaffold interface ($p < 0.01$) and center ($p < 0.001$) whereas macrophage levels were not statistically different. RP-Composite implanted animals demonstrated a lower FBGC response than Gore-Tex operated animals ($p < 0.05$). At the scaffold interface infiltrating macrophages were significantly higher in RP-Composite group ($p < 0.05$) than sham animals. There was a less aggressive cellular response at the RP-Composite center compared to the Gore-Tex implanted animals (Figure 6q).

Collagen maturity and amount were statistically different in sham operated and implanted animals ($p < 0.01$) (Figure 6i-l). RP-Composite implanted animals showed the highest

amount of collagen at both the scaffold interface and center, although maturity was not different from Gore-Tex animals. The number of blood vessels were significantly increased in RP-Composite and Gore-Tex groups (Figure 6m-n, Table 2).

3.3.3.3 Visual representation of scaffold-tissue interface

NanoCT scanning of the scaffold-tissue interface clearly demarcated the scaffold in both Gore-Tex and RP-Composite implanted animals (7a-b). In the Gore-Tex implanted animals, there was virtually no tissue ingrowth at the interface confirming histology (Figure 7a). The layers of SIS were clearly visible as part of the RP-composite which showed areas of separation although there was significantly more tissue integration (Figure 7b).

4. DISCUSSION

Herein we used a growing rabbit model to test patch materials for neonatal diaphragmatic reconstruction. Diaphragms reconstructed with RP-Composite scaffolds have, unlike Gore-Tex, a compliance comparable to native diaphragm. We also found comparable transdiaphragmatic pressure measurements. Remarkably, despite their clinical use all SIS scaffolds failed, whereas reherniation rates of RP-Composite scaffolds were equal to the clinical standard, Gore-Tex.

In children born with CDH requiring defect closure, a Gore-Tex patch is the most commonly used patch (Gasior and St Peter, 2012). This synthetic durable material has a compliance that is different from diaphragmatic tissue, which may hamper fluid diaphragmatic movements (Mayer et al., 2015; Touloukian, 1978). Furthermore, it is not remodelled and does not grow with the neonate, thus potentially leading to re-herniation, chest wall and spinal deformities (Gasior and St Peter, 2012; Takayasu et al., 2016). However, in this rabbit model -similar to several large volume centers- rates of re-herniation following patch repair are low (Jawaid et al., 2013; Tsai et al., 2012). A strategy to prevent complications and improve diaphragmatic function may be the use of acellular collagen scaffolds. Porcine decellularised small intestinal submucosa (SIS) is embedded with active growth factors, non-crosslinked and ideally remodels as it degrades (Badylak et al., 2002; McDevitt et al., 2003). When SIS is used for diaphragmatic defect closure, clinical results are similar to Gore-Tex (Romao et al., 2012). However, when used in our model, it appears that the tissue remodeling at 90 days could not prevent re-herniation or eventration in all animals. As rabbits have a more active collagenase degradation this may not be entirely representative of the clinical situation (Becker et al., 1975). Accepting this limitation, we aimed to improve the

outcomes of 4-ply SIS by adding an additional layer of collagen. The entire scaffold was rendered more resistant to degradation by crosslinking with formaldehyde vapour as well as EDC/NHS. In addition, tissue ingrowth was enhanced by radially orientated unidirectional pores (RP), which as an unsupported scaffold would have inadequate strength (Bozkurt et al., 2007; Riboldi et al., 2008). The combination of SIS with RP was preferred over an EDC/NHS crosslinked scaffold combining RP with Vicryl, because the latter resulted in eventrations in another large animal model (Brouwer et al., 2014c).

With the RP-Composite scaffold we observed similarities in both the number and phenotype of re-herniations to rabbits implanted with Gore-Tex. In terms of adhesions, the pattern was similar to that of Gore-Tex, with minimal adhesions in the thorax, yet strong adhesions to sutures and the abdominal side of the implant, which are also observed clinically (The Myomectomy Adhesion Multicenter Study Group, 1995). Microscopically, the layered collagen scaffold structure of our composite was still recognizable at 90 days. However, the entire scaffold was surrounded by relatively immature collagen with a paucity of inflammatory cells. Conversely, there were more inflammatory cells around the Gore-Tex patch yet similar collagen deposition. NanoCT analysis was used to appreciate ingrowth, however we did not observe a difference between Gore-Tex and RP-Composite. Neither of the two truly integrated; conversely, they became encapsulated. Additionally, macroscopically we observed the radial pore layer separated from the SIS on dissection, suggesting the absence of tissue ingrowth between the different collagen layers of the implant. Gore-Tex with a pore size of $0.2\text{ }\mu\text{m}$ leads to encapsulation rather than tissue ingrowth (Saffarini et al., 2013). Scaffolds with unidirectional pores with a similar magnitude ($20\text{--}50\text{ }\mu\text{m}$) as the radial part of the composite scaffold facilitated the formation of C2C12 derived myotubes in culture (Kroehne et al., 2008). The average “pore” size of SIS is larger

(144 μm), which likely contributed to rapid degradation (Khang et al., 2002). Passive biomechanical testing demonstrated that RP-Composite explants behaved like the diaphragm of sham operated animals, unlike the stiffer Gore-Tex. We also observed transdiaphragmatic pressure of both groups as an *in vivo* proxy to compliance. Gore-Tex reconstructed animals were unable to increase transdiaphragmatic pressure in response to airway occlusion unlike sham operated animals. However, the RP-Composite implanted had pressure changes within the range of shams. We also assessed breathing pattern but did not detect differences between the three groups. Conversely, children with CDH exhibit mild obstructive airway disease and have hyper distended lungs at the age of three and older (Ijsselstijn et al., 1997; Panitch et al., 2015). Obviously, these children have underlying hypoplasia, yet there is limited clinical data of the impact of different patches on pulmonary function.

We also observed a few local side effects. More than half of the RP-Composite scaffolds demonstrated calcifications located on the SIS material as well as the radial pored scaffold. Collagen biomaterials can calcify as previously seen in reconstructed bladders of rabbits (Nuininga et al., 2004). Calcification in SIS scaffolds have been observed before and are suggested to arise from cellular debris, elastin or as a result of an innate response leading to differentiation of macrophages to an osteoblast phenotype (Daamen et al., 2005; Levy et al., 1991; Mohler et al., 1997; Mosala Nezhad et al., 2017; Rieder et al., 2005; Sloff et al., 2016; Woo et al., 2016). The crosslinking process has been previously suggested to cause calcifications (Ancillotti et al., 1977; Huang-Lee et al., 1990; McIntosh, 1992), even though sodium borohydride was used to quench unreacted aldehydes. However, other xenogeneic decellularized grafts, such as cardiac valves (kangaroo and pig to sheep) calcify despite being non-crosslinked (Van Nooten et al., 2006). Regardless, calcifications at this time-point did not affect compliance within our graft, yet that may be the case later on. The *in vivo* detachment

of the RP-Composite from the SIS with several layers visible on nanoCT suggested that although the scaffold was partially resorbed, the tissue remodelling response was adequate to prevent major re-herniations.

We must acknowledge a number of shortcomings in our experiment. Firstly, despite powering our experiment to the primary outcome (compliance), we still used a relatively small number of animals. Secondly, we could have included a control group with crosslinked SIS or RP scaffold alone; the latter we left out as we anticipated that it would not have enough structural strength. We did not investigate an alternate absorbable patch (Alloderm or Permacol) or 8-ply SIS patch, which has been used clinically although no differences in re-herniation rates between the two were reported (Grethel et al., 2006). Thirdly, we did not look at blood oxygen, carbon dioxide or pH levels at the time of harvest. Lastly, we did not include primary defect closure, which is ultimately the commonest defect closure in these children. This was technically and ethically impossible given the large defect size. Furthermore, this model does not mimic the consequences of pulmonary hypoplasia, feeding problems or scoliosis, which are typical for this disease. Unfortunately, rabbit pups with surgically created diaphragmatic hernia *in utero* do not survive more than 30 min post-natally so are not suitable for later patch implantation (Fauza et al., 1994; Flemmer et al., 2017; Russo et al., 2016). Although our model mimics rapid and significant growth, longer term observations would have been informative, to exclude late degradation or to study neural and muscle regeneration with appropriate staining (Claerhout et al., 2008). However, our experiment offers a comprehensive evaluation using a model that seems to resemble clinical outcome as it demonstrated rapid growth and displayed re-herniation.

CONCLUSIONS

In conclusion, the combination of a radial pore orientated collagen scaffold to SIS with additional crosslinking results in avoidance of the re-herniations observed with non-crosslinked SIS alone. Reconstructions with this composite scaffold had a compliance and transdiaphragmatic pressure comparable to the untouched diaphragm in sham operated animals. This RP-composite scaffold offers an absorbable clinical alternative to the traditionally used Gore-Tex, although the impact of calcifications at longer times points and in a larger animal should be further studied.

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Table 1: Summary by mesh implant of all rabbits entering the experiment with survival and weight data at time of implant and at the 90 day time-point. At the 90 day time point the herniation rate and adhesion score at the site of the abdominal scar, implanted mesh and to the left lung

Mesh	Number of Rabbits		Weight			Outcome at 90 days			
	Implanted	90-day survival	Time of Implant (kg)	90 days (kg)	Average Increase (%)	Herniation	Adhesion Score		
							Scar	Mesh	Left Lung
Sham	6	6 (100%)	1.55 (1.2-1.7)	3.4 (3.2-3.6)	140%	-	0,0 (0,0-6,5)	6,35 (0,0-8,0)	0,0 (0,0-0,0)
Gore-Tex	9	6 (67%)	1.2 (0.8-1.4)	3.4 (3.2-4.0)	220%	1 (17%)	8,0 (3,5-9,0)	10,0 (8,0-10,0)	5,0 (4,0-7,0)
SIS	7	7 (100%)	1.6 (1.4-1.8)	3.1 (2.8-3.5)	108%	7 (100%)	7,5 (0,0-8,3)	9,5 (8,0-11,0)	5,0 (1,5-7,0)
Radial Pore-SIS Composite	9	9 (100%)	1.4 (1.3-1.6)	3.9 (3.6-3.9)	155%	2 (22%)	0,0 (0,0-8,5)	10,0 (8,0-10,6)	5,0 (3,25-5,0)
TOTAL	31	29 (94%)	1.4 (1.3-1.7)	3.4 (3.2-3.9)	154%	10 (36%)			

Table 2: Histological outcomes at 90 days. Comparisons are made between groups in each column using one way anova and post testing were indication between a) Sham and other meshes *P<0.05, **p<0.01, ***p<0.001 b) Gore-tex and other meshes †p<0.05, ‡p<0.01. Abbreviations: Encapsulation (*Encaps*), Calcification (*Calcif*)

Mesh Type	Cellular Response						Movat				Blood Vessels		Encaps.	Calcif.
	Polymorphonuclear cells		Foreign body giant cells		Macrophages		Maturity		Amount					
	Interface	Center	Interface	Center	Interface	Center	Interface	Center	Interface	Center	Interface	Center	Number (%)	
Sham	0.2 (0.0-1.0)	0.2 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0 (0.0-0.5)	0.1 (0.0-0.7)	0.0 (0.0-0.0)	0.0 (0.0-3.0)	0.5 (0.0-1.5)	0.0 (0.0-1.0)	0.5 (0.0-1.0)	0.0 (0.0-1.0)	0/6 (0)	0/6 (0)
Gore-Tex	0.0 (0.0-0.0)	0.0 (0.0-0.0)	1.0 (0.75-1.0)**	1.0 (1.0-1.0)***	1.3 (0.85-1.95)	1.7 (1.15-2.4)	3.0 (1.75-3.0)**	2.0 (2.0-2.75)	2.0 (1.75-3.0)	3.0 (2.25-3.0)*	1.0 (1.0-1.0)*	1.0 (0.7-1.15)*	4/6 (67)	0/6 (0)
SIS	0.0 (0.0-0.0)	0.07 (0.0-0.2)	0.2 (0.0-1.0)	0 (0.0-0.2) ‡	1.0 (0.0-1.4)	0.0 (0.0-0.2)	3.0 (1.5-3.0)*	3.0 (1.0-3.0)	2.0 (1.0-3.0)	3.0 (1.0-3.0)	0.8 (0.6-1.0)	0.8 (0.6-1.0)	2/5 (20)	3/5 (60)
RP Comp	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.6 (0.0-1.0)	0.6 (0.0-0.8)*‡	2.0 (1.6-3.0)*	1.0 (0.8-1.2)	2.0 (2.0-3.0)*	3.0 (3.0-3.0)	3.0 (3.0-3.0)***	3.0 (3.0-3.0)**	1.0 (1.0-1.0)*	1.0 (1.0-1.0)**	9/9 (100)	4/9 (44)

Figure 1: Diagrammatic representation of the division of the left hemidiaphragmatic explant for i) biomechanical testing, ii) histological specimens placed in paraformaldehyde (including immunohistochemistry) and snap frozen specimens (PCR and nanoCT).

Figure 2: Scanning electron micrographs of the RP-composite scaffolds with radially oriented pores. a) Overview of the RP-Composite scaffold, showing the SIS layer beneath the collagen scaffold with radial pores. The two layers are microscopically well connected. Non-sterilized scaffold. Bar is 100 μm . b) Higher magnification showing the radial orientation of the pores in the collagen scaffold (sterilized). Bar is 100 μm . c) Higher magnification of the RP-collagen scaffold showing small interconnections between the collagen lamellae. Bar is 10 μm .

Figure 3: Biomechanical mesh properties in vitro (a) and at 90 days post-implantation (b-e). a) Comparison in vitro of RP-Composite 0 and 60, or SIS 0 and 60 (sterilized and tested at implantation or 60 days in PBS). At 90 days biomechanical comparison of: b) left/right diaphragmatic tendon and muscle-tendon interface in sham operated animals, c-f) left diaphragmatic tendon (sham), Gore-Tex and RP-Composite values for c) stress-strain curve, d) comfort zone modulus, e) ultimate tensile strength, and f) elongation to failure. **p<0.01

Figure 4: At 90 days post implant a) Gore-Tex mesh: abdominal side, b) RP-Composite (thoracic side: arrow pointing to area for freezing pole), c) RP-Composite with medial herniation of liver (arrows), d) Mesh separation of RP-Composite, e) Mesh failure of SIS, f) Suture line in SIS g) Eventration in SIS, h) Adhesions between left lung and RP-Composite.

Figure 5: Radiological and functional outcomes at 90 days a) Chest X-Ray demonstrating herniation (arrow) of abdominal organs into the left hemi-thorax in an SIS implanted animal. b-c) Vt in ml and flow rate in ml/s for all implanted groups. d) Changes in transdiaphragmatic pressure (Pdi) before and after tracheostomy occlusion by mesh type (*P < 0.05). e) Pdi increase by mesh type following tracheostomy occlusion (p=0.2211).

Figure 6: Histology at 90 days of sham operated animals (a, e, i, o) or of the central area of the implanted Gore-Tex (b, f, j, n), SIS (c, g, k) or RP-Composite (d, h, l, m, p) scaffolds. Macrophage and foreign body giant cell counts at the scaffold center and interface are given (q). SIS scaffolds were nearly completely reabsorbed (c, g, k). Mature capsule formation shown at higher magnification in RP-Composite (m) and Gore-Tex (n) scaffolds with neovascularisation (arrows). Alizarin red staining showing calcifications (bright red) on both sides of RP-Composite scaffolds (p) compared to sham tendon (o). Scale bars: 100 μ m (a-l, o-p), 50 μ m (m-n). Abbreviations: Abdo (abdominal side of mesh), encapsulation (caps), Li (liver), RP-Composite (RP), small intestinal submucosa (SIS). Symbols: Scaffold (star), calcifications (Δ).

Figure 7: NanoCT scan of the scaffold-tissue interface of a) Gore-Tex scaffold (*) showing clear demarcation (interface: IF) with the tissue b) RP-Composite (star) showing layered SIS implant (two-sided arrow) at 90 days and tissue surrounding the repair (white triangle = tissue), c. RP-Composite showing scaffold encapsulation (arrow).