Effect of long-term polytrauma on ventilator-induced diaphragmatic dysfunction in a piglet model

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Declaration of interests:

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Running head title:

Impact of polytrauma on VIDD

Abstract:

Introduction: Mechanical ventilation is known to activate oxidative stress and proteolytic pathways in the diaphragm. Trauma by inducing inflammation and activating proteolytic pathways may potentiate the effects of mechanical ventilation on the diaphragm. In a blunt chest trauma with concomitant injuries we tested the hypothesis that trauma via inflammation further activates the proteolytic pathways and worsens atrophy in the diaphragm.

Material and Methods: Piglets were separated into 2 groups and underwent 72hrs of mechanical ventilation. One group received a polytrauma (PT) by unilateral femur fracture, blunt chest trauma with lung contusion, laparotomy with standardized liver incision and a predefined haemorrhagic shock. The second mechanically ventilated group (MV) did not receive any trauma. A non-ventilated group (Con) served as control.

Diaphragmatic fibre dimensions, Western Blot analyses of proteolytic pathways and lipid peroxidation and mRNA levels of cytokines and NFkB subunit p65 were measured.

Results: Active Caspase-3 was significantly increased in MV ($p=0.019$), and in PT ($p=0.02$) compared to Con. NFkB subunit p65, was up-regulated in PT ($p=0.010$) compared to Con. IL-6 mRNA increased significantly in PT compared to Con ($p=0.0024$) but did not differ between Con and MV.

Conclusion: Trauma and mechanical ventilation induced proteolysis and atrophy in the diaphragm, but only polytrauma induced an inflammatory response in the diaphragm. The additional traumatic inflammatory stimulus did not increase the levels of the pre-mentioned variables. These data underline that inflammation is not a major contributor to VIDD.

Keywords: Inflammation; mechanical ventilation; polytrauma; diaphragm; proteolysis

Trial registry number: AZ 84-02.04.2014.A265 (Landesamt für Natur-, Umwelt- und Verbraucherschutz, LANUV NRW, Germany)

Introduction

Severely injured patients suffer from primary traumatic tissue lesions, and secondary inflammatory damage following the traumatic insult ¹. The inflammatory response and the subsequent systemic inflammatory response syndrome (SIRS) are linked with organ dysfunction following the initial trauma and are independent of direct physical harm to the organ ¹. Especially lung contusions and chest traumas represent well-known risk factors for posttraumatic pro-inflammatory effects, which contribute to the development of posttraumatic complications like acute respiratory distress syndrome (ARDS) and increase morbidity and mortality of these patients ². Whether this inflammatory stimulus is sufficient to hamper muscle or diaphragm function is not known.

Otherwise, the diaphragm itself can synthesize inflammatory mediators in response to both disuse and overload ^{3,4}. In the diaphragm these pathways include the nuclear translocation of NFkB as an inductor of cytokine production and the activation of caspases, which cleave compounds of sarcomeric structures ^{5,6}. However, it is unclear if the inflammatory stimulus following traumatization may affect the diaphragm ⁷ while triggering ROS generation additionally to the ongoing proteolytic process induced by disuse (ventilator-induced diaphragm dysfunction (VIDD)). ⁸

We hypothesized that the pronounced inflammatory stimulus after trauma in combination with MV may further activate proteolytic pathways and worsen atrophy in the diaphragm via an increase in ROS production compared to MV alone and to non-ventilated, non-traumatized controls. To test this hypothesis, we used a polytrauma model in pigs undergoing 72 hrs of MV to assess diaphragm fibre dimensions, lipid peroxidation, inflammatory cytokines, and markers of the calcium-dependent proteolytic pathways.

Material and Methods:

This study presents a post hoc-analysis from a large animal porcine multiple trauma model. The model has been previously described in detail by Horst et al.⁹. The study has been approved by the appropriate governmental institution (Landesamt für Natur-, Umwelt- und Verbraucherschutz, LANUV NRW, Germany, reference number: AZ 84-02.04.2014.A265). The experiments were conducted in accordance with the principals of Laboratory Animal Care National Research Council (US) and the Committee for the Update of the Guide for the Care and Use of Laboratory Animals (National Academies Press (US), Washington DC, 2011).

Animal model:

Healthy, 30 ± 5 kg, male German landrace piglets were separated into 3 groups. The first two groups underwent 72 hrs of mechanical ventilation with either polytrauma: *PT* ($n=12$) or a sham operation: *MV* ($n=5$) as previously described by Horst et al.⁹; referred to as interventional groups, while the third non-ventilated group was euthanized without mechanical ventilation and served as control (Con, $n=6$ see Supplemental Figure 1, <http://links.lww.com/SHK/A822>).

Anaesthetic treatment:

For pre-medication animals received an intramuscular injection of 4 mg kg^{-1} azaperone (StresnilTM, Janssen Pharmaceutica, Beerse, Belgium). Anaesthesia was induced by intravenous injection of 3 mg kg^{-1} propofol and afterwards maintained via continuous intravenous infusions of propofol and sufentanil as previously described by Horst et al.⁹. All animals were orally intubated and connected to a ventilator (Evita 4, Draeger, Luebeck, Germany). Continuous intravenous fluid (Sterofundin; $2 \text{ ml kg}^{-1} \text{ h}^{-1}$) was supplied to ensure

normovolaemia and diuresis was monitored via a suprapubic catheter (12Fr. Cystofix, B.Braun, Melsungen, Germany).

All intubated animals were mechanically ventilated with volume-controlled and lung-protective ventilation to ensure normocapnia (end-tidal CO₂ (etCO₂) 35-45 mmHg with a tidal volume of 6-8 ml kg⁻¹ bodyweight⁻¹). Body core temperature was maintained at a physiological range of 38.7 to 39.8 °C.

A three-lumen haemodialysis catheter (12.0 Fr., Arrow Catheter, Teleflex Medical, Wayne, PA, USA) was placed into the right femoral vein. Arterial blood pressure was continuously measured via an arterial line (Vygon, Aachen, Germany), placed in the femoral artery.

After this initial preparation, the animals were randomized to one of the interventional groups (*PT*; *MV*).

Surgical instrumentation:

Polytrauma in the PT group was induced by unilateral femur fracture and blunt chest trauma with lung contusion provoked by a standardized bolt gun shot, as previously described⁹. Subsequently a laparotomy with standardized liver incision and a short period of uncontrolled bleeding before liver packing followed by blood withdrawal (via the central venous line; max. 45% of total blood volume) resulting in a predefined haemorrhagic shock with a systemic mean arterial blood pressure of 40 mmHg. Haemorrhagic shock was maintained for 90 minutes. Animals were not prevented from hypothermia in this initial period to mimic pre-hospital trauma circumstances. After this initial phase a 60 minutes resuscitation was performed according to human trauma guidelines. This resuscitation period was followed by surgical stabilization of the fractured femur.

Animals of the MV group did not receive any injury or haemorrhage, but anaesthesia, mechanical ventilation and all instrumentation (including all catheters) was equivalent to PT group, as described above.

After 72 hrs of mechanical ventilation all interventional (MV and PT) animals were euthanized by a lethal overdose of pentobarbital.

Histological measurements:

Costal diaphragm tissue taken from the non-traumatized diaphragm side was embedded and frozen in liquid butane for histological assessment of muscle fibre dimensions by ATPase assay as described before ¹⁰. Fibre cross-sectional areas were examined using immunofluorescence microscopy (approximately 200 fibres per animal) and analysed using the software ImageJ (v1.46k; National Institute of Health, Bethesda, MA, USA) ¹¹.

Biochemical measurements:

Proteins were extracted from diaphragm samples and total concentration was determined by Bradford assay ¹². Proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). Equal loading and efficient protein transfer were confirmed by Ponceau-S staining. To assess lipid peroxidation and proteolysis, western blots were incubated overnight at 4°C with appropriate primary antibodies (4-HNE: #AB-46545, Abcam, Cambridge, UK; Caspase-3: #C9662, Pro-Caspase-3 #9664 Cell-Signaling, Danvers, USA; USA; P50/P65: #4764 and phospho-P65: #3031, Cell-Signaling, Danvers, MA, USA; Calpain1, Genetex, GTX23589, Irvine, USA), Vinculin (#V9131, Sigma-Aldrich, St. Louis, MO, USA) served as loading control and subsequently with the suitable horseradish-peroxidase conjugated secondary antibody (anti-rabbit, #7074, Cell-Signaling technology, Danvers, MA, USA). To visualize proteins enhanced chemiluminescence was used via peroxidase substrate (ClarityTM Western ECL Blotting Substrate, Bio-Rad Laboratories, Hercules, CA, USA) and analysed using the ImageQuant LAS 4000TM System with the Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

RNA preparation and quantitative real-time PCR (qPCR)

RNA from porcine diaphragm samples were extracted with TRIZOL¹³. 2µg purified RNA was reverse transcribed (Maxima H Minus Kit with dsDNase, #K1682, Thermo Fisher Scientific, Waltham, MA, USA). qPCR was run on a StepOne Plus instrument (LifeTechnologies, Carlsbad, CA, USA) using Power SYBR Green Master Mix (#4368706, Applied Biosystems, Foster City, CA, USA) and primers depicted in Table 1. For messenger RNA (mRNA) analysis, inflammatory and proteolytic marker expression was normalized with ribosomal protein S7. RNA expression was analysed by the $\Delta\Delta C_T$ method¹⁴.

Statistical analysis:

In normally distributed data (Kolmogorov-Smirnov test) a one-way ANOVA with a Dunnett's post-hoc test or in not-normally distributed data the Kruskal-Wallis test followed by a Dunn's post hoc test was performed, as indicated in the results section. Values are displayed as mean $\pm$ SD or median $\pm$ interquartile range. In all cases, a level of $P < 0.05$ was considered as statistically significant. Prism 6.0 (Graphpad Software Inc., La Jolla, CA, USA) was used for data analysis.

Results:

Clinical data

Clinical characteristics of shock severity have been reported elsewhere ⁹. In brief, these data indicated the induction of inflammation by blunt chest trauma and the concomitant trauma with significant systemic increases of IL-6 in the trauma group (PT) compared to the non-trauma group (MV) and lung damage.

Histological measurements

Diaphragm type I and II fibre dimensions were significantly reduced to the same extent (-47%) in the PT group ($p=0.0041$ for type I fibres; $p=0.0029$ for type II fibres) compared to Con (See Figure 1). The fibre atrophy in MV group for type I (-29% vs con, $p=0.06$) and type II (-40% vs Con, $p=0.07$) fibres was not significant compared to Con and did not differ from the PT group.

Proteolysis and lipid peroxidation

The mRNA levels of Caspase-3 were significantly and similarly elevated by factor 6 in MV ($p=0.0193$) and factor 6.7 in PT ($p=0.0362$) compared to Con (Figure 2, panel A). Similar results were found at the protein levels where the ratio of active cleaved Caspase-3/Pro-Caspase-3 increased in both intervention groups as a result of enhanced active Caspase-3 protein expression (Figure 2, panel B). The level of calpain-1 protein expression, was enhanced in the PT group compared to Con ($p=0.016$, factor 1.5) but not in the MV group. Compared to Con, lipid peroxidation (assessed via 4-hydroxynonenal; 4-HNE) was significantly and similarly increased in both groups versus Con (PT $p=0.04$; MV $p=0.02$) (Figure 3, Panel A).

Inflammatory signalling via NFkB

Protein expression of the transcription factor NFkB subunit p65, was up-regulated in the PT group (factor 2.9, $p=0.0132$) but not in the MV group compared to Con (Figure 3, panel B), while p50 protein levels remained unchanged in both intervention groups (data not shown).

Interleukin (IL) mRNA expression in the diaphragm

The mRNA level of IL-6, was increased significantly in the PT group only compared to Con (factor 8.5, $p=0.0020$, Figure 4, Panel A), but did not differ from MV. The slight enhanced TNF-alpha mRNA levels in both intervention groups failed to reach statistical significance (Figure 4, panel B). Importantly, the expression of the mRNA coding for IL-1beta upstream to IL-6 was also significantly elevated in PT compared to Con (factor 3.5, $p=0.0273$) (Figure 4, panel C). Consistent with the modulatory function of cytokines, anti-inflammatory cytokine IL-10 mRNA levels were significantly increased in PT compared to Con (factor 4.7 $p=0.0108$) (Figure 4, panel D) and not significantly in MV group compared to Con.

Discussion:

General discussion of the main findings

The data of this study show that polytraumatization and mechanical ventilation led to diaphragmatic atrophy after 72 hrs of mechanical ventilation with a concomitant increase in proteolytic enzyme activity and lipid peroxidation. A significant increase in diaphragm inflammatory response was solely observed in the PT group. This was accompanied by a significant activation of transcription factor NFkB sub-unit P65.

The findings are discussed in detail below:

Impact of polytraumatization and MV on fibre atrophy

72 hrs of mechanical ventilation led to diaphragmatic atrophy of both type I and II fibres although this effect was not significant in the MV group compared to Con. However, the reduction in fibre size by around 30% in type I fibres and 40% in type II fibres in the MV group was impressive and should not be neglected. Our data are in agreement with those reported by Jung and colleagues in piglets in which a reduction of fibre size of around 30-40% in both fibre types was present after 72 hrs of MV compared to supported spontaneous breathing¹⁵. In our study, the PT group also showed significant diaphragm atrophy compared to Con, but this decrease in fibre size was however, not statistically different from that observed in the MV group. Polytraumatization therefore did not induce further diaphragm atrophy as hypothesized.

Both trauma and mechanical ventilation activate proteolytic pathways

Our data reveal an increase in lipid peroxidation and activation of the major protease Caspase-3 in both interventional groups compared to Con, but with no additional increase in the PT group compared to MV alone.

It is well established that oxidative stress is stimulated in both ventilator-induced diaphragm dysfunction and inflammatory mediated processes ^{16,17}. Oxidative stress can induce the proteolytic pathway namely Caspase-3 ¹⁸ and Calpain-1 ¹⁹ and increase the susceptibility of muscle proteins to its cleavage by proteases ^{18,20}.

Besides the oxidative trigger, Caspase-3 activation is linked to inflammatory stimuli and several intrinsic or extrinsic pathways ^{19,21,18}. While the animals in the PT group had to face both disuse and inflammatory stimuli, no additional increase in oxidative stress or Caspase-3 activation was detected in the diaphragm pointing to the possibility that polytrauma did not increase these pathways of trauma-linked inflammation.

However, the levels of Calpain-1 protein were significantly increased in the PT group, but in the MV group the increase was modest and failed to reach statistical significance. Calpain-1 is a powerful proteolytic enzyme able to cleave the sarcomeric proteins for subsequent degradation by the ubiquitin proteasome pathway. It is expected to result in muscle atrophy. Interestingly, Caspase-3 and Calpain-1 function in a cross-linked manner ^{19,22}.

However, these did not result in more fibre atrophy in the PT group compared to the MV group. This suggests that changes induced by diaphragm inactivity are likely to be the major trigger in both interventional groups with no additional effect of traumatic inflammatory stimuli.

Polytrauma induces inflammatory response in the diaphragm

Increases in inflammatory cytokines have been described during MV ⁴ and also when combined with sepsis ¹⁶. In addition, Smuder and colleagues revealed the importance of oxidative stress triggered by NFkB elevation during MV ²³. In addition, the importance of cytokine release for the pathogenesis of VIDD remains unclear. In the current study, pro-inflammatory cytokines IL-6, IL-1beta and the anti-inflammatory cytokine IL-10 as well as the activation of NFkB p65 subunit was solely observed in the PT group while no changes

were found in the MV group. Thus, despite enhanced circulatory IL-6 as previously reported in this polytrauma model ⁹ and elevated cytokine expression in the diaphragm of these animals, no further increase in lipid peroxidation or activation of caspase activation was found in the diaphragm of PT versus MV. These data suggest that the inflammatory stimulus did not further impact these pathways. This is in agreement with the data of Maes and colleagues describing comparable results in their model of mechanical ventilation in septic animals ¹⁶: the inflammation caused by LPS administration to induce sepsis did not cause an additional increase in lipid peroxidation or protease activation in the diaphragm compared to MV alone, although circulating cytokines were obviously present. Our data suggest that impact of inflammation is less compared to that caused by disuse atrophy with MV. The inflammatory stimulus, at least in the magnitude of our chosen model, did not increase diaphragm proteolysis, oxidation or atrophy, contrarily to our hypothesis.

Limitation of the model

We used a complex animal model of PT including 72 hrs of mechanical ventilation that itself may imply the major impact to the diaphragm in this model. During this time period, different effects might have influenced the diaphragm until the time point at which the samples were collected. However, this complexity of damaging events represents the reality in the clinical setting where a pure VIDD (i.e. a single damage by inactivity) is seldom. This variety of influences has been termed ICU-acquired-diaphragmatic weakness; our model may exclude the entity “polytrauma” to this terminology. To distinguish between the inflammatory response of traumatization and MV, a control group lacking mechanical ventilation had been ideal-nevertheless the caused trauma made anesthesia (and subsequent ventilation) necessary for animal care reasons and to uphold gas exchange.

Even if the proteolytic and inflammatory response in pigs may vary compared to humans, the effects of MV or other stimuli on the diaphragm have been demonstrated to be more or minor

equal between the species²⁴⁻²⁶. We did not investigate the cellular immune response (i.e. neutrophil or macrophage invasion) in our experiment, because we are convinced that these data would not change the conclusion of the current study.

We have to acknowledge that the used standardized liver incision and the concomitant standardized bleeding do not completely represent the diversity of blunt traumatic liver lacerations in severe multiple trauma patients, but this standardized procedure was essential to enable a comparable and reproduceable level of traumatization in all animals.

It is important to mention that in this study, we were not interested in the early effects of polytrauma on the diaphragm or in the time course of these effects. The time point of 72h was actually chosen as time point measure because previous literature indicated the presence of VIDD in the piglet model after 72h of mechanical ventilation. This condition was necessary to address whether polytrauma would worsen the effect of mechanical ventilation on the diaphragm.

Conclusion

We show that polytraumatization induces an inflammatory response in the diaphragm but this did not lead to an additional activation of proteolytic pathways or oxidative modifications compared to MV alone. Our data underline that additional inflammation caused by traumatization did not severely impede the diaphragm and disuse caused by MV was the major major insult to the diaphragm in this experiment rather than polytrauma.

ACCEPTED

Details of authors contributions

T.B.:	Study design, Data collection, data analysis, writing of the manuscript, review of the manuscript
C.S.B.:	Study Design, Data collection, data analysis, writing of the manuscript, review of the manuscript
K.H.:	Data collection, data analysis, writing of the manuscript, review of the manuscript
C.T.:	Data collection, data analysis, writing of the manuscript, review of the manuscript
F.H.:	Data analysis, writing of the manuscript, review of the manuscript
S.L.:	Data analysis, writing of the manuscript, review of the manuscript
T.S.:	Data analysis, writing of the manuscript, review of the manuscript
N.S.:	Data analysis, writing of the manuscript, review of the manuscript
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L.M.:	Data analysis, writing of the manuscript, review of the manuscript
D.H.O.:	Data analysis, writing of the manuscript, review of the manuscript
G.M.:	Data analysis, writing of the manuscript, review of the manuscript
T.-P. S.:	Study design, data collection, data analysis, writing of the manuscript, review of the manuscript

All authors have read and approved the final version of the manuscript.

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Abbreviations list:

4-HNE	diaphragmatic 4-hydroxynonenal
ARDS	acute respiratory distress syndrome
Con	control group
etCO₂	endtidal carbon dioxide
hrs	hours
ICU	intensive care unit
IDV	integrated density value
LANUV	Landesamt für Natur-, Umwelt- und Verbraucherschutz
mRNA	messenger RNA
MV	mechanical ventilation; non-traumatized mechanically ventilated group
PT	polytrauma group
PVDF	polyvinylidene fluoride
qPCR	quantitative real-time PCR
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SDS	sodium dodecyl sulfate
VIDD	ventilator-induced diaphragmatic dysfunction

Guarantor statement

Thomas Breuer is guarantor of the content of the manuscript, including data and analysis.

Table1:

The following primers were used:

TNF-alpha for	CCACGTTGTAGCCAATGTC
TNF-alpha rev	GAGTAGATGAGGTACAGCCC
IL 6 for	GGTTCAATCAGGAGACCTGC
IL 6 rev	GCTTTGTCTGGATTCTTTCCC
IL-10 for	GCATCCACTTCCCAACCAG
IL-10 rev	ACCCAGGTAACCCTTAAAGTC
Casp3 for	TCATAATTCAGGCCTGCCGA
Casp3 rev	GCTGCACAAAGTGACTGGA
IL-1beta1 for	TGAAGAAGAGCCCATCATCCT
IL-1beta1 rev	TCATGCAGAACACCACTTCTC
S7 for	AAATAAGCAGAAGCGTCCCA
S7 rev	AATGTTTCAACCTTGTGCTCC

Figure 1: Diaphragmatic cross sectional areas (CSA) in μm^2 of type I and type II fibers White bar: Control group (Con; n=6); grey bar: MV group (MV; n=6); Black bar: Polytraumatized group (PT; n=8). Values are displayed as mean $\pm$ SD. * indicates $p < 0.05$.

Figure 2: *Panel A:* Relative mRNA expression of Caspase-3 normalized to ribosomal protein S7. White bar: Control group (Con; n=5); grey bar: MV group (MV; n=5); Black bar: Polytraumatized group (PT; n=11). *Panel B:* Diaphragm levels of Caspase-3 expressed as the ratio of Caspase-3/Procaspase 3 and Vinculin detected via Western blot technique. White bar: Control group (Con; n=5); grey bar: MV group (MV; n=5); Black bar: Polytraumatized group (PT; n=10). *Panel C:* Assessment of calpain-1 activity as marker of protein breakdown. White bar: Control group (Con; n=6); grey bar: MV group (MV; n=5); Black bar: Polytraumatized group (PT; n=11). All Values are displayed as median $\pm$ interquartile range. Western blot data presented as integrated density value (IDV), mRNA as fold increase to control.

Figure 3: *Panel A:* Diaphragm lipid peroxidation assessed by 4- hydroxynonenal (4-HNE) bands (100, 60, 50, 37kDa) , relative to the global amount of Vinculin. *Panel B:* Diaphragm levels of activated P65 expressed as the ratio of P65 to Vinculin. White bar: Control group (Con; n=6); grey bar: MV group (MV; n=5); Black bar: Polytraumatized group (PT; n=11). Values are displayed as means $\pm$ SD. All data presented as integrated density value (IDV).

Figure 4: *Panel A:* Relative mRNA expression of IL-6 normalized to ribosomal protein S7. *Panel B:* Relative mRNA expression of TNF-alpha normalized to ribosomal protein S7. *Panel C:* Relative mRNA expression of IL-1 β normalized to ribosomal protein S7. *Panel D:* Relative mRNA expression of IL-10 normalized to ribosomal protein S7. White bar: Control group (Con; n=5); grey bar: MV group (MV; n=5); Black bar: Polytraumatized group (PT; n=11). Values are displayed as means $\pm$ SD.