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Effect of High Pressure-Volume and Low Pressure-Volume Mechanical Ventilation on Plasma Concentrations of Inflammatory Markers in Horses during General Anaesthesia

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Abstract

Systemic changes of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), neutrophil elastase (ELT) and myeloperoxidase (MPO) during mechanical ventilation (MV) in horses anaesthetized for surgery are evaluated. Thirty-four client-owned ASA I-II horses randomly received mechanical ventilation (MV) with either a peak inspiratory pressure (PIP) of 30 cm H_2O and tidal volume (VT)>10 mL kg^{-1} (high pressure-volume MV) or PIP of 15 cm H_2O and VT ≤ 10 mL kg^{-1} (low pressure-volume MV) in dorsal or lateral recumbency. Horses were premedicated with acepromazine (0.1 mg kg^{-1} IM) and xylazine (0.6 mg kg^{-1} IV), induced with midazolam (0.06 mg kg^{-1} IV) and ketamine (2.2 mg kg^{-1} IV) and maintained with isoflurane in oxygen 70% plus ketamine-midazolam infusion (1 and 0.02 mg kg^{-1} h^{-1}, respectively). Anti-inflammatory drug and antibiotics were administered before surgery. Plasmatic pro-inflammatory mediator concentrations were estimated by ELISA at the beginning (T_0) and after 60 minutes (T_1) of MV. Mean plasmatic TNF-α, MPO, and ELT concentrations at T_1 were significantly ($p<0.05$) lower than T_0. Mean plasmatic concentration of IL-6 did not significantly change with time. The reduction in plasmatic concentration of pro-inflammatory mediators at T_1 was not linked to ventilation strategy or recumbency. None of the ventilation protocols enhanced systemic inflammatory response during surgery after 1 hour of MV. The anti-inflammatory properties of drugs included in the anaesthesia protocol may have contributed to the overall decreased systemic inflammatory mediator concentrations, despite MV and surgery.

Keywords: Horses; Anaesthesia; Mechanical ventilation; Ventilator-induced lung injury; Biotrauma

Introduction

Mechanical ventilation (MV) may be responsible for lung damage, a phenomenon referred as ventilator-induced lung injury (VILI). The potential for high airway pressure and resulting alveolar overdistention to cause or extend VILI has been extensively documented [1-3]. Further, repeated airway closure and reopening takes place during the tidal cycle when insufficient positive end-expiratory pressure (PEEP) is applied and has been associated with intense shearing forces [4]. These observations are alerting because airway pressure of 20-35 cm H_2O are often applied during MV in anesthetized horses [5] and most of large animal ventilators do not allow the setting of a PEEP. Irrespective of mechanical rupture, tissue stretch caused by MV may induce the release of mediators triggering an inflammatory based injury, called biotrauma [6]. It has been shown that this pulmonary inflammatory response may be activated by MV even without preexisting lung disease [7]. Experimental studies using normal lungs have shown a systemic cytokine response to injurious MV through a mechano-transduction mechanism and a loss of alveolar compartmentalization [6,8,9]. Conventional ventilation also enhances the systemic inflammatory response after major surgical procedures (Michelet et al. 2006). Systemically spread of pulmonary inflammation may possibly delay or preclude the resolution of systemic inflammatory process [10]. Neutrophils play a critical role in biotrauma as manifestations of VILI were almost completely abrogated in neutophil-depleted rabbits [11]. Neutrophil elastase (ELT) is implicated in inflammatory tissue damage [12,13] and it has been shown to cause or amplify acute and chronic lung injury [14-16]. Neutrophil myeloperoxidase (MPO) production, which promotes tissue damage in numerous inflammatory diseases [17] increases after high volume ventilation in rats, mice and humans [18,19]. *In vivo* high tidal volume (VT) and peak inspiratory pressure (PIP) ventilation of healthy lungs is associated with increased levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in broncho-alveolar lavage (BAL) [7,20]. During oesophagectomy in healthy humans, conventional MV induced a persistent elevation in plasmatic concentration of IL-6 compared to protective ventilation with reduced VT and additional PEEP [10]. Besides its beneficial effects, PEEP is known to exacerbate the reduction in cardiac output [21]. This is largely mediated by a reduction in venous return, stroke volume, and blood pressure associated with positive pressure MV, and should be taken into account whenever PEEP is applied. Increased levels of both IL-6 and TNF-α were found in isolated perfused murine lungs [22] and after *in vivo* ventilation of healthy rats with 20 mL kg^{-1} of VT [23]. To our knowledge there are no studies documenting the relation between plasmatic level of pro-inflammatory mediators and ventilation strategies in horses. We investigated the plasmatic change of IL-6, TNF-α, MPO and ELT during MV in systemically healthy horses anesthetized for surgery. The aim of the study was to investigate if conventional MV acts as priming insult to trigger an inflammatory

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response in healthy lung. This in turn could increase the systemic pro-inflammatory cytokine response associated with surgery.

Materials and Methods

Thirty-four client-owned horses referred to the Animal Hospital of University of Liège for soft tissue or orthopedic surgeries lasting more than 45 minutes were included in the study. The study was approved by the Committee for the ethical use of animals of the University of Liège and owners gave consent for the use of the data of their animals in this study. The inclusion criteria during this study were as follows: ASA I-II horses, of all sexes, of all breeds, aged 2-19 years old, of more than 250 kg body weight. Horses were assigned a physical status category according to the American Society of Anaesthesiologists guidelines, based on physical examination and the hematological and biochemical analysis of a venous blood sample. A 14 gauge (GA) catheter (Intraflow, Vygon, Belgium) was placed in the jugular vein for subsequent drugs administration. Antibiotic therapy was administered intravenous (IV) preoperatively and either flunixine meglumine (Emdofluxine, Emdoka, Antwerp, Belgium) at a dose of 1.1 mg kg^{-1} or phenylbutazone (Fenylbutazon, VMD, Arendonk, Belgium) at a dose of 2.2 mg kg^{-1} was given IV before the beginning of anaesthesia. Horses received acepromazine (Placivet, Codifar, Wommelgem, Belgium) 0.1 mg kg^{-1} intramuscular (IM) one hour prior to induction of anaesthesia. Anaesthesia was induced 10 minutes after the administration of xylazine (Proxylaz 2%, Prodivet pharmaceuticals, Belgium) 0.6 mg kg^{-1} IV by intravenous injection of ketamine (Anesketin, Eurovet, Belgium) 2.2 mg kg^{-1} IV and midazolam (Dormicum, Roche, Belgium) 0.06 mg kg^{-1} IV. After induction of anesthesia the horses were intubated orotracheally and positioned in dorsal or lateral recumbency according to the requirement of the surgical technique. Additional boluses of ketamine (0.2-0.5 mg kg^{-1} IV) were given if required to permit orotracheal intubation. Anaesthesia was maintained with isoflurane (IsoFlo, Abbott, Belgium) in a mixture of air and oxygen (inspiratory fraction of oxygen of 70%) at low flow rate using a rebreathing circuit system (DRE Titan Large Animal Anaesthesia Machine, DRE Medical, Inc. Louisville, KY, USA). Within 10 minutes of induction of general anaesthesia isoflurane vaporizer (Dräger 19.1 Isoflurane Vaporizer, Lübeck, Germany) was turned on and a constant rate infusion (CRI) of ketamine and midazolam was started. Ketamine and midazolam were administered at rates of 1 and 0.02 mg kg^{-1} $hour^{-1}$, respectively, using a peristaltic infusion pump (MPIV300 Infusion Pump, Millpledge, UK). CRI was discontinued 20 minutes before the recovery phase. Intermittent positive pressure ventilation (IPPV) started within 10 minutes of induction of general anaesthesia. Surgery started within 25 minutes of the beginning of the maintenance phase. Lactated Ringer's solution was infused at a rate of 10 mL kg^{-1} $hour^{-1}$ during anaesthesia and inotropic support provided with dobutamine (Dobutrex mylan, Mylan, Belgium) to maintain a mean arterial blood pressure (MAP) ≥ 70 mmHg. A 20 or 22 GA catheter (BD Insyte-W, Vialon biomaterial, Belgium) was placed in the facial, transverse facial or metatarsal artery for measurement of systemic arterial blood pressure and for arterial blood samples collection. Arterial blood samples were collected 20 minutes after the beginning of MV and every 20 minutes then. The arterial partial pressure of carbon dioxide ($PaCO_2$), arterial partial pressure of oxygen (PaO_2), alveolar partial pressure of oxygen (PAO_2), alveolar-arterial oxygen gradient ($AaDO_2$), pH, base excess (BE), bicarbonate (HCO_3) and saturation of hemoglobin with oxygen (SpO_2%) were either measured or calculated by the inbuilt software of the blood-gas analyzer (AVL-OMNI blood gas analyser, Roche, France). A base-apex lead electrocardiogram was used to monitor heart rate (HR) and rhythm. Respiratory gas was collected continuously from the circuit at the end of the orotracheal tube, and analyzed by infrared absorption. The end-tidal concentration of CO_2 ($ETCO_2$) and isoflurane (ETISO), the fraction of inspired (FiO_2) and expired oxygen (FeO_2), SpO_2, respiratory rate (RR), HR, and MAP were displayed continuously by an anesthesia monitoring system (Datex Ohmeda S/5™ Anesthesia Monitor, Weregem, Belgium) and recorded every 5 minutes, as well as the PIP, VT and the inspiratory to expiratory time ratio (I:E). Anaesthetic depth was adjusted by altering the inspired isoflurane concentration according to assessment of palpebral and corneal reflex, eyeball position, nystagmus, muscle contraction, swallowing, shivering, stretching, spontaneous movements and cardiovascular status against surgical stimuli. Additional doses of ketamine (0.2-0.5 mg kg^{-1} IV) were administered to maintain a surgical plane of anaesthesia, if necessary. At the end of the surgery isoflurane vaporizer was turned off, RR decreased and IPPV discontinued when spontaneous ventilation resumed. During the recovery period additional bolus of 0.2 mg kg^{-1} of xylazine IV were given.

Ventilation protocol

IPPV was started within 10 minutes of induction of anaesthesia and discontinued at the end of the surgery. MV was provided by a volume targeted, time cycled, double-circuit large animal ventilator fully integrated into the anesthesia machines and designed for use in continuous mandatory ventilation mode (Dräger AVE ventilator, Dräger Medical, Lübeck, Germany). Horses were randomly assigned to receive one of the two protocols of MV using either a PIP of 30 cm H_2O and a VT>10 mL kg^{-1} (high pressure-volume MV) or a PIP of 15 cm H_2O and VT ≤ 10 mL kg^{-1} (low pressure-volume MV). Randomization was realized by computer-generator codes (http://www.randomization.com). Horses received either low pressure-volume MV in dorsal (15D) or lateral (15L) recumbency or high pressure-volume MV in dorsal (30D) or lateral (30L) recumbency. I:E was set at 1:2-1:3 and RR at 4-15 breaths/minute to achieve the desired PIP and VT. Horses showing intra-operative $PaCO_2$ of <30 mmHg or >60 mmHg or a PaO_2 < 80 mmHg, were ventilated conventionally and not included in the study.

Measurements

Plasmatic equine IL-6, TNF-α, MPO and ELT concentrations were measured using enzyme linked immunosorbent assay (ELISA) validated by our group (MPO and ELT) or commercialized (IL-6 and TNF-α) for the analysis of equine samples. After connecting the horse to the circle system and while under spontaneous breathing the first venous blood sample (T_0) was collected in EDTA tube from the catheter in the jugular vein. Thereafter IPPV was immediately started. A second venous sample was then collected from the catheter in the jugular vein 1 hour after the institution of MV (T_1), while horses were still anesthetized with isoflurane and ketamine-midazolam CRI. Plasma was separated by centrifugation of the blood at 1600 g for 3 minutes at room temperature and stored at -20°C within 30 minutes of collection until analyses. Plasma samples were thawed prior to assays. All the samples were analyzed within one month after sampling.

Sandwich ELISA for equine myeloperoxidase (MPO)

Concentration of MPO was determined by a commercially available ELISA (Equine MPO ELISA kit, BiopTis, Belgium) as described previously by Franck et al. [24]. EDTA plasma samples were diluted 40 times. Each sample was assayed twice and the absorbance values were read at 405 nm with the Multiscan Ascent plate reader (Fisher Scientific, Belgium). Concentrations were calculated in reference to the

calibration curve furnished into the kit. The lower limit of detection of the test was 0.50 ng/ml.

Sandwich ELISA for equine elastase (ELT)

A specific ELISA for equine neutrophil ELT (Equine ELT ELISA kit, BiopTis) was used as described previously by De la Rebière de Pouyade et al. [25]. EDTA plasma samples were diluted 6 times. Each sample was assayed twice and the absorbance values were read at 405 nm with the Multiscan Ascent plate reader (Fisher Scientific, Belgium). Concentrations were calculated in reference to the calibration curve furnished by the kit. The lower limit of detection of the test was 0.56 ng/ml.

Sandwich ELISA for equine TNF-α

Plasma TNF-α concentration was determined by a sandwich ELISA kit designed and validated specifically for equine samples (Equine TNF-α screening set, Thermo Scientific). Calibration curves and non-diluted EDTA-plasma samples were incubated 1 hour at 22°C. Microplate wells coated with specific antibodies against natural and recombinant TNF-α were filled with 100 µl TNF-α standards ranging from 15.6 to 1000 pg/ml and EDTA undiluted samples then incubated 1 h at 22°C. After washings, a biotin-conjugated antibody was added into the well and incubated 1 h at 22°C and, after another washing step, the streptavidin conjugated to horse radish peroxidase (HRP) was added and incubated 30 min at 22°C. After a final washing step, the peroxidase activity was detected by incubation (20 min, 37°C, in the dark) with the substrate tetramethylbenzidine (TMB). The reaction was stopped with sulfuric acid solution (0.16 M) and the absorbance of the yellow color was read at 450 nm with the Multiscan Ascent plate reader. Each sample was assayed twice and the mean value was calculated. The lower detection limit of the test was 5 pg/ml.

Sandwich ELISA for equine IL-6

Plasma IL-6 concentration was determined by a sandwich ELISA kit designed and validated specifically for equine samples (ELISA kit for IL-6 from Equus caballus, USCN, Life Science Inc.). A monoclonal antibody specific to equine IL-6 was pre-coated onto microplate wells (NuncMaxiSorp) Cliniplate EB). IL-6 standards ranging from 31.2-2000 pg/mL and EDTA undiluted samples were added (100 µl) into the wells and microplates were incubated 2 h at 37°C. After washings a biotin-conjugated polyclonal antibody was added into the well and incubated 1 h at 37°C and, after another washing step, the streptavidin HRP conjugate was added and incubated 30 min at 37°C. After a final washing step, the peroxidase activity was detected by incubation (20 min, 37°C, in the dark) with the TMB-peroxidase substrate. The reaction was stopped with sulfuric acid solution and the absorbance of the yellow color was read at 450 nm with the Multiscan Ascent plate reader. Each sample was assayed twice and the mean value was calculated. According to the firm, the minimum detectable dose of equine IL-6 is typically less than 14.4 pg/ml.

Statistical analysis

The effects of high pressure-volume and low pressure-volume ventilation were compared in both dorsal and lateral recumbency at T0 and T1. All data were analyzed using SAS commercial software (Statistical Analysis System, SAS Institute GmbH Heidelberg, Germany). After normalization of the data (when necessary), a linear mixed model was used to describe the experiment with a first-order autoregressive structure for the repeated measurements. Fixed effects were for time, recumbency and pressure and their 2-by-2 interactions, anti-inflammatory drugs and antibiotics as main effects, and weight as a covariate. Random effects included horse and repeated measures within horses. The same model was applied to all variables with the exception of the repeated part when the variable was measured once. Analyzed variables were: IL-6, TNF-α, MPO and ELT plasmatic concentration, RR, VT, minute volume, $ETCO_2$, ETISO, HR, MAP, PaO_2, $PaCO_2$, SpO_2. Demographic characteristics and preoperative parameters were compared with one-way analysis of variance (ANOVA) and validated via Bonferroni test. Significance was set at $p<0.05$.

Animals

34 horses were randomized and analyzed. Five horses were included in the group 15D, 11 in 15L, 9 in 30D and 9 in 30L. The horses were a mixed population consisting of 11 mares, 13 geldings, 10 stallions, aged 2-19 years old (median 11 years) and having a body mass of 510 ± 88 kg [mean ± standard deviation (SD)]. Demographic characteristics and preoperative parameters did not differ between groups except for weight (p=0.001), haematocrit (p=0.027) and the number of red blood cells (p=0.002). Details of surgical interventions and preoperative antibiotic and NSAIDs therapy are shown in Table 1. All horses were ventilated for at least 60 minutes and recovered without any problem from anaesthesia.

Group		**15 D**		**30 D**		**15 L**		**30 L**
Number of animals		**5**		**9**		**11**		**9**
Surgery	n=2 n=1 n=1 n=1	castration wound debridment sarcoid tooth extraction	n=1 n=6 n=2	arthroscopy castration castration + sarcoid	n=1 n=3 n=1 n=1 n=1 n=2 n=2	eyelied carcinoma enucleation foot abscess curetting teeth extraction osteotomy sarcoid wound suture	n=1 n=1 n=2 n=1 n=1 n=1 n=1 n=1	abscess curetting arthroscopy enucleation laryngeal tie-forward osteotomy prosthetic eye removal screw's removal wound suture+articular flush
Antibiotic therapy	n=3 n=2	cefquinome penicillin g procaine	n=6 n=3	cefquinome penicillin g procaine	n=5 n=4 n=1 n=1	cefquinome penicillin g procaine marbofloxacin trimethoprim/sulfadiazine	n=7 n=2	cefquinome penicillin-gentamicin
NSAIDs therapy	n=5	flunixin meglumine	n=7 n=2	flunixin meglumine phenylbutazone	n=8 n=3	flunixin meglumine phenylbutazone	n=8 n=1	flunixin meglumine phenylbutazone

15D=low pressure-low volume MV in dorsal recumbency; 30D=high pressure-high volume MV in dorsal recumbency; 15L=low pressure-low volume MV in lateral recumbency; 30L=high pressure-high volume MV in lateral recumbency; n=number of animals; NSAIDs=non-steroidal anti-inflammatory drugs

Table 1: Surgical interventions and preoperative antibiotic and NSAIDs therapy.

Results

Respiratory, hemodynamic and pulmonary variables

All the respiratory, hemodynamic and pulmonary variables at T_0 and T_1 are displayed in Table 2. RR, $PaCO_2$ and $ETCO_2$ were significantly higher in low pressure-volume than in high pressure-volume MV, irrespective of recumbency. The interaction of time and pressure had a significant effect on $ETCO_2$ which was significantly lower at T_1 than T_0. VT did not change with time, but was significantly higher with high pressure-volume MV and in dorsal than in lateral recumbency. The minute volume was significantly higher in dorsal than in lateral recumbency, and did not vary with PIP and time. PaO_2 was only evaluated at T_1 and significantly varied with weight only. MAP was significantly higher at T_1 compared to T_0. The interaction of time and pressure had a significant effect on HR and ETISO.

Plasmatic levels of IL-6, TNF-α, MPO and ELT

On 40 assays performed in duplicate, the means of coefficient of variation (CV) were 18.4 ± 21.3% for IL-6, 6.1 ± 8.1% for TNF-α, 3.2 ± 2.6 for MPO and 8.7 ± 11.1% for elastase. The means CV values for cytokine assays were inferior to 10% except for IL-6. While the kits for TNF-α, MPO and ELT measurements have already been validated for the use in horses [24-26], it seems that the USCN kit for IL-6 measurement has not been validated in equine research. Plasmatic levels of IL-6, TNF-α, MPO and ELT at T_0 and T_1 are shown in figures 1 and 2. There was a high variation in plasma concentration of these inflammatory mediators among horses at both T_0 and T_1. The range of the plasmatic levels recorded in the 34 horses at T0 were as follow: IL-6: 0-961.24 pg/mL (median 26.32 pg/mL); TNF-α: 0-2017.08 pg/mL (median 163.94 pg/mL); MPO: 51.69-1133.25 ng/mL (median 238.29 ng/ mL); ELT: 3.68-203.13 ng/mL (median 42.36 ng/mL).

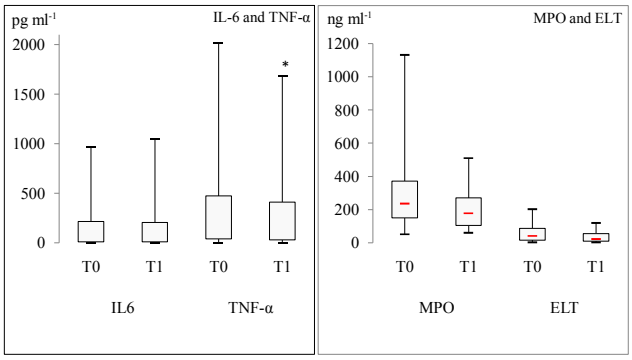
At T_0, 9 of 34 horses [4 of 11 horses in group 15L; 2 of 9 in 30D, 2 of 9 in 30L and 1 of 5 in 15D] had MPO concentrations above the cutoff value of 352.7 ng/mL determined to distinguish between healthy and pathologic horses [24]. While, 22 of 34 horses [7 horses in 15L (63.6%); 5 in 30D (100%), 30L (55.5%) and 15D (55.5%)] had ELT concentrations above the cutoff value of 35.9 ng/mL as determined between healthy and pathologic patients [25]. For the IL-6 and TNF-α assay, we did not found in the literature cutoff values to distinguish between healthy and pathologic horses.

At T1, the range of plasmatic levels were as follow: IL-6: 0-1050.54 pg/mL (median 18.99 pg/mL); TNF-α: 0-1682.10 pg/mL (median 141.01 pg/mL); MPO: 60.79-510.55 ng/mL (median 179.47 ng/mL); ELT: 3.39-119.40 ng/mL (median 23.06 ng/mL). At T1, 4 of 34 horses [1 of 11 horses in group 15L, 1 of 9 in 30D, 1 of 9 in 30L, and 1 of 5 in 15D] and 16 of 34 horses [4 of 11 horses in 15L, 3 of 9 in 30D, 3 of 9 in 30L, and 3 of 5 15D] showed respectively MPO and ELT concentrations superior to the corresponding cutoff values. MPO and ELT concentrations were never below the assay detection limit. Plasmatic levels of IL-6 were undetectable in 1 of 34 horses (30D) at T0 and in 3 of 34 horses (1 horse in 30D; 1 in 30L; and 1 in 15D) at T1.

Group		**15D**	**30D**	**15L**	**30L**
Number of animals		**5**	**9**	**11**	**9**
RR (breaths/minute)	T_0 T_1	10.2 ± 3.2†‡ 11.3 ± 2.3†‡	5.9 ± 1.3 5.3 ± 0.7	9.4 ± 2.6†‡ 10.1 ± 2.5†‡	5.2 ± 0.8 5.1 ± 0.5
VT (ml/kg)	T_0 T_1	9.2 ± 2.3†‡¤ 8.5 ± 2†‡¤	16.6 ± 2.8‡¤# 16.6 ± 2‡¤#	8 ± 1.8†‡# 8 ± 1.8†‡#	14.2 ± 2.3†¤# 15.4 ± 2.8†¤#
Minute Volume (RR x VT)	T_0 T_1	94.4 ± 36.4‡¤ 93.7 ± 19.3‡¤	96.1 ± 20.6‡¤ 86 ± 8.5‡¤	73.4 ± 19.9 80.1 ± 22.6	72.2 ± 13 77.3 ± 14.9
$ETCO_2$ (mmHg)	T0 T_1	43.2 ± 11.4* 40.5 ± 4.8	35.6 ± 4.5* 29.5 ± 3.4	44.4 ± 9.6* 41.8 ± 6.6	33 ± 3.2* 29.1 ± 3.3
ETISO (%)	T_0 T_1	1.3 ± 0.3 1.2 ± 0.3	1.1 ± 0.2 1.1 ± 0.1	1.1 ± 0.1 1.1 ± 0.1	1 ± 0.2 1.1 ± 0.1
HR (beats/minute)	T_0 T_1	40.6 ± 9.6* 41.8 ± 7.1	39.2 ± 4.8* 40.2 ± 3.8	34.4 ± 5.6* 40.2 ± 5.9	36.2 ± 7.5* 39.7 ± 8.6
MAP (mmHg)	T_0 T_1	69.2 ± 21.2* 75.5 ± 16.1	73.4 ± 17.5* 84.7 ± 6.8	77.8 ± 7.9* 84.1 ± 10.3	80 ± 15.8* 86 ± 22.8
PaO_2 (kPa)	T_0 T_1	np 22.8 ± 8.7	np 28.7±17.4	np 31 ± 9.4	np 32.2 ± 6.5
$PaCO_2$ (kPa)	T_0 T_1	np 7.1 ± 0.8	np 5.2 ± 0.4	np 6.8 ± 1	np 5.2 ± 1
SpO_2 (%)	T_0 T_1	np 98.9 ± 1.2	np 98.5 ± 2.7	np 99.7 ± 0.3	np 99.8 ± 0.1
PAO_2 (kPa)	T_0 T_1	np 61±7.7	np 66.6±8	np 62.1 ± 6	np 62.7 ± 5.6
BE	T_0 T_1	np 3.75 (0.5-6.8)§	np 3.25 ± 0.82	np 5.2 ± 1.7	np 4.13 ± 1.1
HCO_3^-	T_0 T_1	np 30.3 ± 3.3	np 27.3 ± 1.2	np 30.7 ± 2.8	np 24.6 ± 5.7
pH	T_0 T_1	np 7.4 ± 0.0	np 7.5 ± 0.0	np 7.4 ± 0.0	np 7.4 ± 0.1

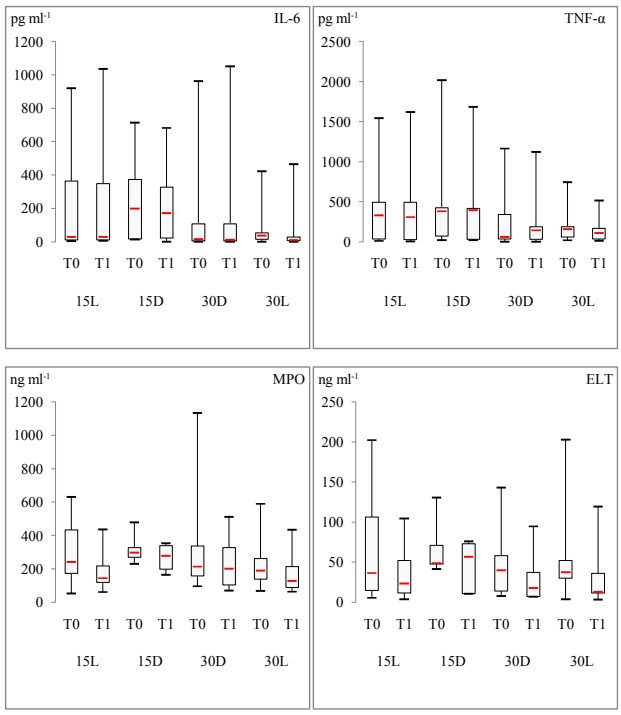
Data are shown as mean ± standard deviation. §Values reported as median and range because they were not normally distributed. Np: not performed. †significantly different ($p<0.05$) from 30D; ‡significantly different ($p<0.05$) from 30L; ¤significantly different ($p<0.05$) from 15L; #significantly different ($p<0.05$) from 15D; *significantly different ($p<0.05$) from T1. 15D: low pressure-low volume MV in dorsal recumbency; 30D: high pressure-high volume MV in dorsal recumbency; 15L: low pressure-low volume MV in lateral recumbency; 30L: high pressure-high volume MV in lateral recumbency; T0: at the beginning of mechanical ventilation; T1: after 60 minutes of mechanical ventilation; RR: respiratory rate; VT: tidal volume; PIP: peak inspiratory pressure; $ETCO_2$: end-tidal carbon dioxide; ETISO: end-tidal isoflurane; HR: heart rate; MAP: mean arterial blood pressure; PaO_2: arterial partial pressure of oxygen; $PaCO_2$: arterial partial pressure of carbon dioxide; SpO_2: saturation of hemoglobin with oxygen; PAO_2: alveolar partial pressure of oxygen; BE: base excess; HCO_3^-: bicarbonate

Table 2: Intraoperative respiratory, hemodynamic and pulmonary variables at T0 and T1.



Box and Whisker Plots show the 25th percentile and 75th percentile (upper and lower limit of the box, respectively), the medianand extreme values (upper and lower limit of the vertical bar). *Significantly different from T_0 ($p<0.05$). T_0=at the beginning of mechanical ventilation; T_1=after 60 minutes of mechanical ventilation

Figure 1: Plasma concentrations of equine IL-6, TNF-α, MPO and ELT at T0 and T1 (n=34).



Box and Whisker Plots show the 25th percentile and 75th percentile (upper and lower limit of the box, respectively), the median and extreme values (upper and lower limit of the vertical bar). 15D=low pressure-low volume MV in dorsal recumbency; 30D=high pressure-high volume MV in dorsal recumbency; 15L=low pressure-low volume MV in lateral recumbency; 30L=high pressure-high volume MV in lateral recumbency; T0=at the beginning of mechanical ventilation; T1=after 60 minutes of mechanical ventilation

Figure 2: Plasma concentrations of equine IL-6, TNF-α, MPO and ELT in 15D (n=5), 15L (n=11), 30D (n=9) and 30L (n=9) at T0 and T1.

Plasmatic TNF-α level was below the assay detection limit (5 pg/ml) in 1 of 34 horses (30D) at both T_0 and T_1. Plasmatic TNF-α, MPO and ELT concentrations decreased significantly at T_1 compared to T_0 ($p=0.001$, $p=0.009$ and $p=0.015$, respectively). Plasmatic level of IL-6, TNF-α, MPO and ELT did not vary significantly with PIP or recumbency or their 2-by-2 interaction. No variation with NSAIDs, antibiotic therapy and weight was found.

Discussion

We investigated the effect of two different ventilation protocols on circulating levels of IL-6, TNF- α, MPO and ELT during general anaesthesia in horses. Plasmatic concentration of inflammatory mediators did not increase after 1 hour of MV with none of the protocols used in this study, and did not change with either PIP or recumbency or their 2-by-2 interactions. The levels of TNF-α, MPO and ELT significantly decreased over time compared to T0, and the number of patients having MPO and ELT concentrations above the cut off values was decreased at T1 in comparison to T0. IL-6 at T1 did not significantly change compared to T0.

Results of this study are unexpected compared to what can be found in the literature in other species, but can be explained by a number of factors related to the protocol and the animals investigated. These factors include the diversity of pre-existing pathologies in the animals. T0 levels of IL-6, TNF-α, MPO and ELT were variable between patients, and 9 and 22 of 34 horses included in this study had MPO and ELT concentrations, respectively above the corresponding cutoff values determined between healthy and pathologic horses [24,25]. To our knowledge, no such cutoff values have been determined for the TNF-α and IL-6 ELISA kits used in our study. All the horses enrolled in the study were classified as free of systemic disease on the basis of a physical examination and routine laboratory tests. However, these tests are probably not sensitive enough to indicate low-grade or subclinical diseases. Even if there was no significant difference in WBC count at the admission to the hospital, and values of inflammatory mediators at T0 did not differ between groups, the different inflammatory state could have influenced the results. Horses with both acute and chronic local inflammation were enrolled. Further, post sampling ex vivo activation of neutrophils and monocytes may have occurred in the sampling tube [27], although samples were carefully handled as described by Franck et al. [24], who have investigated the stability of MPO in samples.

A range of factors related to the study protocol, such as the intensity and the duration of ventilation and the drugs before and during anaesthesia may have led to the unexpected results of this study. The group of animals included was heterogeneous and probably horses experienced different transpulmonary pressure despite MV with the same PIP and VT. In addition to the possible lack of aggressiveness of high-pressure MV, the time of 60 minutes of MV might have been too short to induce systemic changes. Horses in this study were ventilated for only 60 minutes and even if several studies have demonstrated that MV may lead to release of pro-inflammatory cytokines soon after its initiation [8,28,29] in some authors' opinion, one to two hours of MV are insufficient for an adequate inflammatory response [30,31]. Circulating TNF-α levels was shown to peak early with a short half-life after the inflammatory hit [32]. IL-6 is released within the first 60-120 minutes of high ventilation [33] however some works suggest that IL-6 does not always increase after 1-3 hours of non-protective ventilation in healthy humans and mice [34-36]. Systemic detection of inflammatory mediators is variable between studies, especially for TNF-α and IL-6 [7,9,20,22,23,37]. These considerations, along with our results may suggest that a longer lasting MV with pressure higher than that used in our study, presumably associated to a higher RR may be required in healthy horses to induce biotrauma or that the same protocols used may induce biotrauma if applied for more than 60 minutes. This could also explain why there was no correlation between plasmatic pro-inflammatory concentrations and recumbency that has been shown to influence the extent and distribution of VILI in healthy lungs of dogs [38]. A recent study has demonstrated no difference

in circulating inflammatory markers among different ventilation strategies and concluded that systemic response is not correlated to ventilation strategy [39]. Further, postoperative samples would have been useful in order to identify a possible systemic inflammation not detectable at the timing of T_1.

Horses of the present study have received various drugs, which are all known to modify inflammatory parameters. The reduction in systemic concentration of TNFα, MPO and ELT could be the result of intravenous and inhalational anaesthesia effects on cells involved in immune response. Isoflurane attenuates ischemia-reperfusion-induced injury in lungs and ameliorates endotoxin-induced lung injury [32,40,41]. Isoflurane prevents histological signs of lung damage, abolishes neutrophil infiltration, lung inflammation and cytokines release [32,42,43]. IL-6 and TNF-α production was decreased by isoflurane in an acute lung injury model of rats and mice [32] and by subanesthetic doses of ketamine in rats and dogs [44,45]. Ketamine reduces *in vitro* production of TNF-α and IL-6 in an equine macrophage cell line [46], another study, at the contrary, failed in showing a reduction in TNF-α production in horses with experimental endotoxemia receiving ketamine [47]. Ketamine-xylazine attenuates inducible nitric oxide synthase (iNOS) activity in activated alveolar macrophages exposed to lipopolysaccharide [32]. Isoflurane has been also proposed to reduce the activity of this enzyme [48]. Acepromazine has anti-inflammatory properties and modulates polymorphonuclear activation and reactive oxygen species production in horses [49-51]. Benzodiazepines inhibit phagocyte oxidative metabolism and production of TNF-α and IL-6 [52]. Midazolam inhibits iNOS and cyclooxygenase-2 expression, human neutrophil function and endothelial activation thought peripheral benzodiazepine receptor localized in mitochondria of the endothelial cells [53-55]. All the horses have received either flunixine or phenylbutazone, both non-steroidal anti-inflammatory drugs, which are expected to reduce the presence of inflammatory markers. Phenylbutazone has been shown to reduce the production of reactive oxygen species by equine neutrophils [56] and flunixine reduces the expression of TNF- but increases the expression of IL-6 after experimental administration of endotoxins to horses [57]. The use of nonselective COX-inhibitors does not affect the ventilator-induced release of TNF-α and IL-6 in man [58]. This study showed that pretreatment with ibuprofen effectively inhibited eicosanoid synthesis and COX-2 activity, increased survival, and attenuated lung edema and decrement in respiratory mechanics. However, ibuprofen had no modulatory effect on ventilator-induced activation of NF-κB or inflammatory cytokines (TNF-α, IL-1β, IL-6, and growth-related oncogene/keratinocyte chemoattractant). COX activity seems important in the pathogenesis of VILI in the *in vivo* rat. Inhibition of COX provides significant protection (i.e., survival, pulmonary function) in VILI, but without affecting levels of important mediators of activation of NF-κB.

We only investigated systemic concentrations of inflammatory mediators and comparison to broncho-alveolar lavages in order to detect alveolar levels of inflammatory markers has not been performed in the present study. Local inflammation, moreover, is normally accompanied by a compensatory systemic anti-inflammation usually accompanies a local inflammation. It and concentrates activated phagocytes and other effectors at an injured local site while preventing potentially damaging inflammation in uninvolved sites [59,60]. The level of the anti-inflammatory IL-10, which level that increased in mice anaesthetized with isoflurane [47], decrease the TNF-α production [61,62] The physical stress of ventilation also increases catecholamine secretion which may interfere with the production of pro- and anti-inflammatory cytokines. The immunological effects of the anaesthetic drugs and the use of NSAIDs may have then contributed to the observed sum effect of reduced systemic inflammatory mediator concentrations, despite MV and surgery.

Conclusions and Perspectives

Ventilation strategies using a PIP of 15 or 30 cm H_2O, VT between 5-21 mL kg^{-1} and RR of 4-15 breaths/minute did not induce an increase of plasmatic IL-6, TNF-α, MPO and ELT during 1 hour of MV in healthy horses undergoing general anaesthesia for surgery, in either dorsal and lateral recumbency. No differences were found between groups in terms of respiratory parameters and plasmatic levels of inflammatory mediators. Further, a reduction in the plasmatic concentrations of these mediators has been observed 1 hour after the beginning of MV. The anti-inflammatory properties of the drugs included in the anaesthesia protocol may have contributed to this. Although local inflammation of the lung without systemic spill-over of inflammatory mediators cannot be excluded, it seems that conventional short-term MV does not induce a systemic inflammatory reaction in the group of horses investigated here. The heterogeneity of the animals included in this study, the variability of our results and the limitations of our study may reduce the validity of our results, although it seems that short-term conventional ventilation does not induce systemic inflammation in horses anesthetised for various clinical indications. Further studies on a more homogenous group of animals including broncho-alveolar lavages are necessary to clarify the role of short lasting conventional and non-protective MV in a one-hit model of VILI in horses.

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