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Remote lung injury after experimental intestinal ischemia-reperfusion in horses

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Summary. Ischemia followed by reperfusion leads to release of toxic molecules into the circulation, and these molecules may cause injury in remote organs such as the lung. Horses commonly suffer from episodes of intestinal ischemia-reperfusion (IR) due to intestinal twisting/strangulation followed by repair. Because there is no evidence of lung injury associated with IR in horses, we designed a study to characterize the intestinal IR-associated lung inflammation and determine the effect of lidocaine on lung inflammation in IR horses. Lung tissues were collected from non-anesthetized (n=4) and anesthetized (n=4) control horses and horses (n=12) after 70 minutes of ischemia followed by 60 minutes of reperfusion. Horses in IR groups received Lactated Ringer's Solution (LRS; n=6) or lidocaine (n=6) intravenously. Control lungs had normal histology but lungs from IR horses showed moderate accumulation of neutrophils in blood vessels and airways. We found increased staining for TLR4, IL-8, TLR9, and von Willebrand factor (vWF) along with aggregates of vWF-positive platelets in lung vessels of IR horses compared to the controls. Lung TNFα was significantly increased in IR horses compared to the control horses ($P<0.05$). Neutrophil numbers, but not MPO concentrations, were significantly lower, while macrophage numbers were higher in the IR group receiving lidocaine compared to the LRS horses ($P<0.05$). We conclude that intestinal IR leads to remote lung injury characterized by recruitment of inflammatory cells and expression of inflammatory molecules in horses, and lidocaine may ameliorate lung inflammation following intestinal IR.

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Introduction

Ischemia-reperfusion (IR) injury is characterized by an acute inflammatory response resulting in tissue injury. It occurs following restoration of blood flow to an organ after an ischemic event. During IR, several cell-surface receptors of the innate immune system are stimulated, resulting in leukocyte recruitment and the initiation of cellular signaling cascades that regulate transcription of pro-inflammatory cytokines, chemokines and adhesion molecules (Herskowitz et al., 1995). In the lung, this response may in part be mediated by the activation of the Toll-like receptor (TLR)/MyD88 signaling pathway (Victoni et al., 2010) and nuclear factor kappa B (NF-κB) translocation to the nucleus (Ishiyama et al., 2005). Activation of resident alveolar macrophages, resulting in production of pro-inflammatory mediators such as tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 has also been described (Zhao et al., 2006). Moreover, there appears to be a role for the neutrophils since their removal from the circulation or inhibition of their function reduces the degree of IR injury in the lung of rats (Hirayama et al., 2006; Mori et al., 2007). In addition to the inflammatory events taking place in the organ suffering from IR injury, remote tissue injury, particularly in the lung, can also occur (Ishii et al., 2000; Victoni et al., 2010, Lapchak et al., 2012). Based on the studies in rodent models, it is believed that TLRs, neutrophils, and platelets play a role in early inflammatory changes resulting in remote lung injury following intestinal IR injury. However, the precise

mechanisms of remote lung injury associated with intestinal IR are not fully understood.

Gastrointestinal disease such as that associated with intestinal strangulation is common in horses and requires surgical intervention for its correction. Intestinal strangulation causes ischemia and an influx of large numbers of neutrophils into the ischemia region. Upon surgical correction, toxic metabolites released from activated neutrophils and the damaged intestinal tissue enter the circulation and lead to systemic inflammation, leading to pulmonary damage (Cook et al., 2008, 2009). Recently, it was reported that distention of the equine colon with a surgically implanted balloon for a period of 4 hours led to inflammation and accumulation of neutrophils in the lung suggesting communication between the gut and the lungs (Faleiros et al., 2008). Although there are no specific therapeutics to manage injury associated with intestinal ischemia-reperfusion, lidocaine has been tried. It was reported that a concurrent administration of lidocaine and flunixin meglumine reduces the inhibitory effects of flunixin on mucosal healing in horses after ischemic intestinal injury (Cook and Blikslager, 2008; Cook et al., 2008). However, there are very few data on the description of remote lung injury associated with intestinal ischemia reperfusion in horses and on the use of lidocaine to ameliorate inflammation associated with ischemia-reperfusion in horses.

There are currently limited data available on lung inflammation in horses suffering from intestinal ischemia-reperfusion. Therefore, we designed this study to test a hypothesis that remote lung injury in horses is associated with intestinal ischemia-reperfusion and that lidocaine ameliorates lung injury.

Materials and methods

Animals

The experimental methods for this study were approved by the Animal Care Assurance Committee of the University of Saskatchewan in accordance with the Canadian Council on Animal Care Guidelines for the use of animals in research. Samples from healthy, non-anesthetized control horses (n=4; Group 1) and healthy anesthetized control horses (n=4; Group 2) were obtained from horses enrolled in another study. Two groups of horses (n=6 per group) were subjected to experimental intestinal IR and received either intravenous lidocaine (Group 3) or Lactated Ringer's Solution (LRS; Group 4). These horses were of mixed breed and sex ranging in age from 2 to 18 (mean 6.3) years and in body weight from 320 to 550 (mean 425.8) kg. Horses were randomly assigned to either the LRS or lidocaine group. Thorough physical examination, a CBC and biochemistry panel were performed on each horse prior to the study. The CBC was used to rule out systemic inflammation by evaluating the leukocyte count, especially the total neutrophil count. Neutrophil count range was 3.6-13.6x10^9 L (reference range 1.8-7.2x10^9 L). Two horses, one in each group, had neutrophil counts above the reference range; these changes were attributed to stress. The biochemistry panel performed on each horse was to assess liver function, as lidocaine is metabolized primarily by the liver. No significant elevations in liver enzymes were found. The horses were housed in a barn isolated from other horses or animals and were examined daily for any clinical abnormalities. The examination included recording of temperature, heart and respiration rates. The horses were used for experiments after they were found to be clinically normal and acclimatized to the animal care facility.

Treatments

A catheter was placed in the jugular vein. Horses were sedated with xylazine (1.0 mg/kg IV) and general anesthesia was induced with a combination of diazepam (0.1 mg/kg IV) and ketamine (2.0 mg/kg IV). Anesthesia was maintained using isoflurane in oxygen in a semiclosed system with intermittent positive pressure ventilation. Lactated Ringer's solution (LRS) was administered at a rate of 10 ml/kg/hr. Mean blood pressure was maintained at ≥70 mm Hg and monitored by direct arterial catheterization.

Horses were placed in dorsal recumbency and prepared for aseptic ventral midline celiotomy. A 35-40 cm incision was made through the skin and *linea alba* cranial to the umbilical scar. The small intestine was isolated and exteriorized in a routine manner. Next, the small intestine was evacuated by milking of fluid and ingesta into the cecum. The intestine was kept moist with warm LRS solution. Beginning at the distal jejunum, four 40-cm jejunal segments, each supplied by its own jejunal artery and vein, were identified. The identified segments were then surgically isolated from each other by applying nylon wire ties to each end and transecting distally to the ties. This process resulted in each of the four segments being attached solely by the jejunal artery and vein supplying the segment, as well as its corresponding mesentery. The four segments were designated as; control, low-flow ischemia reperfusion (IR), intraluminal distension-decompression (DD), and complete venous occlusion (VO). During the study period, the exteriorized intestine was kept moist with warm LRS and covered with a sterile plastic drape that served as a barrier protecting the intestine from desiccation and serosal irritation.

Ischemia-reperfusion protocol

Low-flow ischemia was created for 70 minutes in the designated jejunal segment. Initially, the jejunal artery supplying the segment was isolated and a perivascular flowprobe (Transonic T106, Transonic Systems Inc., Ithaca, NY) was positioned around the artery to measure baseline/normal blood flow. After

establishing normal jejunal arterial blood flow, a Rummel ligature was placed around the artery to reduce blood flow to 20% of its baseline value. Following 70 minutes of low-flow, the Rummel ligature was removed and with the flowprobe still in place, to monitor the return of blood flow to baseline levels, a period of 60 minutes of reperfusion occurred.

Complete venous occlusion protocol

The complete venous occlusion protocol was identical to the above IR protocol regarding timeline and sample collection. Initially, the jejunal vein of the segment was isolated and a hemostat was used to clamp the vein, achieving complete venous occlusion for a period of 70 minutes. Then, as in the IR protocol, the hemostat was removed to allow for a 60-minute period of normal outflow of blood from the jejunal segment.

Intraluminal distention-decompression protocol

The designated DD section of jejunum was prepared for distention by a stab incision into the lumen near one end of the segment. A 20-Fr Foley (polyethylene) catheter was then inserted into the lumen and secured in place using 2-0 nonabsorbable suture material using a Chinese finger trap. After distending the balloon/cuff, the jejunal lumen was infused with sterile LRS and distended to a pressure of 25 cm H_2O (18.4 mm Hg). A manometer was used to monitor the pressures throughout the 120-minute period of distention. After the 120 minutes of distention, the intestine was decompressed for 60 minutes.

The three protocols were run simultaneously as two surgical teams performed the experimental procedures. In order to coincide sampling periods, the protocols were staggered i.e. the DD model was initiated first while isolation of the jejunal artery and vein were performed for the IR and VO models. With the intention of mimicking a clinical strangulating small intestinal injury, the horses in Group 3 received a loading dose of 1.5 mg/kg IV solution (lidocaine) over a 5 minute period followed by a continuous rate infusion (CRI) of the solution at a rate of 0.05 mg/kg/min IV immediately prior to reperfusion, removal of the ligature, and/or decompression. The horses in Group 4 were not infused with lidocaine but were instead given LRS. The surgical teams were unaware of the treatment groups. The horses were euthanized while under general anesthesia by an overdose of sodium pentobarbital.

Tissue collection and processing

Lungs were collected as described previously (Parbhakar et al., 2004). Briefly, the lungs were fixed *in situ* for 30 min by pouring 5 L of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) into the trachea. The lungs were removed *en bloc* and tissue pieces were collected from each lung and fixed in 4% paraformaldehyde for 24 h at 4°C. The necropsy was conducted to determine any gross abnormalities in the viscera. The lungs were dehydrated, cleared in xylene and embedded in paraffin. Some lung tissues were snap frozen in liquid nitrogen and stored at -80°C.

Histopathlogy

Standard hematoxylin and eosin (H&E) staining was performed for all tissue slides. Macrophage numbers were counted for all groups (15 fields per group) at 40x magnification. Neutrophil numbers were counted for IR groups (10 fields per horse; 40x magnification). Slides were evaluated by two of the authors (B. Singh and J. Montgomery), who were unaware of the treatment group at the time of evaluation.

Immunohistology with anti- TLR4, MAC387, IL-8 and vWF antibodies

Immunohistology with TLR4 (Santa Cruz Biotechnology, SC 12511), MAC387 (MCA 874 Serotec), IL-8 (Santa Cruz Biotechnology, SC 7922) and vWF antibodies was performed on lung sections from each of the horses, as previously described (Singh Suri et al., 2006). Briefly, following dewaxing, rehydration and quenching of endogenous tissue peroxidase, antigens were unmasked through incubation with pepsin (2 mg/mL 0.01N hydrochloric acid) for one hour. Bovine serum albumin (1% in PBS buffer) was applied for 30 minutes to block nonspecific sites and the sections were subsequently treated with anti- TLR4, MAC 387, IL-8, or vWF antibodies, and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were applied for 2 hours and 60 minutes, respectively, at room temperature. The stain reactions were visualized using a color development kit (Vector Laboratories, Ontario, Canada) and counterstained with methyl green (Vector Laboratories). Immunohistochemical controls included incubation with normal goat IgG or omission of primary antibody or both primary and secondary antibodies, as well as staining with vWF as a positive control. While negative controls did not show any staining in the lung tissues (data not shown), vWF stained the vascular endothelium but not the airway epithelium (Fig. 3).

Enzyme linked- immunosorbent assay (ELISA) for TNF-α

ELISA was conducted on the lung tissue from IR horses and non-anesthetized control horses using a rabbit anti equine TNF alpha antibody (Abd serotec AHP 853Z). Frozen lung samples were homogenized in lysis buffer [150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM TRIS (pH 8.0), 5 mM EDTA, and protease inhibitor cocktail (100 μl/10 ml)]. Homogenates were collected after centrifuging the samples at 25,000 g for 20 minutes and

stored at -80°C until further use. For quantification, samples in duplicates, from three animals from each of the treatment groups were used. TNF-α was quantified by sandwich ELISA using antibody pairs and recombinant standard. For the lung homogenate analysis each well was loaded with 20 μg/ml.

Myeloperoxidase (MPO) activity

Myeloperoxidase activity was measured in 100mg of lung sample from each horse undergoing IR. Samples were homogenized in 50 mM of Hepes (pH 8.0). The homogenates were then centrifuged at 10,000g for 20 minutes at 4°C and the supernatant was discarded. The pellet was then resuspended to the original volume in 0.5% Cetyltrimethylammonium chloride solution (Sigma Aldrich). The sample was rehomogenized and centrifuged at 10,000g for 20 minutes at 4°C. Samples were stored at -20°C until further use. The MPO assay was performed using 96 well plates and samples were

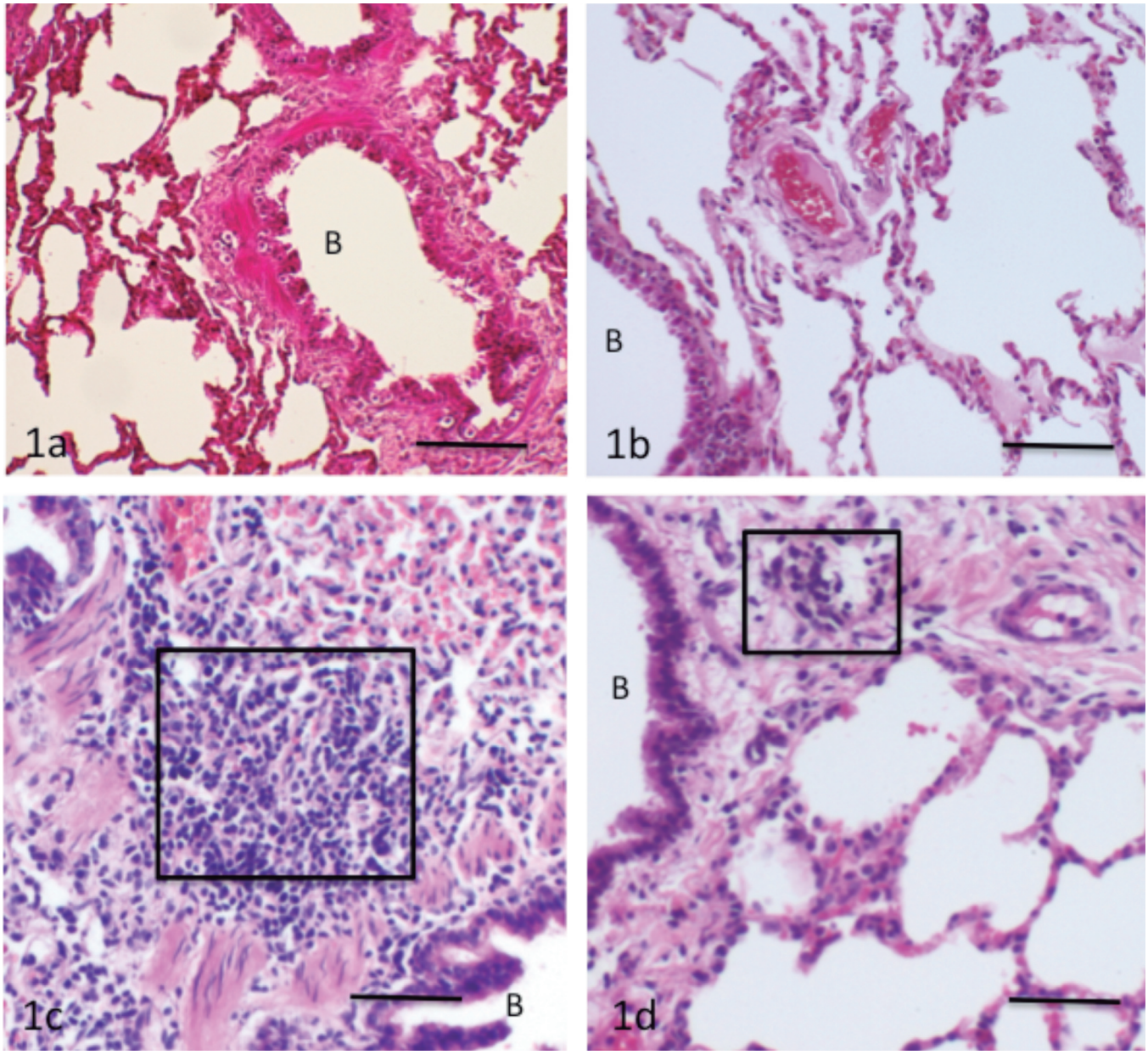

Fig. 1. H&E-stained lung sections from non-anesthetized **(a)** and anesthetized (**b)** horses showed lack of inflammation in alveoli and bronchiles (B). IR horses treated with LRS (**c**) showed larger aggregates of accumulated neutrophils (box) in peribronchial area. IR+lidocaine horses also showed very few neutrophils in peribronchial areas (box). Bar: 50 μm.

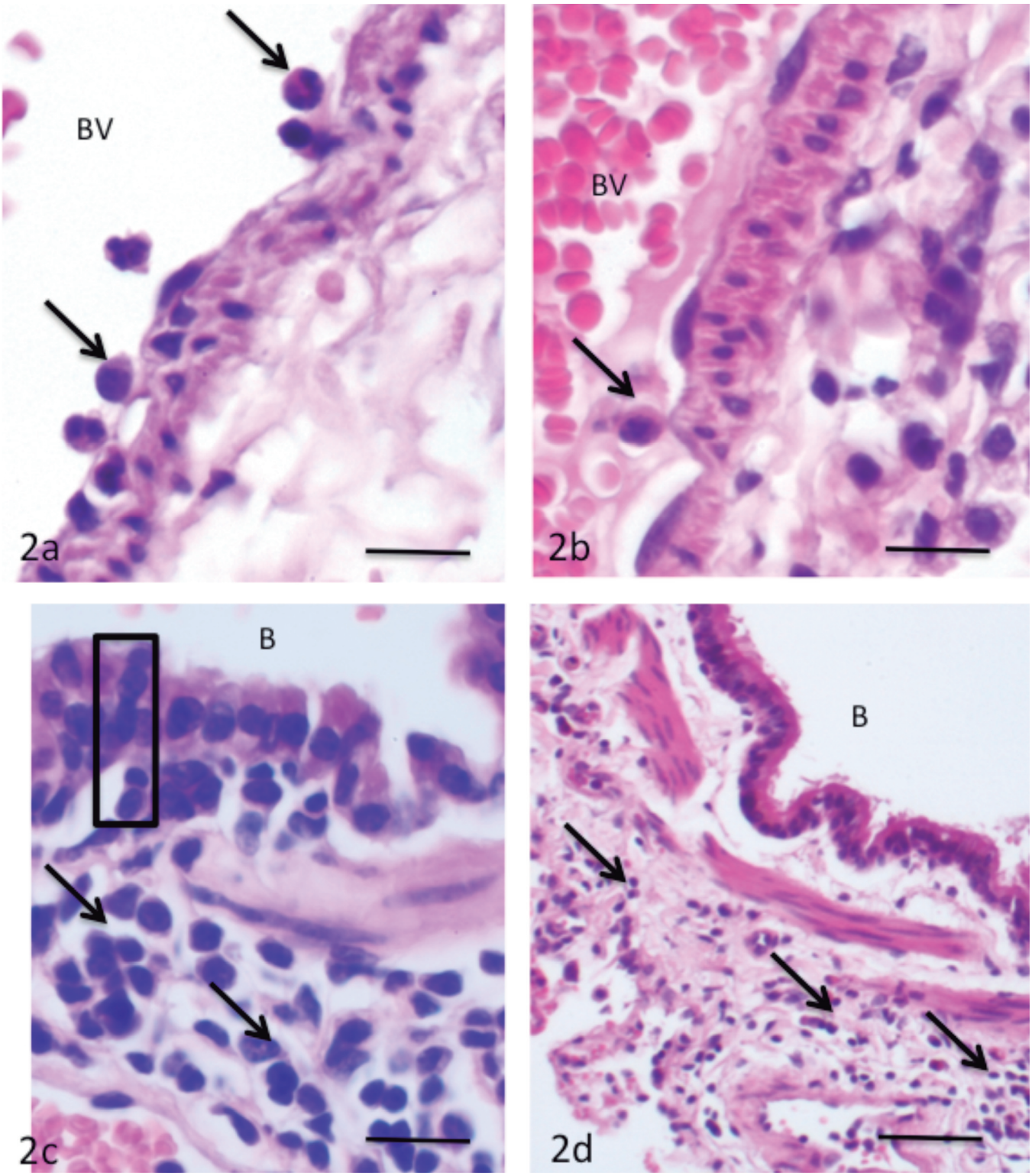

Fig. 2. H&E-stained lung section from an IR+LRS horse (**a**) showed many neutrophils (arrows) attached to endothelium of a vessel (BV) compared to a section from an IR+lidocaine horse (**b**). LRS+IR horses (**c**) showed accumulation of neutrophils (arrows) under the epithelium, including those arranged in a line across the epithelium (box). The bronchial epithelium and subepithelial areas were much less inflamed (arrow) and there were very few neutrophils in subepithelial areas in a lung section from a IR horse treated with lidocaine (**d**). Bars: a-c, 25 μm; d, 100 μm

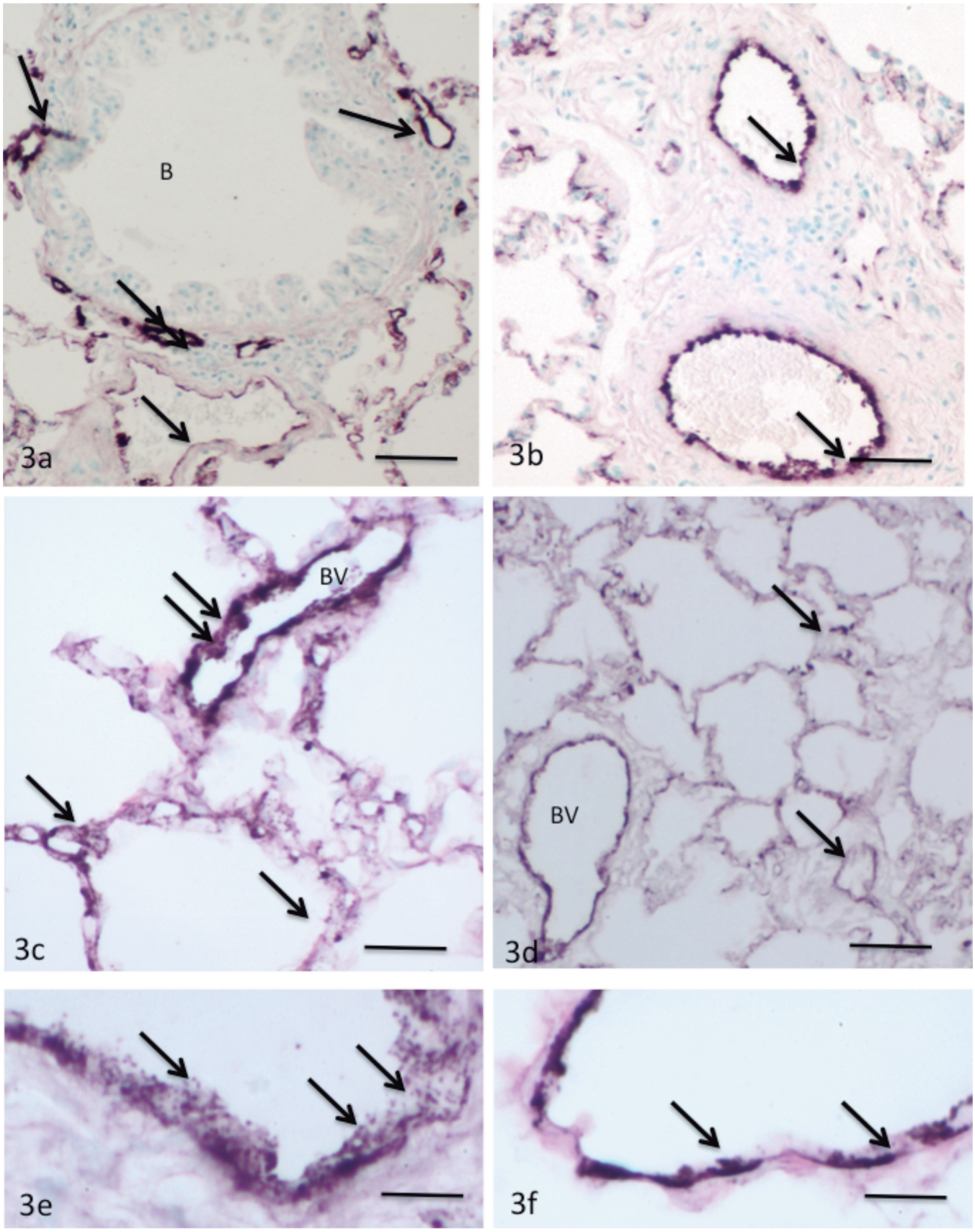

Fig. 3. WF staining is localized in vascular endothelium (arrows) but not in bronchiolar (B) epithelium in lung sections from non-anesthetized (**a**) and anesthetized (**b**) control horses. The staining (double arrows) was more in the endothelium of blood vessels (BV) and alveolar septa (arrow) in IR+LRS (**c**) compared to IR+lidocaine (**d**). Endothelium of blood vessels in lungs of IR+LRS (**e**) had many platelets (arrows) compared to that from IR+lidocaine (**f**). Bars: a-e, 25 μm; f, 50 μm

run in quadruplicates. Stop solution (150 μl of 1M H_2SO_4) was added at minute "0", followed by 75 μl of sample and substrate, respectively. For the 2-minute reading 75 μl of sample were added, followed by 75 μl of substrate. After 2 minutes, 150 μl of stop solution were added. The optical density reading was obtained at 450 nm.

Statistical analysis

Data were analyzed using Graph Pad Prism 5. Comparisons between two groups were made using a two-tailed t-test. Comparisons between three or more groups were performed by one-way analysis of variance followed by Tukey's post hoc comparisons. Differences were considered significant at $P<0.05$.

Results

Characterization of lung inflammation in IR horses

Lung sections from control animals (anesthetized and non-anesthetized controls) showed normal histology including normal alveolar septa and lack of inflammatory cells in alveoli (Fig. 1a,b). In contrast, lungs from IR horses with or without lidocaine showed moderate to severe inflammation, including congestion, leukocyte infiltration in the septa, as well as in peribronchial areas in IR+LRS horses (Fig. 1c) and at a much reduced level in IR+lidocaine horses (Fig. 1d). Lung sections from IR+LRS horses showed many more leukocytes attaching to vascular endothelium (Fig. 2a) and accumulated in subepithelial areas, including those trapped in the epithelium (Fig. 2c) compared to the lung tissues from IR+lidocaine horses (Fig. 2b,d).

We further characterized lung inflammation through staining for vWF, which is an adhesive protein and is highly expressed in acute vascular inflammation. Compared to the lung sections from control horses (Fig. 3a and 3b), vWF staining intensity was increased in the IR lungs (Fig. 3c,d). Furthermore, we noticed more aggregates of vWF-positive platelets in lung vasculature of LRS+IR horses (Fig. e) compared to lidocaine+IR horses (Fig. 3f).

Neutrophil and macrophage recruitment in lungs of IR horses

There was an overall difference between neutrophil numbers in the lungs of IR horses receiving lidocaine when compared to horses receiving LRS with significantly fewer neutrophils in the lidocaine group ($P=0.0223$; Fig. 4a). Overall, neutrophils were significantly more abundant in the airways of IR horses receiving LRS when compared to IR horses receiving lidocaine ($P=0.0051$) (data not shown). There were no differences in MPO activity, which is a surrogate for neutrophil trapped in the interstitial and vascular compartments of the lung in our experiments, between the LRS and lidocaine treated IR horses (Fig. 4b)

To further identify inflammatory cells in the lungs from IR horses, lung sections were stained with an anti-MAC 387 antibody. This antibody recognizes calprotectin, expressed by granulocytes, monocytes, and tissue macrophages (Faleiros et al., 2009; Chiavaccini et al., 2011). In the control groups, only a few cells stained positive with MAC387, all of which were located in the alveolar septa (Fig. 5a,b). Likely these are resident tissue as well as pulmonary intravascular macrophages as described previously (Parbhakar et al., 2004). Following intestinal IR, lungs of horses exhibit an increased number of MAC387-positive leukocytes in both the alveolar septa as well as the alveolar space (Fig. 5c,d). Lung sections did not show differences between the control groups in macrophage numbers but both IR groups had significantly higher numbers of macrophages present in lung tissues compared to the controls ($P<0.05$). Furthermore, macrophage numbers were significantly ($P<0.05$) higher in the IR group receiving lidocaine when compared to the IR group receiving LRS

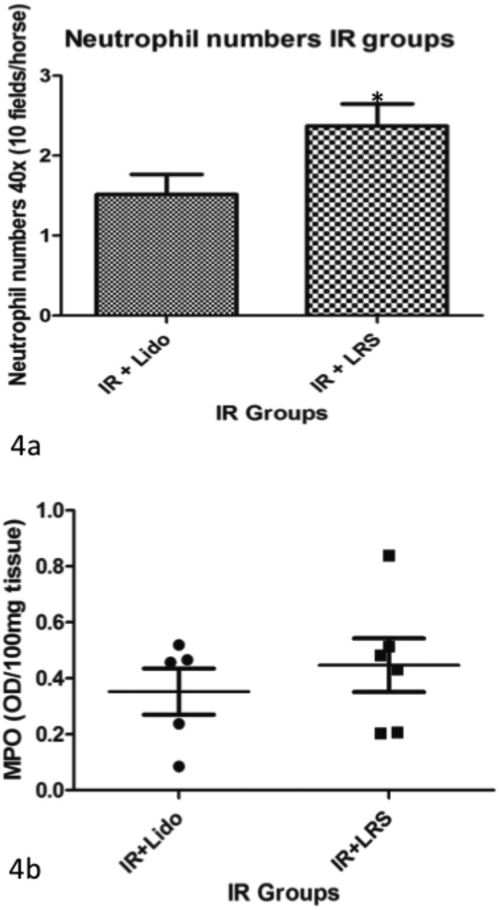

Fig. 4. Lungs of IR horses treated with LRS showed significantly more numbers of neutrophils compared to the IR horses treated with lidocaine (**a**). The MPO concentrations (**b**) in lung tissue homogenates were not different between IR horses treated with lidocaine versus those given LRS.

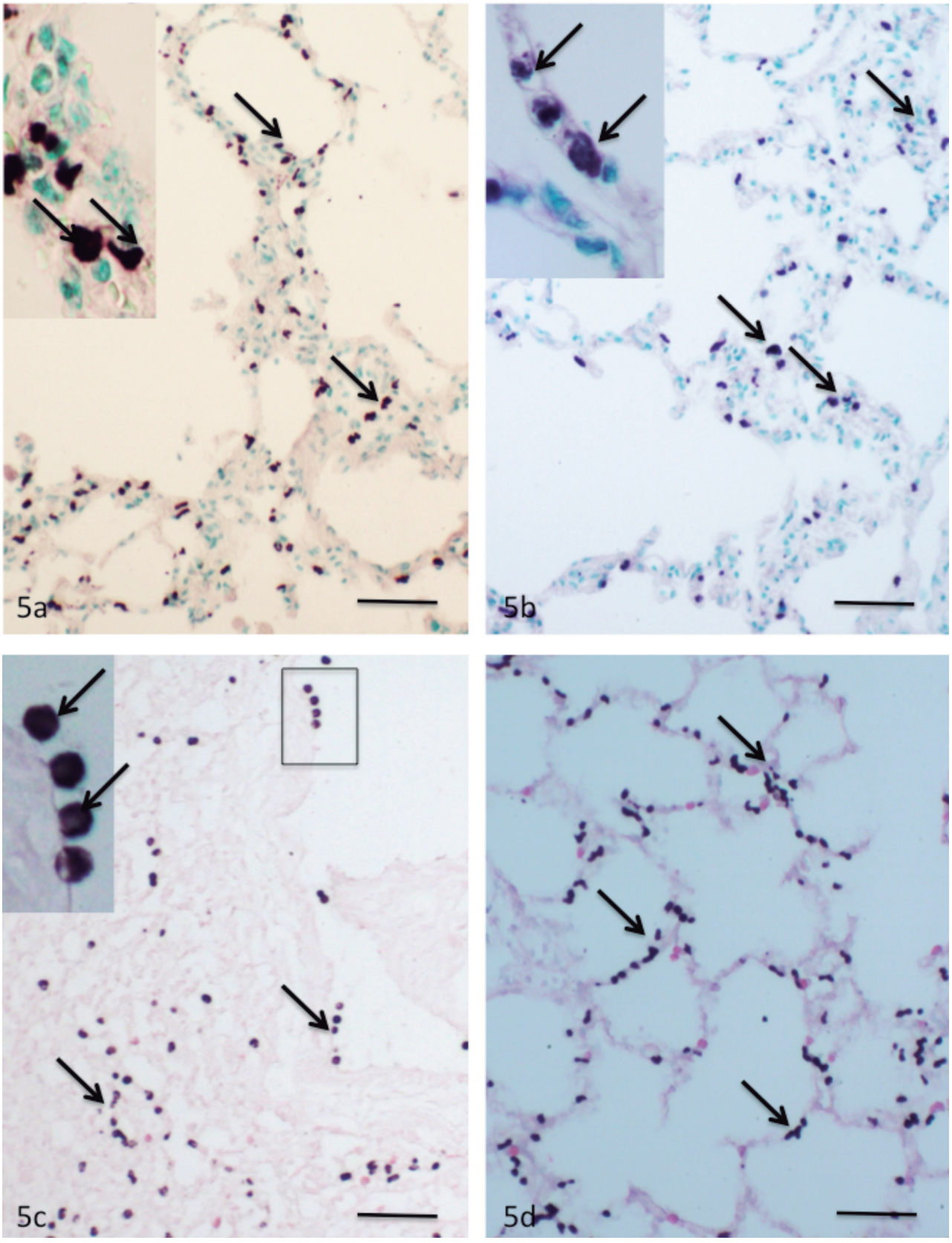

Fig. 5. MAC387-macrophages (arrows) are observed in alveolar septa of lungs from non-anesthetized (**a** and inset) and anesthetized (**b** and inset) control horses. IR horses treated with LRS (**c**) show septal macrophages (arrows) and also macrophages attaching to the vascular endothelium (inset). There is an appearance of increase in MAC-387-positive alveolar septal cells in IR+lidocaine (**d**) horses. Bars: 50 μm

(Fig. 6).

IL8 and TNF-α expression in lungs

We examined the potential role of IL-8, a potent chemokine for neutrophil and monocyte recruitment. While in tissues from untreated control animals, only mild staining was noticed on airway epithelium (Fig. 7a,b), the expression was upregulated in airway epithelium in sections from IR horses from both groups (Fig. 7c,d). Tumor necrosis factor-alpha (TNF-α) concentration was measured using ELISA, except in anesthetized control animals for which we did not have frozen lung samples. The comparison of the remaining three groups showed significantly higher concentrations of TNF-α ($P<0.05$) in both IR groups when compared to the non-anesthetized control group (Fig. 8). There was no difference between the two IR groups.

TLR4 and TLR9 expression in lungs

TLR4, a critical regulator of endotoxin-induced inflammation, showed only weak staining intensity of airway epithelium and vascular endothelium of control horses (Fig. 9a,b). The lung sections from IR horses showed increased staining intensity of TLR4 in alveolar septa and mostly in the cells accumulated in the septa (Fig. 9c,d). Several areas of intense staining were noted at the level of the terminal bronchioles where they open into the alveolar duct area. Additionally, TLR4 expression was increased in airway epithelium, peribronchial area, and endothelium of larger blood vessels of IR horses.

TLR9, which ligates bacterial DNA motifs, was localized in septal cells (10a) and vascular endothelium (10b) in lungs of non-anesthetized control horses (10a and 10b) and the anesthetized controls (data not shown). Increased staining for TLR9 was observed in alveolar septal cells in lungs of IR horses treated with LRS (10c) or lidocaine (10e). Vascular endothelial staining for TLR9 was more intense in LRS-treated IR horses (10d) compared to those administered lidocaine (10f).

Discussion

The first aim of our study was to investigate whether experimental intestinal IR results in remote lung injury in horses. We provide evidence of cellular and molecular inflammatory changes in lungs of horses following intestinal IR injury, when compared to healthy control horses. Our data show evidence of moderate to severe pulmonary inflammation, including recruitment of leukocytes (neutrophils, monocytes/macrophages), platelet accumulation, and increased expression of vWF, TLR4, TLR9, IL-8, and TNFα. A control group of healthy anesthetized horses was included in order to rule out anesthesia-induced pulmonary changes.

To date, knowledge of mechanisms involved in intestinal IR-associated remote lung injury is mostly based on the study of rodent models (Ishii et al., 2000; Victoni et al., 2010; Lapchak et al., 2012). Toll-like receptors have been shown to play a key role in IR injury (Andrade et al., 2006; Shimamoto et al., 2006). Lung IR in TLR4-null (TLR4-/-) mice showed marked reduction in lung myeloperoxidase activity (MPO), leukocyte accumulation, and several pro-inflammatory cytokines (Andrade et al., 2006; Shimamoto et al., 2006). Additionally, TLR4 is known to regulate IR injury responses in the heart (Chong et al., 2004; Oyama et al., 2004), kidney (Wu et al., 2007), and the liver (Jin et al., 2007; Wang et al., 2007). Jin et al. (2007) also found increased expression of TLR4 and 2 in the lung during partial hepatic IR in mice (Jin et al., 2007). Our data also show increased immunohistologic expression of TLR4 and TLR9 in the lungs from IR horses. The increase in protein expression of TLR4 and TLR9 will potentially increase susceptibility of the horse for exaggerated lung inflammation in response to bacterial endotoxin and DNA released into the circulation from the ischemic and reperfused intestine.

In mice, TLR activation seems to be a key factor in remote lung injury following intestinal IR injury, since disruption of the TLR activation pathway results in reduced intestinal and lung injury, as well as decreased endothelial damage following experimental intestinal IR (Victoni et al., 2010). Activation of TLR4 results in production of inflammatory mediators such as TNFα and IL-8, leading to recruitment of neutrophils and monocytes/macrophages (Perros et al., 2011). In addition

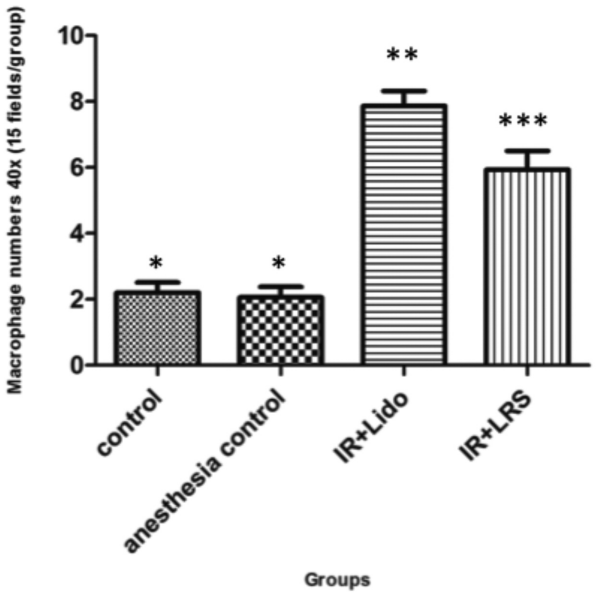

Fig. 6. MAC387 counts representing macrophages were significantly higher in lungs of IR horses compared to both of the controls. The numbers were significantly higher in lungs of IR horses given lidocaine compared to those treated with LRS.

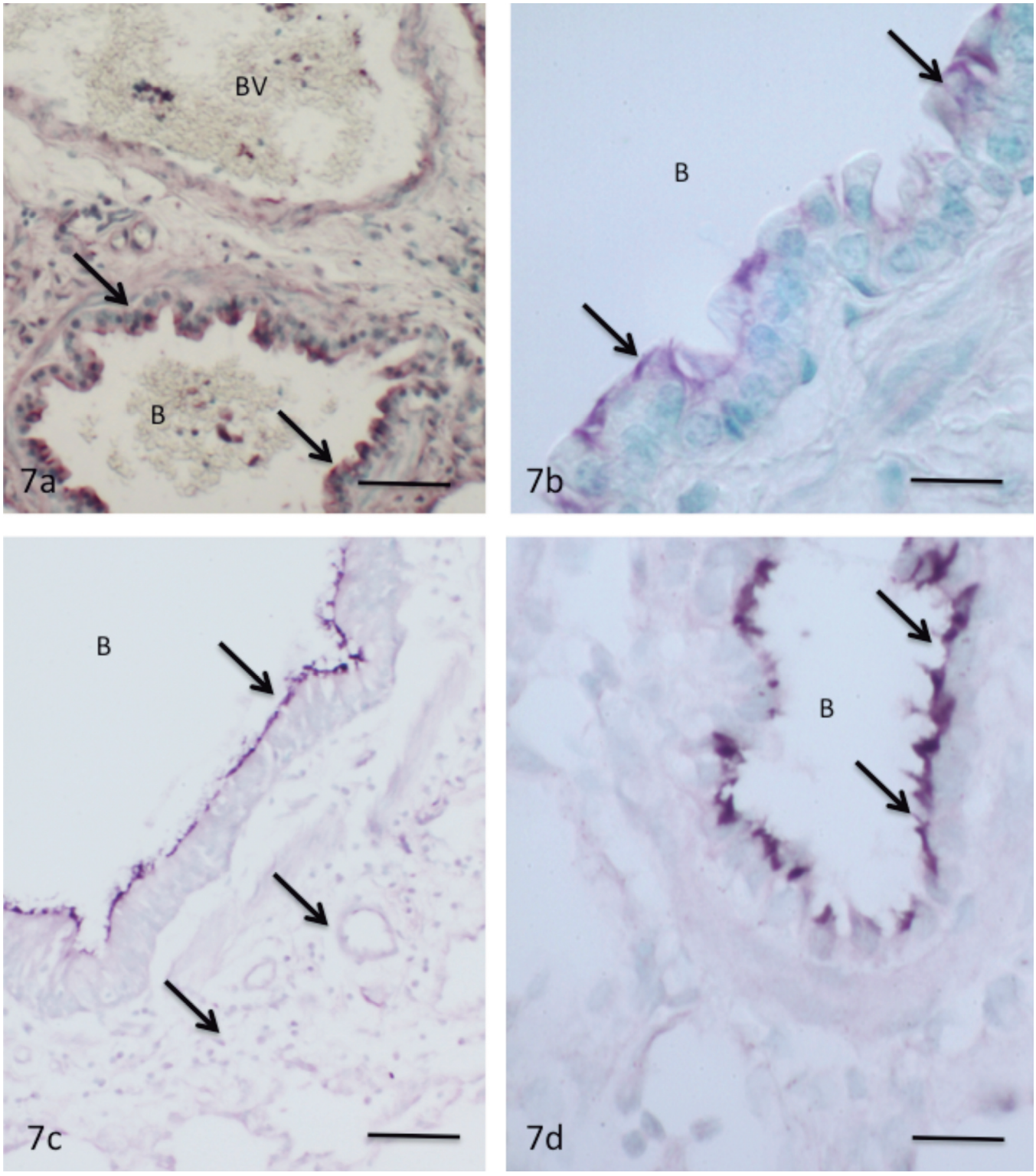

Fig. 7. IL8 is localized on bronchiolar (B) epithelium (arrows) in lungs from all the four groups. Compared to non-anesthetized (**a**), anesthetized (**b**) controls had reduced expression. IL-8 staining (arrows) appears to be increased in bronchioles (B) of IR+LRS (**c**) and IR+lidocaine (**d**) groups. Note presence of many leukocytes (arrows) in the peribronchial area of the lung in IR+LRS (**c**). Bars: 50 μm.

to the increase in TLR4 expression, which can regulate neutrophil migration, we also noticed intense staining for vWF in lung endothelium and IL-8 in airway epithelium. Based on the histologic evidence of attachment and transendothelial migration of leukocytes in IR horses, potentially mediated through vWF and other adhesion molecules, IL-8 expressed on the airway epithelium may facilitate transendothelial migration of neutrophils. The neutrophil recruitment in subepithelial and other parts of the lung observed in our experiments is similar to that described following intestinal IR injury in rats (Lu et al., 1997; Ishii et al., 2000; Sharma et al., 2007). Not only vWF expression was increased in lungs of IR horses, we made an intriguing observation of more accumulation of vWF-positive platelets in lung vasculature of IR+LRS horses compared to IR+lidocaine horses. Recently, it was reported that platelets may play a major role in initiation of remote lung injury after intestinal IR injury in mice (Lapchak et al., 2012). While the precise mechanisms of recruitment of neutrophils and macrophages in lungs of horses suffering from intestinal IR remain to be elucidated, our data create a molecular framework to dissect specific steps in this cascade.

The second aim of our study was to evaluate whether intravenous lidocaine administration has any effect on intestinal IR-associated remote lung injury in horses. While our results do not provide conclusive evidence of the role of lidocaine in IR-associated lung inflammation, lidocaine did result in a significantly lower neutrophil count in lung sections from IR horses receiving lidocaine when compared to IR horses receiving LRS. Intriguingly, the MPO and TNFα concentrations, which are used as surrogates for tissue accumulation of neutrophils, were not different between the LRS and lidocaine-treated IR horses. Interestingly, the macrophage numbers were higher in lidocaine-treated IR horses compared with those administered LRS. One of the effects of lidocaine is to suppress inflammatory functions of neutrophils (Hollmann et al., 2001; Cook and Blikslager, 2008). Even though MPO is commonly used as an indirect measure of neutrophils it is also produced by monocytes/macrophages and an increase in macrophage counts in lidocaine-treated IR horses may have compensated the MPO decrease due to reduced neutrophil counts. The small sample size may have been another reason to not yield a significant difference in MPO concentration. Results from a recent *in vitro* study suggest that lidocaine alters macrophage function, partly through inhibition of the NF-κB signaling pathway (Wang et al., 2011). Lidocaine may also manifest its anti-inflammatory actions by promoting recruitment of macrophages, as observed in our experiments, because macrophage recruitment is associated with resolution of inflammation. Macrophage migration leads to secretion of TGF-beta and phagocytosis of dead neutrophils to restore homeostasis (Murray and Wynn, 2011); this may explain our finding of increased numbers of macrophages in IR horses. Based on our limited knowledge of the effect of lidocaine on IR-injured tissues in horses and contradicting results between study groups for different parameters measured, it is difficult to reach a conclusion as to the significance of lidocaine to horses suffering from IR injury at this point.

Limitations of the study include the wide age range of horses, which may have affected the results. However, all horses were adults (2 years or older) and none of the horses were of an age considered as geriatric (20 years and older). Based on the differences in weight of the horses, the percentage of jejunum affected by ischemia and reperfusion injury may have differed slightly, which could have affected the severity of the inflammatory response in individual animals. A scoring system of the degree of lung injury observed could have been used to further quantify the results. Since secondary lung injury is not yet well described in the horse, we opted for a primarily descriptive study to determine if the results warrant further research in horses.

In conclusion, this is the first study that shows evidence of remote lung injury following intestinal IR injury in horses. The observed inflammatory changes are characterized by recruitment of inflammatory cells, including neutrophils, monocytes/macrophages, and platelets, as well as expression of inflammatory molecules, including TLRs, TNFα, and IL-8, in horses. Lidocaine may ameliorate lung inflammation following intestinal IR injury in horses. This may partially be mediated through a decrease in neutrophil numbers. The data, however, provide only limited and partial understanding of the complex integrative physiology that underlies remote lung injury in association with intestinal IR. The role of pulmonary intravascular macrophages, already established in endotoxin-induced lung inflammation, in intestinal IR-associated lung inflammation may be explored further by depleting them in horses undergoing experimental IR

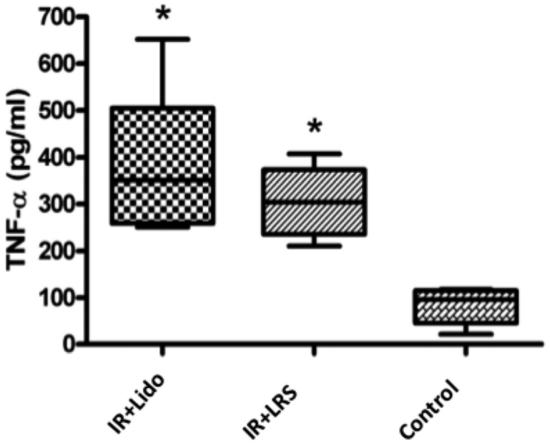

Fig. 8. TNFα concentrations were significantly higher in lung homogenates from both the IR horse groups compared to the non anesthetized controls. However, there were no differences in TNFα concentrations between the IR groups.

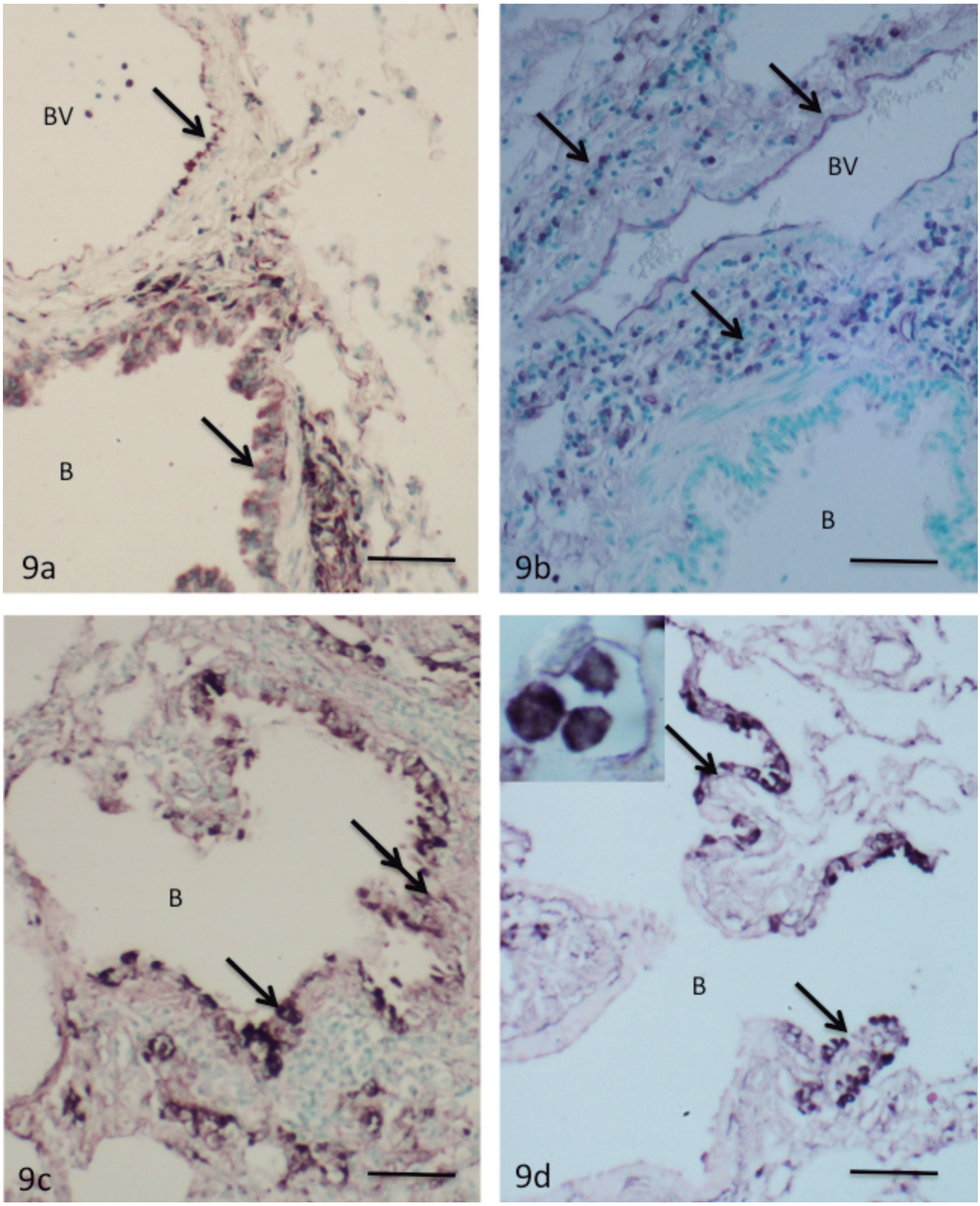

Fig. 9. TLR4 staining is observed in bronchioles (B) and blood vessels (BV) in lungs from non-anesthtized control horses (**a**). While vascular endothelium (BV) and peri-vascular cells (arrows) are positive for TLR4, bronchiole is not (B) in anesthetized control (**b**). Lungs from IR horses treated with LRS (**c**) or lidocaine (**d**) showed intense staining in bronchiolar (B) epithelium (arrows), and macrophages (inset; **d**). Bar: 50 μm.

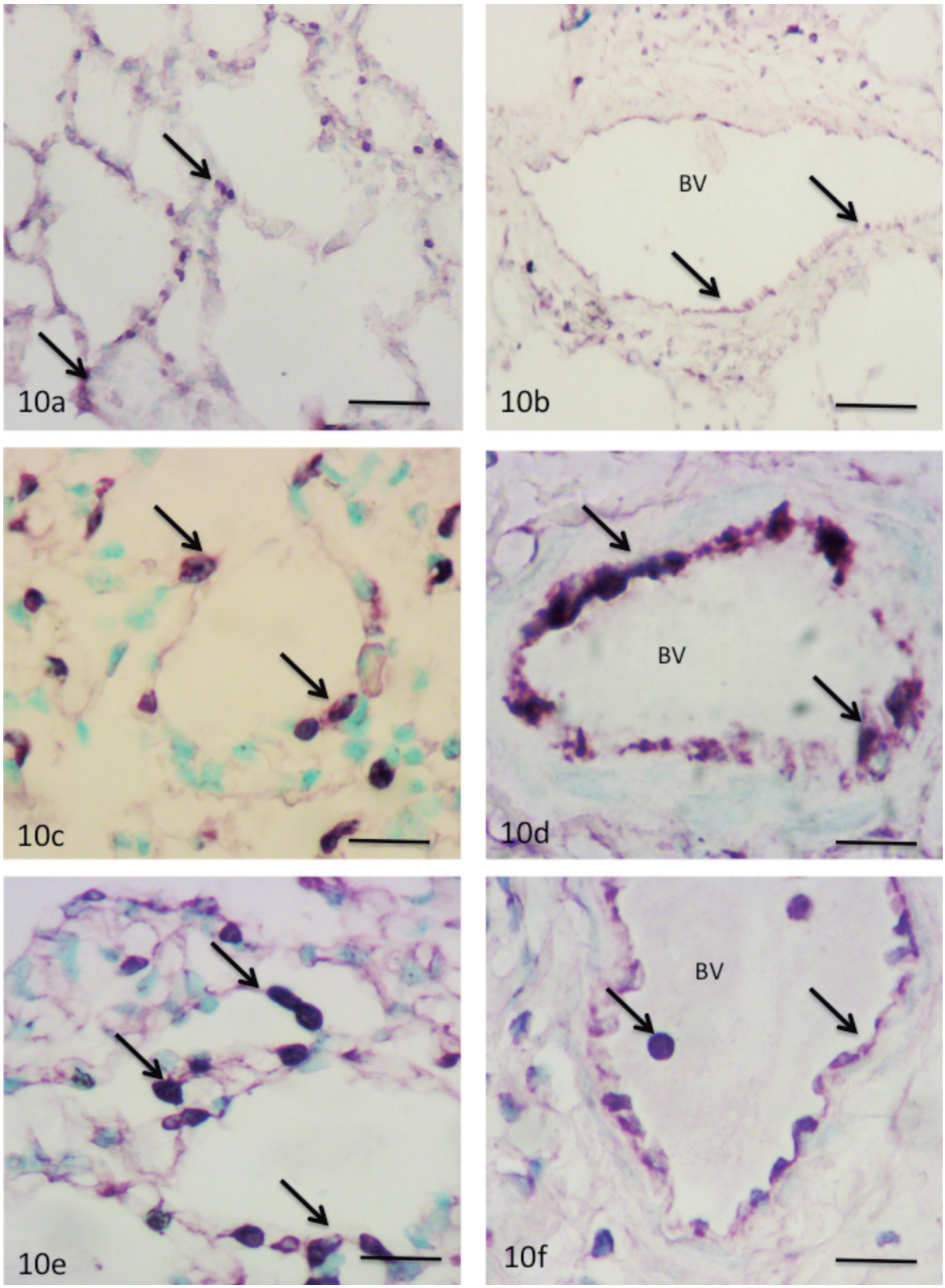

Fig. 10. TLR9 staining (arrows) is localized in alveolar septal cells in lungs from non-anesthetized horses (**a**) and the intensity of staining is increased in septal cells in lungs from IR+LRS (**c**) and IR+lidocaine (**e**) horses. Compared to TLR9 staining in the endothelium (arrows) in blood vessels (BV) in anesthetized control lungs (**b**), increased intensity of staining was seen in the endothelium (arrows) of blood vessels (BV) in lungs from IR+LRS (**d**) and IR+lidocaine (**f**) horses. Bar: 50 μm.

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