Preconditioning with lidocaine and xylazine in experimental equine jejunal ischaemia

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Abstract
Background: Pharmacological preconditioning of dexmedetomidine on small intestinal ischaemia/reperfusion injury has been reported in different animal models including horses.

Objectives: The objective was to assess if xylazine and lidocaine have a preconditioning effect in an experimental model of equine jejunal ischaemia.

Study design: Terminal in vivo experiment.

Methods: Ten horses under general anaesthesia were either preconditioned with xylazine (group X; n = 5) or lidocaine (group L; n = 5). A historical untreated control group (group C; n = 5) was used for comparison. An established experimental model of equine jejunal ischaemia was applied, and intestinal samples were taken pre-ischaemia, after ischaemia and following reperfusion. Histomorphological examination was performed based on a modified Chiu score. Immunohistochemical staining for cleaved caspase-3, TUNEL and calprotectin was performed, and positive cell counts were expressed in cells/mm².

Results: There was no progression of histomorphological mucosal injury from ischaemia to reperfusion, and there were no differences in histomorphology between the groups. After ischaemia, group X had significantly less caspase-positive cells compared to the control group with a median difference of 227% (P = .01). After reperfusion, group X exhibited significantly lower calprotectin-positive cell counts compared to the control group, with a median difference of 6.8 cells/mm² in the mucosa and 44 cells in the serosa (P = .02 and .05 respectively). All groups showed an increase in caspase- and calprotectin-positive cells during reperfusion (P < .05). TUNEL-positive cells increased during ischaemia, followed by a decrease after reperfusion (P < .05).

Main limitations: The small sample size and the use of a historical control group. Preconditioning effects of the tested drugs may be masked by the protective effects of isoflurane in the anaesthetic protocol.

Conclusions: Preconditioning with lidocaine did not have any effect on the tested variables. The lower cell counts of caspase- and calprotectin-positive cells in group X may indicate a beneficial effect of xylazine on ischaemia/reperfusion injury. Due
INTRODUCTION

Small intestinal strangulation with concurrent ischaemia/reperfusion injury is a major cause of mortality in horses. An increasingly popular mechanism for the treatment of ischaemic lesions in human medicine is ischaemic preconditioning. This refers to the activation of intrinsic cell survival programs after exposure to mild ischaemic stimuli or pharmacologic agents, and several studies have demonstrated the beneficial effect on the survival of intestinal tissue.

Besides mechanical ischaemic preconditioning, many different pharmacological agents, like volatile anaesthetics and alpha-2 agonists, have been shown to activate these protective cell responses. Some authors have reported the pharmacological preconditioning effect of the alpha-2 agonist dexmedetomidine on intestinal injury in rabbits and rats. Moreover, a protective effect of dexmedetomidine was identified in an experimental model of equine small intestinal strangulation. This beneficial effect may be due to its anti-inflammatory and antiapoptotic properties. To the authors’ knowledge, there are no reports on a preconditioning effect of xylazine, a more commonly used alpha-2 agonist in horses.

Lidocaine is the most commonly used prokinetic drug in the perioperative management of horses undergoing colic surgery. In experimental models, it has been shown to have a beneficial effect on the intestine after ischaemia and reperfusion, by decreasing intestinal oedema, decreasing COX-2 expression in intestinal mucosa and by limiting the increased gut wall permeability compared to flunixin-meglumine administration alone. In a more recent experimental study, the horses treated with lidocaine did not show a consistent decrease in intestinal neutrophil infiltration compared to the untreated horses. Currently, the exact mechanism of these actions remains unclear. No studies have explored if there is a preconditioning effect of lidocaine in horses.

The aim of this study was to investigate whether xylazine and lidocaine, which are routinely used in the management of small intestinal colic, have a preconditioning effect on equine jejunal ischaemia. The objective was to describe histomorphology, apoptosis and inflammatory cell count in the intestinal tissue undergoing experimental ischaemia, and to compare these results between different treatment groups. We hypothesise that preconditioning with xylazine or lidocaine will ameliorate ischaemia/reperfusion injury. Identifying a preconditioning effect of either pharmacologic agent would support its use in sedation protocols and anaesthetic regimens for horses with colic.

MATERIALS AND METHODS

Animals

For this terminal in vivo experiment, 10 adult Warmblood horses were randomly assigned to a lidocaine (group L; n = 5) or a xylazine (group X; n = 5) group. Group L comprised of three mares, one gelding and one stallion, with an age range of 9-19 years (12 ± 4 years, mean ± SD) and the weight ranging between 540 and 619 kg (573 ± 31 kg). Group X comprised of four mares and one stallion, with an age range of 4-21 years (14 ± 8 years) and the weight between 520 and 705 kg (577 ± 76 kg). A historical control group consisting of five Warmblood horses, with an age range of 2-14 years (5.4 ± 4.9 years) and the weight between 464 and 610 kg (544 ± 53 kg), was used to limit the amount of horses needed. This control group was taken from our previous study evaluating the preconditioning effect of dexmedetomidine, using the same experimental model, anaesthetic regime and sample preparation as the current study. The horses were acquired by the equine hospital for educational purposes and were subjected to euthanasia due to problems unrelated to the gastrointestinal tract, such as orthopaedic disease. All horses were systemically healthy without any signs of gastrointestinal disorders. At least 2 weeks prior to surgery, the horses were stabled and no medication was administered during this time. The horses had free access to hay and water and were hand-walked daily. Feed but not water was withheld before surgery for six hours.

Anaesthetic protocol and monitoring

Before the procedure, a 12-gauge Teflon catheter (Intraflon, Vygon GmbH) was placed in the left jugular vein. In the control group (group C; n = 5), anaesthesia was induced without prior sedation. The horses were infused with 5% guaifenesin (My-50 mg/mL, CP-Pharma GmbH) until ataxia was apparent. At this point, 0.05 mg/kg diazepam (Zlapam 5 mg/kg, EcuPhar GmbH) and 2.5 mg/kg ketamine (Narketan, Vétoquinol GmbH) were administered to induce general anaesthesia. Orotracheal intubation was performed and anaesthesia was maintained with isoflurane (Isofluran CP, CP-Pharma GmbH) in 100% oxygen. The horses in group L were anaesthetised according to the same protocol, and additionally received a loading dose of 1.3 mg kg BW lidocaine (Lidocain 2%, Bela-Pharm GmbH) over 10 minutes prior to induction. Subsequently, a continuous rate infusion (CRI) of lidocaine at a rate of 0.05 mg/kg/min was instituted within 5 minutes after induction. Group X, received a loading dose...
of 1 mg/kg xylazine (Xylavet 20 mg/mL, CP-Pharma GmbH) over 10 minutes prior to induction of anaesthesia, followed by a CRI of 1 mg/kg/h. In group C, lactated Ringer’s solution (Ringer-Laktat EcobagClick, B. Braun Melsungen AG) and dobutamine (Dobutaminratiopharm 250 mg, Ratiopharm GmbH) were given at a constant rate of 5 mL/kg/h and 0.5 µg/kg/min respectively. In groups L and X, lactated Ringer’s solution was started at 5 mL/kg/h and increased stepwise in increments of 5 mL/kg/h to a maximum of 20 mL/kg/h to maintain a mean arterial blood pressure (MAP) above 60 mm Hg. This was supplemented by a CRI of dobutamine at a rate of 0.3 µg/kg/min if the initial increase in intravenous fluid rate did not have an effect, and subsequently titrated with increments of 0.3 µg/kg/min. This stepwise approach was continued until the MAP reached the desired level above 60 mm Hg. If the MAP rose above 80 mm Hg, the rates were decreased in the same manner. During the procedure, cardiovascular and respiratory values were monitored and documented every 10 minutes. Direct arterial blood pressure measurement and arterial and mixed venous blood gas analyses were performed, as well as cardiac output measurement by thermodilution as described previously. The cardiac index (CI) was determined by dividing the cardiac output by the bodyweight, and the oxygen extraction ratio (OER) was calculated as the ratio of the difference between the arterial and mixed venous blood oxygen content to the arterial blood oxygen content.

### 2.3 Surgical procedure and sample collection

After induction of anaesthesia, the horses were positioned in dorsal recumbency. Sixty minutes after the induction of anaesthesia, a routine pre-umbilical midline laparotomy was performed. At 90 minutes, the distal jejunum was exteriorised and a 10-cm intestinal segment located 1 m oral to the jejunooileal junction was excised after ligation of the blood vessels and the intestinal lumen (pre-ischaemia [P] sample). Subsequently, ischaemia was induced in a jejunal segment located 2 m oral to the jejunooileal junction by occluding the intestine and mesentry with umbilical tape under monitoring of tissue blood flow and saturation by micro-lightguide spectrophotometry and laser Doppler flowmetry (O₂C Oxygen to see LEA Medizintechnik GmbH), until the blood flow was reduced to 10% of the preligation measurement. Ninety minutes after initiation of low-flow ischaemia, a 10-cm intestinal segment was excised from the ischaemic area (ischaemia [I] sample). Subsequently, the ligature was released under monitoring with the O₂C® to confirm restoration of blood flow. After 30 minutes of reperfusion, an intestinal segment from the previously occluded area was resected (reperfusion [R] sample). After the last sample was taken, the horses were subjected to euthanasia by intravenous administration of 90 mg/kg pentobarbital (Release 50 mg/mL, WDT eG) without regaining consciousness.

### 2.4 Sample preparation

The intestinal samples were fixed in 4% formaldehyde and embedded in paraffin. Four micrometre thick sections were cut and the slides were stained routinely with haematoxylin and eosin (H&E) for histomorphological examination. Immunohistochemical staining was performed for cleaved caspase-3 as marker for apoptosis, and for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) as marker for late-apoptosis and cell necrosis. The staining for cleaved caspase-3 was performed using commercial antibodies (Cleaved Caspase-3Asp175 antibody, Cell Signaling Technology Europe B.V.). The complete staining protocol is provided in Data S1. A commercial kit was used for the immunohistochemical staining of TUNEL (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Merck KGaA). This was performed according to the manufacturer’s instructions. Furthermore, immunohistochemical staining for cytosolic calprotectin was performed using monoclonal mouse anti-human myeloid/histiocyte antigen (clone MAC 387, DakoCytomation) as described elsewhere. 

### Table 1 Description of the modified Chiu score with a separated villous and haemorrhage score

<table>
<thead>
<tr>
<th>Modified Chiu score</th>
<th>Villi score</th>
<th>Haemorrhage score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal mucosal villi</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Development of subepithelial (Gruenhagen’s) space at the apex of the villus</td>
<td>Dilated capillaries in the lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>Extension of the subepithelial space with moderate lifting of the epithelium</td>
<td>Local haemorrhage in the lamina propria</td>
</tr>
<tr>
<td>3</td>
<td>Severe epithelial separation down the villus sides, until halfway down the villus</td>
<td>Diffuse haemorrhage in the lamina propria</td>
</tr>
<tr>
<td>4</td>
<td>Denuded villi with the lamina propria exposed</td>
<td>Subepithelial haemorrhage</td>
</tr>
<tr>
<td>5</td>
<td>Digestion and disintegration of the lamina propria and ulceration</td>
<td>Massive haemorrhage</td>
</tr>
</tbody>
</table>
2.5 | Histomorphological and immunohistochemical examination

The histomorphological and immunohistochemical examination of all the slides including those of the historical control group was performed by the same observer, after being blinded to the sample type and group assignment. This observer (N.V.) was trained by an experienced histologist (C.P.). The histomorphological properties of the mucosa were assessed by light microscopy (AXIO Scope.A1, Carl Zeiss GmbH) in H&E-stained slides in 10 adjoined high-power fields (HPFs) at a 400-fold magnification using a modified Chiu score.17,18 One slide per time point per horse was assessed. Each field of view was scored individually, and subsequently averaged to make up the final score of each slide. In this modified Chiu score, the villous morphology and the degree of haemorrhage in the tissue were scored separately (Table 1).

The TUNEL- and caspase-3-positive cells in the mucosa were counted in 10 adjoined HPFs per slide. Using a microscope camera and accompanying software (Axiocam 105 color and Software ZEN 2.3, Carl Zeiss GmbH), the exact surface area was determined in mm² and the apoptotic cell count was expressed in cells/mm². For the calprotectin stain, the number of positive cells in the mucosa was counted in five adjoined HPFs per slide and the count was expressed as cells per mm². Additionally, the positive cells within 30 randomly identified submucosal venules were counted. In the submucosa and muscularis, very few positive cells were located outside the vasculature (<1/HPF), hence these layers were not included in the counts. Positive cells in the serosa were counted over the width of five HPFs.

2.6 | Data analyses

Prior to commencing the study, a power calculation was performed with free software (G*Power 3.1.9.2, Heinrich Heine Universität). To detect a difference of 1 grade in the histomorphology score between the treatment groups with a standard deviation of 0.5, based on a power of 0.8 and alpha of 0.05, a total sample size of 10 horses was required.

Statistical analysis and graph design were performed with commercial software (SAS 9.4m5 with the Enterprise Guide Client 7.15 (SAS Institute Inc.) and Graphpad Prism 7.0e, Graphpad Software Inc.). P ≤ .05 was considered significant. Testing for normal distribution was done by visual assessment of the qq-plots of the model residuals and the Shapiro-Wilk test. Variance homogeneity in the groups was assessed by visual assessment of box and whisker plots and Levene’s test (for ANOVA). The normal distributed cardiovascular parameters HR, MAP, CI and OER as well as the end-tidal isoflurane concentration were expressed as mean (±SD). The data that were neither normal nor lognormal distributed (histomorphology scores and the cell counts for TUNEL, cleaved caspase-3 and calprotectin) were expressed as median (min-max). For the latter, distribution-free nonparametric models were used for independent (treatment and control groups) and correlated effects (time points). To correct for the variation in the number of apoptotic cells in the pre-ischaemia samples, the positive cell counts of the TUNEL- and caspase-stained slides were rescaled and expressed as a percentage of the cell count in the pre-ischaemia sample (relative cell count). A Kruskal-Wallis test in combination with a Dunn’s multiple comparisons test was used to compare the results between the different groups at each time point, assessing the histomorphology score, the relative cell counts in percentages in the caspase- and TUNEL-stained slides and the absolute cell counts of the calprotectin-stained slides. For comparing the correlated different time points, a permutation test (as exact Friedman test) was used,19 with the post hoc Sidak test for multiple pairwise comparisons, complying the experimentwise error rate. These calculations were done with the SAS macro RIBDPERM.

Hypocaudal parameters HR, MAP, CI and OER, a two-way analysis of variance (ANOVA) was performed for one independent effect (group), and the time points as repeated effect. This was implemented to compare the values between the different time points and groups, with the horses as subject effect and compound symmetry as covariance structure, taking the interaction term into account. Multiple pairwise comparisons were performed with a post hoc Tukey test. End-tidal isoflurane concentrations, dobutamine rates and intravenous fluid rates were averaged over all time points for each horse, and a one-way ANOVA was used to compare the three groups for these variables.

3 | RESULTS

3.1 | Anaesthetic parameters

The mean end-tidal concentrations of isoflurane (ET iso) to maintain an adequate depth of anaesthesia were 1.45 (±0.07) vol%, 1.21 (±0.1) vol% and 1.18 (±0.13) vol% for groups C, L and X respectively. This was significantly higher in group C compared to group L (P = .008, mean difference 0.24, confidence interval [CI] 0.06-0.41) and group X (P = .004, mean difference 0.27, CI 0.09-0.20). The mean dobutamine rate during the anaesthetic period was 0.5 (±0.00), 0.4 (±0.36) and 0.17 (±0.13) µg/kg/min for groups C, L and X, respectively, without a significant difference between the groups (P = .09). The mean fluid rate was 5.0 (±0.00), 9.82 (SD 3.14) and 8.05 (SD 2.15) mL/kg/h for groups C, L and X respectively. The fluid rate was significantly higher in group L compared to group C with a mean difference of 4.8 mL (CI −8.53 to −1.11, P = .01). Selected cardiovascular and oxygenation variables and comparison between the different groups are listed in Table S1.

3.2 | Histopathological evaluation

Compared to the pre-ischaemia sample, the villous score was significantly increased in all groups after ischaemia and reperfusion (P = .02
for groups C and L, $P = .01$ in group X), but no difference could be detected between ischaemia and reperfusion (Figure 1). One horse from group X had a very low score for villous morphology at both ischaemia and reperfusion, with an average score of $1.2 \pm 0.4$ SD and $1.1 \pm 0.3$ SD respectively. The haemorrhage score was 0 or 1 for all horses in the pre-ischaemia samples, and there was a significant increase of the score after ischaemia ($P = .02$). In group X, the cell count had increased significantly after ischaemia and reperfusion ($P = .02$ and $P = .03$ for group L and $P = .03$ and $P = .02$ for group X) (Figure 1). There was no statistically significant difference for either score between any of the experimental groups.

Calprotectin-positive cells were seen in the mucosa of all slides. Compared to pre-ischaemia, all groups had a higher mucosal cell count after ischaemia ($P = .02$, $P = .02$ and $P = .03$ for groups C, L and X respectively) and reperfusion ($P = .02$, $P = .02$ and $P = .03$ for groups C, L and X respectively) (Figure 2). In group X, the cell count was significantly lower during reperfusion compared to the control group with a median difference of 6.8 cells/mm² ($P = .02$). In the submucosal venules, a range of 0-4 calprotectin-positive cells per slide was found in the pre-ischaemia sample. In groups L and X, the cell count had increased significantly after ischaemia and reperfusion compared to pre-ischaemia ($P = .02$ and $P = .03$ for group L and $P = .02$ and $P = .02$ for group X). There were no statistically significant differences at any time point between the groups. In the serosa, no calprotectin-positive cells were found in the pre-ischaemia samples. After ischaemia, only one horse in group L and one horse in group X had positive cells (two and eight cells respectively). In the reperfusion sample, the cell count was 94 (86-117), 10 (1-105) and 12 (4-44) for groups C, L and X respectively (Figure 3). This was a statistically significant increase compared to both the pre-ischaemia ($P = .02$, $P = .02$ and $P = .03$ for groups C, L and X respectively) and ischaemia ($P = .03$, $P = .03$ and $P = .02$ for groups C, L and X respectively). Group X had a significantly lower serosal cell count after reperfusion compared to group C ($P = .05$). No significant difference could be detected between group L and the two other groups ($P > .99$ and $P = .07$ for comparison with groups X and C respectively).

The pre-ischaemic samples all revealed caspase-3- and TUNEL-positive cells (Table 2). After ischaemia, the caspase cell count increased significantly in groups C and L ($P = .02$ and $P = .007$ respectively) (Figure 4). Group X had a significantly lower relative cell count compared to the control group at this time point, with a median difference of $227\%$ ($P = .01$) (Figure 4). During reperfusion, the caspase-positive cell counts increased significantly in all groups ($P = .02$) (Figure 5). For the TUNEL-positive cells, a significant increase between pre-ischaemia and ischaemia was noted in all groups ($P = .03$, $P = .02$ and $P = .03$ for groups C, L and X respectively), as was a significant decrease between ischaemia and reperfusion ($P = .02$, $P = .03$ and $P = .02$ for groups C, L and X respectively) (Figure 6). There were no significant differences between the groups at any time point. The cell debris at the mucosal surface of the ischaemia samples contained only a very low number of TUNEL-positive cells (<5 cells/HPF), whereas the debris of the reperfusion samples contained many positive cells (>50 cells/HPF).

**FIGURE 1** Box-plot diagram of the separated Chiu score for villous histomorphology and haemorrhage. The horizontal bar displays the median, the interquartile range is represented by the box and the minimum and maximum by the whisker plots. There were no significant differences between the groups. Significant differences ($P < .05$) between different time points within the groups are marked with an asterisk. P, pre-ischaemia; I, ischaemia; R, reperfusion.

**FIGURE 2** Box-plot diagram of the calprotectin-positive cells in the mucosa, expressed as cells per mm². The horizontal bar displays the median, the interquartile range is represented by the box and the minimum and maximum by the whisker plots. Significant differences ($P < .05$) are marked with an asterisk. P, pre-ischaemia; I, ischaemia; R, reperfusion.

**FIGURE 3** Microscopic images of the serosa with an original magnification of 400 (see scale bar on images). These sections were immunohistochemically stained for cytosolic calprotectin, and represent the reperfusion sample of a horse belonging to group C (A) and a horse in group X (B). The arrows indicate representative examples of calprotectin-positive cells, and the arrowheads indicate the border between the tunica serosa and tunica muscularis.
DISCUSSION

This is the first study investigating the preconditioning effect of lidocaine and xylazine in experimental small intestinal ischaemia in horses. No significant differences in histomorphologic changes could be detected between the groups C, L and X at any time point. The mucosal damage induced during ischaemia did not progress after reperfusion, but the number of mucosal apoptotic cells and serosal inflammatory (calprotectin-positive) cells did increase during reperfusion. The main finding was that, compared to the control group, xylazine treatment resulted in a lower apoptotic cell count after ischaemia, and fewer inflammatory cells in the mucosa and serosa after reperfusion.

These results might indicate a protective effect of xylazine on ischaemia/reperfusion injury, even though this was not supported by fewer histomorphological injury. A protective effect has also been described for the more selective alpha-2 agonist dexmedetomidine.6 The use of xylazine for clinical cases may be more feasible, as this drug is licensed for horses and already part of many established anaesthetic regimens. Other alpha-2 agonists, like detomidine and

| TABLE 2 | Cleaved caspase-3 and TUNEL-positive cells |
|------------------|------------------|------------------|
|                  | Cleaved caspase-3 | TUNEL            |
|                  | Group C         | Group L         | Group X         | Group C         | Group L         | Group X         |
| Pre-ischaemia    | 10.4 (1.8-12.0) | 4.7 (1.7-8.4)   | 5.8 (3.0-10.4)  | 15.8 (13.1-20.0)| 11.2 (5.0-16.6)| 8.0 (2.1-27.7) |
| Ischaemia        | 32.9 (13.4-41.4)| 8.7 (5.1-15.2)  | 4.6 (3.5-14.2)  | 37.5 (22.7-44.0)| 21.2 (9.7-22.3)| 11.3 (10.0-28.8)|
| Reperfusion      | 80.9 (37.0-156.5)| 113.3 (27.7-143.0)| 37.8 (7.4-117.6)| 18.7 (15.7-22.4)| 9.0 (0.9-13.8)| 5.9 (5.5-11.9) |

Note: The positive cell count for the cleaved caspase-3 and TUNEL immunohistochemistry of the different groups and time points, expressed as positive cells per mm² (median, minimum-maximum). Group C is the control group, and groups L and X are preconditioned with lidocaine and xylazine respectively.

**Figure 4** Box-plot diagram of the cleaved caspase-3-positive cells in the mucosa, expressed as percentage of the cell count in the pre-ischaemia sample. The horizontal bar displays the median, the interquartile range is represented by the box and the minimum and maximum by the whisker plots. Significant differences (P < .05) are marked with an asterisk. P, pre-ischaemia; I, ischaemia; R, reperfusion

**Figure 5** Microscopic images of the intestinal mucosa after immunohistochemical staining for cleaved caspase-3 in an ischaemia (A) and reperfusion (B) sample of the same horse. Original magnification 100× (see bars on images). The arrows indicate representative examples of caspase-positive cells.

**Figure 6** Box-plot diagram of the TUNEL-positive cells in the mucosa, expressed as percentage of the cell count in the pre-ischaemia sample. The horizontal bar displays the median, the interquartile range is represented by the box and the minimum and maximum by the whisker plots. Significant differences (P < .05) are marked with an asterisk. P, pre-ischaemia; I, ischaemia; R, reperfusion.

4 | DISCUSSION
romifidine, might be capable of inducing comparable effects; however, no studies on the preconditioning effect of these drugs have been performed so far.

The current study did not demonstrate an influence of lidocaine preconditioning on any of the tested intestinal variables. Other studies have reported beneficial effects of lidocaine on the intestine after ischaemia/reperfusion injury, considering different measurements like intestinal permeability and oedema.\textsuperscript{8,10} The effect of lidocaine on intestinal neutrophilic inflammation in horses has not been consistent in previous studies. One author reported reduced mucosal neutrophil counts when treatment with flunixin-meglumine was combined with lidocaine administration,\textsuperscript{9} while another found no consistent decrease in neutrophil tissue infiltration.\textsuperscript{11} The exact mode of action of lidocaine on inflammatory cells has not been clarified; however, a recent study using murine neutrophils discovered that lidocaine influences the pivotal function of neutrophil sodium channels, thereby inhibiting their adhesion and migration.\textsuperscript{20} On the contrary, an in vitro experiment on equine neutrophils found that lidocaine did not inhibit neutrophil migration or adhesion at therapeutic concentrations, and even increased migration and adhesion at higher concentrations.\textsuperscript{21}

The MAC387 stain used in the current study to identify inflammatory cells is not specific for neutrophils, as macrophages and monocytes also express cytosolic calprotectin during inflammation.\textsuperscript{22} However, a significant correlation between neutrophils identified histomorphologically and calprotectin-positive cells has been found in the equine colon.\textsuperscript{23} Therefore, it is believed that this immunohistochemical stain provides a good estimate of the neutrophil count in the equine intestine. In the current study, a significant increase in calprotectin-positive cells was noted during reperfusion. Neutrophilic inflammation, initiated by the presence of superoxide radicals during reperfusion, has been indicated as a cause of reperfusion injury.\textsuperscript{2} On the contrary, the influx of neutrophils may be part of normal tissue repair, and one cannot assume a direct relationship between higher inflammatory cell counts and increased ischaemia/reperfusion injury.

The occurrence of reperfusion injury in small intestinal strangulation in horses is under debate, and it has been suggested that this does not contribute significantly to injury in clinical cases.\textsuperscript{24} The results of the caspase-3 immunohistochemistry could indicate an effect of reperfusion in the current model, although this did not appear to cause mucosal changes on histological examination. It has been suggested that reperfusion injury may be more likely to occur in models of low-flow ischaemia, where the blood flow is typically reduced to 20%.\textsuperscript{25} In the current study, the flow was reduced to 10%, verified by Doppler flowmetry. This model was chosen to induce significant injury without causing severe intestinal necrosis, because the latter would preclude any benefit from protective effects. The venous and arterial blood flow in clinical strangulating obstructions may depend on the type and the duration of the strangulation, and larger clinical studies on blood flow are lacking. Therefore, it remains difficult to establish a solid comparison between experimental ischaemia and equine strangulating colic.

Anaesthetic maintenance with isoflurane could have influenced the results by masking or diminishing preconditioning effects of the tested drugs. The protective actions of volatile anaesthetics like isoflurane and sevoflurane on ischaemia/reperfusion injury have been reported in the literature in different organs including the intestine.\textsuperscript{26} In the current study, isoflurane could have ameliorated ischaemia/reperfusion injury across the groups, which might explain the lack of progression of histomorphological damage from ischaemia to reperfusion. Considering all horses were anaesthetised with isoflurane, this effect would be present across all groups. There is evidence indicating that the protective effect of isoflurane is dose-dependent.\textsuperscript{27} In the current study, the mean isoflurane concentration was higher in the control group; however, this group did not have better results for any of the tested variables.

The anaesthetic protocols of this study are not feasible in a clinical situation, as general anaesthesia was induced without a sedative premedication in the control group and in the lidocaine group. This set-up was chosen to avoid the influence of an additional sedative on the studied variables. Proper handling and quiet surroundings provided an acceptable quality of induction in all horses. Moreover, possible stress factors like instrumentation with the cardiac catheters were postponed until after induction of anaesthesia. The cardiac output and central venous blood gas values were not determined in the control group, therefore, CI and OER could not be calculated for these horses. When assessing the cardiovascular variables, the xylazine group had a significantly lower CI compared to the lidocaine group during ischaemia and reperfusion. However, there were no significant differences in OER at these time points, indicating that the oxygen supply was not significantly affected by this difference in CI. Group L received more intravenous fluids than group C, which is most likely a consequence of the fixed fluid rate in group C. Because there were no histomorphological or immunohistochemical differences between these two groups, the relevance of this finding remains unclear. Dobutamine was administered at relatively low dosages of ≤0.5 µg/kg/min in all groups. According to a study assessing different dobutamine rates in horses, infusion of 0.5 µg/kg/min did not significantly affect the intestinal microperfusion.\textsuperscript{28} Therefore, it is unlikely that minor differences in the dose range between the groups have affected the results.

One horse in group X did not show the same histomorphological changes as the others, with almost no changes in the villous structure during ischaemia and reperfusion. The micro-lightguide spectrophotometry and Doppler flowmetry measurements indicated correct placement of the ligature, and the apoptotic cells and calprotectin-positive cell counts of this horse were comparable to the other individuals of the group. Taking these observations into account, this horse was not excluded from the study.

Caspase- and TUNEL-positive cells could be detected in all slides, which is to be expected as apoptosis occurs as a part of the intestine’s normal function. Cleaved caspase-3 is activated during early apoptosis and plays an important role in the cascade leading to DNA-cleaving, whereas TUNEL is a less specific indicator that detects the cleaved DNA that is associated with nuclear change during apoptosis or cell...
necrosis. Considering the short time span and the lack of a comparable rise in caspase-positive cells, the high TUNEL-positive cell count in the ischaemia sample may represent necrotic cells rather than apoptotic cells. The decrease in this cell count during reperfusion may be explained by the observed increase in TUNEL-positive cell debris, possibly indicating the separation of these cells. The low TUNEL cell count in the reperfusion sample compared to the increased caspase cell count may reflect the difference in the apoptotic stage that is detected, because these cells may not have reached the phase of DNA cleavage yet.

Other limitations of this study are the small sample size and the use of a historical control group. Both were the consequences of limiting the amount of horses needed, and a power analysis performed prior to the study indicated sufficient power with this number of horses. We believe that the results of the historical group are comparable with the results of the test groups, as all aspects of the experiment were performed according to the same protocol and under guidance of those involved in the previous study. With regard to the comparability of the horses in the different groups, the horses in the control group appear to be younger than those in groups L and X. Even though a statistically significant difference could not be detected, an effect of age on the test results cannot be excluded. Another limitation of the study design was that only the short-term effects of preconditioning could be examined, and thereby limiting this to the early phase of protection. Furthermore, only one observer graded the histology, and the site of tissue sampling was not randomised.

The occurrence of equine strangulating colic is unpredictable, thereby precluding the application of preconditioning before the ischaemic insult has commenced. Nevertheless, there may still be blood flow to the tissue in the early stages of ischaemia, presenting the opportunity to precondition the tissue incorporated within the lesion. For the type of strangulating obstructions where increasingly more intestine is incorporated over time, the surrounding intestinal segments may be preconditioned before their blood supply is affected. Furthermore, several studies have found that prestenotic and remote intestinal segments also sustain injury. Therefore, there are several situations where the concept of preconditioning could be a feasible therapeutic strategy to reduce intestinal ischaemia/reperfusion injury in colic horses.

In conclusion, the results of this study indicate a beneficial effect of xylazine on apoptosis rate and inflammation. A concurrent reduction in mucosal histomorphological injury could not be found; therefore, the clinical significance of these findings remains uncertain. Preconditioning with lidocaine did not have any effect on the tested variables. The results may support the use of xylazine in sedative analgesia and anaesthetic protocols for horses with strangulating small intestinal lesions. The administration of xylazine for this indication may be most appropriate in the time frame between the diagnosis of suspected small intestinal strangulation and surgical correction of the lesion. Further research assessing the long-term effects on intestinal injury and survival is necessary to establish the value of preconditioning in clinical cases.

ETHICAL ANIMAL RESEARCH
The study was reviewed by the Ethics Committee for Animal Experiments of Lower Saxony, Germany, and approved according to §8 of the German Animal Welfare Act (LAVES 33.8-42502-04-17/2595).

OWNER INFORMED CONSENT
Not applicable.

DATA ACCESSIBILITY STATEMENT
The data that support the findings of this study are openly available under the following reference: Verhaar, Nicole (2019), ‘Preconditioning with lidocaine and xylazine in experimental equine jejunal ischaemia’, Mendeley Data, v1 http://dx.doi.org/10.17632/bg7f74ns6z.1

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AUTHOR CONTRIBUTIONS
All authors contributed to the manuscript. N. Verhaar contributed to the study design and execution, and performed the data analysis and interpretation. C. Pfarrer and S. Kästner contributed to the study design as well as the data analysis and interpretation. N. Neudeck, L. Twele and K. König contributed to the study design and its execution. K. Rohn contributed to the data analysis.

CONFLICT OF INTEREST
No competing interests have been declared.

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REFERENCES

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.