

The use of slaughterhouse-obtained small intestinal tissue as control material in histological studies should be applied with prudence

K. De Ceulaer¹, C. Van Ginneken^{1,2}, C. Delesalle^{2,3}, L. Van Brantegem^{1,2}, P. Deprez² and A. Weyns¹

¹Laboratory of Veterinary Anatomy, Embryology and Pathology, Department of Veterinary Sciences, University of Antwerp, Antwerp, Belgium, ²Department of Large Animal Internal Medicine and Clinical Biology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium and ³Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Summary. This study aimed to evaluate the reliability of slaughterhouse-obtained small intestinal tissue as control material in equine colic research where molecular stress responses in small intestinal tissue are investigated. For this purpose, small intestinal samples from colic horses were collected during surgery or immediately after euthanasia at the oral border of strangulation resection sites and routinely processed for histopathology (i.e. rinsed with 4°C Krebs' solution, fixated overnight with 4% neutral buffered formaldehyde (FH) at room temperature). Control samples consisted of pieces of mid-jejunum, collected at the slaughterhouse and routinely processed for histopathology under 4 different conditions. The 4 conditions differed with regard to incubation and fixation temperature and whether or not oxygenated Krebs' solution was used. Histological scoring revealed that slaughterhouse samples had a higher mean lesion score ($P < 0.001$) than colic samples. In addition, more slaughterhouse samples had a higher mean inflammation score than colic samples ($P = 0.001$). The inflammatory cells in the small intestine consisted mostly of eosinophils and as such were very suggestive for parasitic infestation. Hypoxia-inducible factor-1 α (HIF1 α) nuclear immunoreactivity was more pronounced in slaughterhouse tissue, probably as a result of the delay between slaughter and sampling ($P = 0.034$). The histopathological score ($P = 0.291$), the inflammation score ($P = 0.248$) and the HIF1 α nuclear immunoreactivity ($P = 0.538$) did not differ between the different collection protocols. It is concluded that slaughterhouse-obtained small intestinal tissue shows distinct alterations and that its use as control tissue when evaluating

molecular stress responses should be applied with prudence.

Key words: Horse intestine, Slaughterhouse, HIF1 α , Histology

Introduction

Horses with small intestinal strangulation often need surgery and intestinal resection, with subsequent ileus as an important post-operative complication (Holcombe et al., 2009). Post-operative ileus has numerous causes, amongst which the inflammatory and molecular stress reactions in the remaining intestines are viewed as important. Surgical handling, partial ischemia and distension of the remaining bowel are known to be important triggering factors for these reactions (Dabareiner et al., 2001; Holcombe et al., 2009).

Lack of oxygen is without any doubt the most important trigger of molecular stress pathways (Malago et al., 2002). In order to detect intestinal ischemia, hypoxia-inducible factor 1 α (HIF1 α) can be used as a marker (Koury et al., 2004). HIF1 consists of 2 subunits: HIF1 α and HIF1 β . HIF1 β is a constitutively expressed protein but HIF1 α is regulated depending on the presence of oxygen. When oxygen is present, HIF1 α is continuously synthesized and degraded by ubiquitination. In the absence of oxygen HIF1 α and HIF1 β form a complex, which translocates to the nucleus where it binds to a hypoxia response element (HRE). The HIF1 α /HIF1 β /HRE complex initiates the transcription of molecules involved in the survival of hypoxic cells by stimulating angiogenesis, glucose transport and anaerobic metabolism (Semenza, 1999,

2007; Masson et al., 2001). Besides the clear relationship between HIF1 α upregulation and hypoxia, HIF1 α is also known to be upregulated by cytokines, growth factors and tumour oncogenes (Kong et al., 2006).

In equine gastrointestinal research, slaughterhouse tissue or tissue collected from horses suffering from diseases not related to gastrointestinal problems, is used as control material for e.g. histomorphology (Domeneghini et al., 2004; Chiocchetti et al., 2009). For ethical and economic reasons the use of slaughterhouse material provides a convenient alternative to sacrificing horses to obtain control samples for research purposes. However, one can expect that slaughter procedures can influence both the quality and viability of the collected intestinal material. In this study the slaughter protocol involved stunning of the horse, followed by carotid artery and jugular vein transection. There was a time lapse of 30 to 60 min between stunning of the horse and subsequent evisceration. The latter could entail the onset of ischemic damage to the intestine and subsequent activation of molecular stress pathways rendering the tissue unsuitable as control material. Therefore our aim was to evaluate the suitability of small intestinal tissue collected at slaughter as control material to study histological alterations and signs of ischemia at the macroscopically normal, oral border of resected strangulated small intestinal segments of colic horses. Additionally it was investigated whether different protocols for collecting slaughterhouse intestinal samples influenced histological quality and HIF1 α expression.

Materials and methods

Sample collection

Intestinal samples of mid jejunum (7 cm in length) of 31 horses were taken approximately 45 min after stunning at the moment of evisceration at a local slaughterhouse. Samples were rinsed immediately in Krebs' solution (77.7g NaCl, 3.5g KCl, 2.0g MgCl₂·6H₂O, 3.7g CaCl₂·2H₂O, 1.9g NaH₂PO₄·4H₂O, 13.7g NaHCO₃, 15.5g glucose). As temperature and fixation are important factors in the preservation of tissue, tissue was collected under differing conditions. One set of samples (n=8) was briefly rinsed in Krebs' solution at room temperature (RT) and fixated by immersion in 4% neutral buffered formaldehyde (FH) (Acros Organics, Geel, Belgium) at RT overnight (ON). A second set of samples (n=10) was briefly rinsed in cooled Krebs' solution (4°C) and fixated by immersion in 4% FH at 15°C ON. A third set (n=3) was rinsed in cooled (4°C) oxygenated Krebs' solution and fixated by immersion 4% FH ON at 15°C. A last set of samples (n=10) was rinsed in cooled (4°C) oxygenated Krebs and fixated in 4% FH ON at 15°C by immersion and by filling the lumen with fixative under light pressure. After fixation, several 8 mm diameter samples were taken both

at the mesenterial and anti-mesenterial side. These samples were rinsed using 0.01 M phosphate buffered saline (pH=7.4) and subsequently paraffin embedded.

The macroscopically normal oral borders of resected strangulated small intestinal loops of surgical colic cases (n=18) were collected at Ghent University (Department of Large Animal Internal Medicine, Belgium) during surgery or immediately after euthanasia.

The horses' age ranged from <1 year to 25 years old. All horses were warmblood horses with the exception of 1 Friesian, 1 pony and 1 Haflinger. The population consisted of 6 geldings, 11 mares and 1 stallion. Samples were rinsed in cooled (4°C) Krebs' solution and fixed in 4% FH ON at RT. After fixation, 8 mm samples were taken, rinsed with 0.01 M phosphate buffered saline (pH=7.4) and paraffin embedded.

Histological evaluation

Paraffin sections (4 μ m) were made, rehydrated and routinely stained with haematoxylin (Sigma Aldrich, Saint Louis, Missouri, USA) and eosin (HE) (Merck KGaA, Darmstadt, Germany). The lesion scoring system of Chiu et al., (1970) was adapted: grade 0 entailed no lesions; grade 1 indicated the presence of subepithelial lifting of the enterocytes at the villus tip; grade 2 was characterized by the absence of enterocytes at the villus tip; in grade 3 the enterocytes were absent halfway up the villus; grade 4 showed a denuded lamina propria, and in grade 5 the lamina propria was collapsed.

In addition, sections were scored for the presence and distribution of inflammatory cells. Most inflammatory cells encountered were eosinophils, therefore our grading system was limited to eosinophil counting. Grade 1 consisted of up to 10 inflammatory cells per high power field (HPF; 400x) (Fig. 1A); grade 2 included more than 10 but less than 40 inflammatory cells per HPF (Fig. 1B) and grade 3 included more than 40 inflammatory cells per HPF (Fig. 1C). As the highest density of inflammatory cells was encountered in the mucosa and submucosa, 3 HPF were chosen at random in that region and the average number was used to score the samples.

Immunohistochemistry

From each tissue sample, 4 μ m paraffin sections were deparaffinised, rehydrated and processed for HIF1 α immunohistochemistry. Sections were pre-treated for epitope retrieval in a Tris-EDTA buffer (pH 9) (Dakocytomation, Glostrup, Denmark) in a microwave at 90W for 10 min followed by a cool down period of 10 min. Non-specific binding was inhibited by incubating for 30 min in 1.5% H₂O₂ diluted in a 50% methanol/demineralised water solution. Primary antibody (anti-HIF1 α ; Abcam, Cambridge, United Kingdom) was applied for 20 h at 4°C in a concentration of 1:100, diluted in Trisphosphate buffered saline (TBS, pH=7.5, 121.14g Tris, 87.66 NaCl) enriched with 1% bovine

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serum albumin (Sigma Aldrich, Saint Louis, Missouri, USA) and 0.1% triton X (Sigma Aldrich, Saint Louis, Missouri, USA). After rinsing, an enzyme-conjugated secondary antibody (Envision anti-mouse; Dakocytomation, Glostrup, Denmark) was applied for 30 min. Staining was developed using 3-amino-9-ethyl-carbazole (AEC, Dakocytomation, Glostrup, Denmark). Sections were scored (grade 0 or grade 1) for absence or presence of mucosal nuclear HIF1 α immunoreactivity. The same protocol was applied in negative control tissue except for omitting the primary antibody; positive controls consisted of using the same protocol on tissue material from ischemic rat small intestine.

Statistical analysis

Results were expressed as means \pm standard error of the mean (SEM). For the statistical evaluations, SPSS[®] 18.0 (SPSS Inc, Chicago Illinois, USA) was used. All

data consist of categorical data and were analysed using a Chi-square test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Histological lesion score

The lesion score distribution of the samples collected at the slaughterhouse was 3.2% (n=1) with a lesion score of 0, 3.2% (n=1) with a lesion score of 1, 38.7% (n=12) with a lesion score of 2, 48.4% (n=15) with a lesion score of 3, 3.2% (n=1) with a lesion score of 4 and 3.2% (n=1) with a lesion score 5. Colic samples had, in general, a better lesion score compared to control samples ($P < 0.001$): 55.6% (n=10) in lesion score 0, 16.7% (n=3) in lesion score 1, 5.6% (n=1) in lesion score 2, 5.6% in lesion score 3, 11.1% (n=2) in lesion score 4 and 5.6% in lesion score 5. Tissues collected

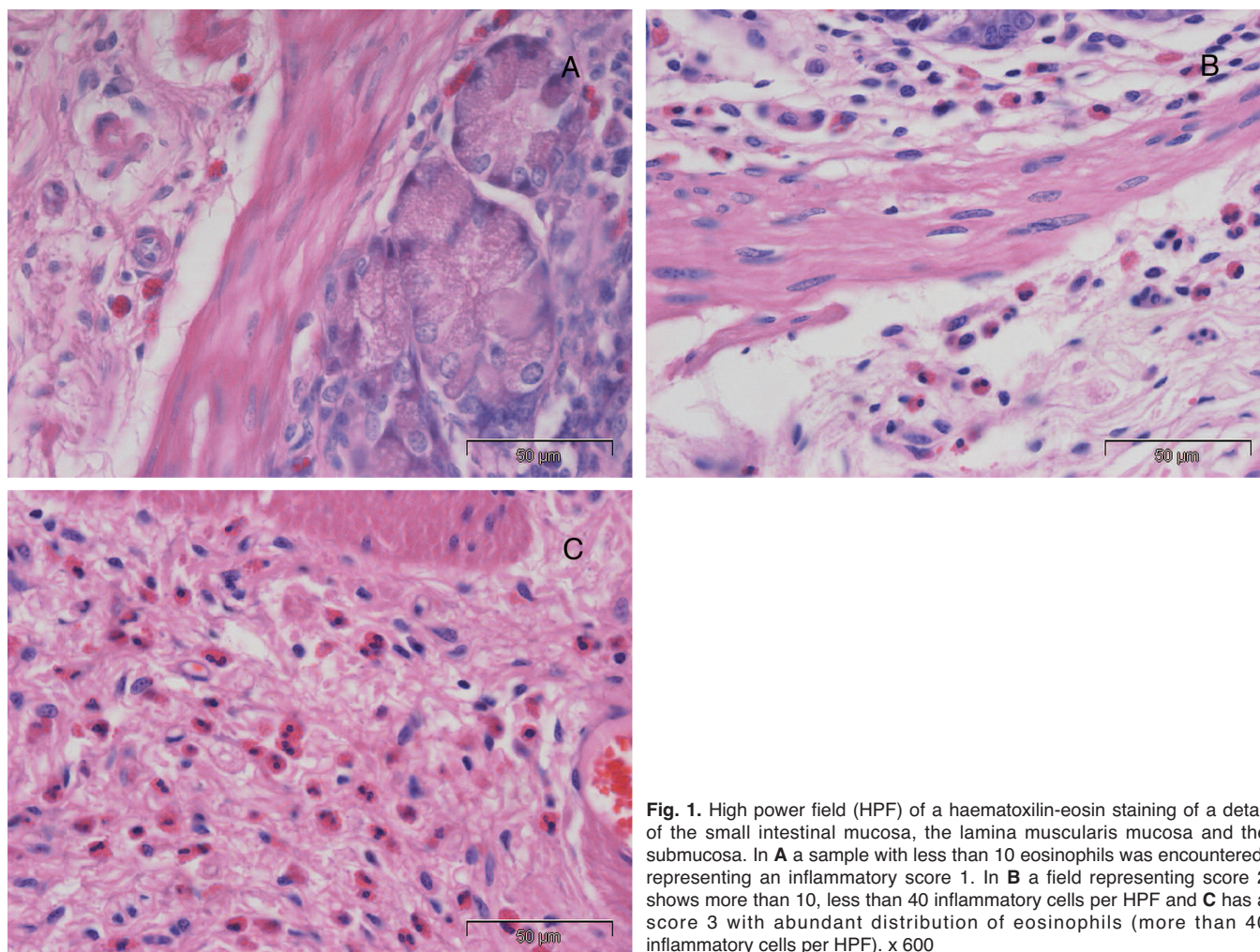


Fig. 1. High power field (HPF) of a haematoxylin-eosin staining of a detail of the small intestinal mucosa, the lamina muscularis mucosa and the submucosa. In **A** a sample with less than 10 eosinophils was encountered, representing an inflammatory score 1. In **B** a field representing score 2 shows more than 10, less than 40 inflammatory cells per HPF and **C** has a score 3 with abundant distribution of eosinophils (more than 40 inflammatory cells per HPF). x 600

Table 1. The distribution of the lesion score and the presence of HIF1 α -IR in control samples according to the fixation method.

	Fixation 1	Fixation 2	Fixation 3	Fixation 4
Score 0	0%	10%	0%	0%
Score 1	12.5%	0%	0%	0%
Score 2	37.5%	60%	0%	30%
Score 3	25%	30%	100%	70%
Score 4	12.5%	0%	0%	0%
Score 5	12.5%	0%	0%	0%
HIF1 α -IR negative	0%	10%	0%	0%
HIF1 α -IR positive	100%	90%	100%	100%

with the different collection protocols did not differ with regard to lesion score (Table 1) ($P=0.291$).

Inflammation score

The inflammation score was based on a semi-quantitative estimation of the amount of inflammatory cells present in the mucosa and submucosa of the small intestinal samples. In control samples 22.6% ($n=7$) had score 1, 32.3% ($n=10$) had score 2, and 45.2% ($n=14$) had score 3. The inflammation score did not differ between the collection protocols for the slaughterhouse samples ($P=0.248$). In colic samples, 72.2% ($n=13$) had score 1, 27.8% ($n=5$) had score 2 and no animals had an inflammation score of 3. All colic samples were infiltrated with inflammatory cells to a lesser extent than control samples ($P=0.001$).

The inflammatory cell population consisted predominantly of eosinophils with only very few neutrophils and lymphocytes. The eosinophils were mostly encountered in the submucosa with slight infiltration in the mucosa.

HIF1 α

HIF1 α -immunoreactivity (IR) was encountered in 96.8% ($n=30$) of the control samples. HIF1 α -IR was mainly seen in the enterocytes of the mucosa where it was distributed in the nucleus and/or in the cytoplasm. HIF1 α -IR enterocytes were mainly observed at the villus tips. Their amount decreased towards the crypts. HIF1 α -IR did not differ between collection protocols ($P=0.538$, Table 1).

In colic samples HIF1 α -IR was observed in 77.8% ($n=14$) of cases. The distribution of HIF1 α -IR was similar in colic and control samples. In colic horses significantly more HIF1 α -immunonegative samples were found compared to controls ($P=0.034$).

Discussion

In equine gastrointestinal research, the use of slaughterhouse tissue as a control is common practice (Domenechini et al., 2004; Chiochetti et al., 2009).

Indeed, in view of both ethical and economic considerations slaughterhouse material provides a useful alternative for the need to sacrifice healthy horses. Additionally, animals presented at the slaughterhouse are deemed to be free of disease and treatment, making this source of control tissue even more interesting. However, one should realize that some tissues are more resilient to transient hypoxia that occurs in between stunning and tissue collection, than others. Intestinal tissue is known to be highly susceptible to damage caused by ischemia. In sheep for example, small intestinal epithelial lifting was reported to occur already 14 to 16 min after death (Nicholls and Lee, 1989). In a letter to the editor, Angus and co-workers (1972) stated that post mortem lesions in the small intestine of lambs were a result of anoxic stagnation and that exsanguination accelerated this process. In our study intestinal tissue samples could only be collected approximately 45 min after stunning. Apparently, this period of time is long enough to cause important post mortem changes since in the majority of slaughterhouse samples in our study the enterocytes were absent halfway up the villus (grade 3) or had a denuded lamina propria (grade 4). In contrast, colic samples showed a lesion score between 0 and 1 in the majority of cases.

The damage seen in the slaughterhouse samples typically started with a contraction of the lamina propria creating a subepithelial space at the villus tip. Subsequently, this advances further into disruption of the epithelial basement membrane leading to shedding of the epithelial mucosal cells. Similar intestinal lesions were described by Fabiny (1959) as a result of tissue fixation with formaldehyde (FH). However, in our study FH was used as a fixative in both slaughterhouse and colic samples, which means that fixation alone cannot be held responsible for the extensive lesions seen in the slaughterhouse material.

Since preservation in a cool environment shows better post-mortem histomorphology (Gelpi et al., 2007), we tried to compare different collection protocols, such as using cooled Krebs' solution, performing FH fixation at low temperature and instilling FH under light pressure. Also, the effect of oxygenating the Krebs solution used to rinse the slaughterhouse obtained intestinal samples was evaluated. However, no difference in lesion score distribution could be observed between the applied collection protocols, which emphasises in turn the importance of the effects of the slaughter protocol on sample quality.

Intestinal ischemia-reperfusion injury is manifested as increased microvascular and mucosal permeability, and mucosal necrosis. Inflammatory cells, predominantly neutrophils, are recruited into tissues during ischemia-reperfusion (Moore et al., 1995; Gerard et al., 1999; Little et al., 2005). However, in our study predominantly eosinophil infiltration was found in the mucosal and submucosal layers of the slaughterhouse samples. Mild eosinophilic infiltration in the lamina propria of the midjejunum of healthy horses has already

been demonstrated and is to be considered as a component of the immune cell population. It can be upregulated as the result of a parasitic infestation (Packer et al., 2005). In our study this infiltration was much more pronounced in horses presented at the slaughterhouse (and presumed healthy) than in colic horses. Therefore, most probably this eosinophil infiltration was the result of parasitic infestation of the slaughterhouse horses. This finding underlines an extra disadvantage of the use of slaughterhouse obtained small intestinal samples, since anti-parasitic prophylaxis in these horses might be less rigid than in horses kept for sport purposes or as companion animals, which are the more likely candidates for colic surgery. In addition, sheep infected with the stomach parasite *Haemonchus contortus*, started to shed their epithelial cells already 6 min after slaughter (Nicholls and Lee, 1989). Apparently intestinal parasitic infestation can accelerate the onset of post-mortem intestinal tissue damage.

HIF1 α is mostly expressed as a result of oxygen deprivation (Semenza, 1999). Exsanguination results in acute generalized tissue hypoxia triggering protective pathways, of which HIF1 α is a very important molecule. Almost all slaughterhouse collected tissue samples expressed HIF1 α . This is in contrast to the colic samples, where less samples showed HIF1 α -IR. This suggests that the cranial border tissue of resected colic tissue suffered to a lesser extent from hypoxia or other stress factors than small intestinal samples collected at the slaughterhouse.

It is concluded that slaughterhouse obtained small intestinal tissue shows more pronounced alterations compared to material obtained during surgery or shortly after euthanasia and that its use as control tissue when evaluating molecular stress responses should be considered with prudence. The slaughter procedure seems to play a determining role in the quality of intestinal tissue. Ideally, prior histological appraisal of these samples should be performed, or the introduction of a second set of control samples obtained from horses euthanized for other reasons than gastrointestinally related problems should be considered.

Acknowledgements. We would like to thank the Department of Large Animal Internal Medicine of Ghent University for the collection of colic tissue. We would like to thank Katty Huybrechts and Gunther Vrolix for their technical assistance.

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