SCIENTIFIC ARTICLE

Comparison between the techniques of inclusion in glycol methacrylate (GMA)-based plastic resin and paraffin for evaluation intestinal morphometry in horses

Comparação entre as técnicas de inclusão em resina plástica à base de glicol metacrilato (GMA) e inclusão em parafina para avaliação da morfometria intestinal em equinos

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Abstract

To determine the effect of the inclusion method on the histomorphometric evaluation of the gastrointestinal mucosa of horses, jejunum samples were collected using flank laparotomy. Sixteen mixed breed healthy adult horses, including four males and 12 females, aged 4–14 years with an average body weight of 248.40 ± 2.28 kg, were used. Jejunal biopsies were collected and analyzed by light microscopy using two methods: group 1 comprised biopsies fixed using 10% neutral formalin and embedded in paraffin; biopsies in group 2 were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, followed by inclusion in glycol methacrylate (GMA)-based plastic resin. Intestinal villi height, crypt depth, glandular mucosa thickness, total mucosal thickness, and villus/crypt ratio were then evaluated. For all the variables studied, with exception of the villus/crypt ratio, a significant difference ($p < 0.05$) was found between samples in groups 1 and 2. Processing samples for embedding in plastic resin was quicker and easier to perform compared to that for paraffin embedding. In addition, the epithelial lining of tissues in group 2 showed better resolution for conducting cytological studies under a light microscope. The difference between the studied variables can be attributed to tissue retraction caused by conventional processing for inclusion in paraffin. Therefore, the method of inclusion in GMA described in the present study appears to be a more reliable choice for morphometric evaluation of the intestinal mucosa of horses.

Keywords: biopsy, equine, intestinal mucosa, small intestine, villi height.

Resumo

Para determinar o efeito do método de inclusão sobre a avaliação histomorfométrica da mucosa gastrointestinal de equinos foram coletadas amostras do jejuno por laparotomia pelo flanco. Foram utilizados 16 equinos adultos hígidos, sem raça definida, de ambos os sexos, quatro machos e 12 fêmeas, com idade variando entre quatro e 14 anos, e peso corporal médio de 248,40 ⁺ 2,28kg. Amostras do jejuno foram coletadas e processadas para biopsia em microscopia de luz sob dois métodos: grupo 1 - fixação em formalina neutra tamponada a 10% e inclusão em parafina, grupo 2 - fixação em glutaraldeído 2,5% em tampão fosfato 0,1M pH 7,2, seguido de inclusão em resina plástica à base de glicol metacrilato. Os seguintes parâmetros foram avaliados: altura das vilosidades intestinais, profundidade de cripta, espessura da mucosa glandular, espessura total da mucosa e relação vilo/cripta. Para todas as variáveis estudadas, exceto relação vilo/cripta, foi encontrada diferença significativa ($p<0,05$) entre os dois métodos. O processamento para inclusão em resina plástica foi rápido e de fácil execução quando comparado à inclusão em parafina. Além disso, o epitélio de revestimento apresentou melhor resolução das células para estudo histológico ao microscópio de luz. A diferença entre as variáveis pode ser atribuída a retração do tecido provocada pelo processamento convencional por inclusão em parafina. Portanto, o método de inclusão em GMA mostrou-se, no presente estudo, uma escolha mais fidedigna para as avaliações morfométricas da mucosa intestinal de equinos.

Palavras-chave: biópsias, equino, mucosa intestinal, intestino delgado, altura de vilosidade.

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Introduction

The use of histological processing to assess the intestinal mucosa has been described in horses, poultry, and in humans (Lima et al, 2020; Milne et al., 2010; Rocchigiani et al., 2021). This technique is used to evaluate the responses of the intestinal mucosa to nutritional support, congenital disorders (Ruemmele et al., 2006), treatment of gastrointestinal disorders, and morphometric assessments. Intestinal biopsies obtained via endoscopy are useful for diagnosis of equine inflammatory bowel diseases. However, a definitive diagnosis using a rectal biopsy can be performed in one-third of the animals. However, the best samples to diagnose these changes are biopsies comprising all layers of the affected intestinal portions, sampled using a flank laparotomy or ventral midline (Kalck, 2009).

The large and small and large intestines are composed of concentric layers, namely after the mucosal, submucosa, muscular, and a serous layers. The intestinal mucosa comprises of the epithelial lining, the lamina propria, and the mucosal muscle, which generally consist of a thin circular inner layer and a longitudinal outer layer of smooth muscle (Bezerra et al., 2016; Junqueira & Carneiro, 1999).

In the small intestine, the process of digestion of food and its unabsorbed products takes place in the small intestine. Due to the presence of folds called villi and microvilli, absorption increases considerably on the intestinal surface. It is estimated that the presence of villi increases by eight times and that of microvilli by more than 20 times the intestinal surface, making a total of 160 times (Junqueira & Carneiro, 1999).

In addition to using an appropriate technique to collect the biological material, another important factor for histological evaluation is the choice of fixative and sample inclusion method for processing intestinal biopsy samples. Intestinal biopsy processing techniques have remained the same in recent years and include fixation in 10% formalin and embedding in paraffin (Lima et al., 2020; Rocchigiani et al., 2021). This technique has been widely used in research and intestinal morphometry in horses (Melo et al., 2014; Mendes et al., 2009). However, the inclusion of biological tissues in plastic resin is advantageous when compared to embedding them in paraffin, which is one of the techniques of choice for processing tissue samples for research (Chiarini-Garcia et al., 2011).

The aim of this study was, therefore, to compare the effects of two different types of histological processing on the preservation and evaluation of intestinal morphometry in healthy horses.

Material and methods

Sixteen healthy adult mixed breed horses, including four males and 12 females, aged 4–14 years with an average body weight of 248.40 ± 2.28 kg, were used. For screening and selection of animals, a complete clinical examination and blood count, as well as an agar gel immunodiffusion test were performed to check for the diagnosis of equine infectious anemia.

Fasting samples were collected using flank laparotomies for histomorphometric evaluation of the intestinal mucosa. The horses were tranquilized with 0.05 mg/kg body weight of acepromazine hydrochloride (Acepran 1%, UNIVET S/A Indústria Veterinária, São Paulo/SP, Brasil) and sedated with 1.0 mg/kg body weight of xylazine hydrochloride (Sedomin, König do Brasil Ltda, Santana do Parnaíba/SP, Brasil). They were kept in a containment trunk, and local anesthesia was performed with an inverted "L" in the skin, subcutaneous and muscle layers with 2% lidocaine hydrochloride. After preparing the operative field, a vertical skin incision, approximately 15 cm in length, was made on the flank, at the central point equidistant from the costal margin, transverse from the lumbar vertebrae and the coxal tuberosity. Subsequently, the external oblique abdominis muscle was incised in the same direction as the skin incision. This was followed by a blunt divulsion of the internal oblique abdominis and transversus abdominis muscles and then incising the peritoneum. Through internal palpation, the small intestine was identified for biopsy. The jejunum was gently exteriorized for sample collection via an enterectomy. After obtaining the sample, enterorrhaphy and continuous suture of the abdominal wall were performed.

The biopsy samples were processed using two methods. Group 1 samples were embedded in paraffin, whereas group 2 samples were embedded in plastic resin glycol methacrylate (GMA).

The paraffin embedding method followed the protocol described by Luna (1968), while GMA embedding followed the protocol described by Amaral et al. (2004) and Chiarini-Garcia et al. (2011).

Intestinal morphometry was performed on fasting fragments using a micrometric ruler coupled to an optical microscope eyepiece (Nikon, model Eclipse E200). Villi height, crypt depth, glandular mucosa thickness, total mucosal thickness, and villus/crypt ratio were studied in the mucosa of the jejunum at 40x magnification. Ten measurements were performed for each variable in each sample.

The experimental design was completely randomized with 12 replicates to evaluate the intestinal morphology under light microscopy. Data were tabulated in an Excel® spreadsheet and the means were calculated using the statistical program Statistical Analyses System (SAS, 2000). The procedures PROC MEANS, PROC UNIVARIATE, and PROC NPARIWAY were used at a significance level of 95% ($p < 0.05$). To assess the homoscedasticity of the responses, the means obtained were processed using PROC UNIVARIATE to assess normality using the Shapiro-Wilk test. Logarithmic transformation ($\log x + 1$) was then applied to the data, and the means were also compared using a Wilcoxon test.

Results

All the morphometric variables evaluated, with the exception of the villus-crypt relationship, showed significant differences between the two groups ($p < 0.05$) (Table 1).

Table 1. Mean values + standard deviation of the variables measured in the jejunum mucosa of healthy horses, according to the method of inclusion.

Variable (μm)	Group	
	Plastic resin	Paraffin
Villous height	455.37 ± 8.35^{A}	386.69 ± 12.80^{B}
Villous width	165.50 ± 1.84^{A}	144.57 ± 3.77^{B}
Crypt depth	181.00 ± 4.99^{A}	141.94 ± 4.90^{B}
Glandular mucosa thickness	181.00 ± 4.99^{A}	141.94 ± 4.90^{B}
Total mucosal thickness	636.12 ± 10.88^{A}	528.89 ± 15.95^{B}
Villous/crypt relationship	2.78 ± 0.08^{A}	2.94 ± 0.11^{A}

Means after transformation, followed by different capital letters on the line differ ($p<0.05$, Wilcoxon test).

Discussion

When comparing the inclusion techniques performed in this study, processing for inclusion in GMA was found to be faster and easier to handle. In addition, the ability to create thinner sections (3 μm) improved the visualization of cellular structures in group 2 samples (Figure 1). Moreover, in these samples, there was no overlapping of structures, which is a common occurrence in sections made from paraffin. When using GMA inclusion in renal, skin, lymph node, and thyroid tissue samples in 0.5–2 μm thick sections, Flax and Caulfield (1962) observed that methacrylate increases the possibility of visualizing the detailed morphology with little distortion of the structures. This is mainly due to the thinner sections. Furthermore, Flax and Caulfield (1962) found that semi-thin sections were relatively free from tissue retraction, as observed in this study.

Another factor that may have influenced the quality of the samples included in the GMA was the fixative used. In this case, 2.5% glutaraldehyde in a 0.1 M phosphate buffer of pH 7.2 was used instead of 10% neutral formalin used as a fixative for inclusion in paraffin. Both fixatives used were efficient in maintaining tissue characteristics, but fixation with 2.5% glutaraldehyde in a 0.1 M phosphate buffer at pH 7.2, followed by inclusion in GMA resulted in better quality histological sections, as evidenced by qualitative assessment (Figure 1).

Histology is an extremely relevant tool for the diagnosis of certain diseases. Therefore, tissue fixatives play an important role in the quality of histological processing, with direct consequences for microscopic analysis. The fixation step comprises the use of chemical procedures to stabilize

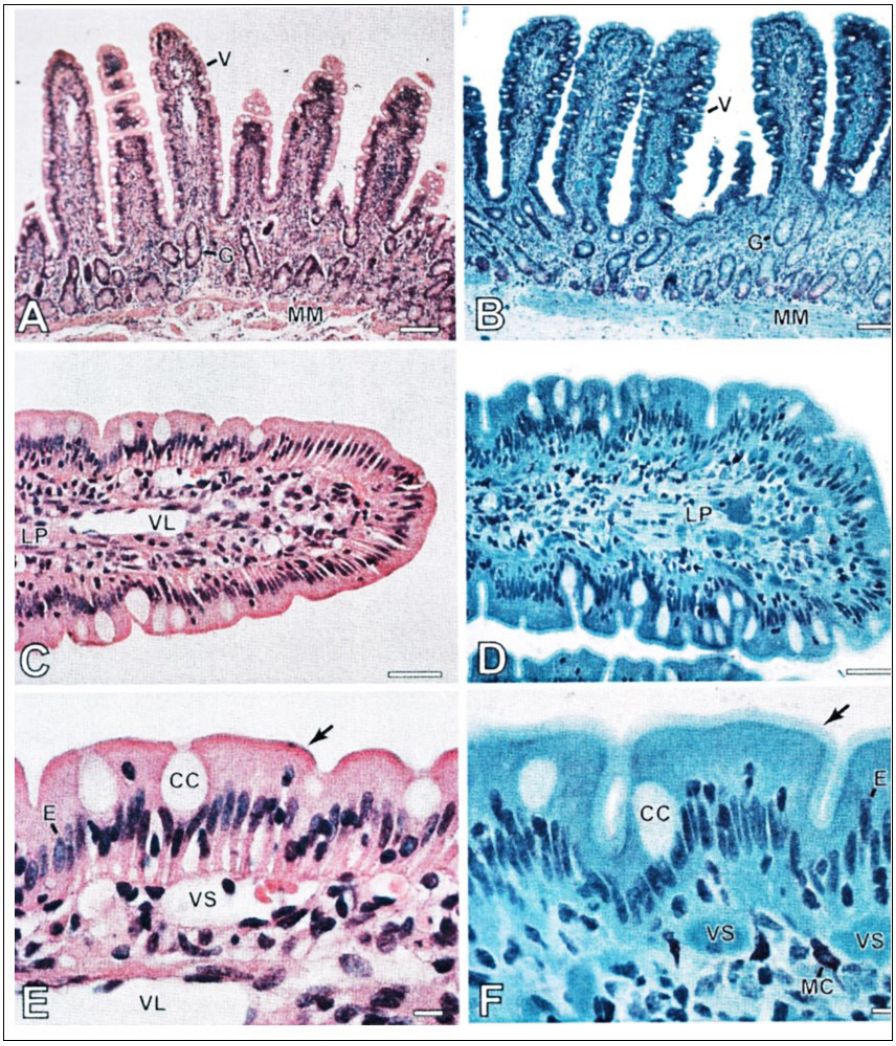

Figure 1. Histological sections of the intestinal mucosa of healthy horses processed by different inclusion methods. A, C and E: intestinal mucosa of the jejunum, paraffin embedded and hematoxylin-eosin stained. B, D and F: intestinal mucosa of the jejunum, inclusion in GMA and toluidine-sodium borate blue staining.

V: intestinal villi; G: likerbuin gland or crypts; MM: mucosal muscle; VL: lymphatic vessel; LP: own blade; CC: goblet cell; E: enterocytes; VS: blood vessels; MC: macrophages (arrows). Bars in A and B: 100 μm; C and D: 50 μm; and E and F: 10 μm.

and provide resistance for the other tissue processing steps used to make histological slides. In addition it also delays the onset of postmortem changes and maintains tissue architecture (Campos et al., 2016).

The effectiveness of glutaraldehyde as a fixative is attributed to its ability to efficiently cross-link proteins and amino acids to form a bifunctional molecule, both in the cytoplasm and cytoplasmic membranes (Bozzola & Russell, 1998; Kirkeby & Moe, 1986). Furthermore, glutaraldehyde has less specificity for lipids, carbohydrates, and nucleic acids. Therefore, its specificity within the cell is not restricted to proteins (Bozzola & Russell, 1998).

However, formaldehyde-based fixatives interact with amino acids and do not cause protein precipitation. Although they do not preserve free fats, they bind to complex lipids, causing slight precipitation of other cellular constituents and therefore lead to better tissue preservation. They are, however, not preferred fixatives for carbohydrates (Carvalho, 2009).

In addition, the use of a buffer system associated with the fixative causes changes in tissue pH, which normally decreases drastically during the fixation process, allowing it to remain within physiological limits. This considerably reduces the possibility of artifacts due to pH changes (Bozzola & Russell, 1998). According to the results from this study, in samples processed by inclusion in

GMA, using a buffer system in the fixation step is suggested to improve sample preservation for the evaluation of morphometry and morphology of the intestinal mucosa.

In previous reports, histomorphometric evaluation of the intestinal mucosa of horses through routine paraffin embedding has been recorded (Batista et al., 2005; Faleiros et al., 2007; Lucas et al., 2005; Mendes et al., 2009; Milne et al., 2010). However, GMA has several advantages over conventional paraffin-based methods (Amaral et al., 2004). One of the main disadvantages of paraffin inclusion is shrinkage or contraction associated with tissue distortion. When comparing the inclusion techniques using GMA and paraffin in small intestine samples, Tacha and Richards (1981) observed cell overlap along the crypts associated with cell retraction and distortion. In addition, the ability of GMA to prevent tissue retraction facilitates the study of intestinal crypt kinetics.

The biological material fixatives used also determine the final quality of histology. Some fixatives, such as 10% formalin, when used at varying times and temperatures, can influence the appearance of artifacts and compromise the final quality of histological processing. Tissues fixed in 10% neutral buffered formalin for long periods can lead to excessive hardening of the tissue and, consequently, to its fragmentation during processing, causing retraction or folding. This can be aggravated in cases of smaller tissues, where the tissues are extracted using biopsies (Campos et al., 2016).

In this study, the difference between the studied variables was likely due to tissue retraction caused by routine paraffin processing. Therefore, fixation in glutaraldehyde and subsequent inclusion in GMA should be the technique of choice for evaluating intestinal morphometry in equine species.

Conclusion

Histomorphometric evaluation of the intestinal mucosa of horses using the GMA embedding technique showed more reliable results than the conventional paraffin embedding technique. It should, therefore, be the technique selected for morphological studies of the intestinal mucosa in horses.

Ethics statement

This experiment was approved by the Animal Experimentation Ethics Committee (CETEA/UFMG) under the number 0063/2011.

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None.

Conflict of interests

There were no conflicts of interest in carrying out this study or in the publication of its results.

Authors' contributions

CF, MSP, UPM, HCG, and VAG - Development of methodology; preparation and writing the initial draft. RPAM and FOPL - Development of methodology.

Availability of complementary results

N.A.

The work was carried out at Clínica Médica de Equinos da Escola de Veterinária, Departamento de Clínica e Cirurgia Veterinárias, Universidade Federal de Minas Gerais – UFMG, Belo Horizonte, MG, Brazil.

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