http://dx.doi.org/10.1590/1678-4162-12895

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Arq. Bras. Med. Vet. Zootec., v.75, n.3, p.455-466, 2023

Effects of time-dependent degradation and freezing on biomechanical properties of healthy bovine jejunum

[*Efeitos da degradação dependente do tempo e do congelamento sobre as propriedades biomecânicas do jejuno bovino saudável*]

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ABSTRACT

Degradation of bovine small intestine and respective effects on biomechanics have not been described to date. Biomechanical testing of intestinal tissues is often carried out within a few hours of donor death and tissue deterioration is not accounted for. Freezing is efficient for the preservation of several tissues; however, it may cause cellular damage. This study investigated the morphologic and biomechanical changes of bovine jejunum at different *postmortem* moments. Effects of freezing and thawing on morphology and biomechanical behavior were also examined. Macroscopic changes were first noted within eight hours of death. At this time, histologic changes also started to set in, and biomechanical tests revealed lower bursting pressure (203.10±46.14mmHg). At 12 hours, tissue rearrangement was noted, and bursting pressure increased (238.43±31.04mmHg). A second drop in pressure was detected at 18 hours (235.20±38.21mmHg), followed by a progressive drop until the end of the experimental period. Histologic changes revealed progressive deterioration. Mechanical resistance did not differ between thawed and fresh specimens. It was concluded that bovine jejunal specimens retain biomechanical resistance up to 6 hours after death. Freezing and thawing did not affect the mechanical resistance of the intestinal wall in this experimental model.

Keywords: intestinal preservation, cryopreservation, biomechanical, Histology, jejunum

RESUMO

A degradação do intestino delgado de bovinos e seu efeito biomecânico não são descritos na literatura. Trabalhos que envolvem a biomecânica intestinal realizam os ensaios em poucas horas após o óbito dos doadores, sem avaliação de seu estado de deterioração. O congelamento é eficiente na conservação de vários tecidos, porém pode provocar danos em nível celular. Este estudo teve por objetivo avaliar a degradação morfológica e biomecânica do jejuno de bovinos em diferentes tempos post mortem*. O efeito do congelamento e do descongelamento sobre essas características também foi avaliado. As primeiras alterações macroscópicas foram observadas oito horas após o óbito. No mesmo momento, se iniciaram as alterações histológicas e a menor pressão suportada ao teste biomecânico (203,10±46,14mmHg). A partir de 12 horas, o tecido sofreu um rearranjo, suportando maior pressão (238,43±31,04mmHg). Uma segunda queda foi detectada após 18 horas (235,20±38,21mmHg), seguida de redução progressiva até o término do experimento. As lesões histológicas foram progressivas e graduais. As amostras testadas após descongelamento não apresentaram diferença estatística quanto à resistência mecânica em comparação com os espécimes frescos. Concluiu-se que as amostras mantêm sua resistência biomecânica até 6 horas após o óbito. Também se mostrou que o congelamento e o descongelamento, nas condições testadas, não alteraram a resistência mecânica da parede intestinal.*

Palavras-chave: preservação intestinal, criopreservação, biomecânico, histologia, jejuno

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Submitted: August 16, 2022. Accepted: January 3, 2023.

INTRODUCTION

In the last few years, several studies investigating the biomechanical behavior of intestinal tissues in different species have been performed (Bracamonte *et al*., 2018, Risselada *et al*., 2015, Sokolis, 2017). In most cases, fresh specimens were used, and experimental procedures carried out shortly after death (Auletta *et al*., 2011, Gandini *et al*., 2013, Lee *et al*., 2012). The impact of *postmortem* time on biomechanical properties and the time limit for storage of intestinal specimens before the onset of autolytic changes remains to be determined.

Freezing is a well-established, user friendly and low-cost preservation method, which is widely used for preservation of soft tissues (Stemper *et al*., 2007). However, tissue damage may result from excessively slow or rapid freezing (Fuller *et al*., 2004).

The cold storage time of the intestine prior to transplantation is 12 hours (Lewis *et al*., 2016). Hence, under controlled conditions, it may be assumed that intestinal tissues retain their original histologic characteristics and biomechanical properties for longer than reported in prior studies investigating mechanical resistance.

The high cellularity and water content of the intestinal mucosa and muscle layers suggest these layers are highly sensitive to freezing, whereas the submucosa and the serosa, which consist primarily of collagen fibers, tend to be less prone to freeze-induced damage. In the human small intestine, the mechanical stability, strength and resistance of the intestinal wall to large and long-lasting strains is provided by the submucosa (Egorov *et al*., 2002). Therefore, the biomechanical resistance of intestinal specimes is unlikely to be affected by freeze-thaw procedures.

This study was designed to investigate the impacts of tissue degradation and freeze-thaw procedures on the morphology and biomechanical behavior of cadaveric jejunal specimens harvested from healthy adult cattle. Findings of this study may contribute to future research into intestinal biomechanics.

MATERIAL AND METHODS

This study included two phases. In the first phase, intestinal specimens were submitted for histological analysis at different time points. In the second phase, the effect of *postmortem* time and freeze-thaw procedures on mechanical resistance was determined. Experimental procedures were approved by CEUA (protocol No. 1605230818).

In the first phase, Intestinal specimens used in this study were obtained from adult Nelore cattle (6). Donor animals weighed 450 to 550kg and were slaughtered at *Fernando Costa* plant (University of São Paulo). Intestinal segments measuring approximately 50 cm in length were harvested from the distal jejunum immediately after death. Only segments with no gross abnormalities were used.

Intestinal contents were gently evacuated. Specimens were washed in running water, stored in plastic bags containing lactated Ringer's solution and placed on crushed ice for transportation to the laboratory. Specimens were then kept at room temperature (20 to 25°C) until processing and formaldehyde fixation.

Intestinal wall fragments were obtained and fixed in 10% formalin solution at different time points, as follows: zero (fresh tissue sample), two, four, six, eight, 12, 18, 24, 36 and 48 hours (T0, T2, T4, T6, T8, T12, T18, T24, T36 and T48 respectively).

Prior to processing, tissue segments (0.5cm wide) were resected from one end of each intestinal specimen and discarded. Segments (1.5cm wide) were then resected and the mesentery dissected away. These tubular segments were cut, and a flat tissue fragment was obtained to facilitate fixation. Tissue fragments were attached to an expanded polyethylene plate using metallic pins to prevent shrinking during the fixation process.

Formalin-fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were examined using a bifocal microscope (BX – 60, Olympus®, Japan) connected to a digital camera (Axio Cam HRc, Zeiss®, Germany). Images were analyzed using software (Zen Blue, Zeiss®, Germany).

In the second phase of the study, segments measuring approximately 1.5m in length were harvested from the jejunum 18 Nelore steers of similar body weight and processed as previously described. Each segment was split into 10 specimens measuring approximately 15 cm in length. Of these, nine were submitted to biomechanical testing at different time points (T0, T4, T6, T8, T12, T18, T24, T36 and T48). The remaining segments were placed in plastic bags containing lactated Ringer's solution and stored in a domestic freezer (-20°C) for seven to 15 days.

Specimens were thawed and submitted to biomechanical testing within one hour of thawing or less. Thawing procedures consisted of immersion in lactated Ringer's solution and exposure to temperatures ranging from 20 to 25°C in an air-conditioned room.

Prior to biomechanical testing, samples were collected from thawed specimens for histological analysis. These samples were used to investigate differences in biomechanical resistance between frozen and thawed specimens and potential correlations with histologic findings.

An adapted version of the method described by Egglestone (2004) was used for biomechanical testing. One hose was connected to each end of intestinal specimens for air infusion and intraluminal pressure measurement (proximal and distal end respectively). Hoses were secured using nylon clamps and attached to a plastic sealing cap designed to provide an air-tight system. Specimens were placed in jars and submerged in lactated Ringer's solution. The jars were tightly sealed, and a third hose attached to the jar lid. This hose served as an outlet for the fluid displaced by the distented intestinal segments to prevent external compression (Fig. 1).

The air infusion hose was connected to an air compressor (Pratic Air, Schulz®, Brazil). Air was then delivered at a constant rate (600 ml/min) and the flow controlled using a flowmeter (Fig 1). Intraluminal pressure was measured using an electronic sensor (MPX5700/D, Motorola®, United States) connected to an independent microcontroller (Arduino®, Italy). Pressure data was sent to a portable computer and stored for processing (Fig. 1).

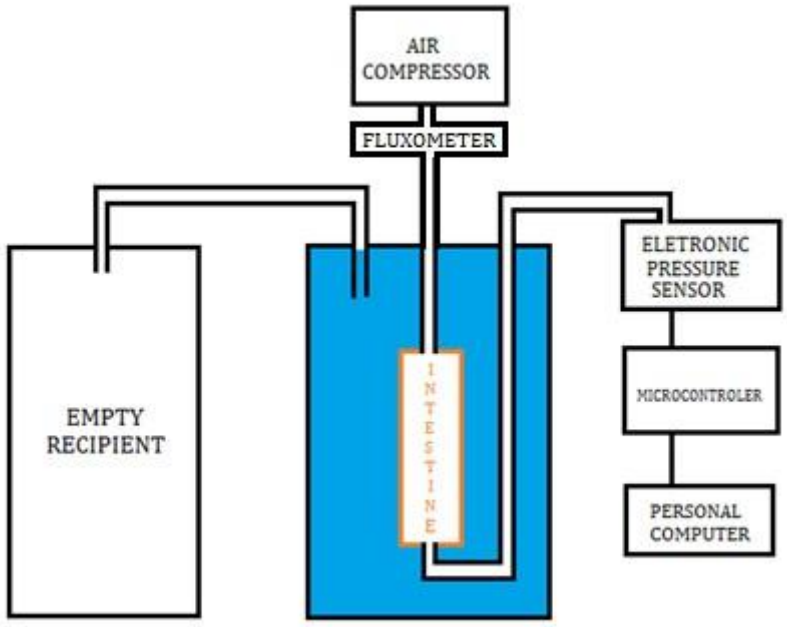

Figure 1. System used for compressed air infusion and intraluminal pressure measurement in biomechanical tests.

Bursting was defined as air leakage, intestinal rupture, or sudden drop in pressure. Trials were filmed and images stored for analysis.

Following test completion, specimens were examined, and rupture sites recorded. Intestinal segments were divided into six regions, as follows: upper, middle, and lower, and mesenteric and antimesenteric (longitudinal plane and cross-sectional plane respectively) (Fig 2).

Statistical analysis was carried out using GraphPad Prism 6.01 software (Prism, United States). Data were tested for normality using the Kolmogorov-Smirnov and the D'Agostino & Pearson omnibus test. Biomechanical data were analyzed using repeated measures Anova (analysis of variance). Significant differences were submitted to the Tukey test. The level of significance was set at 5% ($p<0.05$).

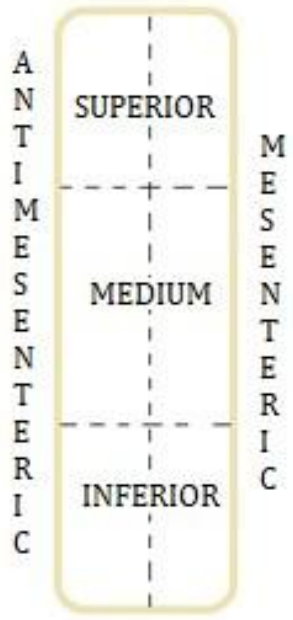

Figure 2. Representative illustration of the division used for rupture site description.

RESULTS

At harvest (T0), specimens had normal odor, texture, consistency, color, and wall thickness. The first gross changes were noted within eight hours of death (T8). These consisted of mild changes in the consistency of the intestinal mucosa and presence of gelatinous material in the intestinal lumen. At T12, intestinal segments were softer, had thinner walls and contained large amounts of gelatinous material in the lumen. At T18, friable consistency, foul odor and green discoloration were noted. At T24, mucosal folds were less distinct and flaccid. At T36, the intestinal wall was significantly thinner and extremely friable. Green discoloration persisted and a putrid odor developed. At T48, specimens were in an advanced stage of decomposition.

At T0, histological analysis revealed normal intestinal layers, except for mild epithelial sloughing (Fig. 3). Secretory glands were organized, rounded, and had a well-defined lumen. The inner circular and outer longitudinal muscle layres and the serosa were in close apposition and well aligned. No changes in tissue morphology were noted at T2 and T4 (Figs. 3 and 4). At T6, intracellular vacuoles were seen in the mucosa, particularly around secretory glands, whereas remaining layers remained intact (Fig. 4). At T8, changes in the mucosa had progressed. The submucosa was thicker and separated from the inner muscle layer. The outer longitudinal muscle layer and the serosa were also separated (Fig. 5). These changes were more pronounced at T12. At this time point, the distance between longitudinal muscle layer fibers and bundles had increased and serous membrane and muscle layers were no longer in apposition (Fig. 5). T18 marked the onset of serous membrane disorganization (Fig. 6), with progressive disarray of the intestinal wall architecture from T24 to T48 (Figs. 6 and 7).

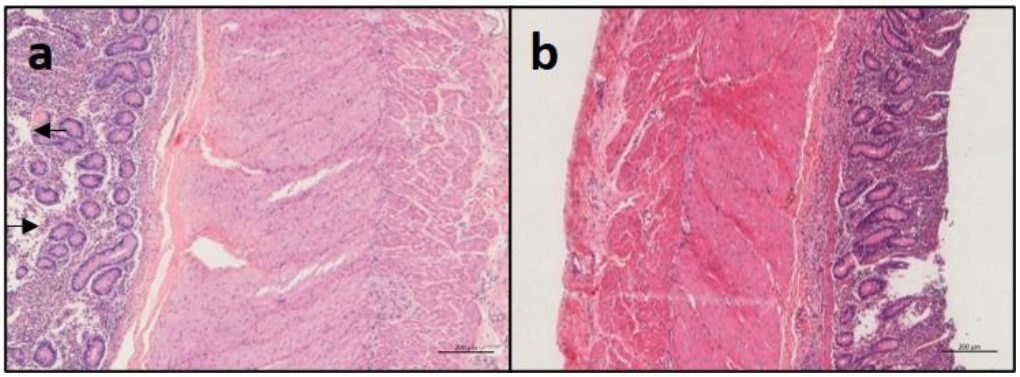

Figure 3. (**a**) Specimen No. 3, T0. Note normal morphology of intestinal wall layers and sloughing of the epithelium in intestinal villi (arrows). Bovine, Nelore, hematoxylin-eosin, 10 x magnification; (**b**) Specimen No. 4, T2. All intestinal wall layers are well preserved. Bovine, Nelore, hematoxylin-eosin, 10 x magnification.

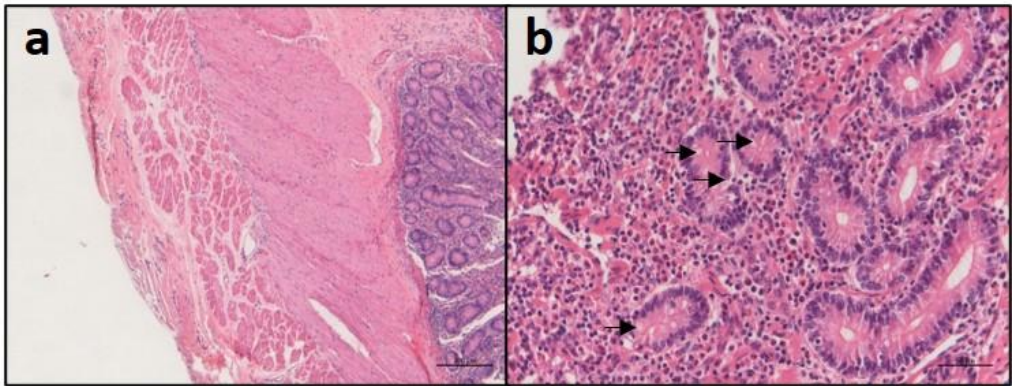

Figure 4. (**a**) Specimen No. 2, T4. Note normal distribution of intestinal wall layers. Bovine, Nelore, hematoxylin-eosin, 10 x magnification; (**b**) Specimen No. 3, T6. Note intracellular vacuoles around secretory glands (arrows). Bovine, Nelore, hematoxylin-eosin, 40 x magnification.

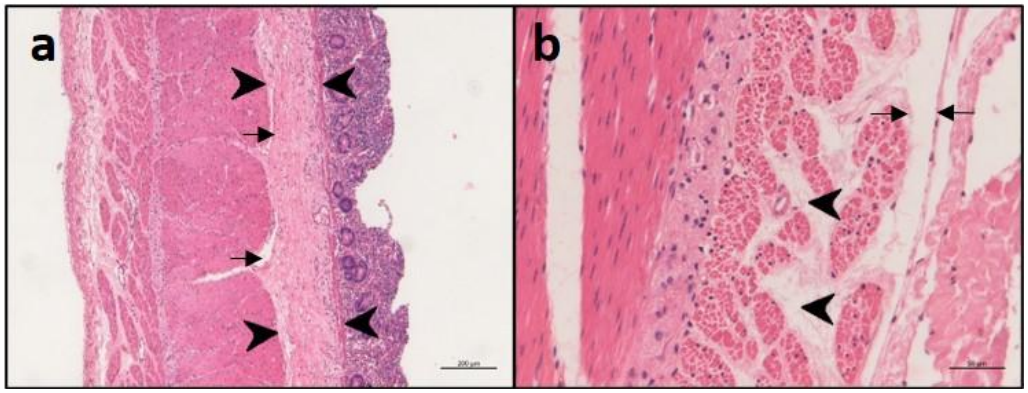

Figure 5. (**a**) Specimen No. 2, T8. Note thickening of the submucosa (arrow heads) and detachment of the submucosa and inner circular layer (arrows). Bovine, Nelore, hematoxylin-eosin, 10 x magnification. (**b**) Detachment of outer longitudinal muscle layer fibers (arrow heads) and spacing between the outer muscle layer and the serosa (arrows). Bovine, Nelore, Hematoxylin-eosin, 40 x magnification.

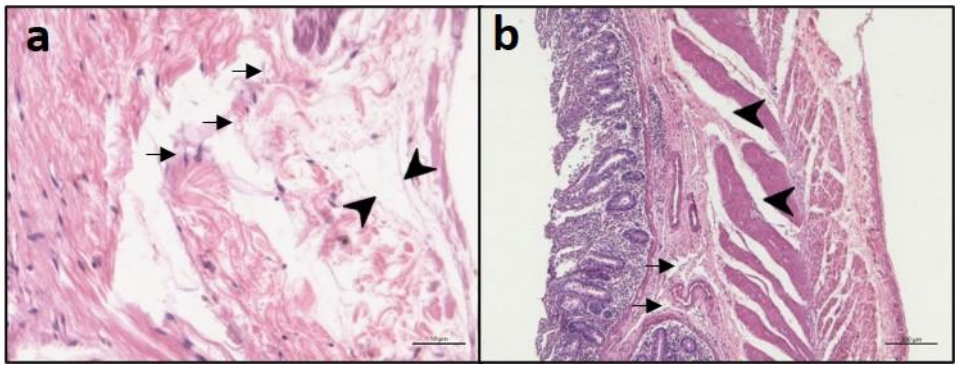

Figure 6. (**a**) Specimen No. 1, T18. Note tearing of outer longitudinal layer muscle fibers (arrows) and separation from the serosa (arrow heads). Bovine, Nelore, hematoxylin-eosin, 40 x magnification. (**b**) Specimen No. 4, T24. Note decondensation of the submucosa (arrows) and separation of muscle fiber bundles in the inner circular layer (arrow heads). Bovine, Nelore, hematoxylin-eosin, 10 x magnification.

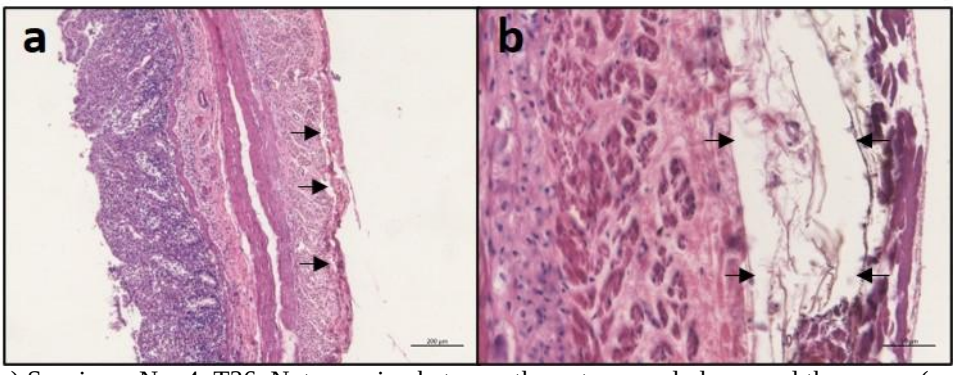

Figure 7. (**a**) Specimen No. 4, T36. Note spacing between the outer muscle layer and the serosa (arrows). Hematoxylin-eosin, 10 x magnification. (**b**) Specimen No. 6, T48. Note detachment and decondensation of the serosa (arrows). Bovine, Nelore, hematoxylin-eosin, 40 x magnification

Thawed specimens had a similar odor and gross appearance to fresh specimens. However, these specimens had friable consistency, less distinct mucosal folds and contained gelatinous material in their lumen.

Histological analysis revealed moderate to severe changes. Secretory glands had ill-defined shape or unrecognizable architecture. The submucosa was highly edematous and detached from adjacent layers. Collagen fibers in this layer were disorganized and spaced apart. Muscle fibers were torn, misaligned and disorganized. Cell rupture and tissue retraction caused muscle fibers and bundles to separate. The distance between the muscle and adjacent layers was also larger. The serosa was irregular, had a frayed appearance and contained misaligned collagen fibers. In some specimens, serosa was totally disconnected from the longitudinal muscle layer (Fig. 8).

Bursting pressure dropped significantly from T6 to T8 (242.17 ± 29.81 mmHg and 203.10±46.14 mmHg respectively, p = 0.0049) and increased significantly from T8 to T12 (203.10±46.14mmHg and 238.43±31.04mmHg respectively, p=0.0178). Bursting pressure decreased progressively after T12. However, only values recorded at T8 and T18 were significantly different (203.10±46.14mmHg and 235.20±38.2mmHg respectively, p=0.0479). Changes in bursting pressure in this trial are shown in Fig 9.

Bursting pressure did not differ significantly between thawed and fresh (T0) specimens (230.01±29.61mmHg and 224.18±38.69mmHg respectively, p=0.9999).

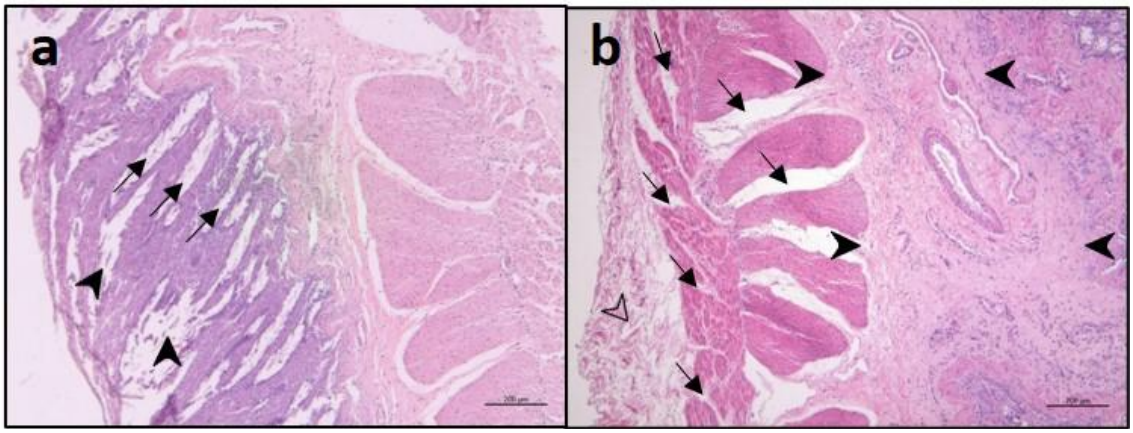

Figure 8. (**a**) Specimen No. 6 (post-thawing). Note epithelial sloughing (arrowhead) and ill-defined shape of secretory glands (arrow). Bovine, Nelore, hematoxylin-eosin, 10 x magnification. (**b**) Specimen No. 12 (post-thawing). Note thickening of the mucosa (arrow heads), spacing of muscle fibers and serosal decondensation (hollow arrowhead). Bovine, Nelore, hematoxylin-eosin, 10 x magnification

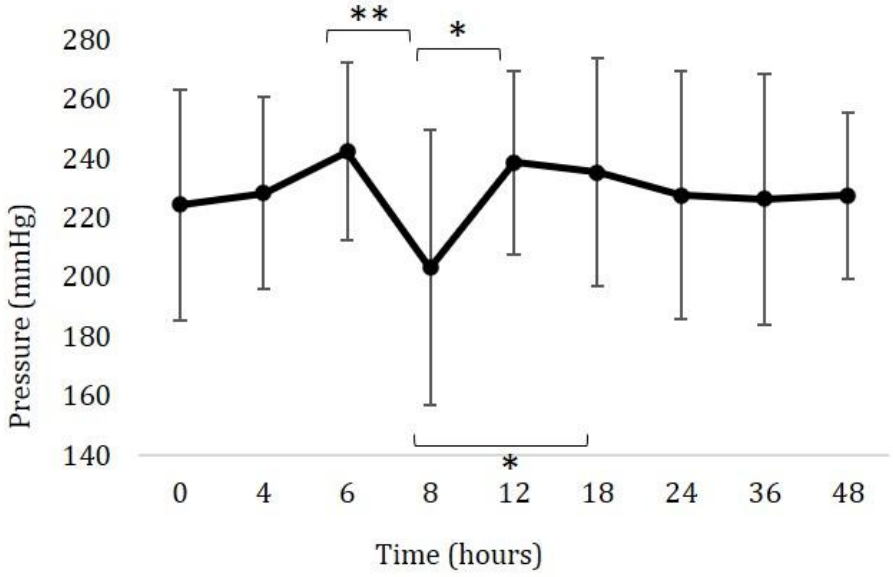

Figure 9. Variation in maximum bursting pressure over the course of the degradation period. * $p < 0.05$. ** $p < 0.01$.

Overall, there was a short period of system accommodation between T0 and T8. During this period, tracings revealed a small pressure plateau or subtle pressure elevation (25 to 50mmHg). This was followed by a slow and progressive rise in pressure and a sudden drop at the time of bursting. A different pressure profile was observed between T18 and T48. During this period, the accommodation phase lasted much longer, as shown by pressure plateaus (50 to 75 mmHg). A sharper pressure increase followed by a sudden pressure drop in pressure occurred at the time of bursting. Pressure curve changes over time are represented in Fig. 10.

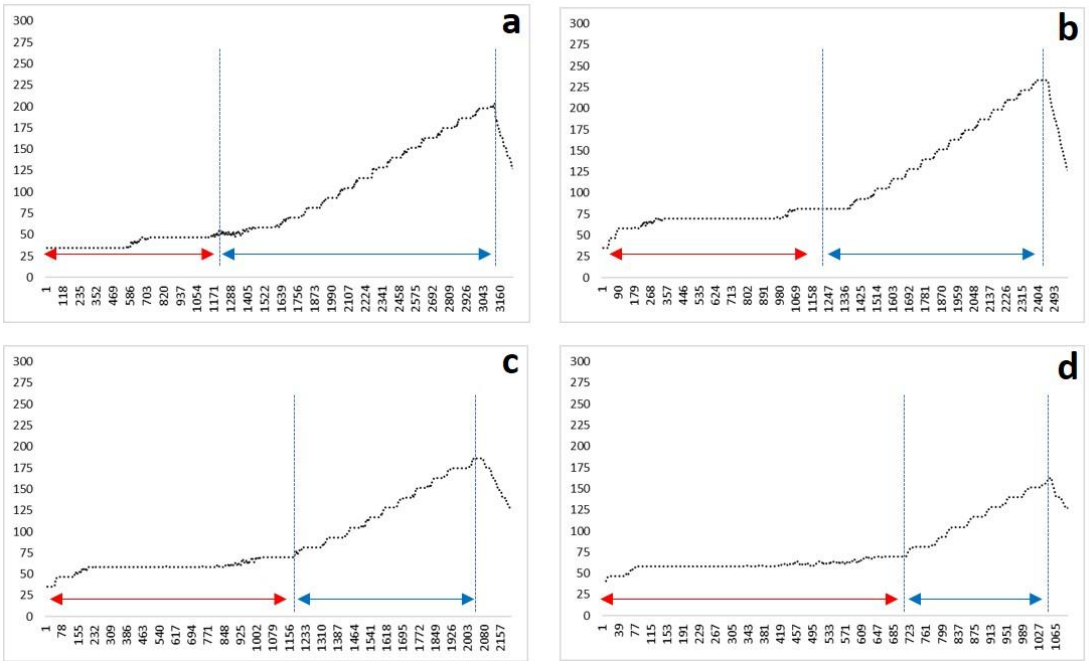

Figure 10. (**a**) T0; (**b**) T8; (**c**) T18; (**d**) T48. System accommodation (red arrows) and intestinal specimen distension (blue arrows).

In the longitudinal plane, bursting occurred primarily at mesenteric region (178 times and twice, mesenteric and antimesenteric region respectively). In this plane, the upper mesenteric region was the most common site of rupture, followed by the middle and lower regions. Large ruptures involving more than one region were also common. The frequency of ruptures per region is shown in chart 1.

Segment	Total Number	Percentage
Proximal	66	36,66
Medium	46	25,55
Distal	27	15,00
Proximal and medium	28	15,55
Medium and distal	10	5,55
Proximal, médium and distal	3	1,66

Correlations between *postmortem* time and rupture (i.e., specimen failure) were investigated. Ruptures involving more than one region were more common between T0 and T6. The number of regions affected decreased between T6 and T8 and gradually increased after T8. The number of rutures and regions involved is shown in Fig 11.

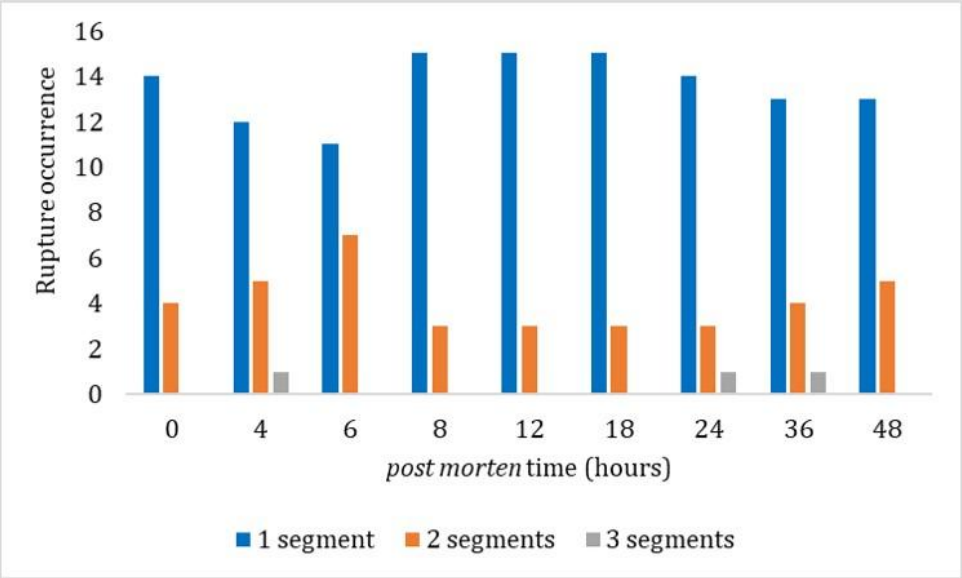

Figure 11. Relation between degradation time and rupture size

DISCUSSION

In studies investigating intestinal tissue morphology and biomechanics, specimen harvesting and processing deserves special attention. Use of specimens obtained from slaughterhouses is recommended to eliminate potential impacts of factors such as age (Zao and Gregersen, 2015, Chantereau *et al*., 2014), pre-existing disease (Loehry and Creamer, 1966) and diet (Nakajima *et al*., 2008, Liu *et al*., 2017a, Liu et al., 2017b) on intestinal tissue characteristics. Also, slaughtered animals are eviscerated within a few minutes of death and specimens can be quickly harvested and washed, preventing tissue degradation by the intestinal microbiota.

Cooling has been used in different studies to extend the durability of anatomical specimens (Stemper *et al*., 2007, Sokolis, 2017; Bracamonte *et al*., 2018). Massalou and coworkers (2016) performed biomechanical trials using cadaveric human colon samples stored at 1^{o}C for 21 days on average. In this study, specimens were first chilled on crushed ice, then kept at room temperature to allow for potential logistic constraints, such as large distances between harvesting and testing facilities.

Most studies investigating biomechanical characteristics of soft tissues to date employed uni (Walsh *et al*., 2014; Massalou *et al*., 2016) or biaxial (Khoiy *et al.*, 2018) tension and passive tests (Sokolis, 2017). Distension tests generate tension in different directions at the same time. Given the anisotropic behavior of the intestinal wall (Fung, 1993), these tests were selected for this study.

As expected, no significant gross or histologic changes, or variations in bursting pressure were detected between T0 tand T4. These findings support the reliability of data derived from biomechanical trials carried out with specimens harvested within four hours of donor death (Sherlock *et al*., 2011, Lee *et al*., 2012, Gandini *et al*., 2013) or less (Bracamonte *et al*., 2018).

Despite mild histologic changes in the mucosa, lack of significant differences in bursting pressure between specimens tested within six hours of death (T6) is in keeping with data reported elsewhere. In a study carried out by Chen and coworkers (2008), absence of intestinal villi had no impact on the behavior of intestinal specimens in distension tests. These findings suggest mucosal integrity does not affect biomechanical behavior.

In this study, gross and histologic changes, such as thickening and separation of the submucosa from the inner muscle layer, were first noted within eight hours of death (T8). Given the mechanical resistance of the intestinal wall is provided primarily by the submucosa (Egorov *et al*., 2002; Gandini, 2010), the disarray of

collagen fibers in the submucosa may explain the drop in bursting pressure between T0 and T8.

Increased intestinal wall stifness in response to *rigor mortis* may also explain the drop in bursting pressure at T8. This *postmortem* phenomenon results from depletion of glycogen and ATP, leading to irreversible muscle contraction (Myers and McGavin, 2009). *Rigors mortis* starts two to four hours after death and is fully established within six to 12 hours of death. Increased intestinal wall stiffness may have affected tissue elasticity and distensibility, resulting in lower resistance to pressure. Inverse correlations between intestinal wall stifness and distensibility have been described (Sokolis, 2017).

The significant increase in bursting pressure and tissue degradation at T12 may have reflected the gradual dissipation of *rigor mortis* as tissue autolysis progressed. Muscle fibers lose their ability to contract due to protein denaturation during autolysis (Vleet, 2000). The fact that the first signs of muscle layer degradation were noted at T12 suggests bursting pressure variations at this time point were also due to *rigor mortis* development.

Studies investigating the occurrence, effects, and duration of *rigor mortis* in smooth muscles are lacking. However, since smooth and striated muscle contraction share a common physiological basis, it is plausible to assume that *rigor mortis* also occurs in smooth muscles and leads to changes in intestinal wall stiffness and biomechanical resistance.

Changes detected at T12 were even more pronounced at T18. At this time point, muscle fiber disarray also coincided with increased mechanical resistance. At T18, mechanical resistance was significantly higher relative to T8, but only numerically higher relative to T12, suggesting further tissue rearrangement was no longer possible. In skeletal muscles, *rigor mortis* may last from 24 to 48 hours. However, several factors affect *rigor mortis* duration, such as glycogen depletion, muscle mass and speed of autolysis (Myers and McGavin, 2009).

Bursting pressure dropped progressively after T18. However, despite marked histological changes, bursting pressure differences were non-significant. The flabby consistency detected on gross examination may have reflected the architectural disarray of intestinal tissues, leading to increased specimens distensibility, and rising bursting pressure.

In this study, intraluminal pressure was measured using electronic sensors. These devices have been employed elsewhere (Bracamonte *et al*., 2018 Nieto *et al*., 2006). and have some advantages to mercury (Auletta *et al*., 2011, Gandini, 2006, 2010) and electronic manometers (Rosser *et al*., 2012), in particular the ability to record real-time changes in pressure. Different from other studies, use of electronic sensors enabled constant monitoring of specimen behavior during biomechanical testing in this trial.

Pressure curve analysis suggests the firmness and resistance of the intestinal wall allowed for slow, gradual distention until failure occurred in earlier stages (T0 to T8). In contrast, from T18 to T48 resistance to distension decreased due to progressive loss of rigidity and structural integrity. Pressure remained unchanged in response to the rapid luminal volume increase. However, when intestinal tissues reached their stretching limit, pressure increased sharply, and rupture ensued.

Lack of studies addressing pressure curves in distension tests preclude comparative analysis of these findings. Still, it is interesting that, despite significant differences in bursting pressure, samples of different time points responded to increasing pressures in a similar manner. This probabbly reflected changes in intestinal wall elasticity. Sadly, this variable could not be investigated in this experimental model.

Ruptures occurred primarily at mesenteric aspect of intestinal segments. According to Khoiy and coworkers (2018), the distal portion of the mesentery is stiffer and less distensible, and hence more prone to tearing when submitted to tension. Increased stiffness in areas subjected to greater tension is also thought to be a significant factor in intestinal wall collapse. The strong attachment of the mesentery to the intestinal wall in cattle and the greater stiffness of the distal portion of the mesentery may explain why ruptures tended to occur at the mesenteric aspect in this trial. Lack of flexibility may have

prevented the mesentery from stretching in response to distension. The inability of the intestinal wall to adapt to rising intraluminal pressure is the most likely reason why ruptures occurred almost exclusively at the mesenteric aspect.

In the longitudinal plane, ruptures involving a single site occurred primarily in the upper region, whereas those involving more than one segment were more common in the upper and middle regions. Intestinal specimens were not secured to the glass jar in this trial. Therefore, folds resulting by specimen distension may have generated uneven pressure distribution along intestinal segments. Attachment of the air infusion hose to the proximal end of intestinal specimens may also have accounted for higher pressures at this site.

In this study, specimens were stored in a domestic freezer. This storage method was selected for practical reasons and low cost, and to facilitate the replication of this experimental model in future studies. Immersion in lactated Ringer's solution was aimed at preventing potential tissue damage and cell rupture induced by dehydration (Fuller *et al*., 2004).

Given the high water content and cellularity of intestinal tissues, severe histologic changes were expected, particularly due to ice crystal formation (Gage and Baust, 1998). However, the use of frozen intestinal specimens in biomechanical testing has not been described to date. Positive results obtained with other tissues (Stemper *et al*., 2007 Wex *et al*., 2014) encouraged the use of freezing for tissue preservation in this study.

Despite gross and histologic tissue changes, bursting pressure did not differ significantly between fresh (T0) and frozen-thawed specimens. These findings support the hypothesis that freeze-thaw procedures do not affect the biomechanical resistance of intestinal tissues to intraluminal pressure.

Preservation of other organs using cryoprotectants or liquid nitrogen vapour has been described (Fahy *et al*., 2009; Brockbank *et al*., 2015). However, these methods require dedicated equipment, and their applicability is limited by factors such as high costs and the need for trained personnel. The volume of large animal bowels is yet another limitation.

CONCLUSION

Findings of this study suggest that intestinal specimens retain their original gross, and histologic characteristics, as well as biomechanical properties, for up to six hours *postmortem*. Freeze-thaw procedures had no significant effects on bursting pressure. Therefore, harvesting and preservation methods described in this study can be used to prepare intestinal specimens for biomechanical testing. However, potential impacts of gross and histologic changes on other variables of interest should be accounted for in future studies.

ACKNOWLEDGEMENTS

This study was funded by *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil* (CAPES) - Financing code 001.

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