Hystopathological study of the intestinal epithelium submitted to different times of programmed ischemia and twenty four hours reperfusion[1]

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ABSTRACT:

PURPOSE: To investigate the changes in the intestinal epithelium of rats, when submitted to the ischemia of varied time and scheduled reperfusion.

METHODS: For this purpose, the following groups were established six groups: control, 10, 20, 30, 40, 60 minutes of ischemia and reperfusion time (24 hours). Their intestines being resected for histopathological analysis: the length and width of the villus, and mitotic index. The nonparametric test Kruskal-Wallis and multiple comparisons test were applied.

RESULTS: The qualitatively the intestinal epithelium is regenerated when submitted to different ischemia, and 24 hours reperfusion time. It was observed that morphometry analyzing taking villus length and width and mitotic index as parameter, meaningful alterations were detected.

CONCLUSION: The epithelium is regenerated, when subjected to different time planned of ischemia and reperfusion. It was observed: decrease the length and increase the width of the villi, when compared Group VI with I and II, and Group VI with I; increased mitotic index when compared Groups III with I. Every analysis was statistically significant.

Key works: Ischemia. Reperfusion. Intestinal Mucosa. Rats.

Introduction

Changes that occur in the intestinal epithelium due to ischemia and reperfusion (IR) have been the subject of countless studies. Among these studies, the histopathological changes that occur in the intestinal epithelium after periods of IR have been described by several authors[1]. It should be emphasized, however, that most of these studies aimed the evaluation of the changes and their consequences in programmed ischemia and reperfusion in short periods of time and always less than 24 hours.

Other studies have demonstrated the importance of biochemical processes knowledge for a better

understanding of the reactions triggered after a period of ischemia and reperfusion[2]. More recently, these studies made possible the use of drugs that could act systematically in the intestine minimizing the harmful consequences caused by the IR[3].

According to evidence, it is relevant to evaluate the qualitative and quantitative (morphometric) histopathological changes of the villus, as well as the occurrence of the intestinal epithelium reconstitution by the mitotic index, after using the process of different times of ischemia and 24 hours reperfusion.

Acute mesenteric ischemia is the absence or severe reduction of blood flow due to arterial, venous or intestinal microcirculation damage**.** Through reperfusion and an increase in cell permeability, oxygen becomes available again causing oxidation in the metabolites and the emergence of free radicals which leads to tissue damage and other injuries in the system[4,5].

The study of various authors about the mechanism of ischemia and reperfusion injury and histological changes has showed that injuries caused by the decrease of the flow occurred from the villus to the crypt, and also that the villus had greater sensitivity to ischemic process, with injuries to more serious levels[5-10].

So in 1982, Parks[11]observed that the top of the villus of the intestinal mucosa has greater sensitivity to the free radicals action than the phenomenon hypoxic itself. Horton *et al*.[12] noted that oxygen free radicals produced during ischemia and reperfusion contribute to lipid oxidation of cell membrane and prevent increased permeability of the mucosa, possibly making bacterial translocation and sepsis more potent.

Grisham[13]and Kubes[14] found evidence of biochemical changes and the presence of an inflammatory response of the intestinal epithelium compared to the IR process due to a significant adhesion and extravasation of leukocytes. Given this verified fact, some authors studied the importance and influence of oxygen free radicals in the injury occurred due to the ischemia and reperfusion.

The use of drugs in mesenteric ischemia after mesenteric artery occlusion: angiotensin II, chelants of free radicals, vasopressin, allopurinol (GII), superoxide dismutase (GIV), tocopherol, ascorbate, adenosine (ADO), heparin (HEP), ketamine and oxipurinol prevent damage caused during ischemia and also extend the bowel tolerance to ischemia and reperfusion[3,15-28].

The importance of experimental studies with Wistar rats is evidenced by the description of many mechanisms involved in the lesions and histopathological changes caused by ischemia and reperfusion. The aim of this study is to observe the changes in the intestinal epithelium of rats, when submitted to the ischemia of varied time and scheduled reperfusion

Methods

The maintenance of experimental animals in the Animal Center of Experimental Medicine and Surgery - NMCE School of Medical Sciences, followed the criteria of scheduled replacement of beds, balanced feed and water *ad libtum* in conventional environment, with controlled temperature according to good practices in animal houses.

The Ethics Committee of Animal Experimentation - EAEC INSTITUTE OF BIOLOGY approved the experimental procedures under the protocol number 133-1.

The experimental protocol used 30 Wistar heterogeneous rats, divided into six groups of five male animals, with mean age of 39.7 days and an average weight of 244 grams. The animal selection was judicious and it excluded those who had physical changes compatible with possible undesirable conditions such as peeling of the skin, eye discharge, hair loss and behavior not compatible to animal welfare.

The animals were randomly divided into six groups of five animals:

GROUP I - Control
GROUP II - Group 10 minutes ischemia
GROUP III - Group 20 minutes ischemia
GROUP IV - Group 30 minutes ischemia
GROUP V - Group 40 minutes ischemia
GROUP VI - Group 60 minutes ischemia

All animals underwent surgery consisted of intestinal ischemia by placing a vascular hemostatic clamp in the superior mesenteric artery, according to predetermined times for each group. Following this procedure the reperfusion of the intestine was performed, just after the removal of clamps and release of the mesenteric artery flow. After the period of 24 hours, the animals were euthanized with an intestinal biopsy for collection of

pathologic analysis.

The experiment was conducted in the animal house of Microprocedures and Vascular Research Center for Experimental Medicine and Surgery, Medical Sciences School, State University of Campinas (UNICAMP). The preparation of the operative field included shaving hair and antisepsis with iodine alcohol 2% after the anesthetic procedure and fixing plate heated to 37°C.

The anesthetic used was sodium Thiopental <2% at a dose of 30 mg / kg / body weight, intravenous flow, emphasizing that the same anesthetic was used in all groups, so it was not necessary to perform anesthetic supplementation.

The operative procedure was carried out through laparotomy, opening the plans of the wall, anterior and posterior peritoneum (Figure 1).

FIGURE 1 – Opening of Animal Model with abdominal cavity.

The handles were exposed with exteriorization of the abdominal cavity. Once located, the superior mesenteric artery dissection was performed. Vascular hemostatic forceps were used for clamping the superior mesenteric artery according to the predetermined duration of ischemia for each group respectively (Figures 2 to 5).
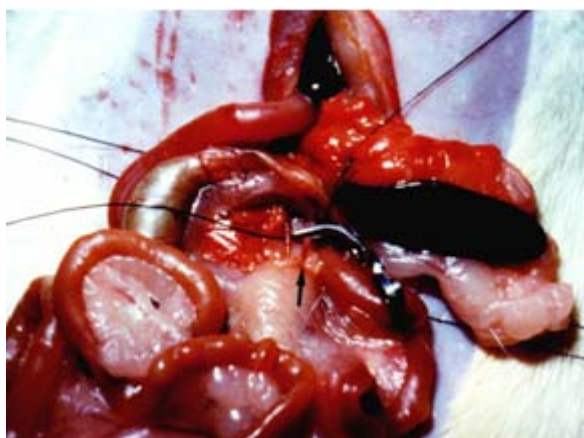

FIGURE 2 – Isolation and clamping the superior mesenteric artery TROUGH VASCULAR a hemostat - seta.

FIGURE 3 – 10 MINUTES ISCHEMIA - RED WINE STAIN of the proximal small bowel (arrow).

FIGURE 4 – SMALL INTESTINE AFTER 40 minutes of ischemia - STAIN dark (arrow).

FIGURE 5 – SMALL INTESTINE AFTER 60 minutes of ischemia.

After determining clamping time, vascular hemostatic clamp was withdrawn from the superior mesenteric artery with replacement of the bowel into the abdominal cavity and closure of the peritoneum, aponeurosis and skin, in layers.

During the intra-operative phase, mesenteric ischemia was performed with pre-determined times to the different groups. In Group I - considered the control group - we only performed the opening of the abdominal cavity in layers of exposure of the handles, dissection of the artery and closure in layers. In Group II, we did the same surgical procedure than in group I, but we did the dissection and placement of hemostatic clips in superior

mesenteric artery for 10 minutes. After this time, we made the unclamping of the superior mesenteric artery with subsequent closure in layers.

In Groups III, IV, V and VI we followed the same technique of the previous groups except for group I, with clamping of the superior mesenteric artery during 20, 30, 40, 60 minutes respectively.

The animals were kept in individual cages with solid and liquid diet *ad libitum*. After completing 24 hours of the first surgery, they were anesthetized via inhaled ether (update anesthetic methodology abolishing the use of ether-2000) and reoperated by the same team, adopting the same technique in order to make the collection of the necessary material for evaluation.

After re-laparotomy the identification of the cecal ileum valve was carried out and subsequent bowel resection. The collection of all pieces of intestine (jejunum) always obeyed the anatomical arrangement of the irrigation of vascular arcades, from caudal to cranial and from ileum to jejunum. The material was preserved in buffered formalin.

After collection and biopsies, the animals were sacrificed with a lethal dose of ether, as recommended by the Brazilian College of Animal Experimentation, within ethical standards (Update methodology abolishing the use of anesthetic ether).

The fragments of intestine were carefully selected according to the description of the item "collection of material ", washed in 0.9% saline solution, opened in their anti-mesenteric border and stretched on small cork plates with the mucosa exposed and their four borders tied up with pins. After these procedures, the fragments were placed in Carnoy solution for six hours to be fixed. Following that, the fragments were processed according to routine methods for optical microscopy. Histological sections of five micrometers thick were dyed by hemalúmen and then by eosin.

The cuts assembly on each slide was made as follows: we chose the first cut of paraffin queue then we discarded the subsequent five cuts and we set up the seventh. This procedure was successively done until obtaining five cuts per slide. The purpose of this assembly was the detection of microscopic findings distinct from one another, for morphometric evaluations.

Qualitative histological evaluation

During ischemia and the period of blood flow restoration, intestinal mucosal lesions (reperfusion injury) were evaluated by analysis of histological sections, at different times of ischemia and reperfusion, in all animals belonging to six groups - DOUBLE-BLIND. Inflammation and necrosis of intestinal villus and crypts were graded according to the classification proposed by Granger *et al.*[25] as described below:

a - Villus

0 - no injury

1 – lesion on the tip of rare villus

2 - injury at the end of most villus

3 - injury at the end of most villus and the measuring middle portion of 10 villus

4 - injury in the end of middle and lower portions of most villus

b - Crypts

0 - no injury

1 - injury of rare crypts (<10%)

2 – injury of sparse crypts (> 10% and <75%)

3 - injury of many crypts (> 50% and <75%)

4 - injury of most crypts (75%)

Quantitative histological evaluation

With the aid of an ocular 8x, a graduated scale and a 10x objective, the measurement was made in ten well-oriented villus (cut across its length) in each of the animals at different times of ischemia.

After measuring these structures, we proceeded calibrating the scale of the ocular with a slide fitted with special divisions of 0.01 mm (10um), in order to transform the measures (taken in units of the ocular) in micrometers so we could properly express the dimensions of the structures mentioned in the metric system.

The mitotic index (MI) is the percentage of nucleus in mitosis of a given cell population compared to those in inter-phase. This was obtained by counting 100 cells in the middle and/or bottom of the intestinal glands (intestinal crypts) and writing down how many nucleus of these 100 cells were in mitosis. For each animal of the different groups were counted 500 cells of five microscopic fields, randomly taken.

Statistical analysis

To evaluate possible differences between the groups previously defined for the various lengths, widths and mitotic indexes of the villus, we used a nonparametric test for Kruskal-Wallis averages. It should be noted that when there was statistical

significance, we supplemented this test adopting the test of multiple comparisons.

The significance level for the rejection of the nullity hypothesis was set, always in a value less than or equal to 0.05 (5%).

When the calculated statistic presented significance, we used an asterisk (*) to characterize it.

The averages were calculated and presented for information purposes only. With the use of nonparametric statistical test, we assumed that the variables under study do not behave like a Gaussian curve. So, it makes sense to calculate the standard deviation[25,26].

Results

Qualitative histological evaluation

In the intestinal samples belonging to different groups of animals we verified a complete regeneration of the intestinal mucosa after a 24 hours reperfusion (Figure 6).
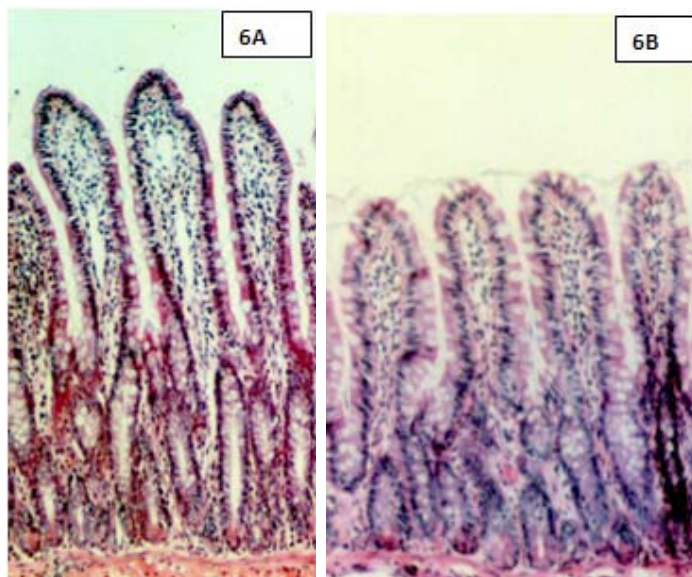

FIGURE 6 – Photomicrography. **A.** Intestinal villus of animals from Group I (Control) with hematoxylin-eosin (HE) coloration (100x). **B.** Intestinal villus of animals from Group III submitted to 20 minutes of ischemia (HE, 100x).

Quantitative histological evaluation (morphometry)

In the morphometric analysis of the intestinal villus of the different groups we observed a reduction in its length, as we increased the time of ischemia. This reduction in villus length was statistically significant when comparing to the villus that underwent 60 minutes of ischemia (group VI) in relation to the

control group (no ischemia) and to the villus that underwent 10 minutes of ischemia (group II). (Tables 1 and 2, Figures 7 and 8).

TABLE 1 - Length of intestinal villus (μm) of animals submitted to progressive increase of ischemia time according to group I (control), group II (10 minutes), group III (20 minutes), group IV (30 minutes), group V (40 minutes) and group VI (60 minutes).

Group	I	II	III	IV	V	VI
	287	275	249	193	211	166
	275	277	216	196	219	172
Length of intestinal villus in μm	286	251	176	181	190	170
	274	243	203	187	166	174
	281	261	245	ND	ND	169
Average	280.60	261.40	217.80	189.30	196.50	170.20

ND = Not determined
μm = milimicras
The dates were been submitted to statistical analysis for the Kruskal-Wallis test and multiple comparisons test. The Kruskal-Wallis test showed these results:
Hcrit = 11.07 (critical length)
Hcalc = 22.54 (calculated length estimated ≥ critical statistically significant)
Hcorr = 22.55 (corrected length)

Multiple comparison tests have been done to complete the analysis of the results of this experimental study and demonstrated statistical significance difference.

TABLE 2 – Multiple comparison test analyzing the morphometry compared of the villus.

Pairs	VADMP	MSD
I, II	4.00	15.27
I, III	10.90	15.27
I, IV	15.30	16.20
I, V	14.93	16.20
I, VI	21.40*	15.27
II, III	6.90	15.27
II, IV	11.30	16.20
II, V	10.93	16.20
II, VI	17.40*	15.27
III, IV	4.40	16.20
III, V	4.03	16.20
III, VI	10.50	15.27
IV, V	0.38	17.07
IV, VI	6.10	16.20
V, VI	6.48	16.20

VADMP = calculated difference between the median posts
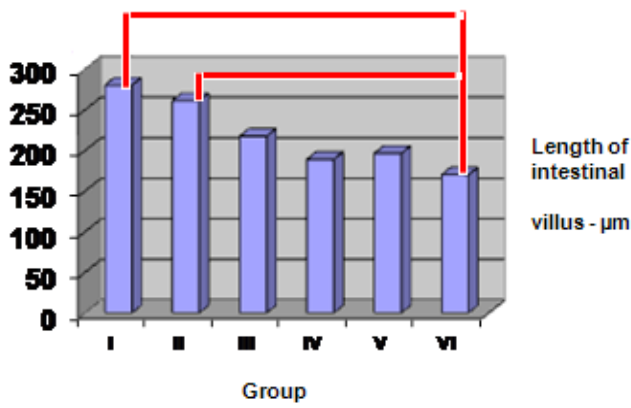MSD = minimum significant difference

FIGURE 7 - Average length of intestinal villus - µm (in relation to groups).
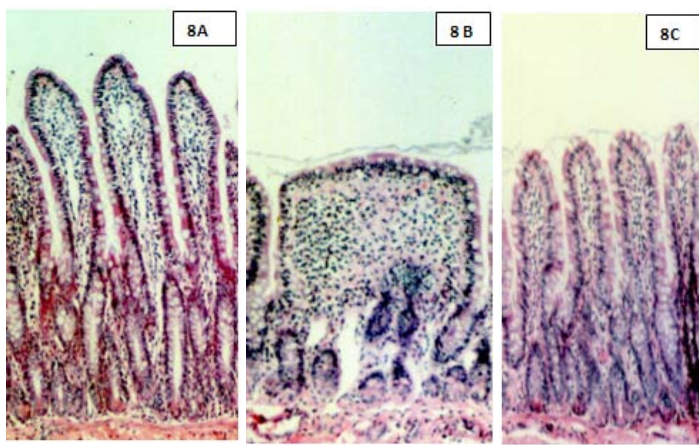

FIGURE 8 - **A.** Intestinal villus of animals from group I (control) with hematoxylin-eosin (HE) coloration; **B.** Intestinal villus of animals from group VI submitted to 60 minutes of ischemia (HE, 100x); **C.** Intestinal villus of animals from group II submitted to 10 minutes of isquemia (HE, 100x).

Comparing Group IV (30 minutes ischemia) and Group V (40 minutes ischemia) to the control group, the results also suggest that the value of the statistic is close to the critical value: (15.3 to 16.2) and (14.93 to 16.2) respectively.

When analyzing the width of the intestinal villus, we observed an enlargement of its area. It should be highlighted that such enlargement is significant when group VI (60 minutes ischemia) was compared to the control group (group I) (Tables 3 and 4, Figures 9 and 10).

TABLE 3 - Intestinal villus width (µm) of animals submitted to a progressive increase in ischemia time, according to group I (control), group II (10 minutes), group III (20 minutes), group IV (30 minutes), group V (40 minutes) and group VI (60 minutes).

Group	I	II	III	IV	V	VI
	77.00	99.00	89.00	89.00	75.00	90.00
Width of the intestinal villus	76.00	78.00	87.00	88.00	77.00	91.00
	75.00	83.00	90.00	87.00	93.00	89.00
	73.00	78.00	78.00	82.00	93.00	91.00
	75.00	80.00	80.00	ND	ND	90.00
Average	75.00	83.60	84.80	86.50	84.50	90.00

ND = Not determined
The dates were been submitted to statistical analysis for the Kruskal-Wallis test and multiple comparisons test. Kruskal-Wallis Test showed this results:
Hcrit - 11.07 (critical width)
Hcalc = 13.52 (calculated width estimated $\geq$ critical statistically significant)
Hcorr = 13.60 (corrected width)

TABLE 4 - Multiple comparison Test analyzing the width of the villus.

PAIRS	VADMP	MSD
I, II	10.60	15.27
I, III	11.70	15.27
I, IV	12.43	16.20
I, V	11.93	16.20
I, VI	8.70*	15.27
II, III	1.10	15.27
II, IV	1.82	16.20
II,V	1.32	16.20
II,VI	8.10	15.27
III,IV	0.72	16.20
III,V	0.22	16.20
III,VI	7.00	15.27
IV, V	0.50	17.07
IV, VI	6.27	16.20
V. VI	6.77	16.20

VADMP = calculated difference between the median posts
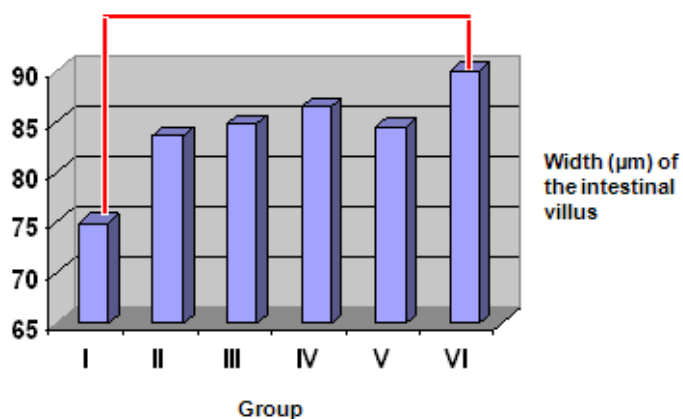MSD = minimum significant difference

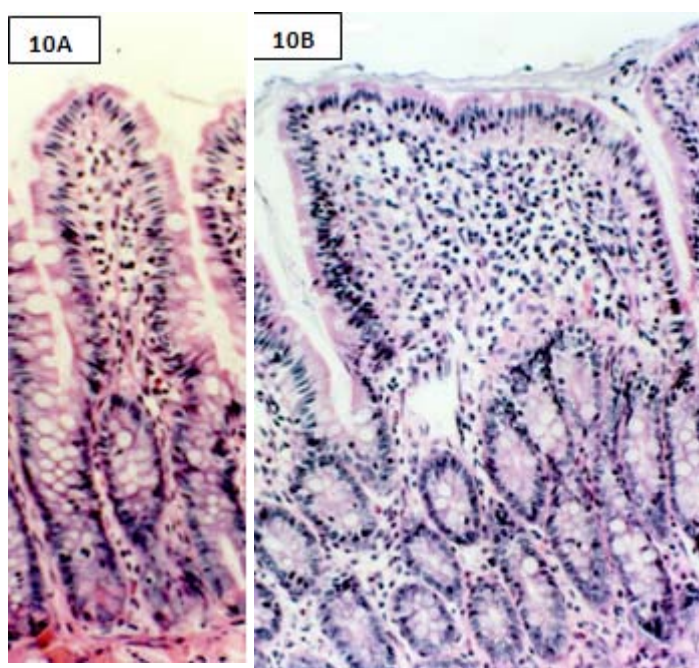**FIGURE 9** - Average width (μm) of the Intestinal villus in relation to groups.

FIGURE 10 – A. Intestinal villus of animals from group I (control), absence of ischemia (HE, 256x). **B.** Intestinal villus of animals from group VI submitted to 60 of ischemia (HE, 256x).

When observing the mitotic index of the villus from different groups, we found an increased percentage of nucleuses in mitosis in the groups that suffered ischemia when compared to the control group (Tables 5 and 6, Figures 11 and 12).

However, statistical analysis found a significant difference in the comparison between group III (20 minutes ischemia) and the control group.

TABLE 5 - Mitotic index of the intestinal villus (%) of animals submitted to a progressive increase in ischemic time, according to group I (control), group II (10 minutes), group III (20 minutes), group IV (30 minutes, group V (40 minutes) and group VI (60 minutes).

Group	I	II	III	IV	V	VI
	4.00	9.00	10.00	9.00	9.00	9.40
Mitotic index of the intestinal villus	3.40	9.60	9.40	10.40	9.80	10.40
	4.80	9.20	10.40	11.00	10.60	11.00
	3.40	9.20	10.20	10.40	11.00	10.40
	4.60	11.00	11.20	ND	ND	9.40
Average	4.04	9.68	10.24	10.20	10.10	10.12

ND = Not determined
The dates were been submitted to statistical analysis for the Kruskal-Wallis test and multiple comparisons test.
Hcrit - 11.07 (critical mitotic index)
Hcalc = 12.96 (calculated mitotic index estimated ≥ critical statistically significant)
Hcorr = 13.11 (corrected mitotic index)

TABLE 6 - Multiple-comparison test of the Mitotic Index.

Pairs	VADMP	DMS
I, II	10.70	15.27
I, III	15.40*	15.27
I, IV	15.13	16.20
I, V	14.63	16.20
I, VI	14.50	15.27
II, III	4.70	15.27
II, IV	4.43	16.20
II, V	3.93	16.20
II, VI	3.80	15.27
III, IV	0.27	16.20
III, V	0.77	16.20
III, VI	0.90	15.27
IV, V	0.50	17.07
IV, VI	0.63	16.20
V, VI	0.13	16.20

VADMP = calculated difference between the median posts
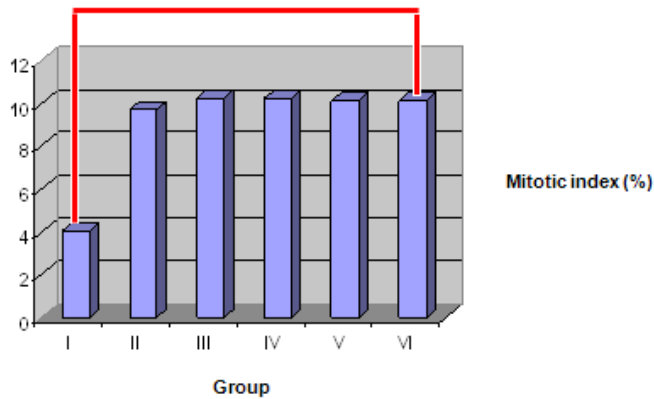MSD = minimum significant difference

FIGURE 11 – Average of the Mitotic Index (%) of intestinal villus towards the groups.
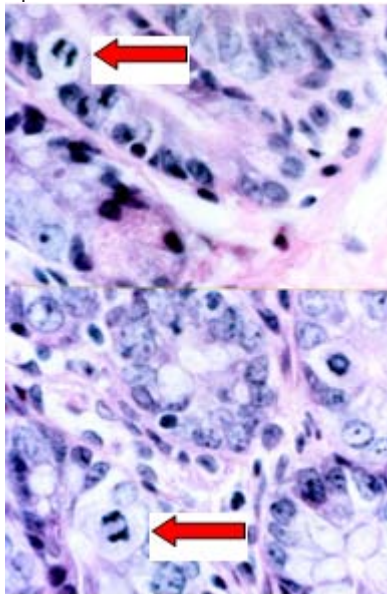

FIGURE 12 - Intestinal villi Animal Group III undergoing 20 minutes of ischemia (HE, 1024x). Myths in figures (arrow).

Discussion

The changes caused by ischemia and reperfusion process in the intestine made several authors develop studies on this topic. These studies found that lesions in the intestinal epithelium and their histopathological changes are proportional to the time of exposure of these tissues to ischemia[4,27-31].

Haglund *et al*.[5] found it possible to detect changes in the endoplasmic reticulum with ischemia of 10 minutes. After 15 minutes of ischemia the mucosa of the intestinal epithelium showed changes in its structure and after 30 minutes of ischemia basal membranes were compromised, indicating a rise in the intra and extra-cellular spaces, with intense lesions at the top of the villus.

The qualitative changes that occurred in the intestinal epithelium after the ischemia process were observed by some authors. According to these authors, the injury caused by the process of ischemia – reperfusion would follow the direction intestinal villus to the crypt, and that the villus showed a greater sensitivity to ischemia[4-6] .Serious injury occurs, also, during the reperfusion, more intensive and in more levels[7-9] . Lundgren[10]suggest that the injury caused by ischemia would be more intense in the villus because the decreased blood flow and oxygenation occurs from the top of the villus to the crypt. Bounus[32] observed that the reduction of the mucosa is proportional to ischemia time, and more intense during the reperfusion phase. Thus, it is known that the epithelium is reconstructed in the reverse direction, in other words, from the crypt to the villus.

The intestinal villus is the last portion of the epithelium to regenerate after ischemia, when analyzed qualitatively. It is known that regeneration of the epithelium is intense and that for a scheduled 10-minute ischemia and it was found almost complete regeneration around three hours of reperfusion[14,26,33].

In the presented work, the exposure of the intestinal epithelium was performed at various times of ischemia, increasing it gradually. Reperfusion was maintained without variation for the different groups (24 hours). Initially, the regeneration was qualitatively evaluated and the result was that according to the classification of Granada the regeneration was full in the different groups of ischemia. However, when considering the morphometric parameters significant changes were observed such as decreased length of villus, increased width and increased mitotic index.

When evaluating the length of the intestinal villus, we observed that the villus in group II (10 minutes ischemia), in group III (20 minutes ischemia), in group IV (30 minutes ischemia) and in group V (40 minutes ischemia) showed no statistically significant decrease in villus length when compared to group I (control). But group VI (60 minutes ischemia) presented significant decrease of the length of the villus, when compared to group I. In this morphometric analysis, it was also observed that when comparing the reduction of the length found in group IV (30 minutes ischemia) and group V (40 minutes ischemia) to the control group (no ischemia), these values are close to the value of statistical significance (15.30 to 16.20) and (14.93 to 16.20), respectively, suggesting a significant decrease in length. When evaluating the decrease in intestinal villus length between groups subjected to ischemia and no more to the control group we observed that: group II (10 minutes ischemia) presents no statistical significance difference in the length of the villus when compared to the group III (20 minutes), group IV (30 minutes)

and group V (40 minutes). However, when comparing the length of the villus in group II (10 minutes) to group VI (60 minutes), the latter had a statistically significant decrease in the length of the villus. Group III (20 minutes ischemia) showed no significant difference in the length of the villus when compared to the other groups (II, IV, V, VI). Group IV (30 minutes ischemia) showed no statistically significant difference compared to groups (III, IV and V), however, as given above, it has presented a significant decrease in the length of the villus when compared to the vilus of group II.

When the width parameter was analyzed, it was observed that the villus of the group VI showed a statistically significant increase in width when compared to group I (control). The villus in groups II, III, IV and V did not show a statistically significant increase when compared to group I (control). By comparing the width measurement between the groups subjected to ischemia, excluding the control, no significant differences were found.

The present study showed that the quantitative analysis presented morphological changes of intestinal villus after ischemia and reperfusion, and that these alterations were not detected in the qualitative analysis, although it was demonstrated that the epithelium is regenerated from the qualitative point of view. When quantitatively analyzed, it was detected significant histopathological changes after 24 hours reperfusion, such as decreased length and increased width. It is important the fact of finding an increased percentage of nucleuses in mitosis (mitotic index) in epithelia submitted to ischemia and reperfusion, after 24 hours, in the process of regeneration. It should be emphasized that it is statistically significant when group III was compared to group I (control). In this way, the findings disorders of mesenteric lymph flow and in vivo intestinal lymphocytes migration and the accompanying increase of endotoxin and TNF-alpha levels in mesenteric lymph, and the lymph drainage could block the "gut-lymph" pathway, improve intestinal barrier function, and attenuate distant organ injury in the early stage of gut I/R injury[34,35]. The intestinal post ischemic perfusion with hypertonic saline through the superior mesenteric artery followed by blood flow restoration is a protective procedure that could be used for the prevention of intestinal ischemia and reperfusion injury[36].

Through comparisons between groups, it can be inferred that the villus regeneration by mitosis begins immediately after ischemic injury caused in the villus. In addition to the qualitative and quantitative histopatological changes already observed, several studies showed biochemical and inflammatory alterations after ischemia and specially reperfusion[14,27,37-40] Some authors think that the preconditioning protection revealed significant

evidence against small bowel wall of ischemia-reperfusion injury and attenuate the permeability of the small intestines after limb ischemia-reperfusion. The protective mechanism of ischemic post-conditioning may be related to inhibition of oxygen free radicals and inflammatory cytokines that cause organ damage[41-45].

The studies of Parks *et al.*[11], Horton *et al.*[12] and Van Ye *et al.*[15]about the importance of free radicals in the injuries caused by ischemia and reperfusion allowed the understanding of the process of these changes, and contributed to an effective action to reduce the harmful effects triggered by them.

Among these performances several drugs have been and are being used, such as "chelating" free radicals and antioxidants substances which provide evidence in reducing injuries caused by the ischemia and reperfusion, as well as extending the length of bowel tolerance time to this process[3,17-19,40,46]. Also the hyperbaric oxygen therapy can limit tissue damage due to ischemia/reperfusion injury, by inducing reparative signaling pathways[47].

Recently the studies are demonstrating the use of gene therapy submucosal infusion of mesenchymal stem cells (MSCs) might exert to show how it is possible to obtain benefits in the prevention of injuries brought about by the process of ischemia and reperfusion[48,49].

The present study demonstrated the importance of performing quantitative analysis to evaluate the changes caused by ischemia and reperfusion, whereas the qualitative analysis may not be sufficient to define these changes. It should be stressed the need to consider morphological changes (quantitative) and mitotic index to evaluate the results of various forms of treatment that can minimize the consequences of the ischemia and reperfusion.

Conclusion

The epithelium is regenerated, when subjected to different time planned of ischemia and reperfusion. It was observed: decrease the length and increase the width of the villi, when compared Group VI with I and II, and Group VI with I; increased mitotic index when compared Groups III with I. Every analysis was statistically significant.

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