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Intestinal fatty acid-binding protein, as a marker of anastomotic leakage after colonic resection in rats

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Abstract

Aim: The aim of this experimental study was to determine if the type of termino-terminal anastomosis of the colon affect the process of healing of the intestinal mucosa and if the severity of the anastomotic leakage (AL) can be predicted based on the circulating level of intestinal fatty acid-binding protein (I-FABP). *Materials and Methods*: In 18 healthy Wistar rats, two types of open colon resection with termino-terminal anastomosis were performed: group A (*n*=9) – without inversing the vascularization and group B (*n*=9) – with inversing the vascularization. To assess the intestinal barrier function, circulating level of I-FABP was measured pre- and post-operatively. I-FABP tissue expression was immunohistochemically assessed in the anastomotic and perianastomotic colonic mucosa. The rats were sacrificed at three, five, and seven days after surgery. *Results*: In both groups, the post-operative serum level of I-FABP increased 3–4 times at 3–5 days and seven times in the seventh post-operative day. In the six cases that showed AL, the increased level was significantly higher: seven times at three days (*n*=2) or five days (*n*=2) and 30 times at seven days (*n*=2). The I-FABP epithelial expression was lost in all cases from group B (as result of prolonged ischemia) and in cases with AL from group A. The I-FABP was translocated in the intraglandular mucus. *Conclusions*: The post-operative level of I-FABP can be appreciated based on the pre-operative value only. A 7–8 increased value in first five days might indicate a risk of AL. After seven days, a seven times increased value is an indicator of a proper healing process but an increasing amount higher that 30 times might predict risk for AL, fistula, peritonitis and septic shock. The risk of AL does not depend on the anastomotic method, although the level of ischemia is higher in anastomoses that involve vascular damage.

Keywords: colon resection, anastomotic leakage, serum FABP2, immunohistochemistry.

Introduction

Digestion and transport of lipids is realized through a water-soluble membrane and transport proteins due to their hydrophobic properties. Cytosolic fatty acid-binding proteins (FABPs) are small transport proteins that are highly expressed in tissues with active metabolism of fatty acids (FAs) [1–3]. The FABP family was discovered in the 1970s and comprises proteins with a molecular weight of 14–45 kDa and common tertiary structure, which is composed from a hydrophobic center, with two alpha chains surrounded by a hydrophilic peripheral zone, formed by 10 anti-parallel beta chains [3]. They are members of intracellular lipid-binding proteins (iLBPs) family [1, 2]. The main function of FABPs is to facilitate the intracellular transport (mitochondria, peroxisomes, endoplasmic reticulum and nucleus) of long chain FAs, bile salts and eicosanoids. FABPs are also involved in modulation of cell growth and proliferation [1, 2].

Nine isoforms of FABPs were described in tissues that exhibit high FAs metabolic activity [4]. They are named based on the first tissue in which they were described, as follows: liver (L-FABP or FABP1), whole intestine (I-FABP or FABP2), heart (H-FABP), ileum (Il-FABP or FABP6), adipose tissue (A-FABP), brain (B-FABP), myeloid tissue (M-FABP), epidermis (E-FABP) and testis (T-FABP) [3, 5]. The ileal FABP is also known as bile acid-binding protein (BABP) [3]. However, their expression is not completely specific for an organ [4, 5]. For example, L-FABP is expressed in liver, intestine and kidney [3].

In the small intestine, three isoforms were described to be expressed in the enterocytes (L-FABP/FABP1, I-FABP/FABP2 and Il-FABP/FABP6) [3–6]. Both I-FABP and L-FABP was shown to especially mark the absorptive intestinal villous cells and are at the limit of detection in crypt cells [3, 7]. I-FABP is mainly involved in intracellular transport and intestinal absorption of lipids. It is highly selective and specific for long chain free FAs [3, 6]. To realize a proper metabolism of dietary lipids, I-FABP can bind the free FAs from cytosol and sequestrate them in enterocytes [6]. L-FABP is mostly expressed in the duodenum and jejunum, whereas I-FABP is more expressed in jejunum [3]. Decreased level of I-FABP and/or L-FABP in enterocytes was previously identified as being a possible indicator of malabsorption [3]. Il-FABP is involved in the metabolism of unsaturated FAs and bile acids [3, 6].

In colonic mucosa, both I-FABP and L-FABP proved to be present in fetal and adult period [7]. L-FABP plays role in cells growing and differentiation [3, 6]. Using immunohistochemical (IHC) methods it was shown a decreased expression of L-FABP in colorectal neoplastic cells, compared to healthy mucosa, being suggested that L-FABP may serve as a marker of dysplastic or malignant cells [6]. There are hypotheses that mention involvement of I-FABP in protection integrity of damaged intestinal mucosa but the exact mechanism is still unknown. In human intestine, L-FABP is more expressed, compared with I-FABP but both isoforms are expressed in mice [3]. Human I-FABP exhibits 78% identical amino acid sequences with mice, 82% with rats and 86% with dogs [6].

I-FABP is also considered a marker of mucosal injury and ischemia and serum I-FABP level is used as a tissue damage indicator [8–12]. Based on the above-mentioned aspects, the aim of this experimental study was to examine the possible correlation between IHC expression of I-FABP in damaged colonic mucosa (after two types of termino-terminal anastomosis of the colon) and serum level of I-FABP in rats. Our hypothesis was that the restore of colonic mucosa integrity depends on the type of anastomosis and the serum value of I-FABP might have predictive value in the evaluation of the anastomotic healing process. This is the first study showing how many times the I-FABP should be rose in serum to predict risk of improper anastomotic healing.

⊟ Materials and Methods

An experimental model for intestinal injury and ischemia was created using two types of open colon resection in rats. The serum I-FABP level was determined before and after surgical intervention and IHC expression of I-FABP in peri-anastomotic tissue was evaluated in surgical specimens.

This experimental study was approved by the Ethical Committee of University of Medicine and Pharmacy of Tîrgu Mureş, Romania.

Surgical technique

Eighteen healthy Wistar rats weighing between 350–450 g were included. Two groups of rats were used, based on the type of colon resections: group A (n=9) – sectioning of the colon, without inversing the vascularization, followed by anastomosis, and group B (n=9) – large segmental resection of the colon, with inversing the vascularization, followed by anastomosis. The restoration of the colon continuity was performed *via* termino-terminal anastomosis with separate or continuous polydioxanone suture (PDS) 5.0. The surgical intervention was performed under anesthesia, *via* open laparotomy. Then, the rats had a normal habit. Three rats from each group have been randomly selected to be sacrificed at three, five, and seven days after surgery.

I-FABP in serum

Pre-operative blood samples (2 mL) were collected from each rat, on the day before surgery, from the dorsal tail vein on anticoagulant support. On the three, five, and seven post-operative days, before sacrification, another 2 mL of blood were collected from each rat. The serum level of I-FABP was determined using Quantikine® ELISA Mouse/Rat FABP1/L-FABP Immunoassay Kit.

The normal serum value was calculated based on the pre-operative levels of I-FABP. As the range interval between minimal and maximal pre-operative value was quite large and an increased post-operative serum I-FABP was detected in all of the cases, we did not use a standard normal value but established an individual one. For any rat, the pre-operative value was considered as the standard normal/individual value (Table 1).

Table 1 – *The pre-* versus *post-operative serum values of I-FABP in the two groups of rats*

	No. of case	Post-operative day of sacrifice	Pre-operative value [pg/mL]	Post-operative value [pg/mL]	Increased value
	1.		6.35	22.53	3.5×
	2.	3	**3.57**	**28.89**	**8×**
	3.		15	44.09	3×
Group A	**4.**		**25.36**	**188.79**	**7.5×**
	5.	5	12.92	43.65	3.5×
	6.		2.86	5.97	2×
	7.		25.55	178.62	7×
	8.	7	**4.31**	**133.89**	**31×**
	9.		6.27	39.96	6×
	10.		5.65	23.62	4×
	11.	3	**3.99**	**29.19**	**7×**
	12.		14.21	43.84	3×
Group B	**13.**		**27.9**	**187.74**	**7×**
	14.	5	13.31	39.83	3×
	15.		3.28	5.9	2×
	16.		26.55	177.49	7×
	17.	7	**4.09**	**133.66**	**33×**
	18.		6.95	43.57	6×

I-FABP: Intestinal fatty acid-binding protein.

IHC expression of I-FABP in intestinal mucosa and peri-anastomotic tissue

For histological and IHC examination, the colon was formalin-fixed and tissue from anastomotic and peri-anastomotic area was paraffin-embedded (two blocks for each rat). The histological specimens were evaluated at three, five, or seven days after surgical intervention based on Hematoxylin–Eosin (HE) staining. Assessment of the healing process took into account the amount and type of inflammatory cells, presence or absence of granulation tissue, characterized by formation and remodeling of the collagen matrix and new vessels formation [11], and presence or absence of fibrosis. Mucosal regeneration at the anastomotic site was also evaluated.

IHC staining with rat anti-I-FABP antibody (clone ab60272, Abbott) diluted at 1:100 was then performed. Citrate buffer was used for heating antigen retrieval and 3,3'-Diaminobenzidine (DAB) was used to perform developing.

The absorptive cells of the rat colon were used for positive external control (Figure 1). The rat used for positive control was a healthy Wistar rat weighing 420 g that was sacrificed without performing surgical intervention. Post-operatively, the I-FABP expression was checked in the anastomotic leakage (AL) but also in normal mucosa adjacent to the resected areas.
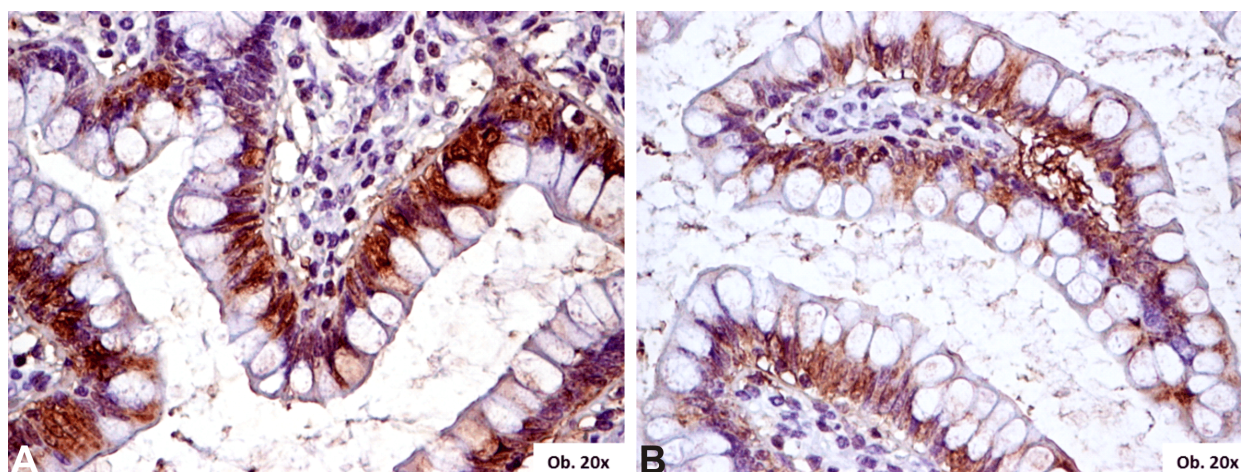
Figure 1 – *(A and B) Cytoplasmic expression of I-FABP in normal colonic mucosa of rats. Anti-I-FABP antibody immunomarking, ×200. I-FABP: Intestinal fatty acid-binding protein.*

Statistical analysis

Statistical analysis was performed using GraphPad system. Data distribution per group was analyzed using analysis of variance (ANOVA) test and simple or large contingency tables. Wilcoxon signed-rank tests were used to compare the median values. $P<0.05$ with 95% confidence interval was used for considering statistical significant values.

⊟ Results

Clinicopathological evolution

All of the rats survived without showing signs of damaged general status. During sacrifice, AL was observed in cases Nos. 2, 4 and 8 from group A and also Nos. 11, 13 and 17 belonging to group B (Table 1).

I-FABP in serum

Comparing with the pre-operative serum I-FABP level that was 11.56±9.032 pg/mL (ranging 2.86 and 27.9 pg/mL), independently from the day of sacrificed rats or the belonging group, the post-operative median value was increased (Table 1) until a median value of 79.18± 68.3 pg/mL (ranging 5.9 and 188.79 pg/mL).

The circulating level of I-FABP was significantly increased post-operatively in both group A and group B (Figure 2). For group A, the pre-operative median value was 11.35±8.991 pg/mL (ranging 2.86 and 25.55 pg/mL), with post-operatively increasing until 76.27±23.56 pg/mL (ranging 5.97 and 188.79 pg/mL). For group B, the values were 11.77±9.61 pg/mL (ranging 3.28 and 27.9 pg/mL) and 76.09±23.38 pg/mL (ranging 5.9 and 187.74 pg/mL), respectively.

For both groups, gradual increase of serum I-FABP level was noted at three, five, and seven days after surgery (Figure 3). No differences were observed between post-operative values in the group A *versus* group B analyzed as median values (p=0.99) or based on the day of sacrifice (Figure 4).

Taking into account data from Table 1, it is important to notice the individual differences among the rats. A large contingency table analysis proved that this individual difference is extremely significant ($p<0.0001$). Raising I-FABP values pre- *versus* post-operatively, independently from the surgical method, were 2–4 times for the post-operative day 3 or day 5 (except cases Nos. 2 and 4 from group A and Nos. 11 and 13 from group B, with increasing of 7–8 times compared with the pre-operative level). As regarding the seventh post-operative day, the I-FABP value increased 6–7 times, except case No. 8 from group A and No. 17 from group B that showed 31 and 33 times increased value compared with the pre-operative level (Table 1).
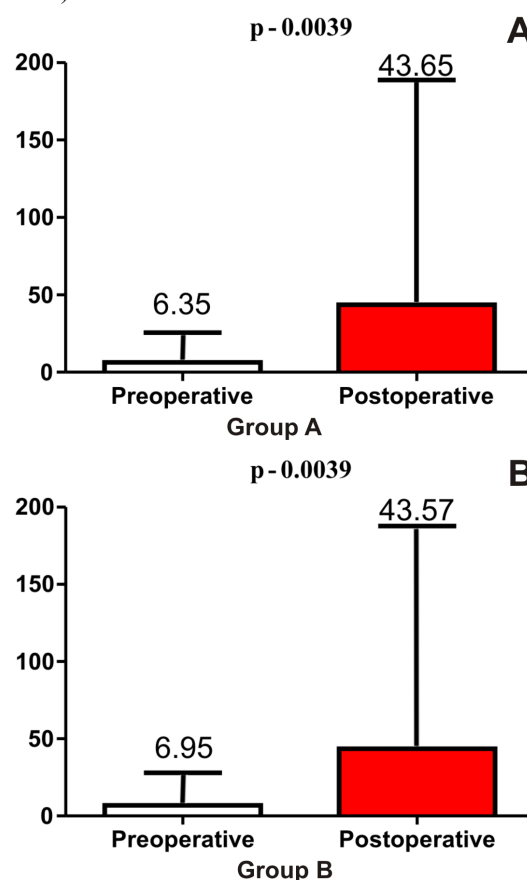

Figure 2 – *(A and B) The pre- versus post-operative serum values of I-FABP in the group A and group B of rats. I-FABP: Intestinal fatty acid-binding protein.*
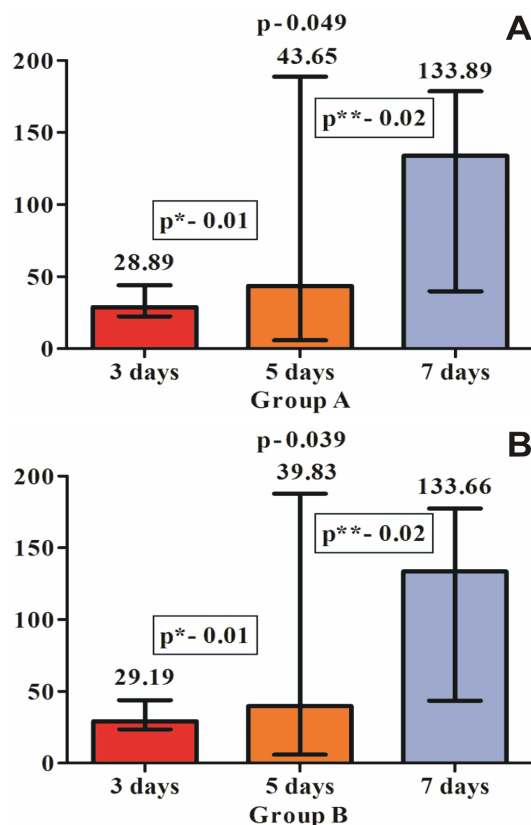
Figure 3 – *(A and B) The post-operative serum values of I-FABP in the group A and group B of rats, based on the day of sacrifice (*3 vs. 5 days and **5 vs. 7 days). I-FABP: Intestinal fatty acid-binding protein.*

Histological features

For the first 3–5 days, the differences between rat intestine in groups A and B were not significant. In all animals, a large amount of inflammatory infiltrate was noted in the anastomotic area, with tendency of mucosal regeneration in animals sacrificed in the day 5 but not in day 3. In the cases Nos. 2 and 4 from group A and Nos. 11 and 13 from group B (Table 1), AL was present, with large and deep fistula involving the entire intestinal layer. In the cases No. 6 from group A and No. 15 from group B, the I-FABP serum value increased two times only. In both of the cases, an excessive amount of granulation tissue, which was composed by newly formed capillaries and fibroblast, was observed, without fistula.

In animals sacrificed in the seventh post-operative day, fibrosis was not complete but mucosa was partly regenerated, except cases No. 8 from group A and No. 17 from group B (Table 1). In both of these cases, AL was large, with transmural inflammation, without regeneration.

IHC expression of I-FABP

In normal mucosa, the absorptive but not goblet cells showed cytoplasmic positivity for I-FAPB (Figure 1). In cases from group A, the I-FABP cytoplasmic expression was diffuse or focally shown by the normal intestinal epithelium from the peri-anastomotic zones and was also shown by the intraglandular mucus. In cases Nos. 2 and 4 that showed high I-FABP serum level, the intestinal mucosa was negative. In the seventh day, independently from the serum level of I-FABP, the epithelial expression was absent (Figure 5).
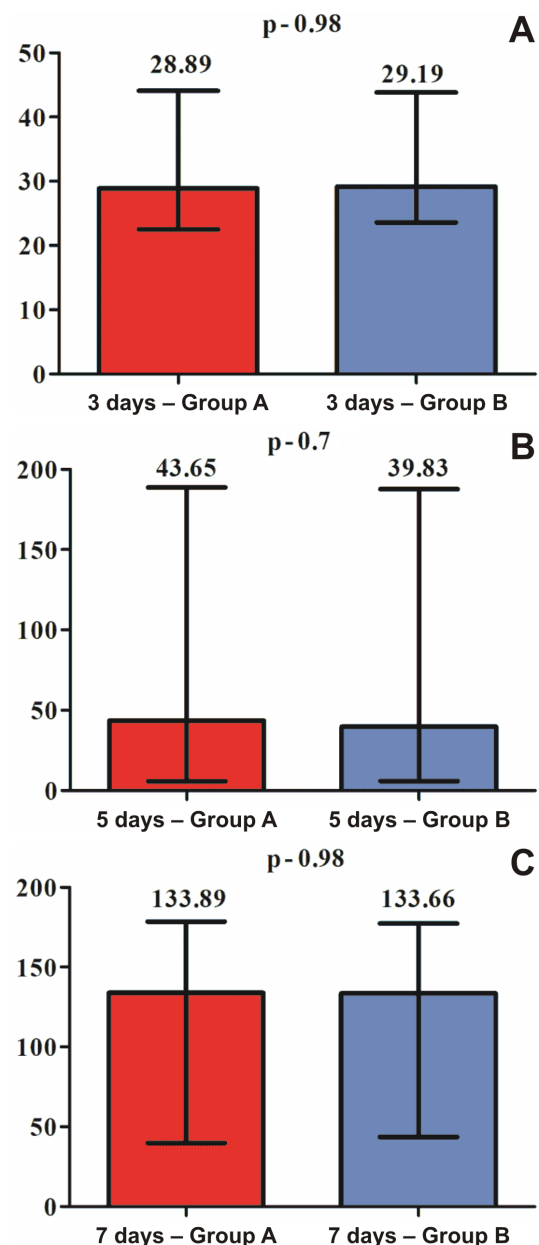

Figure 4 – *The post-operative serum values of I-FABP in the group A and group B of rats sacrificed at 3 days (A), 5 days (B), or 7 days (C) after surgical intervention. I-FABP: Intestinal fatty acid-binding protein.*

In all of the cases from group B, independently from the day of sacrifice and/or the I-FABP serum level, the intestinal mucosa was negative and a significant I-FABP translocation from the epithelial cells insight the glandular lumen was observed (Figure 6).

⊡ Discussions

In some of the previous studies, it was shown that the intestinal barrier function might be compromised by ischemia that also induces bacteria translocation and risk for septicemia [11]. One of the markers that is supposed to be an indicator of colonic mucosa ischemia is the I-FABP, that is codified by the *FABP2* gene, located on the q28–q31 region of chromosome 4 [6, 7, 11]. It can be measured in urine or in serum, as in the present study. Its increased serum value might serve as an indicator of intestinal ischemia as well as tissue damage [8, 11, 13].
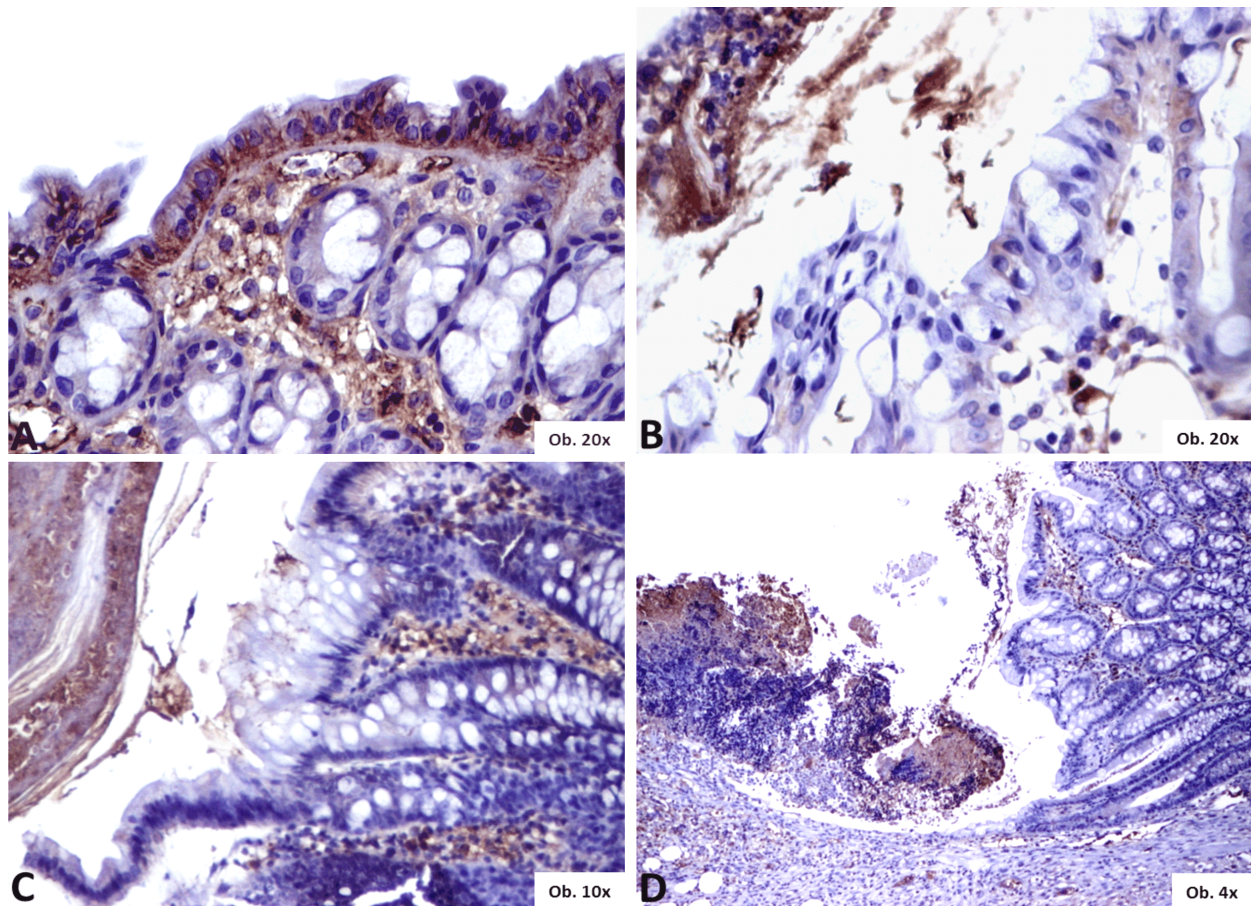
Figure 5 – I-FABP in rats from group A, can be seen in the colonic mucosa in the third post-operative day (A), but is translocated in mucus in the post-operative days 5 (B) and 7 (C) and in cases with anastomotic leakage (D). Anti-I-FABP antibody immunomarking: (A and B) ×200; (C) ×100; (D) ×40. I-FABP: Intestinal fatty acid-binding protein.

Figure 6 – I-FABP in rats from group B does not mark the colonic mucosa and is released in the mucus in the post-operative days 3 (A), 5 (B) and 7 (C) and in cases with anastomotic leakage (D). Anti-I-FABP antibody immunomarking: (A and C) ×100; (B) ×20; (D) ×40. I-FABP: Intestinal fatty acid-binding protein.
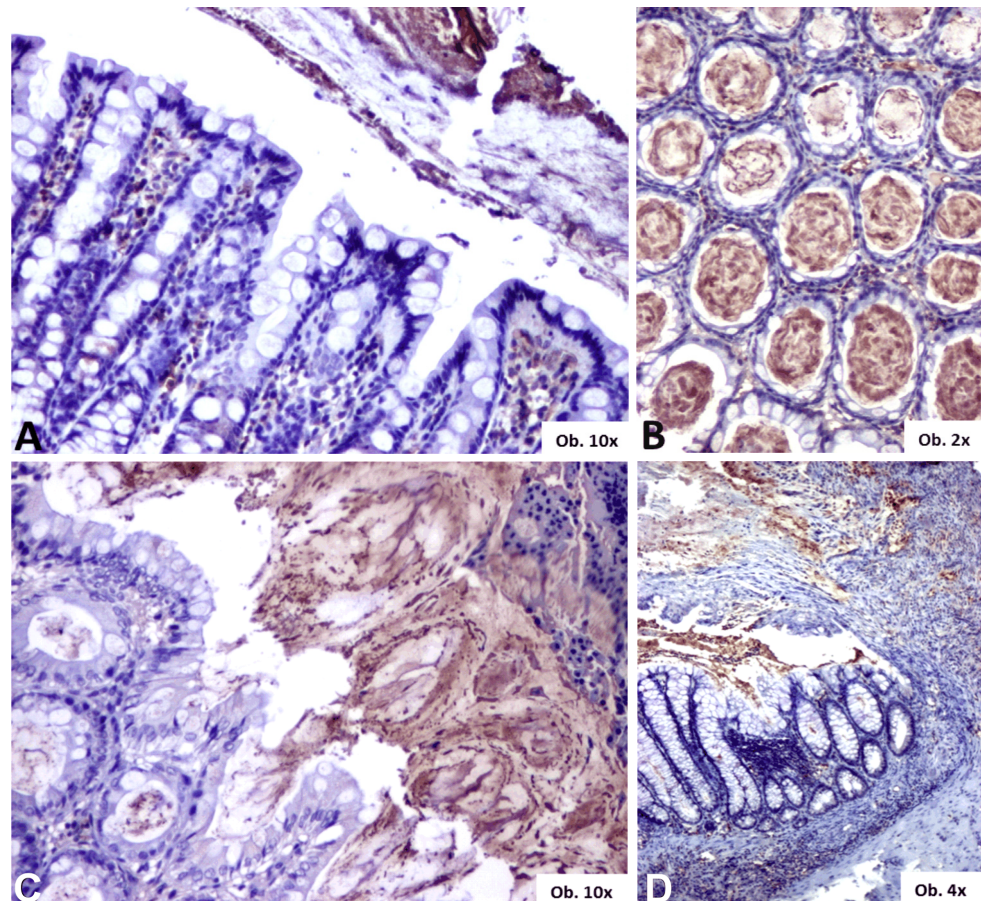
After surgical interventions, such as aortic surgery, it was proved that the severity of intestinal ischemia might be predicted by a significant serum I-FABP level [14]. In polytrauma patients, elevated serum I-FABP was correlated with the severity of the abdominal lesions [15]. In patients with high intra-abdominal pressure and compartment syndrome, significant elevation of serum I-FABP levels was also recorded [16].

In open surgery for colorectal cancer, increased plasma I-FABP level was an indicator of bacterial translocation, severe sepsis and post-operative pneumonia [11, 17]. Based on this fact, it was supposed that, as I-FABP is a small molecule, it can be early released from necrotic intestinal cells, which are affected by ischemia, leading to its increased serum levels [9]. Similar to our data, in both *in vivo* and *in vitro* experiments, I-FABP circulating level was proved to be correlated with the severity of intestinal mucosa damage [10].

As the AL following abdominal surgery is a critical determinant of post-operative recovery and the etiology is frequently unknown [12], in this experimental study we have proposed to evaluate the role of I-FABP in prediction of the severity of AL. For this aim, an AL was created in the rat colonic mucosa using two types of anastomoses: without harvesting the vascularization (group A) and resection with larger vascular resection (group B). Then, the healing process was evaluated under microscope at three, five, and seven days after surgical intervention. The results were correlated with the circulating level of I-FABP.

Our study confirmed data revealing that the risk of AL might be predicted by a significant increased serum level of I-FABP [12]. The I-FABP serum level value might be related on the length of damaged mucosa but also with the time of mucosal-induced ischemia [11]. To suspect an AL, we have proved that I-FABP values should be more than four times at five days and 30 times at seven days after surgery.

Histological findings demonstrated that ischemic intestinal cells present reduction or absence of I-FABP expression, leading to a more easily identification of the ischemic zones [13]. In this experimental study, it was proved that harvesting of the vessels increased the mucosal ischemia and the epithelial cells do not express I-FABP, independently from the serum level of this marker. In cases with anastomosis performed without vascular damage, the I-FABP loss in the epithelium is correlated with significant increasing of I-FABP in serum at 3–5 days after surgery, but its tissue level decreases at seven days, in parallel with the regenerative processes. Although in human adult colonic mucosa, I-FABP was mainly expressed in the lower half of the crypt and a lower intensity was shown for the upper crypt and surface epithelial cells [7], the distribution is probably species-dependent [7] and is not specific for rats.

⊟ Conclusions

As the individual specimen-related serum value of I-FABP is particular, the post-operative level can be appreciated based on the pre-operative value only. A 7–8 increased value in first five days might indicate a risk of AL. After seven days, a seven times increased value is an indicator of healing process but an increasing amount higher that 30 times might predict risk for AL, fistula, peritonitis and septic shock. The risk of AL does not depend on the anastomotic method, although the level of ischemia is higher in anastomoses that involve vascular damage.

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