

# Chronic morpho-functional damage as a consequence of transient ischemia/reperfusion injury of the small bowel

Sergio Morini<sup>1</sup>, Georg Elias<sup>2</sup>, Melisa Brown<sup>2</sup>, Vladimir Subbotin<sup>3</sup>, Cristiana Rastellini<sup>4</sup> and Luca Cicalese<sup>4</sup>

<sup>1</sup>Department of Biomedical Research (CIR), University Campus Bio Medico, Rome, Italy, <sup>2</sup>Division of Transplantation, Department of Surgery, University of Massachusetts, Worcester, MA, USA, <sup>3</sup>Mirus Bio Corporation, Madison, WI, USA and <sup>4</sup>Division of Transplantation, Department of Surgery, University of Texas Medical Branch, Galveston, TX, USA

**Summary.** Introduction: The prevailing notion is that ischemia reperfusion injury of the small bowel induces transient changes that resolve within a few days post-occurrence. However, chronic injury has been described following a single ischemia reperfusion in the kidney. We proceeded to ascertain if a similar outcome is also witnessed in the small bowel. Materials and methods: ACI rats (n=32) underwent 1, 2 or 3 episodes of ischemia reperfusion by clamping the superior mesenteric artery for 45 minutes at 7-day intervals. Control groups included sham-operated (n=6) or non-operated (n=5) rats. Morphology was examined at day ninety post-ischemia reperfusion and immunostaining was used to evaluate macrophage infiltration, microvascular distribution, and apoptosis. RT-PCR was used to evaluate expression of Inter-Cellular Adhesion Molecule-1 (ICAM-1), transforming growth factor- $\beta$  (TGF- $\beta$ ), Insulin Growth Factor-I (IGF-1), and Insulin Growth Factor-I Receptor (IGF-R). Intestinal function was evaluated by D-xylose performed 24 hours and 4, 8, and 12 weeks after reperfusion. Results: Chronic morphologic changes were observed with degeneration of crypts, endothelial damage, matrix degeneration, and heightened lymphocyte degeneration within the Payer's patches. Major structural changes were characterized by villous atrophy from partial to total. The grade of histological injuries was significantly increased ( $P<0.001$ ) after multiple ischemia reperfusion episodes. A higher number of apoptotic cells ( $P<0.001$ ) and a prominent macrophage infiltration ( $P<0.05$ ) was also witnessed. Altered expression of ICAM-1, TGF- $\beta$ , and IGF-1 was observed. At 24 hours after ischemia reperfusion D-xylose absorption was diminished, returning to baseline values within 4 weeks and

becoming abnormal again at 8 and 12 weeks ( $P<0.05$ ). CONCLUSIONS: Unlike the prevailing conviction, these data demonstrate that transient ischemia reperfusion repeated injuries of the small bowel result in chronic intestinal damage.

**Key words:** Chronic intestinal injury, Intestinal transient ischemia, Transient ischemia/reperfusion, Small bowel, Intestine

## Introduction

Transient ischemia reperfusion (IR) injury of the small bowel is common to several pathological conditions. Historically, IR has been mostly associated with macrovascular blood flow alterations, such as mesenteric vascular insufficiency. Shock and severe hypotension are associated with gut IR for the reduced splanchnic flow due to a physiologic central redistribution of the haematic mass (Morini et al., 2000). Intestinal IR can be surgically induced, when the mesenteric flow is reduced due to temporary clamping during vascular reconstructions (Jarvinen et al., 1999). During small bowel transplantation, the graft is harvested after exsanguination, stored in cold preservation solution then revascularized in the recipient. These unavoidable events lead to IR or preservation injury (Massberg and Messmer, 1998). Moreover, there are several conditions where intestinal IR mechanisms have not been fully understood and often involve the microcirculation. Trauma and burn can generate oxygen free radicals and angiotensin II which can induce vasoconstriction and ischemia (McCord and Roy, 1982; Tadros et al., 2000). Laparoscopy has been associated with intestinal IR (Kleinhaus et al., 1978; Sternberg et al., 1998; Emir et al., 2001). Furthermore, acute rejection of intestinal grafts is associated with a

decreased mucosal blood flow and transient IR (Tabasco-Minguillan et al., 1995; Dindelegan et al., 2003; Oltean et al., 2005). Inflammatory bowel disease, such as Crohn's disease, is accompanied by microvascular injury and it has been associated with IR (Brown et al., 1934; Thornton and Solomon, 2002). Radiation therapy can also cause intestinal microvascular injury (Langberg et al., 1994).

It is a common tenet that IR causes a series of events that produce injury followed by a complete regeneration of the mucosa within a few days without long-term consequences (Robinson et al., 1974; Muller et al., 1994; Takeoshi et al., 2001). Nonetheless, in organs such as the kidney, a single ischemic event leads to chronic morphological changes, fibrosis and nephropathy. Moreover, IR injury has been recognized as an important etiologic component of chronic nephropathy following renal transplantation (Bursztyn et al., 2001).

Most of the research has been focused on studying the events and mechanisms associated with acute intestinal IR injury, while long term studies have been precluded by this notion. However, considering that mucosal blood flow is significantly reduced during each IR event, repeated re-acutezation in chronic bowel diseases, or repetitive acute rejection in intestinal transplantation (Grant et al., 2005), or radiation therapy, could contribute to producing chronic damage, finally affecting intestinal morphology and function. These observations lead to the hypothesis that single or multiple transient IR events in the small intestine might exacerbate chronic changes, similarly to what has been observed in the kidney. Starting from this hypothesis, we designed a novel small animal model that mimics the clinical scenario where the bowel is subject to repetitive transient IR events and where the long term effects of these can be investigated.

## Materials and methods

### *Study design and surgical procedure*

Male ACI rats (Sprague-Dawley Inc., Indianapolis, IN) weighing 150 to 200 g were used for the study. Rats used in the study group (total number =32) underwent I/R by clamping the superior mesenteric artery (SMA) for 45 minutes either once, twice or three times at 7-day intervals. Two additional control groups included sham-operated (n=6) and not operated (n=5) rats. All animals were sacrificed 90 days after the last surgery. Survival and body weight were evaluated throughout the study. Animals were maintained in accordance with the *Principles of Laboratory Animal Care*. Rats were anesthetized using inhalation of methoxyflurane (Pittman Moore Inc., Mundelein, IL) for induction and intraperitoneal injection of 25 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, IL) for maintenance. Through a midline incision, the small intestine was mobilized cephalad and was protected with a sponge moisturized with sterile saline solution and the SMA was identified and isolated. Transient ischemia

was accomplished occluding the SMA for 45 minutes with a small atraumatic vascular clamp. Successful occlusion was confirmed by absence of pulsation at the periphery of the mesentery and by blanching of the intestine. After this period of ischemia the clamp was removed, the intestine was perfused again, and the abdomen was closed. Animals were fed immediately after surgery. Animals receiving multiple episodes of IR were re-operated using the same technique at 7 day intervals.

### *Histopathology*

For the histopathologic study the animals were sacrificed 90 days after the third IR episode, while the sham operated animals were sacrificed after a corresponding period of time from the surgical procedure. Samples, of 2 cm in length, of the small intestine were taken every 10 cm aboral from the ileocecal valve. The samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin; 4  $\mu$ m thick sections were cut and stained with haematoxylin-eosin (H&E) and Verhoeff-Van Gieson's Trichromic. All microscopic examinations were performed using an Olympus BX40 microscope in blinded fashion.

### *Microscopic evaluation*

From each animal 6 different samples were considered in order to evaluate the degree of intestinal tissue injury. A grade corresponding to the damage was attributed to all the villi held in each sample. The damage was evaluated following the microscopic criteria in the grading of intestinal injury previously described by Chiu et al. (1970) and later adapted to rats (Haglund et al., 1980; Park et al., 1990), as follows: Grade 0: normal mucosal; Grade 1: subepithelial space at villus tip; Grade 2: more extended subepithelial space; Grade 3: epithelial lifting along villus sides; Grade 4: denuded villi; Grade 5: loss of villus tissue; Grade 6: crypt layer infarction; Grade 7 and 8: transmucosal and transmural infarction.

These criteria were originally used to evaluate acute IR injury. In order to adapt to non-acute damage, we considered to be within grades 1-3 the presence of necrotic matter in the subepithelial space, even though apparent integrity of the epithelium could be observed.

An amount of more than a thousand villi were considered for each group of animals. For every group of animals the number of villi found within each single grade was normalized to the percent number of the villi considered. The results of the grading of mucosal injury were expressed as median and range. Statistical analysis was performed using Mann-Whitney-U test for independent data (software SPSS, Chicago, IL).

### *Immunohistochemistry*

Four micrometer paraffin sections were used for  $\alpha$ -smooth muscle actin and for in situ apoptotic cell immunostaining. Monoclonal mouse anti-human  $\alpha$ -

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smooth muscle actin antibody (DAKO Corporation, Carpinteria, CA) was used. As control we used normal and grafted aortas. This antibody does not react with actin present in fibroblasts, but does react with actin present in smooth muscle cells and pericytes (Skalli et al., 1986, 1989).

For macrophage detection specimens of small bowel were embedded in O.C.T. (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Four micrometer frozen sections were mounted on the charged precleaned slides (Fisher Scientific, Pittsburgh, PA), air dried at room temperature, fixed in cold acetone for 10 minutes and air dried. Monoclonal mouse anti CD163, also known as ED-2, antibody (Accurate Chemical Corporation, Westbury, NY) was used. This antibody recognizes membrane antigens of tissue macrophages. Positive and negative controls were performed on rat spleen sections.

The indirect immunohistochemical technique was used (Sternberger, 1979). After blocking the endogenous peroxidase activity, Avidin/Biotin Blockage (Vector Laboratories, Burlingame, CA) serum was applied to reduce nonspecific background staining. Specimens were then incubated overnight with the primary antibody in a humidity chamber at 4°C. After washing in phosphate buffer saline (PBS), the biotinylated secondary antibody (Vector Laboratories) was applied. The sections were then incubated with streptavidin-peroxidase complex (Vector Laboratories), and the subsequent development was performed with 3-amino-9-ethylcarazole (DAKO). Sections were counterstained with haematoxylin (BioGenex, San Ramon, CA) to facilitate nuclear identification.

For the detection of in situ apoptotic cells, the ApopTag<sup>®</sup> Peroxidase Kit (Serologicals Co., Pensacola, FL) was used. We followed the indirect method: in brief, nucleotides labelled with digoxigenin were enzymatically added to the DNA by terminal deoxynucleotidyl transferase enzyme (TdT). The labelled DNA was detected using an anti-digoxigenin antibody peroxidase conjugated fragment. Detection was then performed using 3-3' diaminobenzidine (DAKO) as chromogen.

In order to avoid any ambiguity regarding the

interpretation of possible false positive results and to confirm the apoptotic mode of cell death, the morphology of respective cells was carefully examined microscopically.

The number of both ED2 positive macrophages and apoptotic cells was counted on sham operated and treated animals. 3-5 different samples from each animal were considered. The count of ED2 positive cells was performed at high magnification (40x), choosing randomly at least 6 different fields for each sample. Data are expressed as mean number of positive cells/field  $\pm$ SEM. For the count of apoptotic cells we used the same method, but considering only the crypt area. The values obtained were then converted in percent of positive cells/per field, and the mean and standard deviation (SEM) were calculated for each group. Statistical analysis was performed using Student's t test.

### RT-PCR

Samples of gut from test and control animals were snap-frozen in liquid nitrogen. Total RNA was extracted from frozen tissue using Ultraspec reagent (Biotecx, Houston, TX), following the manufacturer's protocol. Total RNA was treated with RNase-free DNAase (Gibco, Grand Island, New York) and 5.0  $\mu$ g of RNA was converted to cDNA using Superscript II preamplification kit (Gibco). The primers used for PCR are shown in table 1. The reaction mixture contained 1x PCR buffer (Gibco), 1.75 mM MgCl<sub>2</sub>, 0.25 mM dNTP, 20 pico moles of each of the primers, cDNA template equivalent to 250 ng of total RNA and one unit of Taq DNA polymerase (Gibco) in a total volume of 50  $\mu$ l. The reaction was performed using a GeneAmp Thermal Cycler (PEC, Foster City, CA) programmed at (a) 94°C for five minutes (one cycle), (b) 94°C for 30 sec, 55-58°C for 30 sec and 72°C for 30-90 sec (30-35 cycles), (c) 72°C for five minutes. The number of cycles in step II was determined based on control experiments to prevent product saturation. The products were electrophoretically resolved in 1.4% agarose gels and visualized after staining with SYBR green I (BMA, Rockford, IL). The bands were transferred onto nylon

**Table 1.** PCR primers used.

Primer	Sequences	Annealing temp (°C)	Product size (bp)	Specificity
TGFbF TGFbR	5'ACGTCAGACATTCGGAAGCAGTG3' 5'GCAAGGACCTTGCTGTACTGTGTG3'	57	615	Rat TFb cDNA
ICAM-1F ICAM-1R	5'GGTGTGATATCCGGTAGACA3' 5'GCACCCTGACCTGTGGAGAA3'	55	265	Rat ICAM-1 cDNA
IGFF IGFR	5'ACAGCCGGACCAGAGACCCT3' 5'CGCTGGGCCCGGATGGAAC3'	58	237	Rat IGF cDNA
IGFrF IGFrR	5'GTGCGTGGGTGTGTGCTCACT3' 5'GAATGAGGAGACTCAATTCTC3'	56	277	Rat IGF receptor cDNA
$\beta$ -ActinF $\beta$ -ActinR	5'TTCTACAATGAGCTGCGTGTG3' 5'TTCATGGATGCCACAGGATTC3'	55	565	Mammalian $\beta$ -Actin cDNA

membrane (Amersham-Pharmacia, Piscataway, NJ) and then hybridized with  $^{32}\text{P}$  labelled DNA probes using standard techniques. After extensive washes, the membrane was exposed to Kodak Xomat AR film (Eastman Kodak, Rochester, NY). The band intensity was measured using a scanner. The ratio of band intensity for TGF $\beta$ , ICAM-1, IGF or IGF-R and  $\beta$ -Actin for control and test rats was determined and compared.

#### Intestinal absorption

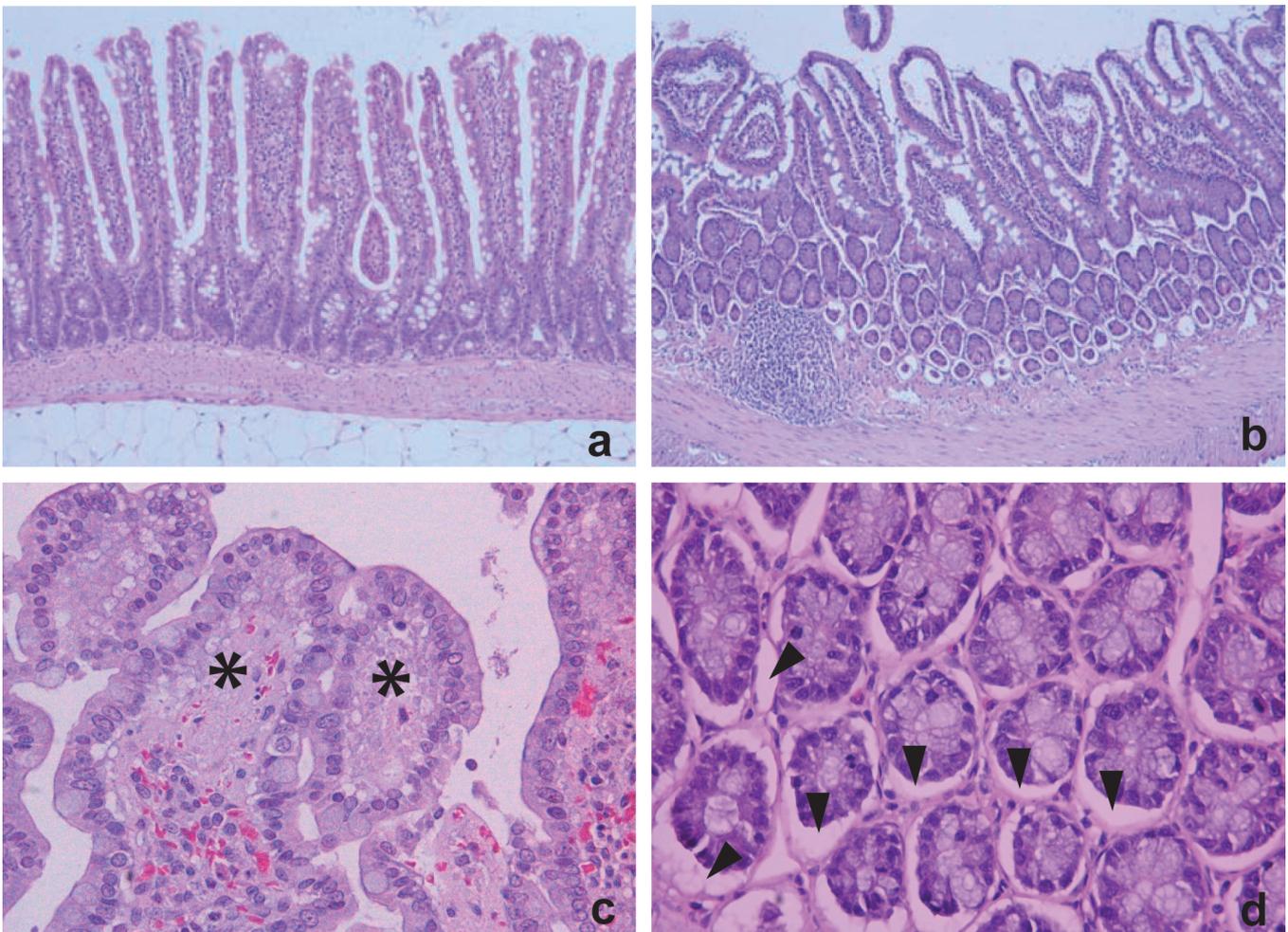
Gut absorptive capacity was determined using the D-Xylose absorption test. Rats were put in metabolic cages and fasted overnight. Baseline blood sample was obtained prior to D-Xylose administration; serum was separated off and stored in a freezer until the assay.

Intragastric 5% solution (1 mL per 100 g body weight) of D-Xylose (0.5 g/kg body weight) was administered to the animals by oral gavage using a syringe with an atraumatic tipped needle. Animals were

kept without food and allowed only to drink water. Two hours after D-Xylose administration, a serum sample was obtained and 2 ml of sterile saline was given to the animals subcutaneously for hydration. The urine was collected for a total of 5 hours. The D-Xylose concentration in urine and serum samples was measured by an independent laboratory (ARUP Laboratories, Salt Lake City, UT).

#### Results

All animals survived in the control and sham operated groups. Animals undergoing IR showed different mortality rates. After a single episode of IR only 1 animal died, and the mortality was 12,5%. Two animals died after 2 IR episodes (mortality rate 22%). Animals undergoing 3 episodes of IR experienced mortality over 50% (8 animals died over a total of 15 animals), suggesting that the experiment be stopped at this point. While mortality occurred within 72 hours



**Fig. 1.** Histological appearance of the intestinal mucosa. **a.** Sham control; 90 days following multiple IR events. **b.** Absence of the stromal portion of the lamina propria in the apical portion of the villi and abnormal crypts. **c.** Amorphous necrotic matter (\*) fills the subepithelial space at the tip of the villi. **d.** Diffuse epithelial lifting and separation from the lamina propria (arrowheads) at crypt level. H&E. a, b, x 100; c, d, x 400

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after IR, samples from these animals were not included in the study and the number of rats was restored by other animals until reaching the expected number ( $n=7$  in each group). In animals undergoing repeated operations, adhesions in abdomen were not found, except for delicate brindle close to the wound of the wall.

The rats showed significant weight loss early after the IR episodes especially if multiple clamping was performed. In the group undergoing 3 IR episodes, the mean percentage of weight was  $*87.6\% \pm 4.0$  after the last IR,  $*84.6\% \pm 6.6$  ( $*p < 0.05$ ) 1 week later,  $91.6\% \pm 7.4$  after 2 weeks returning to baseline values approximately 3 weeks after surgery ( $100\% \pm 4.6$ ) [data are expressed as mean percentage of weight from pre-surgical measurements  $\pm$  SD].

### Histopathologic findings

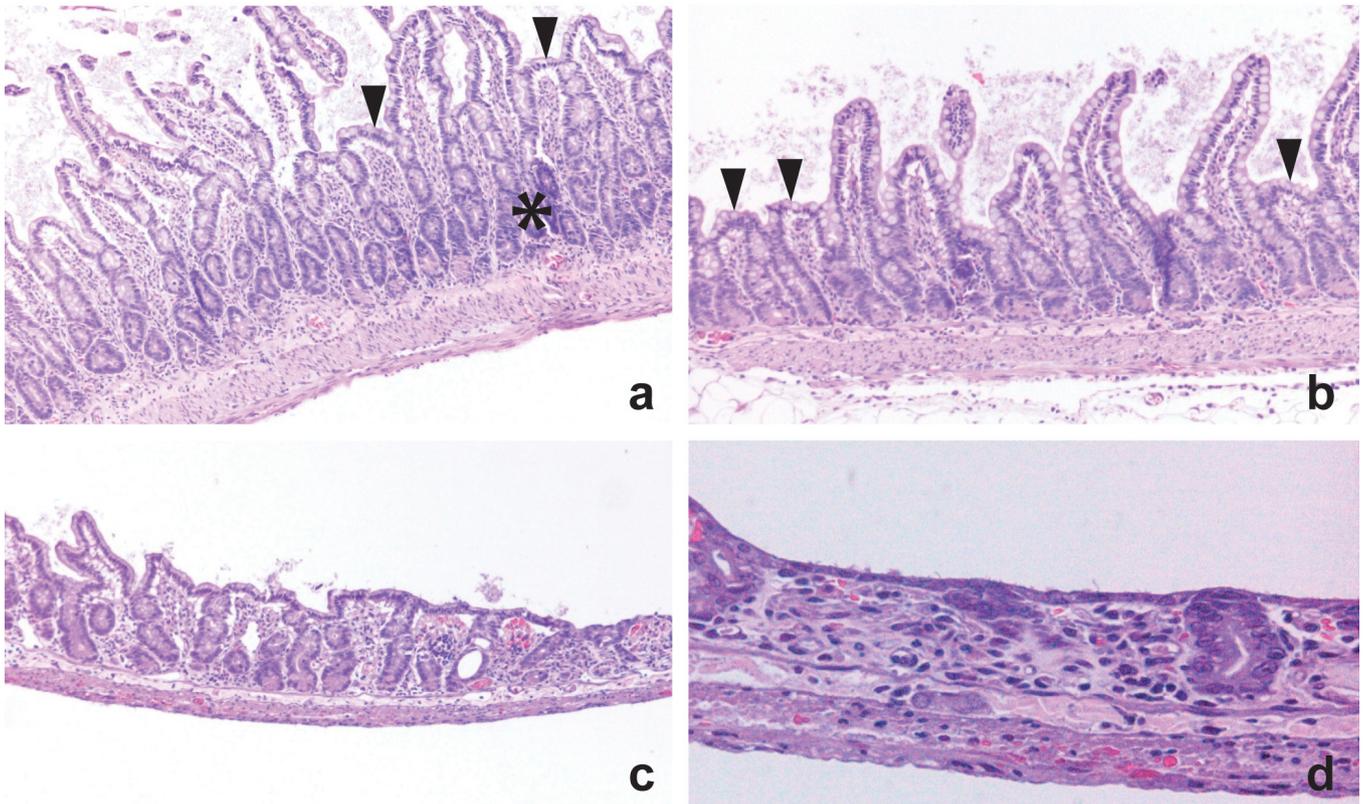
Observation with light microscopy in the study groups revealed diffuse lifting and separation of the epithelium from the lamina propria when compared to sham operated animals that appeared to have normal morphology. The extent of the lifting was variable ranging from the apical portion to the entire villus. Within the same animal different intensities of injury

could be observed, although these changes were progressively increased in the animals subject to multiple, compared to single IR episodes.

In the single IR group mostly normal mucosa or mild damage of the villi was observed. Animals subject to multiple IR events showed changes characterized by the absence of the stromal portion of the lamina propria in the apical portion of the villi (Fig. 1a,b). In the subepithelial space at the tip of the villi amorphous necrotic matter containing lymphocytes, leukocytes and mononuclear cells in various stage of degeneration was commonly seen (Fig. 1c). Damage of the crypts was also observed, extending from the occasional finding in animals subject to a single IR event to widespread degeneration after multiple IR events (Fig. 1d).

However, in some cases major structural changes could be observed. These were characterized by hyperplasia of the crypts and villous atrophy from partial to total. In selected zones total mucosal atrophy with flattened epithelium, absence of goblet cells, complete loss of the villi and progressive degeneration and loss of the crypts was present (Fig. 2).

The score of injury in controls and in sham operated animals ranged from 0 to 1 (mean  $0.01 \pm 0.1$  in controls;  $0.12 \pm 0.4$  in sham operated); in single IR it ranged from 0



**Fig. 2.** Histological appearance of the intestinal mucosa 90 days following multiple IR events. Major damage represented by: crypt hyperplasia and initial villous atrophy (arrowheads) (a); partial villous atrophy, characterized by villi showing normal length and shortened villi (arrowheads) (b); total villous atrophy and reduction of thickness of the intestinal wall (c); complete atrophy of the mucosa with disappearance of the crypts, flattened epithelium and absence of goblets cells (d). H&E. a-c, x 100; d, x 400

to 4 (mean  $0.42 \pm 0.93$ ,  $p < 0.01$  vs. sham operated), and in multiple IR it ranged from 0 to 6 (mean  $2.0 \pm 1.44$ ,  $p < 0.001$  vs. sham operated). Figure 3 reports the grade of injuries in all the groups of animals and the results of the statistical analysis. Observation of the samples stained for collagen fibres (Van Gieson's trichromic stain) confirmed that in the animals subject to multiple IR the collagen of the lamina propria extended from the basal portion of the villus without reaching the apex (Fig. 4a-d).

The distribution of collagen fibres in the submucosa appeared more pronounced, with increased collagen distribution. Particularly, in the Payer's patches thickened distribution of collagen fibres was evident throughout the patches when compared to controls, where a fine reticulum was observed (Fig. 4e,f). Furthermore, there was evidence for matrix degeneration, loss of the germinal centres and heightened lymphocyte degeneration within the Payer's patches.

#### Immunohistochemistry

Observation of the sections stained for  $\alpha$ -actin confirmed that in the animals subject to multiple IR the smooth muscle cells in the lamina propria extended from the basal portion of the villus without reaching the apex. In control samples,  $\alpha$ -actin positive cells were distributed throughout the villus, always reaching the apex (Fig. 5).

The distribution pattern of smooth muscle cells was superimposable to the one previously described for collagen. The muscularis mucosa also appeared slightly thickened near the basal portion of the lamina propria of the villi paralleling the changes seen in collagen distribution.

The distribution of ED-2 positive cells (macrophages) was homogeneous within the areas where collagen was present in all animals. However, the number of these cells was increased after a single IR episode and almost doubled in animals undergoing multiple IR events when compared to controls. Sham control:  $7.1 \pm 1.2$ ; single IR:  $*10.3 \pm 0.6$ ; multiple IR:  $*13.9 \pm 2.1$  ( $*p < 0.05$  vs. control).

The number of epithelial cells staining positively for apoptosis was increased in the apical portion of the villi in animals subject to multiple IR compared to sham controls. This phenomenon was particularly evident where the epithelium presented with lifting. Additionally, apoptotic cells were increased in the crypts. Sham Control:  $1.5 \pm 1.0$ ; single IR:  $*11.5 \pm 1.5$ ; multiple IR:  $*39 \pm 12.5$  ( $*p < 0.05$  vs. control).

#### RT-PCR results

Expression of selected growth factors/mediators: The levels of mRNA for TGF $\beta$ , ICAM-1, IGF were elevated in rats undergoing IR compared to controls. The increase was significant among rats with two or more episodes of IR injury (Fig. 6A,B). However, the level of

IGF $\alpha$  mRNA remained unchanged even after three episodes of IR.

#### Intestinal absorption

At 24 hours after the last IR episode D-xylose absorption was significantly diminished in animals undergoing IR compared to controls, but it returned to the baseline values within 4 weeks. However, at 8 and 12 weeks D-xylose absorption became abnormal again, reaching a stable lower value of D-xylose absorption. Baseline values (control animals):  $11.7 \pm 1.8$ ; 24 hours after 3 IR episodes:  $*3.8 \pm 1.6$ ; 4 weeks:  $12.7 \pm 4.9$ ; 12 weeks:  $*4.0 \pm 0.7$  ( $*p < 0.05$  vs. control).

#### Discussion

This study performed on an animal model provides the first evidence of a long-term effect of transient IR on the small bowel. With this model, the investigators intended to reproduce the clinical situation in which an episode of intestinal IR occurs either once or multiple times, and it is followed by a period in which the intestinal blood flow is normalized. In our opinion, this model may allow the study of the consequences of the ischemic effect that accompanies either macro or microvascular disorders of the small bowel.

Several disorders are associated with transient intestinal IR. These conditions can be associated with necrosis that represents the maximum extent of injury observed. However, for intestinal IR the common tenet is that the bowel can undergo necrosis after prolonged IR, although a shorter event results in temporary injury

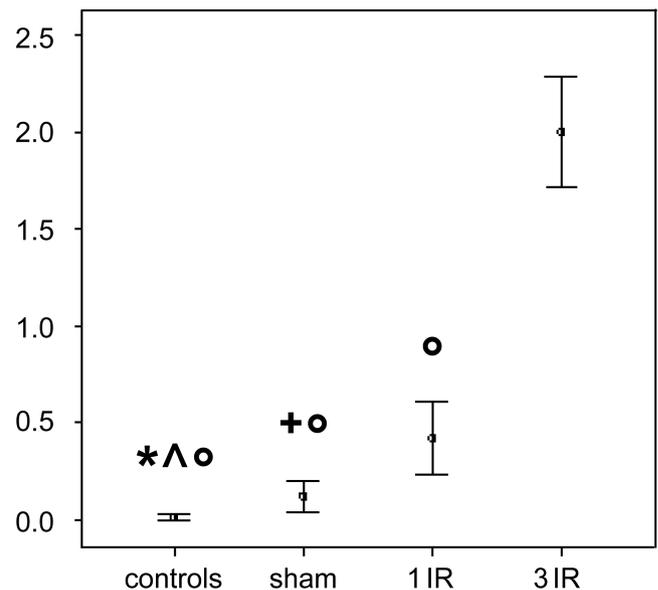


Fig. 3. The graphic reports the mean values, with the 95% confidence interval on the mean, attributed to the histopathologic score (Y axis) of the injury for all the groups. \*:  $p < 0.01$  vs. sham; ^:  $p < 0.001$  vs. 1 IR; o:  $p < 0.001$  vs. multiple IR; +:  $p < 0.01$  vs. 1 IR.

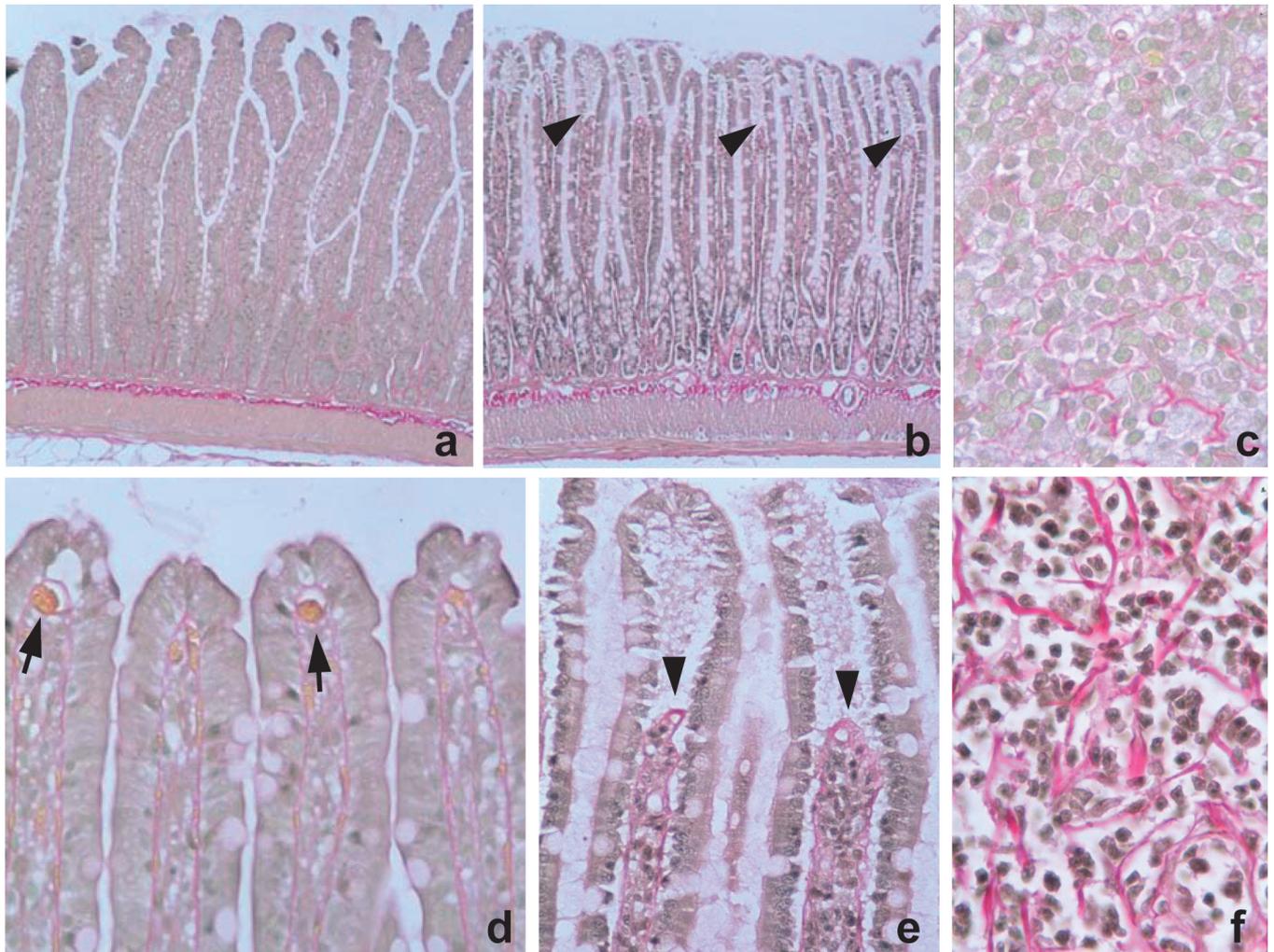
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followed by restoration of morpho-functional integrity without long-term consequences. In a clinical setting it is difficult to obtain samples from the small bowel and these are often endoscopically procured biopsies and the morphologic modifications are scarcely evident when samples are observed using conventional staining. Furthermore, procedures such as intestinal transplants and the research associated with it only recently provided additional insight into intestinal pathophysiology. Moreover, these factors and the inhomogeneous distribution of these changes could explain why in many years of clinical observations these chronic changes following IR have not been clearly identified.

To develop our model we considered that intestinal ischemia of 45 minutes was most appropriate. This was chosen based on the knowledge that less than 30 minutes

of ischemia in rats can cause too limited damage, while longer than 60 minutes will most likely result in massive necrosis that is associated with high mortality rate (Levy et al., 1990). Following IR, in animals undergoing multiple events, we chose to provide an interval between them while feeding the animals for a week in order to minimize mortality and provide sufficient time for animal recovery.

Structural modification of the stromal portion of the villi is prominent in the observation of samples obtained from our model. To better characterize the damage present in our model, and to permit statistical analysis, we used the classical Chiu/Park system for the evaluation of the histologic grading injury (Chiu et al., 1970; Haglund et al., 1980; Park et al., 1990). This system was recognized as the most suitable to be recommended as a standard scoring scale for histological

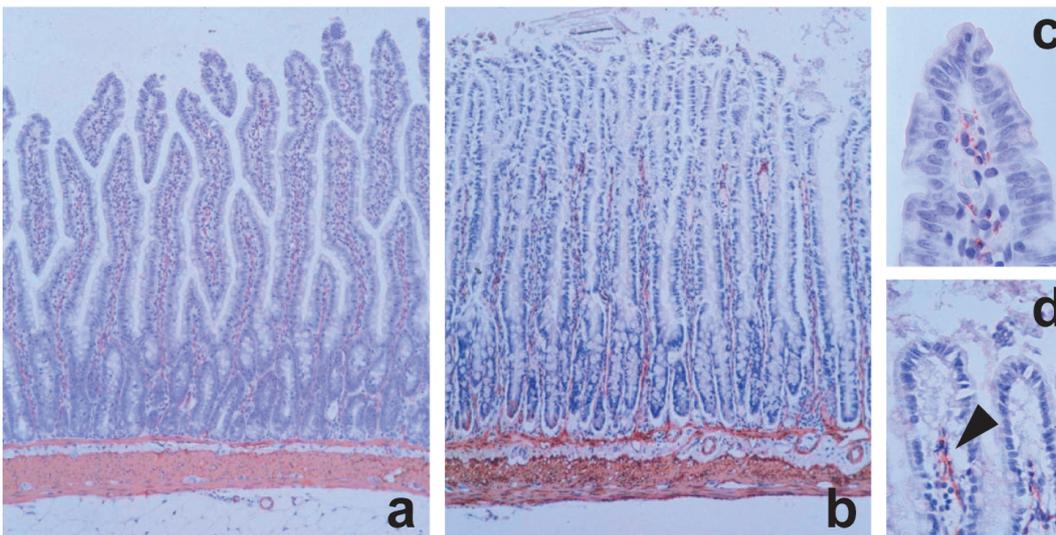


**Fig. 4.** Collagen fibres (red stained) distribution in the lamina propria extended from the basal portion of the villus reaching the apex in sham controls (**a** and **d**) while in animals subject to multiple IR the collagen do not reach the apex (see arrowheads in **b** and **e**). Red blood cells (arrows) reaching the apex of villus were evident only in control samples (**d**). close to the Payer's patches thickened distribution of collagen fibres was evident throughout the patches (**f**) in animals undergoing multiple IR when compared to controls (**c**) where a fine reticulum was observed. Van Gieson's trichomic. a, b, x 100; c-f, x 400.

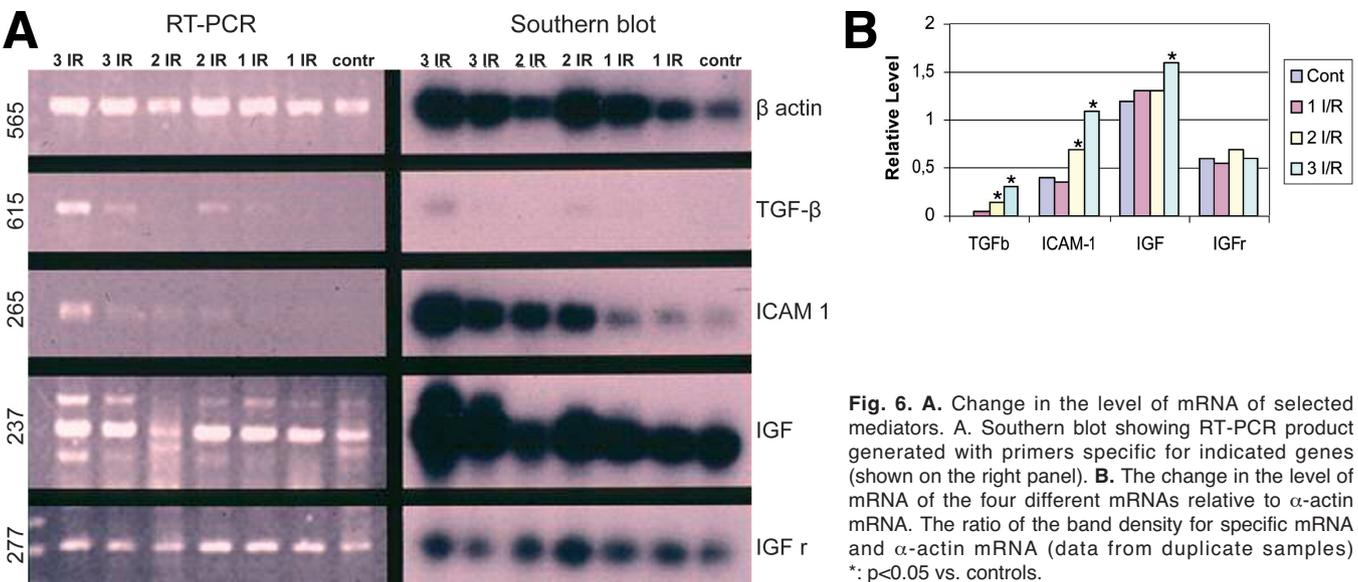
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evaluation of IR damage (Quaedackers et al., 2000). However, these criteria, originally used to evaluate acute IR injury, were not completely explicative of the histologic damage found in our model which reproduces non acute damage. In fact, although most of the damage was similar to that originally reported, the presence of necrotic matter in the subepithelial space was frequently found within grades 1-3. Moreover, the most advanced damage showed various level of mucosal atrophy. These histopathological features appeared non-specific, and could resemble the one described by Marsh in the celiac disease (Marsh, 1992; Green et al., 2005). However, the difference from the Marsh classification was the absence of the massive lymphocyte infiltrate in the lesion after multiple IR. Collagen fibres are missing at the apex, as

well as the smooth muscle fibres, suggesting that the structure supporting the epithelium in the apical portion of the villus is lost. Apoptosis of numerous enterocytes and other cell types of the epithelium is present. This is particularly evident in the portion of the villus missing the stromal component. This could represent the consequence of the persistent reduced ability of these cells to receive the necessary nutrients, since lack of smooth muscle cells and erythrocytes suggests a lack of microvascular support to the area. Moreover, the missing stromal portion of the villus suggests a lack of integrity of the basal membrane at the same level. This could also be responsible for the apoptosis observed (Mayhew et al., 1999; Zbar et al., 2004; Bouchard et al., 2008). An increased amount of apoptosis is observed also in



**Fig. 5.** The smooth muscle cell (brown stained) distribution in the lamina propria was extending from the basal portion of the villus to the apex in controls (a and c) but it did not reach the apex (arrow-head) in animals following multiple IR (b and d). Immunohistochemistry with anti- $\alpha$ -actin smooth muscle cells antibody. a, b, x 100; c, d, x 400



**Fig. 6. A.** Change in the level of mRNA of selected mediators. A. Southern blot showing RT-PCR product generated with primers specific for indicated genes (shown on the right panel). **B.** The change in the level of mRNA of the four different mRNAs relative to  $\alpha$ -actin mRNA. The ratio of the band density for specific mRNA and  $\alpha$ -actin mRNA (data from duplicate samples) \*:  $p < 0.05$  vs. controls.

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the crypts, suggesting that injury is diffuse rather than localized at the villus apex. However, the ability to regenerate the epithelial layer is evident since this was found intact in most of the sections observed.

The collagen thickening, observed throughout the sections, provides additional evidence of the diffuse distribution of these chronic alterations. The mechanisms for this abnormal collagen distribution were not specifically investigated in this study. Nevertheless after repeated IR we observed an increased number of resident macrophages, and an over expression of ICAM-1 at gene level. ICAM-1 is commonly expressed by macrophages and neutrophils, and its over expression after repeated IR could be related to the increased number of inflammatory cells (Danese et al., 2005). This finding demonstrated a stable increase of resident macrophages and other inflammatory cells, which are known to support fibrosis in the tissues.

In fact, macrophage production of fibrogenic cytokines such as transforming growth factor beta (TGF $\beta$ ) and its effects have been described (Tsunawaki et al., 1988; Wahl, 1994; Letterio and Roberts, 1998). The up-regulation of TGF $\beta$  at the genetic level was documented in our model, providing initial evidence that the collagen abnormalities observed could be related to an increased TGF $\beta$  production by activated macrophages.

An additional genetic up-regulation in this model was insulin like growth factor 1 (IGF-1). IGF-1 can be produced locally in intestinal tissue by myofibroblasts (Mourelle et al., 1998; Pucilowska et al., 2000; Simmons et al., 2002). IGF-1 has been demonstrated to induce type 1 collagen synthesis and to stimulate macrophage, enterocyte, fibroblast and crypt cell proliferation (Dahly et al., 2002, 2003; Chetty et al., 2006). IGF-1 also exerts an anti-apoptotic effect on intestinal cells (Dahly et al., 2002, 2003). Its over expression could be an attempt to support the tissue repair after repeated injuries, when apoptosis drastically increases. However, overexpression of IGF-1 is not followed by IGF-1 receptor increase, likely invalidating its long term proliferating and anti-apoptotic effect.

There is also evidence that both TGF $\beta$ , and IGF-1 are up-regulated in myofibroblasts at sites of fibrosis in enterocolitis and Crohn's disease (Pucilowska et al., 2000; Simmons et al., 2002). Furthermore, preserved TGF $\beta$  function, in spite of a reduction of its inhibitor (EGF), has been associated with chronic rejection of intestinal grafts, but was found to be equally increased following allogeneic and syngeneic transplants (Walgenbach et al., 1996; Kouwenhoven et al., 1999). Since TGF  $\beta$  increase is not specific for the allogeneic combination, this suggests that it is consequent to graft preservation (i.e. IR) associated with the procedure, and not to acute rejection or other immunologic factors. Shock is also associated with increased TGF  $\beta$ . However, a protective role has been suggested for its effect on inducible nitric oxide synthase regulation (Perrella et al., 1996; Taylor et al., 1998).

Finally, diffuse damage and atrophy of the mucosa

with marked reduction of the intestinal surface can justify the diminished intestinal absorption. The gut absorptive capacity was apparently restored some weeks after the last IR, coinciding with the attempt to tissue repair. However, after about 12 weeks, we observed heavier damage with a stable decrease of the absorption. This finding suggests the failure of the repair mechanism and further supports the hypothesis of a progressive damage, up to atrophy of the tissues, as a long term consequence of acute injuries.

In conclusion, we demonstrated that IR triggers long term chronic morphologic changes in the small intestine in our rat model. The histopathological features of the damage appear non-specific and are increased in intensity in animals subject to multiple IR events, ranging from minimal damage to total atrophy of the mucosa. These morphologic changes are associated with increased mortality and a reduced absorption of D-Xylose in our animal model.

## References

- Brown P.W., Barger J.A. and Weber H.M. (1934). Chronic inflammatory lesions of the small intestine (regional enteritis). *Am. J. Dig. Dis.* 1, 426-431.
- Bouchard V., Harnois C., Demers M.J., Thibodeau S., Laquerre V., Gauthier R., Vézina A., Noël D., Fujita N., Tsuruo T., Arguin M. and Vachon P.H. (2008). B1 integrin/FAK/Src signaling in intestinal epithelial crypt cell survival: integration of complex regulatory mechanisms. *Apoptosis* 13, 531-542.
- Burszty M., Flyvbjerg A., Weiss O., Mekler J. and Raz I. (2001). Atrophy or hypertrophy in chronic renal ischemia: role of the IGF-I system. *Am. J. Hypertens.* 14, 1211-1218.
- Chetty A., Cao G.J. and Nielsen H.C. (2006). Insulin-like Growth Factor-I signaling mechanisms, type I collagen and alpha smooth muscle actin in human fetal lung fibroblasts. *Pediatr. Res.* 60, 389-394.
- Chiu C.J., McArdle A.H., Brown L., Scott H.J. and Gurd F.N. (1970). Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch. Surg.* 101, 478-483.
- Dahly E.M., Guo Z. and Ney D.M. (2003). IGF-I augments resection-induced mucosal hyperplasia by altering enterocyte kinetics. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 285, R800-808.
- Dahly E.M., Guo Z. and Ney D.M. (2002). Alterations in enterocyte proliferation and apoptosis accompany TPN-induced mucosal hypoplasia and IGF-I-induced hyperplasia in rats. *J. Nutr.* 132, 2010-2014.
- Danese S., Semeraro S., Marini M., Roberto I., Armuzzi A., Papa A. and Gasbarrini A. (2005). Adhesion molecules in inflammatory bowel disease: therapeutic implications for gut inflammation. *Dig. Liver Dis.* 37, 811-818.
- Dindelegan G., Liden H., Kurlberg G., Oltean M., Nilsson O., Aneman A., Lycke N. and Olausson M. (2003). Laser-Doppler flowmetry is reliable for early diagnosis of small-bowel acute rejection in the mouse. *Microsurgery* 23, 233-238.
- Emir H., Akman M., Belce A., Gümüftaflı K. and Söylet Y. (2001). Is intestinal ischemia a risk of laparoscopy? An experimental study in rabbits. *Eur. J. Pediatr. Surg.* 11, 158-162.
- Grant D., Abu-Elmagd K. and Reyes J. (2005). 2003 report of the intestine transplant registry: a new era has dawned. *Ann. Surg.* 241, 607-613.

- Green P.H., Rostami K. and Marsh M.N. (2005). Diagnosis of coeliac disease. *Best Pract. Res. Clin. Gastroenterol.* 19, 389-400.
- Haglund E., Haglund U., Lundgren O., Romanus M. and Scherstén T. (1980). Graded intestinal vascular obstruction: I. Description of an experimental shock model in the rat. *Circ. Shock* 7, 83-91.
- Jarvinen O., Laurikka J., Salenius J.P. and Lepäntalo M. (1999). Mesenteric infarction after aortoiliac surgery on the basis of 1752 operations from the National Vascular Registry. *World J. Surg.* 23, 243-247.
- Kleinhaus S., Sammartano R. and Boley S.J. (1978). Effects of laparoscopy on mesenteric blood flow. *Arch. Surg.* 113, 867-869.
- Kouwenhoven E.A., Stein-Oakley A.N., Jablonski P., de Bruin R.W. and Thomson N.M. (1999). EGF and TGF-beta1 gene expression in chronically rejecting small bowel transplants. *Dig. Dis. Sci.* 44, 1117-1123.
- Langberg C.W., Hauer-Jensen M., Sung C.C. and Kane C.J. (1994). Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. *Radiother. Oncol.* 32, 29-36.
- Letterio J.J. and Roberts A.B. (1998). Regulation of immune responses by TGF- $\beta$ . *Annu. Rev. Immunol.* 16, 137-161.
- Levy P.J., Krausz M.M. and Manny J. (1990). Acute mesenteric ischemia: improved results – a retrospective analysis of ninety-two patients. *Surgery* 107, 372-380.
- Marsh M.N. (1992). Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 102, 330-354.
- Massberg S. and Messmer K. (1998). The nature of ischemia-reperfusion injury. *Transplant. Proc.* 30, 4217-4223.
- Mayhew T.M., Myklebust R., Whybrow A. and Jenkins R. (1999). Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol. Histopathol.* 14, 257-267.
- McCord J.M. and Roy R.S. (1982). Pathophysiology of superoxide: Roles in inflammation and ischemia. *Canad. J. Physiol. Pharmacol.* 60, 1346-1351.
- Morini S., Yacoub W., Rastellini C., Gaudio E., Watkins S.C. and Cicalese L. (2000). Intestinal microvascular patterns during hemorrhagic shock. *Dig. Dis. Sci.* 45, 710-722.
- Mourelle M., Salas A., Guarner F., Crespo E., García-Lafuente A. and Malagelada J.R. (1998). Stimulation of transforming growth factor beta1 by enteric bacteria in the pathogenesis of rat intestinal fibrosis. *Gastroenterology* 114, 519-526.
- Muller A.R., Langrehr J.M., Nalesnik M., Hoffman R.A., Lee T.K., Lee K.K. and Schraut W.H. (1994). Mucosal glutaminase activity and histology as parameters of small bowel preservation injury. *J. Surg. Res.* 56, 207-215.
- Oltean M., Aneman A., Dindelegan G., Mölne J., Olausson M. and Herlenius G. (2005). Monitoring of the intestinal mucosal perfusion using laser Doppler flowmetry after multivisceral transplantation. *Transplant. Proc.* 37, 3323-3324.
- Park P.O., Haglund U., Burkley G.B. and Fält K. (1990). The sequence of development of intestinal tissue injury after strangulation ischemia and reperfusion. *Surgery* 107, 574-580.
- Perrella M.A., Hsieh C.M., Lee W.S., Shieh S., Tsai J.C., Patterson C., Lowenstein C.J., Long N.C., Haber E., Shore S. and Lee M.E. (1996). Arrest of endotoxin-induced hypotension by transforming growth factor beta1. *Proc. Natl. Acad. Sci. USA* 93, 2054-2059.
- Pucilowska J.B., McNaughton K.K., Mohapatra N.K., Hoyt E.C., Zimmermann E.M., Sartor R.B. and Lund P.K. (2000). IGF-I and procollagen alpha1(I) are coexpressed in a subset of mesenchymal cells in active Crohn's disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279, G1307-322.
- Quaedackers J.S., Beuk R.J., Bennet L., Charlton A., Oude Egbrink M.G., Gunn A.J. and Heineman E. (2000). An evaluation of methods for grading histologic injury following ischemia/reperfusion of the small bowel. *Transplant. Proc.* 32, 1307-1310.
- Robinson J.W.L., Haroud M., Winistörfer B. and Mirkovitch V. (1974). Recovery of function and structure of dog ileum and colon following two hours' acute ischemia. *Eur. J. Clin. Invest.* 4, 443-452.
- Skalli O., Ropraz P., Trzeciak A., Benzonana G., Gillesse D. and Gabbiani G. (1986). A monoclonal antibody against alpha-smooth muscle actin: a probe for smooth muscle differentiation. *J. Cell Biol.* 103, 2787-2796.
- Skalli O., Pelte M.F., Peclat M.C., Gabbiani G., Gugliotta P., Bussolati G., Ravazzola M. and Orci L. (1989). Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37, 315-321.
- Simmons J.G., Pucilowska J.B., Keku T.O. and Lund P.K. (2002). IGF-I and TGF-beta1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283, G809-818.
- Sternberg A., Alfici R., Bronek S. and Kimmel B. (1998). Laparoscopic surgery and splanchnic vessel thrombosis. *J. Laparoendosc. Adv. Surg. Tech. A* 8, 65-68.
- Sternberger L.A. (1979). The unlabelled antibody peroxidase-antiperoxidase (PAP) method. In: *Immunocytochemistry*. 2nd ed. Cohen S. and McCluskey R.T. (eds). John Wiley and Sons. New York. pp 104-169.
- Tabasco-Minguillan J., Cicalese L., Lee R.G. and Rakela J. (1995). Mucosal perfusion and reactivity of the rat small intestinal allograft. *Transplantation* 60, 1572-1577.
- Tadros T., Traber D.L., Heggers J.P. and Herndon D.N. (2000). Angiotensin II inhibitor DuP753 attenuates burn- and endotoxin-induced gut ischemia, lipid peroxidation, mucosal permeability, and bacterial translocation. *Ann. Surg.* 231, 566-576.
- Takeoshi I., Zhang S., Nomoto M., Zhu Y., Kokudo Y., Suzuki T., Hamada N., Nemoto A., Starzl T.E. and Todo S. (2001). Mucosal damage and recovery of the intestine after prolonged preservation and transplantation in dogs. *Transplantation* 71, 1-7.
- Taylor B.S., Alarcon L.H. and Billiar T.R. (1998). Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry (Mosc.)* 63, 766-781.
- Thornton M. and Solomon M.J. (2002). Crohn's disease: in defense of a microvascular aetiology. *Int. J. Colorectal Dis.* 17, 287-297.
- Tsunawaki S., Sporn M.B., Ding A. and Nathan C. (1988). Deactivation of macrophages by transforming growth factor- $\beta$ . *Nature* 334, 260-262.
- Wahl S.M. (1994). Transforming growth factor  $\beta$ : the good, the bad and the ugly. *J. Exp. Med.* 180, 1587-1590.
- Walgenbach K.J., Heeckt P.F., Stanson J.D., Whiteside T.L. and Bauer A.J. (1996). Increased expression of transforming growth factor-beta during chronic rejection. *Transplant. Proc.* 28, 2450.
- Zbar A.P., Simopoulos C. and Karayiannakis A.J. (2004). Cadherins: an integral role in inflammatory bowel disease and mucosal restitution. *J. Gastroenterol.* 39, 413-421.