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Trehalase as a possible marker of intestinal ischemia — reperfusion injury

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Background: Different pathological affections of the small intestine cause corresponding morphological and functional changes. The present study was aimed to assess intestinal trehalase activities during ischemia and following reperfusion, correlate them with the pathological changes and determine whether trehalase could be used as a biochemical marker of the intestinal ischemia, ischemia — reperfusion injury. Material and methods: Wistar rats, randomly divided into 5 experimental groups (IR) (each n=15), were subjected to one hour mesenteric ischemia followed by 0, 1, 4, 12 and 24 hours of reperfusion. As a control group sham operated animals were used (n=15). The activity of trehalase was determined using an adapted Dahlqwist method. The range of intestinal injury was determined using histological (histopathological injury index and goblet cell quantification) and immunohistochemical (Ki67, *InSitu* TUNEL) methods. Results: The highest activities of trehalase were recorded in the control group (C=4.42±0.373 μmol/mg/h). The most altered intestinal histology detected in group IR1 was accompanied by the lowest trehalase activity (IR1=0.97±0.209 μmol/mg/h; $p<0.001$ C *vs*. IR1). Improved histological structure in the remaining reperfusion periods correlated with increase in trehalase activity. Almost normal mucosal histological architecture and 72% of the enzymatic activity were restored after 24 hours of reperfusion (IR24=3.20±0.266 μmol/mg/h; $p<0.01$ IR1 *vs*. IR24). Conclusion: The correlation between intestinal histology and trehalase activities during intestinal injury has been shown. Trehalase activity is closely associated with the status of the histological architecture of the small intestine.

Key words: detection; injury; ischemia — reperfusion; small intestine; trehalase

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INTRODUCTION

Intestinal ischemia — reperfusion injury (IRI) is a frequent phenomenon entailing high morbidity and mortality (Grootjans *et al.,* 2010). It represents a pivotal complication in many clinical cases and surgical procedures such as: cardiopulmonary bypass, cardiac insufficiency, organ transplantation, stroke hemorrhage, severe burn injuries and shock conditions (Collard & Gelman, 2001). According to our previous experimental study, the major source of intestinal graft damage in the early stages was preservation injury (ischemia) (37.98%), reperfusion (53.91%) and the minor part was due to mechanical manipulation (8.11%) (Varga *et al.,* 2009). It is clear that splanchnic hypoperfusion involves reduction in intestinal motility and enzymatic activities, immunoinflammatory responses (Verma *et al.,* 2013) and causes morphological as well as functional changes (Morini *et al*., 2010). The nonspecific signs and symptoms of mesenteric vascular disease can significantly delay diagnosis (Sandeep & Shashank, 2009). High morbidity and mortality rates create the need to develop diagnostic methods to facilitate earlier recognition and detection (Kimizuka *et al.,* 2004). The brush border disaccharidases (sucrase, maltase, trehalase and palatinase) are considered to be the accurate markers of enterocyte maturity and functional capacity (Cuong *et al.,* 2011). Disaccharidases reside on the villus (Dahlqvist & Nordström, 1966), which is well known to be very sensitive to blood supply (Varga *et al.,* 2010), so the deepening ischemic - reperfusion injury should be reflected in a proportional decrease in disaccharidase activity.

The aim of this study was to analyze trehalase activity (TA) at various degrees of injury induced experimentally by ischemia and followed by reperfusion, and to correlate TA with the histopathological and immunohistochemical findings. Furthermore, we endeavored to determine the possible clinical role of trehalase assessment from mucosal scrapes as a novel biochemical method for rapid detection and continual monitoring of injured small intestine.

MATERIAL AND METHODS

Experimental animals and grouping. Adult male Wistar rats (mean body weight 280±22 g, total n=90) used for the experiments were housed under standard conditions and maintained at a temperature of 22±2°C with a 12 h/12 h light/dark cycle. Food and water were provided *ad libitum*; only water was given for 12 h prior to surgery. General anesthesia was induced by intraperitoneal application of ketamine 60–80 mg/kg (Narketan 10 inj. ad us. vet., Vétoquinol S.A., Lure Cedex, France), and xylazine 8–10 mg/kg (Xylariem inj. ad us. vet.,

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Abbreviations: IRI, ischemia — reperfusion injury; TA, trehalase activity; CMA, cranial mesenteric artery; HII, histopathological injury index; GC, goblet cell; HE, hematoxylin-eosin; TUNEL, terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate in situ nick end-labeling.

RiemserArzneimittel, Greifswald-InselRiems, Germany). Normothermic conditions (37°C) were monitored using a microthermistor placed in the ear. Temperature was maintained using a homeothermic blanket.

Animals were randomly divided into the following groups:

Sham control (C, n = 15): animals were subjected to laparotomy and isolation of cranial mesenteric artery (CMA) without its clamping.

Experimental groups (IR, n = 75): Experimental animals were subjected to ischemic-reperfusion injury. IRI was induced by total occlusion of the CMA for a 60 min period. At the end of ischemia the vascular clamp was removed and reperfusion started for the predetermined time for the groups, i.e. 0, 1, 4, 12 and 24 hours, respectively (IR0, IR1, IR4, IR12, IR24).

After the surgical procedures the abdominal cavities of the animals were closed in two layers with Silon 2.0 EP suture (Chirmax, Prague – Modřany, Czech Republic).

Sampling. In accordance with the experimental groups' reperfusion times, the animals were sacrificed by means of a lethal dose of the anaesthesiological mixture. Samples were taken from the proximal jejunal part 10 cm from the Ligament of Treitz. For histological examination a 2 cm segment of the jejunum was removed. Harvested bioptic samples were immediately rinsed in cold saline and fixed in 4% *p*-formaldehyde. Samples for biochemistry were obtained as a scrape of the intestinal mucosa from a transversally opened segment of the intestine located also about 10 cm from the Ligament of Treitz.

Enzymatic assay. Collected samples were homogenized by a mechanical homogenizator (T10 basic ULTRA- TURRAX, IKA, Germany) in 500 µl of 0.9% NaCl on ice. The homogenized samples (120 µl) were diluted in 1020 µl of 0.9% NaCl and they were frozen at –70°C until analyzed. TA was determined using the adapted method of Dahlqvist (1970) based on incubating 50 µl aliquots of the diluted sample with 50 µl of maleate buffer, pH 8.0, containing trehalose, for 20 min in a 37°C degree preheated thermostat. After this time the reaction was stopped by increasing the temperature to t = 100°C for 5 min. A glucose-oxidation method (Bio-La-Test, Lachema, Brno) was used to determine glucose concentration. Protein concentration in the samples was established using the Bradford method (1976) and specific activities were calculated as the activity of each sample divided by the protein concentration and reported as µmol/h/mg; micromoles of substrate hydrolyzed per hour at 37°C per milligram of protein.

Histopathological analysis. For detecting the range of histopathological damage, expressed as the histopathological injury index (HII), Hematoxylin-Eosin (HE) histological staining was used. HE-stained sections of the intestinal tissue were scored using a semi-quantitative Park/Chiu grading system adapted from Quaedackers *et al.* (2000).

The population of goblet cells (GC) present in the intestinal epithelium was detected using the Alcian blue histochemical staining method. Alcian blue 8GX solution (pH 2.5, Sigma-Aldrich, St. Louis, MO, USA) stains both sulphated and carboxylated acid mucopolysaccharides and sulphated and carboxylated sialomucins (glycoproteins). Alcian blue/nuclear red stained tissues were acquired and the number of Alcian blue positive GC was determined in 10 intestinal villi and corresponding intestinal crypts in each sample.

Immunohistochemical analysis. A primary Ki67 polyclonal antibody, clone MIB-5 (Millipore Bioscience Research Reagents, Billerica, MA, USA) was used for detecting proliferation activity. Primary antibody was labeled using a two-stage indirect immunoperoxidase technique. Positive cell populations were visualized with diaminobenzidine, DAB (Fluka) and counterstained with Mayer's heamatoxyline. Omitting of the primary antibody was considered as the negative control. The apoptotic cells in the epithelium were detected using the terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate in situ nick end-labeling (TUNEL) method to assess DNA fragmentation in the intermediate and late stages of apoptosis (*In situ* Cell Death Detection Kit, Fluorescein, Roche, Germany).

Statistical analysis. Two independent pathologists made immunohistochemical and histopathological analysis of each slide. The statistical analysis was performed using GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA). The semi-quantitative results (histopathological injury index) were determined using the non-parametric Kruskal-Wallis test and Dunn's *post hoc* test for multiple comparisons. The quantitative results (goblet cell quantification, apoptotic, proliferation and disaccharidase activities) were determined using one-way ANOVA with a multiple comparison Tukey-Kramer *post hoc* test. All the results are expressed as mean ± S.E.M. *p* values less than 0.05 were considered significant.

Ethics. This experiment was approved by the Committee for Ethics on Animal Experiments at the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and the experimental protocol was approved by the State Veterinary and Food Administration of the Slovak Republic No. 2843/08-221a.

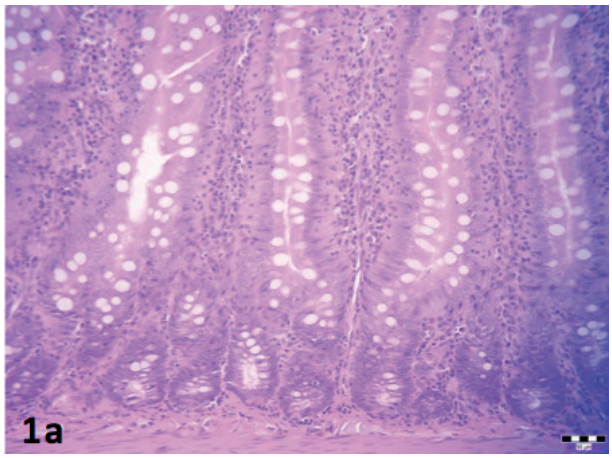
RESULTS

Characteristics of changes in trehalase activity

The highest TA was recorded in the control group (C = 4.42±0.373). A significant decrease in enzymatic

Table 1. The values of measured parameters of histologically or biochemically determined injury. (p*<0.05; ***p*<0.01 and ****p*<0.001)**

Groups	Activity of trehalase	Histological assessment			
		HII	GC	*InSitu* TUNEL	Ki67 Mucosa
C	4.42±0.373	0.18±0.09	52±2.3	1.5±0.42	1.7±0.25
IR0	2.52±0.261**	1.66±0.21***	32.32±1.2***	2.2±0.5	2.8±0.31
IR1	0.97±0.209***	5.52±0.17***	17.22±1.5***	3.5±0.42***	4.9±0.26***
IR4	1.48±0.408	5.0±0.36	22.3±2.5	2.8±0.36**	3.8±0.36
IR12	1.53±0.136	3.92±0.32	30.46±3.2	2.1±0.7	3.67±0.33
IR24	3.20±0.266**	0.85±0.05**	37.25±1.8	0.85±0.32	1.93±0.15



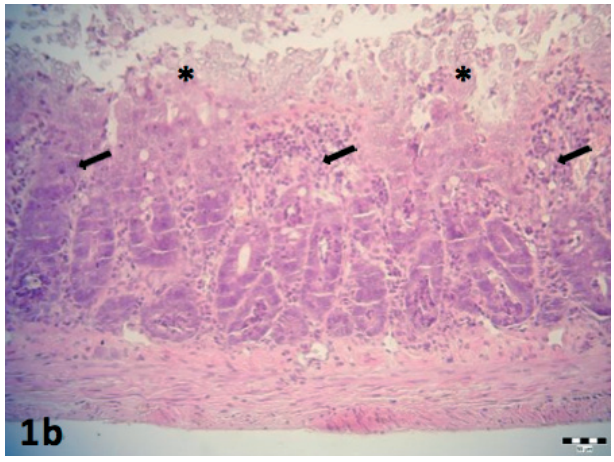
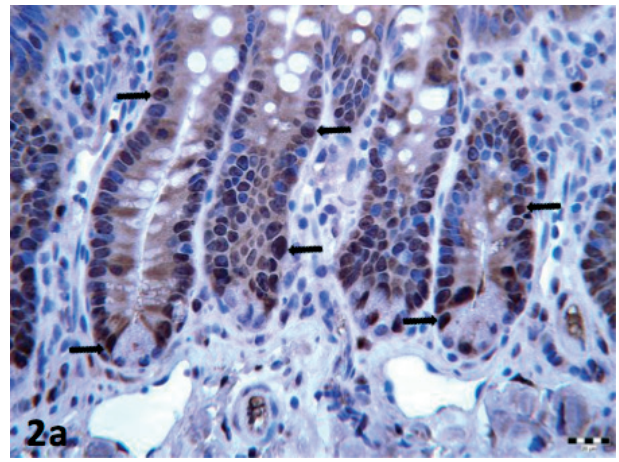


Figure 1. Representative photomicrographs illustrating histopathological changes of the jejunal biopses.
Haematoxylin and eosin (H&E) staining. Normal tissue architecture of jejunum in control groups (Fig. 1a, 400x). Massive destruction and disintegration of villous lamina propria (arrows), jejunal mucosa fragments (asterisk) in the lumen 1 hour after ischemia and 1 hour of reperfusion (Fig. 1b, 400x)



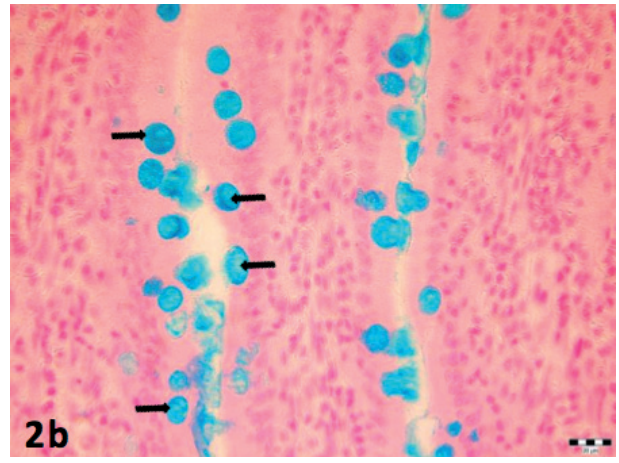


Figure 2. Representative photomicrographs illustrating changes in the intestinal mucosa — Ki67 immunohistochemical staining, positive nuclei — arrows (Fig. 2a, 1000x); Histochemical detection of goblet cell population by the Alcian Blue&Nuclear Red staining method, positive cytoplasm — arrows (Fig. 2b, 1000x).

activity was recorded immediately after 1 hour of mesenteric ischemia (IR0 = 2.52±0.261; $p<0.01$ IR0 *vs.* C). The activity continued to decrease with the time of reperfusion with the lowest value measured after the first hour of reperfusion (IR1 = 0.97±0.209; $p<0.001$ IR1 *vs.* C). An increase in TA was subsequently detected after longer reperfusion periods and 24 hours after the start of reperfusion the activity of trehalase reached 72% of that in group C (IR24 = 3.20±0.266, $p<0.01$ C *vs.* IR24; IR1 *vs.* IR4; $p<0.01$ IR1 *vs.* IR24) (Table 1).

Histopathological injury index

Almost intact jejunal mucosa was detected in control group C (C = 0.18±0.09; Fig. 1a). One hour of anoxia caused moderate histological damage in the intestinal mucosa (IR0 = 1.66±0.21, $p<0.001$ IR0 *vs.* C). Statistically important damage was observed 1 hour after reperfusion started, when compared to the control group (IR1 = 5.52± 0.17; $p<0.001$ C *vs.* IR1; Fig. 1b). Thereafter improved jejunal histological architecture was observed in the remaining groups with an almost normal structure after 24 hours of reperfusion. (IR24 = 0.85± 0.05; $p<0.001$ IR1 *vs.* IR12; $p<0.01$ C *vs.* IR24; IR1 *vs.* IR24; IR4 *vs.* IR24) (Table 1).

Histochemical quantification of Goblet cell population

One hour of ischemia caused significant decrease in GC numbers in comparison to sham- operated group C, where physiological amounts were detected (C = 52±2.3; IR0 = 32.32±1.2; $p<0.001$ IR0 *vs.* C). A significant decrease in GC numbers to the lowest values was observed after one hour of reoxygenation (IR1 = 17.22±1.5; $p<0.001$ IR1 *vs.* C; IR0 vs. IR1). Thereafter a progressive increase in GC numbers was recorded, and insignificant difference was detected in group IR24 (IR24 = 37.25±1.8) (Table 1; Fig. 2b).

Ki67 immunoreactivity

The proliferation intensity i.e., the proportion of Ki67-positive cells, was measured in the intestinal epithelial layer. The highest level of proliferation was found in the IR1 experimental group IR1. The obtained values were statistically different when compared to the control group (C = 1.7±0.25; IR1 = 4.9±0.26 $p<0.001$ IR1 *vs.* C). Decrease in the number of Ki67-positive cells was then detected in the remaining groups, with an almost normal level in the IR24 group (IR24 = 1.93±0.15; $p<0.001$ IR1 *vs.* IR24) (Table 1; Fig. 2a).

In Situ TUNEL positivity in the intestinal epithelium

The number of apoptotic cells after 1 hour of mesenteric ischemia (IR0 = 2.2±0.50) was moderately higher

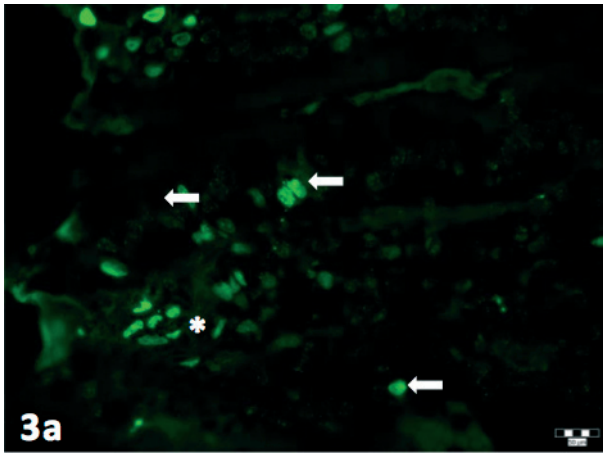


Figure 3. Representative photomicrographs illustrating apoptotic changes in jejunal biopsies.
In Situ TUNEL Fluorescein method staining — TUNEL immunoreactive nuclei in epithelial lining — arrows, in lamina propria villi _ asterisk (Fig. 3a, 1000x).

than in the control group (C = 1.5±0.42). A significant increase was observed after 1 hour of reoxygenation (IR1 = 3.5±0.42; $p<0.001$ IR1 *vs.* C), with a significant decrease 4 hours after removal of the clamp (IR4 = 2.8±0.36; $p<0.01$ IR1 *vs.* C). Almost physiological values, statistically different from those obtained after 4 hours, were detected after 24 hours of reperfusion (IR24 = 0.85±0.32; $p<0.01$ IR4 *vs.* IR24) (Table 1; Fig. 3).

Juxtaposition of biochemical and histological analyses

Correlation between TA and HII, GC number and Ki67+ immunoreactive cell number was observed. A distinct increase in the histopathological injury grade was observed immediately after declamping of the CMA. It was manifested biochemically by decreasing TA, histochemically by a decreasing number of goblet cells and histopathologically by increasing HII and an increasing number of Ki67+ and apoptotic TUNEL+ cells. The injury propagation due to reperfusion continued until one hour of reoxygenation, which was confirmed by distinct alterations in the intestinal mucosa and a decrease in TA activity. Improved morphology of the jejunum in the remaining groups was confirmed by a decrease in HII, Ki67+ and TUNEL+ cell numbers and by a moderate increase in GC population. Similarly, a gradual increase in TA activity was seen in groups IR4, IR12 and IR24 (Fig. 4).

DISCUSSION

Viability of the small intestine must be evaluated frequently during abdominal surgery. Intestinal hypoperfusion in the surgical anastomosis area can result in the leakage, stricture and prolonged hospitalization, significant postoperative morbidity and mortality (Urbanavičius *et al.,* 2011). In the clinical praxis, the intestinal microcirculation and viability is usually estimated visually from the color of the serosal surface, the presence of bowel peristalsis, pulsation and bleeding from the arteries. This method is subjective and based only on the clinical experience of the surgeon. In some cases even the normal color of the serosal surface could be associated with intestinal hypoperfusion while the dark blue color with transient venous insufficiency (Páral *et al.,* 2009). Plenty of diagnostic methods were highlighted in preclinical/clinical studies: pulse oximetry, polarographic measurement of oxygen tension, near-infrared and visible light spectrophotometry, doppler ultrasound, fluorescence or radioisotope studies etc. (Urbanavičius *et al.,* 2011). Among all the methods for assessment of the intestinal viability during surgery, only the fluorescence spectroscopy and the doppler ultrasound are clinically used (Páral *et al.*, 2009). These methods require specific equipment or preparation, therefore their use is restricted by time and financial limitations. Detection of the graft viability prior to the small intestine transplantation plays a crucial role for the success of the entire procedure (Varga

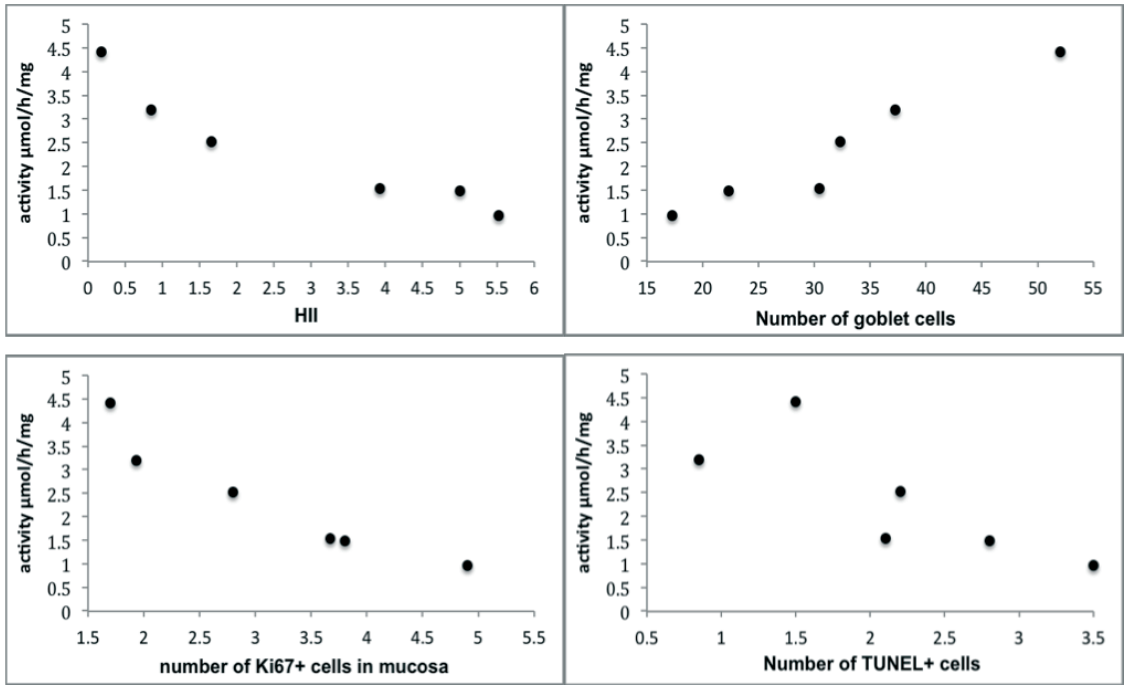


Figure 4. Correlation of trehalase activity with various histological parameters

et al., 2009). Fluorescence spectroscopy and the doppler ultrasound diagnosis reveal only the presence/absence of the blood flow but not the kinetics of the pathological changes, or the vulnerability, therefore they cannot be used for rapid detection of the intestine graft viability.

There is still no routinely usable specific biochemical marker of intestinal damage (Kanda *et al.,* 2011). Various biochemical parameters of the small intestine ischemic injury were tested such as: intestinal diamine oxidase, hyaluronic acid, intestinal fatty acid-binding protein, adenine nucleotide metabolism, mucosal glutaminase activity etc. However, none of these parameters is currently suitable for clinical practice (Matia *et al.,* 2004; Páral *et al.,* 2009). In our previous experimental study we described functional as well as morphological changes in the injured intestines. Activities of sucrase and maltase were determined from mucosal scrapes and from homogenates of the entire intestinal wall. In that previous study we highlighted significant alterations in the sucrase and maltase activities in the injured intestine relative to the time of reperfusion. We underlined also the importance of being able to correlate histological changes with disaccharidase activities because of the persistent discrepancy in the relation of sucrase and maltase activities *vs.* morphological status of the small intestines (Varga *et al.,* 2012).

Nowadays, the TA changes have been mentioned only in a few experimental studies on animal models and humans (Oku *et al.,* 2011; Murray *et al.,* 2000). The correlation between TA and the histological outcome and its significance still remains unclear (Gupta et al., 1999). Trehalose, a non-reducing disaccharide, can be found in mushrooms, algae and insect hemolymph (Sugimoto, 1995). In comparison with other disaccharides present in food or in digestion products, the amount of trehalose in the diet is significantly lower. This property makes trehalase unique among other disaccharidases because of the minimal impact of carbohydrates present in the diet on its enzymatic activity.

Most of the disaccharidase studies in adults have focused on chronic conditions such as the alcohol intake effect on the brush border enzymes, their contribution to malnutrition or their role in infants with portal venous obstruction or chronic diarrhea (Sidhu *et al.,* 2010). Whilst in the human population, sucrase and maltase show consistent activity that of lactase varies depending on several factors, for example the age. These facts have been confirmed by the previously undertaken studies, in which differences in lactase activity were verified while in the case of sucrase and maltase consistent values were detected (Grundmann *et al.,* 2011; Vieira *et al.,* 2000; Mądry *et al.,* 2010).

Several studies have highlighted a gradual decrease in sucrase and maltase activity during small intestine injury, with mutual correlation with the degree of injury quantified by a routine histological analysis and other histopathological techniques. Other studies, however, have produced no evidence of correlation (Heitlinger *et al.,* 1991; Shulman *et al.,* 1991; Tori *et al.,* 2007). Murray *et al.* (2000) described significant changes in intestinal sucrase, maltase as well as trehalase activities related to the suspected malabsorption and coeliac disease. Shulman *et al.* (1991) mentioned that it would be advantageous if disaccharidase activity could be extrapolated from the histological assessment of the small intestinal mucosa, but their study showed that disaccharidase activity (sucrase, maltase, lactase) did not correlate with the histological findings obtained from morphometric analysis. The observations made in this study are in conflict with those of Berg *et al.* (1973) and Heitlinger *et al.* (1991). The results of Heitlinger *et al.* (1991) suggest that disaccharidase activity in intestinal mucosal biopsies is diminished in the presence of mucosal injury, and correlates inversely with the degree of injury determined histologically. There is, however, a limitation for the clinical usage in the low specificity rates, with 51% for maltase and 43% for lactase. The study of Kaufman et al. aimed to determine whether concurrent determination of mucosal disaccharidase activities (maltase, sucrase, lactase, and palatinase) and histological assessment improves the accuracy of the diagnosis of rejection of the intestinal graft following small intestine transplantation (Kaufman *et al.,* 1998). The correlations of histological parameters with disaccharidase activities were found but the results of their investigation show that determination of the mucosal disaccharidase activity provides no additional useful information concerning the efficacy of the anti- rejection therapy as compared to the histological analysis alone (Kaufman *et al.,* 1998).

The acute ischemic-reperfusion experimental model used in our study is suitable for simulating various severities of the intestinal injury depending on the times of reperfusion. Reoxygenation of the ischemic intestine is a major source of damage (Varga *et al.,* 2010). The injury increases with the time of reperfusion, and the maximum is reached in the first four hours of reoxygenation (Tóth Jr. *et al.,* 2013). In the current study, the effects of one hour of anoxia were manifested in two ways: morphologically, by a slight increase in HII, and biochemically, by a significant decrease in TA. Regarding TA in the obtained samples, a decrease of 60% compared to the control group was observed immediately after 1 hour of ischemia while quantification of HII showed only moderate insult. This indicates the importance of TA assessment in the early stages of the intestinal injury since other histopathological parameters can be normal or show only moderate alterations. One hour of reperfusion induces the highest HII and also the lowest activities of the trehalase. A decrease in TA could be associated with significant histophysiological changes occurring in cells of the intestinal epithelial lining during ischemia. After the first hours of reperfusion the number of TUNEL-positive cells in the intestinal epithelium significantly increases. This fact is connected with the activation of various pathways for programmed cell death as well as elimination of damaged epithelial cells. One hour of ischemia and one hour of reperfusion results in the disintegration of the lamina propria of the intestinal wall but the intestinal glands, or crypts of Lieberkühn with undifferentiated stem cell population are mostly intact. Accordingly, the highest apoptotic activity could be accompanied by the activation of the proliferative capacities of stem cells located in the Lieberkühn crypts. The increase in the number of Ki67-possitive cell at longer reperfusion times was coupled with a gradual increase in TA. After 24 hours of reperfusion the numbers of Ki67-positive cells reached almost normal values and the numbers of TUNEL-positive cells were even lower than in the control group. The persistent effect of IRI on the intestine was manifested as a lower number of GC. Significant depletion of the goblet cells one hour after reperfusion may be associated with subsequent rapid turnover. Secretory products released by GC population are essential for the protection and stimulation of the repair/regeneration of the injured intestinal mucosa (Kjellev, 2009). After 24 hours of reperfusion IRI was still significantly mirrored in the altered functional capacity of the intestine. Trehalase activity (TA) reached near-

ly 72% of the physiological value while other histological tissue parameters were close to the normal values.

In conclusion, our experimental results demonstrated a possible relationship between trehalase activities and the histological status of the small intestine and similarities in the chronologies of their changes. Detection of TA is based on enzymatic methods, the cost of which is comparable with the blood glucose and protein concentration assessment. The activity of trehalase showed a slight decrease allowing to more accurately distinguish the early and late phase of ischemia in comparison with the activity of sucrase and maltase, which exhibited a rapid decrease already at the early stages. According to the obtained results, which can be collected in a short time, we suggest possible further clinical application of the trehalase activity assay. This possible diagnostic method can be used for rapid assessment of the intestinal viability and its continual monitoring mainly during surgical procedures such as small intestine transplantation. For the use in human clinical practice, further clinical studies are needed.

Acknowledgements

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