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DSC EXAMINATION OF INTESTINAL TISSUE FOLLOWING WARM ISCHEMIA AND REPERFUSION INJURY

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The fact that small bowel is extremely sensitive to ischemia/reperfusion (I/R) injury had encouraged us to compare the conventional histology and differential scanning calorimetry (DSC) methods in intestinal structural changes following experimental warm I/R models. Our histological findings showed that longer warm I/R period caused more severe damage in structure of mucosa and crypts, but there were no changes in the muscular layer. According to our DSC data (transition temperature, calorimetric enthalpy) suggest that the thermal destruction of mucosa, muscular layer and total intestinal wall following I/R injury revealed significant differences compared to normal bowel structure.

Keywords: *DSC, intestine, ischemia/reperfusion*

Introduction

The ischemia and reperfusion (I/R) syndrome performs a fundamental role in the pathophysiology of several clinical and surgical conditions and may be caused by intestinal invagination, acute mesenteric arterial occlusion, and hemodynamic shock. Lesions caused by mesenteric I/R can also occur in transplants of the small intestine [1, 2]. I/R of the mesenteric blood vessels is the initial stages of multi-organ failure, conditions which are associated with high rates of morbidity and mortality [3]. The qualitative as well as quantitative analyses are essential for the determination of potential mechanisms underlying injury and for the development of treatment strategies in the clinical practice.

Several studies demonstrated that mesenteric I/R can be evaluated by the detection of various products resulting from injury, using laboratorial and histomorphological methods [4, 5]. The I/R injury of the gut is most often assessed by histologic evaluation on hematoxylin and eosin (H and E) stained tissue sections. From different systems have been described the Park's scoring system is the most suitable to be recommended as a standard scoring scale for histological evaluation of intestinal I/R damage [6]. Advantages of this scoring system are that it grades the progression of morphologic injury from mild to severe, showing the best correlation with clinical outcome [7]. However, lack of this evaluation that it does not describe the delicious details in the tissue structures.

Besides the well-established morphological methods during intestinal I/R injury, differential scanning calorimetry (DSC) is a new approach in this field. It allows demonstrating the thermal consequences of local and global conformation changes in the structure of different tissue elements [8–14].

Aim of the study

We wanted to compare the standard histology and DSC to define details of intestinal structural changes following warm I/R injury.

Experimental

Materials and methods

Animal preparation and anaesthesia

Adult male Wistar rats (250–300 g) were purchased from the Laboratory Animal Centre of University of Pécs, housed under pathogen-free conditions and were fasted for 24 h preoperatively, but had free access to water. Rats were anaesthetized with intramuscular ketamine hydrochloride (0.01 mg g^{-1} of body mass) and diazepam (0.01 mg g^{-1} of body mass) (Richter Gedeon, Budapest, Hungary). All procedures were performed in accordance with the ethical guidelines of NIH and guidelines approved by the University of Pécs (BA02/2000-20/2006) to minimize pain and suffering of the animals.

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Ischemia/reperfusion model

Warm I/R groups were established, where ischemia was induced by clamping the superior mesenteric artery. In Group I (GI, n=10) ischemia maintained for 1 h and reperfusion kept for 3 h. In Group II (GII, n=10) 3 h ischemia and 1 h reperfusion were applied. Intestinal biopsies were collected after laparotomy (control), at the end of the ischemia and the reperfusion periods.

Histology

The bowel tissues were processed using standard histologic techniques including formalin fixation, dehydration and paraffin embedding, then cut in 4 μm sections and stained with H and E. Structural damage was assessed in a 'blind' manner with two observers using Park's histologic classification of intestinal injury grading from 0 to 8 (Nicon Eclipse 80 Light Microscope, Kingston, England) (original magnification ×100) [6]. Mucosa thickness, depth of the crypts and muscular layer thickness were quantitative analyzed using the software Scion Image (Scion Corporation, Maryland, USA). The number of square pixels was counted in 5 fields per sections at ×400 magnification and the length was given in micrometer.

DSC measurements

The thermal unfolding of the intact intestine, its mucosa and muscle components were monitored by Setaram Micro DSC-II calorimeter. All experiments were conducted between 0 and 100°C. The heating rate was 0.3 K min^{-1} in all cases. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μL sample volume (samples plus buffer) in average. Typical sample wet masses for calorimetric experiments were between 100–150 mg. Tissue samples were in Dulbecco's Modified Eagle's Medium (DMEM/F12) containing 10% fetal bovine serum (FBS) and 1% PS (penicillin/streptomycin) (Sigma-Aldrich Co., St. Louis, MO, USA) stored, and this buffer was used as a reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the point of view of heat capacity between sample and reference vessels. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve. Calorimetric enthalpy was calculated from the area under the heat absorption curve by using two-point setting Setaram peak integration.

Statistical analysis

Results are expressed as mean values±SEM. Data were analyzed with one-way analysis of variance (ANOVA). The level of significance was set at P<0.05. The MicroCal Origin 6.0 program (Microcal Software Inc., Northampton, USA) was used for graphical presentation.

Results and discussion

Histology

According to Park's classification, the highest grade of injury was observed in GII during reperfusion, whereas the lowest grade of injury was found in GI following ischemia. The control group showed an injury Grade 0, corresponding to normal bowel structure. GI tissues showed the best maintenance of mucosal morphology after 1 h ischemia showing minor clefting with the villus epithelium adjacent to the crypts intact (Grade 2). While the histological findings were corresponding to an injury Grade 4 at the end of 3 h reperfusion, characterized by massive epithelial lifting and villus tip denudation. In ischemia-end samples of GII the injury showed denuded and loss of the villi and crypt layer injury (Grade 6). Moreover, the structural injury raised to Grade 8 by the end of the reperfusion in this group, where transmural infarction (i.e., necrosis of the muscularis propria and the mucosa), eventually de-structured intestinal tissue was observed (Fig. 1).

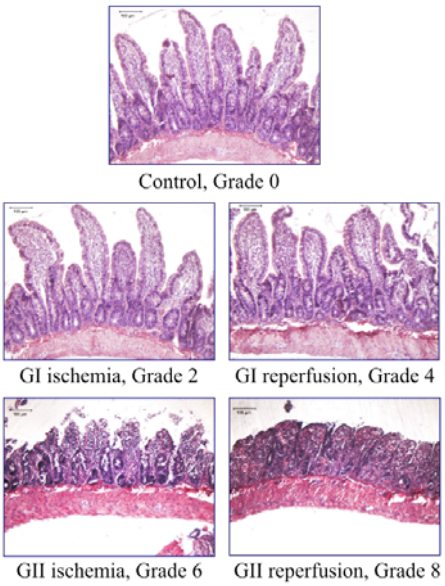

Fig. 1 Standard histology of I/R injuried small bowel on H and E stained sections (Park's score)

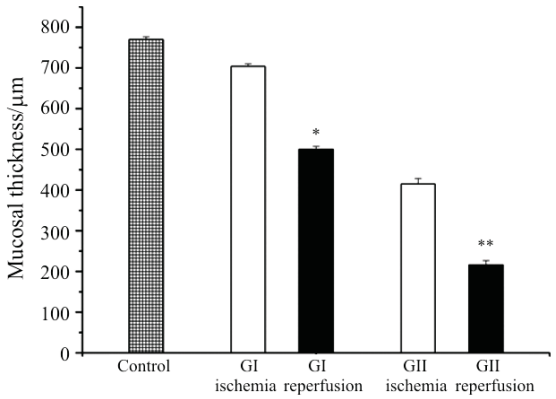

Fig. 2 Quantitative analysis of mucosal thickness following I/R injury of small intestine. Data are presented as mean±SEM. *P<0.05 *vs*. control; **P<0.01 *vs*. control

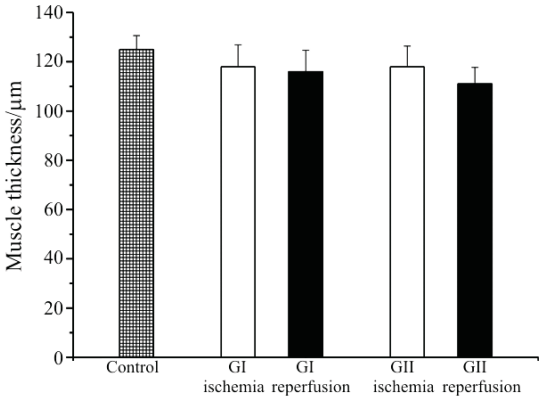

Fig. 4 Quantitative analysis of muscle thickness after I/R injury of the small intestine. Data are presented as mean±SEM

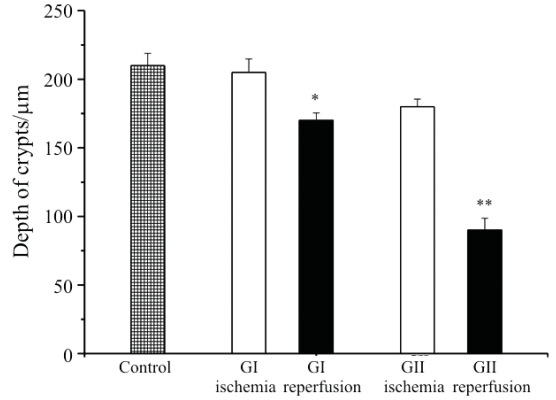

Fig. 3 Quantitative analysis of crypts' depth following I/R injury of the small bowel. Data are presented as mean±SEM. *P<0.05 *vs*. control; **P<0.01 *vs*. control

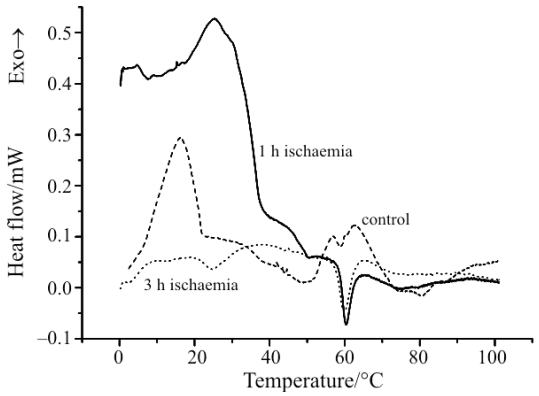

Fig. 5 Thermal denaturation of intact rat intestine in different physiological states

By Scion Image quantitative analysis, mucosal thickness decreased significantly in GI and GII reperfusion-end samples compared to control (Fig. 2). Similarly, depth of crypts decreased significantly by the end of the reperfusion periods in each group (Fig. 3). In contrast, muscle thickness showed mild decrease in all groups compared to controls, but these changes were not significantly different by the end of I/R periods (Fig. 4).

DSC measurements

In Fig. 5 can be seen the thermal denaturation of intact rat intestine. The scan is very complex, but the effect of ischemia is significant: the pronounced low temperature exotherm in case of control is shifted to higher temperature after 1 h treating while the answer for 3 h ischemia was an exoterm with two states and lower calorimetric enthalpy (ΔH), followed by an endotherm. The main melting of control sample contains more endotherms, these in case of 1 h ischemia appear as a well cooperative melting around 60°C with a pre- and post-transitions. After 3 h ischemia these additional meltings practically disappear.

The clean mucosa is the inner surface of intestine. Its DSC scans (Fig. 6) show significant damage during ischemia. The definite exotherm around 55°C could be the sign of a crystallization like structural rearrangement. Its transition temperature and calorimetric enthalpy are ischemia time dependent, and in case of 3 h treatment the change in ΔH is about 40% of control one.

The muscle constituent of the intestine, similar to the intact intestine exhibits the greatest alteration after 1 h ischemia (Fig. 7). The main T_m and ΔH are the greatest in his case, and these parameters decrease for 3 h ischemia and control samples. The internal structure of DSC scans also shows the internal rearrangements during ischemia treatments: in the control muscle we can assume at least 4 different thermal domains, these appear after 1 h ischemia too (in less pronounced manner), but in case of 3 h ischemia only the main denaturation peak could be identified.

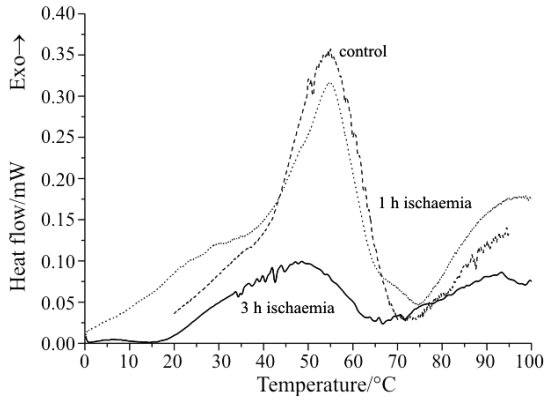

Fig. 6 DSC scans of mucosa

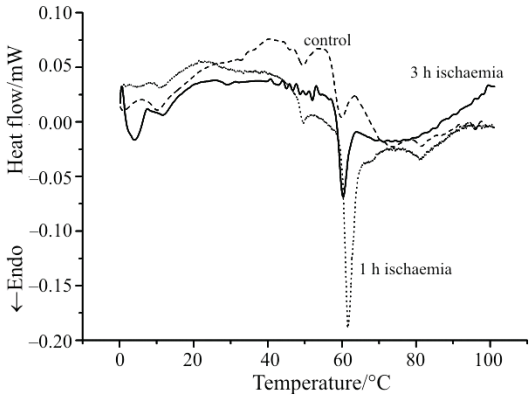

Fig. 7 Thermal transition of muscle component of rat intestine

An intact intestinal structure is of vital importance for efficient assimilation of ingested nutrients, but it also serves as a barrier that limits access of enteric bacteria and other noxious stimuli to the systemic circulation. One condition is associated with a disrupted mucosal barrier, is warm ischemia followed by reperfusion [15]. Intestinal cell damage following warm ischemia is a biphasic process. Ischemia initiates the injury by depriving cells of the energy needed to maintain homeostasis. Reperfusion exacerbates this damage by triggering an inflammatory reaction involving oxygen free radicals (OFRs). During I/R OFRs can attack any biochemical component of cells, inducing peroxidation of membrane lipids, destruction of carbohydrates, proteins and DNA-strand scission, thus lead to tissue damage [16, 17].

The initial injury is merely functional, firstly causes increased capillary and then mucosal permeability. But, as the extent and duration of I/R increases, morphologically detectable injury is caused in a continuous spectrum from superficial mucosal injury to transmural infarction. The morphologic injury is characteristically first is a subepithelial space developed right at the villus tip (Grade 1). This space is more extended in Grade 2 and there is a massive epithelial lifting down the sides of the villi in Grade 3. In the following grade (Grade 4) the epithelial covering is lost, and the villi desintegrate (Grade 5). Even with this severe form of villus injury, the deeper layers of the intestinal wall remain microscopically intact. The characteristic feature of Grade 6 is injury of the crypt layer. In Grade 7 transmucosal injury is seen and in Grade 8 transmural infarction [6].

In this study, intestinal I/R injury is defined by histological method. This damage correlated to the duration of warm I/R, with the highest destruction observed in bowel following 3 h ischemia and 1 h reperfusion periods. Both qualitative and quantitative analysis demonstrated that mucosal thickness, and depth of crypts were better maintained by the end of 3 h reperfusion followed 1 h ischemia. Several studies have described similar results [18–20]. In contrast, despite of transmural infarction (Grade 8) the muscle thickness showed mild decrease without significant changes by the end of I/R periods. Presumably the reason of this discrepancy follows from the lack of these morphological evaluations.

The thermal parameters of I/R injury are the mirror of histological results. The most significant changes are manifested in the mucosa (Table 1). The half width of melting temperature increases from the control 20 to 30°C in case of 3 h ischemia, indicating a loosening its structure (with lower cooperativity), and simultaneously the denaturation tempera-

Table 1 The thermal parameters of denaturation of different parts of intestine

Sample	T_m/°C		$T_{1/2}$/°C		ΔH/J g^{-1}	
	1 h	3 h	1 h	3 h	1 h	3 h
Control	55.6±0.4 (mucosa) 58.9±0.5 (muscle) 50.1±0.3 (intact)		20.3±0.2 2.8±0.1 15.1±0.2		4.1±0.22 0.11±0.01 0.42±0.05	
Mucosa	55.6±0.4	48.7±0.3	27.3±0.3	30.2±0.3	3.41±0.3	2.67±0.2
Muscle	61.05±0.5	61.7±0.4	3.2±0.1	3.1±0.1	0.33±0.03	0.18±0.02
Intact intestine	60.2±0.4	59.8±0.3	3.1±0.1	2.9±0.1	0.28±0.02	0.33±0.03

mean±s.d., T_m – melting temperature, $T_{1/2}$ – half width of melting and ΔH is the calorimetric enthalpy change of endotherm process normalized on wet mass of samples

ture decreases from 55.6 to 48.7°C. In contrast, the muscle component became more 'rigid', its melting temperature varied from 58.9 (control) to 61.7°C (3 h ischemia). Using the denaturation data achieved in case of rabbit psoas muscle [21, 22] we can suppose that the myosin system underwent to a significant structural rearrangement during the ischemia with different time duration.

In summary, this is the first report compared the standard histology and thermal changes by DSC on intestinal warm I/R. These thermal parameters indicate the thermodynamic consequences of structural destruction rearrangement, which provides basis for further investigation in different intestinal stress models.

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