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DSC examination of intestinal tissue following cold preservation

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ABSTRACT

The fact that small bowel is extremely sensitive to cold preservation had encouraged us to compare the conventional histology and differential scanning calorimetry (DSC) methods in intestinal structural changes following experimental cold storage models. Our histological findings showed that longer cold preservation 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 preservation injury revealed significant differences compared to normal bowel structure.

1. Introduction

Small bowel transplantation has progressively improved with modern immunosuppressive strategies. The deleterious effects of cold ischemia and reperfusion are major problems that affect clinical outcomes after small bowel transplantation. Intestinal ischemia/reperfusion (I/R) can lead to oxidative injury and loss of intestinal barrier function [1,2]. Although cold preservation is employed to reduce tissue degeneration, there is a progressive deterioration of cellular function over time [3,4]. The current clinical standard for small bowel preservation is intravascular flushing and cold static preservation using the University of Wisconsin (UW) solution. 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 [5].

Several studies demonstrated that cold preservation can be evaluated by the detection of various products resulting from injury, using laboratorial and histomorphological methods [6,7]. The injury of the gut is most often assessed by histological evaluation on hematoxylin and eosin (H&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 damage [8]. Advantages this scoring system is, that it grades the progression of morphologic injury from mild to severe, showing the best correlation with clinical outcome [9]. However, lack of this evaluation that it does not describe the delicious details in the tissue structures.

Differential scanning calorimetry (DSC) is a thermoanalytical technique which monitors small heat changes between a sample and reference as a function of temperature. As numerous articles illustrated DSC is a validly efficient method for the demonstration of structural changes not only in the molecules, but in the structure of different tissue elements in biological systems [10–15]. To the knowledge of the authors, there is no previous study performed with the application of DSC in the field of monitoring the effect of cold preservation on the intestinal tissue. Besides the well-established morphological methods during intestinal preservation injury, the main goal of this study was to measure the structural changes by DSC technique following experimental small bowel cold storage.

2. Materials and methods

2.1. 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 anesthetized with intramuscular ketamine hydrochloride (0.01 mg g^{-1} of body weight) and diazepam (0.01 mg g^{-1} of body weight) (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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2.2. Cold preservation model

Cold I/R groups were designed with small intestine cold preservation (4 °C) in UW solution (Viaspan, Bristol-Myers Squibb GesmbH, Wiena, Austria) for 1 h (GI, $n = 5$), for 3 h (GII, $n = 5$), and for 6 h (GIII, $n = 5$). Small bowel biopsies were collected after laparotomy (control) and at the end of preservation periods.

2.3. Histology

The bowel tissues were processed using standard histological techniques including formalin fixation, dehydration and paraffin embedding, then cut in 4 μm sections and stained with H&E. Structural damage was assessed in a 'blind' manner with two observers using Park's histological classification of intestinal injury grading from 0 to 5 (Nicon Eclipse 80 Light Microscope, Kingston, England) (original magnification 100×) [16]. Mucosa thickness, depth of the crypts and muscular layer thickness were quantitative analyzed using the software Scion Image (Scion Corporation, MD, USA). The number of square pixels was counted in 5 fields per sections at 400× magnification and the length was given in micrometer.

2.4. DSC measurements

The thermal unfolding of the total intestinal wall, 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 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 and 150 mg. Tissue samples were stored in UW solution, and this solution was used as a reference sample. The sample and reference samples 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 samples. 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.

2.5. Statistical analysis

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

3. Results

3.1. Histological results

According to Park's classification, the highest grade of injury was observed in GIII, whereas the lowest grade of injury was found in control sample (Grade 0), corresponding to normal bowel structure. GI tissues showed the best maintenance of mucosal morphology after 1 h cold preservation showing minor clefting with the villus epithelium adjacent to the crypts intact (Grade 2). While the histological findings were corresponding to an injury Grade 3 at the end of 3 h storage, characterized by epithelial lifting and villus tip denudation. In GIII the injury showed denuded and loss of the villi and crypt layer injury (Grade 4) (Fig. 1).

By Scion Image quantitative analysis, mucosal thickness decreased significantly in GII and in GIII samples compared to control (Fig. 2). Similarly, depth of crypts decreased significantly by the end of the preservation period in GIII (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 cold preservation periods (Fig. 4).

3.2. DSC results

In Fig. 5 the thermograms of mucosa are shown following 1, 3 and 6 h cold preservation. The control sample exhibited exoterm maxima with $T_m = 53.6$ °C as well as $\Delta H = 5.94$ calorimetric enthalpy. The effect of cold preservation is manifested after 1 h treatment in splitting into two coagulation peaks with 30.4 and 59.3 °C transition temperatures and a decreased calorimetric enthalpy (3.72 J g^{-1}). After 3 and 6 h preservation both transitions are moved to higher temperatures (32.6, 59.7, 47.9, and 58.8 °C with decreasing enthalpies).

In Fig. 6 the consequences of thermal coagulation of the muscular layer can be seen after cold preservation periods. The time

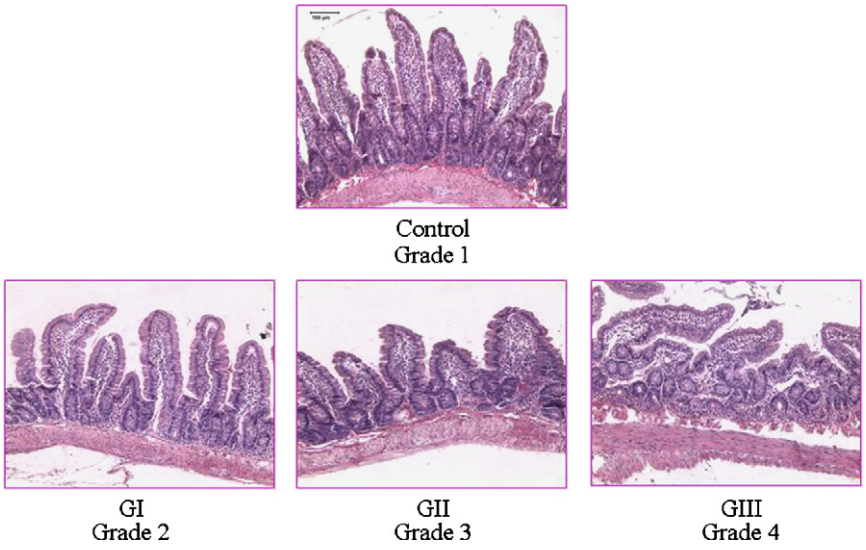

Fig. 1. Conventional histology of preserved small bowel on HE stained sections (Park's classification).

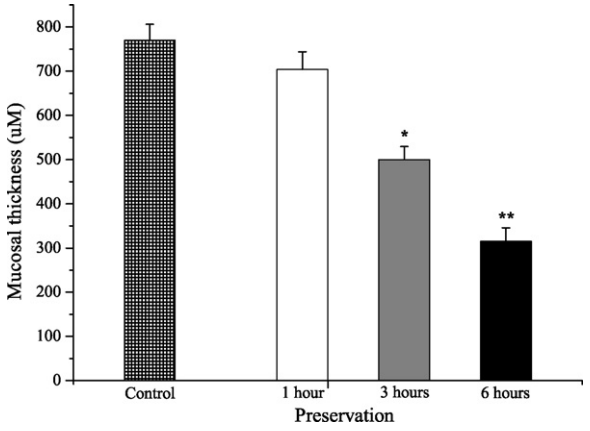

Fig. 2. Quantitative analysis of mucosal thickness following small bowel preservation. Data are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

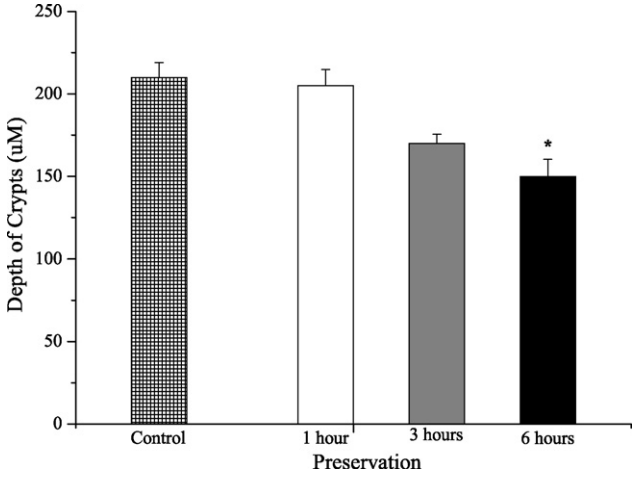

Fig. 3. Quantitative analysis of crypts' depth following the small bowel preservation. Data are presented as mean ± S.E.M. $^{*}P < 0.05$ vs. control.

effect of ischemia could be followed in these scans. In case of control we can distinguish at least 3 different thermal domains with T_m 52.9, 58.1 and 59.9 °C, which could be assigned to the myosin (52.9, 58.1 °C) and actin (59.9 °C) compounds of muscle. After 1 h preservation the two melting assigned to myosin are changed to 53.5 and 56 °C and the contribution of actin cannot be resolved at its original place. The effect of 3 and 6 h treatment is different significantly only in the calorimetric enthalpy (see Table 1).

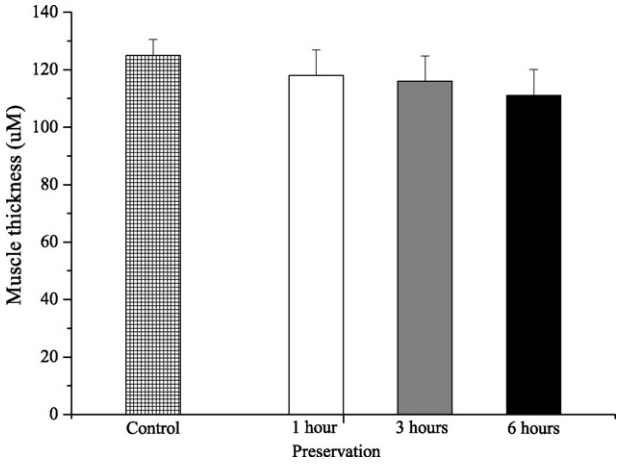

Fig. 4. Quantitative analysis of muscle thickness after small bowel preservation. Data are presented as mean ± S.E.M.

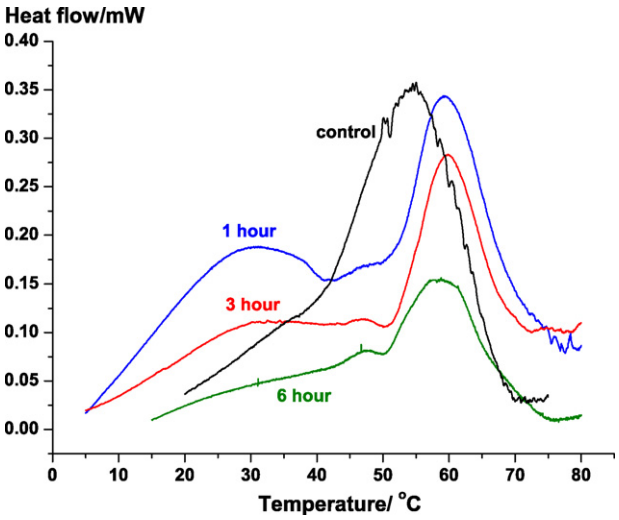

Fig. 5. DSC analysis of mucosa following 1, 3 and 6 h cold preservation of the small bowel. The upward deflection of DSC scans means an exotherm process, indicating the coagulation of tissue elements.

In Fig. 7 the results can be seen in cases of total intestinal wall following preservation periods. The time effect of preservation is markedly pronounced in the contribution of mucosa (the transition temperatures are shifted towards to the higher range) and first coagulation temperature of the main transition is decreasing with the similar change of calorimetric enthalpy (see Table 1).

The thermal parameters of different intestinal layers are given in Table 1, indicating the mean values and standard errors (in each group 5 different samples) of transition temperature and calorimetric enthalpy.

4. Discussion

Intestinal transplantation is inevitably accompanied by cold preservation. Although injury to the intestinal mucosa is evident throughout the period of cold storage, this damage is exacerbated on the reintroduction of oxygen and with reperfusion of the organ. The intestine is one of the most sensitive tissues to

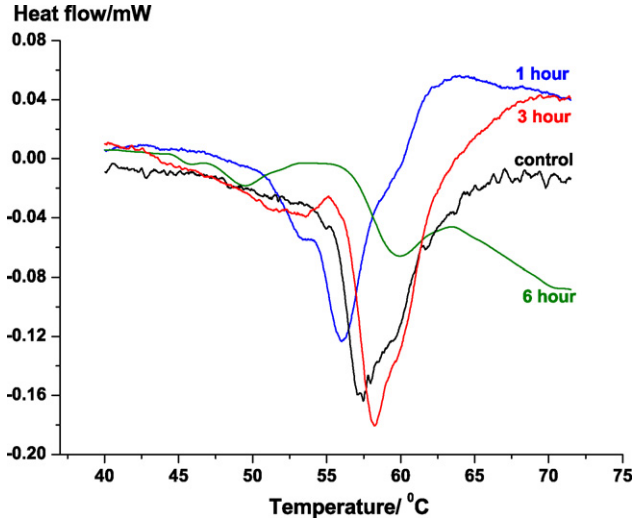

Fig. 6. DSC curves of the muscle layer following 1, 3 and 6 h small bowel cold preservation. Downward deflection represents endotherm process.

Table 1
DSC data ($n = 5$ in each group, with mean ± S.E.M.) of mucosa, muscular layer and total intestinal wall after 1, 3 and 6 h of small bowel cold preservation (symbols: transition temperature: T_m/°C; calorimetric enthalpy: ΔH/J g^{-1}; normal letter represents exotherm, bold one endotherm transition).

	Mucosa		Muscle		Total intestinal wall	
	T_m/°C	ΔH/J g^{-1}	T_m/°C	ΔH/J g^{-1}	T_m/°C	ΔH/J g^{-1}
Control	53.6 ± 0.2	5.944 ± 0.4	52.9, 58.1, 59.9 ± 0.2	**0.58 ± 0.04**	40, 49, 59, 75.6 ± 0.2	**0.46 ± 0.03**, **0.45 ± 0.03**
GI	30.4, 59.3 ± 0.2	3.72 ± 0.2	53.5, 56, 58 ± 0.2	**2.21 ± 0.2**	54, 56, 60, 75 ± 0.2	**2.33 ± 0.15**
GII	32.6, 59.7 ± 0.2	1.9 ± 0.15	53.6, 58.3,61 ± 0.2	**1.58 ± 0.1**	53, 55, 59.6, 76 ± 0.2	**1.764 ± 0.1**
GIII	47.9, 58.8 ± 0.2	2.96 ± 0.2	53.6, 57.9, 60 ± 0.2	**1.434 ± 0.1**	51.6, 57.9, 62, 76 ± 0.2	**0.58 ± 0.04**

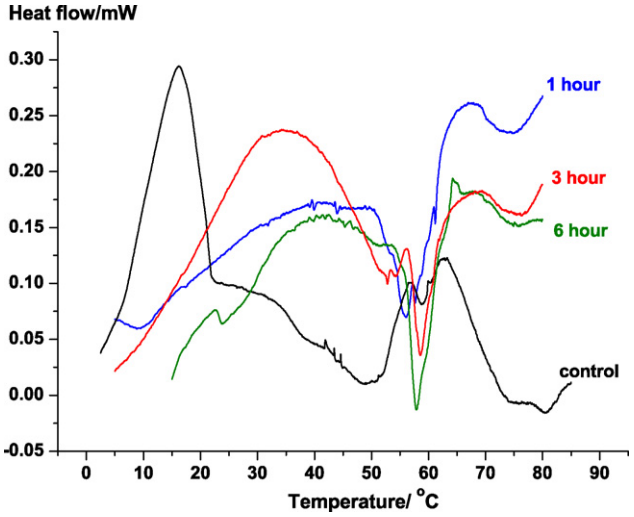

Fig. 7. DSC scans of the total intestinal wall after 1, 3 and 6 h cold preservation.

I/R injury and oxidative stress. On this basis, the development of preservation solution that reduces substantially or even eliminates mucosal injury incurred during cold storage represents an important step toward improving outcomes of intestinal transplantation in the clinic [16]. The initial injury is merely functional, firstly causes increased capillary and then mucosal permeability. But, as the extent and duration of ischemia increases, morphologically detectable injury is caused in a continuous spectrum from superficial mucosal injury to transmural infarction. According to Park's grading system 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 an 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 bowel wall remain microscopically intact [8].

In this study, intestinal cold preservation caused injury is defined by Park's histological method. This damage correlated to the duration of cold storage time, with the highest destruction observed in bowel following 6 h preservation on 4 °C. Both qualitative and quantitative analysis demonstrated that mucosal thickness and depth of crypts were better maintained by the end of 1 h cold ischemia. Several studies have described similar results [17–20]. In contrast, the muscle thickness showed mild decrease without significant changes by the end of preservation periods. Presumably the reason of discrepancy follows from the lack of these morphological evaluations encouraged us to measure the structural changes by DSC in the different layers of the small intestine.

Recently, there are continuous research efforts to examine the structural and functional changes in biological systems with biophysical methods. One of this is DSC technique, as a new and well-applied method in research of orthopedics, gynecology, thoracic surgery, gastroenterology, and recently in the oncology [10–12,14,21,22]. These indicate great potential for the application of DSC as a clinical diagnostic tool, for example during disease grading and staging processes.

The thermal parameters of cold injury are the mirror of histological results in the cases of mucosa. In good agreement with histological findings, the injury of mucosa can be seen from DSC scans as a time dependent process. While in case of control mucosa one can see a single coagulation at around 53 °C, after 1 h storage it was splitting in two transitions with decreased calorimetric enthalpy. After 3 and 6 h preservation enthalpies decreased more, indicating further mucosa injury.

DSC showed the most significant changes in the muscle layer. Despite of the lack of histological abnormalities the time effect can be seen in the shape of DSC scans as well as in the thermodynamic parameters. Using the denaturation data achieved in case of rabbit psoas muscle [23–25], we can suppose that the actomyosin system underwent to a significant structural rearrangement during cold ischemia with different time duration. This is important for two reasons during cold storage and intestinal transplantation. Firstly, the oxygen free radicals (OFRs) are able to modify the catalytic centre of myosin after ischemic insult. The extension of modification depends on time, concentration and chemical structure of OFRs. Secondly, this oxidative injury can cause impairment in the ATP hydrolysis cycle and force-generation of motile systems [26]. Thus, cold preservation, which is meant to reduce ischemic injuries, can cause structural and functional changes in the muscle layer, resulting severe post-transplant peristaltic problems [27,28]. But, in the background of this functional injury histological discrepancy have not been yet.

Thermal behaviour of total intestinal wall was extremely complex. On the DSC scans both the typical curves of mucosa and the muscular layer are found. These thermal components showed that total intestinal wall damaged following preservation periods with increasing cold ischemia time. But, the DSC results from separated bowel layers are shown clearly more.

5. Conclusion

In summary, this is the first report compared the standard histology and thermal changes by DSC on intestinal cold preservation. Our results showed the thermal parameters of cold injury are the mirror of histological results in the cases of mucosa. Its injury can be seen as a time dependent process by DSC scans also. DSC showed the most significant changes in the muscle layer. Despite of the lack of histological abnormalities the time effect can be seen in the shape of DSC scans as well as in the thermodynamic parameters. These parameters indicate the thermodynamic consequences of structural destruction rearrangement, which provides basis for further investigation in different intestinal stress models in the future.

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