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DSC, as a new method to verify the exact warm and cold ischemic injury during small bowel surgery

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ABSTRACT

The fact that small bowel is extremely sensitive to ischemia/reperfusion injury had encouraged us to compare the influences of warm and cold ischemia on the intestinal structural changes by differential scanning calorimetry (DSC) method. Warm and cold ischemia groups were established on Wistar rats with 1, 3 and 6 h ischemic times. Intestinal biopsies were collected after laparotomy and at the end of the ischemia periods. DSC measurement was performed on mucosa, on muscular layer and on the total intestinal wall. Our DSC data confirmed that longer warm ischemia period caused more severe damage in the structure of mucosa and muscular layers. According to the results of transition temperature and calorimetric enthalpy suggest that these changes reduced by cold ischemic procedure in University of Wisconsin solution. However, the thermal destruction of each layers following cold preservation injury revealed significant differences compared to normal bowel structure.

1. Introduction

Intestinal warm ischemia/reperfusion (I/R) is an important factor associated with a high morbidity and mortality in some critical clinical settings including hemorrhagic shock, strangulation obstruction, cardiovascular surgery, and severe trauma or combustion. These warm intestinal ischemic states through the mesenteric infarction cause abdominal emergency. The mortality of patients who experience mesenteric infarction continues to be high, with up to 80% of such patients dying. In the background of this severe clinical condition stand several molecular processes, including production of oxygen free radicals (OFRs), oxidative stress, inflammatory processes, and irreversible cell death. An intact intestinal mucosa 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. Disruption of the mucosal barrier results in the absorption of nutrients and if the lesion is severe, it can lead to sepsis, to multi organ failure and finally to death [1,2]. Moreover, few study in the literature showed that, I/R significantly alters intestinal motility function. The generation of OFRs and disruptions in the calcium homeostasis also play an important role in the pathogenesis of muscular layer's damage [3].

During small bowel transplantation to minimize warm ischemic damage of intestine cold preservation has been applied in the clinical practice. This procedure can reduce cellular damages, but it is inevitably accompanied by cold ischemic injury. Although tissue injury is evident throughout the period of cold storage, this damage is exacerbated on the reintroduction of oxygen and with reperfusion of the organ in the recipients. To prevent oxidative damage, the University of Wisconsin (UW) solution is the first choice for small intestinal cold preservation, which contains among others OFRs scavengers (glutathione and allopurinol), and energy source (adenosine) [4–8].

Several experiments demonstrated that ischemia can be evaluated by the detection of various products resulting from injury, using laboratorial and histopathological methods. Numerous different histological grading systems have been described, namely the Park's, the Chiu's, the Parks's and the Sonnino's systems. From these, the Park's and Chiu's methods for grading injury are the most suitable as a standard scoring scale for histological evaluation of intestinal damage. Advantages these scoring system are, that they grades the progression of morphologic injury from mild to severe, showing well-correlation with clinical outcome. However, the disadvantages of them are firstly, they do not describe the delicious details in some tissue structures; secondly, as in a qualitative methods the diagnosis (degree of injury) are closely depend on the person of the pathologists, thus not free from the examiner-based differences; thirdly, these classifications alone are not able to take the exact tissue injury into account [9]. Unfortunately, there is no consensus exists on how the injury should be graded.

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Differential scanning calorimetry (DSC) is a thermoanalytical technique which monitors small heat changes between a sample and a reference. DSC examination is a validly efficient method for the demonstration of structural changes not only in the physical sciences, but also in numerous biological systems. It allows demonstrating the thermal consequences of local and global conformation changes in the structure of different tissue elements [10–14]. The goal of this study was to evaluate and to compare the exact and quantitative morphological changes in each layer of the intestinal tissue by DSC technique after 1, 3 and 6 h warm and cold ischemic periods.

2. Materials and methods

2.1. Animal preparation and anaesthesia

Adult male Wistar rats (250–300 g, $n = 30$) 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 (BA02/2000-9/2008) to minimize pain and suffering of the animals.

2.2. Warm and cold intestinal ischemia models

After median laparotomy warm ischemia groups were established. In these groups ischemia was induced by clamping the superior mesenteric artery for 1, 3 and 6 h. In cold ischemia groups median laparotomy was followed by small bowel resection from the ligament of Treitz to the ileocoecal part. Grafts were perfused and stored in 4 °C UW solution (Viaspan, Bristol-Myers Squibb GesmbH, Vienna, Austria) for 1, 3 and 6 h. Small bowel biopsies were collected after laparotomy (control) and at the end of the warm and cold ischemia periods.

2.3. DSC measurements

The thermal unfolding of the total intestinal wall, its mucosa and muscle components were monitored by SETARAM Micro DSC-II calorimeter. We have listed our samples in Table 1. In our case "sample" means separate mucosa, muscle and total intestinal wall. We have used for 6 h ischemia 5-5, in other cases 4-4 samples. We have chosen a relatively longer bowel; half of it was separated into mucosa and muscle, and a remaining one was the total intestinal wall from the same rat. The total intestinal wall is the "intact" bowel. It is not the mixture of mucosa and muscle. In case of mucosa we have invaginated the bowel, and scraped off from its inner surface the mucosa (other way it is impossible to separate it because of its very thin layer thickness), and the remaining part was the muscle sample. The fashion of the DSC curves was reproducible. The onset and the end point of denaturation were determined by the change of the slope of scans comparing with the native (onset) and the denatured state (end point) slopes (in the figures it cannot be seen). The precision of enthalpy determination was between 5% and 10%.

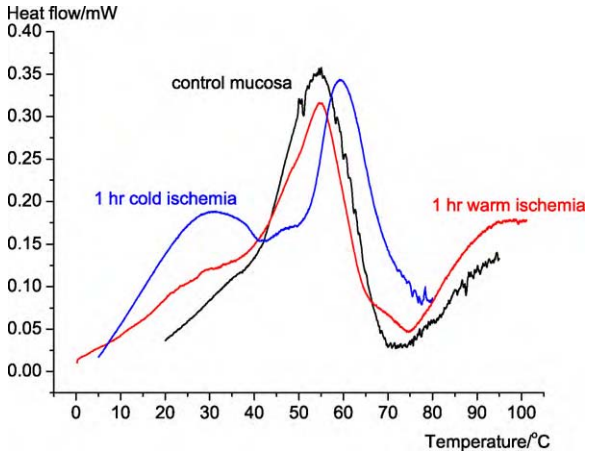

Fig. 1. DSC analysis of mucosa following 1 h intestinal warm ischemia and cold preservation. The upward deflection of DSC scans means an exotherm process, indicating the coagulation of tissue elements.

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. Reference sample was normal saline (0.09% NaCl) at the measurement of warm ischemia tissue samples. At cold ischemia groups 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.

3. Results

Our DSC results are presented according to the all parts of the intestinal wall and the duration of ischemia times. As it can be seen in Figs. 1–3 the mucosa has undergone significant changes in a time-dependent manner during different types and durations of ischemia. The control (untreated) sample has an exotherm with T_ms

Table 1
DSC data of mucosa, muscle and total intestinal wall after 1, 3 and 6 h of small bowel warm ischemia and cold preservation (transition temperature: T_m (°C); calorimetric enthalpy: ΔH (J/g), average ± s.e.).

	Mucosa		Muscle		Total intestinal wall	
	T_m (°C)	ΔH (J/g)	T_m (°C)	ΔH (J/g)	T_m (°C)	ΔH (J/g)
Control	55.6 ± 0.2	−4.1 ± 0.22	52.8; 58.1; 59.9 ± 0.2	0.59 ± 0.03	17.5; 49.5 ± 0.2	0.46; 0.45 ± 0.03
1 h warm ischemia	55.6 ± 0.4	−3.41 ± 0.3	49.5; 62 ± 0.5	1.4 ± 0.2	23; 50.5; 60.2 ± 0.4	1.1 ± 0.07 0.28 ± 0.02
3 h warm ischemia	48.7 ± 0.3	−1.7 ± 0.2	60.7 ± 0.4	0.8 ± 0.06	59.8 ± 0.3	0.28 ± 0.02
6 h warm ischemia	41.1; 46.3 ± 0.2	−5.2 ± 0.3	52; 59 ± 0.5	3.1 ± 0.2	58 ± 0.3	0.58 ± 0.03
1 h cold ischemia	30.4; 59.3 ± 0.2	−5.94 ± 0.4	53.5; 56 ± 0.2	2.21 ± 0.2	56 ± 0.2	0.38 ± 0.02
3 h cold ischemia	32.6; 59.7 ± 0.2	−2.67 ± 0.2	53.6; 58 ± 0.2	3.4 ± 0.1	53; 55; 59.6 ± 0.2	0.76 ± 0.06
6 h cold ischemia	47.9; 58.8 ± 0.2	−1.96 ± 0.2	53.6; 57.9 ± 0.2	3.8 ± 0.1	41; 57.9; 58.6 ± 0.2	0.4 ± 0.02

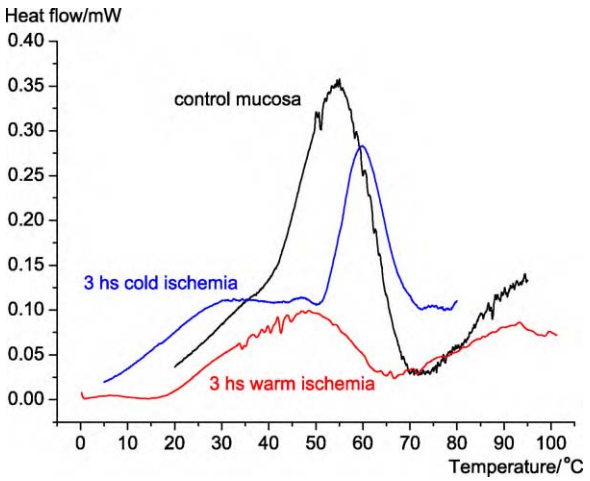

Fig. 2. DSC analysis of mucosa following 3 h intestinal warm ischemia and cold preservation. The upward deflection of DSC scans means an exotherm process.

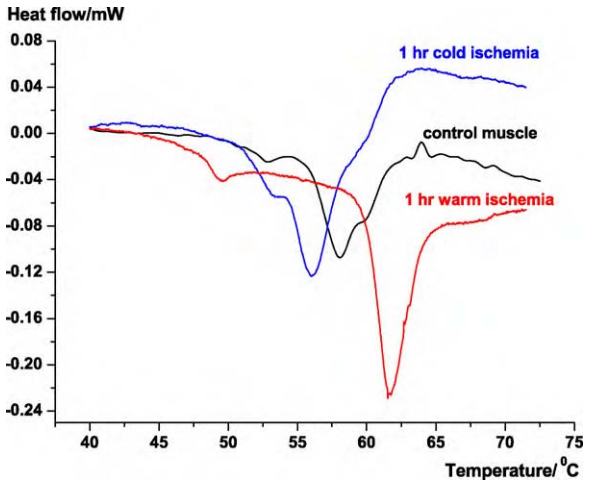

Fig. 4. DSC curves of the muscle layer following 1 h small bowel warm ischemia and cold preservation. Downward deflection represents endotherm process.

53.6 °C with a total calorimetric enthalpy change −4.1 ± 0.22 J/g. Applying warm ischemia a decreasing trend in T_ms and ΔH can be seen except of the case of 6 h intervention, where ΔH is greater than in control and in the other warm ischemia. In case of cold ischemia of different time durations after 1 h treatment a lower denaturation appeared with a T_m = 30.4 ± 0.2 °C and the higher transition shifted to 59.3 ± 0.2 °C with a total calorimetric enthalpy of −5.94 ± 0.4 J/g. The 3 h treatment resulted different transition temperatures and calorimetric enthalpies: in case of warm ischemia the T_m and the enthalpy decreased to 48.7 ± 0.2 °C and −1.7 ± 0.2 °C J/g, while the cold treatment increased the first transition by 2.2 °C compared with the 1 h treatment with a decreased enthalpy (−2.67 J/g). After 6 h cold ischemia the enthalpy decreased further but the warm ischemia exhibited a close doubled thermal transition with extreme high enthalpy (see Table 1).

In case of the muscle compound of intestinal wall the control exhibited a denaturation transition with T_ms 52.8 (myosin head), 58.1 and 59.9 °C (myosin tail and actin [according to [15–17]]) together with a 0.59 J/g calorimetric enthalpy (Fig. 4). In case of 1 h warm treatment the main denaturation peak shifted to the conventional actin denaturation range with (62 °C) a narrower half-width. It means that in the actomyosin complex the contribution of myosin tails cannot be resolved, that is it exhibits more significant structural effect with smaller ΔH (1.4 J/g) as the cold preservation with same time duration (53.5; 56 °C, 2.21 J/g, see Fig. 4). This cold preservation also indicates a significant structural change in myosin thermal domains as well as in the actin-myosin interaction. During 3 h warm ischemia the muscle exhibited an increase in T_m but a decrease in the calorimetric enthalpy (Fig. 5) while the cold treatment revealed decrease in denaturation temperatures but an increase in calorimetric enthalpy (53.6; 58.3 °C, ΔH = 3.4 J/g). The effect of 6 h warm ischemia was the most striking one (Fig. 6). It showed nearly the same thermal parameters as the proper 6 h cold intervention.

The effect of ischemia in case of total intestinal wall has shown a big variety. Applying 1 h ischemia (Fig. 7) the cold preservation shifted the main transition temperature into the higher range, and the contribution of mucosa cannot be seen. During warm treatment the contribution of myosin and actin compounds separated more significantly with increased main melting temperature (50.5; 60.2 °C), where the myosin rod contribution could not be separated from actin. The thermal contribution of mucosa can be seen clearly in this latter case (23 °C). The effect of 3 h treatment was more definite in mucosa (Fig. 8), and it was more pronounced in cold ischemia. In case of muscle proteins the cold ischemia caused the biggest structural change (53; 55; 59.6 °C). The 3 h warm treatment exhibited only one main transition (59.8 °C) and we could see the contribution of mucosa. After 6 h treatment in case of cold ischemia

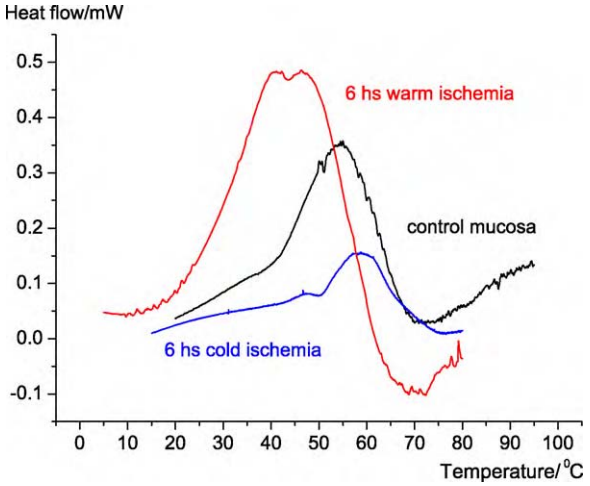

Fig. 3. DSC analysis of mucosa following 6 h intestinal warm ischemia and cold preservation. The upward deflection of DSC scans means an exotherm process.

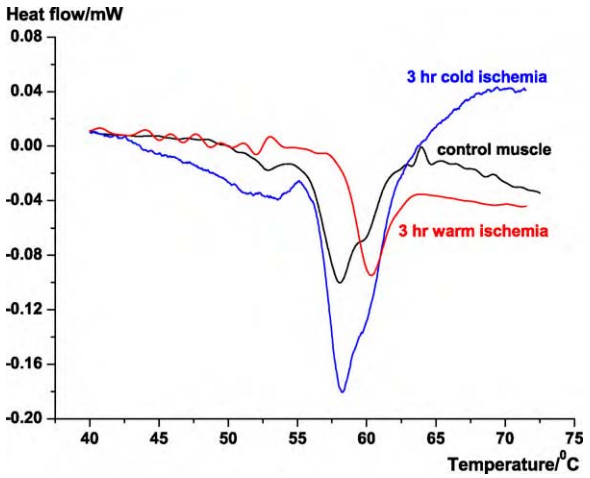

Fig. 5. DSC curves of the muscle layer following 3 h small bowel warm ischemia and cold preservation. Downward deflection represents endotherm process.

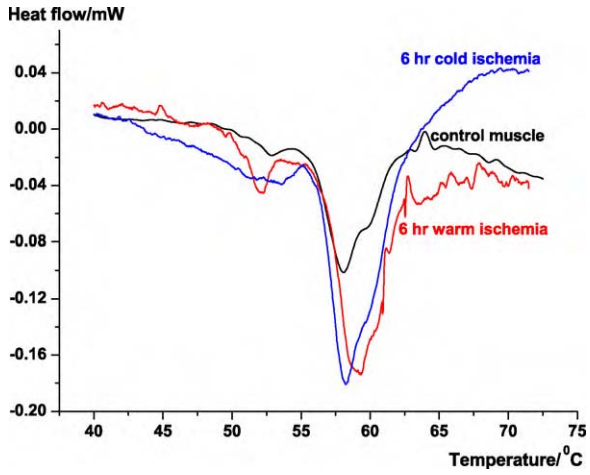

Fig. 6. DSC curves of the muscle layer following 6 h small bowel warm ischemia and cold preservation. Downward deflection represents endotherm process.

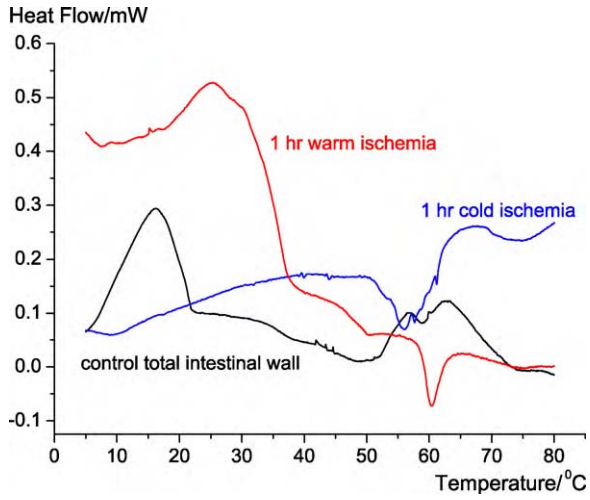

Fig. 7. DSC scans of the total intestinal wall after 1 h warm ischemia and cold preservation.

the mucosa contribution can be seen clearly and it made the most significant alterations in the actin/myosin contribution. Moreover, the warm ischemia resulted in the biggest calorimetric enthalpy of actomyosin system (Fig. 9 and Table 1).

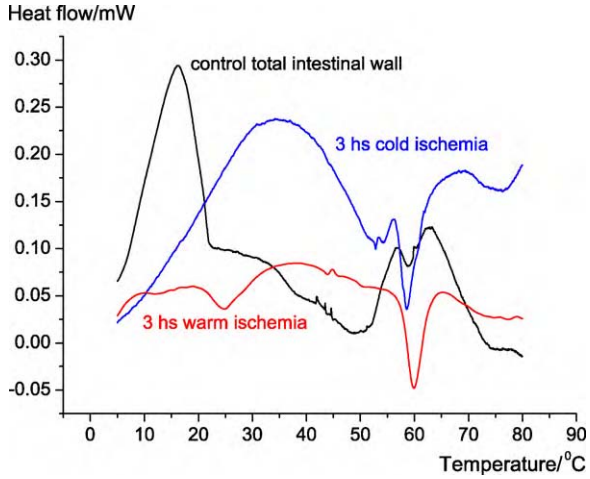

Fig. 8. DSC scans of the total intestinal wall after 3 h warm ischemia and cold preservation.

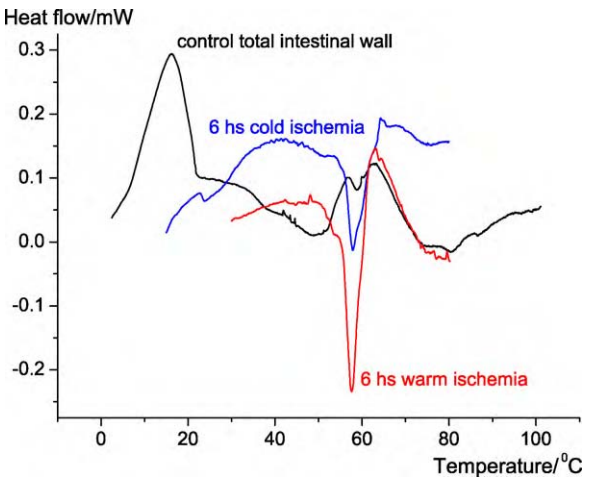

Fig. 9. DSC scans of the total intestinal wall after 6 h warm ischemia and cold preservation.

4. Discussion

Intestinal injury following warm or cold ischemia is a common clinical complication that is most often assessed by histological evaluation of standard hematoxylin and eosin stained tissue sections. For this purpose, many different grading systems for the bowel injury have been described. Instead, most studies have used their own (qualitative or semi quantitative) systems, thus this is difficult to compare these results of studies [18,19]. However, quantitative comparison of structural damage is essential for the development of understanding of the mechanisms of ischemic injury and the development of treatment strategies. In our previous studies, we examined the correlation between the histological and the DSC data of the warm and cold intestinal ischemic injuries. We concluded that DSC data reflected the Park's score grades in the mucosa, but DSC showed more details about the seriousness of structural injury in the smooth muscle layers [20–23].

In this study, we compared the effect of warm and cold ischemia following 1, 3 and 6 h in the different layers of the intestinal wall by DSC method. Our measurements showed that mucosa has undergone significant changes in a time-dependent manner during different types and durations of ischemia. After warm ischemia periods severe structural damage could be seen compared the ΔHs with the calorimetric enthalpy of the control tissue. In cases of cold preservation in UW solution the structure of mucosa could sustain the "normal physiological activity"; it is more saved if we compare them to the same warm ischemic periods. Moreover, its thermal contribution could be separated also in case of total intestinal wall denaturation. These calorimetric results were correlated to numerous experimental and clinical observations being in the literature, namely, as the extent and duration of warm or cold ischemia increases, morphologically more detectable injury results in the mucous layer [7–9].

It has been extensively described that under normal circumstances the turn over of the mucosa cells approximately 3–4 days, when they have been dying during apoptotic processes [24]. In fact, the variability in the mesenteric occlusion times used and qualitative description of the warm ischemic injury make interpretation of the data difficult. Moreover, no consensus exists how long the cold ischemic time has been not caused irreversible damage in the mucosa structure yet. For example, the warm ischemic period is varying from 15 to 90 min, while preservation period was suggested be last shorter than 6–8 h [7–9,25–27]. Present calorimetric data indicating structural injury

confirmed our previous biochemical measurements, where we assessed the degree of oxidative injury by measuring the level of lipid peroxidation, and on the other hand the status of endogenous scavenger and antioxidant capacity was determined. Moreover, our surgical observations showed that serious mucosa damage develops after 3 h warm ischemia and 6 h cold preservation period. [4–6].

According to our previous studies the muscle thickness showed mild decrease following warm ischemia and cold preservation compared to controls, but these changes were not significant [20,21]. Several experiments confirmed that appreciable changes in the muscular layer have not been seen by histological measurements [2,3,26,27]. Contrarily, comparison of DSC data showed that in case of cold ischemia the contribution of different muscle proteins could be more definitely separated as in warm treatment. The biggest change can be seen in the interaction of actomyosin complex. The contribution of myosin and actin cannot be so easy resolved as in case of cold preservation, and the heat capacity change (the baseline shift between the native and denaturated states) as well as the increase of melting temperature are also greater in warm ischemia, which could be the sign of greater loosening of bound water and this way the more dense "packaging" of protein system. These calorimetric data showed that cold preservation in UW solution ameliorated the structural injury in the muscular layer also. However, there is no other DSC data on the effect of warm or cold ischemia against tunica muscularis of the intestine. But, these structural injuries correlated to the pathophysiological results of the ischemic smooth muscle studies [28]. These are demonstrated that warm ischemia decreased the number of ganglionic cells in the myenteric plexus and they did not recover in the postoperative period, which may have the reason of alterations in intestinal motility [29]. In the background of these functional damages stand several processes, including oxidative injury, disruption in the cellular calcium homeostasis, and evoked inflammatory responses. This last factor occurs of great importance during cold preservation and subsequent intestinal transplantation to result in a significant reduction in muscular contractility [30].

Thermal behaviour of total intestinal wall has been shown a big variety, which arises from the nature of the intestine, namely it contains different structural layers. But, the above mentioned thermal events for mucosa and smooth muscle can be followed clearly in data of total intestinal wall too. Although, the histological studies are used in assessing the severity of the intestinal wall injury, our results strongly confirmed hat DSC more definitive way to judge the small bowel structural damage during small bowel surgery.

5. Conclusion

In summary, DSC measurements provide a new and well-useable approach to examine and compare the intestinal structural changes following warm ischemia and cold preservation. These thermal parameters indicated the thermodynamic consequences of structural destruction rearrangement, which provides basis for further investigation. Furthermore, DSC analysis can be used with reliability in quantitatively evaluating the degree of bowel ischemic injury, allowing a more objective and reproducible measurement if ischemic changes.

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