

Effects of ischemic preconditioning associated to different preservation solutions in protecting the intestinal graft¹

Efeitos do condicionamento isquêmico associado a diferentes soluções de preservação na proteção do enxerto intestinal

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ABSTRACT

PURPOSE: To evaluate the effects of ischemic preconditioning (IPC) associate with different preservation solutions, in the protecting of gut.

METHODS: Four groups of 14 rats underwent laparotomy and collecting 20 cm of ileum, for preservation, at 4°C, in Belzer (Belz), Ringer (RL), Celsior (Cs) and Custodiol (Cust) solutions, for 24 hours. Prior to collection, half of the animals in each group were subjected to IPC. During preservation, in the periods of zero, 12, 18 and 24 hours, were conducted evaluating the degree of mucosal injury and dosage of malondialdehyde acid (MDA).

RESULTS: In all periods the RL group, with and without IPC, presented MDA values higher than the Belz and Cs. The degree of mucosal injury in the non-ipc RLgroup with 12h preservation was higher than the others; with 18 and 24h, the RL and Cust had higher degrees of damage than Cs and Belz. With IPC, in all periods, the group Cs and Belz had lower degrees of injury.

CONCLUSION: The Celsior and Belzer solutions had better protective effects on the gut and these effects were enhanced by IPC.

Keywords: Organ Preservation Solutions. Ischemic Preconditioning. Intestine, Small. Rats.

RESUMO

OBJETIVO: Avaliar os efeitos do condicionamento isquêmico (PCI) associado a diferentes soluções de preservação, na proteção do intestino delgado.

MÉTODOS: Quatro grupos de 14 ratos Wistar, foram submetidos à laparotomia e coleta de 20 cm de íleo, para preservação, a 4°C, nas soluções de Belzer (Belz), Ringer (RL), Celsior (Cs) e Custodiol (Cust) por 24 horas. Previamente à coleta, em metade dos animais de cada grupo, o intestino foi submetido ao PCI. Durante a preservação, nos períodos de Zero, 12, 18 e 24 horas, foram realizados avaliação do grau de lesão da mucosa e dosagem do ácido malondialdeído (MDA).

RESULTADOS: Em todos os períodos o grupo RL, sem e com pci, apresentou valores maiores de MDA do que o Belz e Cs. O grau de lesão da mucosa nos grupos sem pci com preservação de 12h, no grupo RL, foi maior que nos demais; com 18h e 24h o grupo RL e Cust apresentaram maiores graus de lesão do que Cs e Belz. Com o pci, em todos os períodos, os grupos Belz e Cs apresentaram menores graus de lesão

CONCLUSÃO: As Soluções Celsior e Belzer tiveram melhores efeitos na proteção do intestino e estes efeitos foram incrementados pelo condicionamento isquêmico.

Descritores: Soluções para Preservação de Órgãos. Precondicionamento Isquêmico. Intestino Delgado. Ratos.

Introduction

The small bowel transplantation (SBT) is the proposed treatment for various intestinal disorders. However, in despite of significant advances, the bowel graft preservation is still a challenge. The SBT is indicated especially for patients who underwent extensive small bowel resection and evolved with short bowel syndrome and intestinal failure. In such cases the patient usually becomes dependent of parenteral nutrition (TPN) for survival. The maintenance of patients with venous nutrition therapy, for long times, presents a number of complications and the alternative, in some cases, is the transplantation^{1,2}.

Currently the SBT is technically well established but the results are still lower than the other organs and the morbidity and mortality remain high. The complications in most patients, are related to the occurrence of primary graft dysfunction, induced by injuries during the period of cold ischemic preservation, reperfusion injury, rejection and infection. Advances in immunosuppressive therapy, like the use of tacrolimus, have provided improvements in rates of rejection and infection, resulting in a decrease in mortality rates, which nevertheless still remain around to 50% at 5 years post-transplant^{1,3}.

Preservation injury is caused primarily by ischemia and hypothermia. The small intestine is the most perfused organ, receiving around 25% of blood pumped by the heart and of that amount 90% are consumed in the mucosa and submucosa, which are the areas earlier affected by ischemia. The interruption of oxygen supply to tissues leads to inhibition of mitochondrial oxidative phosphorylation and a fall in production and supply of adenosine triphosphate. Hypothermia, which is indispensable to the preservation is another factor that, paradoxically, provides breakdown in cell physiology. The main side effects of hypothermia are cellular swelling, acidosis, and generation of free radicals such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radical (-OH)^{2,4}.

Fundamentally the proper preservation depends on actions against the deleterious effects of ischemia and hypothermia. The first preservation solution formulated were Collins and Eurocollins, developed for use in preserving renal hypothermic. In the early 70's, a new solution, the Custodiol, also known as HTK (histidine-tryptophan-ketoglutarate), initially indicated to cardioplegia in heart surgery, began to be tested also in the preservation of kidneys and digestive organs⁴. In 80's, discovery of immunosuppressants became possible to perform transplants of liver, pancreas and intestine and at that time, a new solution was developed in the University of Wisconsin (Belzer's Solution), for

the preservation of the liver. In the last years various solutions have been introduced, as Celsior, IGL-1 (developed at the Institute George Lopez - Lyon / France) and Polysol, among others^{4,5}.

In general, the solutions are formulated to reduce the harmful effects of hypothermia and provide energetic substrate. Thus, the solutions contain, for example, impermeants like histidine and lactobionate, buffer as KH₂PO₄, and antioxidants such as glutathione, which act by eliminating free radicals^{4,5}.

Specifically in relation to the small intestine, there are a variety of clinical and experimental studies, comparing the protective effects of different preservation solutions⁶. The results are quite different and in reality, to date, a preservation solution specific to gut protection, was not yet developed. The small bowel is difficult to be preserved and the maximum time of preservation, with current methods, according to some authors, is around 10 hours².

A perspective came when some authors observed experimentally that the myocardium of dogs, previously submitted to short cycles of ischemia and reperfusion, became more resistant to the prolonged ischemia, including reduction of necrotic area⁷. This protection mechanism was known as ischemic preconditioning (IPC), leading some researchers to study the phenomenon in other organs such as lung, liver and intestine^{2,7,8}. The results of these studies generally showed that the IPC confers some protection to the organs evaluated, with reduction of injuries due to ischemia and reperfusion.

The mechanisms by which the IPC acts remain not clear, however, its effects may be mediated by endogenous production of nitric oxide (NO). IPC possibly acts by reducing the conversion of ATP to hypoxanthine, decreasing the ability of free radical production and stimulating the production of antioxidants^{8,9}.

There is no consensus as to which solution is best suited to preserve the intestine; there is neither experimental studies evaluating the effects of IPC in association with different preservation solutions.

The aim of this study was assess the effects of ischemic preconditioning associated with Celsior, Custodiol, Belzer and Ringer-Lactate solutions in the protection of the small bowel during hypothermic ischemic preservation phase and assess which of the preservation solutions, alone, is more effective in protecting the gut during hypothermic preservation.

Methods

This research was approved by the Ethics Committee of the Federal University of São Paulo (UNIFESP) with Reference CEP N° 1278/08.

Experimental design

It was used 56 rats and 4 types of preservation solutions. The animals were randomly assigned to 4 groups of 14 animals each, according to the type of solution: Groups Ringer lactate, Celsior, Custodiol and Belzer. Half the animals in each group underwent IPC forming a subgroup with and another without preconditioning.

The Celsior and Belzer solutions contain the antioxidant glutathione and Custodiol, also named HTK, is mainly composed by histidine, tryptophan and ketoglutarate. The full composition of the solutions is shown by Weil *et al.*⁵.

All rats were anesthetized intramuscularly with a solution of ketamine (90mg/kg) and xylazine (20mg/kg), laparotomy and collection of ileal segment for preservation for 24 hours, in the solution corresponding to the group. During the period of preservation, intestinal samples were collected for analysis and evaluation of which solutions, in combination with IPC, obtained better performance in intestinal preservation. The parameters analyzed were score of the degree of mucosal injury according to scheme proposed by Park *et al.*¹⁰ and quantification of malondialdehyde acid (MDA) by the thiobarbituric acid test (TBARS)¹¹.

It were used Wistar rats, adult, male, weighting between 250 and 300g, coming from the Central Animal Laboratory, Faculty of Medicine, Federal University of Mato Grosso. In the period before the experiment, the animals were kept in individual cages, fed with a proper diet for the species (Purina®) and free access to water. The temperature and the cycles of light and dark in the laboratory environment were controlled, so that the conditions remain suitable for the animals in the experiment.

Surgical procedure

The surgery consisted of midline incision, opening the peritoneum, exposing of the bowel, opening the retroperitoneum, isolation of the aorta and superior mesenteric artery (SMA). In the subgroups without IPC, after 20 minutes of observation, the aorta was cannulated just below the SMA and it was infused 40 ml of the respective preservation solution, corresponding to the group. In animals of subgroups with IPC, it was performed the occlusion of the SMA, with bulldog clamp type, for 10 minutes followed by reperfusion for the same period. At the end of the period established for the IPC, the aorta was cannulated and the solution perfused. Immediately at the start of perfusion, right thoracotomy was performed, section of the inferior vena cava and drainage the

solution infused into the mesenteric arcade. The euthanasia was performed by exsanguination.

Collection of intestinal segments for preservation

At the final period of vascular perfusion, it was collected 20 cm of ileal segment, measured from 2 cm of the ileocaecal valve. One sample of 5 cm was immediately separated for histological analysis (zero hour sample) and MDA dosage. The rest, 15 cm, was placed in a vial containing 50 ml of preservation solution referring to the group and preserved for 24 hours at 4°C. Intestinal samples were collected when completing the periods of 12, 18 and 24 hours of preservation to morphological and biochemical (measurement of MDA) analysis.

Measurement of MDA

Samples collected were submerged in liquid nitrogen for freezing and sent to determination of MDA content that was performed by thiobarbituric acid test (TBARS)¹¹. This test is based on the reaction of MDA with thiobarbituric acid at low pH and high temperature, forming the MDA-TBA complex, with its own color and absorption, which can be detected by spectrophotometer. The samples, still frozen, were macerated in an ice bath and suspended in a saline solution from which was taken 100 ul, added to sodium sulfate solution, 2.5 ml of TBA, and reagent buffer (acetic acid, thiobarbituric acid and sodium hydroxide). The tubes were covered and incubated at 95°C for 1 hour, cooled, and centrifuged for 15 minutes at 3000 rpm. The supernatant was removed, prepared and analyzed in a spectrophotometer with a wavelength of 532 nm.

Morphological analysis

To assessment of mucosal injury, samples of 2 cm were collected, placed in formaldehyde at 10% and sent to Laboratory for preparation of the paraffin blocks. After paraffin embedding, ileum fragments were sectioned to 4µm-thick microtome, attached on slides and stained by hematoxylin-eosin (H.E). The analysis of injury degrees were performed under a light microscope by pathologist blinded to the project data. The degree of mucosal injury was classified according to the criteria proposed by Park *et al.*¹⁰.

Statistical analysis

For data analysis, it was used the statistical software SPSS version 15.0 for Windows. The p<0.05 or 5% was considered statistically significant. The descriptive analysis of continuous

variable was described by mean and standard deviation. For the inferential analyze of mucosal injury degree were applied the nonparametric Kruskal-Wallis test to verify the hypothesis of equality between the four groups and Mann-Whitney, to check the equality of groups 2 to 2, when difference was detected. To analyze of MDA were applied the analysis of variance (One-way ANOVA) and Tukey test, to check the equality of groups 2 to 2, when difference was detected.

Results

In the evaluation of MDA was observed that those subgroups without IPC and preserved with Ringer-Lactate showed higher values of MDA denoting greater degree of lipid peroxidation of cell membranes. The other groups showed similar results. In groups with IPC, within 12h of preservation, the ileum preserved with Custodiol had significantly higher values of MDA and thus more cell membrane injury than those preserved with Belzer solution. After 18h of preservation, there is progressive decrease of MDA in all groups (Table 1, Figures 1 and 2).

TABLE 1 - Mean and standard deviation of MDA value, in nmol/mg tissue, groups Ringer, Celsior, Custodiol and Belzer, without and with ischemic preconditioning, at zero, 12, 18 and 24 hours of preservation.

		Ringer	Celsior	Custodiol	Belzer	p
Zero	without	2.40±0.5	1.52±0.9	2.30±0.4	1.57±0.8	0.104
	with	2.87±1.0	1.50±0.8	2.80±1.2	1.50±0.6	0.015
12	without	3.56±1.1	2.33±1.6	2.90±1.1	1.70±0.7	0.053
	with	3.10±0.8	1.92±0.8	2.80±0.6	1.60±0.5	0.002
18	without	4.40 ± 1.6	3.50±1.4	2.37±0.6	2.70±0.9	0.030
	with	4.30±1.4	3.10±0.8	3.03±0.5	2.30±0.7	0.007
24	without	4.21±1.0	3.26±1.4	2.0±1.0	2.52±0.6	0.005
	with	3.90±1.5	2.20±0.7	2.0±0.7	1.47±0.8	0.001

Statistical calculation comparing the four groups without and with IPC: ANOVA

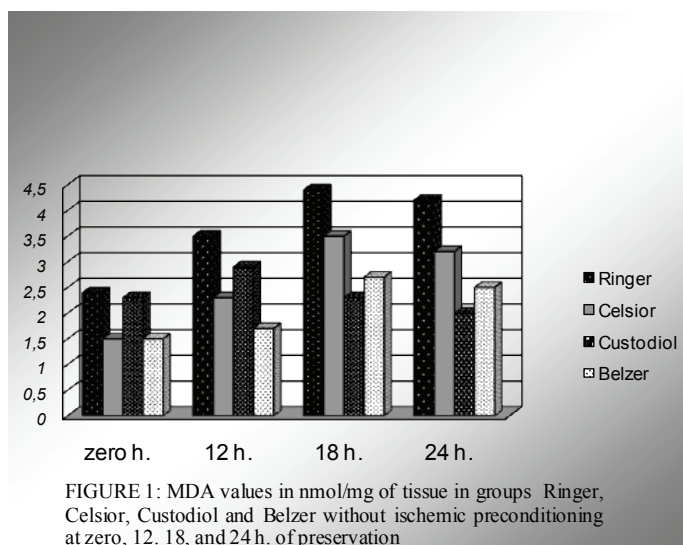
NS=without significance ; *= statistically significant

Tukey test: groups without IPC:

Zero NS ; 12h: NS

18h RL=Cs (p=0,542); *RL>Cust (p=0,028);
RL=Belz (p=0,102); Cs=Cust (p=0,358);Cs
=Belz (p=0,715);Cust=Belz (p=0,927).

24h RL=Cs (p=0,376);*RL>Cust
(p=0,005);*RL>Belz (p=0,035); Cs=Cust
(p=0,168); Cs=Belz (p=0,582);Cust=Belz
(p=0,825).



Tukey test: group with IPC

Zero: NS

12h *RL>Cs (p=0,016); RL=Cust(p=0,784); *RL>Belz
(p=0,003); Cs=Cust (p=0,121); Cs=Belz (p=0,904);
*Cust>Belz (p=0,029).

18h RL=Cs(p=0,129); RL=Cust (p=0,086);
*RL>Belz(p=0,004); Cs=Cust (p=0,997);
Cs=Belz(p=0,407); Cust=Belz(p=0,529).

24h *RL>Cs(p=0,020);*RL>Cust
(p=0,012);*RL>Belz(p=0,001); Cs= Cust (p=0,996);
Cs=Belz(p=0,524);Cust=Belz(p=0,658).

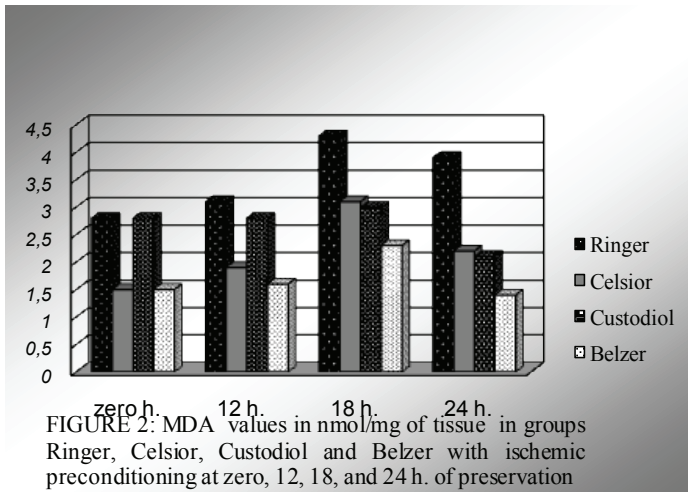


FIGURE 2: MDA values in nmol/mg of tissue in groups Ringer, Celsior, Custodiol and Belzer with ischemic preconditioning at zero, 12, 18, and 24 h. of preservation

Degree of mucosal injury

The results regarding the grades of mucosal injury showed progression of lesions over time and decrease with the achievement of IPC. The Ringer group, at all times, presents greater degree of mucosal injury. With 12, 18 and 24 hours of preservation, the Custodiol group showed higher levels of damage than the groups Celsior and Belzer and these results became even more evident and more significant with the implementation of IPC (Table 2, Figures 3 and 4).

TABLE 2 - Mean, standard deviation and median of the degree of mucosal injury with Zero, 12, 18 and 24 hours of preservation, groups Ringer, Celsior, Custodiol and Belzer, without and with ischemic preconditioning.

	Ringer	Celsior	Custodiol	Belzer	p	
Zero	without	1.1±0.6 / 1	0.5±0.5 / 1	0.8±0.3 / 1	0.4±0.5 / 0	0.124
	with	1.0±0.8 / 1	0.1±0.3 / 0	0.6±0.5 / 1	0.1±0.3 / 0	0.042
12	without	4.0±0.5 / 4	2.2±0.7 / 2	3.0±0.8 / 3	2.1±0.7 / 2	0.002
	With	3.4±0.5 / 3	1.4±0.5 / 1	2.4±0.5 / 2	1.5±0.5 / 2	0.001
18	without	5.4±0.5 / 5	3.2±0.4 / 3	4.4±0.7 / 4	3.4±0.7 / 3	0.001
	With	4.4±0.5 / 4	2.5±1.1 / 2	3.7±0.7 / 3	2.2±0.4 / 2	0.001
24	without	6.5±0.5 / 7	4.0±0.8 / 4	5.5±1.1 / 5	3.8±0.6 / 4	0.001
	with	6.4±0.5 / 6	3.5±0.7 / 3	5.0±0.5 / 5	3.7±0.7 / 4	0.001

statistical test: kruskal-Wallis; NS=without significance ; *= statistically significant

Mann-Whitney test: group without IPC

Zero NS

12h *RL>Cs(p=0,002); *RL>Cust(p=0,027); *RL>Belz(p=0,002); Cs=Cust (p=0,133); Cs=Belz(p=0,674); Cust=Belz (p=0,065).

18h *RL>Cs(p=0,001); *RL>Cust(p=0,09); *RL>Belz(p=0,002); *Cust>Cs(p=0,014); Cs=Belz(p=0,087); *Cust>Belz(p=0,040).

24h *RL>Cs(p=0,001); RL=Cust (p=0,079); *RL>Belz(p=0,001); *Cust>Cs (p=0,017); Cs=Belz(p=0,728); *Cust>Belz (p=0,008).

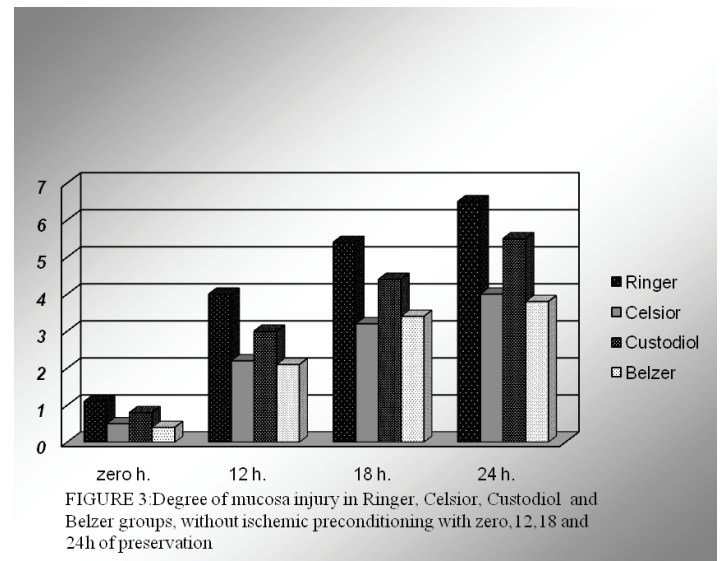


FIGURE 3: Degree of mucosa injury in Ringer, Celsior, Custodiol and Belzer groups, without ischemic preconditioning with zero, 12, 18 and 24h of preservation

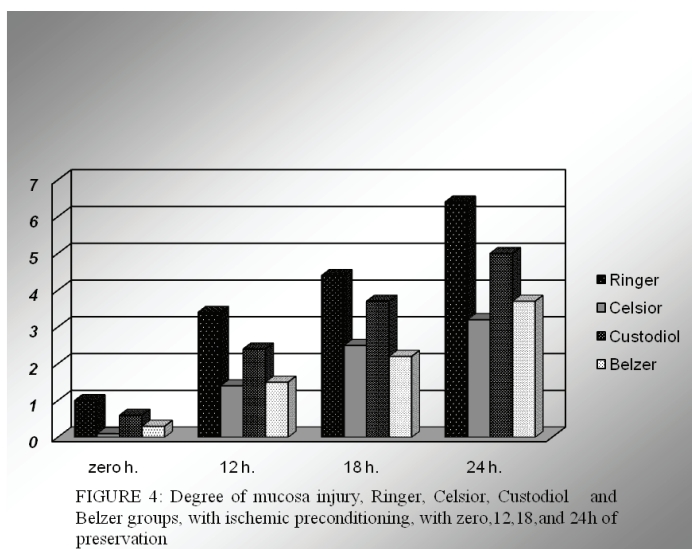
Mann-Whitney test: group without IPC

Zero *RL>Cs(p=0,031); RL=Cust(p=0,293); *RL>Belz(p=0,031); Cs=Cust (p=0,107); Cs=Belz(p=1,000); Cust=Belz(p=0,107).

12h *RL>Cs(p=0,001); *RL>Cust(p=0,010); *RL>Belz (p=0,001); *Cust>Cs(p=0,010); Cs=Belz(p=0,606); *Cust>Belz (p=0,018).

18h *RL>Cs(p=0,011); RL=Cust (p=0,071); *RL>Belz(p=0,001); *Cust>Cs(p=0,028); Cs=Belz(p=0,872); *Cust>Belz(p=0,0040).

24h *RL>Cs(p=0,001); *RL=Cust(p=0,002); *RL>Belz (p=0,001); *Cust>Cs(p=0,007); Cs=Belz(p=0,674); Cust>Belz(p=0,008).



Discussion

The morphological integrity of the transplanted small bowel is essential for the full success of the transplant. The proper preservation of the intestinal graft is essential to prevent complications, such as bacterial translocation and sepsis, the leading cause of mortality after transplantation, accounting for approximately 60 to 70% of deaths. The hypothermic preservation of the small intestine was one of the first techniques used to maintain the viability of the graft. It seeks to extend the period

of preservation infusing and immersing the graft in the cold preservation solution^{2,4}.

These liquid solutions emerged as a mechanism to minimize the side effects of hypothermia. The Belzer's solution, formulated at the University of Wisconsin (UW) in late 1980 is still considered, according Oltean *et al.*², as the gold standard for abdominal organ preservation. The UW solution has a high concentration of potassium (130mmol/l), containing also: lactobionic acid and raffinose, impermeant of high molecular weight that prevent intracellular edema; hydroxy-ethyl-starch (HES), which gives oncotic activity; and glutathione, antioxidant introduced to attenuate oxidative stress. The Custodiol, a preservation solution alternative, has a buffer system composed of histidine/histidine chloride and impermeant (ketoglutarate), but in electrolyte content is similar to the extracellular medium unlike Belzer's solution. There is growing evidence suggesting that Custodiol has good results in the preservation of liver and pancreas, but the results for intestinal preservation still need further studies to be proved^{4,5}. Celsior is another solution that has been tested in intestinal preservation. Its formulation appears as a hybrid of the previous two, containing reduced glutathione; mannitol and lactobionic acid; high sodium concentration (100 mmol/l) with low potassium, similar to UW; and histidine, that also composes the Custodiol⁶.

It has been postulated that an ideal preservation solution should preserve the structure of the intestinal epithelium during the cold ischemic storage. Regarding the effects of preservation solutions there are several studies and results are quite controversial⁵. Straatsburg *et al.*⁶ evaluated apoptosis and necrosis in rat liver after hypothermic preservation, concluded that Celsior and UW were more effective than HTK. Wei *et al.*⁵ investigated the effects of a new solution, Polysol, in intestinal preservation and concluded that this solution was superior to Custodiol and Celsior, which was considered superior to UW. Mangus *et al.*¹², in a clinical study, which compared Custodiol and Belzer in intestinal and multivisceral transplant, assessed the survival rate of graft and patients, observing no differences between solutions.

In addition to hypothermia and liquid solutions, it has been investigated other methods to improve preservation as the ischemic preconditioning (IPC), which involves subjecting the graft to short cycles of ischemia and reperfusion before the harvest⁷. Many experimental studies in rats have shown that IPC reduces the intensity of the ischemic lesions^{8,13}. Clinically, in hepatic surgery, some authors as Clavien *et al.*¹⁴ observed lower enzyme changes and less endothelial apoptosis of liver cells in patients submitted to the IPC, when compared to the group not

subject to the procedure. The mechanisms by which the IPC acts, still not fully understood. A greater maintenance in energy cellular occurs with IPC by decreasing of the conversion of ATP to hypoxanthine and thereafter, by decreasing production of oxygen free radicals (OFR). Moreover there is an increased production of antioxidant and nitric oxide (NO), which seems to be a key facilitator of the process⁹. The present study speculated that the IPC in combination with the various preservation solutions would present different results. The methods employed to assess the intestinal preservation were analyze of mucosal damage and MDA content in intestinal tissue. The molecule of MDA results of cell membrane destruction by OFR, which occurs in tissues under oxidative stress. This process, known like lipid peroxidation, resulting in a chain reaction mediated by OFR with the oxidative deterioration of polyunsaturated fatty acids, the major components of the lipid bilayer, comprising the cell membrane.

The results of MDA values in the non-PCI groups, with zero hours of preservation, showed no statistically significant difference. With 12, 18 and 24 h., in the Ringer group, the MDA content were significantly higher than others. This indicates that lipid peroxidation was more intense in these groups. Ringer lactate actually has not composition suitable for tissue preservation and possibly the effects observed in this group are due solely to hypothermia. It should be noted also that the Celsior and Belzer solutions contain the antioxidant glutathione and it possibly should have reduced the peroxidation of the cell membrane in these groups. To making the IPC, was observed a decrease in the MDA in all groups. This study, however, did not perform the comparison between groups with and without IPC, because this comparison is beyond the scope of this research, besides the influence of IPC in ischemia/reperfusion and ischemia itself is well established in the literature.

The analyze of mucosal injury is considered a gold standard method when it wants to examine the effectiveness of preservation methods and is the most commonly used parameter to assess the quality of the intestinal graft under cold storage.

Balaz *et al.*⁸, comparing the effects UW and Custodiol solutions in intestinal hypothermic preservation, assessing the degree of injury and the concentration of serotonin in the mucosa concluded that the UW solution had better effect protector. Tesi *et al.*¹⁵, in a clinical study evaluated the histological changes in the small intestine preserved in viaspan solution[®] (UW) concluded that the morphology maintains near normal by 6h and that after 9h of ischemia, severe mucosal injury occurs with total detachment of the epithelial layer.

In the present research, analysis of the degree of mucosal

injury in the samples with zero hour of preservation in the non-IPC groups, showed no statistically significant differences, but on performing the IPC, the Celsior, Belzer and Custodiol group had lower injuries than the Ringer. With 12h of preservation in the intestines without IPC, all groups, except Ringer, had equal severity of damage, but when performing IPC, Celsior and Belzer groups began to show lower scores of injuries than the Ringer and Custodiol. With 18h of preservation in the groups without IPC, Celsior and Belzer had also similar results, with less pronounced lesions than another. With the IPC, the Ringer group showed the same lesion score of Custodiol, and both higher than the Celsior and Belzer. Completed 24h, the severity of injuries continued progress; however, the groups maintained the same pattern and the same proportionality in intensity of injuries. When performed IPC, there was overall reduction of injuries and Custodiol group also began to show a lower degree of injury than the Ringer. According to experimental model and methodology proposed in this research and considering the results obtained, the solution Celsior and Belzer associated with the IPC showed similar results and better than Ringer lactate and Custodiol.

Concerning the IPC, whose beneficial effects on preservation of liver, intestine, and other organs, are widely supported in the literature, there is agreement with the results of this study. The IPC increased positively the effects of preservation solutions, and therefore can be considered as an important adjunct in improving the quality of intestinal preservation. The clinical application of this technique can be evaluated when the intestinal transplants are more widely spread³, enabling the use of new techniques such as this widely based on experimental studies.

Conclusion

The intestinal ischemic preconditioning associated with Belzer and Celsior solutions were more effective in protecting the intestine from the oxidative stress and mucosal injury.

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