

USE OF HYPERTONIC SOLUTIONS FOR LIVER PRESERVATION IN RATS*Uso de soluções hipertônicas para preservação hepática em ratos*Vitor Nagai **YAMAKI**, Renan Kleber Costa **TEIXEIRA**, Marcus Vinicius Henriques **BRITO**

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ABSTRACT - Background - The success of a transplant depends mainly on the viability of the graft, which is currently the main point of difficulty focuses on the triad preservation-rejection-infection. There are several specific components of preservation solutions that could prevent certain tissue damage. From these components, the osmotic factor has been highlighted as a factor in preventing edema and subsequent cell death, suggesting a possible advantage in the use of hypertonic solutions for organ preservation. **Aim** - To compare different hypertonic solutions as alternative to liver preservation. **Method** - A total of 105 Wistar rats were divided in Standard Group (GP, n=5 rats), to verify the normal range of the study, and five experimental groups of 20 rats each, according to the preservation solution used: Group Eurocollins (GE), Group Saline 0.9% (GF), Group Glucose 50% (GG), Group Mannitol 20% (GM), Group Salty - NaCl 7.5% (GS). All animals in experimental group were also divided into four subgroups according to the time of collection in: 0 h, 2 h, 6 h and 12 h. Was assessed cell viability by the reaction with Methyl Blue Thiazolyl (MTT) and the dosages of lactate and alanine aminotransferase (ALT). **Results** - Regarding the lactate level, was observed a relative improvement of hypertonic solutions compared to eurocollins, and in 12 h, the GE and GS showed no statistically significant difference ($p > 0.05$). When assessed cell viability, absorbance at MTT also demonstrated favorable results to the GS, since no statistically significant difference in relation to GE. **Conclusion** - The 7.5% NaCl solution showed promising results for organ preservation, presenting parameters and capability comparable to eurocollins preservation solution.

HEADINGS - Hepatectomy. Neoplasm metastasis. Portal Vein

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DESCRITORES - Solução salina hipertônica. Transplante. Preservação de órgãos. Fígado. Ratos.

RESUMO - Racional - O sucesso de um transplante depende, principalmente, da viabilidade do enxerto, sendo que, atualmente, o maior ponto de dificuldade concentra-se na tríade preservação-rejeição-infecção. Existem diversos componentes específicos das soluções de preservação que previnem danos. Destes componentes o fator osmótico tem se destacado como de prevenção do edema e posterior morte celular, sugerindo possível vantagem na utilização de soluções hipertônicas para preservação de órgãos. **Objetivo** - Comparar soluções hipertônicas como alternativas para preservação hepática. **Método** - Foram utilizados 105 ratas Wistar distribuídas em Grupo Padrão (GP, n=5) para controle e cinco grupos experimentais com 20 animais cada, a saber: Grupo Eurocollins (GE), Grupo Soro Fisiológico 0,9% (GF), Grupo Glicose 50% (GG), Grupo Manitol 20% (GM), Grupo Salgado - NaCl 7,5% (GS). Todos os animais dos grupos experimentos foram ainda distribuídos em quatro subgrupos de acordo com o seu tempo de coleta em: 0 h, 2 h, 6 h e 12 h. Foram avaliados a viabilidade celular a partir da reação com Methyl Thiazolyl Blue (MTT), além das dosagens de lactato e de alanino amino-transferase (ALT). **Resultados** - Em relação à dosagem de lactato, foi possível observar relativa melhora das soluções hipertônicas em comparação à eurocollins, sendo que em 12 h, o GE e o GS não apresentaram diferença estatisticamente significante ($p > 0,05$). Quando avaliada a viabilidade celular, as absorbâncias ao MTT também demonstraram resultados favoráveis ao GS, visto que, não apresentou diferença estatística em relação ao GE. **Conclusão** - A solução de NaCl 7,5% apresentou resultados mais promissores para preservação de órgãos com capacidade de preservação comparável à solução de eurocollins.

INTRODUCTION

The success of a transplant has as primary factor the organ viability¹⁶. The present difficulties are not based on surgical technique, but in the preservation and handling of the procedure¹².

The preservation is an update issue, since the hepatic ischemia syndrome and reperfusion, trigger the release of oxygen free radicals and inflammatory reaction mediated by Kupffer cells, leading to necrosis, apoptosis, microcirculation failure and parenchymal sinusoidal endothelium injury, culminating in graft loss or reduction of its function¹⁷.

The basic process involves organ removal, solution preservation for storage, temperature and time of organ function, excluding contamination throughout the process¹². Variations in the methods of preservation involve the temperature (cryopreservation normothermia and hypothermia), the solution components and different regimes of organ perfusion in the presence or absence of oxygen².

Aiming to improve methods for preservation, numerous investigators have devoted themselves to this issue¹, with emphasis to increase storage time⁸. The first major step in the study of solutions of conservation took place in 1987 when Folkert Belzer in University of Wisconsin presented solution called the University of Wisconsin^{8,19}. Later it was named Viaspan[®]. This solution would preserve the liver for a period up to three times greater than that of the previous solutions by passing the cold ischemia time of eight to 24 h without affecting significantly the organ viability⁷. Although clinical success, several other alternatives have been introduced.

For many doctors the solution University of Wisconsin is considered the gold standard for organ preservation. However, it has disadvantages due to its high viscosity, reducing the capacity of penetration into the microcirculation and bile ducts and particularly its high cost^{18,20}.

From existent solutions, the eurocollins has been for a long time the most accessible. Although not presenting viscosity as the others, it has good capacity to reduce the liver surface temperature, with good preservation in experimental studies¹³.

When formulating new solutions to prevent tissue acidosis, interstitial edema, damage by free radicals and depletion energy phenomena triggered by the process of ischemia and subsequent reperfusion must be in mind¹¹.

Of these, the colloid-osmotic action is presented as an important prognostic factor of organ viability, since the solution diffuses rapidly

into the interstitial space, leading to tissue edema and microcirculation irrigation difficulties. Therefore, the preservation solution should contain a substance with a colloid-osmotic interstitial space preventive expansion, and subsequent cellular swelling, proving the advantage of the use of hypertonic solutions¹². Studies on preservation solutions are still far from reaching the "ideal solution". Currently, success is reached on the use of hypertonic solutions in the liver preservation⁹.

The objective of this study is to compare solutions of 7% hypertonic NaCl, 50% glucose and 25% mannitol as cost-effective alternative solutions for liver preservation instead of eurocollins.

METHOD

A hundred and five Wistar rats (*Rattus norvegicus*) weighing 210-250 g from the Instituto Evandro Chagas (Belém, PA) were used. The animals were adapted to the Laboratory of Experimental Surgery, University of Pará (UEPA), Belém, Brazil, for 15 days before the experiment receiving water and food ad libitum, and maintained under temperature and light control.

The animals were randomly divided into six groups, namely: 1) Standard Group (GP) with five animals that were used to verify the normal range of biochemical and cell viability study; 2) Eurocollins Group (EG) with 20 animals, using eurocollins solution for liver preservation; 3) Group Saline (GF) with 20 animals, using saline 0.9% solution; 4) Glucose Group (GG) with 20 animals using solution with 50% of glucose, 5) Mannitol Group (GM) with 20 animals using 20% of mannitol solution; 6) Group Salty (GS) with 20 animals using solution of NaCl 7.5%.

All groups, except the GP, were divided into four sub-groups, with five animals each, differentiated by the time of organ collection, among zero, two, six and 12 hours; the GP was only evaluated at 0 h. In the research protocol was adopted anesthetic combination of ketamine hydrochloride and xylazine intraperitoneally, used at doses of six and 60 mg / kg, respectively.

After trichotomy of thoracoabdominal region, was initiated a transverse incision in the abdominal region, at the level of the costal margin, with extension to the thoracic region, followed by location, dissection and splenic artery ligation. Identification, dissection and false ligation of inferior vena cava were performed, followed by dissection and ligation of mesenteric vein. Dissection of the portal vein was done, with false ligation of the caudal and cephalic portions.

Hemi-section of the portal vein with the aid of iris scissors was done. This vein was cannulated

with venous access catheter 20 in the cephalic-caudal direction, which was fixed with the same thread of false ligation previously performed. To this catheter was connected the test solution at 4°C, with continuous perfusion pressure of 60 cm H₂O. The first solution used to rinse the liver in all groups, except the GP, was 0.9% NaCl, followed by infusion of the solution related to each study group.

Immediately after perfusion with the test solution, the liver was isolated and removed from its natural position. Three fragments of 1x1 cm, weighing approximately 200 mg - one for the study of cell viability, the second to lactate and the third for alanine aminotransferase (ALT) dosage - were removed. Fragments of groups 2, 6 and 12 hours were stored in the study solution for further analysis. The fragments 0 h were immediately sent for analysis. After the uptake of the liver, the animals were euthanized.

For measurement of lactate and transaminase, 200 mg liver parenchyma was collected, macerated in a test tube with 2 ml of distilled water for 15 seconds, microfiltered with microfilter of 0.22 A and packed respectively in Vacutainer® and test tube containing 2 ml of macerate in each. The lactate and transaminase dosage were performed at the Laboratory of Biochemistry, University of Pará, according to the standardization of kits lactate (Katal®) and ALT (Katal®). To check the cell viability was standardized removal of a segment of 200 mg of the median lobe of the liver on the right side of the median fissure. Each piece was introduced in Eppendorf test tube with 1.5 cc containing 1100 microliters (ul) of phosphate buffered saline (PBS) pH 7.2, to which were added 105 µl of solution of Methyl Thiazolyl Blue (MTT) (Sigma) prepared by adding 0.005 g of MTT salt in 1 ml of PBS.

The test tubes were kept in a water bath at 37°C for two hours. At the end of this period was added 800µl of isopropyl alcohol (isopropanol) in a test tube and waited five minutes to tissue digestion in order to perform maceration and homogenization of the solution with electrical homogenizer (T-8 ika®) pipe assay. After 30 minutes of addition of isopropyl alcohol, 2 ml of the solution were taken with a spectrophotometer (Espec®20 mVolts) calibrated at a wavelength of 570 nm. A total of five measurements were recorded in the protocol. The arithmetic mean was adopted as the result of absorbance in each animal.

The results were evaluated using the normality test of Kolmogorov-Smirnov test to examine the Gaussian distribution of the samples, and ANOVA followed by Tukey correction for variance analysis; significance was at $p < 0.05$.

RESULTS

According to liver lactate levels at time 0 h was only significant the difference between the GE (-0.2 ± 0.14) and GP (0.68 ± 0.10). However, after 2 h in conservation, significant ($p < 0.05$) improvement happened with other solutions. All groups except the GF showed statistically lower lactate levels compared to GP. At the time of 6 h, the animals of GG and GS were statistically different to GP. The other groups had similar results ($p > 0.05$). After 12 h all solutions showed beneficial effects with lower levels of lactate compared to GS ($p < 0.05$). The GE and GS had no statistically significant difference ($p > 0.05$).

TABLE 1 - Liver lactate levels at different time periods

Group/Analysis time	0 h *	2 h **	6 h ***	12 h #
Eurocolins	-0,2	-0,04	-0,16	-0,74
SF 0,9%	0,27	0,06	0,22	0,12
Glucose	-0,04	-0,68	-0,34	-0,04
Mannitol	-0,06	-0,2	0,08	-0,18
Hypertonic	-0,02	-0,24	-0,24	-0,32
Standart	0,68	0,68	0,68	0,68

Fonte: Research Protocol

* $p < 0,05$ (ANOVA) Eurocolins x Standart Group

** $p < 0,05$ (ANOVA) Standart Group X eurocolins; glucose; mannitol and hypertonic

*** $p < 0,05$ (ANOVA) Standart Group X glucose and hypertonic

$p < 0,05$ (ANOVA) Standart Group X eurocolins; glucose; mannitol; SF 0,9 and hypertonic

With respect to ALT levels, the GF presented transaminase levels high during all times of preservation, when compared to other solutions. The lower ALT levels was observed in GE.

In the mean absorbance of MTT by hepatic mitochondria, it could be seen basically the same pattern found in many other markers in the analysis. At time 0 h, only the GM showed statistically superior to the GP, but when assessed after effect of these solutions, in time of 2 h, was found that the GP showed statistically different compared to GS and GP ($p < 0.05$). All other groups showed no statistically significant difference. At the time of 6 h all groups were similar ($p > 0.05$); however, when posterior effect was checked after 12 h it was observed that the 7.5% NaCl solution was statistically superior to GP.

TABLE 2 - Mean absorbance of MTT by hepatic mitochondria in different time periods

Group/Analysis time	0 h *	2 h **	6 h	12 h ***
Eurocolins	0,446	0,353	0,201	0,374
SF 0,9%	0,301	0,960	0,233	0,339
Glucose	0,209	0,454	0,364	0,499
Mannitol	0,747	0,415	0,583	0,534
Hypertonic	0,337	0,531	0,658	0,658
Standart	0,122	0,122	0,122	0,122

Fonte: Research Protocol

* $P < 0.05$ (ANOVA) Standard X mannitol

** $P < 0.05$ (ANOVA) Standard X eurocolins

*** $P < 0.05$ (ANOVA) Standard X hypertonic

DISCUSSION

The preservation of the tissue has been shown to be a determining factor in the success of liver transplants. In this regard, various pharmacological agents have been tested to offer greater protection against the lesions triggered during ischemia³. Several preservation solutions have been proposed as a way to avoid deleterious effects of normothermic ischemia edema preventing cellular damage caused by free radicals, and energy depletion, resulting in irreversible damage with consequent loss of organ function¹³. However, the ideal solution is still far from being discovered, indicating the need for more research¹².

For the purposes of preserving the Solution of the University of Wisconsin is considered by many to be the most effective in transplants. However, it still has some disadvantages to the body, besides being difficult to acquire due to its high cost⁶. Therefore, it is of great importance to study alternative preservation solutions that can provide efficiencies similar to it, but more affordable. Thus, appear as options the hypertonic solutions of NaCl (7.5%), glucose (50%) and mannitol.

One of the parameters used to infer the ability of various preservation solutions was the intracellular lactate indicating, indirectly, the lactic acid production due to a process of anaerobic respiration in ischemic conditions. The structural changes that accompany cellular ischemia are well known to affect mitochondria, nucleus, endoplasmic reticulum, lysosomes, and finally the cell membranes, which harbor the molecular changes underlying ischemic cause^{7,19}. In analyzing the effect of different solutions in times of evaluation (0, 2, 6, 12 h) in liver preservation, the solution eurocollins (GE) and NaCl (7.5%) showed the highest indices of lactate (negative) and may indicate, since very early periods, beneficial feature of the solutions in maintaining low levels of lactate in the intracellular environment. This fact can be explained by the decrease in arterial pH and loss of bases observed directly after reperfusion, probably triggered by the onset of anaerobic glycolysis due to oxygen deficiency. Whereas acidic pH can protect against cell death by anoxia, ischemic reperfusion cell in acidotic pH leads to severe cell death (pH paradox)^{6,10}.

In contrast, the GF answered negatively relative to lactate levels and preservation with saline (0.9%) due to their character hypotonic in hepatic acidosis, leading to edema⁵. Solutions like physiologic solution and ringer lactate are crystalloids without any additional drug. The buffering capacity in general does not lead to

good response preservation solutions, except for purpose of hypothermic perfusion in order to cool the hepatic tissue metabolism and reducing the need for oxygen. Kept at 28° C, cell metabolism and oxygen demand is still approximately 50% of normal function².

Isotonic crystalloid solutions, whose chemical compositions are incompatible with the preservation of organs, have no buffering capacity, energy supplements and additives, membrane stabilizers and mainly become hypotonic compared with organs subjected to oxidative stress, leading to intensify tissue process of "swelling"⁵.

Regarding the levels of AST groups presented with very heterogeneous and disparate results with the literature. This fact has been observed in the study by Siqueira et al.¹⁵ showing marked change in behavior in observation times. Failing to have statistical analysis of this group, these results were not considered relevant for this search.

In order to estimate the rate of cell viability, colorimetric method was used to evaluate the survival of cells after a noxious event such as ischemia. It is a salt (Methyl Blue-Thiazolyl C₁₈H₁₆N₅SBr) that detects only metabolically active cells due to its mechanism acting on the mitochondrial structure⁵. Based on this parameter was observed that in the initial days, the GE presented itself with better rates of cell viability, agreeing well with data found in the intracellular lactate. This data could suggest good performance with solution EC, especially in the early hours of ischemia, with gradual decrease in their ability to preserve. Indeed, suggested by Ochoa et al.¹⁴, preservation period of more than six hours of ischemia, supports the hypothesis that the solution of eurocollins should be used for "flushing" the livers in situ, prior to use of another preservative solution more effective by or, for preservation in reduced times¹⁴.

In general, the parameters to evaluate the solutions in the early stages of ischemia show poor outcome, suggesting that hypothermia in itself, since the beginning of this process, is an aggressive factor to liver tissue, as noted by Smith et al¹⁹ and lead to small changes in the biochemical and enzymatic liver.

CONCLUSION

It is promising the use of 7.5% NaCl as a preservation solution for organs; it is effective and has characteristics to prevent further damage to the organ during uptake till inclusion on the recipient.

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