

Comparison of the solution of histidine-tryptophan-alfacetoglutarate with histidine-tryptophan-glutamate as cardioplegic agents in isolated rat hearts: an immunohistochemical study

Comparação da solução de histidina-triptofano-alfacetoglutarato com histidina-triptofano-glutamato como agentes cardioplégicos em corações isolados de ratos: estudo imuno-histoquímico

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Abstract

Introduction: Cardiac arrest during heart surgery is a common procedure and allows the surgeon to perform surgical procedures in an environment free of blood and movement. Using a model of isolated rat heart, the authors compare a new cardioplegic solution containing histidine-tryptophan-glutamate (group 2) with the histidine-tryptophan-alfacetoglutarate (group 1) routinely used by some cardiac surgeons.

Objective: To assess caspase, IL-8 and KI-67 in isolated rat hearts using immunohistochemistry.

Methods: 20 Wistar male rats were anesthetized and heparinized. The chest was opened, cardiectomy was performed and 40 ml/kg of the appropriate cardioplegic solution was infused. The hearts were kept for 2 hours at 4°C in the same solution, and thereafter, placed in the Langendorff apparatus for

30 minutes with Ringer-Locke solution. Immunohistochemistry analysis of caspase, IL-8, and KI-67 were performed.

Results: The concentration of caspase was lower in group 2 and KI-67 was higher in group 2, both $P < 0.05$. There was no statistical difference between the values of IL-8 between the groups.

Conclusion: Histidine-tryptophan-glutamate solution was better than histidine-tryptophan-alfacetoglutarate solution because it reduced caspase (apoptosis), increased KI-67 (cell proliferation), and showed no difference in IL-8 levels compared to group 1. This suggests that the histidine-tryptophan-glutamate solution was more efficient than the histidine-tryptophan-alfacetoglutarate for the preservation of hearts of rat cardiomyocytes.

Descriptors: Heart Arrest, Induced. Myocardial Ischemia. Heart.

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Abbreviations, acronyms and symbols

ATP	Adenosine triphosphate
BSA	bovine serum albumin
MOD	mean optical density
HTG	histidine-tryptophan-glutamate
HTK	histidine-tryptophan-ketoglutarate
IL	Interleukin
IP	Intraperitoneal
AU	Arbitrary units

Resumo

Introdução: A parada do coração durante a cirurgia cardíaca é procedimento comum e permite que o cirurgião realize os procedimentos cirúrgicos em ambiente isento de sangue e movimento. Os autores comparam, em modelo de coração isolado de rato, uma nova solução cardioplégica com histidina-triptofano-glutamato (grupo 2) com a histidina-triptofano-alfacetoglutarato (grupo 1) já utilizada de rotina por alguns cirurgiões cardíacos.

Objetivo: Avaliar por análise imuno-histoquímica a caspase, a IL-8 e KI-67 em corações isolados de ratos.

Métodos: 20 ratos machos de raça Wistar foram anestesiados e heparinizados. O tórax foi aberto, realizado cardiectomia e infundido 40 ml/kg de solução cardioplégica apropriada. Os corações foram mantidos por 2 horas na mesma solução a 4°C e, após esse período, colocados em aparato de Langendorff por 30 minutos com solução de Ringer Locke. Foram feitas análises imuno-histoquímicas para caspase, IL-8 e KI-67.

Resultados: A concentração de caspase estava menor no grupo 2 e da KI-67 estava mais elevada no grupo 2, ambos com $P < 0,05$. Não houve diferença estatística entre os valores de IL-8 entre os grupos.

Conclusão: A solução com histidina-triptofano-glutamato foi melhor que a com histidina-triptofano-cetoglutarato, pois reduziu a caspase (apoptose), aumentou o KI-67 (proliferação celular) e não apresentou valores diferentes de IL-8 (inflamação e necrose) que no grupo 1. Isso sugere que a solução histidina-triptofano-glutamato foi mais eficiente que a histidina-triptofano-cetoglutarato na preservação dos cardiomiócitos dos corações de ratos.

Descritores: Parada Cardíaca Induzida. Isquemia Miocárdica. Coração.

INTRODUCTION

Induction of temporary arrest of the heart during cardiac surgery is a relatively common procedure that allows the surgeon to perform procedures in an environment free of blood and movement [1-3]. One of the cardioplegic agents is histidine-tryptophan-ketoglutarate (HTK) solution.

The HTK was tested by Bretschneider et al. [4] in Germany, 1975. Its mechanism of action comes from the absence of calcium, which prevents its influx into the cell by type "L" calcium channel in the plateau phase of the potential action, inhibiting the release of calcium from the sarcoplasmic reticulum over the myocyte, resulting in inactivation of myofilaments [5,6].

This mechanism is complemented by cellular protection given by the constituents of this solution, whose main functions include: 1 - histidine: temperature-dependent buffer system, inhibitor of matrix metalloproteinases and cell impermeant [7] 2 - tryptophan: acts in maintaining the integrity of cell membrane [8], and 3 - ketoglutarate: improves maximum developed pressure and prevents increased creatine kinase MB fraction [8].

According Pisarenko et al. [9], the substitution of *alpha*-Ketoglutaric acid by glutamate bring some advantages

to myocyte: the decrease in lactate and raising the pH in the mitochondrial matrix, even in ischaemia, avoiding intracellular acidosis and edema, and contributing to the maintenance of intracellular adenosine triphosphate (ATP), protecting the myocyte of ischemia - reperfusion lesion.

In turn, the reduction of reperfusion injury cause decrease of caspase [10-12] and IL- 8, due to the reduction in cellular apoptosis and necrosis, respectively [13,14]. Nevertheless, the reduction of reperfusion injury may not be acting alone on behalf myocyte. Proliferative proteins such as KI- 67, could be re-coded, thus contributing to the reduction of the cell death and formation of new myocardial fibers [15,16]. This study assess HTG solution as a cardioplegic agent in isolated rat heart, considering immunohistochemical analysis of caspase markers, IL-8 and KI-67.

METHODS

After approval by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of São José do Rio Preto (authorization number 015/2012), 20 male Wistar rats (10 in each group) were used, weighing 280±29 grams.

All animals received care according to the recommendations of the Committee on Care and Use of Laboratory An-

imals - Institute of Laboratory Animal Resources (ILAR) -
 National Research Council, United States [17].

Experimental Protocol

The animals were anesthetized with an injection of 65 mg/kg intraperitoneal sodium pentobarbital and received IP systemic heparin (500 IU/kg). After opening the chest, cardiectomy was performed. Hearts received Ringer's lactate solution to "wash" the coronary tree and then cardioplegic solution according to the corresponding group.

The hearts in this phase of the experiment were divided into 2 groups. In group 1, was used HTK solution at 4°C and in Group 2, solution of histidine-tryptophan-glutamate (HTG) at 4°C. Table 1 shows the composition of each solution. In all cases, the infusion of cardioplegia was performed as a single dose 40 ml/kg at the aortic root, followed by immersion of the organ in the same solution for 2 hours at 4°C.

After this time, the hearts were placed in a Langendorff system and perfused with oxygenated Locke Ringer buffer under normothermy and constant pressure of 100 cm H₂O for gravitational method for 30 minutes. The drainage of the right ventricle was performed by opening the pulmonary artery, and the right atrium was maintained intact in order to preserve the sinus node [18].

Three threads of epicardial pacemaker were inserted at equidistant points of the ventricles for electrocardiographic documentation of cardiac events. The time of onset of ventricular fibrillation and the first heartbeat counted from the start of infusion of Ringer Locke solution was noted.

After 30 minutes of infusion of Ringer Locke, the experiment was discontinued. The hearts were removed from the Langendorff system and fragments of the cardiac apex, which were stored in sterile Falcon type tubes containing 10% formalin for subsequent histological and immunohistochemical preparation.

Table 1. Composition of solutions used.

Substance	HTK (g/L)	HTG (g/L)
Sodium chloride	0.8766	0.8766
Potassium chloride	0.671	0.671
Magnesium chloride	0.8132	0.8132
Calcium chloride	0.0022	0.0022
Potassium-hydrogen-2-ketoglutarate	0.1842	---
Glutamate	---	0.1842
Histidine	27.9289	27.9289
Histidine chloride, H ₂ O	3.7733	3.7733
Tryptophan	0.4085	0.4085
Mannitol	5.4651	5.4651
Water for injection	a 1000 ml	a 1000 ml

HTK: Histidine-tryptophan ketoglutarate; HTG: histidine-tryptophan-glutamate

Histological and immunohistochemical technique preparation

Initially, the material was embedded in paraffin, a procedure that provides resistance allow for cutting thickness of 3µm and placed on silanized slides. The silanization of the blades consisted in preparing these with an adhesive that fixes the fragment to the blades, preventing their detachment during the immunohistochemical procedure. For this, they were immersed in acetone P.a. (2 minutes), 4% silane solution diluted with acetone (2 minutes) and again in acetone P.a. (4 to 5 dips). The drying of the slides was performed in an oven at 60°C.

The block was attached to the microtome, the slice thickness was set to 3 µm and the cuts placed on silanized identified and left in an oven at 60°C for 24 hours. The blade went through the process of deparaffinization in xylene, followed by hydration in absolute alcohol I, II and III, finishing with six dives in tap water, incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase.

Antigen retrieval was performed in the steamer with specific buffer for each antibody for 30 minutes (Table 2). Then the slides were covered up with a solution containing fetal bovine serum (BSA) and incubated with the primary antibody.

Table 2. List of antibodies used.

Antibody	Specificity	Dilution	Buffer	Lab
anti-Ki-67	Monoclonal	1:200	Citrato pH6	Biocare Medical
anti-Caspase 3	Polyclonal	1:1000	Citrato pH6	Abcam
anti-IL-8	Monoclonal	1:50	Citrato pH6	Santa Cruz

After this step, the slides were washed in PBS solution and incubated for 15 minutes with Starr Trek Universal HRP Detection kit (Biocare Medical®), which consisted in secondary antibody biotinylated for 1 hour and streptavidin-peroxidase complex for 30 minutes, followed by washing with PBS for 15 minutes. The revelation was performed with substrate chromogen (DAB Betazoidchromogen) of the Starr Trek Universal HRP Detection kit (Biocare Medical®) for 2 to 5 minutes, and counterstained with Harrys hematoxylin for 40 seconds. The tissues were dehydrated in alcohol in ascending degree and bathed in xylene before mounting the slides on ERV-MOUNT amid (Erviagas®).

Negative control reactions were obtained by omitting the primary antibody. Tonsil tissue was used for Ki-67 reactions and Caspase 3 and as positive control breast tissue for IL-8 reaction.

Quantification of immunohistochemical staining

Slides were photographed and quantified by enzyme AxioVision software on X40 magnification microscope Zeiss Axioskop 2. For each sample, three regions of cardiac tissue and 20 points of myocardial cell were marked in each region were selected. Thus, 60 different points on each sample were assessed by obtaining the average relative intensity of immunoreactivity. The values were obtained in arbitrary units

(AU). The average optical density (AOD) was obtained with the aid of the following formula:

$$\text{AOD} = 255 - \text{AU}$$

This formula showed the intensity of immunostaining specifically in immunoreactive areas.

Statistical Analysis

The data were subjected to the Kolmogorov-Smirnov test and subsequently the parametric analysis by unpaired Student's t test or non-parametric by Mann-Whitney test when appropriate, and Fisher's exact test for categorical data. Results were expressed only in mean \pm standard deviation due to the fact all variables behave as continuous quantitative with Gaussian distribution. *P* values were presented, and those who were less than 0.05 were considered significant. The GraphPad InStat and Prism 6.0 softwares of statistical analysis, both for Windows® were used.

RESULTS

The average weight of the animals was 277.4 ± 24.6 g (group 1) and 288 ± 34.5 g (group 2), respectively, with no significant difference between groups ($P=0.4396$). Regarding the average volume of Ringer Locke collected from coronary sinus after 30 minutes (363.1 ± 177.3 ml and 277.4 ± 33.7 ml, respectively), there was no significant difference between groups ($P=0.1923$).

Findings during perfusion with cardioplegic solution and Ringer Locke

All hearts showed adequate perfusion of cardioplegia and Ringer Locke, evidenced by clear staining in the ventricular wall. The average heart rate after 5 minutes of perfusion (233 ± 36 and 188 ± 53.4 beats per minute, respectively), showed a significant difference ($P=0.0086$). The time of onset of ventricular fibrillation (49 ± 28.2 and 45 ± 17 seconds, respectively) and time to first heartbeat (153 ± 78 and 117 ± 96.8 seconds respectively) showed no significant difference ($P=0.5869$ and $P=0.187$, respectively).

Immunohistochemical findings

After 2 hours of ischemia and 30 minutes of reperfusion, caspase activity was significantly lower in group 2 ($P<0.0001$), the activity of KI-67 was higher in group 2 ($P<0.0001$) and IL-8 was not different between groups (Figure 1).

DISCUSSION

Myocardial ischemia causes various cardiac effects, such as decreases force of contraction; increases diastolic pressure, indicating contraction of myofibrils in isovolumic conditions, causes a decline in phosphocreatine and ATP; de-

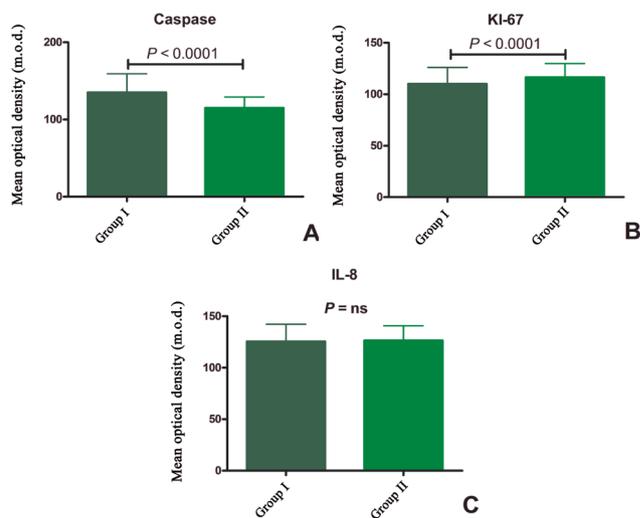


Fig. 1 - Histograms showing mean optical densities of: (A) caspase, (B) KI-67 and (C) IL-8. Group 1: solution with histidine-tryptophan-ketoglutarate, Group 2: solution with histidine-tryptophan-glutamate

creases contraction, and glutamate and aspartate; increases lactate, pyruvate, alanine and succinate [9]. According Pisarenko et al. [9] the addition of glutamate in the perfusate keeps the intracellular ATP and decreases both lactate and pyruvate as that contribute to acidosis. These effects contribute to improve cardiac function recovery after ischemia. Our results show similar behavior in the two solutions studied concerning the time and duration of ventricular fibrillation first beat, however, it was better for group 2 concerning heart rate, which was lower, which can be correlated with lower acidosis, probably myocyte.

Another process that is intrinsically related to ischemia-reperfusion injury is apoptosis [10,11], characterized by morphological changes such as chromatin condensation, fragmentation of nuclei and formation of "apoptotic bodies". These changes are made by a family of proteases called caspases [12]. The degree of caspase activation is directly related to the degree of apoptosis, which plays a critical factor in the recovery of cardiac function [13].

Our results demonstrate less caspase activity in group 2, suggesting a potential protective for myocardial function.

In contrast to apoptosis, necrosis is an irreversible process of cell death due to the breakdown in cellular homeostasis. There is disruption of the cell membrane, with leakage from the cytosol to the extracellular medium, leukocyte margination and activation of the inflammatory cascade [13,14]. Anselmi et al. [14] have described IL-8 peak at 35 minutes of reperfusion and IL-6 in 75 minutes. Lee et al. [13] stated that the HTK solution inhibits increase of interleukin. Thus, as in this study there was no significant difference in IL-8 between groups 1 and 2, and we can infer that the anti-inflammatory

protection provided by HTG solution was not different from that given by the HTK solution.

Mammalian hearts have low proliferative capacity after birth. One of the markers used to assess cell proliferation is the Ki-67 [15]. With this marker, Walsh et al. [16] demonstrated that 12% to 23% of fetal rat cardiomyocytes exhibit proliferative activity, from 1% to 8% up to the 7th day and virtually undetectable from the 14th day. In our study, there was a significant increase of the KI-67 in group 2, demonstrating early proliferative activity of HTG solution guaranteed with 2 hours of ischemia.

Associated with this, Walsh et al. [16] also draw the attention to the KI-67 activity also be inversely related to apoptosis, which is also confirmed in our study, in which caspase is lower in group 2 than in 1. However, the increased activity of this marker is worrisome because it has been associated with myxomas [19,20] and cardiac sarcomas [21].

Although the results obtained here are consistent with the literature, they are not definitive regarding the replacement of *alpha*-Ketoglutaric acid by glutamate. Quantitative analysis with ATP and other nuclear markers for cell proliferation should be used to target a more comprehensive and safe conclusion. Another relevant aspect is the concentration of glutamate. Would have the same protective effect on the heart if we change its concentration? Further studies are needed to answer these questions.

CONCLUSION

Immunohistochemical analysis of replacement of *alpha*-Ketoglutaric by glutamate in cardioplegia with histidine and tryptophan showed that heart muscle cells showed no greater incidence of necrosis, since when measuring IL-8 they presented a lower incidence of apoptosis, confirmed by the lower values of in group 1 and caspase best proliferative activity, with higher values of Ki-67 compared to group 1. This suggests that HTG solution was more efficient than the HTK in preserving cardiomyocytes of rat hearts.

Authors' roles and responsibilities	
MABO	Study design, execution of experiments, analysis of results and writing of the manuscript
LCF	Assistance in immunohistochemical techniques
DAPCZ	Assistance in immunohistochemical techniques
ACB	Participation in the preparation of the final text
CAS	Participation in the preparation of the final text
PHHB	Participation in drafting the final text
OP	Review of the version
DMB	Study design, analysis of results and writing of the manuscript

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