

Comparison of fractal dimension and Shannon entropy in myocytes from rats treated with histidine-tryptophan-glutamate and histidine-tryptophan cetoglutarate

Comparação da dimensão fractal e entropia de Shannon em miócitos de ratos submetidos à cardioplegia utilizando solução histidina-triptofano-cetoglutarato e histidina-triptofano com glutamato

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Abstract

Introduction: Solutions that cause elective cardiac arrest are constantly evolving, but the ideal compound has not yet been found. The authors compare a new cardioplegic solution with histidine-tryptophan-glutamate (Group 2) and other one with histidine-tryptophan-cetoglutarate (Group 1) in a model of isolated rat heart.

Objective: To quantify the fractal dimension and Shannon entropy in rat myocytes subjected to cardioplegia solution using histidine-tryptophan with glutamate in an experimental model, considering the caspase markers, IL-8 and KI-67.

Methods: Twenty male Wistar rats were anesthetized and heparinized. The chest was opened, the heart was withdrawn and 40 ml/kg of cardioplegia (with histidine-tryptophan-cetoglutarate or histidine-tryptophan-glutamate solution) was infused.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 min with Ringer-Locke solution. Analyzes were performed for immunohistochemical caspase, IL-8 and KI-67.

Results: The fractal dimension and Shannon entropy were not different between groups histidine-tryptophan-glutamate and histidine-tryptophan-cetoglutarate.

Conclusion: The amount of information measured by Shannon entropy and the distribution thereof (given by fractal dimension) of the slices treated with histidine-tryptophan-cetoglutarate and histidine-tryptophan-glutamate were not different, showing that the histidine-tryptophan-glutamate solution is as good as histidine-tryptophan-cetoglutarate to preserve myocytes in isolated rat heart.

Descriptors: Heart Arrest, induced. Apoptosis. Myocardial Ischemia.

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Abbreviations, acronyms & symbols

HTG	Histidina-triptofano-glutamato
HTK	Histidina-triptofano-cetoglutatarato
IP	Intraperitoneal

Resumo

Introdução: As soluções que provocam parada cardíaca eletiva estão em constante evolução, porém, o composto ideal ainda não foi encontrado. Os autores comparam uma nova solução cardioplégica com histidina-triptofano-glutamato (Grupo 2) com histidina-triptofano-cetoglutatarato (Grupo 1) em modelo de coração isolado de rato.

Objetivo: Quantificar a dimensão fractal e entropia de Shannon em miócitos de rato submetidos à cardioplegia utilizando solução histidina-triptofano com glutamato em modelo experimental, considerando-se os marcadores caspase, IL-8 e Ki-67.

Métodos: Vinte 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 imunohistoquímicas para caspase, IL-8 e KI-67.

Resultados: A dimensão fractal e a entropia de Shannon dos corações submetidos à parada cardíaca eletiva nos grupos 1 e 2 não foram diferentes.

Conclusão: A quantidade de informações avaliada pela entropia de Shannon e a distribuição das mesmas (dada pela dimensão fractal) nas lâminas de coração de rato submetidas à cardioplegia com solução histidina-triptofano-acetoglutatarato ou histidina-triptofano-glutamato não foram diferentes, o que mostra que a solução de histidina-triptofano-glutamato é tão boa quanto a histidina-triptofano-cetoglutatarato na preservação dos miócitos em modelo de coração isolado de rato.

Descritores: Parada Cardíaca Induzida. Apoptose. Isquemia Miocárdica.

INTRODUCTION

During cardiac surgery it is usual the temporary arrest of the heart, allowing the surgeon to perform the surgery within the cardiac cavities environment free of blood and movement. Before there was any solution that produces safe cardiac arrest, it was by Gibbon in 1953 the merit of using a technique described by Senning in experimental atrial septal defect closure in dogs using ventricular fibrillation^[1].

In 1955, Melrose et al.^[2] introduced the concept of chemical stopping using solution containing 2.5% potassium citrate, which depolarizes the cell membrane and the conduct of the action potential. However, the high concentration of potassium caused focal myocardial necrosis and death in many patients, resulting in discontinuation of hyperkalemic cardioplegia as protective solution for nearly 20 years. In the mid 70s, alternative cardioplegia containing less potassium than Melrose's solution were successfully introduced as the solution of St. Thomas^[3], low-volume blood cardioplegia^[4,5] and solution of histidine-tryptophan-ketoglutarate (HTK)^[6]. Despite these advances, the ideal cardioplegic solution has not yet been developed^[7,8].

The substitution of glutamate ketoglutarate has impact still uncertain on the behavior of the myocardial muscle, but it will be an effective way to indirectly assess their incorporation into the Krebs cycle, removing the mitochondrial pyruvate, thus preventing acidosis which is notoriously damaging both to the cell as their enzymatic mechanisms^[9].

Cardioplegic solutions with low calcium concentration as the HTK can cause the so-called "calcium paradox", destabilizing the cell membrane, which culminates in necrosis, leukocyte margination and apoptosis^[10]. Histological changes induced by cardioplegic solutions could generate change in the amount and distribution of the information contained on the blade. It is well known that tissue structural changes can be quantified by the fractal dimension and Shannon entropy^[11,12].

The analysis of fractal dimension and Shannon entropy have been recently used in several areas of medicine such as cardiology, neurology, ophthalmology and radiology^[11,13] and are useful in characterizing irregular and complex^[11,13,14] structures. Using fractal analysis, Arruda et al.^[11] and Douglas et al.^[12] correlated the degree of dedifferentiation and tumor invasiveness in prostate cancer and degree of rejection of cardiac tumors, respectively.

This study aims to assess if the study solution histidine-tryptophan-glutamate (HTG) is better than HTK (standard solution) by fractal dimension and Shannon entropy in rat myocytes, considering the caspase markers, IL-8 and KI-67.

METHODS

After approval by the Research Ethics Committee on Animal Experimentation of the Faculty of Medicine of São José do Rio Preto (Protocol 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 Animals - Institute of Laboratory Animal Resources (ILAR) - National Research Council, United States^[15].

Experimental Protocol

The animals were anesthetized with an injection of 65 mg/kg intraperitoneal (IP) of sodium pentobarbital and received 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 their group.

The hearts in this phase of the experiment were divided into two groups. Group 1 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 taken 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 Locke Ringer buffer oxygenated normothermic solution and a 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 preserved the right atrium in order to preserve the sinus node^[16].

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. H2O	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 ketogluturate; HTG: histidine-tryptophan-glutamate

Three threads of epicardial pacemaker were inserted at equidistant points from the ventricles to the 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 solution, the experiment was discontinued. The hearts were removed from the Langendorff system and fragments of the cardiac apex, which were stored in sterile Falcon tubes containing 10% formalin for subsequent histological and immunohistochemical preparation.

Histological and immunohistochemical technical preparation

Initially, the material was embedded in paraffin, a procedure that provides resistance allowing for cutting thickness of 3 m and placed on silanized slides. The silanization of the blades consisted in preparing these with an adhesive fixing the fragment to the blades preventing their detachment during the immunohistochemical procedure. For this, they were immersed in acetone PA (2 minutes), 4% silane solution diluted with acetone (2 minutes) and again in acetone PA (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 m and the cuts placed on silanized blade 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.

After this step, the slides were washed in PBS and incubated for 15 minutes with Starr Trek Universal HRP Detection kit (Biocare Medical®), which consisted in biotinylated secondary antibody for 1 hour and streptavidin-peroxidase complex for 30 minutes, followed by washing with PBS for 15 minutes. The revelation was performed with substrate

Table 2. Relation of antibodies used

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

chromogen (DAB Betazoidchromogen) Starr Trek Universal HRP Detection kit (Biocare Medical®) for 2 to 5 minutes and counter-stained with Harrys hematoxylin for 40 seconds. The tissues were dehydrated in alcohol and bathed in ascending degree in xylene before mounting the slides in ERV-MOUNT amid (Erviogas®).

Negative control of 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.

Slides were photographed and enzyme quantified by AxioVision software on X40 magnification Axioskop 2 Zeiss microscope. For each sample, three regions of cardiac tissue were selected.

Fractal dimension and Shannon entropy

The photographed slides were then binarized for reading of fractal dimension and Shannon entropy. They were estimated by the Box-counting method with the aid of the ImageJ software of the US National Institute of Health (NIH), widely used in the literature and available for free on the Internet (<http://rsbweb.nih.gov/ij/>).

This program considers the Box-counting in two dimensions, allowing the quantification of the distribution of pixels in space, thus not considering the image texture. The influence of this is that two images with the same distribution of pixels, a binarized and one in gray levels, possess the same DF. With this, the DF calculated with ImageJ will be always between 0 and 2, not distinguishing different textures.

Statistical Analysis

The data were subjected to the Kolmogorov-Smirnov test and subsequently the parametric analysis by Student's t test or nonparametric Mann-Whitney test and Fisher exact test for categorical data. Results were expressed as mean \pm standard deviation or median (25.75 percentile), when necessary. *P* value <0.05 was considered significant. The program GraphPad Instat statistical calculations and Prism 6.0, both for Windows ® were used.

RESULTS

The average weight of the animals was 277.4 ± 24.6 (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 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, demonstrated 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 the time of first heartbeat (153 ± 78 and 117 ± 96.8 seconds respectively) showed no significant difference (*P*=0.5869 and *P*=0.187, respectively).

Analysis of fractal dimension and Shannon entropy

The fractal dimension using the caspase marker was 1.59 ± 0.09 (no unit) for group 1 and 1.55 ± 0.13 for group 2, respectively (*P*=0.4400). KI-67 1.53 ± 0.13 and 1.54 ± 0.18 , respectively (*P*=0.9595) and IL-8, 1.52 ± 0.15 and 1.51 ± 0.12 , respectively (*P*=0.9164) (Figure 1).

The Shannon entropy with the caspase was 0.4 ± 0.07 bits for group 1 and 0.38 ± 0.08 bits for group 2 (*P*=0.5487). KI-67 0.36 ± 0.1 bits and 0.37 ± 0.13 bits, respectively (*P* = 0.9149), and IL-8 0.35 ± 0.11 bits and 0.35 ± 0.08 bits, respectively (*P*=0.9678) (Figure 2).

DISCUSSION

Although the effect of replacing ketoglutarate by glutamate in solution with histidine and tryptophan is still not known, glutamate has well-documented role when placed as a constituent of cardioplegic solution. The addition of glutamate in the perfusate maintains intracellular ATP and decreases both lactate as pyruvate, which would contribute to acidosis. Exogenous glutamate and its transamination products normally restore its contents decreased in the hypoxic myocardium, increase the concentration of succinate, which also leads to increased formation of ATP through anaerobic mitochondria pathway, thereby increasing the resistance to ischemia myocytes^[9].

Another process that is intrinsically related to ischemia-reperfusion injury is apoptosis^[17,18]. These changes are made by a family of proteases called caspases^[19]. The degree of caspase activation is directly related to the degree of apoptosis^[20]. In contrast to apoptosis, necrosis is an irreversible process of cell death in which there is disruption of the cell membrane overflow with the overflow of cytosol to the extracellular environment, leukocyte margination and inflammatory cascade activation^[20,21]. In contrast to the cell death the mammalian hearts show little proliferative capacity after birth. One of the markers used to assess cell proliferation is the Ki-67^[22]. With this marker, Walsh et al.^[23] show that 12%-23% of fetal rat cardiomyocytes exhibit proliferative activity, going to 1%-8% by the 7th day and virtually undetectable from the 14th day. Thus, we selected the analysis of caspase-8 and IL-67 KI for assessment of apoptosis, necrosis, cell proliferation, respectively.

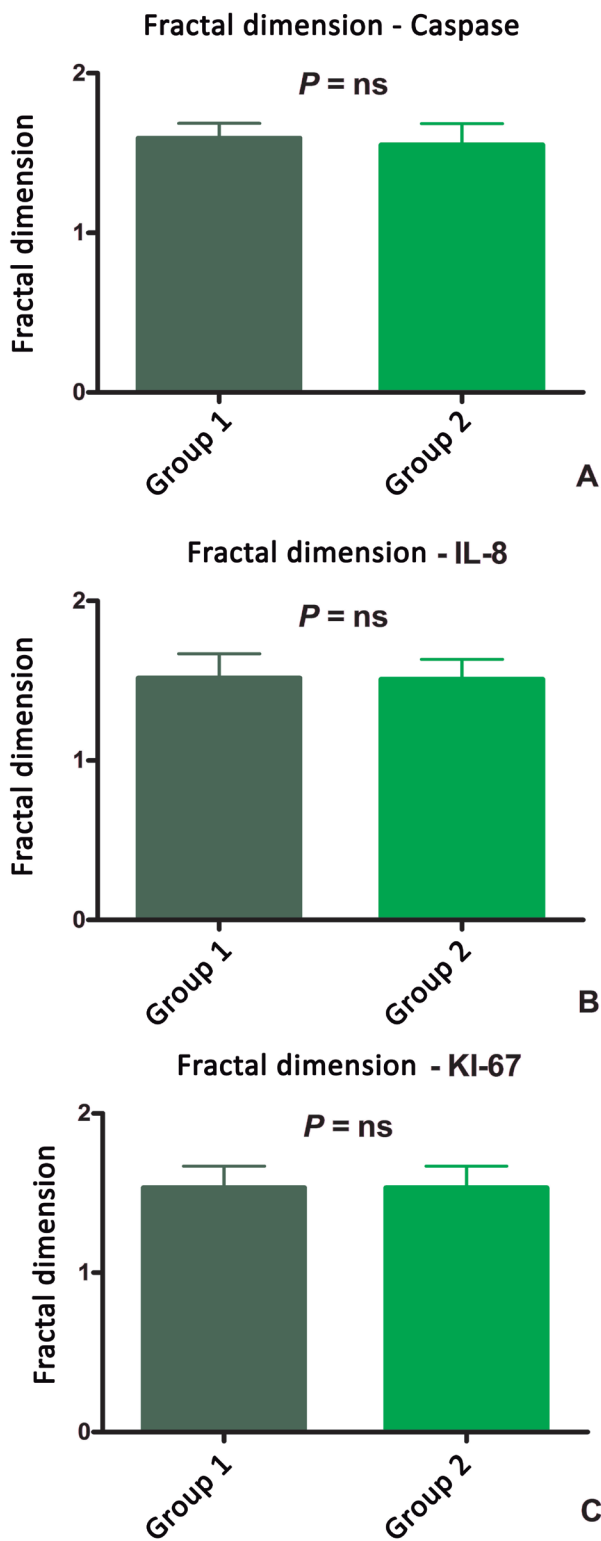


Fig. 1 - Histograms showing distribution of fractal dimension in: (A) caspase. (B) IL-8 and (C) KI-67. Group 1: solution with histidine-tryptophan-ketogluturate. Group 2: solution with histidine-tryptophan-glutamate.

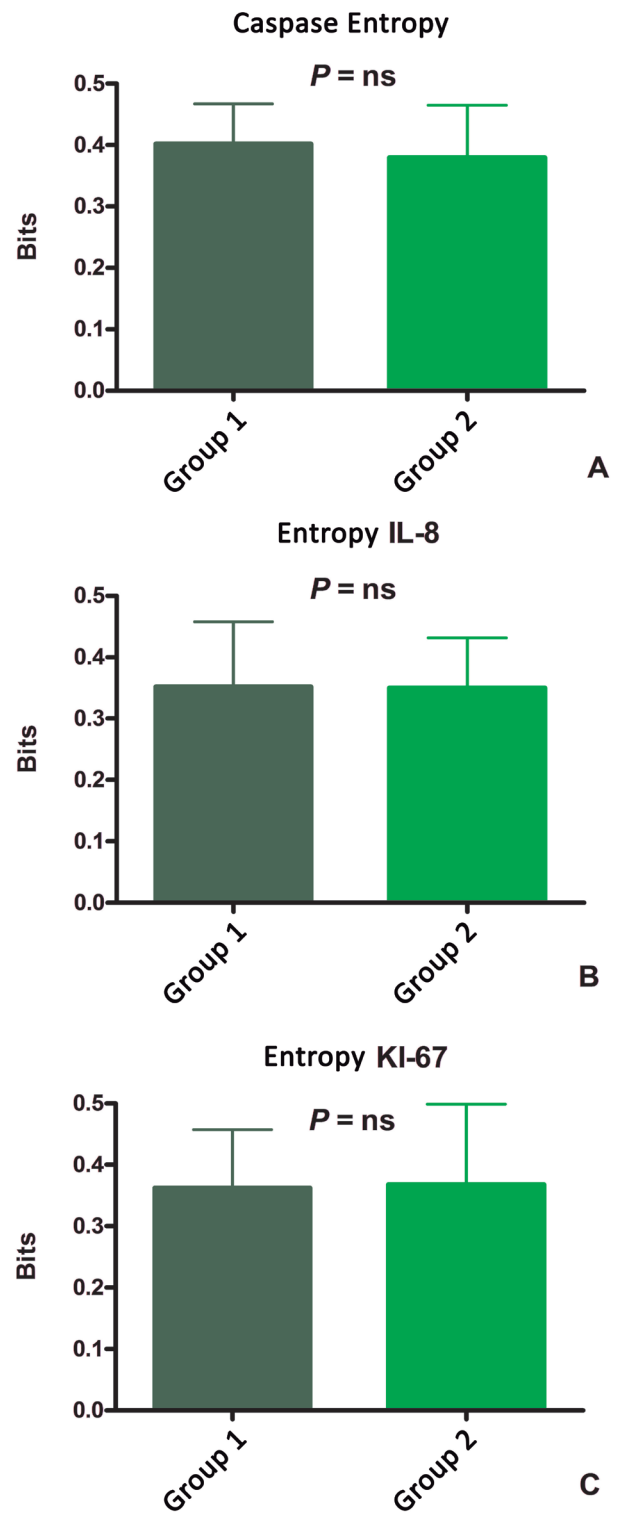


Fig. 2 - Histograms showing distribution of entropy in: (A) caspase. (B) IL-8 and (C) KI-67. Group 1: solution with histidine-tryptophan-ketogluturate. Group 2: solution with histidine-tryptophan-glutamate.

The fractal dimension is a useful parameter for the characterization of complex irregular structures. but when viewed mathematically. its analysis denotes regular figures with self-similarity features. or that is. resemble themselves when observed in different scales^[24].

The fractal dimension of the object account the effective number of degrees of freedom in the dynamical system. and therefore quantifies its complexity. Thus. it appears that images showing higher fractal dimensions are consequently more complex. However. we cannot quantify this complexity only by the visual aspect. The fractal dimension would then remedy this difficulty by adding a numeric value ^[12].

The size of the boxes for calculating the fractal dimension in the Box-counting method was standardized at 4. 8. 16. 32 and 64 pixels. It is known that the pixel size depends on the degree of resolution used. As commented by Tambasco et al. ^[24]. the size of the box used to bear a certain relationship with the studied structure. because it can be so small that. in fact. were being evaluating the subcomponents of the structure or so great that. in fact. were being included in the measurement of components surrounding the structure of interest and not the structure itself. These values. however. are the default values used in the literature and therefore probably not caused interference in the results^[12].

In our study. there was no significant difference in fractal dimension between groups. Thus. we consider that the distribution of the information contained in the slides of hearts treated with HTK solutions or HTG were not different.

An important contribution in Information Theory introduced by CE Shannon in 1948 was the concept of entropy as the amount of information in a system^[25]. It is noteworthy that one should not confuse “entropy state” of Thermodynamics with “entropy concept” of the Information Theory^[26].

According to Shannon. if X is the set of all messages of x. and p (x) is the probability (ranging from 0 to 1) of a message x. then the entropy of X will be^[26]:

The Shannon entropy quantifies the degree of “uncertainty” or degree of “complexity” of information. With this formulation it is easy to understand that if the probability of occurrence of a particular event is 100%. or that is. if P=1 (no uncertainty). then the entropy contained in the message is zero (log 1 = zero)^[26].

$$H(X) = - \sum_{x \in X} p(x) \log p(x)$$

Considering that an image is the result of a stochastic process in which the pi probability of the calculation of Shannon entropy may correspond to the probability of a pixel presents a given intensity or gray color (ranging from 0 to 255). This

probability of each intensity of gray can be obtained easily by constructing a histogram of frequencies^[26].

Zero entropy of an image is obtained when all the pixels have the same color or the same amount of gray (100% probability. or that is 1; log 1=0). On the other hand. the maximum entropy may occur when the image contained the same amount of pixel for each intensities presented. Thus. we demonstrate that entropy is not related to the spatial layout of information. Two images may have the same number of pixels with the same intensity and therefore the total entropy is the same. but spatially distributed [26] in a different manner.

In our study. there was no significant difference between the Shannon entropy between groups. Thus. we consider that the information contained in the slides of hearts treated with HTK or HTG solutions were not different.

The literature shows several studies in which there is a statistical difference between groups assessed with fractal dimension and Shannon entropy. drawing the reader’s attention on its imaging discriminative character. but presenting no relevance when the results found did not reach statistical significance. as occurred in our study. The interpretation of the results of this study is that the HTG group did not alter the amount or distribution of the imaging information of rat hearts when compared to those treated with HTK.

Shannon entropy and the fractal dimension quantifies the distribution and the degree of complexity of the image. respectively. Thus. this technique is not comparable with Western-Blot or PCR. as these quantify the total amount of proteins studied and not their distribution or degree of complexity in the tissue.

CONCLUSION

The amount and distribution of the information assessed by the Shannon entropy and fractal dimension on blades of rat heart undergoing cardioplegia with HTK or HTG solutions were not different. which shows that the HTG solution is as good as HTK in preserving myocytes in isolated rat heart model.

Authors’ Roles & Responsibilities

MABO	Main Author
ACB	Elaboration of Graphics
CAS	Elaboration of Graphics
PHHB	Handling of animals
JLLC	Handling of animals
MFG	Co-advisor and aid in final writing
DMB	Advisor and aid in final writing

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