

# Development of cardioplegic solution without potassium: experimental study in rat

*Desenvolvimento de solução cardioplégica sem potássio: estudo experimental em ratos*

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## Abstract

**Introduction:** Myocardial preservation during open heart surgeries and harvesting for transplant are of great importance. The heart at the end of procedure has to resume its functions as soon as possible. All cardioplegic solutions are based on potassium for induction of cardioplegic arrest.

**Objective:** To assess a cardioplegic solution with no potassium addition to the formula with two other commercially available cardioplegic solutions. The comparative assessment was based on cytotoxicity, adenosine triphosphate myocardial preservation, and caspase 3 activity. The tested solution (LIRM) uses low doses of sodium channel blocker (lidocaine), potassium channel opener (cromakalin), and actin/myosin cross bridge inhibitor (2,3-butanedione monoxime).

**Methods:** Wistar rats underwent thoracotomy under mechanical ventilation and three different solutions were used for "in situ" perfusion for cardioplegic arrest induction: Custodiol (HTK), Braile (G/A), and LIRM solutions. After cardiac arrest, the hearts

were excised and kept in cold storage for 4 hours. After this period, the hearts were assessed with optical light microscopy, myocardial ATP content and caspase 3 activity. All three solutions were evaluated for direct cytotoxicity with L929 and WEHI-164 cells.

**Results:** The ATP content was higher in the Custodiol group compared to two other solutions ( $P<0.05$ ). The caspase activity was lower in the HTK group compared to LIRM and G/A solutions ( $P<0.01$ ). The LIRM solution showed lower caspase activity compared to Braile solution ( $P<0.01$ ). All solutions showed no cytotoxicity effect after 24 hours of cells exposure to cardioplegic solutions.

**Conclusion:** Cardioplegia solutions without potassium are promised and aminoacid addition might be an interesting strategy. More evaluation is necessary for an optimal cardioplegic solution development.

**Descriptors:** Heart arrest, induced. Ischemia. Myocardial ischemia.

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#### Abbreviations, acronyms & symbols

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
G/A	Glutamate/aspartate cardioplegic solution
HE	Hematoxylin and eosin
HTK	Histidine-tryptophan cardioplegic solution

#### Resumo

**Introdução:** Preservação do miocárdio durante cirurgias cardíacas abertas e de colheita para transplante são de grande importância. O coração ao final do processo tem de retomar as suas funções, logo que possível. Todas as soluções cardioplégicas são baseadas em potássio, para indução de parada cardioplégica.

**Objetivo:** Comparar a uma solução cardioplégica sem adição de potássio à sua fórmula com duas outras soluções cardioplégicas disponíveis comercialmente. A avaliação comparativa foi baseada na citotoxicidade, preservação miocárdica (adenosina trifosfato, ATP) e atividade da caspase 3. A solução testada (LIRM) utiliza baixas doses de bloqueador de canal de sódio (lidocaína), abridor do canal de potássio (cromacalina) e inibidor da ponte actina / miosina (2,3-butanodiona monoxima).

**Métodos:** Ratos Wistar foram submetidos à toracotomia sob ventilação mecânica e três soluções diferentes foram utilizadas para perfusão *in situ* para a indução de parada cardioplégica: soluções Custodiol (HTK) Braile (G/A) e LIRM. Após parada cardíaca, os corações foram retirados e mantidos em câmara fria por 4 horas. Após esse período, o coração foi avaliado com microscopia de luz ótica, o conteúdo de ATP miocárdico e atividade da caspase 3. Todas as três soluções foram avaliadas quanto à citotoxicidade direta com células L929 e WEHI-164.

**Resultados:** A quantidade de ATP foi maior no grupo Custodiol em comparação às com outras duas soluções ( $P<0,05$ ). A atividade de caspase foi menor no grupo HTK quando comparado às soluções LIRM e G/A ( $P<0,01$ ). A solução LIRM demonstrou menor atividade da caspase em comparação à solução Braile ( $P<0,01$ ). Todas as soluções não mostraram qualquer efeito de citotoxicidade após 24 horas de exposição das células às soluções cardioplégicas.

**Conclusão:** Soluções cardioplégicas sem potássio são uma perspectiva e a adição de aminoácido pode ser uma estratégia interessante. Mais avaliações são necessárias para o desenvolvimento ideal da solução cardioplégica.

**Descritores:** Parada cardíaca induzida. Isquemia. Isquemia miocárdica.

## INTRODUCTION

Elective cardiac arrest was first performed by global myocardial ischemia with aortic cross-clamping in combination with hypothermia, as reported by Lewis & Taufic [1]. Since then, complex open-heart surgeries with longer aortic cross-clamp periods have been developed. However, the use of longer cross-clamp periods has increased the incidence of ischemia/reperfusion injury. In 1955, Melrose et al. [2] introduced the concept of pharmacologic cardiac arrest, named cardioplegia, which could be obtained by using a solution with a high potassium concentration. Cardioplegic solutions with moderate potassium concentrations were introduced into surgical practice in the mid-1970s and have remained the gold standard for myocardial protection [3,4]. Today, most cardiac surgeries are performed by cardiopulmonary bypass with pharmacologic cardioplegic arrest.

The elevated extracellular potassium level provided by the cardioplegic solution shifts the resting myocyte membrane potential from -85 mV to a range between -65 and -40 mV. This shift inactivates the fast sodium channels, thereby blocking conduction of the myocardial action potential and inducing a “depolarized” arrest. However, an inward non-inactivating sodium “window” current occurs at these higher membrane potentials [5,6]. This condition can lead to intracellular sodium loading and calcium overload of the myocyte, resulting in contracture and cell death [7].

The relative benefits provided by different cardioplegic solutions remain unclear. We idealized a new, potassium-free cardioplegic solution, named LIRM solution, containing: cromakalim, a potassium adenosine triphosphate (ATP)-channel opener that causes hyperpolarization of the cell membrane and coronary artery vasodilation [8]; lidocaine, a sodium-channel blocker that inhibits sodium influx into the myocyte and depolarization of the cell membrane [9]; and 2,3-butanedione, a direct myofilament inhibitor that prevents myocyte contraction by desensitizing the myofilament calcium [10]. In the present study, we tested the myocardial protection afforded by the LIRM solution in terms of the ATP myocardial content and caspase 3 activity, which are important factors for short- and long-term outcomes for heart transplant and conventional open-heart surgeries [4,11-13]. We compared the myocardial protection results among the LIRM, histidine-tryptophan (HTK), and glutamate/aspartate (G/A) cardioplegic solutions after 4 hours of cold storage.

## METHODS

### Surgical protocol

Wistar male rats (250-350 g) were anesthetized by intraperitoneal injection of sodium thiopental (150 mg/kg). The protocol design in this study was intended to recreate the heart situation after aortic clamp release and right before heart reperfusion. The animals underwent tracheostomy and were mecha-

nically ventilated (Minivent, Harvard Apparatus, Holliston, MA, USA). The chest was opened with a median sternotomy, the right carotid artery was catheterized, and the transverse aortic arch was isolated between the brachiocephalic artery and the left carotid artery. The transverse arch was tied, and cardioplegic solution was injected at an infusion rate of 5 mL/min. The total dose for arresting the heart was recorded. Three different cardioplegic solutions were evaluated.

The heart was excised and kept at 4°C for 4 hours, simulating the period of arrest for a transplant or a long period of aortic cross-clamp time. At the end of this period, the heart was snap-frozen in liquid nitrogen. A sample tissue of each group was evaluated with hematoxylin and eosin (HE) staining for gross assessment of the cell anatomy.

All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Brazilian Council in Animal Experimentation (COBEA).

### Cardioplegic solutions

The compositions of the tested cardioplegic solutions are shown in Table 1. The G/A solution (Braile Biomédica, São José do Rio Preto, Brazil) was diluted to 1% before delivery to the heart.

### Determination of the myocardial ATP and caspase 3 activity levels

Myocardial tissue was removed from the liquid nitrogen, kept in the same proportion of extraction buffer, and homogenized with 25 mM Tris-HCL, 150 mM NaCl, 1% NP-40,

1% sodium deoxycholate, and 0.1% SDS. The homogenized tissue was centrifugated at 10,000 × g for 40 minutes at 4°C. The supernatant was used for ATP assessment. Myocardial ATP levels were assessed with the ENLITEN ATP assay system (Promega, Madison, WI, USA) and a Glomax 20/20 (Promega) for bioluminescence quantification, according to the manufacturer's instructions. The ATP level was measured relative to ATP standards provided by the manufacturer. The caspase 3 activity (in relative units of activity) was assessed with the Caspase-Glo 3 Assay (Promega), according to the manufacturer's instructions.

### Determination of cytotoxicity

L929 cells (Genetech Inc. South San Francisco, CA, USA) were incubated at 37°C under an atmosphere of 5% CO<sub>2</sub>. Cardioplegic solutions were added to the cultured cells at progressive dilutions, and the cells were evaluated 24 hours later. The cell viability was assessed with the MTT (dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. Readings were performed at a wavelength of 550 nm [14].

### Statistical analysis

All values were expressed as the mean ± standard deviation (SD). Variables were tested for normal distribution. One-way analysis of variance (ANOVA) or Kruskal-Wallis test was applied where appropriate. Differences with a *P*-value < 0.05 were considered statistically significant. All graphs and statistical analyses were performed with the GraphPad Prism version 6 software package for the Mac OS X (GraphPad Software, La Jolla, CA, USA).

Table 1. Compositions of the tested cardioplegic solutions.

Components	HTK solution	LIRM solution	G/A (stock solution)
Na <sup>+</sup>	15	15	—
K <sup>+</sup>	10	—	75
Mg <sup>2+</sup>	4	4	4
Ca <sup>2+</sup>	0.015	0.015	—
Cl <sup>-</sup>	50	30	34
Histidine	198	—	—
Tryptophan	2	—	—
Glutamate	—	—	30
Aspartate	—	—	30
Mannitol	30	—	—
Cromakalin	—	0.001	—
Lidocaine	—	100	—
2,3-Butanedione	—	30	—
Phosphoric acid	—	1.4	—
pH (at 18°C)	7.10	8.65	6.90

All concentrations are in mmol/L

## RESULTS

The animals showed comparable weights. Compared to the HTK and G/A solutions, less volume of LIRM solution was needed to achieve cardiac arrest ( $P=0.008$ ) (Table 2).

### Histological findings

The HE plates of the three cardioplegic solutions showed similar findings, with no gross disruption of the cell architecture or edema after 4 hours of cold preservation (Figure 1).

### ATP myocardial content and caspase 3 activity levels

The ATP myocardial content after 4 hours of cold storage was higher in the HTK solution group, with no differences between the LIRM and G/A groups (Figure 2A). The caspase 3 activity was higher in the G/A group compared to that of the LIRM and HTK groups, whereas the LIRM group showed higher caspase activity compared to the HTK group (Figure 2B).

### Cell viability

None of the solutions showed cytotoxicity after contact with L929 cells for 24 hours (Figure 3).

Table 2. Animal weights and cardioplegic solution volumes required for total cessation of heart activity

Solution	HTK (n = 5)	LIRM (n = 5)	G/A (n = 5)	P-value
Weight (mg)	314.4 ± 72.5	306.2 ± 69.8	310.2 ± 81.3	0.983
Dose for heart activity cessation (mL)	15.8 ± 8.5	2.6 ± 2.5 *	15.8 ± 4.1	0.008

\* Compared to HTK and G/A solutions by one-way ANOVA

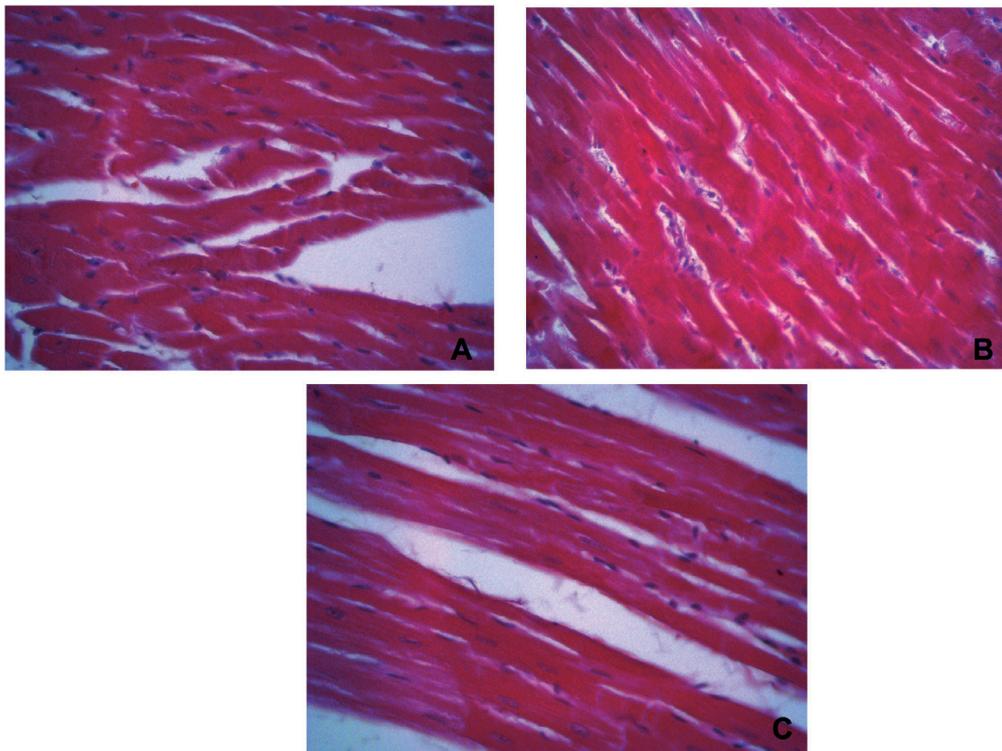


Fig. 1 - Representative left ventricle tissue sections stained with hematoxylin and eosin, showing similar findings of cell preservation in all three groups: (A) HTK, (B) LIRM, and (C) A/G solutions

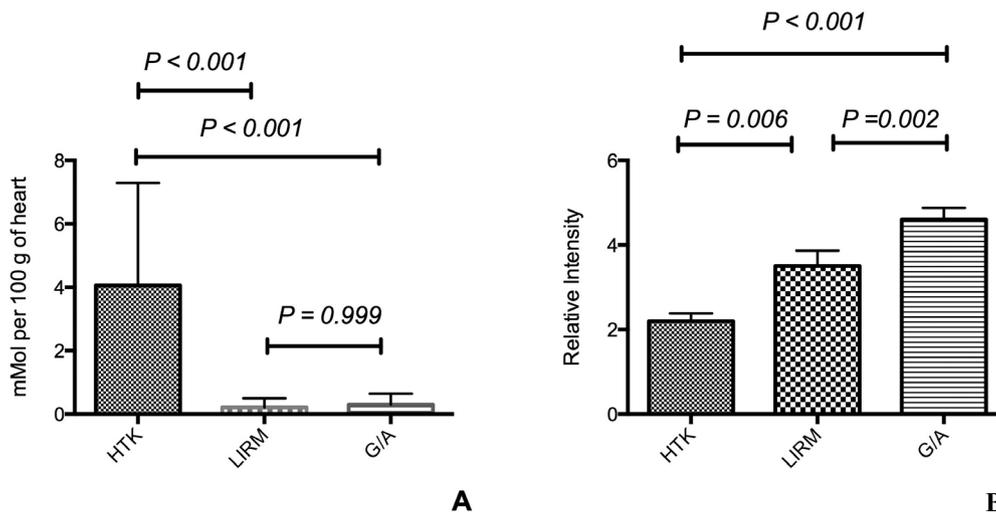


Fig. 2 - The ATP myocardial content was higher in the HTK group compared to the other groups (A). Comparison of caspase 3 activity after 4 hours of cold storage (B). All values are expressed as the means; bars indicate standard error of the mean. Differences were assessed by one-way ANOVA. n=5 animals per group

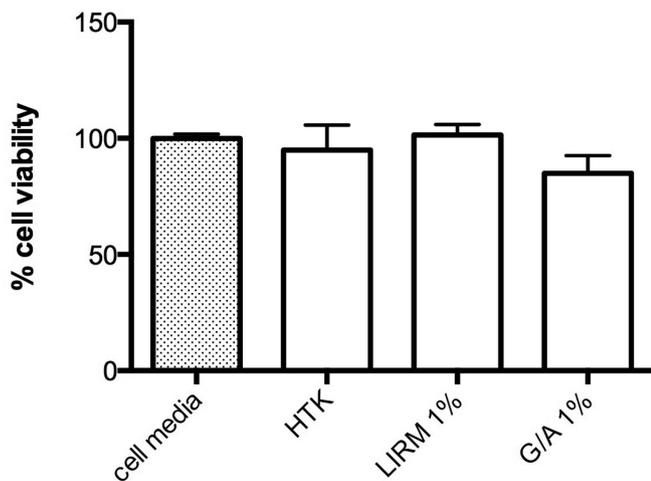


Fig. 3 - Cell viability of L929 cells after incubation with cardioplegic solution for 24 hours. None of the solutions showed cytotoxicity during the observation period

## DISCUSSION

In this study, we evaluated the effect of three different cardioplegic solutions on myocardial ATP content and caspase 3 activity after 4 hours of cold conservation. We also evaluated cell cytotoxicity after 24 hours of exposure to the cardioplegic solutions. The ATP myocardial content was higher in the HTK group compared to the G/A and LIRM groups. The caspase 3 activity was the lowest in the HTK and highest in

the G/A group. Peculiarly, the caspase 3 activity showed an intermediate activity with the LIRM solution compared to the two other solutions. None of the solutions demonstrated cytotoxicity in cell culture, a finding that is not often reported in the literature regarding cardioplegic solutions [15,16].

Our findings confirm the effectiveness of HTK solution as an organ preservation solution, as demonstrated by ATP conservation. Another important finding was the lower activity of caspase 3 with HTK solution, which is not often reported [17]. The LIRM solution showed a beneficial effect on caspase 3 activity and a very effective ability to arrest the heart without potassium. The ideal cardioplegic solution, which has yet to be determined, should allow a rapid and effective induction of diastolic arrest, should minimize ischemia/reperfusion injury, and should have no deleterious effects on other organs [18]. The rapid and effective induction of cardiac arrest may minimize myocardial ATP depletion and contribute to the protective effects during the reperfusion period [18-20]. In the present study, the LIRM solution displayed a pronounced capacity to promote cardiac arrest compared to the other two solutions. However, the myocardial ATP content was very similar when the LIRM and G/A solutions were used. Regardless of the cause, the caspase 3 activity of the LIRM solution was lower than that of the G/A solution, which might represent an improvement during the reperfusion period. An analysis of the details of this concept is beyond of the scope of the current study.

The HTK solution effectively reduced the energy requirements, as observed in this and previous studies [21]. Howe-

ver, other reports have shown an inability of HTK solution to reduce endothelial dysfunction after long periods of cold storage [22]. The LIRM solution contains cromakalin, which has beneficial effects on endothelial function and coronary vasodilation. Nevertheless, the beneficial effects of this agent were not tested in the present study design [23].

To induce a pharmacologic arrest, the arresting agents of a cardioplegic solution must interact with some targets involved in excitation-contraction coupling. This effect can be reached by inhibiting the myocardial action potential propagation and/or inhibiting calcium activation of the myofilaments. The LIRM solution has components to induce cardiac arrest by both of these mechanisms. The LIRM solution contains three different agents (cromakalin, 2,3-butanedione, and lidocaine) at very low concentrations to induce cardioplegic arrest without hyperkalemia. All three components were added to the initial formula for sum effect and to avoid any deleterious effects of the higher concentration of one isolated component. The other solutions tested in this report induce cardiac arrest by different mechanisms. The G/A solution induces cardiac arrest by inhibiting the action potential by hyperkalemia, leading to depolarization of the cell membrane. The HTK solution does the same, inhibiting the myofilament action by providing a very low calcium concentration.

Although universally used, the strategy of depolarized arrest with hyperkalemia has distinct disadvantages. In particular, cellular ionic currents are maintained during the ischemia/arrested period, which can lead to adverse effects [18,24]. Hyperkalemia shifts the membrane potential of the myocytes to a range between -65 and -40 mV. At this voltage, not all of the sodium channels are inactivated. Sodium influx by non-inactivated sodium channels [5,6] can lead to the activation of the sodium-hydrogen exchanger and intracellular acidosis [7,25,26]. Consequently, acidosis and ischemia lead to inhibition of the sodium/potassium-ATPase [26] and further increase the intracellular sodium. Sodium overload causes the sodium/calcium exchanger channel to act in reverse mode, increasing the calcium loading of the myocyte and leading to contracture and cell death [7].

One potential disadvantage of the LIRM solution compared to the HTK and G/A solutions is the inexistence of components that might supply the Krebs cycle, such as tryptophan in the HTK solution or aspartate and glutamate in the G/A solution. The absence such components might be responsible for the lower myocardial ATP content observed with the LIRM solution. The addition of some precursors of the Krebs cycle to the LIRM solution might improve the energy maintenance during cold storage, as assessed by the myocardial ATP content.

#### Study limitations

We did not evaluate hemodynamic data, such as left ventricle systolic and diastolic pressures, dP/dt maximum and mi-

nimum, and contractility indexes. Conclusions were limited to the period before reperfusion, but we were able to show a higher myocardial ATP content with the HTK solution, different caspase activities in all three groups, and higher effectiveness of the LIRM solution in achieving cardiac arrest.

#### CONCLUSIONS

The HTK solution was more effective in promoting higher levels of myocardial ATP content compared to the two other solutions. The LIRM solution was very effective in promoting cardiac arrest and reducing caspase 3 activity compared to the A/G solution. These preliminary data concerning the use of different pharmacological agents for cardiac arrest are promising. In the future, cardioplegic solutions containing Krebs cycle substrates, such as tryptophan and histidine, might be considered.

#### Author's roles & responsibilities

KR	Implementation, writing and discussion
HRPC	Implementation, writing and discussion
FL	Execution discussion
AGT	Writing and discussion
KASV	Design, execution, writing and discussion
PPMO	Discussion and manuscript review
LMSF	Discussion and manuscript review
ESBOS	Discussion and writing
OP	Design, writing, analysis

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