

Long-Term Protective Effects of Single-Dose Cardioplegic Solutions in Cell Culture Models

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Abstract: Despite the popularity of single-dose cardioplegic techniques, the time window and targeted population for successful reperfusion remain unclear. We tested currently available techniques based on cell viability and integrity to demonstrate long-term cardioprotection and clarify whether these solutions were performed on neonatal/adult endothelium and myocardium by examining different cell lines. Cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test proliferation assay and membrane integrity with the lactic dehydrogenase (LDH) cytotoxicity test were documented in a cell culture/microscopy setting on adult (human umbilical vein endothelium [HUVEC]), neonatal (H9C2-cardiomyocytes), and myofibroblast (L929) cell lines. Apoptotic cell activity and necrosis were evaluated by acridine orange/propidium iodide (AO/PI) staining. Twenty-four hours after seeding, cells were incubated in control (Dulbecco's modified Eagle), St. Thomas and blood cardioplegia (4:1), histidine–tryptophan–ketoglutarate (HTK), and del Nido solutions at 32°C followed by an additional 6, 24, and 48 hours in standard conditions (37°C, 5% CO₂).

Experiments were repeated eight times. In MTT cell viability analysis, HTK protection was significantly better than the control medium in L929 cell lines at 48th hours follow-up and acted markedly better on the HUVEC cell line at 24th and 48th hours. del Nido and HTK provided significantly better protection on H9C2 (at 24th and 48th hours). Apoptotic and necrotic cell scoring as a result of AO/PI staining was found consistent with MTT results. The LDH test demonstrated that the level of cell disruption was significantly higher for St. Thomas and blood cardioplegia in H9c2 cells. Experimental studies on cardioplegia aimed at assessing myocardial protection use time-consuming and often expensive approaches that are unrealistic in clinical practice. We have focused on identifying the most effective cell types and the direct consequences of different cardioplegia solutions to document long-term effects that we believe are the most underestimated ones in the cardioplegia literature. **Keywords:** cardiopulmonary bypass, cardioplegia, cell biology/culture, myocardial protection, myocardial injury. *J Extra Corpor Technol. 2020;52:279–88*

Adequate myocardial protection during cardiac surgery is essential for successful clinical outcomes

Despite a multitude of commercially available cardioplegic solutions, no clear consensus has been reached on the optimal composition or technique for using them. Heterogeneity in delivery temperature, dosing frequency, and substrate

composition makes it difficult to evaluate these solutions in a clinical setting (1). Experimental work on cardioplegia aiming to assess myocardial protection use time-consuming, invasive, and often expensive approaches such as continuous monitoring of intra-myocardial pH, myocardial lactate production, or myocardial biopsy for adenosine-triphosphate dosage, which are unrealistic in routine clinical practice (2–3).

There is momentum to incorporate myoprotective methods that extend the safe ischemic time, reducing the need for cardioplegia redosing. These techniques increase the time between dosing, which may lower cross-clamp times and, ultimately, the time on cardiopulmonary bypass, both of which are linked to improved outcomes. This is

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Table 1. Composition of solutions studied.

Solution	Content
DMEM	1,000 mg/L glucose, glutamine, HCO ₃ Pyridoxine
St. Thomas solution	Na: 110 mmol/L K: 16 mmol/L Mg: 16 mmol/L Ca: 1.2 mmol/L Cl: 160 mmol/L HCO ₃ : 10 mmol/L
HTK cardioplegia	Na: 15 mmol/L K: 9 mmol/L Mg: 4 mmol/L Ca: .015 mmol/L Histidine: 198 mmol/L Tryptophan: 2 mmol/L Mannitol: 30 mmol/L
del Nido cardioplegia	Base solution (mmol/L) (Na: 140, K: 5, Mg: 3) K, 7.5%: 26 mL Mannitol 20%: 16.3 mL Mg 50%: 4 mL HCO ₃ 8.4%: 13 mL Lidocaine 1%: 13 mL Mixed with blood (1:4)
Blood cardioplegia	Base: St. Thomas solution K: 40 mmol/L NaHCO ₃ : 10 mmol/L Mannitol: 10 mmol/L Mixed with blood (4:1)

especially attractive in patients with valvular lesions where the administration of cardioplegia may require the use of specialized delivery cannulae or obscures the operative field, extending ischemic time. When minimally invasive techniques are used, there is limited access to coronary anatomy, which reduces access for the delivery and makes repeated dosing difficult. Such challenges have led to an evaluation of a newer generation of cardioplegic solutions that were initially developed for pediatric patients for its potential use in adult cardiac surgery (4).

Because single-dose cardioplegic techniques were initially designed for pediatric use, there is still a lack of quality human research and subsequent publications that prevent best practices from being used in adult surgery. Therefore, the central debate of preservation on which cell population (adult or pediatric), the duration of myocardial protection, and long-term outcomes is not solved.

The purpose of the current study was to compare single-dose cardioplegic techniques with the conventional methods based on cell viability and integrity to demonstrate long-term cardioprotection and clarify

which of these solutions performed better on neonatal/adult endothelium or myocardium by examining different cell lines.

MATERIALS AND METHODS

This study was approved by the Institutional Board of Experimental Sciences Ethics Committee (August 18, 2019).

Experimental Design

Cardioplegic application methods and dosing were simulated from an 80-kg patient (approximate cell count in the heart) (5) undergoing cardiac surgery at moderate hypothermia (32°C) as follows:

Group 1: St. Thomas cardioplegia (4°C, 15 mL/kg initial dose delivered in 3 minutes followed by half dosing every 25 minutes)

Group 2: Cold blood cardioplegia (4:1) (4°C, 15 mL/kg initial dose delivered in 3 minutes followed by half dosing every 25 minutes)

Group 3: Histidine–tryptophan–ketoglutarate (HTK) cardioplegia (single dose; 4°C, 20 mL/kg delivered in 6 minutes)

Group 4: del Nido cardioplegia (1:4) (conventional formula, single dose; 4°C, 20 mL/kg delivered in 4 minutes).

Blood cardioplegia/del Nido samples were obtained from the bags prepared in the operating room with heparinized blood of the patient during surgery and transferred to the experimental area within 5 minutes within an insulated bag. To overcome the limitation of the variability of fresh patient blood or degree of hemodilution, study volumes of solutions were used from one single bag for all related specimens. The composition of the solutions used is summarized in Table 1.

Cultured L929 (2×10^4 cells/mL), human umbilical vein endothelial cells (HUVEC) (2×10^4 cells/mL), and H9c2 (5×10^3 cells/mL) were seeded in 96-well plates for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and in 24-well plates for acridine orange (AO) and propidium iodide (PI) double staining. The characteristics of the selected cell lines and represented tissue are described in Table 2.

After 24 hours of incubation, before applying experimental protocol, all cell types were washed with phosphate buffered saline (PBS) and incubated in a control

Table 2. Selected cell lines and the represented tissue on protection.

Cell Line	Characteristics	Represented Protection
H9C2	Primary neonatal cardiomyocytes	Neonatal/pediatric heart
HUVEC	Human umbilical vein endothelial cells	Adult heart/endothelium
L929	Myofibroblasts	Fiber skeleton of the heart

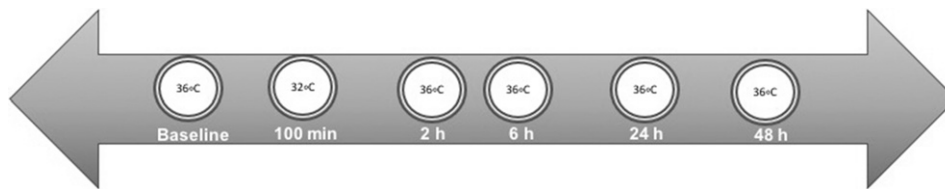


Figure 1. Protocol of cell culture follow-up.

culture medium Dulbecco's modified Eagle's medium (DMEM). Cell culture plates were set up to 32°C in a water bath. HTK and del Nido cardioplegic solutions (200 mL/well) (4°C) were added and kept for 100 minutes on the cells in the water bath (32°C). St. Thomas and cold blood cardioplegia (4:1) solutions (4°C) were also added on the related wells (150 mL/well) in the water bath (32°C), and half dose (75 mL) was repeated three times every 25 minutes to a total of 100 minutes.

Experiments were repeated eight times. During this period, the temperature was monitored by an MP35 data acquisition system (BIOPAC System, Inc., Commat Ltd., Ankara, Turkey). After 100 minutes, all groups (control culture medium, St. Thomas, blood cardioplegia, HTK, and del Nido) were washed with PBS and incubated with pre-warmed control culture medium for additional follow-up of 6, 24, and 48 hours in standard conditions (37°C, 5% CO₂) (Figure 1). Laboratory evaluation tests and projected clinical settings are summarized in Table 3.

Chemicals and Solvents

Penicillin–streptomycin (P/S) (Chemical Abstracts Service [CAS] Number: 3810-74-0), MTT (CAS Number: 298-93-1), AO solution (CAS Number: 65-61-2), and PI solution (CAS Number: 25535-16-4) were obtained from Sigma Aldrich (Germany). DMEM (Cat Number: FG 0415) and fetal bovine serum (FBS) (Cat Number: S 0415) were purchased from Biochrom AG, Germany. All chemicals and solvents were obtained as cell culture grade.

Myofibroblast cell line (L929), HUVEC, and primary neonatal cardiomyocyte cell line (H9c2) were purchased from the American Type Culture Collection (ATCC). Samples from the cardioplegic solutions were obtained from the cardiovascular surgery perfusion services.

Cell Culture

L929 cells were cultured in standard conditions using DMEM/12 containing 10% FBS and 1% P/S. HUVEC were cultured at 37°C in a humidified atmosphere (5% CO₂ in air) using modified endothelial medium (DMEM/12) supplemented with 20% FBS, 100 µg/mL heparin, and 1% P/S. The H9c2 neonatal cardiomyocyte cell line was maintained in ATCC–formulated DMEM/12 supplemented with 10% non–heat-inactivated FBS and 1% P/S in an incubator. In all cell lines, culture mediums were refreshed every 2 days and sub-cultured until the experimental protocol was applied.

Cell Viability via MTT Test

At each time point, the viability of cells was measured by using the MTT test. MTT assay is a colorimetric method standardized by Mosmann et al. (6) for defining viability. At the end of the designed incubation periods, MTT was added to the cells in an amount of 10% of fresh culture medium and incubated for 4 hours in an incubator. Isopropanol alcohol containing .1 N hydrochloric acid was used to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm on a spectrophotometer microplate reader

Table 3. Definition of laboratory tests, interpretation, and clinical projection.

Laboratory Test	Tool to Demonstrate	Interpretation	Clinical Projection
MTT test	Cell viability vs. control	% Of viable cells with respect to control starting at the end of cardioplegic protection (80 minutes) until 48 hours	Long-term protection capacity of cardioplegic solutions on pediatric/adult/fibrous skeleton of the heart
AO/PI staining	Viable cells Early apoptosis Late apoptosis Necrosis	Demonstration of the % viable cells with respect to control until 48 hours with further classification of <ul style="list-style-type: none"> • Viable cells (uniform green nucleus) • Early apoptosis (non-uniform bright green nucleus) • Late apoptosis (non-uniform orange nucleus) • Necrosis (uniform orange nucleus) 	<ul style="list-style-type: none"> • Viable cells (well-protected heart) • Early apoptosis (stunning myocardium) • Late apoptosis (hibernating myocardium) • Necrosis (infarcted myocardium)
LDH test	Cytotoxicity vs. control	Toxic effects of cardioplegic solutions at microscopic levels with prolonged contact (cell membrane destruction) vs control	Demonstrate any toxic effect of the components of cardioplegic solutions in prolonged contact

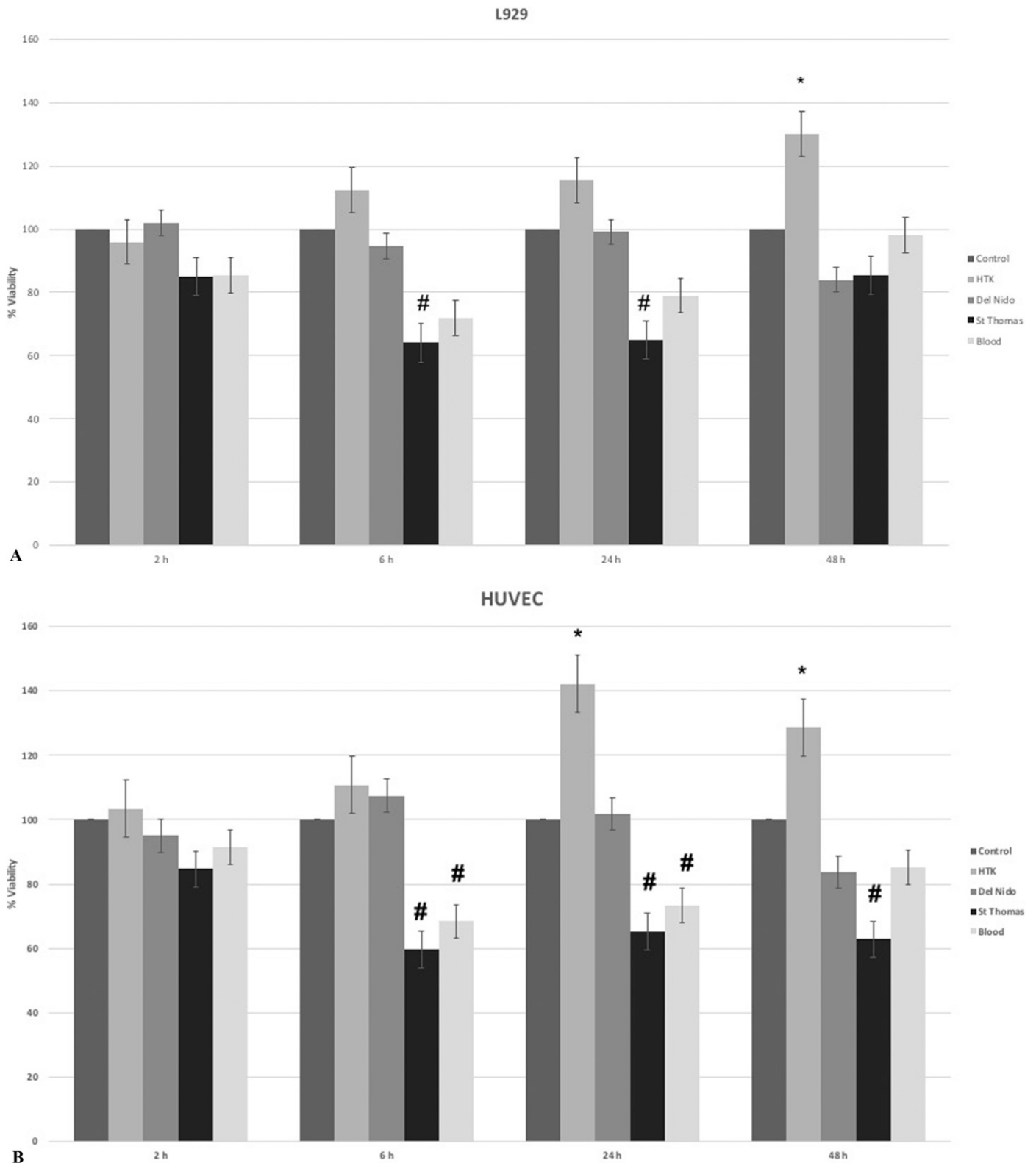


Figure 2. MTT results of L929 (A), HUVEC (B), and H9C2 (C) after treatment with control medium, HTK, del Nido, St. Thomas, and blood for 2, 6, 24, and 48 hours. Results are shown as mean \pm SD, derived from at least three replicates. Levels better/worse compared with control were accepted as significant because the control solution is a medium for routine cell culture (*: $p < .05$ better with respect to control; #: $p < .05$, worse compared with control).

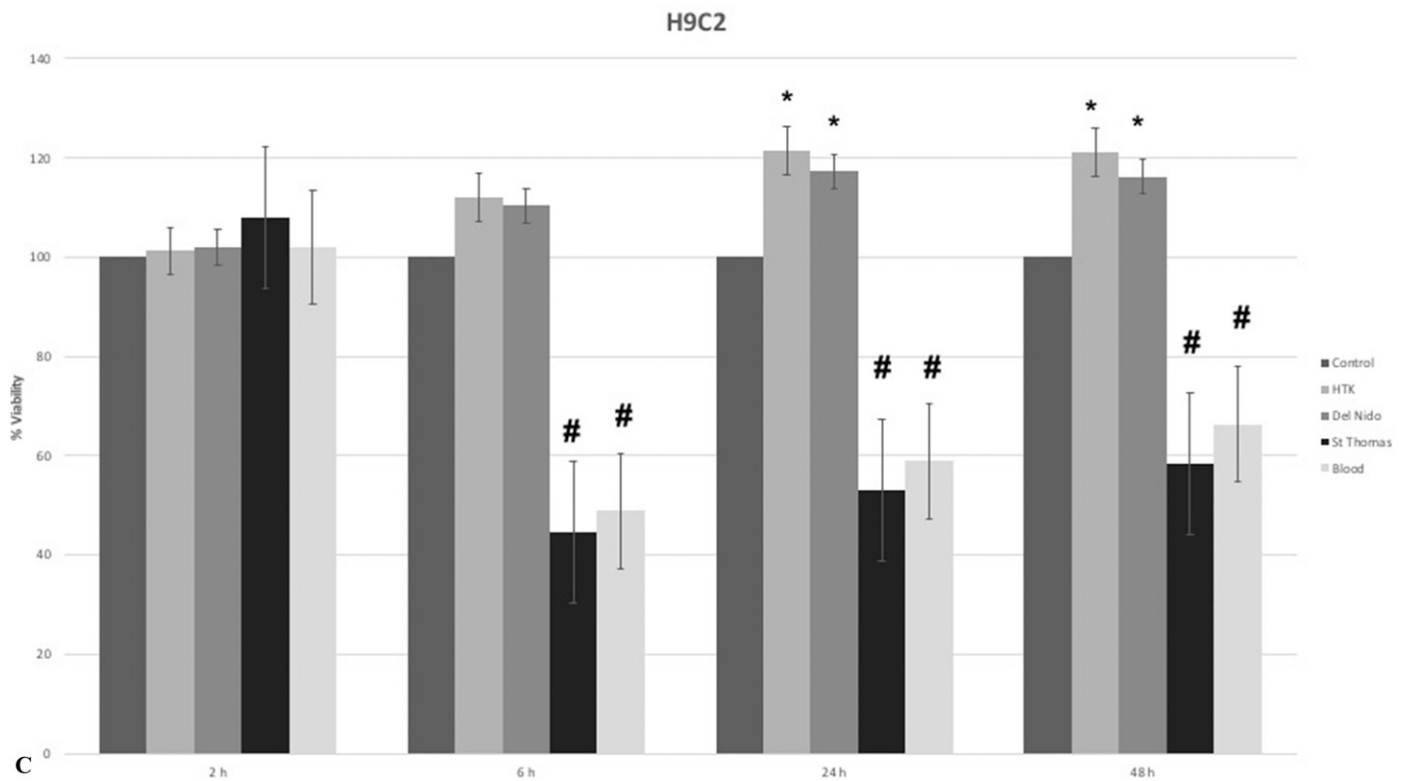


Figure 2. Continued.

(μ QuantTM, Biotek[®] Instruments Inc.). Each experiment was repeated eight times separately. In all groups, viability was given as a percentage of the untreated control group. The cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \left(\frac{A_{\text{treated}}}{A_{\text{control}}} \right) \times 100,$$

where A_{treated} and A_{control} are the absorbance of the treated and untreated cells, respectively.

Apoptotic Cell Analysis

For viability determination at each time point, AO/PI double staining was performed. AO is a stain that is permeable to viable cells and can stain the cell's DNA directly. This dye emits a green fluorescence once it is excited. PI, on the other hand, is a dye that is impermeable to viable cells. It can bind to DNA only when the cells are dead; it emits a red-orange fluorescence instead. After removing the solutions and culture medium, cells were washed twice with PBS. Cells were stained with an equal volume combination of AO and PI for 20 seconds and washed twice with PBS for removing dyes. The stained sample was viewed under a fluorescent microscope (Olympus IX70, Japan).

After AO/PI staining, cells were counted according to the method described in previous studies to determine the potential of different cardioplegic solutions to induce apoptosis and necrosis on other cells (7–9). According to the previous studies, the criteria are as follows: uniform green nucleus with organized structure, intact plasma membrane, orange or green cytoplasm, viable cells 1), bright green areas of chromatin condensation in the nucleus, early apoptosis 2), dense orange areas of chromatin condensation, late apoptosis 3), orange intact nucleus, and necrosis 4). For each test group, three fluorescence microscope images with a magnification of $\times 20$ were analyzed by examining and counting cells manually. The quantification of apoptotic and necrotic cells was calculated according to the following equations.

$$\text{Apoptotic cells (\%)} = \frac{\text{Total number of apoptotic cells (early or late)}}{\text{Total count cell}} \times 100$$

$$\text{Necrotic cells (\%)} = \frac{\text{Total number of necrotic cells}}{\text{Total count cell}} \times 100$$

Lactic Dehydrogenase (LDH) Cytotoxicity Test

For the determination of LDH levels, L929 and HUVEC were seeded into 96-well plates at a density of 2×10^4 cells/mL, and H9c2 cells were seeded at a density of 5×10^3 cells/mL, and all groups were allowed to attach for 24 hours. After incubation with experimental solutions, all wells were changed with normal culture medium and incubated for another 24 hours for LDH testing. At the end of the incubation time, LDH release was measured using the Pierce LDH cytotoxicity assay kit according to the manufacturer's protocols (Thermo Scientific). The absorbance was measured at 490 nm using the scanning multi-well spectrophotometer (μ QuantTM, Biotek[®] Instruments Inc.). Also, % cytotoxicity was calculated according to the manufacturer's protocols:

$$\% \text{Cytotoxicity} = \frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100$$

Statistical analysis

Results of all the studies were statistically evaluated using GraphPad Prism 5.00 program. Data were presented with mean \pm SD for all experimental protocols. Student's *t*-test was used for statistical comparison between groups. A *p*-value less than .05 was considered statistically significant.

RESULTS

MTT Test

Cell viability via MTT test in different cell lines is demonstrated in Figure 2A, B, and C. On cells representing the fibrous skeleton of the heart (L929-myofibroblasts), HTK protection was significantly better than control medium at 48th hours follow-up. St. Thomas group was considerably less viable than control at 6th and 24th hours. HTK acted markedly better than control medium on HUVEC cell line representing adult endothelial cells at 24th and 48th hours. St. Thomas and blood cardioplegia groups were significantly less viable than control at 6th and 24th hours. del Nido and HTK had significantly better protection on H9C2 cells expressing neonatal cardiomyocytes at 24th and 48th hours. At the same time, St. Thomas and blood cardioplegia groups were significantly less viable than control at 6th, 24th, and 48th hours.

Apoptotic Staining and Cell Scoring

During apoptosis, cells undergo significant morphological changes, such as cytoskeleton deformation, cell contraction or membrane swelling, plasma membrane and nucleus condensation, or DNA lysis. AO/PI is the most common method of staining the morphological changes in the nucleus because of apoptosis and necrosis. AO/PI

double staining can also be used to distinguish whether apoptotic cells are in early or late apoptosis and to distinguish necrotic cells. The results obtained from fluorescence images of L929, HUVEC, and H9c2 cells after apoptotic staining are represented in Figure 3A, B, and C, respectively.

Apoptotic and necrotic cell scoring as a result of AO/PI staining was found consistent with MTT results (Figure 4A, B, and C). It was observed that St. Thomas significantly triggered late apoptosis and necrosis in all cell types, especially in the 24th and 48th hours applications. HTK and del Nido solutions were found to cause significantly less cell death on H9c2 cells. HTK worked considerably well in late follow-ups with HUVEC and L929 cell lines. Blood cardioplegia performed well, demonstrating compatible preservation on apoptosis testing in L929 and HUVEC cell lines.

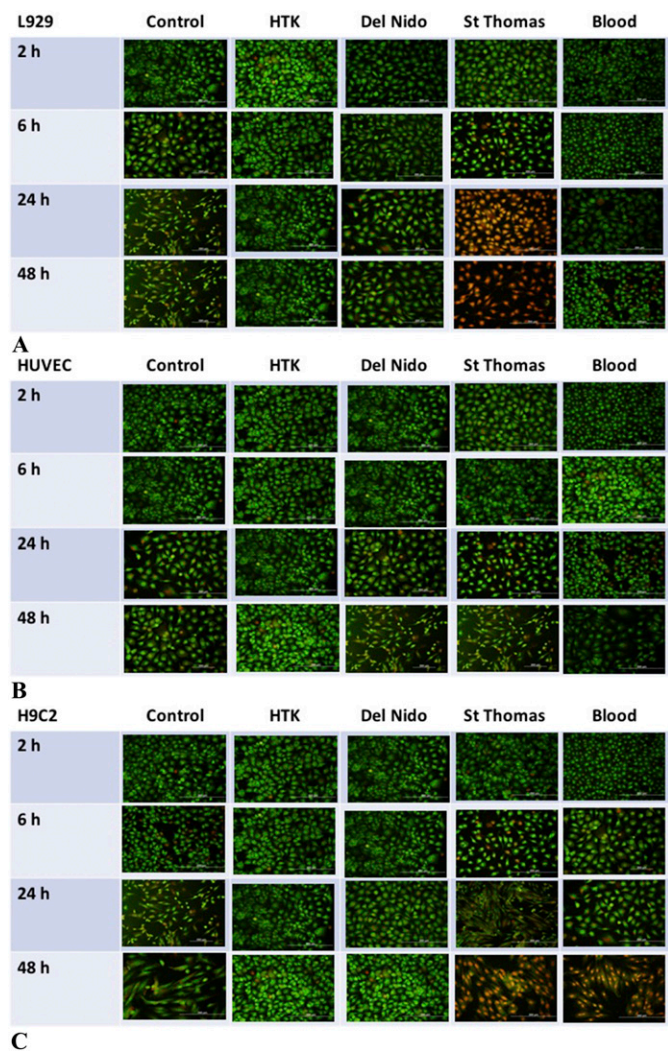


Figure 3. AO/PI double-stained L929 (A), HUVEC (B), and H9C2 (C) cells after treatment of cardioplegic solutions. The fluorescence images of cells are $\times 20$ magnifications.

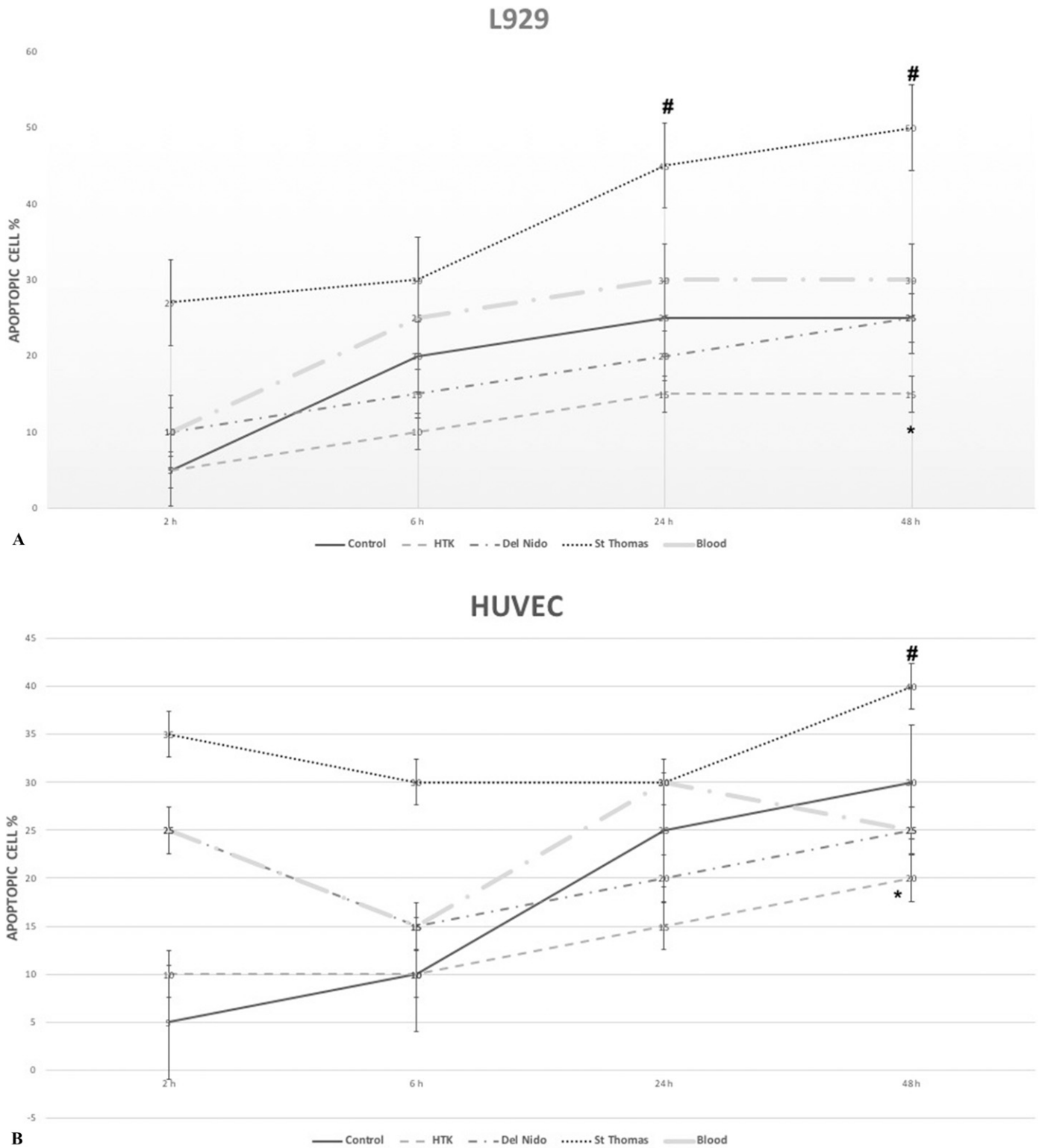


Figure 4. Apoptotic cell percentage of L929 (A), HUVEC (B), and H9C2 (C) cell lines after treatment with different cardioplegic solutions (*: $p < .05$ better with respect to control; #: $p < .05$, worse compared with control).

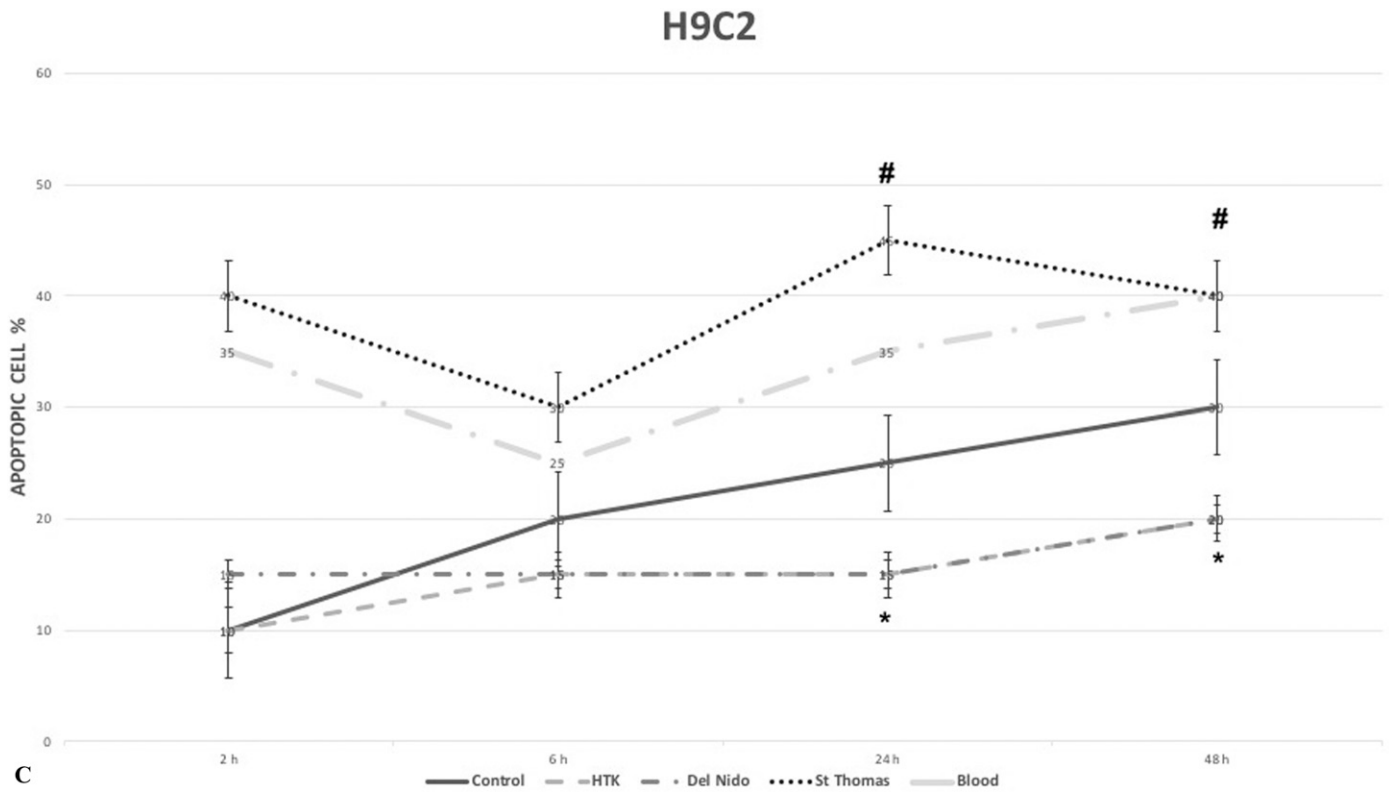


Figure 4. Continued.

LDH Cytotoxicity Test

The LDH test did not differ significantly between groups in L929 and HUVEC cell lines (Figure 5). The level of cell disruption was substantially higher for St. Thomas and blood cardioplegia than the control medium in H9c2 cells. Higher levels mean higher cell lysis (#: $p < .05$ compared with control).

DISCUSSION

Despite extensive research on the topic, the issue of myocardial protection methodology remains unresolved and is largely a matter of surgeon preference and comfort. Because new solutions have become more prominent, they

LDH Assay Results

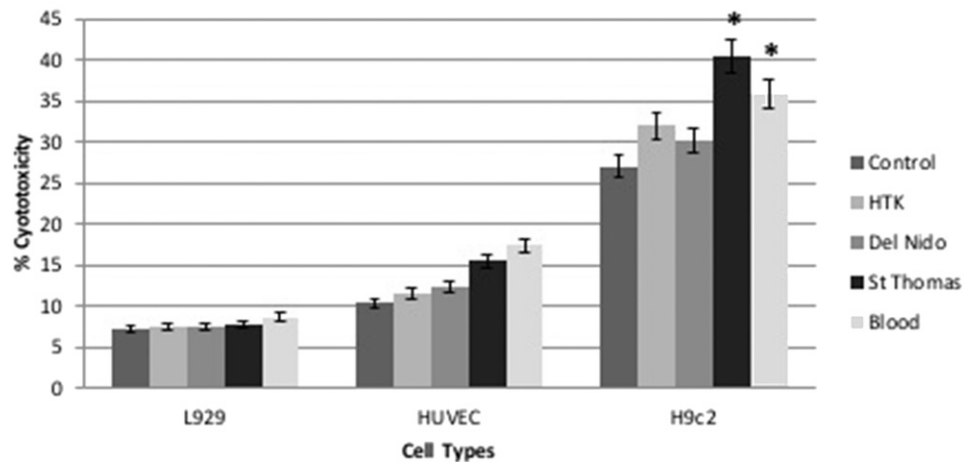


Figure 5. LDH test in three different cell lines treated with other cardioplegic solutions (#: $p < .05$ with respect to control).

have failed to demonstrate a clear clinical advantage or detriment when studied (10). An issue of paramount importance in cardiac surgery is that new cardioplegia solutions require rigorous testing to ensure safety and clinical efficacy (2).

Recently, there has been growing interest in the use of single-dose cardioplegia in adults (11,12). With encouraging *in vivo* animal data and demonstrated safe use in pediatric patients, HTK and del Nido solutions provide longer durations of myocardial arrest. They are believed to reduce myocardial damage through improved preservation of the intracellular milieu (13). Although comparative studies have demonstrated equivalent clinical outcomes to traditional multidose cardioplegia, these studies were small and retrospective, with only indirect benefits described inconsistently (14,15).

Many debates, therefore, remain for which solution, in which operation would work best, and how long an ischemic time is safe. Although these factors have been the focus of many recent meta-analyses, no approach has convincingly demonstrated superiority. Most trials investigating the long-term impact of cardioplegic solutions failed to detect differences in patients' clinical outcome because of the limited direct implications of cardioplegia on hard clinical endpoints (16,17).

Our initial aim was to provide evidence-based explanations to the two most essential dilemmas in the current cardioplegia debate: is there a better solution for pediatric/adult patients, and is it safe to extend ischemic time of single-dose techniques to around 90–100 minutes.

We set up a design mimicking a routine cardiac surgery patient (adult/pediatric) with an additional focus on the connective tissue/myocardium. We applied the solutions as we do in clinical practice. We tested viability in the long-term (MTT analysis), especially the degree of protection via demonstration of apoptosis and necrosis (AO/PI staining). We hypothesized that an ideal cardioplegic solution would improve cell viability beyond the control wells because cardioplegia would better protect the cells than any simple cell medium solution.

Blood cardioplegia was even equivalent/better in some measurements. This may suggest caution in the use of del Nido solution for 100-minute long ischemic times. Using del Nido single dose with a target time shorter than 100 minutes and redosing may be suggested as an option, but this protocol should also be further studied.

In the pediatric setup, both del Nido and HTK worked fine with 15–20% apoptosis/necrosis levels. LDH cytotoxicity testing was mainly used to detect any negative impact of components in the solutions, mainly lidocaine or amino acids. We expect to see toxic effects in 2/6-hour measurements. We did not demonstrate any toxic outcomes in the early measurements. Late-term cell lysis by St. Thomas/blood cardioplegia may be due to the insufficiency

of protection, rather than toxicity. We are studying the redosing effect of cardioplegic solutions in our current studies. The LDH test would be more helpful in demonstrating any toxicity in this sense.

Our findings, although providing novel insights into the cellular pathways of cardioplegic ischemia, do have some limitations. We studied cardiomyocytes in an *in vitro* cell culture model mimicking temperature/delivery/ischemic time conditions during clinical applications. Incubating the cells in the solutions for 100 minutes is similar to how cardioplegia is applied in clinical use. Still, the creation of an ischemic challenge to the cells was not wholly mimicked because this would require a 100% nitrogen chamber. Without an ischemic challenge, the benefits of redosing with cold, oxygenated blood cardioplegia may be reduced such that those solutions might be disadvantaged by this protocol. Again, the advantages of redosing with St. Thomas/blood cardioplegia, which keeps the heart colder, do not fit the clinical setup in this *in vitro* model. We need to overcome this main limitation via using different tests focused on similar parameters, mostly verified with each other.

Interpretation of a basic science study of *in vivo*/animal study nature is always challenging. One issue is that healthy animal hearts were arrested, but not stressed further in terms of a surgical insult. The additional time of on-pump reperfusion also presents a departure from the norm in cardiac surgery, calling into question how well the model approximates the real world (18).

The data presented here clearly show that single-dose cardioplegic techniques exert additional protection for cardiomyocytes, especially in neonatal/pediatric cell lines. del Nido in the adult setting fails to reach satisfactory protection in the long-term. Blood cardioplegia with 25-minute intervals can provide adequate protection in adult cell lines.

The high-quality data obtained from prospective, multicenter trials will help determine which solution is effective in protecting all types of patients undergoing all types of adult/pediatric cardiac surgeries under all conditions. Until then, the jury is still out regarding the role of techniques in clinical practice.

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