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Hypertonic Saline Modulates Heart Function and Myocardial Inflammatory Alterations in Brain-Dead Rats

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ABSTRACT

Background: Brain death (BD) in potential organ donors is responsible for hemodynamic instability and organ hypoperfusion, leading to myocardial dysfunction. Hypertonic saline (HS) is a volume expander with positive effects on hemodynamics and immunomodulation and was tested in this study to prevent left ventricular (LV) dysfunction and myocardial injury.

Methods: BD was induced in anesthetized Wistar rats by inflating a subdural balloon catheter, except in sham-operated animals ($n = 6$). After BD induction, Control animals received only normal saline solution (NaCl 0.9%, 4 mL/kg; $n = 6$), and treated animals were divided to receive HS (NaCl, 7.5% 4 mL/kg) at 1 min (HS1, $n = 6$) or 60 min (HS60, $n = 6$) thereafter. We continuously assessed cardiac function for 6 h with LV pressure-volume analysis. Inflammatory response, markers of myocardial injury, and cellular apoptosis –related proteins were investigated.

Results: BD was associated with decreased LV systolic and diastolic function. In comparison with the Control group, HS treatments improved LV ejection fraction (HS1, 51% [40-66]; HS60, 71% [28-82]; Control, 46% [23-55]; $P < 0.05$) and other parameters of LV systolic function 6 h after BD induction. However, no ventricular relaxation advantages were observed during the same period. HS treatments increased antiapoptotic protein expression and decreased vascular adhesion molecule and tumor necrosis factor alpha expression. No significant differences in histologic or structural protein changes were observed between groups.

Conclusions: The observed data suggest that HS ameliorates LV systolic dysfunction and seems to reduce myocardial tissue compromise in BD rats, even when the treatment is performed during the process triggered by this event.

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Introduction

Hemodynamic instability and primary cardiac dysfunction are important limiting factors for organ availability and result in exclusion of more than 25% of potential heart donors from transplantation.[1] In this regard, brain death (BD) induces a catecholamine storm with an extreme circulatory overload, followed by neurohormonal dysfunction.[2-4] BD in the potential organ donor also causes systemic inflammatory and microcirculatory changes that compromise organ function and promote apoptosis.[2,4] Moreover, during the ischemia/reperfusion period related to a transplantation procedure, the myocardium is also subjected to additional biochemical and metabolic aggression.[5]

Hypertonic saline (HS) is a volume expander that can restore hemodynamics in hypotensive conditions such as hemorrhagic shock[6] and has been shown to induce immunomodulatory effects.[7-9] We previously demonstrated that HS improves microcirculation and reduces inflammation after BD induction, opening the possibility that the use of HS in BD donors may improve organ viability and transplantation results.[10] Importantly, HS infusion before prolonged ischemia and reperfusion has been demonstrated to protect against myocardial stunning and to prevent apoptosis in a heart transplantation model in pigs,[5] despite only being equivalent to isotonic saline in the pretreatment of lung donors with hemorrhagic shock.[11] In the present study, we tested the hypothesis that treatment with HS would prevent left ventricular (LV) dysfunction and myocardial injury caused by BD itself in a rodent model.

Materials and methods

Animals

A total of 48 male Wistar rats weighing 250 to 350 g were used in the experiments. The protocol was approved by the Animal Subject Committee of Sao Paulo University Medical School. All experiments were performed under the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation and rats received humane care in compliance with the 2011 "Guide for the Care and Use of Laboratory Animals", recommended by the U.S. National Institutes of Health.

Study design

After stabilization and baseline measurements, animals were randomly assigned to 4 groups as follows: (1) sham-operated animals (Sham, $n = 12$)—without BD induction; (2) control group (Control, $n = 12$)—after BD induction, injected intravenously with 4 mL/kg normal saline (NaCl 0.9%) as a bolus; (3) hypertonic saline 1 min (HS1, $n = 12$)—immediately after BD induction, injected intravenously with 4 mL/kg HS (NaCl 7.5%) as a bolus; and (4) hypertonic saline 60 min (HS60, $n = 12$)—60 min after BD induction, injected intravenously with 4 mL/kg HS as a bolus. All animals were blind-evaluated for 6 h after the induction of BD. The animals were then exsanguinated from the abdominal aorta and the heart was removed for histopathological, immunochemistry and enzyme-linked immunosorbent assay (ELISA). Six rats per group were used for pressure-volume analysis and another six per group were used for serum and cardiac tissue analysis to avoid any bias related to long-term hemodynamic monitoring.

Anesthesia and surgical preparation

After induction of anesthesia with 5% isoflurane and orotracheal intubation, the animals were ventilated mechanically in a rodent ventilator (Harvard Apparatus, model 683). Anesthesia was maintained with 2% isoflurane inhalation until BD induction and during the entire experiment in the Sham group. The caudal artery was cannulated for continuous mean arterial pressure (MAP) monitoring and blood sampling. A central venous catheter was inserted for continuous infusion of 0.9% saline solution (2 mL/h) to minimize dehydration.

BD was induced as previously described.[2] Using a surgical drill (Dentsclear, São Paulo, Brazil), a small hole was drilled through the skull and a 4-F Fogarty balloon catheter (Edwards Lifesciences LLC, Irvine, CA) was inserted subdurally. BD was induced by rapid inflation with 0.5 mL saline; then, anesthesia was immediately interrupted. BD was confirmed through mydriasis, apnea, absence of reflexes, and a drop in the MAP.

Hemodynamic measurements

In all groups, a 2-F microtip pressure-volume conductance catheter (SPR-838 AD Instruments Inc, Colorado Springs, CO) was inserted into the right carotid artery and advanced into the LV to assess cardiac function during a 6-h period. Except in sham-operated animals, BD was induced after a 5-min period of stabilization. Using an acquisition system (MPVS, AD Instruments Inc) coupled to pressure-volume analysis software (LabChart 8, AD Instruments Inc), LV end-systolic pressure (Pes), LV end-diastolic pressure (Ped), maximum rate of rise of LV pressure (dP/dt_{max}), LV end-diastolic volume (EDV), ejection fraction (EF), stroke volume (SV), and stroke work (SW) were calculated. Ventricular relaxation was assessed by the time constant of LV pressure decay (Tau) and maximum rate of fall of LV pressure (dP/dt_{min}).

Histopathologic examination

Heart samples from each group were obtained at the end of the experimental protocol and fixed in buffered paraformaldehyde solution (10%) and embedded in paraffin. Then, 4-μm-thick sections were stained with hematoxylin and eosin. Inflammation and edema were evaluated by two blinded researchers and the score used to measure the intensity of tissue alterations was 1, 2, 3, or 4 (absent, slight, moderate, and intense, respectively).

Immunohistochemistry analysis

Frozen serial 8-μm-thick sections of heart tissue samples were placed on organosilane (Sigma Chemical Co, St. Louis, MO)–coated slides and fixed in cold acetone for 10 min.

ICAM-1 and VCAM-1
For intercellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1) immunodetection, monoclonal antibodies were used (Abcam Inc, Cambridge, MA).

Bcl-2 and caspase-3
Polyclonal antibodies (Abcam Inc) were used for Bcl2 and caspase-3 immunodetection.

Alpha-actin
For alpha-actin detection, antialpha-actin antibodies (Dako North America Inc, Carpinteria, CA) were used.

In all cases, image acquisition was processed by a digital camera DS-Ri1 (Nikon, Tokyo, Japan), attached to a fluorescence microscope (Nikon). An image analysis software (NIS-Elements-BR—Nikon) was used to obtain marked area percentage values.

Corticosterone and troponin-I analyses

Samples of centrifuged blood were obtained from the abdominal aorta at the end of the experimental protocol. Serum corticosterone and troponin-I concentrations were determined from supernatant with ELISA (R&D Systems Inc, Minneapolis, MN).

Biochemical examination

Interleukin-1, interleukin-10, cytokine-induced neutrophil chemoattractant 1, and tumor necrosis factor alpha (TNF-α) quantification in cardiac tissue was performed with ELISA using DuoSet kits (R&D Systems Inc).

Statistical analysis

Data were analyzed using GraphPad Prism 6.1 software (GraphPad Software Inc, La Jolla, CA). Results are expressed as median and variation of minimum to maximum values. The data were analyzed using the Kruskal–Wallis test, followed by Dunn's test for multiple comparisons; or 2-way analysis of variance, followed by Bonferroni tests for multiple comparisons, after rank transformation of the absolute values. P-values <0.05 were considered significant.

Results

There were no deaths, and the overall volume of fluid administered during the experiments was similar between the groups. In all groups, no differences were observed in blood gases, cell counts, or basal electrolyte levels (data not shown). The blood sodium levels remained stable in the Sham group throughout the experiment, but were elevated in the Control group at 6 h relative to basal levels and were compared with the Sham group. In animals treated with HS, significant hypernatremia was observed at 3 and 6 h when compared with basal levels and the Sham and Control groups (Table 1).

Heart rate and mean arterial pressure

Before BD induction, no significant baseline differences were measured in heart rate (HR) and MAP between experimental groups. After BD induction, MAP showed a constant decrease in BD groups when compared with the Sham group. In the Control group, MAP reached the same levels as in the Sham group after 3 h of experimentation and then progressively declined, becoming significantly decreased at 6 h. Compared with the Control group, animals treated with HS demonstrated different results. From 2 to 4 h of experimentation, the HS1 group showed a significant sustained MAP elevation when compared with the Control group. In the HS60 group, significant MAP elevation was observed from 5 h to the end of the experiment. HR remained constant in the Sham group; however, a decline in HR was observed in BD animals (Table 2).

Left ventricular pressure-volume analyses

Systolic LV function
Basal pressure-volume parameters were similar in all groups before BD induction. After BD induction, the following changes were observed (Fig. 1): end-systolic pressure—when compared with the Sham group, Pes reduction was observed in all BD animals at 30 min. Although HS1 did not show any difference when compared with the Control group, an elevation in Pes was observed in the HS60 group that appeared consistent after 4 h of experimentation (Fig. 1A).

End-diastolic pressure
No differences were observed in Ped among experimentation groups (data not shown). dP/dt_{max}: significant reduction was observed in BD animals that persisted for up to 2 h, when dP/dt_{max} reached Sham group levels. After 6 h of experimentation, a new significant decrease in dP/dt_{max} was detected in the Control group when compared with Sham and HS groups (Fig. 1B).

Table 1 – Serum sodium levels in brain dead rats treated with hypertonic saline.

Time(min)	Sham	Control	HS1	HS60
0	139 (137-143)	141 (139-144)	138 (137-142)	142 (139-144)
180	146 (139-157)	152 (148-159)	158* (155-163)	157* (151-165)
360	142* (139-146)	150 (147-159)	148 (143-161)	159* (151-166)

Data are expressed as median and variation of minimum to maximum values for 12 animals per group.
*$P < 0.05$ compared with Control.

Table 2 – Heart rate and median arterial pressure in brain dead rats treated with hypertonic saline.

Time (min)	Sham		Control		HS1		HS60	
	MAP	HR	MAP	HR	MAP	HR	MAP	HR
0	93 (75-115)	335 (282-397)	98 (79-111)	361 (318-417)	99 (86-111)	371 (352-412)	100 (89-112)	376 (330-425)
30	90* (54-138)	320 (271-404)	54 (43-62)	279 (211-371)	55 (52-66)	302 (248-362)	55 (43-69)	238 (227-331)
120	84 (45-129)	326 (269-379)	70 (54-83)	272 (157-364)	89* (69-163)	272 (208-296)	79 (58-106)	208 (173-299)
240	75 (47-114)	319 (270-366)	72 (50-105)	266 (134-395)	101* (88-115)	246 (196-302)	83 (54-94)	190* (152-276)
360	91* (46-116)	318* (277-406)	60 (48-88)	276 (125-350)	84 (73-96)	227 (83-326)	86* (75-99)	186 (140-280)

Data are expressed as median and variation of minimum to maximum values for 12 animals per group.
*$P < 0.05$ compared with Control.

End-diastolic volume

After BD induction, reduction in EDV was observed in the Control group. The early-treated group (HS1) showed a significant elevation of EDV when compared with the Control group, whereas the HS60 group showed the same pattern as the Control group (Fig. 1C).

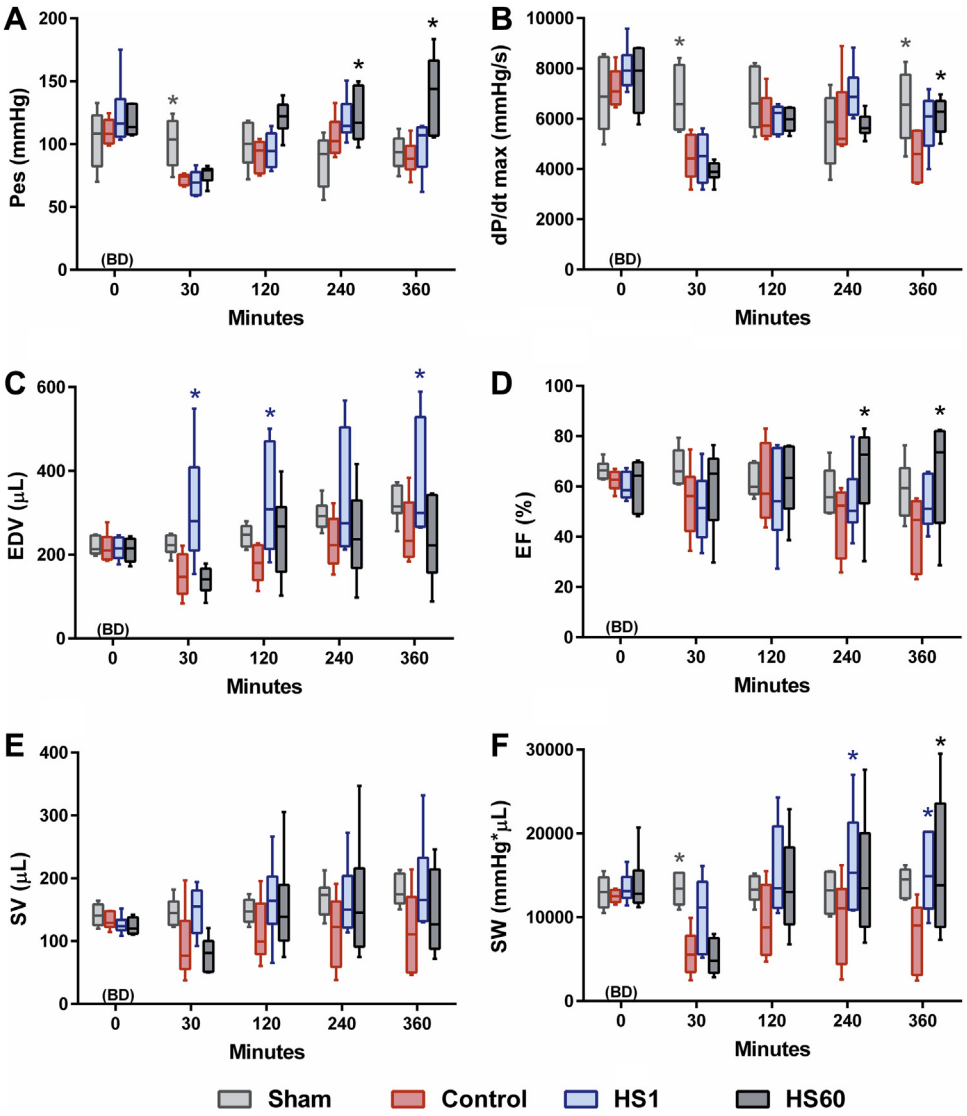

Fig. 1 – Time-course influence of brain death on left ventricular (LV) systolic function. (A) Left ventricular end-systolic and end-diastolic pressure (Pes/Ped), (B) maximum rate of rise of LV pressure (dP/dt max), (C) LV end-diastolic volume (EDV), (D) LV ejection fraction (EF), (E) LV stroke volume (SV), and (F) LV stroke work (SW). Data are expressed in box plots as median, quartiles, and min to max values for six animals per group, *$P < 0.05$ *versus* Control. (Color version of figure is available online.)

Ejection fraction
An increase was observed after 4 h up to the end of the experiment in the HS60 group when compared with the Control group. No statistically significant differences were detected among the other groups (Fig. 1D).

Stroke volume
Despite divergence between curves, no statistically significant differences were observed during the 6 h of experimentation, (Fig. 1E).

Stroke work
Significant reduction in SW was observed after BD induction in the Control group when compared with the Sham group. Two hours after BD induction, a sustained recovery in SW was observed in HS groups, and these groups showed a significant increase in SW when compared with the Control group after 6 h of experimentation (Fig. 1F).

Diastolic LV function
No differences in basal relaxation parameters were observed between groups. After BD induction, differences were observed between groups (Fig. 2) as follows: dP/dt_{min}: from the time of BD induction, a significant increase in dP/dt_{min} was detected in BD groups. HS groups were not significantly different from the Control group (Fig. 2A).

Tau
From the time of BD induction, divergences between Tau curves were observed. However, after 2 h of experimentation, a significant difference was detected in the Control group when compared with the Sham group; this difference persisted with time. Groups treated with HS showed had curves like that in the Control group, whereas the HS60 group showed a significant increase in Tau even when compared with the Control group after 4 h (Fig. 2B).

Cardiac tissue analysis

Cardiac histology
After 6 h of BD induction, no significant differences were observed between groups regarding cell infiltrate and edema (data not shown).

Leukocyte adhesion molecule expression
BD groups showed a significant increase in VCAM-1 expression when compared with the Sham group. When compared with the Control group, HS treatments caused a significant decrease in VCAM-1 expression (Fig. 3A). On the other hand, ICAM-1 expression was similar between groups (Fig. 3B).

Bcl-2 and caspase-3 expression
A significant increase in Bcl-2 expression was observed in HS groups when compared with the Control group (Fig. 3C). Caspase-3 expression was not significant in any tissue samples.

Alpha-actin expression
No difference was observed in alpha-actin expression between groups (Fig. 3D).

Corticosterone and Troponin-I

Table 3 shows a significant decrease in serum corticosterone in BD groups when compared with the Sham group after 6 h of experimentation, but no differences were detected among BD groups. Troponin-I levels were not different among groups when compared with the Sham group.

Biochemical examination

When compared with the Control group, a statistically significant reduction in TNF-α was observed in HS groups. No statistically relevant differences were detected in cytokine levels after BD induction (Table 4).

Discussion

The present study examined HS effects on hemodynamics, LV function, and myocardial tissue alterations after BD induction in rats. Although no significant differences in histological or tissue injury markers were observed between groups, HS caused an increase in antiapoptotic molecule expression and a decrease in inflammatory markers, mainly in early-treated animals. Moreover, HS had a positive impact on maintenance of LV functional parameters in BD animals for 6 h of experimentation.

Organ perfusion and oxygenation depend on the integrity of the microcirculation and persistent microcirculatory dysfunction is fully established 30 min after induction of BD in male rats.[2,4] This mechanism is associated with systemic inflammation and compromised multiorgan function. As

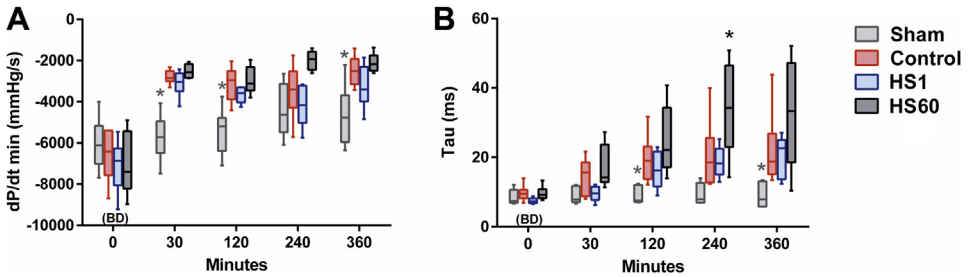

Fig. 2 – Time-course influence of brain death on left ventricular (LV) diastolic function. (A) maximum rate of fall of LV pressure (dP/dt min) and (B) time constant of LV pressure decay (Tau). Data are expressed in box plots as median, quartiles, and min to max values for six animals per group, *P < 0.05 *versus* Control. (Color version of figure is available online.)

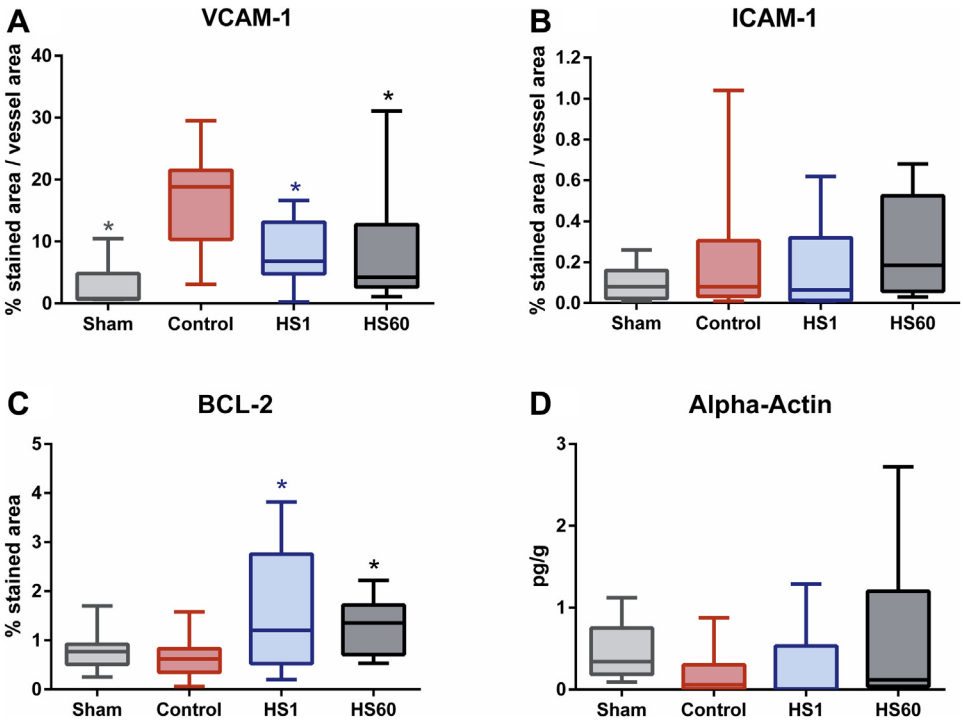

Fig. 3 – Cardiac tissue analysis by immunohistochemistry in brain dead rats. (A) Vascular adhesion molecule (VCAM-1), (B) intercellular adhesion molecule (ICAM-1), (C) antiapoptotic molecule (Bcl-2), and (D) alpha-actin. Data are expressed in box plots as median, quartiles, and min to max values for six animals per group, *$P < 0.05$ *versus* Control. (Color version of figure is available online.)

demonstrated by Li *et al.*,[12] BD causes a progressive decrease in MAP and myocardial contractility, characterized by decreases in EF, ventricular elastance, and preload-dependent recruitable SW, as well as worsening diastolic function, demonstrated by dP/dt_{min} and Tau. In our study, we observed similar LV functional behavior in the Control animals as compared with the Sham animals. However, by contrast, we observed transient MAP and LV functional recovery approximately 2 h after BD induction. These differences were potentially related to diverse anesthetic protocols used in each study. While intraperitoneal phenobarbital used by Li *et al.* has longer effects on peripheral vascular resistance reduction, inhaled isoflurane used in our study rapidly disappears after discontinuation. However, our findings agree with those obtained by Sebening *et al.*[3] in dogs and can be explained by the biphasic catecholamine release pattern found by Chiari *et al.*[13] in their study.

HS use, with or without colloids, is well established in a hemorrhagic shock model,[6,14] showing excellent results in hemodynamic and microcirculatory recovery, with consequent organ perfusion improvement and inflammation reduction.[8] Sztark *et al.*[15] were the first to use HS in a BD model. In a clinical trial, they demonstrated that HS can improve cardiac output and oxygen transportation in potential organ donors. In the present study, as also demonstrated by those authors, HS used shortly after BD induction caused an increase in SV and EF due to cardiac preload augmentation, as compared with controls. Similar changes were also documented when HS was infused during the process triggered by BD, and these events were followed by preservation of LV systolic function during the overall follow-up. The mechanisms involved in these positive changes after HS use seem to be related to more than volume expansion properties alone, as all groups received similar amount of fluids.

According to Herijgers *et al.*,[16] BD reduces myocardial perfusion and contractility through vasospasm and endothelial dysfunction, which leads to tissue hypoxia. HS demonstrated benefit in organ hypoperfusion conditions such as

Table 3 – Serum corticosterone and troponin levels after 6 h in brain dead rats treated with hypertonic saline.

Variables	Sham	Control	HS1	HS60
Corticosterone (pg/mL)	2.09* (1.61-5.65)	0.66 (0.01-1.11)	0.56(0.32-1.2)	0.44(0.18-1.06)
Troponin (pg/mL)	0.08 (0.01-0.35)	0.05 (0.03-0.15)	0.04 (0.02-0.16)	0.06 (0.03-0.36)

Data are expressed as median and variation of minimum to maximum values for six animals per group.
* $P < 0.05$ compared with Control.

Table 4 – Myocardial tissue concentration of TNF-α and cytokines after 6 h in brain dead rats treated with hypertonic saline.

Variables	Sham	Control	HS1	HS60
TNF-α (pg/mL)	301 (182-433)	392 (270-431)	252* (177-291)	420 (362-526)
Cinc-1 (pg/mL)	441 (237-567)	383 (189-517)	341 (310-1000)	427 (267-1000)
IL-1 (pg/mL)	837 (508-1312)	1388 (919-1296)	1083 (439-1296)	1489 (996-1752)
IL-10 (pg/mL)	1333 (610-2132)	1795 (1190-2279)	1053 (759-2228)	1042 (1228-2252)

Data are expressed as median and variation of minimum to maximum values for six animals per group.
TNF-α = tumor necrosis factor alpha; Cinc-1 = cytokine-induced neutrophil chemoattractant 1; IL-1 = interleukin-1; IL-10 = interleukin-10.
*$P < 0.05$ compared with Control.

hemorrhagic shock and ischemia-reperfusion models. This fact is particularly important in cardiac transplantation, in which time and organ ischemic preservation are crucial to improved outcomes. Badiwala *et al.*[5] demonstrated in pigs that HS used before the transplantation ischemia period causes improvement in hemodynamic parameters and LV contractility after transplantation. In cardiac ischemia-reperfusion animal models, HS used before ischemia induction produces contractile recovery improvement after reperfusion, as demonstrated by Waagstein *et al.*[17] Using isolated dog hearts, Harada *et al.*[18] also showed early positive results of HS on hemodynamic response in an ischemia-reperfusion model. In our study, the improvement of LV systolic function observed after HS treatment may have contributed to improved coronary flow, thereby avoiding late deterioration found in the Control group.

As previously demonstrated,[12] BD causes significant diastolic dysfunction, represented by increased dP/dt_{min} and Tau values, which agrees with our study findings. Although HS treatment improves LV systolic function in shock models,[19] it did not improve diastolic function.[20] Similarly, the HS groups had equal diastolic function results when compared with the Control group in the present study.

In addition to classical hemodynamic manifestations, BD also produces a range of neurohumoral and inflammatory effects that culminate in tissue injury.[4] Studies have shown that serum troponin I elevation in potential organ donors is related to poor prognosis after transplantation.[21,22] In our study, no significant changes in histopathological profile were identified when comparing different groups. No changes in alpha-actin expression or plasma troponin-I increase were recognized. Despite a relatively long experimental time, this protocol may have been insufficient to detect myocardial tissue alterations in this model.

On the other hand, mechanisms involving functional worsening and graft deterioration in heart transplantation are generally linked to cell death caused by apoptosis.[23] Adrie *et al.*[24] demonstrated an increase in proapoptotic protein expression and inflammatory cytokine elevation in patients with BD. Belhaj *et al.*[25] verified that methylprednisolone reduces, but does not abolish, the BD inflammatory reaction and apoptosis. In our study, no apoptosis evidence was detected because caspase-3 was not detected in any group. On the other hand, in agreement with HS shock models,[26,27] HS increased Bcl-2 tissue expression, establishing a mechanism of apoptosis prevention.

Besides hemodynamic collapse, BD also causes an augmented inflammatory response. BD animal serum shows increased levels of inflammatory cytokines and TNF-α, as previously demonstrated in several studies.[2,25,28] Inflammation is closely related to BD organ dysfunction, as proven by interleukin inhibitor in experimental studies.[25] Moreover, HS has proven immunomodulatory and anti-inflammatory effects, as evidenced by numerous studies.[9,10,29] Other authors[30] demonstrated that HS can reduce leukocyte adhesion molecule expression in shock models. In the present study, HS was specifically able to decrease leukocyte adhesion molecule expression as demonstrated by VCAM-1, and, when used early, reduced TNF-α levels, indicating a positive immunomodulatory effect on the heart. This tendency to decreased inflammatory response in the early treated group indicates that HS benefits seem to be dependent on the timing of its infusion in relation to BD manifestation. Although some preliminary reports indicate a poor transplant outcome associated with hypernatremia after HS injection, a multicenter study showed a better post-transplant outcome in patients with higher serum sodium levels.[31]

This study has some limitations. Despite the use of a follow-up time of 6 h, which is normally the standard in this experimental model, the observation of the benefits of HS in LV dysfunction and myocardial tissue compromise in BD rats was restricted to initial histologic and inflammatory findings. In addition, because we focused on the early mechanisms involved in the development of heart dysfunction associated with BD, the time points used to initiate HS treatment were only immediately after BD induction and 60 min thereafter, when progressive reduction in MAP and myocardial contractility have been described. Performing HS administration shortly after BD is unfortunately not practically possible in most clinical instances, which represents a limitation to extrapolate the current results to an application in a clinical setting.

BD is a multifactorial systemic condition related to early organ damage, which is worsened by inadequate potential organ donor management. The best strategy for control of hemodynamic instability caused by BD remains unclear. HS use is an easy and inexpensive method of hemodynamic recovery that was able to maintain LV systolic function and mitigate late heart deterioration in BD animals, as demonstrated in this study. Furthermore, HS reduces inflammatory activity and apoptosis. All these effects may be advantageous in potential organ donor maintenance, representing a new

perspective on HS use, that could be started as soon as BD is determined.

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Authors' contributions: D.M.S.M., F.L.Z., and C.J.C. performed all the experiments with the collaboration of R.S. and R.G.F.S.; D.M.S.M. and L.F.P.M. analyzed the data. D.M.S.M. drafted the article and was advised by P.S. and L.F.P.M.

All authors read and approved the final article.

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Disclosure

The authors disclose any financial and personal relationships with other people or organizations that could potentially and inappropriately influence (bias) their work and conclusions.

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