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Anti-Apoptotic Protection Afforded by Cardioplegic Celsior and Histidine Buffer Solutions to Hearts Subjected to Ischemia and Ischemia/Reperfusion

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ABSTRACT

Cardiomyocytes undergo apoptosis in response to ischemia and ischemia/reperfusion (I/R). During heart preservation, inhibition of apoptosis is critical to avoid organ failure. We aimed to compare the protection afforded by Celsior (Cs) and Histidine buffer solution (HBS) against apoptotic signaling in hearts subjected to moderate (4 h) versus severe (6 h) ischemia alone or followed by 30 min reperfusion. The impact of gender on cardioplegic protection was also explored. Hearts from male and female Wistar–Han rats were divided by gender in distinct groups: control, perfusion_control, ischemia, and I/R. Ischemia and I/R groups were divided in subgroups Cs or HBS, and subjected to 4 or 6 h ischemia alone or followed by reperfusion. Proteins involved in apoptotic signaling (p53, Bax, Fas, FasL, and Flip) were quantified by Western blot in mitochondria and/or whole tissue. Caspases 3, 8, and 9-like activities were measured and hemodynamic parameters were monitored. Ischemia activated p53/Bax signaling. After I/R, HBS-preserved hearts had lower p53/Bax content in mitochondrial fractions than Cs-preserved hearts. The p53/Bax decrease in tissue samples was mostly observed in females. Caspase 3-like activity was increased in HBS-preserved male hearts. The heart rate was decreased in Cs and HBS-preserved hearts. Fas protein levels remained unaltered in all conditions but soluble FasL increased from 4 to 6 h preservation in Cs and HBS. Hearts from male rats were more prone to apoptosis and myocardial dysfunction. HBS and Cs were not effective in inhibiting apoptotic signaling although HBS presented best overall results. Both solutions should be improved to prevent apoptosis and myocardial dysfunction after I/R. J. Cell. Biochem. 112: 3872–3881, 2011.

KEY WORDS: ISCHEMIA; REPERFUSION; APOPTOSIS; HEART PRESERVATION

Heart preservation for transplantation purposes is limited to periods no longer than 6 h in cold ischemia [Muhlbacher et al., 1999]. During transplantation procedures, the heart is maintained at 4°C in cardioplegic solutions and then introduced in the recipient. During this process, lethal injury resulting from apoptosis or necrosis may occur as a result from storage (ischemia) or from the sudden reflow of oxygen (reperfusion) [Gottlieb et al., 1994]. Whether necrosis or apoptosis represent different events or a continuum process contributing to cardiac injury after ischemia–reperfusion (I/R) is still unknown but recent studies point to an increase of both processes during I/R. Nevertheless, the apoptotic process can be decreased by caspase inhibition [McCully et al., 2004] suggesting that this is a possible therapeutic strategy to inhibit I/R damage.

Cardiomyocytes undergo apoptosis in response to a variety of stimuli that occur during ischemia, especially when followed by reperfusion [Kang et al., 2000]. It is known that I/R promotes the permeabilization of mitochondrial membranes and the release of several pro-apoptotic factors, including cytochrome *c* [Krysko et al., 2001]. The formation of the apoptosome in the cytosol results in the activation of caspases, specialized proteins that amplify and execute the programmed steps in the apoptotic pathway [Riedl and Salvesen, 2007]. p53 is a key mediator of the apoptotic process. During stress signaling, p53 accumulates in the cells, and, when activated, initiates a cascade of events that results in apoptosis. Cellular death may be directly activated by p53 translocation to mitochondria [Arima et al., 2005], resulting in cytochrome *c* release, collapse of the mitochondrial membrane potential and caspase 3 activation

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[Marchenko et al., 2000]. Studies in p53-null mice showed a slight improvement of functional recovery after reperfusion [Toth et al., 2006] and others also have demonstrated that pifithrin-α, a p53 inhibitor, attenuated I/R-induced infarct size [Mocanu and Yellon, 2003; Liu et al., 2008] which suggest that p53 is a key mediator in I/R injury.

I/R may also lead to downregulation of Fas inhibitors such as cFLIP, conditioning the activation of the extrinsic apoptotic pathway. cFLIP is highly expressed in the heart under normal physiological conditions but is degraded after I/R [Rasper et al., 1998]. Upregulation of Bcl-2 family proapoptotic (Bak, Bax) and anti-apoptotic (Bcl-2, Bcl-xL) proteins has also been reported during heart failure in humans [Latif et al., 2000]. The balance between anti- and pro-apoptotic proteins decides the fate of the myocardium after I/R. Hence, in order to minimize the loss of cardiac cells during I/R, several steps in the apoptotic process must be inhibited.

We have described in previous studies [Alves et al., 2009, 2011a] the mitochondrial protection afforded by both Celsior (Cs) and Histidine buffer (HBS), as well as how both solutions regulate substrate preference during I/R. In both studies, it was concluded that HBS proved to be a good alternative to Cs. Nevertheless, those studies raised very relevant scientific questions: Can HBS be an alternative to normally used cardioplegic solutions? If so, can the protective effect include an inhibition of the apoptotic process mediated by mitochondria?

The aim of the present work was to compare the protection afforded by two cardioplegic solutions: the worldwide clinically used Cs, and an emerging solution in clinical and experimental research, HBS [Takeuchi et al., 1999a,b; Alves et al., 2009], against several apoptotic signaling events in hearts subjected to ischemia alone and I/R. Two distinct preservation periods were investigated to compare the effect of moderate (4 h) versus severe ischemia (6 h). Our hypothesis was that both cardioplegic solutions would be able to inhibit or at least delay apoptotic signaling cascades, thus explaining the cardioprotection achieved by both in previous studies [Sumimoto et al., 1991; Tolba et al., 2000]; also male and female Wistar rats were used to determine if the apoptotic signaling can be responsible for the better protection previously described in hearts from females using the same I/R model [Alves et al., 2009, 2011a].

METHODS

CHEMICALS

Ketamine was obtained from Merial (Lyon, France). Cs was obtained from IMTIX SANGSTAT (Lyon, France). All other reagents were of analytical grade and purchased from Sigma–Aldrich (Roedermark, Germany).

ANIMALS

Age-matched male and female Wistar–Han rats (Charles River Laboratories, Barcelona), weighing 260 ± 22 and 220 ± 19 g respectively, were housed in our credited animal colony (Laboratory Research Center, University of Coimbra). Animals were group-housed in type III-H cages (Tecniplast, Italy) and maintained in specific environmental requirements (22°C, 45–65% humidity, 15–20 changes/h ventilation, 12 h artificial light/dark cycle, noise level <55 dB) with free access to standard rodent food (4RF21 GLP, Mucedola, Italy) and water.

All the experiments complied 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).

CARDIOPLEGIC SOLUTIONS

Celsior solution was purchased (in mM: KCl, 15; $MgSO_4$, 13; $CaCl_2$, 0.24; NaOH, 100; glutamic acid, 20; lactobionic acid, 80; histidine, 30; mannitol, 60; glutathione, 3; pH = 7.3). HBS (in mM: NaCl, 80; KCl, 22.5; $MgSO_4$, 6; $CaCl_2$, 0.1; histidine, 100; mannitol, 20; glucose, 11; adenosine 5, plus 100 mg/L of lidocain and 10 UI/L of insulin; pH = 7.6) was prepared in our laboratory and filtered (0.2 μm, Millipore).

STUDY GROUPS

The animals were divided in two major groups according to their gender, males (M) and females (F), and were then randomly divided in four subgroups: control (Ctrl), perfusion control (Ctrl_P), ischemia, and I/R. Control animals were sacrificed and cardiac mitochondria were immediately isolated or heart tissue was collected before any ischemic insult or reperfusion. Hearts from Ctrl_P were only subjected to 30 min perfusion prior to tissue removal and mitochondrial isolation. Ischemia and I/R groups were divided into two subgroups depending on the preservation solution used and subjected to 4 or 6 h ischemia (subgroups Cs and HBS).

Ischemia was performed according to procedures already described [Ackemann et al., 2002; Alves et al., 2009]. Briefly, after peritoneal anesthesia, ketamine (50 mg/kg) plus chlorpromazine (10 mg/kg), animals were placed on a sacrifice board and an incision was made on the abdominal area. The thorax area was opened and the heart excised and carefully placed in the respective cardioplegic solution. The hearts were flushed with the respective cardioplegic solution in order to remove blood from the coronaries and to avoid the formation of blood clots and were then stored in 10 ml of the cardioplegic solution for 4 or 6 h at 4°C.

PERFUSION APPARATUS

For perfusion studies, a Langendorff perfusion system was used. Hearts were cannulated via the aorta and were perfused during 30 min at constant pressure, 100 cm H_2O, for 30 min at 37°C with a Krebs–Henseleit solution (in mM: NaCl, 118; KCl, 4.4; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaHCO_3$, 25; lactate, 1.2; pyruvate, 0.12; glucose, 5.5; octanoate, 0.3; pH = 7.6) gassed with 95% O_2 and 5% CO_2. For the Ctrl_P group, hearts were immediately cannulated in the Langendorff column after removal.

The perfusate was collected during the perfusion period, the volume registered every 10 min, and the coronary flow (ml/min) was determined. For the measurement of left ventricular developed pressure (LVDP) and heart rate throughout the perfusion, a catheter-tip pressure transducer inside a water-filled latex ballon was placed inside the left ventricle through the left atrium and the volume was adjusted at the beginning of each experiment to achieve an end-diastolic ventricle pressure of 5–10 mmHg. The hemodynamic parameters were digitally recorded using a standard routine WinDaq

DI-720 recording software (DataQ Instruments, Akron, OH). The hemodynamic parameters were converted in percentage relatively to the perfusion control to describe the relative changes in cardiac function.

ISOLATION OF CARDIAC MITOCHONDRIA

Heart mitochondria (HM) were isolated according to standard procedures in our laboratory [Oliveira et al., 2000]. Briefly, the hearts were placed in homogenization medium (HOM; in mM: sucrose, 250; HEPES, 10; EGTA 0.5; pH = 7.4), washed and cut in small pieces. The suspension was homogenized in a Potter-Elvejhem (Thomas C740) containing 0.5 mg of protease type VIII (Sigma, P5390) and 0.1% fatty acid-free BSA. The solution was centrifuged at 9,500*g* during 10 min. The supernatant was discarded and the pellet ressuspended in HOM. After a manual homogenization, with a loose-fit Potter-Elvejhem, the solution was centrifuged 10 min at 900*g*. The supernatant was collected and centrifuged again for 10 min at 9,500*g* and the pellet was suspended in washing medium (WM; in mM: sucrose, 250; HEPES, 10; pH = 7.4) and centrifuged 10 min at 9,500*g*. The pellet was ressuspended in 500 μl of WM and the protein was quantified by Biuret method using BSA as standard.

WESTERN BLOT ANALYSIS

Frozen mitochondrial or whole heart tissue preparations were homogenized in lysis buffer (1 M urea, 10 mM Tris, 2% SDS, pH 7.5) and boiled for 10 min at 60°C.

Aliquots of 50 μg were fractionated in 12% poliacrylamyde gels and electrophoresis was carried out for 1 h with 30 mA per gel. The separated proteins were afterwards transferred to polyvinylidene difluoride membranes and blocked for 1 h in a 5% non-fat milk solution at 37°C. The membranes were then incubated overnight at 4°C with goat polyclonal anti-p53 (1:1,000, Sc 6243; Santa Cruz Biotechnology), rabbit anti-Bax (1:5,000, #2772; Cell Signaling), rabbit polyclonal anti-Flip (1:500, Sc 8347; Santa Cruz Biotechnology), or rabbit polyclonal anti-Fas (1:100, Sc 7886; Santa Cruz Biotechnology). Rabbit anti-cytochrome oxidase, subunit IV (COXIV, 1:1,000, #4844; Cell Signaling) and mouse monoclonal anti-actin (1:1,000, A 5441; Sigma) were used as protein loading control for mitochondrial fraction and heart tissues, respectively. The immunoreactive proteins were detected separately and visualized with rabbit anti-goat IgG-AP (1:5,000, Sc 2771; Santa Cruz Biotechnology), goat anti-rabbit IgG-AP (1:5,000, Sc 2007; Santa Cruz Biotechnology), or goat anti-mouse IgG-AP (1:5,000, Sc 2008; Santa Cruz Biotechnology). Membranes were reacted with ECF™ (GE Healthcare), a fluorescent substrate for alkaline phosphatase-based detection, and read with the Versa Doc imagining system (Bio-Rad). Densities from each band were obtained with Quantity One Software (Bio-Rad) according to standard methods. The band density attained was divided by actin (tissue samples) or COXIV (mitochondrial samples) and expressed in percentage versus the control group.

CASPASE-LIKE ACTIVITY ASSAYS

The activity of caspases 3, 8, and 9 was spectrophotometrically assessed by determining the cleavage of the respective colorimetric substrate [Sardao et al., 2009]. Heart tissue was homogenized in nitrogen and resuspended in collecting buffer (mM: 20 HEPES, pH = 7.5; 250 sucrose; 10 KCl; 2 $MgCl_2$; 1 EDTA) supplemented with 2 mM DTT and 100 μM PMSF. Protein content was assayed by using the Bradford method for measurement of protein concentration in the samples [Bradford, 1976]. To measure caspase-like activities, 50 μg aliquots were incubated with the reaction buffer (25 mM HEPES, pH = 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS) and 100 μM of caspase substrate for 2 h at 37°C. The substrates used were Ac-DEVD-*p*NA for caspase 3, Ac-LEHD-*p*NA for caspase 9, and Ac-IETD-*p*NA for caspase 8. The caspase-like activities were determined by detection of the chromophore *p*-nitroanilide, measured at 405 nm in a spectrophotometer, after the cleavage of the labeled substrate. The method was calibrated with known concentrations of *p*-nitroanilide. The attained activities were expressed in percentage versus the control group (hearts excised and processed after animal sacrifice).

DATA PRESENTATION AND ANALYSIS

The results are presented as mean ± SEM (n = 5 for each experimental condition). To normalize the results attained from Western blot we measured the abundance of COXIV or actin for mitochondrial or tissue samples, respectively. All the results are presented in percentage relatively to the control condition, when animals were sacrificed and hearts immediately processed. Statistical comparison between selected experimental groups and/or subgroups was performed by using a one-way ANOVA, followed by Newman–Keuls post hoc test, to examine about gender differences and time-course and between the three solutions and time-course (GraphPad Software Inc.). $P < 0.05$ was considered significant.

RESULTS

p53–Bax APOPTOTIC SIGNALING IS ACTIVATED IN HEARTS FROM FEMALES AFTER ISCHEMIA ALONE

p53 is responsible for the activation of several pro-apoptotic proteins of the Bcl-2 family, including Bax, resulting in activation of the mitochondrial apoptotic signaling pathway [Chipuk et al., 2004]. The question here was to verify if p53 signaling, involving both p53 itself and Bax, was activated during ischemia alone.

In our experimental conditions, isolated mitochondrial fractions harvested from male and female hearts subjected to ischemia alone, did not show any significant differences regarding p53 or Bax protein content (data not shown).

Regarding intact cardiac tissue, although ischemia per se did not increase p53 or Bax protein in hearts from males, it did cause an increase of both proteins in cardiac tissue from females preserved in HBS and Cs during 6 h (Fig. 1A,B).

Hearts from males preserved in HBS presented lower p53 protein content than those preserved in Cs (Fig. 1A). Bax protein levels in hearts from males were decreased after 4 h preservation in HBS when compared with hearts preserved in Cs. A significant increase in Bax protein levels in all female groups was observed when compared to the control but only HBS-preserved hearts from females presented

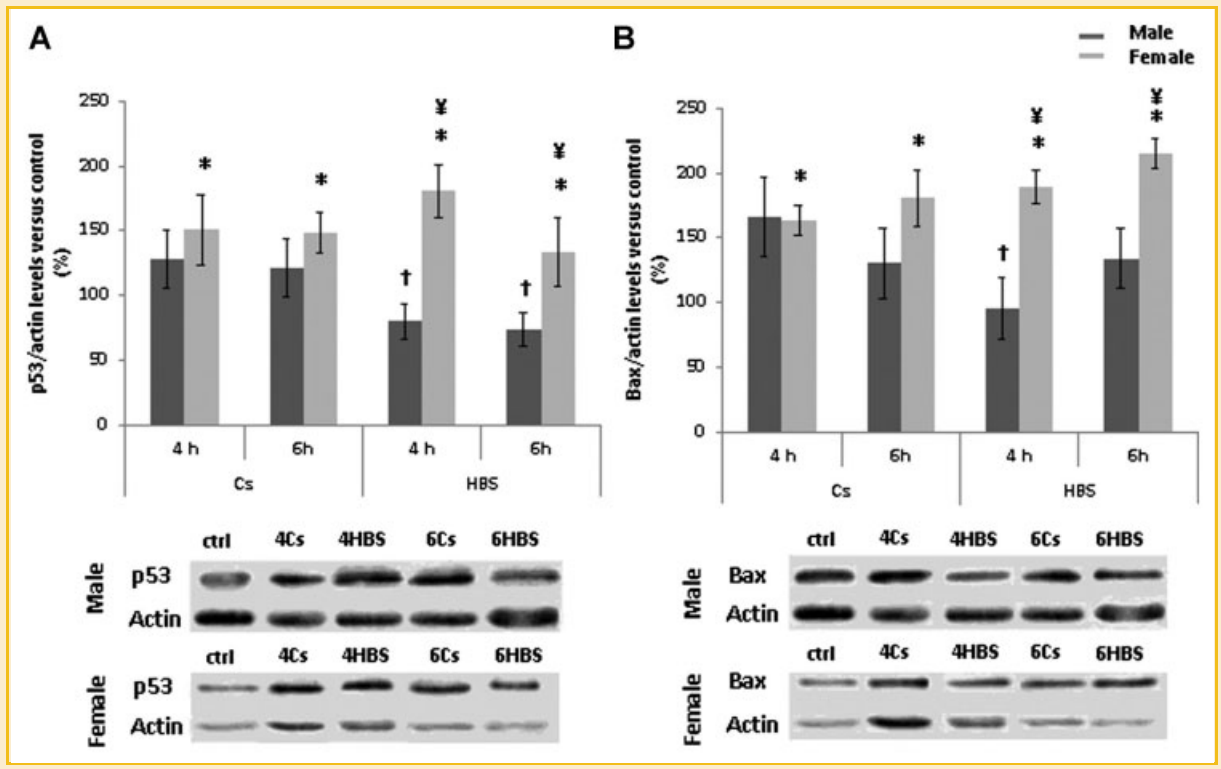

Fig. 1. p53 (part A) and Bax (part B) measured by Western blot in tissues from hearts subjected to ischemia only. Light gray—hearts from females; dark gray—hearts from males. The results are presented as mean ± SEM ($n = 5$ for each experimental condition) and normalized as percentage of control values. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; †, Celsior solution; ¥, male.

significantly higher Bax protein levels than hearts from males preserved in the same solution (Fig. 1B).

p53-Bax APOPTOTIC SIGNALING AFTER I/R

Reperfusion is reported as the main step capable of inducing lethal lesions to cardiomyocytes [Gateau-Roesch et al., 2006]. In our experimental conditions, heart perfusion (Ctrl_P) was not able, per se, to increase mitochondrial p53 protein content in hearts from males and females. In hearts from males, ischemic preservation in the two cardioplegic solutions resulted in an increase in p53 protein levels when compared with the control and Ctrl_P but this increase was smaller when HBS was used. Hearts from females preserved in Cs presented significantly higher p53 content than HBS-preserved hearts (Fig. 2A).

In hearts from females, Ctrl_P increased Bax protein levels when compared to the no-perfusion control. Hearts from males preserved in both, Cs and HBS, presented less mitochondrial Bax accumulation than hearts from females (Fig. 2B).

The tissue content in p53 was not altered after 4 or 6 h preservation in Cs or HBS in hearts from males. Hearts from females had, in general, higher p53 content than cardiac tissues from male hearts. In hearts from females, an increase of p53 protein levels was observed from 4 to 6 h preservation in Cs while preservation in HBS resulted in decreased p53 levels (Fig. 3A).

Bax content was increased in tissue samples obtained from male hearts preserved during 6 h in both Cs and HBS while hearts from females had decreased Bax protein levels after 6 h preservation in HBS. Tissue samples of hearts from females had, in general, lower Bax protein levels than the tissues of hearts from males (Fig. 3B).

EXTRINSIC APOPTOTIC SIGNALING AFTER I/R

Several proteins involved in the extrinsic apoptotic pathway were measured in tissue samples, including Fas and its ligand (FasL). FasL can exist under two distinct forms, one membrane-bound (40 kDa) and in a soluble form (26 kDa). When measuring the death receptor Fas, its content remained unaltered in all experimental conditions (data not shown). Perfusion was not able, per se, to induce changes in FasL protein. Hearts from males showed increased FasL content when ischemia was extended from 4 to 6 h in both Cs and HBS groups (Fig. 4A). Soluble FasL decreased from 4 to 6 h in HBS-preserved hearts from females (data not shown). Another protein involved in the extrinsic apoptotic pathway is the Flice inhibitory protein (Flip), which is expressed as a long ($Flip_L$) or short isoform ($Flip_S$), acting in different steps to inhibit Fas-induced apoptosis. In hearts from males, $Flip_s$ protein levels were decreased after preservation in Cs, while $Flip_L$ protein levels were increased for all experimental conditions. Hearts from females presented no relevant differences in $Flip_s$ and $Flip_L$ protein levels (Fig. 5A,B)

CASPASE ACTIVATION AFTER I/R

Caspases are critical mediators of the apoptotic process. With the alterations in apoptotic signaling verified in previous sections, the next logical step would be to verify if caspase activation was

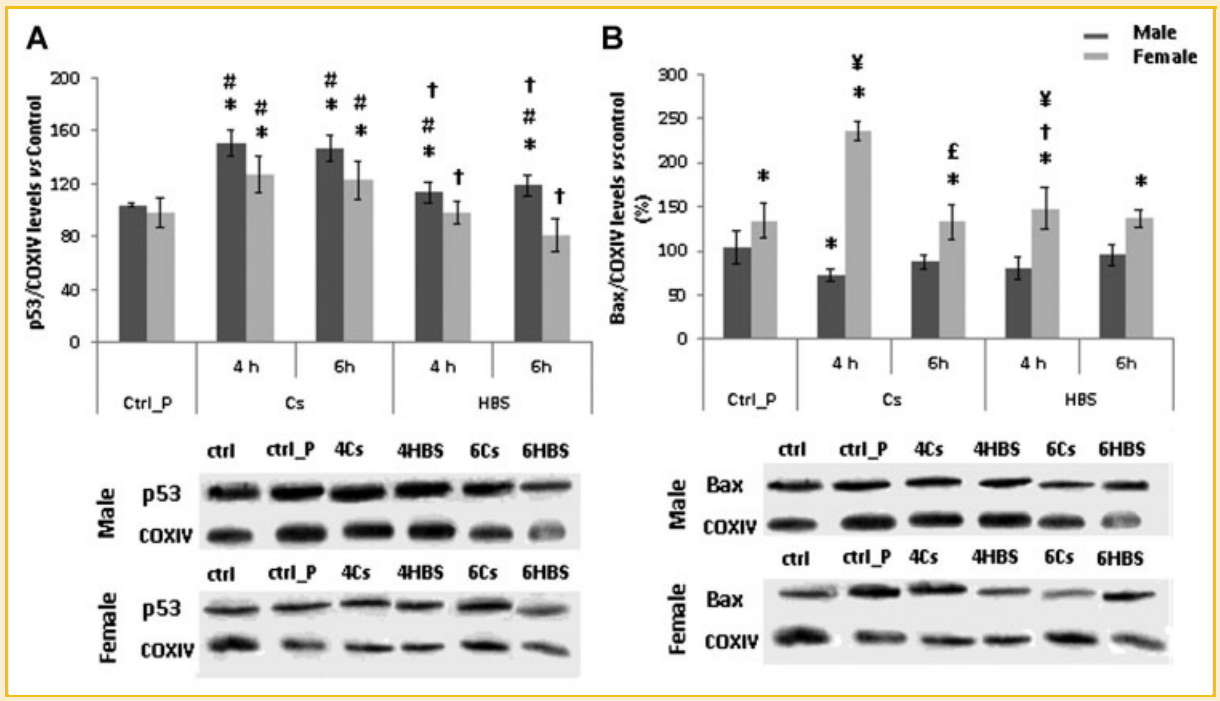

Fig. 2. p53 (part A) and Bax (part B) measured by Western blot in mitochondrial samples from hearts subjected to I/R. Light gray—hearts from females; dark gray—hearts from males. The results are presented as mean ± SEM (n = 5 for each experimental condition) and normalized as percentage of control values. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; #, perfusion control; £, 4 h preservation; †, Celsior solution; ¥, male.

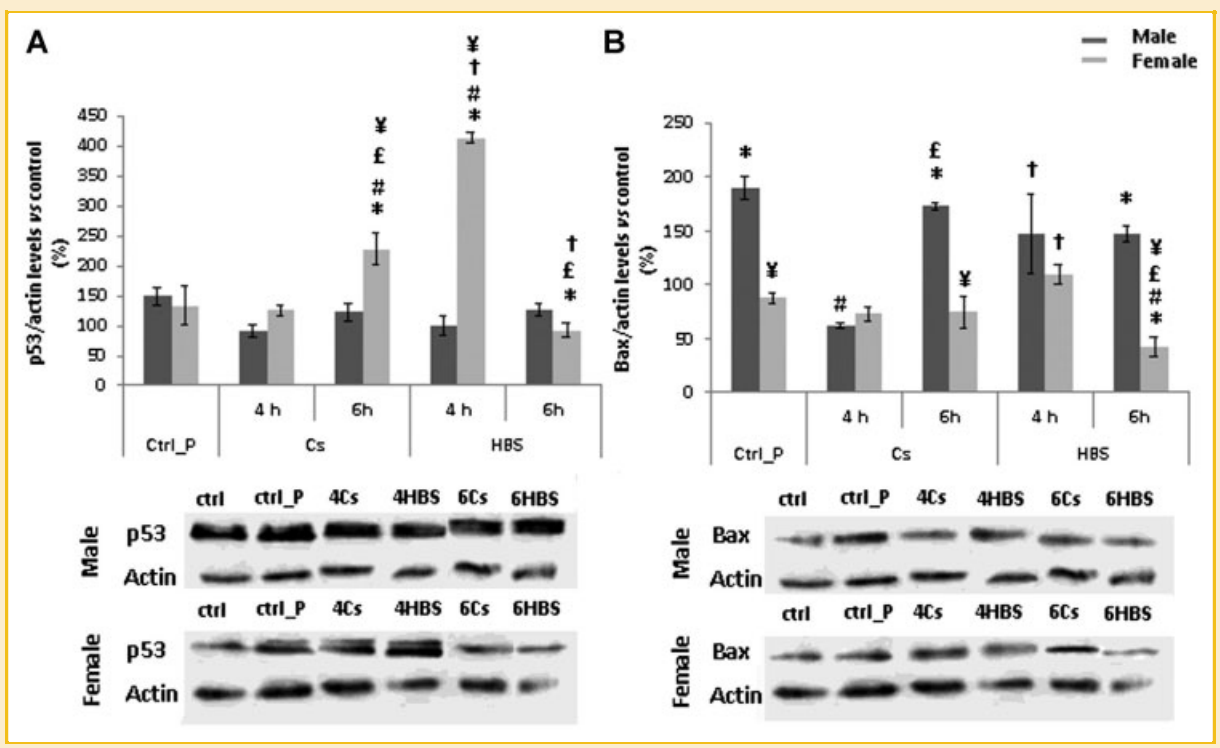

Fig. 3. p53 (part A) and Bax (part B) measured by Western blot in tissues from hearts subjected to I/R. Light gray—hearts from females; dark gray—hearts from males. The results are presented as mean ± SEM (n = 5 for each experimental condition) and normalized as percentage of control values. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; #, perfusion control; £, 4 h preservation; †, Celsior solution; ¥, male.

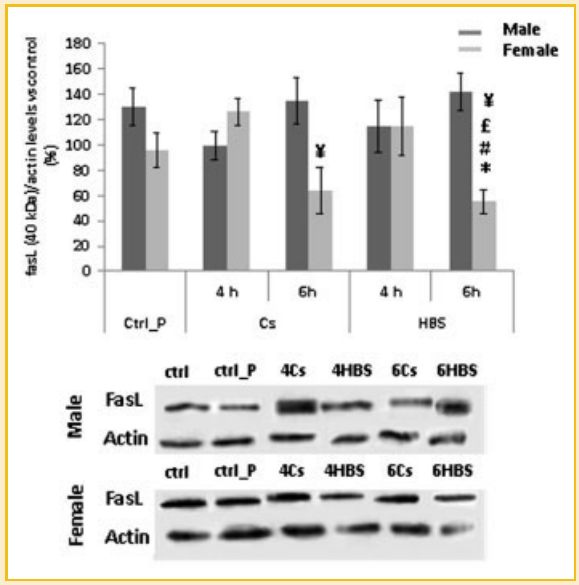

Fig. 4. Membrane-bound FasL (40 kDa) protein measured by Western blot in hearts from males (dark gray) and females (light gray) subjected to I/R. The results are presented as mean ± SEM (n = 5 for each experimental condition) and normalized as percentage of control values. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; #, perfusion control; £, 4 h preservation; ¥, male.

occurring after I/R, which would signal downstream to cause apoptosis induction.

Perfusion was not capable, per se, of altering caspases 3 and 8-like activities in hearts from males or females but clearly, gender plays an important role in the differential activation of caspases and in the protective effect of cardioplegic solutions. The only increase in caspase 8-like activity observed in hearts from males occurred when hearts were preserved in Cs (6 h) and HBS (4 and 6 h; Table I).

Caspase 9-like activity increased in all male and female experimental groups, with none of the cardioplegic solutions providing a protective effect. Higher caspase 9-like activity in the hearts from males and females was correlated with an increase in caspase 9 active fragment as measured by Western blotting (data not shown).

The results for caspase 3-like activity were not as evident, possibly because the values for the Ctrl_P were higher than the control itself, although not reaching statistical significance. Despite this, values for caspase 3-like activities were higher in HBS-preserved hearts from males (Table I).

ENDPOINTS FOR CARDIAC FUNCTION

Next and final question of the present work regarded the functional implications of the different apoptotic pathways activation during preservation in the cardioplegic solutions used.

Cardiac mechanic performance was assessed by measuring coronary flow, heart rate, and LVDP. The coronary flow during

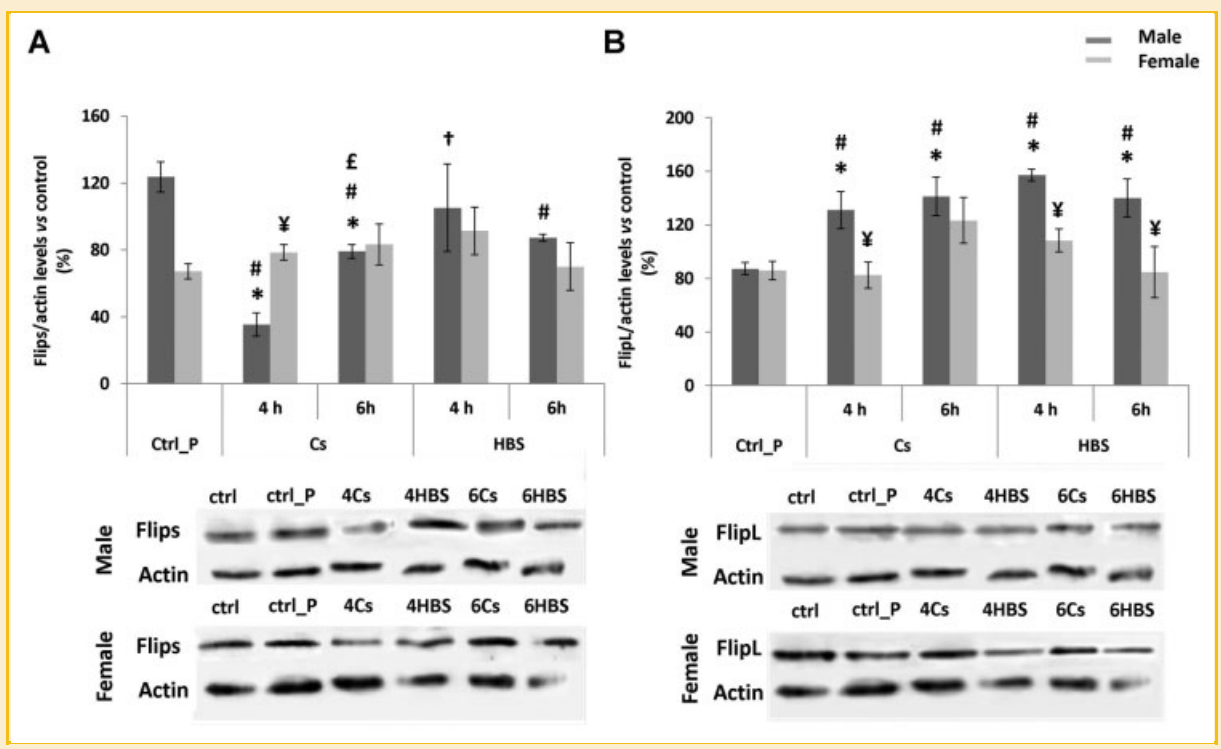

Fig. 5. Flips (part A) and FlipL(part B) measured by Western blot in tissues from hearts subjected to I/R. Light gray—hearts from females; dark gray—hearts from males. The results are presented as mean ± SEM (n = 5 for each experimental condition) and normalized as percentage of control values. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; #, perfusion control; £, 4 h preservation; †, Celsior solution; ¥, male.

TABLE I. Caspases 3, 8, and 9-Like Activities in Tissues From Hearts Subjected to I/R

	Caspase 3 activity (% of control value)		Caspase 8 activity (% of control value)		Caspase 9 activity (% of control value)	
	♂	♀	♂	♀	♂	♀
Ctrl_P	131 ± 16	140 ± 10	97 ± 14	132 ± 12	102 ± 2	180 ± 7*
Cs						
4 h	136 ± 11	103 ± 11	154 ± 14	162 ± 13	219 ± 18*,#	180 ± 16*
6 h	94 ± 15	122 ± 17	295 ± 30*,#,£	127 ± 10¥	303 ± 16*,#,£	202 ± 7*,¥
HBS						
4 h	178 ± 20*	160 ± 18	213 ± 22*,#,†	155 ± 14	300 ± 27*,#	222 ± 19*,¥
6 h	149 ± 17*	138 ± 11	253 ± 26*,#	123 ± 15¥	256 ± 17*,#	173 ± 10*

The results are presented as mean ± SEM (n = 5 independent samples for each experimental condition) and normalized in percentage versus control value. Significantly different results ($P < 0.05$) are as indicated relatively to: *control; #perfusion control, £4 h preservation; †Celsior solution; ¥male.
Ctrl_P, perfusion control; Cs, celsior; HBS, histidine buffer solution; ♂, males; ♀, females; 4 h, 4 hours ischemia + 30 min reperfusion; 6 h, 6 hours ischemia + 30 min reperfusion.

reperfusion only had slight variations between 9.3 ± 1.5 and 11.2 ± 1.8 ml/min in all experimental conditions.

The heart rate decreased in hearts from males preserved during 4 h in Cs and HBS. Hearts from males and females presented a heart rate of 58 ± 8% and 92 ± 10% of the control value after 4 h preservation in Cs (Fig. 6A). When preserved during 4 or 6 h in HBS, hearts from males and females presented lower heart rates, although in females, the decrease was lower in HBS-preserved hearts than in Cs-preserved (Fig. 6A).

The LVDP was not significantly altered in all experimental conditions, except in hearts from females preserved 6 h in HBS, which presented a significant increase in this parameter (Fig. 6B).

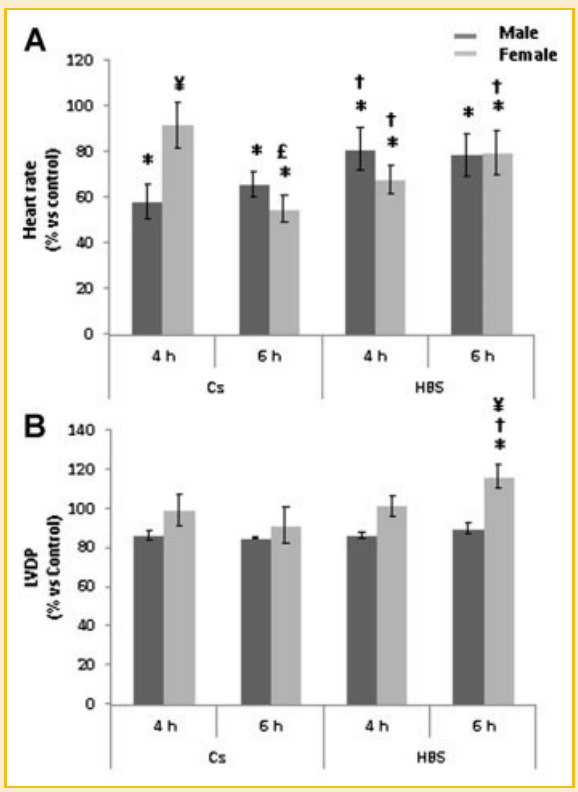

Fig. 6. Heart rate (part A) and left ventricular developed pressure (LVDP; part B) measured in hearts from males (dark gray) and females (light gray) subjected to I/R. The results are presented as mean ± SEM (n = 5 independent assays for each experimental condition) and normalized as percentage of control values. Absolute values for the perfusion control were 238 ± 21 bpm for HR, and 105 ± 8 mmHg for LVDP. Significantly different results ($P < 0.05$) are as indicated relatively to: *, control; #, perfusion control; £, 4 h preservation; †, Celsior solution; ¥, male.

DISCUSSION

Mitochondria play a critical role in several cellular death pathways and apoptotic signaling associated with I/R which is responsible for the loss of myocyte viability and transplantation failure. Not only apoptosis is involved in ischemia and I/R damage, but necrosis can also be responsible for organ deterioration during cardioplegic arrest [Sayk et al., 2004]. Both processes occur during I/R and whether they occur in separate or in overlapping events is unknown [McCully et al., 2004]. Nevertheless, our work was focused on the apoptotic process and on the ability of both Cs and HBS, to inhibit or at least delay the apoptotic signaling cascades since it has been proposed that the effects of apoptosis can be significantly decreased by caspase inhibition [McCully et al., 2004]. In fact, a specific alteration of current cardioplegic solutions aiming to delay or prevent completely the apoptotic process may be a possible therapeutic strategy to improve the cardioprotective potential of these solutions.

HBS had a marginal effect in preventing the increase of p53/Bax content in males (p53) and females (Bax) after ischemia-only conditions (Fig. 1). Concerning I/R conditions, a significant mitochondrial accumulation of p53 in males and Bax in females was detected (Fig. 2). No single solution afforded a complete degree of protection against the activation of the p53/Bax signaling pathway after I/R, although the preservation in HBS yielded overall better results than the preservation in Cs. Upon stimulation, p53 translocation occurs and precedes Bax activation [Chipuk et al., 2004], cytochrome *c* release as well as caspase 3 activation ensuing cell death [Marchenko et al., 2000] and ultimately heart failure. Myocardial dysfunction was assessed by measuring major determinants of myocardial work and viability such as heart rate and LVDP [Griffin et al., 2000; Robinet et al., 2005]. Cs-preserved hearts presented a lower heart rate upon reperfusion than those preserved

in HBS (Fig. 5), which is in the same line of the data obtained for measurement of cell death markers that were more extensive in Cs-preserved hearts.

HBS contains insulin, glucose, adenosine, and lidocain that are ascribed to enhance metabolic recovery after I/R [Albacker et al., 2007], which is critical to avoid apoptosis. Also, calcium has a crucial role in triggering apoptosis [Murphy et al., 1987]. When mitochondria are exposed to high calcium concentrations, the inner mitochondrial membrane becomes permeable to small molecules [Bouchier-Hayes et al., 2005] due to the formation of pores known as permeability transition pores (PTP). One of those small molecules that can be released is the cytochrome *c* [Krysko et al., 2001]. Cytosolic cytochrome *c* reacts with other proteins and activates pro-caspase 3 triggering apoptosis [Han et al., 2006]. Insulin, which is present in HBS, is known to exert protection against I/R injury by inhibiting intracellular calcium transients [Yu et al., 2008], which may explain the overall higher protection against mitochondrial-mediated apoptotic signaling in HBS-preserved hearts.

Regarding the involvement of the extrinsic apoptotic pathway, Fas and FasL increased in hearts from males after I/R (Fig. 4). The protein levels of Flip were not affected in hearts from females. However, in hearts from males, $Flip_L$ protein levels were increased. In Fas-mediated apoptosis, both isoforms $Flip_s$ and $Flip_L$, play a crucial role preventing the initial cleavage step of caspase 8 activation between p10 and p20 subunits of the caspase homology domain. Since the $Flip_L$ isoform is responsible for blocking the apoptotic process at the p10 step [Krueger et al., 2001], we can hypothesize that this pathway is suppressed in hearts from males during I/R conditions. Nevertheless, although the p10 step is suppressed, the extrinsic apoptotic signaling pathway is increased in hearts from males, which suggests that the pathway develops through alternative signaling pathways.

Caspase 3-like activities increased in HBS-preserved hearts from males. The endpoints for cardiac function were not particularly affected by the increase of the ischemia period or by the storage in the different cardioplegic solutions which can explain the lack of an effective anti-apoptotic protection by any of the cardioplegic solutions (Cs or HBS). The hemodynamic recovery of the heart is often related not only to apoptosis but also to necrosis [Wilhelm et al., 2003] and although in a model of local myocardial ischemia in rats, apoptosis represented the major initial form of myocyte death after 6 h ischemia [Kajstura et al., 1996], we cannot exclude the role of necrosis in cardiac dysfunction in our model.

We have previously described gender-related differences regarding the susceptibility to mitochondrial failure, oxidative stress, and metabolic performance after I/R [Alves et al., 2009, 2011a] thus, gender-related differences in apoptotic signaling were expected. Regarding p53/Bax, both proteins were increased in females after ischemia alone while after I/R, only p53 was increased in females. It is unclear at this point if increased p53 protein content has a negative or positive impact on the viability of hearts from females, although enhanced p53 apoptotic signaling in females would contradict the higher protection after I/R described for this gender.

When measuring caspases 3, 8, and 9-like activities, we can conclude that females are less prone to apoptotic signaling since the activities of the executioner caspase 3 are decreased. The hemodynamic data also show gender-related differences. When the heart rate decreases, such as in hearts from males preserved in Cs during 4 h before reperfusion, the cardiac output falls because a maximal filling volume has been achieved and any further reduction in the heart rate cannot be compensated by further increase of stroke volume. The LVDP is maintained which elicited a positive atrial chronotropic response and not a ventricular effect in all conditions except in hearts from females preserved for 6 h in HBS. The difference between males and females regarding the cardiac function measured are in fact in the same line as the results obtained for measurement of cell death markers, which were generally more extensive in males. In fact, the difference between FasL bound to membrane (40 kDa) and soluble (26 kDa) indicates that hearts from males, which presented high quantities of soluble FasL, are more prone to cellular death after I/R. When soluble FasL binds to the membrane receptor, the death-inducing signaling complex is formed, transducing a downstream signal cascade that results in apoptosis. Hearts from males also present higher activities for the executioner caspase 3 and dysfunctional hemodynamic data. Females have been described to have a higher content in ATP-sensitive K^+ channels, and the opening of these channels shortens the myocardial action potential during repolarization and the calcium influx into the myocardium decreases [Ranki et al., 2001]. Since ATP-sensitive K^+ channels avoid mitochondrial calcium overload and subsequently PTP opening and cytochrome *c* release, these channels are considered a good protective mechanism against apoptotic signaling.

The present study provides new evidence that (1) during ischemia-only conditions, hearts from females activate the p53/Bax axis, although the mechanism is not clear; (2) Cs and HBS provide some degree of protection against alterations of apoptotic signaling but were not fully effective; (3) HBS appears to have a more positive effect against apoptotic signaling in the cardiac tissue after I/R; and (4) hearts from males were more prone to cellular death associated with I/R. The possible anti-apoptotic effect observed in hearts from females should deserve more attention in the future. The possible role of necrosis during the I/R setting used may also be responsible for the described differences between hearts from males and hearts from females, and between both solutions. Thus, future works should disclose the role of necrosis in order to understand the undesirable effects occurring during organ storage in Cs and HBS as a priority to evaluate HBS as a good alternative to Cs and/or to establish advantageous alterations in the cardioplegic solutions tested.

From the present data, it is also concluded that the composition of Cs and HBS still needs to be improved in order to counteract the apoptotic signaling pathways and attenuate cold storage and reperfusion-induced injury. Possible inclusions to ameliorate cardioplegic solutions can be: (1) NaHS, a H_2S donor, that improved the mitochondrial phosphorylative system, stimulated glycolysis, and prevented apoptosis in the same I/R model [Alves et al., 2011b]; (2) L-carnitine and analogous compounds, which are capable of protecting the heart against I/R injury due to inhibition of the activity of caspases 8 and 3 [Mutomba et al., 2000]; or (3) diazoxide, a potassium channel activator, which is also known to prevent cell death by inhibiting the mitochondrial permeability transition pore

and cytochrome *c* release [Korge et al., 2002]. The inclusion of one or more of these compounds in HBS or Cs may improve the ability of these cardioplegic solutions to prevent cell death during I/R.

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