ORIGINAL PAPER

Gender differences in apoptotic signaling in heart failure due to volume overload

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Abstract This study examined sex differences in the regulation of pro- and anti-apoptotic proteins in cardiac hypertrophy and heart failure due to volume overload induced by arteriovenous (AV) shunt in rats. General characteristics and hemodynamic assessment revealed the presence of cardiac hypertrophy at 4 weeks of AV shunt in male ($n = 12$) and female ($n = 12$) rats, whereas heart failure was seen at 16 weeks in male rats only. Although a decrease in apoptosis was seen in hearts of both sexes at 4 weeks, an increase in apoptosis in males and a reduction in the female heart were observed at 16 weeks of AV shunt. Unlike females, increases in the pro-apoptotic proteins, BAX, caspases 3 and 9 were seen in 16 weeks post-AV shunt in male rats. While an increase in phospho-Bad was detected, phospho-Bcl-2 protein was decreased in males. Females showed an increase in only phospho-Bcl-2 protein at 16 weeks post-AV shunt. Ovariectomy ($n = 12$) abolished the increase in phospho-Bcl-2 protein, but this was restored by treatment with 17-β estradiol. These data suggest that downregulation of phospho-Bcl-2 and an upregulation of BAX may play a major role in cardiomyocyte apoptosis in heart failure due to volume overload in male rats. Furthermore, upregulation of phospho-Bcl-2 in the heart due to estrogen may confer resistance against cardiomyocyte apoptosis in females.

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Introduction

Cardiomyocyte apoptosis is an important mechanism that may contribute to the development of congestive heart failure (CHF) due to several etiologies [1–7]. While cardiomyocyte apoptosis is known to occur during CHF due to myocardial infarction [4, 6, 7], pressure overload [2, 5], as well as in other non-ischemic models of heart failure [3] in male animals, gender differences in the magnitude and occurrence of apoptosis have been reported in spontaneously hypertensive rat [8] and in ischemia–reperfusion [9]. Although apoptosis has also been observed to occur in failing hearts due to volume overload in male rats [1], there is no information regarding gender differences in cardiomyocyte apoptosis following the induction of volume overload. Furthermore, gender differences in the status of pro- and anti-apoptotic proteins involved in cardiomyocyte apoptosis have not been investigated in CHF due to volume overload. It is pointed out that different proteins such as BAX, Bcl-2 and Bad as well as caspases 3 and 9 play a critical role in the development of apoptosis [5, 10, 11]. It should also be noted that BAX is a member of the "BH3-domain-only" subgroup of the Bcl-2 family of proteins. This group is responsible for the proximal death signal to the core apoptotic pathway [12, 13]. Recent studies suggest that BAX enter the outer mitochondrial membrane and subsequently forms a large conductance channel, which allows the release of cytochrome C [12, 14] and subsequent caspase activation [11]. Furthermore, BAX also can induce transport of cytochrome C in liposomes [15]. On the other hand, Bcl-2 and other members of the anti-apoptotic family

are required for the binding of glucokinase to the mitochondrial complexes to protect the release of cytochrome C [16]. Bad is a pro-apoptotic Bcl-2 family protein, and is considered to be intimately involved with Bcl-2 in the genesis of cell death or survival; where phosphorylation of Bad inhibits its binding to and inactivation of anti-apoptotic Bcl-2 [17]. Thus, increases in phosphorylation of Bad and/or expression of Bcl-2 are considered to prevent myocardial apoptosis. Since the risk of developing cardiovascular disease is considerably less in premenopausal females than in age-matched males [18, 19]. The present study was undertaken to gain some insight into the mechanisms of gender differences with respect to the extent of apoptosis and development of CHF due to volume overload. Accordingly, we tested the hypothesis that female rats are resistant to apoptosis during hypertrophic and heart failure stages due to volume overload induced by arteriovenous (AV) shunt. In view of the role of estrogen in maintaining cardiovascular function in females [20], the effect of estrogen in preventing apoptosis due to volume overload in females was tested by employing ovariectomized rats treated with or without 17-β estradiol.

Materials and methods

Experimental animals

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines established by the Canadian Council on Animal Care. An AV shunt was performed in male ($n = 12$) and female ($n = 12$) Sprague–Dawley rats (weighing 150–200 g) as described previously [10]. Briefly, the animals were anesthetized with 5% isoflurane with a flow rate of oxygen (2 l/min) and an abdominal laparotomy was performed. Following exposure of the abdominal aorta and inferior vena cava between the renal arteries and ileac bifurcation, the descending aorta and the iliac bifurcation was temporarily occluded proximal to the intended puncture site. An 18-gauge needle was inserted and withdrawn across the medial wall of the descending aorta three times to ensure the size and presence of the shunt and finally withdrawn. The puncture site was then immediately sealed with a drop of isocynate (Instant Krazy glue, OH, USA). The creation of the shunt was visualized by the pulsatile flow of oxygenated blood into the vena cava from the abdominal aorta. Throughout the operative procedure, the rats were maintained on 2.5% isoflurane in 2 l/min of oxygen. Age-matched, sham operated animals served as controls and were treated similarly, except that the puncture into the descending aorta was not performed. The animals were allowed to recover

and were maintained on food and water ad libitum for either 4 or 16 weeks. The circulation system was only occluded for about 1 min and the entire procedure was finished within 10 min. Ovariectomized females were purchased from Charles River Laboratories International, Inc. (MA, USA). It should be noted that the female Sprague–Dawley rats were ovariectomized by the supplier in 6 week old (150–200 g) animals and were shipped on the 5th day after surgery. Half of these animals ($n = 12$) were used for inducing AV shunt and the remaining ($n = 12$) underwent sham operation. Animals in each group, received an implantable 90 day slow release 17-β estradiol pellet (1.5 mg) that delivered a daily dose of 17 µg/day to each female rat as described elsewhere [20]. These rats were allowed to recover and maintained on food and water ad libitum for 16 weeks. It should also be pointed out that after 60 days; a second implantable 17-β estradiol pellet was inserted such that estrogen was administered for the full 16 weeks time period. Mortality of the control group was 0% and the mortality rate of the AV shunt animals was less than 4% during 6 h after the surgery; thereafter no mortality was seen in either group as the result of the surgical procedure.

Hemodynamic studies in vivo

The LV function of animals from the sham-operated and AV shunt groups was assessed. Rats were anesthetized by an injection of ketamine-xylazine (100:10 mg/kg; ip.) The right carotid artery was exposed, and a micromanometer-tipped catheter (2-0; Millar SPR-249) was inserted and advanced into the LV. The catheter was secured with a silk ligature around the artery, and after a 15-min stabilization of the heart function, LV pressures and maximum rates of isovolumic pressure development ($+dP/dt$) and decay ($-dP/dt$) were recorded as described earlier [10]. Hemodynamic data was computed instantaneously and displayed on a computer data-acquisition workstation (Biopac, Harvard Apparatus). It is pointed out that after hemodynamic assessment, the left ventricle (LV) tissue was frozen in liquid N_2 and stored at −80°C. At the time of sacrifice, heart weight, LV weight and right ventricle (RV) weight were measured. Blood samples were collected by ventricular puncture for preparing serum.

Preparation of solubilized membrane protein fraction and Western blot analysis

Left ventricle tissue (50 mg) was homogenized with a Polytron (2 × 10; 13,000 RPM) in buffer containing 50 mmol/l Tris–HCl, 0.25 mol/l sucrose, 10 mmol/l EGTA, 4 mmol/l EDTA, and a protease inhibitor cocktail, pH 7.5 and 1% Triton X-100. The homogenate was incubated on ice for 60 min to solubilize membrane proteins and then centrifuged at

100,000g for 60 min. The supernatant was assigned as the solubilized membrane protein fraction. Protein content was measured by the Lowry method as described elsewhere [13]. Pre-stained protein ladder (Invitrogen, CA, USA) and 20 μg of solubilized membrane proteins were separated on a 10% SDS–PAGE as described previously [13]. Separated proteins were then transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membrane at 200 V for 1 h at 4°C. The PVDF membrane was blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 5% skim milk and probed with rabbit polyclonal antibody for BAX (1:1000), Bcl-2 (1:1000), phospho-Bcl-2 (1:1000), Bad (1:1000), phospho-Bad (1:1000), caspase 3 (1:1000) and caspase 9 (1:1000) (Cell Signaling, MA, USA) according to the manufacturer's instructions. Horseradish peroxidase-labeled anti-rabbit IgG (Bio-Rad, ON, Canada) was diluted 1:1,000 in TBS-T and used as secondary antibody. Protein bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, NJ, USA). Band intensities were quantified using a Bio-Rad GS-800 Calibrated Densitometer. PVDF membranes were exposed to Amido black to determine equivalence of protein loading. The data were normalized to loading controls.

Detection of cardiomyocyte apoptosis

Cardiomyocyte apoptosis was measured using a Cell Death Detection ELISA Plus kit (Roche Applied Science, PQ, Canada) as previously described [10]. Briefly, 20 μl of homogenate sample and 80 μl of immunoreagent (supplied by manufacturer) were loaded into each well. The plate was then covered with foil and incubated under gentle agitation for 2 h at 15–25°C. The samples were aspirated and the wells were rinsed three times with 250–300 μl of incubation buffer (supplied by manufacturer); the buffer was removed each time by suction. One hundred microliters of ABTS solution (supplied by manufacturer) was added to each well and incubated for 15 min at room temperature under gentle agitation. The degree of apoptosis was colorimetrically assessed at 405 nm against ABTS solution as the blank (reference wavelength approximately 490 nm).

RNA isolation and determination of mRNA levels

Left ventricle tissue from sham control, 4 weeks AV shunt and 16 weeks AV shunt animals was processed for the isolation of total RNA according to procedures described elsewhere [21]. mRNA levels for caspases 3 and 9 were measured by semi-quantitative Reverse Transcription-Polymerase Chain Reaction and using Superscript Preamplification System for first strand cDNA synthesis (Life Technology, ON, Canada). For the purpose of normalization of the data, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used to amplify the GAPDH gene as a multiplex with the target genes. The PCR

Table 1 General characteristics and hemodynamic assessment of male and female sham and arteriovenous shunt groups at 4 and 16 weeks

	BW (g)	HW (mg)	HW/BW (mg/g)	LVEDP (mmHg)	LVSP (mmHg)	+dP/dt (mmHg/s)	−dP/dt (mmHg/s)
Male							
4 Weeks							
Sham	412 ± 9[#]	1,144 ± 9[#]	2.8 ± 0.3	4.3 ± 0.5[#]	122 ± 7	6,100 ± 112[#]	4,650 ± 134[#]
AV	422 ± 8[#]	2,320 ± 11*[#]	5.5 ± 0.4*	12.2 ± 0.2*[#]	117 ± 4	6,123 ± 117[#]	4,200 ± 113[#]
16 Weeks							
Sham	623 ± 29[#]	1,690 ± 12[#]	2.7 ± 0.1	5.1 ± 0.2	126 ± 6	8,600 ± 100[#]	7,230 ± 134[#]
AV	603 ± 9[#]	2,023 ± 11*[#]	3.4 ± 0.2*[#]	14.3 ± 0.1*	105 ± 4*	6,010 ± 123*	4,880 ± 98*[#]
Female							
4 Weeks							
Sham	281 ± 9[#]	877 ± 8[#]	3.1 ± 0.2	6.7 ± 0.4[#]	105 ± 4[#]	5,200 ± 98[#]	3,856 ± 123[#]
AV	272 ± 6	1,251 ± 9*[#]	4.6 ± 0.4*	8.5 ± 0.2*[#]	110 ± 6	5,076 ± 101[#]	3,540 ± 145[#]
16 Weeks							
Sham	332 ± 12[#]	996 ± 8[#]	3.0 ± 0.4	6.3 ± 0.5[#]	117 ± 12	5,890 ± 90[#]	5,300 ± 112[#]
AV	371 ± 10[#]	1,754 ± 10*[#]	4.7 ± 0.3*[#]	7.1 ± 0.3[#]	112 ± 8	6,112 ± 104	5,450 ± 123[#]

Data are mean ± SE of 12 animals for each group

Sham age-matched controls, *BW* body weight, *HW* heart weight, *LVEDP* left ventricular end diastolic pressure, *LVSP* left ventricular systolic pressure, *+dP/dt* rate of pressure development, *−dP/dt* rate of pressure decay, *AV* arteriovenous shunt

* $P < 0.05$ versus corresponding sham control values

[#] $P < 0.05$ versus corresponding value for other gender

products were analyzed by electrophoresis in 2% agarose gels as previously described [21]. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) and expressed as a ratio of the target gene over GAPDH. Primers used for amplification were synthesized as follows: GAPDH: TGAAGGTCGG TGTGAACGGATTTGGC (forward); GCATGTCAGATC CACAACGGATAC (reverse), Caspase 3: ACCGATGT CGATGCAGCTAA (forward); GCATGAATTCCAGCTT GTGC (reverse) and Caspase 9: TGGCTTCATTCTTGCA AAGC (forward); TGAAGAGTTTCGGCTTTCCAG (reverse).

Statistical analysis

All values are expressed as mean ± SE. The differences between two values were evaluated by Student's *t*-test. One-way Analysis of variance (ANOVA) followed by Newmans–Keuls test was used for comparing more than two values. A probability of 95% or more ($P < 0.05$) was considered significant.

Results

General characteristics and hemodynamic alterations in male and female rats at 4 and 16 weeks post AV shunt

Several differences were observed in the basal hemodynamic assessment between male and female rats (Table 1). In this regard, +d*P*/d*t* and −d*P*/d*t* were significantly lower in the female control rats as compared to the male counterparts at 4 and 16 weeks time points; however, LVSP was only lower in the female rats at 4 weeks. In contrast, the LVEDP was seen to be higher in the female control rats at both 4 and 16 weeks time points. Gender differences were also evaluated for general characteristics and hemodynamic alterations at 4 and 16 weeks post-AV shunt male and female rats. Both male and female animals showed increases in heart wt and heart wt to body wt ratio indicating the development of cardiac hypertrophy at 4 and 16 weeks post-AV shunt. The LVEDP in male rats was significantly increased at 4 and 16 weeks of AV shunt whereas no changes were evident in LVEDP in females at 16 weeks post-AV shunt. LVSP was significantly decreased in males at 16 weeks following induction of volume overload. Values for both +d*P*/d*t* and −d*P*/d*t* were unaltered in both male and female rats at 4 weeks of AV shunt whereas these parameters of cardiac performance were significantly depressed in males, unlike females, at 16 weeks of AV shunt (Table 1). It should be noted that the data on cardiac hypertrophy and function in female rats is in accordance with other reported studies [22, 23].

Furthermore, our results for male rats are similar to those reported earlier from our laboratory [10, 21] suggesting that, unlike males, cardiac function is maintained in female rats upon inducing AV shunt for 16 weeks. It is pointed out that the same size of needle was used for creating AV fistula in male and female animals and the patency of the shunt was visually confirmed at the beginning and at 4 and 16 weeks of the study. It is unlikely that the differences in cardiac function in male and female rats is due to differences in the size of the fistula and degree of AV shunt because the increases in HW/BW ratio, as an index of cardiac hypertrophy, were 96 and 48% at 4 weeks and 26 and 57% at 16 weeks in male and female rats, respectively.
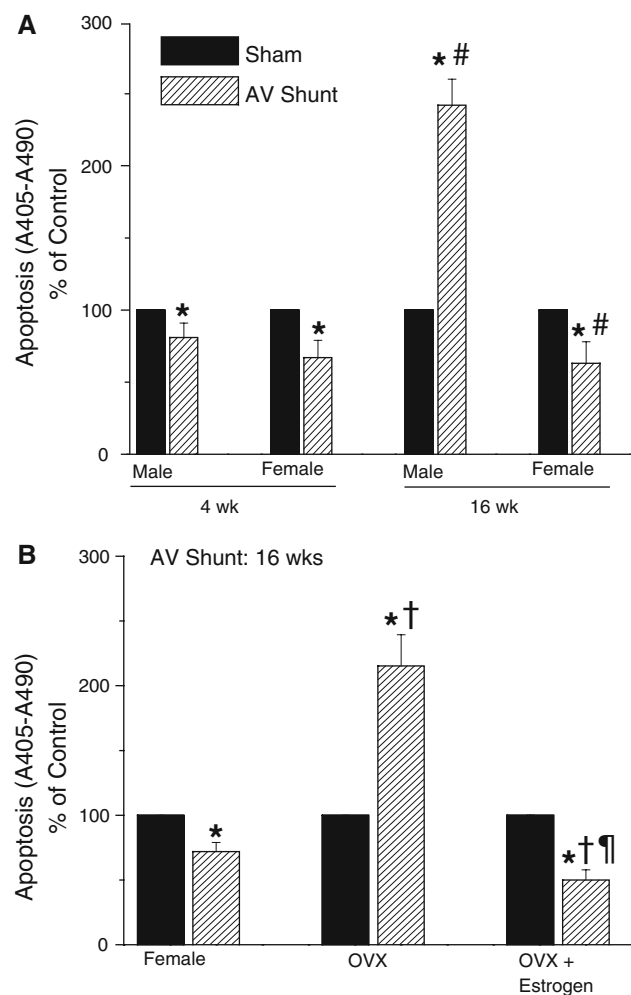

Fig. 1 Cardiomyocyte apoptosis in male and female rats at 4 and 16 weeks post AV-shunt (**a**). Cardiomyocyte apoptosis in female rats with or without ovariectomy at 16 weeks post AV-shunt (**b**). Data are presented as mean ± SE of three animals for each group; *OVX* ovariectomized. * $P < 0.05$ versus corresponding sham control values; # $P < 0.05$ versus corresponding value for other gender; † $P < 0.05$ versus corresponding value for intact female; ¶ $P < 0.05$ versus corresponding value for ovariectomized female without 17-β estradiol treatment

Apoptosis in male and female rats at 4 and 16 weeks post AV shunt

Apoptosis in both male and female rat hearts was studied in hypertrophic and failing heart stages of volume overload induced by AV shunt for 4 and 16 weeks, respectively, and the results are shown in Fig. 1. At 4 weeks post-AV shunt both males and females experienced a reduction in apoptosis; however, at 16 weeks post-AV shunt males exhibited a significantly higher level of apoptosis as compared to their sham control. In contrast to males, 16 weeks post-AV shunt females showed a marked decrease in apoptosis (Fig. 1a). In view of the critical role of caspases for the development of apoptosis, we examined changes in mRNA levels for caspases 3 and 9 in both male and female rats at 4 or 16 weeks of inducing AV shunt and the blots and quantitative analysis of the data are shown in Fig. 2. At 4 weeks of inducing AV shunt there was no change in mRNA level for either caspase 3 or caspase 9 in both male and female rats (Fig. 2a–c); however, at 16 weeks of inducing AV shunt, mRNA levels for both caspase 3 and caspase 9 were increased in male rats, unlike female rats (Fig. 2d–f). Since no changes in mRNA levels for these caspases in both male and female rats were seen at 4 weeks of AV shunt, all other experiments in this study were carried out for studying the effect of volume overload due to 16 weeks of AV shunt.

Alterations in signaling mechanisms for apoptosis

Since different signal transduction pathways are involved in the regulation of apoptosis, the present study was

Fig. 2 mRNA levels for caspases 3 and 9 in male and female rats at 4 and 16 weeks post-AV shunt. Representative blots of caspases 3 and 9 at 4 weeks (a). Quantified data of caspase 3 (300 bp) (b) and caspase 9 (510 bp) (c) at 4 weeks. Representative blots of caspases 3 and 9 at 16 weeks (d). Quantified data of caspase 3 (e) and caspase 9 (f) at 16 weeks. Values are mean ± SE of three experiments. Ratios of target gene to GAPDH are expressed as % of control values. * $P < 0.05$ versus corresponding sham control values; # $P < 0.05$ versus corresponding value for other gender
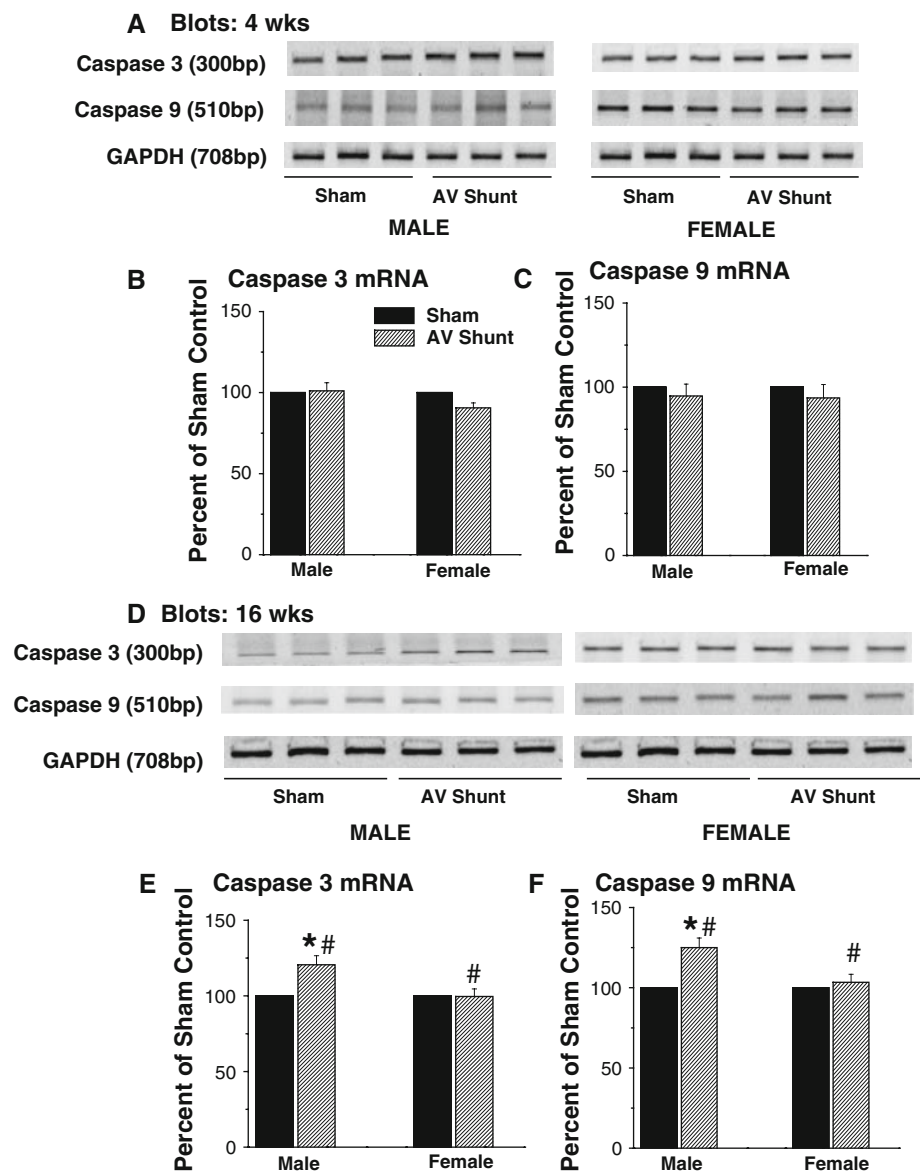
focussed in determining the status of Bad, BAX and Bcl-2 in this process. The basal levels of pro- and anti-apoptotic proteins were determined in male and female control animals. It can be seen from Fig. 3b and d that the protein levels of the pro-apoptotic factor, phospho-Bad were lower in the female heart, whereas levels of the anti-apoptotic protein, Bcl-2 in its phosphorylated form, were higher in the heart of the control female rat. While non-phosphorylated and phosphorylated Bad protein contents were found to be increased in males 16 weeks post-AV shunt, no changes were observed in the female group (Fig. 3a, b). There was no change in the protein content of non-phosphorylated Bcl-2 for both males and females (Fig. 3c), however; phospho-Bcl-2 was significantly decreased in males and increased in females (Fig. 3d) at 16 weeks post-AV shunt. These results indicate that the phospho-Bcl-2:Bcl-2 ratio is decreased in the 16 weeks male AV shunt group and significantly increased in the 16 weeks female AV shunt animals. The pro-apoptotic factor Bax was found to be increased in the 16 weeks male AV shunt animals, but remained unchanged in the females (Fig. 4a).

Effects of estrogen on apoptosis signaling mechanisms

In view of the role of estrogen in maintaining cardiac function on subjecting animals to volume overload, we sought to determine the effects of estrogen on apoptosis induced by AV shunt for 16 weeks. In female rats at 16 weeks post-AV shunt there was a significant decrease in apoptosis, however; upon ovariectomy and subsequent induction of volume overload there was an increase in apoptosis at 16 weeks post-AV shunt (Fig. 1b). Treatment of ovariectomized rats with 17-β estardiol showed a decrease in apoptosis similar to that in intact females (Fig. 1b). The protein content of non-phosphorylated Bad was found to be only significantly decreased due to AV shunt in the ovariectomized treated with estrogen group (Fig. 5a); however, phosphorylated Bad protein content was significantly increased in the ovariectomized group upon inducing AV shunt (Fig. 5b). Anti-apoptotic Bcl-2 was decreased in the ovariectomized group in the non-phosphorylated form (Fig. 5c) and also in the phosphorylated form due to AV shunt in this group (Fig. 5d). On the
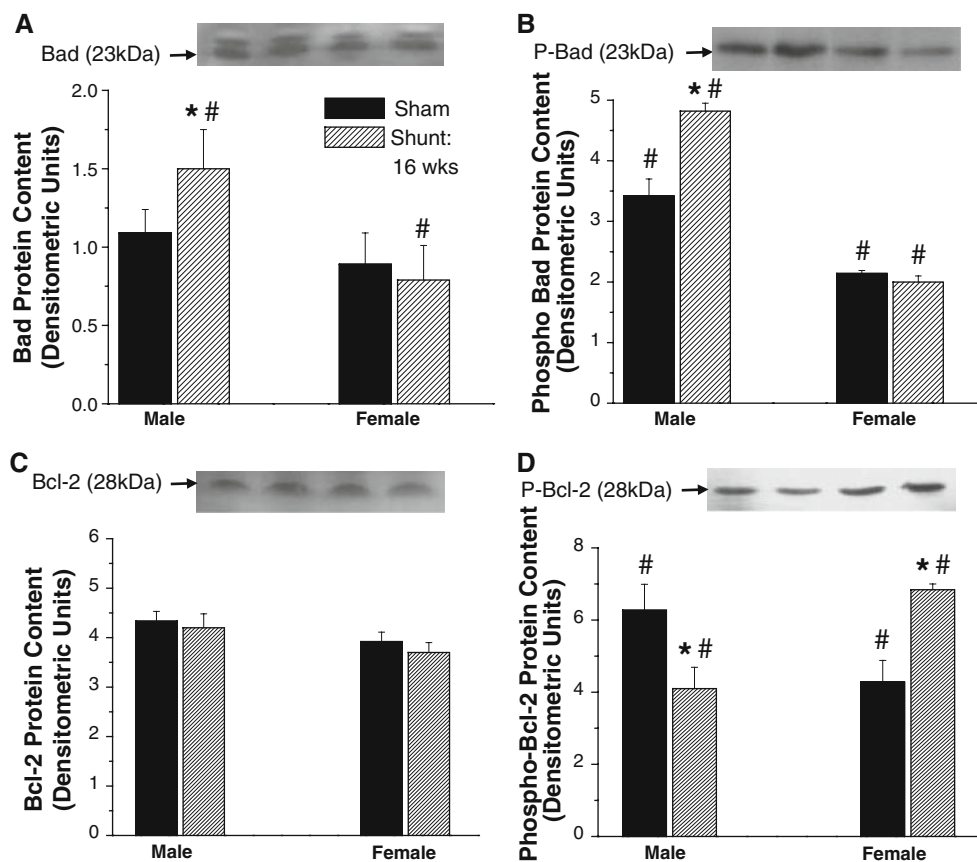

Fig. 3 Bad and Bcl-2 protein content in male and female rats at 16 weeks post-AV shunt assessed by Western blotting. Representative blots and quantified data of Bad (a), phospho-Bad (b), Bcl-2 (c) and phospho-Bcl-2 (d) protein content. Values are mean ± SE of five experiments performed with five different preparations. * $P < 0.05$ versus corresponding sham control values; # $P < 0.05$ versus corresponding value for other gender. *P-Bad* phosphorylated-Bad, *P-Bcl-2* phosphorylated-Bcl-2
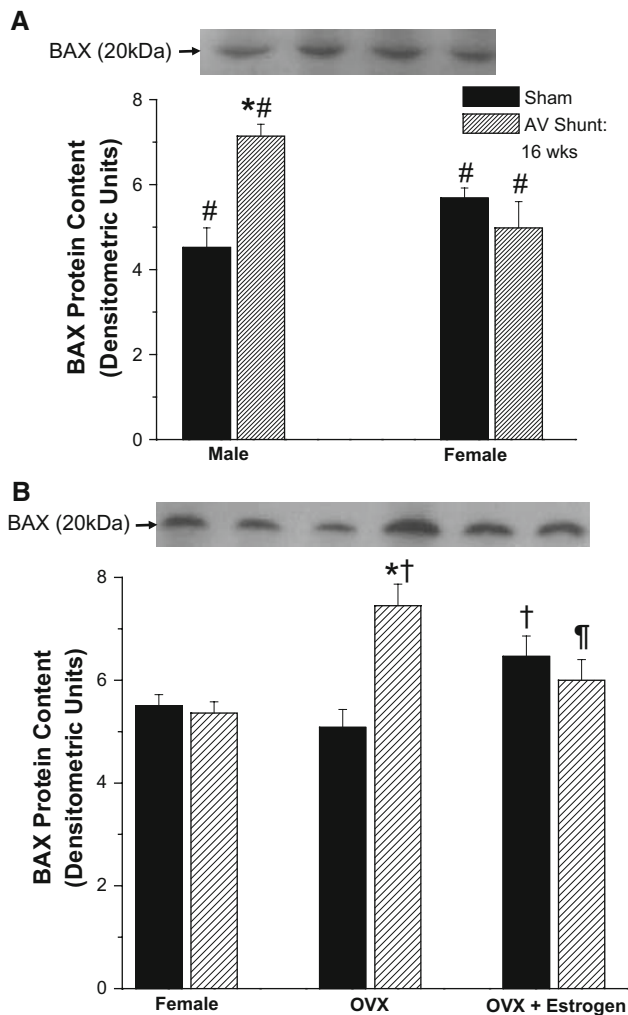
Fig. 4 BAX (20 kDa) protein content assessed by Western blotting at 16 weeks post-AV shunt. Representative blots and quantified data of BAX protein content in male and female rats (**a**). Representative blots and quantified data of BAX protein content in female rats with or without ovariectomy (**b**). Values are mean ± SE of five experiments performed with five different preparations. * $P < 0.05$ versus corresponding sham control values; # $P < 0.05$ versus corresponding value for other gender; † $P < 0.05$ versus corresponding value for intact female; ¶ $P < 0.05$ versus corresponding value for ovariectomized female without 17-β estradiol treatment. *OVX* ovariectomized

other hand, an increase in the phosphorylated Bcl-2 was observed in the intact females and those treated with slow release estrogen (Fig. 5d). Pro-apoptotic BAX protein content was significantly increased due to AV shunt in the ovariectomized group, but not in those treated with estrogen (Fig. 4b). In contrast to intact female rats, AV shunt for 16 weeks increased protein content for caspase 3 without any change in the protein content for caspase 9 in ovariectomized rats (Fig. 6a, b). Furthermore, treatment of ovariectomized animals with estrogen decreased protein content for both caspase 3 and caspase 9 due to AV shunt (Fig. 6a, b). It is noted that ovariectomy alone was

observed to increase the protein content of phospho-Bcl-2 and decrease caspase 3 protein content in female rats (Figs. 5d, 6a). On the other hand, treatment of ovariectomized sham control rat with estrogen decreased protein content for phospho-Bad and non-phosphorylated Bcl-2 (Figs. 4b, 5b, c). It is also pointed out that 17-β estradiol levels in the serum, as determined by radioimmunoassay, were 49.3 ± 0.3, 11.2 ± 1.1 and 33.0 ± 2.7 pg/ml in intact female, ovariectomized rats and estrogen treated ovariectomized animals ($n = 6$ for each group), respectively.

Changes in hemodynamic function in ovariectomized female rats treated with and without estrogen

The results in Table 2 show that in contrast to intact female rats, AV shunt produced significant depressions in +dP/dt, −dP/dt and LVSP and a marked increase in LVEDP in ovariectomized rats; these alterations were either fully or partially prevented by estrogen treatment. Although ovariectomy decreased LVEDP, unlike +dP/dt, −dP/dt and LVSP, this change was not affected by estrogen treatment (Table 2). It should be noted that the hemodynamic function of the ovariectomized rats was comparable to male rats following volume overload induced by AV shunt. Thus, we believe that the observed gender differences are not due to differences in the size of the fistula and degree of AV shunt. Furthermore, cardiac function in females with an AV fistula is dependent on functioning ovaries and that the above noted differences may be due to differences in the type of cardiac remodeling as a consequence of ovarian hormones.

Discussion

Cardiomyocyte apoptosis has been implicated to play a role in cardiac dysfunction in CHF as well as in the transition from cardiac hypertrophy to heart failure due to both ischemic and non-ischemic etiology [1–7]. In view of the fact that there is limited ability for self renewal of the myocardium, cardiomyocyte loss through apoptosis may profoundly influence cardiac structure and function (cardiac remodeling). Since, there is a lower incidence of heart failure in women, it can be suggested that there would also be a corresponding lower incidence of cardiomyocyte apoptosis. Interestingly, differences in the pro- and anti-apoptotic proteins between male and female control rats were observed in the present study. In this regard, the protein levels of the pro-apoptotic factor, phospho-Bad were lower in the female heart, whereas levels of the anti-apoptotic protein, Bcl-2 in its phosphorylated form, were detected to be higher in the heart of
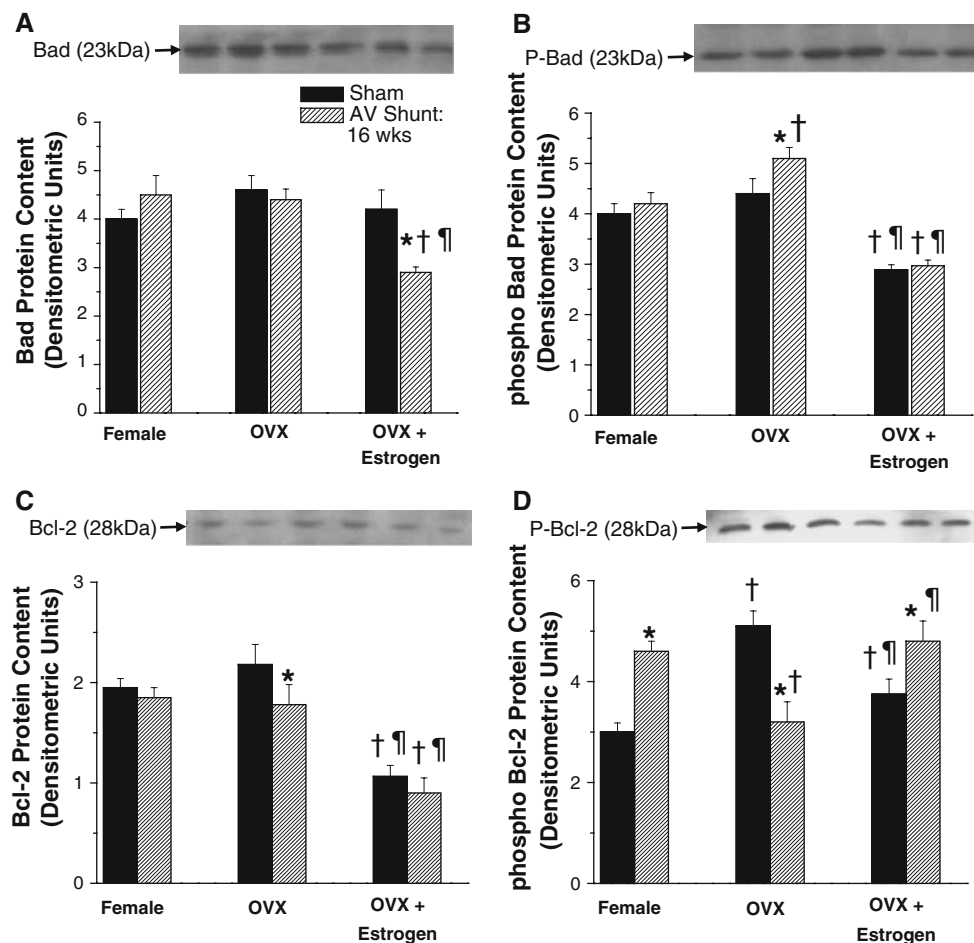
Fig. 5 Protein content for Bad and Bcl-2 in female ovariectomized rats treated with or without estrogen at 16 weeks post-AV shunt assessed by Western blotting. Representative blots and quantified data of Bad (**a**), phospho-Bad (**b**), Bcl-2 (**c**) and phospho-Bcl-2 (**d**). Values are mean ± SE of experiments performed with three different preparations. * $P < 0.05$ versus corresponding sham control values; † $P < 0.05$ versus corresponding value for intact female; ¶ $P < 0.05$ versus corresponding value for ovariectomized female without 17-β estradiol treatment. *OVX* ovariectomized, *P-Bad* phosphorylated-Bad, *P-Bcl-2* phosphorylated-Bcl-2

the control female rat. These observations are suggestive that the potential for the occurrence of apoptosis in the female heart is less than in the male heart. Although our results regarding the occurrence of apoptosis due to volume overload in male rats confirm our previous observations [10], the present study reports the gender differences with respect to apoptosis at the hypertrophic as well as the failing stages after the induction of volume overload. During cardiac hypertrophy we found a decrease in the amount of apoptosis in both males and females, which may be seen as an initial adaptive response to volume overload; however, in failing heart there was a clear gender difference in the extent of apoptosis. In this regard, males develop a significant increase in apoptosis while the females continue to have a lesser extent of apoptosis at 16 weeks post AV shunt. This is in accordance with a study in which explanted hearts in patients undergoing cardiac transplantation were observed to show that

myocyte necrosis and apoptosis were markedly lower in women than in men [24]. In this study, higher myocyte death in men was associated with shorter duration of myopathy and earlier onset of heart failure [24]. Interestingly, unlike in the female heart, a fourfold increase has been reported in the senescent male monkey, indicating the role of aging in cardiomyocyte apoptosis [25]; however, in the human heart it has been suggested that aging does not influence the percentage of cardiomyocyte apoptosis, but gender appears to be an important determinant for the occurrence of apoptosis [26]. Since we did not determine the percentage of cardiomyocytes undergoing apoptosis due to AV shunt in male and female rats, we are unable to indicate whether there is any gender differences in this aspect. In addition, the quantitative aspects of the cell death kit employed in this study does not necessarily reflect cell numbers and thus some caution must be exercised in the interpretation of our data.
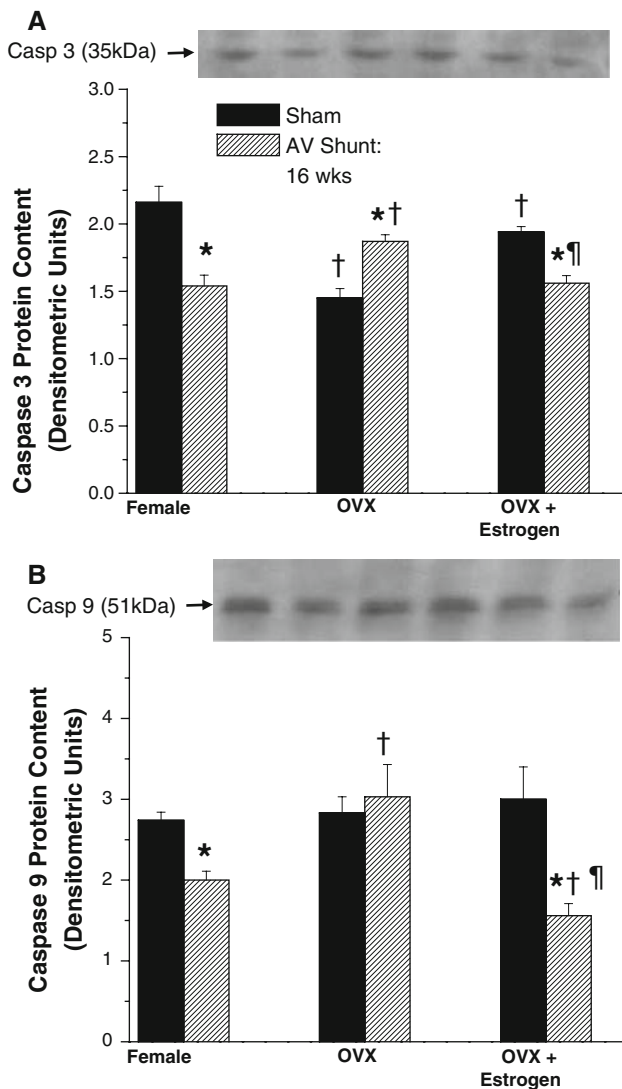
A

Casp 3 (35kDa) →

B

Casp 9 (51kDa) →

Fig. 6 Protein content for caspases 3 (35 kDa) and 9 (51 kDa) in female, ovariectomized rats treated with or without estrogen at 16 weeks post-AV shunt assessed by Western blotting. Representative blots and quantified data of caspase 3 (**a**) and caspase 9 (**b**). Values are mean ± SE of experiments performed with three different preparations. * $P < 0.05$ versus corresponding sham control values; † $P < 0.05$ versus corresponding value for intact female; ¶ $P < 0.05$ versus corresponding value for ovariectomized female without 17-β estradiol treatment. *OVX* ovariectomized

BAX is a member of the pro-apoptotic family that is responsible for the proximal death signal to the core apoptotic pathway [12], whereas Bcl-2 and other members of the anti-apoptotic family are required for the binding of glucokinase to the mitochondrial complexes to protect the release of cytochrome C [27]. Expression of Bcl-2 appears to be inversely related to the extent of apoptosis [28]. In contrast, the pro-apoptotic factor BAX binds to Bcl-2 and inhibits its protective effect [29]. It is pointed out that Akt is considered to play a central role in cell survival and resistance to apoptosis [30] and an increase in the activity

of which has been reported in females [4, 31]. We have previously shown that there occurred a downregulation in the Akt dependent survival signal involving Bcl-2 whereas the pro-apoptotic protein BAX was upregulated in the male heart and these alterations may play a role in the occurrence of cardiomyocyte apoptosis seen in heart failure due to volume overload [10]. Females have also been shown to be protected against ischemia–reperfusion injury and this protection may be mediated by an increase in phosphorylated Akt and PKC ε levels [32]. Recently, Moorjani et al. [33] have found that activation of the cardiomyocyte apoptotic cascade occurs during the development of volume overload induced heart failure in humans; specifically, BAX, p53 and caspases 3, 8 and 9 increased progressively, however, there was no completion to DNA fragmentation [33]. In our study a significant increase in the pro-apoptotic factors phospho Bad and BAX and a significant decrease in the anti-apoptotic factor phospho Bcl-2 were seen in the male heart. There was also an increase in the gene expression of caspase 3 and 9 at 16 weeks post AV shunt in male rats. In contrast, no significant difference in the expression of pro-apoptotic factors phospho Bad and BAX were seen in the AV shunt female rats, but there was a significant increase in the expression of phospho Bcl-2. These data are consistent with a lesser degree of apoptosis in the female AV shunt rat. There are many triggers to induce apoptosis such as angiotensin II (Ang II)/Ang II type 1 receptor (AT$_1$R); in fact, Ang II induced a significant increase in BAX protein where Bcl-2 was decreased [34, 35]. Losartan and AT$_1$ receptor blocker alone or in combination with simvastatin blocked the increase in BAX and caused an increase in Bcl-2 [30]. It is also well known that activation of Ang II/AT$_1$R pathway can cause an increase in intracellular Ca^{2+}, stimulate DNAse I and induce cardiomyocyte apoptosis [36]. It is also known that hyperactivity of the sympathetic nervous system (SNS) leads to cardiotoxicity and cardiomyocyte death by apoptosis. Norepinephrine is able to induce cardiomyocyte apoptosis via different adrenergic receptor-coupled signaling pathways [37]. In this regard, experiments conducted in our laboratory have indicated a differential activation of both SNS and renin-angiotensin system in male and female rats at 4 and 16 weeks post-AV shunt (M. R. Dent, P. S. Tappia, and N. S. Dhalla, unpublished observation), which may play a role in the gender differences in apoptosis seen in the present study.

It is relevant to question whether estrogen is the key player in the lower susceptibility to death signaling in the female heart and able to defend against pathological cell death. The mechanism by which estrogen is thought to reduce the apoptotic cascade is through phosphorylation of insulin-like growth factor-1 receptors [38]. In addition to a decrease in the levels of pro-apoptotic factors such as BAX

Table 2 Hemodynamic changes in female ovariectomized rats treated with or without estrogen at 16 weeks post AV shunt

	BW (g)	HW (mg)	HW/BW (mg/g)	LVEDP (mmHg)	LVSP (mmHg)	+dP/dt (mmHg/s)	−dP/dt (mmHg/s)
Female							
16 Weeks							
Sham	321 ± 12	946 ± 19	2.8 ± 0.3	6.3 ± 0.2	119 ± 7	5,982 ± 214	5,574 ± 251
AV	349 ± 9	1,698 ± 11*	5.2 ± 0.6	6.9 ± 0.4	116 ± 8	6,001 ± 228	5,624 ± 182
OVX							
16 Weeks							
Sham	378 ± 10[†]	875 ± 10[†]	2.3 ± 0.2	5.1 ± 0.2[†]	130 ± 8	6,015 ± 128	5,412 ± 245
AV	365 ± 12	1,341 ± 10*[†]	3.7 ± 0.4*[†]	14.9 ± 0.4*[†]	112 ± 9	4,852 ± 156*[†]	3,895 ± 166*[†]
OVX + estrogen							
16 Weeks							
Sham	318 ± 15[¶]	752 ± 13[†¶]	2.4 ± 0.3	4.9 ± 0.3[†]	117 ± 6	5,891 ± 198	4,954 ± 265
AV	306 ± 8[†¶]	1,432 ± 10*[†¶]	4.7 ± 0.2*[¶]	10.5 ± 0.3*[†¶]	120 ± 7	5,995 ± 254[¶]	5,029 ± 221[¶]

Data are mean ± SE of 12 animals for each group

Sham age-matched controls, *BW* body weight, *HW* heart weight, *LVEDP* left ventricular end diastolic pressure, *LVSP* left ventricular systolic pressure, *+dP/dt* rate of pressure development, *−dP/dt* rate of pressure decay, *AV* arteriovenous shunt, *OVX* ovariectomized

* $P < 0.05$ versus corresponding sham control values

[†] $P < 0.05$ versus corresponding value of intact female

[¶] $P < 0.05$ versus corresponding value for ovariectomized female without estrogen treatment

[24], the enhancement of this system leads to increased expression of anti-apoptotic factors such as Bcl-2 and Bcl-$_{XL}$. IGF-1 is also able to activate the PI3 kinase/Akt pathway which may contribute to the suppression in the cardiomyocyte death [39]. We have found that ovariectomy results in a significant increase in cardiomyocyte apoptosis as well as reduced +dP/dt and −dP/dt, at 16 weeks post-AV shunt, which were similar to the observations in the male 16 weeks post AV shunt animals. However, when the ovariectomized female rats were treated with slow release estrogen pellets apoptosis was significantly depressed at 16 weeks post AV shunt similar to control females, which was associated with improved cardiac contractile function. These results are in accordance with others that have shown that estrogen is protective in such models as pressure overload [40] and myocardial infarction [41]. It has been suggested that the beneficial effects of estrogen may be through a reduction in NAPDH oxidase activity and reactive oxygen species production and activating the antioxidative thioredoxin reductase as shown in a genetic model of CHF [42]. In the present study, we found that ovariectomy lead to an increase in phosphorylated Bad and BAX protein content, as well as a decrease in phosphorylated Bcl-2 amounts; however, treatment with estrogen resulted in a decrease in Bad and an increase in phosphorylated Bcl-2 protein contents. Caspase 3 protein content was also elevated in the ovariectomized group whereas treatment with estrogen decreased protein expression of both caspase 3 and 9. However, while estrogen normalized caspase 3 protein levels, caspase 9 protein content was

decreased to below the protein levels seen in the intact female AV shunt animal. This may reflect an over compensation in response to the marked increase in apoptosis in the ovariectomized AV shunt rat. It is interesting to note that caspase 3 protein level was significantly less in ovariectomized sham animal as compared to the intact female sham. Since treatment of the ovariectomized rat with estrogen resulted in an increase in the basal level of caspase 3 protein, it can be suggested that caspase 3 protein level is dependent on functioning ovaries. In fact, Wang et al. [43] have recently reported that myocardial protection following ischemia–reperfusion by upregulating the PI3K/Akt pathway whereas decreased caspase 3 and 9 and increased Bcl-2 in female hearts is mediated through estrogen receptor, ERβ. In summary, this is the first study to clearly identify gender differences in cardiomyocyte apoptosis and pro- and anti apoptotic factors in hearts subjected to volume overload. These findings may provide possible mechanisms to explain the gender differences in the myocardial remodeling and cardiac function following volume overload, which could prompt the use of female myocardial progenitor or stem cells for cell replacement therapy in cardiac failure, on the premise of a greater protection from myocardial apoptosis and unfavourable remodeling in women [44]. Since the intact female rat produces estrogen as well as other sex hormones (progesterone and testosterone, caution must be exercised on the interpretation of our data since we treated ovariectomized female rats with estrogen only. Nonetheless, the role of estrogen in preventing apoptosis is evident from our

observation that ovariectomy decreased the protein content for phospho-Bcl-2 while treatment of ovariectomized animals with 17-β estradiol produced an increase in phospho-Bcl-2 protein levels in female hearts.

Conflict of interest statement The authors declare that they have no conflict of interest.

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