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**Postconditioning cardioprotection against infarct size and post-ischemic systolic dysfunction is influenced by gender**

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**Running head:** Postconditioning cardioprotection and gender

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## **Abstract**

Whether cardioprotection by postconditioning (PostC) is gender dependent is not clear. We studied the effect of PostC in terms of both infarct size (IS) and post-ischemic systolic dysfunction (PSD) reduction.

Isolated male and female rat hearts were subjected to 10- or 30-minutes of global ischemia and 120-minutes of reperfusion, with or without PostC (i.e., 5 cycles of 10-s reperfusion/ischemia immediately after the ischemia).

Surprisingly, after 10-min ischemia, IS and PSD were greater in female than male hearts (IS:  $21\pm 2\%$  vs  $11\pm 2\%$ ;  $p < 0.01$ ), while PostC attenuated IS and PSD in female hearts only.

After 30-min ischemia IS was smaller in female than male hearts ( $52\pm 2\%$  vs  $61\pm 3\%$ ;  $p < 0.05$ ), whereas PSD was similar in these two groups. PostC reduced IS in both genders, though the effect was smaller ( $p < 0.05$ ) in females. Yet, PostC reduced PSD in female, but not in male hearts. Contracture development paralleled IS in all groups. To check the effects of buffer perfusion over heart function, additional hearts underwent 150-min buffer perfusion only. Contractile function of these hearts was not significantly affected over time.

In conclusion IS, contracture and PSD are differently affected by gender, depending on ischemia duration. Yet, reduction of IS induced by PostC depends on the extension of IS induced by index-ischemia. While in female hearts reduction of PSD paralleled IS reduction, in male it does not occur. Results suggest that improvement of systolic function is mainly due to the anti-necrotic rather than to the anti-stunning effect exerted by PostC.

**Word count: 247**

**Keywords:** Contracture; Infarct size; Ischemia/reperfusion; Myocardial stunning; Systolic function.

## Introduction

Two strategies for protecting the heart are ischemic preconditioning and postconditioning, which imply the cardioprotection obtained from applying brief episodes of myocardial ischemia and reperfusion either before or after the index-ischemia, respectively. Much progress has been made in elucidating the signal transduction pathway behind their protective role [e.g. 4,6,7,8,11,13,17,19-24,27-29,34-42,45,47,51,52].

The role of “Ischemic Postconditioning” against *infarct size* was first described by Vinten-Johansen’s group [52]. In such a study the Postconditioning (PostC) protocol was 30 s of reperfusion followed by 30 s of coronary occlusion, which were repeated for three cycles at the onset of reperfusion. In general, PostC can be defined as intermittent interruption of coronary flow in the very early phase of a reperfusion, which leads to cardioprotection. In their study Vinten-Johansen and co-workers [52] reported that PostC causes massive salvage of the myocardium. The infarct size was reduced by ~45% in PostC group compared with a group subjected to an abrupt and complete reperfusion. In multiple species and models, PostC reduced infarct size by ~20-70% versus matched controls with matched risk areas [16,49,51,52]. Studies from our and other laboratories confirmed the infarct size reduction in isolated rat heart model [24,36-42,46].

Virtually in all of the species in which different PostC protocols have been tested against infarct size, they have been protective [7,13,22,24,34,49,51,52], including humans [46], with the exception of some works as, for example, a recent work conducted on a *rodent model* by Dow et al. [12]. However, many other groups observed a cardioprotective effect of PostC in rodent models [24,25,36,39-41,47], including mice lacking connexin-43 [21]. We showed that in isolated hearts perfused with constant flow the infarct size reduction by PostC is greater than that observed

in the same model perfused at constant pressure [39]. Aging was also considered a condition that reduces PostC effectiveness against infarct size extension [4].

Yet, it is unclear if there is a beneficial impact of PostC on *systolic function* and which are the putative determinants. Studies report either an improvement [45,51,52] or no effect [9,39,42] on cardiac performance by PostC. To date very few studies tested whether PostC attenuates myocardial stunning in the absence of necrosis. In an *in vivo* study, using models of short ischemias (i.e. 10-min ischemia) that usually induce stunning with little or no cell death, Couvreur *et al.* [9] report that PostC does not prevent myocardial stunning. Yet, in an *ex vivo* preparation of human atrial appendages, hypoxic PostC seems to attenuate post-ischemic dysfunction [45].

In many animal studies, including the rat, no *gender* difference in ischemia/reperfusion injury is observed; however, in other animal studies, particularly in the rat, females show less ischemia/reperfusion injury following infarcting ischemia [15,30-32,50]. Very few studies tested the differences between genders with regard to PostC effectiveness. In a study specifically designed to compare PostC in male and female hearts, Crisostomo *et al* [10] reported PostC protective effect against post-ischemic dysfunction in isolated male rat hearts after either 20 min or 25 min ischemia. This protective effect was also present in female rat hearts exposed to 20 min of ischemia, but absent in those exposed to 25 min ischemia. However, Crisostomo *et al* [10] did not measure infarct size. In this respect, it is also particularly intriguing that the above mentioned study of Dow *et al* [12] in which PostC was ineffective in reducing infarct size was conducted on female rats. In such a study it was suggested that gender may be a confounder in PostC scenario [12]. Another confounder, which may influence the outcome of PostC, may be the end-point considered (e.g. infarct size, contracture development, and post-ischemic systolic dysfunction [17,29,39,42]). Therefore it is necessary to study multiple end-points simultaneously. These is

particularly important if different end-points may influence each other during the period of observation.

We hypothesize that PostC effectiveness is different against infarct size reduction in female and male hearts and that this differences may explain the gender difference in post-ischemic recovery of heart function, as observed in the study of Crisostomo *et al.* [10].

To test this hypothesis we studied the effects of PostC against infarct size and myocardial post-ischemic dysfunction in groups of hearts isolated from both female and male rats, which underwent either long infarcting or short-lasting periods of ischemias.

## **Materials and Methods**

### ***Animals***

Five month old male (n=72; body weight 410–530 g) and female (n= 72; body weight 275–385 g) Wistar rats received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and in accordance with Italian law (DL-116, Jan. 27, 1992).

### ***Isolated heart perfusion***

The methods were similar to those previously described [6,35,37-42]. In brief, each animal was anesthetized with urethane (1 g/kg i.p.). The chest was opened 10 min after heparin treatment and the heart was rapidly excised. Isolated rat hearts were retrogradely perfused with oxygenated Krebs-Henseleit buffer (127 mM NaCl, 17.7 mM NaHCO<sub>3</sub>, 5.1 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.26 mM MgCl<sub>2</sub>, 11 mM D-glucose and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). A constant coronary flow (CF) was adjusted with a proper pump to obtain a typical coronary perfusion pressure (CPP) of 80-85 mmHg during the initial part of stabilization. Thereafter the same flow level (9±1 ml/min/g) was maintained throughout the experiment. The hearts were electrically paced at 280 bpm and kept in a temperature-controlled chamber (37°C).

A small hole in the left ventricular wall allowed drainage of the thebesian flow, and a polyvinyl-chloride balloon was placed into the left ventricle and connected to an electromanometer for recording of left ventricular pressure (LVP). The balloon was filled with saline to achieve an end-diastolic LVP of 5 mmHg. CPP, CF and LVP were monitored to assess the preparation conditions and to study heart function in pre- and post-ischemic periods. LVP trace was used to measure end-diastolic LVP and to calculate developed LVP as the difference between systolic LVP and end-diastolic LVP.

#### ***Experimental protocols (Fig. 1)***

After stabilization period (20 min), hearts underwent 10- or 30-min of global no-flow ischemia. In all groups ischemia was followed by 120 min of reperfusion, which followed 10- or 30-min of global no-flow ischemia. Pacing was discontinued on initiation of ischemia and restarted after the third minute of reperfusion in all groups [24,39-42].

#### **Short-lasting ischemia**

##### ***Male and Female rat hearts exposed to 10-min ischemia (Groups 1-4)***

While myocardial stunning is the post-ischemic contractile dysfunction in the absence of an increase in cell death, global post-ischemic systolic dysfunction in our model may be the result of both stunning and cell death [2,9]. It has been suggested that 10-min ischemia may induce post-ischemic myocardial stunning with no or little necrosis [2,9] thus, in order to study functional recovery after a *short-lasting ischemia*, hearts of the Group 1 (*Male-Short-lasting-Ischemia-group*, n= 10) and hearts of Group 2 (*Female-Short-lasting-Ischemia-group*, n= 10) were exposed to 10-min ischemia and then to 120 min reperfusion only.

In Group 3 (*Male-Short-lasting-Ischemia+PostC-group*; n=10) and Group 4 (*Female-Short-lasting-Ischemia+PostC-group*; n=10) after the 10-min global ischemia, the hearts



immediately underwent a protocol of postconditioning. This consisted of five cycles of 10-s reperfusion and 10-s ischemia [36,39-42].

### **Infarcting ischemia**

#### ***Male and Female rat hearts exposed to 30-min ischemia (Groups 5-8)***

It has been shown that 30-min ischemia followed by reperfusion may induce I/R injury, which lead to a large infarct size in isolated rat hearts [3,6,13,36-42]. Therefore, in order to study functional recovery after an *infarcting ischemia*, hearts of the *Male-Infarcting-group* (Group 5, n= 22) or *Female-Infarcting-group* (Group 6, n= 22) were exposed to 30-min global ischemia and then to 120 min reperfusion only.

In Group 7 (*Male-PostC-group*; n=22) and Group 8 (*Female-PostC-group*; n=22) after the 30-min ischemia, the hearts immediately underwent a protocol of postconditioning. Also in this case PostC consisted of five cycles of 10-s reperfusion and 10-s ischemia [36,39-42].

#### ***Additional controls hearts (Groups 9 and 10)***

In order to check time-dependent deterioration of the experimental preparation, and to check whether or not significant cardiodepression occurs during oxygenated buffer perfusion, hearts of male (*Sham Male group*; Group 9, n=8) and female (*Sham Female group*; Group 10, n=8) rats underwent 150 min of perfusion only.

In all hearts, pressures were monitored throughout the experiments and infarct area assessed at the end of the experiment.

### ***Infarct size***

At the end of the experiment stained and unstained areas were assessed, as previously described, using a solution of nitro-blue tetrazolium in phosphate buffer [6,36-42]. In brief, immediately after reperfusion each heart was rapidly removed from the perfusion apparatus and

the left ventricle (LV) was dissected into 2–3 mm circumferential slices. Following 20 min of incubation at 37°C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, unstained tissue was carefully separated from stained tissue by an independent observer. In fact, while unstained tissue represents the amount of death cells, the stained tissue represents the viable cells. The unstained mass was expressed as a percentage of total left ventricular mass. In fact, the total left ventricle mass also corresponds to the risk area because a global ischemia was performed.

Reagents necessary to assess myocardial infarction were purchased from Merck (USA). Other chemicals were purchased from Sigma (USA).

#### *Statistical analysis*

All data are expressed as means±S.E.M. One-way ANOVA and Newman-Keuls Multiple Comparison Test (for post-ANOVA comparisons) have been used to compare infarct size. One-way ANOVA for multiple measures (Bonferroni Post test) has been used for the analysis of LVP data. Linear and non-linear fit are assessed between infarct size and pressures. A p value <0.05 was considered statistically significant.

## Results

### *Cardiac weight, cardiac to body weight ratio and risk area in male and female rats*

Male cardiac weight ( $1482 \pm 21$ ; range 1270-1790 mg, n =72) and the cardiac to body weight ratio ( $2.67 \pm 0.012$ ; range 2.24 – 2.99 mg/g) were similar in all groups of experiments (infarcted and stunned hearts). Importantly, the risk area, i.e., LV mass, was also similar among groups (LV weight was  $905 \pm 17$ ; range 590 – 1135 mg).

Female cardiac weight ( $1117 \pm 33$ ; range 1005 – 1580 mg, n =72) and the cardiac to body weight ratio ( $3.66 \pm 0.010$ ; range 3.03 – 4.08 mg/g) were similar in all groups of the two series of experiments (infarcted and stunned hearts). The risk area, i.e., LV mass, was also similar among groups (LV weight was  $717 \pm 12$ ; range 510 – 1003 mg).

Comparison between all male and female hearts (Table 1) revealed that the latter had smaller cardiac weight and risk area ( $p < 0.01$  for both), but higher cardiac to body weight ratio ( $p < 0.01$ ).

### *Infarct size (Figs 2 and 3)*

As can be seen in Fig 2, in hearts of *Male-Short-lasting-Ischemia group* (Group 1) and in those of *Male-Short-lasting-Ischemia+PostC group* (Group 3), which were subjected to 10-min ischemia, infarct areas were similar ( $11 \pm 2\%$  and  $12 \pm 2\%$  of risk area, respectively;  $p =$  Non significant vs each other).

For comparative purpose, we also measured the infarct size in the sham group hearts perfused with buffer only (*Sham groups*). In these hearts we found an infarct size of  $7 \pm 2\%$  in male group and  $8 \pm 2\%$  of risk area in female group ( $p =$  Non significant vs each other and vs *Male-Short-lasting-Ischemia groups*). These infarct areas are similar to those reported in a similar model of male sham buffer-perfused rat [44] and rabbit hearts [23].

However, surprisingly, in hearts of *Female-Short-lasting-Ischemia* group (Group 2), which were subjected to 10-min ischemia only, infarct area (21±2% of risk area) was significantly higher than infarct size observed in *Sham* groups, in *Male-Short-lasting-Ischemia* and *Male-Short-lasting-Ischemia+PostC* groups ( $p < 0.01$  for all). Yet, in *Female-Short-lasting-Ischemia+PostC* group (Group 4), PostC significantly ( $p < 0.01$  vs *Female-Short-lasting-Ischemia* group) reduced cardiac infarct size to 12±2% of risk area (NS vs Sham groups).

In hearts subjected to 30-min ischemia (Fig. 3) infarct size was slightly smaller ( $p < 0.05$ ) in female than male hearts, being 52±2% of risk area in *Female-Infarcting* group (Group 6) and 61±3% in *Male-Infarcting* group (Group 5). PostC reduced cardiac infarct size both in *Female-PostC* group (Group 8 = 40±3% of risk area;  $p < 0.01$  vs *Female-Infarcting* group) and *Male-PostC* group (Group 7 = 29±3% of risk area;  $p < 0.001$  vs *Male-Infarcting* group). However, while about 50% lesser infarct size between *Male-PostC* and *Male-Infarcting* groups was observed, a reduction of about 20% only was observed in *Female-PostC* with respect to *Female-Infarcting* group ( $p < 0.05$ ).

#### ***End-diastolic LVP (Table 2; Fig 4)***

Contracture development can be defined as an increase in intrachamber pressure of 4 mmHg above pre-ischemic (baseline) end-diastolic LVP values [3, 35]. Contracture has been suggested as a very good indicator of I/R injury [17,28]. In this respect, it is paradigmatic that *Male-Short-lasting-Ischemia* group (Group 1; Fig 4-Panel B) did not show contracture development, whereas contracture was increasingly higher in the following groups: *Female-Short-lasting-Ischemia* group (Group 2; Fig 4-Panel A), *Female-Infarcting* group (Group 6; Fig 4-Panel C) and *Male-Infarcting* group (Group 5; Fig 4-Panel D), which also showed increasing infarct areas (Figs 2 and 3).

PostC reduced cardiac contracture either after 10- or 30-min ischemia in female hearts (groups 4 and 8; Fig 4 - Panels A and C, respectively), as well as in male hearts subjected to 30-min of ischemia (group 3; Fig 4 – Panel D): *i.e.* contracture was reduced in groups in which PostC reduced infarct size.

In hearts subjected to 30-min ischemia, while about 50% lesser contracture between *Male-PostC* and *Male-Infarcting groups* was observed (Fig 4 – Panel D), a reduction of about 25% only was observed in *Female-PostC* with respect to *Female-Infarcting groups* (Fig 4 – Panel C). In other words, PostC protective effects against I/R injury (both infarct size and contracture) seem stronger in male than female hearts subjected to 30-min ischemia.

In hearts subjected to 10-min ischemia, as said, an higher infarct area was observed in female hearts, which also showed contracture development. Only in this *Female-Short-lasting-Ischemia group*, PostC reduced contracture by about 10% (*Group 4 vs Group 2; Fig 4 – Panel A*). No effects of PostC are observed over contracture development in male hearts subjected to 10-min ischemia (*Fig 4 – Panel B*): in this group infarct size was not increased by the 10-min ischemia and was not affected by PostC.

#### ***Developed LVP (Table 2; Fig 5)***

Stunning can be defined as “*the post-ischemic contractile dysfunction that persists following reperfusion in the absence of irreversible damage and despite the restoration of normal or near-normal coronary blood flow*” [2]. Post-ischemic systolic dysfunction may be a result of stunning and necrosis [2]. Post-ischemic recovery of systolic function was evaluated as % of developed LVP recovery during reperfusion. As can be seen in Fig. 5, either 10- or 30-min ischemia depressed contractile function in all ischemic groups as compared to buffer perfused (non-ischemic, Sham) hearts. In particular, in *Female-Short-lasting-Ischemia groups* (10-min ischemia; Fig 5 - Panel A) at the end of reperfusion the developed LVP recovered by  $62\pm 18\%$

only in *Female-Short-lasting-Ischemia* group and by  $81\pm 11\%$  in *Female-Short-lasting-Ischemia+PostC* group (i.e.  $-38\%$  and  $-19\%$  developed LVP vs baseline level, respectively;  $p < 0.05$  vs baseline for both, and  $p < 0.05$  vs each other). It is likely that this difference in developed LVP recovery is due to the different final infarct size in these two groups. As a matter of fact, in *Male-Short-lasting-Ischemia* and *Male-Short-lasting-Ischemia+PostC* groups in which infarct size was small and not different between each other, at the end of reperfusion the post-ischemic depression of systolic function was limited and similar in the two groups (by about  $-17\%$  at the end of reperfusion for both;  $p < 0.05$  vs baseline level for both; Fig 5; Panel B). Therefore, these data suggest that PostC does not protect against stunning (*ad litteram*).

Yet, in the hearts that underwent 30-min ischemia, the post-ischemic recovery of systolic function was improved by PostC in female, but not in male. In particular, in female groups (Fig 5 – Panel C) at the end of reperfusion developed LVP recovered by  $58\pm 18\%$  in *Female-PostC* group vs  $38\pm 10\%$  recovery in *Female-Infarcting* group (i.e.  $-42\%$  and  $-62\%$  developed LVP vs baseline, respectively;  $p < 0.01$  vs baseline for both, and  $p < 0.01$  vs each other). In male groups (Fig 5 – Panel D), despite marked difference in infarct size, the developed LVP at the end of reperfusion was similar in *Male-PostC* and *Male-Infarcting* groups.

Figure 6, summarize the effects of ischemia/reperfusion and PostC at the end of reperfusion. As can be seen, contracture parallels infarct size in all cases. However, post-ischemic systolic dysfunction seems not correlated to infarct size. Yet, in female hearts, PostC improvement of systolic function seems stronger than the effects on infarct size. Whereas the opposite holds true in the male hearts subjected to 30 min ischemia. In other words, systolic dysfunction is not correlated to the limitation of necrosis, but may be correlated with the persistence of stunning of viable cardiomyocytes. In fact, data show that a strong linear-correlation ( $r^2 = 0.823$ ;  $p < 0.0001$ ) exist between infarct size and end-diastolic LVP (contracture)

at the end of reperfusion (Fig 7A). Yet, a linear correlation ( $r^2 = 0.5621$ ;  $p < 0.001$ ) between infarct size and developed LVP at the end of reperfusion is less evident (Fig 7B). A better fit ( $r^2 = 0.7033$ ) between infarct size and developed LVP can be obtained with a non-linear regression (Boltzmann equation).

## **Discussion**

### ***Implication of the findings***

We found that infarcts were larger in female than male hearts subjected to 10-min ischemia and smaller in female than male hearts subjected to 30-min ischemia. Moreover, PostC protection seems both gender- and infarct size-dependent. In fact, after 10-min ischemia, PostC reduces infarct size and improves systolic function only in female hearts, in which a larger infarct size is observed; in male hearts, in which a limited infarct size is observed, infarct size and post-ischemic systolic function are not affected by PostC. Yet, after 30-min ischemia, PostC reduces infarct size more in male than female hearts, but improves systolic function in female hearts only.

When both stunning and large necrosis are induced by 30-min/120-min I/R protocols, it is hard to distinguish whether the impairment of global function is due to necrosis and/or to stunning of viable tissue. It is likely that an intricate interrelation between the two effects (necrosis and stunning) influences heart performance. Thus recovery of function may not be an appropriate end-point to consider when large infarct size extension is induced by the experimental maneuvers. In fact, in hearts subjected to 30-min ischemia, both in female and male hearts, PostC protects against infarct size extension, but heart contractile function is improved in female hearts only, in which infarct size is smaller. This is also in line with the low level of linear-correlation between infarct size and developed LVP during reperfusion (Fig 7B).

In fact, the more robust end-point in analyzing I/R injury is infarct size [17,28]. Therefore myocardial damages/protection are better evaluated by infarct size assessment. As for preconditioning [17,28], systolic function (developed LV pressure) may not be an appropriate end-point to study PostC protection. The improvement of function during reperfusion in *pre-conditioned* rat hearts has been attributed to the reduction of adenosine release during this phase [17]. On the contrary, in *post-conditioned* hearts an accumulation of adenosine has been reported



[24]. These differences may explain our findings of unclear effect on systolic function by PostC treatment in infarcted groups (i.e. after 30-min ischemia). Adenosine accumulation in the post-ischemic phase may affect systolic function because may lead to excessive ROS formation. In fact in post-ischemic phase adenosine by-products are metabolized by xanthine-oxidase and produces ROS [17]. As a matter of fact, stunning is mainly due to ROS and acidosis [2], which are also integral to the PostC protective signaling [14,26,40,41,48]. Thus, it is not surprising that PostC does not protect against stunning. Of course, in the long term, when stunning spontaneously recovers after ischemia, the reduced necrosis by PostC persistently improves the heart function [33,34].

In order to understand the role of PostC on myocardial stunning appropriate studies are required, in which infarct size is kept at minimum. Our experiments with short-lasting ischemia aimed to ascertain the effect of PostC on myocardial stunning. Surprisingly, in our model 10-min ischemia increased infarct size in female hearts. Yet, PostC reduced infarct size and improved post-ischemic systolic function in female hearts only. Therefore data suggest that the improvement of function is due to the reduction of infarct size rather than stunning. This seems to hold true both in female and male hearts. In fact, PostC reducing necrosis, but not stunning, may (in females) or may not (in males) improve global left ventricle systolic function during 2-hours reperfusion. Moreover, the fact that at the end of reperfusion both the infarct size and systolic dysfunction are greater in *Female-Short-lasting-Ischemia* group than other hearts subjected to 10-min ischemia, and the fact that both infarct size and systolic depression are similar in the *Female-Short-lasting-Ischemia+PostC* group, *Male-Short-lasting-Ischemia* group and *Male-Short-lasting-Ischemia+PostC* group support the idea that the recovery of function is due to the anti-necrotic effect observed in female hearts rather than to an anti-stunning effect.

Our data are in line with those of Couvreur *et al.* [9] which used 10- or 30-min ischemia in canine (gender non specified) and male rabbit models. These authors reported that PostC does not protect against myocardial stunning which follow short period of ischemia (in this case of 10-min ischemia the authors did not check for necrosis). However, their PostC protocol protected against infarct size which followed the 30-min ischemia, but in this last case the authors did not check for post-ischemic function. Here we go further showing that infarct size and post-ischemic systolic dysfunction can act simultaneously, influencing each other.

The higher is the infarct size the more intricate is the interrelation between infarct size and post-ischemic function. In the presence of infarct size higher than 30% of left ventricle mass no-correlation exists between infarct size and global systolic function (Fig 7B); the absence of correlation might depend on the variable degree of myocardial stunning of the viable tissue.

Both contracture and infarct size may be due to calcium-overload, which is reduced by PostC [49]. It has been suggested that in rodent models contracture development, rather than systolic function, may be a more appropriate end-point to study the protective effects of cardioprotective maneuvers [17]. The present study confirms this point of view; in fact contracture development correlates very well with infarct size in all the experimental conditions (Fig 7A). Our study is also in line with that of Kin *et al* [24] conducted in isolated mouse hearts, in which PostC reduced contracture during both early and late reperfusion. In such a study, PostC also hastened the early recovery developed pressure, but this improvement did not persist at 30-min reperfusion.

#### **Comparison with previous studies on female hearts**

Several studies tested the differences in I/R susceptibility between male and female hearts. The majority of studies report a better tolerance to I/R by female hearts, which has been attributed to the effects of estrogens [e.g. 15, 30-32,50]. Yet, no differences in infarct size or in the

incidence of arrhythmias have been reported *in vivo* and *in vitro* models of myocardial ischemia/reperfusion [5,27,43]. Recent studies have also demonstrated differences in post-ischemic myocardial function and infarct size between female and males [1,2,16,32,46] in isolated rat hearts subjected to I/R. It seems that there is a sex difference in the utilization of specific cardioprotective signaling pathways [5,32]. In our study we stress the importance of the duration of index ischemia: 30-min ischemia damages less the female than male hearts; the opposite occurs in response to 10-min ischemia. We do not know the exact mechanism(s) for this difference. We know that after shorter ischemia, reoxygenated cardiomyocytes are in acute jeopardy of Ca<sup>2+</sup> overload-induced contracture and necrosis. Yet, after prolonged ischemia, the ability of mitochondria to rapidly restore a normal cellular state of energy upon reoxygenation is reduced. Thus mechanisms related to the control of pH, Ca<sup>2+</sup> homeostasis or myofibrillar Ca<sup>2+</sup> sensitivity and mitochondria failure become progressively more important. It is likely that cardiomyocytes of pre-menopausal females may be more vulnerable to the early ischemic events and more protect against late ischemic injury. The effect of estrogen on nitric oxide synthases and/or PI3-kinase are likely involved [1-5,32]. Also, estrogens have been shown to reduce ischemia/reperfusion injury under conditions of increased contractility [15,32]. They may up-regulate nitric oxide signaling leading to S-nitrosylation of L-type calcium channels thereby reducing calcium loading during ischemia and early reperfusion [15,32]. These may also explain gender differences in ischemia/reperfusion injury, in post-ischemic systolic recovery and in PostC protection. The relationship between estrogen and these mechanisms, and their role in cardioprotection will require further study.

Very few studies tested the differences between male and female hearts with regard to PostC effectiveness. Crisostomo *et al.* [10], analyzed post-ischemic function and reported that PostC is more protective in male than female rat hearts. In particular, they showed an improved

systolic function in male hearts after either 20 min or 25 min ischemia. This protective effect was also present in female rat hearts exposed to 20 min of ischemia, but absent in those exposed to 25 min ischemia [10]. Whether these protocols of ischemia/reperfusion induced also infarct size was not tested by the authors. From our data, it can not be excluded that the differences in heart recovery are differently and non-linearly influenced by different effects on infarct size in the two genders. In the work conducted by Dow *et al.* [12] on female rats PostC was ineffective in reducing cardiac infarct size induced by either 30- or 45-min ischemia. In our model, after 30-min ischemia PostC is less effective in female than in male hearts in reducing infarct size. We can not exclude that this reduced effectiveness in female hearts may be one of the reason of the negative results in the study of Dow *et al.* [12]. In fact these authors suggested that gender may be a confounder in PostC. We should also keep in mind that 1) infarct size is different in the two genders even when the same duration of ischemia is used [present study], 2) PostC effectiveness against infarct size critically depends on the duration of the preceding period of index ischemia rather than on the PostC algorithm/protocol [present study and 29], and 3) PostC may even be detrimental depending on the duration of the index ischemia [29]. Therefore gender, duration of index of ischemia, development of necrosis and PostC algorithm may be strictly controlled in assessing effectiveness of protective effects of postconditioning against the studied end-points of I/R injury. As a matter of fact in a 2004 editorial Heusch [20] commented that in cardioprotection studies it is mandatory “to carefully control for confounding variables ... such as the size of the area at risk, the duration of the preceding ischemic insult, and collateral status. Neglect of these confounding variables has probably contributed to the failure of translation of experimentally validated principles of cardioprotection to the clinical arena.”

### ***Methodological considerations***

By reducing the number of independent variables to a reasonable minimum, the isolated and isovolumically beating rat heart can be used to study the acute effects of I/R on heart contractility and on infarct size [19,35-41,47]. This model excludes other potential confounders such as collateral vessel (global ischemia), temperature, preload, after-load and heart rate variations. However, the buffer perfusion may compromise heart function over the time and even sham-perfused hearts may develop some degree of necrosis (about 10% of risk area in buffer perfused rat [44, and present study] and rabbit [23] hearts). In our hands, sham perfused hearts show that during the period of observation significant effects on heart function are absent, despite an area of necrosis similar to that of male hearts subjected to 10 min ischemia and 120 reperfusion. Since the latter group of hearts develop systolic dysfunction during reperfusion, it is likely that this dysfunction is due to stunning of viable tissue.

By reducing the number of potential confounders, the overall results suggest that gender exerts an appreciable influence on PostC cardioprotection (evaluated in terms of both infarct size and post-ischemic systolic dysfunction). Yet, the model allowed to show the importance of the duration of index ischemia, since 30-min ischemia damages less the female than the male hearts, the opposite occurring in response to 10-min ischemia.

### ***Conclusions***

Taken together, our data confirm that gender plays an important role as I/R injury determinant. Our data also show, for the first time, that gender plays a pivotal role in PostC effectiveness against necrosis. While PostC-dependent infarct size reduction seems stronger in males than females, myocardial stunning seems not to be affected by PostC in both female and male hearts.

That is, the extension of infarct size depends on the duration of index ischemia, but the outcomes of ischemias of identical durations are different in the two genders: short duration may be more detrimental in females, whereas longer duration may be more detrimental in males. Contracture development and infarct size are linearly correlated both in female and male hearts. Yet, the post-ischemic systolic dysfunction of the left ventricle is a result of both stunning and necrosis and, therefore, the reduction of only one of these two detrimental effects may not be appreciated in terms of developed LVP improvement. In fact PostC reducing necrosis, but not stunning, may or may not improve left ventricle systolic function during 2-hours reperfusion.

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## FIGURE LEGENDS

**Fig. 1. Experimental design.** The isolated, Langendorff-perfused hearts were stabilized (Stab) and subjected to 10 min or 30 min of normothermic global ischemia followed by 120-min of reperfusion. Sham Groups underwent 150-min perfusion only. PostC = postconditioning; R= 10 s reperfusion; I = 10 s ischemia. For further explanation see text.

**Fig. 2. Infarct size is expressed as the percent of left ventricle which is considered as risk area.** The isolated, Langendorff-perfused hearts were subjected to 10 min of normothermic global ischemia followed by 120 min. Male-Short-lasting-Ischemia group and Male-Short-lasting-Ischemia+PostC group show a similar and limited infarct size. Female-Short-lasting-Ischemia group shows a significantly larger infarct size, which was significantly reduced by postconditioning (PostC). NS= non significant; Female-Short-lasting-Ischemia group vs Female-Short-lasting-Ischemia + PostC group and vs both Male groups = \*\* p<0.001 for all.

**Fig. 3. Infarct size is expressed as the percent of left ventricle which is considered as risk area.** The isolated, Langendorff-perfused hearts were subjected to 30 min of normothermic global ischemia followed by 120 min of reperfusion. Postconditioning (PostC) reduces infarct size more in male than female hearts. PostC vs Infarcting Group of the same gender: \* p<0.01, \*\* p<0.001. Male vs female groups: # p<0.05.

**Fig. 4. Left ventricular end-diastolic pressure (LVEDP) during ischemia and reperfusion.** For ischemic groups time 0 marks the onset of 120 min reperfusion, which follow 10 min (Panels A and B) or 30 min (Panels C and D) global ischemia (ischemia begins at time -10 or -30, respectively). Data are expressed as mmHg. Values represent means±SE.

Female-Short-lasting-Ischemia group vs both Sham and Female-Short-lasting-Ischemia+PostC groups: \* \*\* $p < 0.001$  (Panel A). Female-Infarcting group vs Female-PostC: \*  $p < 0.05$  (Panel C). Male-Infarcting group vs Male-PostC: # # #  $P < 0.001$  (Panel D).

**Fig. 5. Developed left ventricular pressure (LVP) during ischemia and reperfusion.** For ischemic groups time 0 marks the onset of 120 min reperfusion, which follow 10 min (Panels A and B) or 30 min (Panels C and D) global ischemia (ischemia begins at time -10 or -30, respectively). Data are expressed as % change from pre-ischemic baseline. Values represent means $\pm$ SE.

Female-Short-lasting-Ischemia group vs Sham: \*\*  $p < 0.01$  (Panel A); Female-Short-lasting-Ischemia group vs Male-Short-lasting-Ischemia+PostC group: \*  $p < 0.05$  (Panel A). Male-Short-lasting-Ischemia group and Male-Short-lasting-Ischemia+PostC group vs Sham: #  $p < 0.05$  (Panel B). Female-Infarcting group vs Female-PostC: \* \* $P < 0.01$  (Panel C).

**Fig. 6. Effects of ischemia/reperfusion and PostC at the end of 120 min reperfusion: infarct size (Panel A), contracture (Panel B) and post-ischemic systolic function (Panel C).** I/R = groups which underwent ischemia/reperfusion only; PostC = groups which underwent postconditioning protocols and 120-min reperfusion after ischemia. PostC reduces infarct size in all groups, except 10-min-Male group which already shows the smallest infarct size. Contracture development parallels infarct size in all cases. Post-ischemic systolic dysfunction is affected by PostC in female only and also in this case parallels infarct size reduction. Left ventricular end-diastolic pressure (LVEDP).

**Fig. 7. Linear and non-linear (Boltzman equation) correlation between Infarct size and End-diastolic left ventricular pressure (LVP) (Panel A) and between Infarct size and Developed LVP (Panel B) at the end of reperfusion.** In Panel A, a very good fit is obtained with both linear and non-linear correlation. On the contrary, in Panel B, a better fit is obtained with the non-linear correlation. Left ventricular end-diastolic pressure (LVEDP). For further explanation see text.



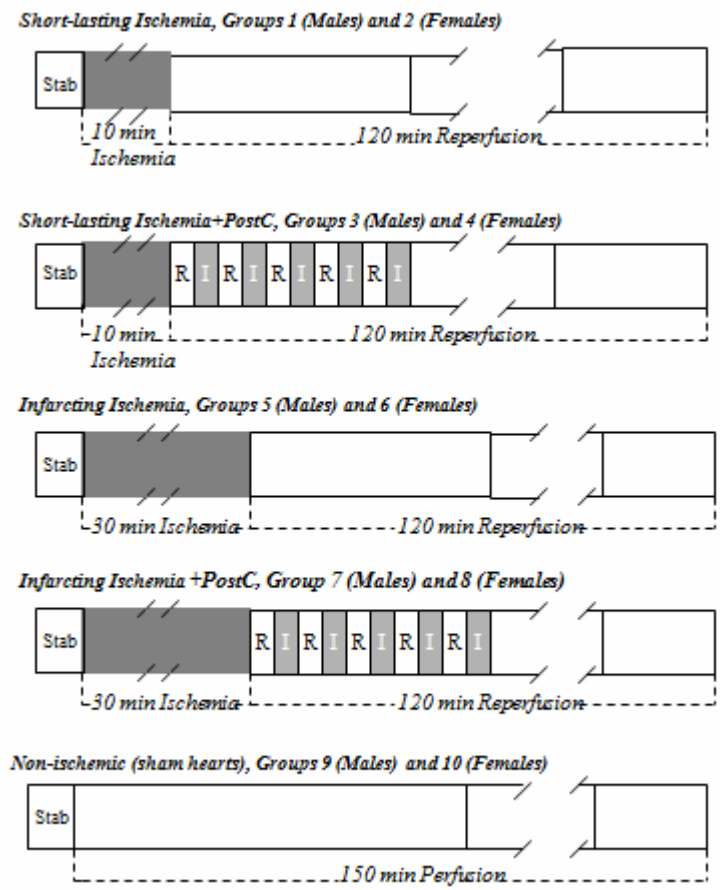


Fig.1

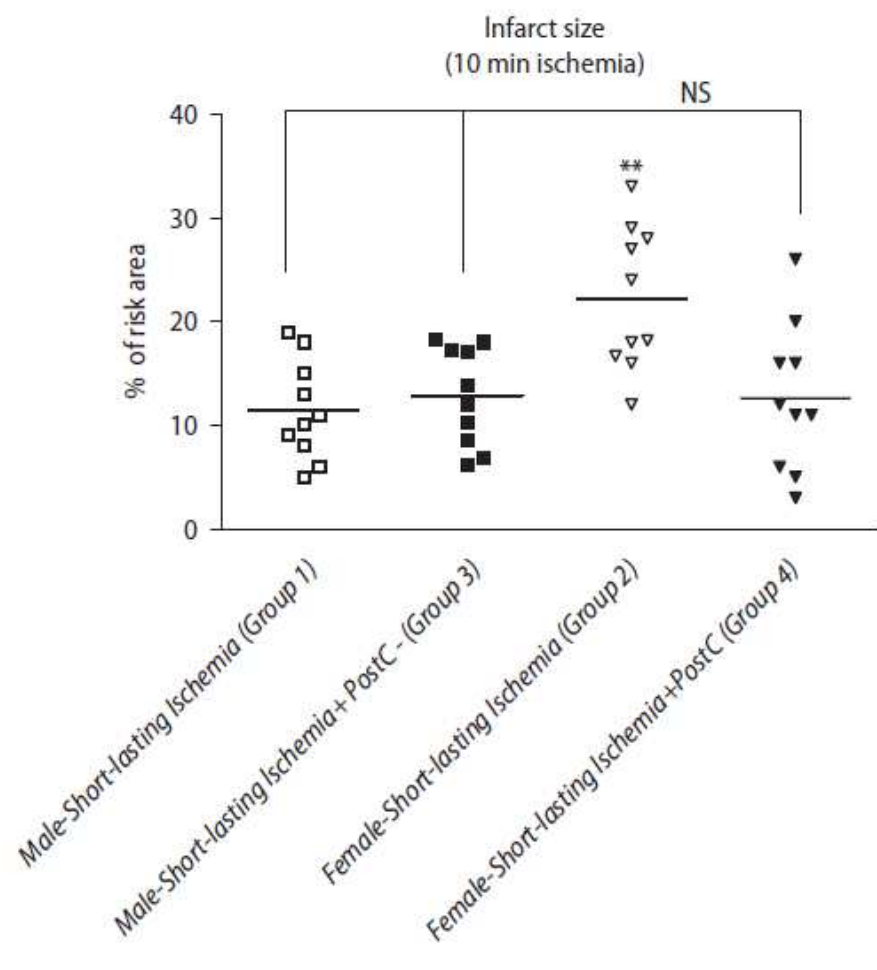


Fig.2

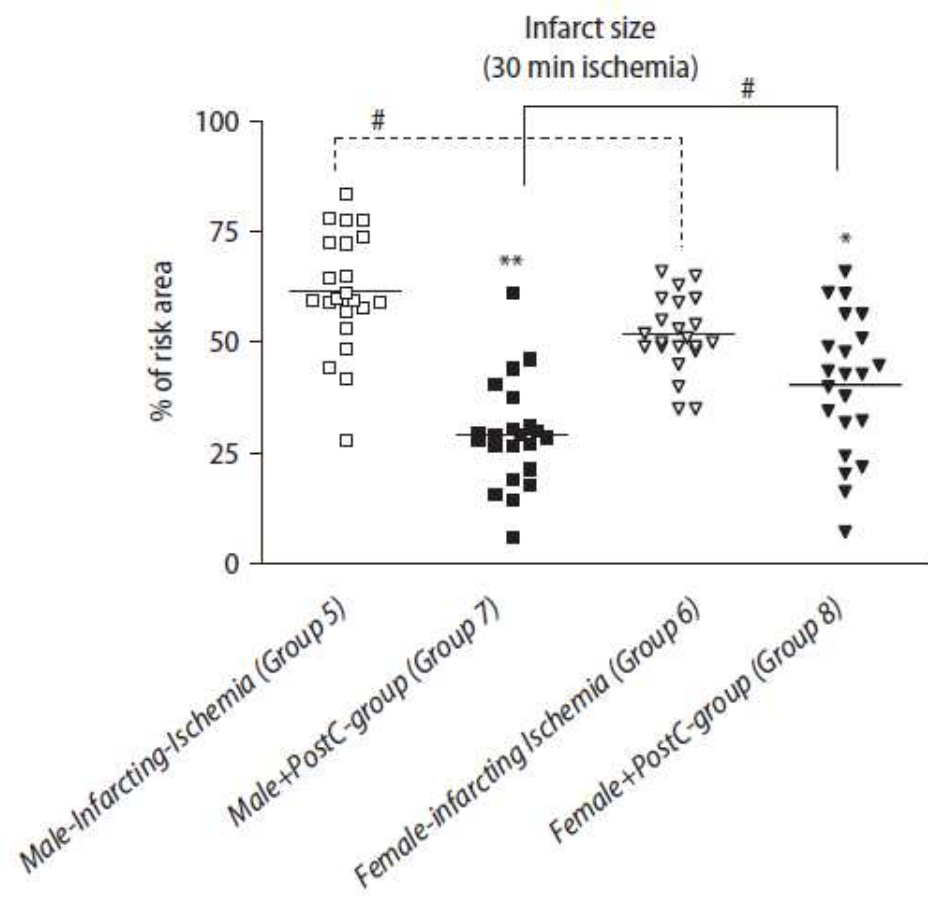


Fig.3

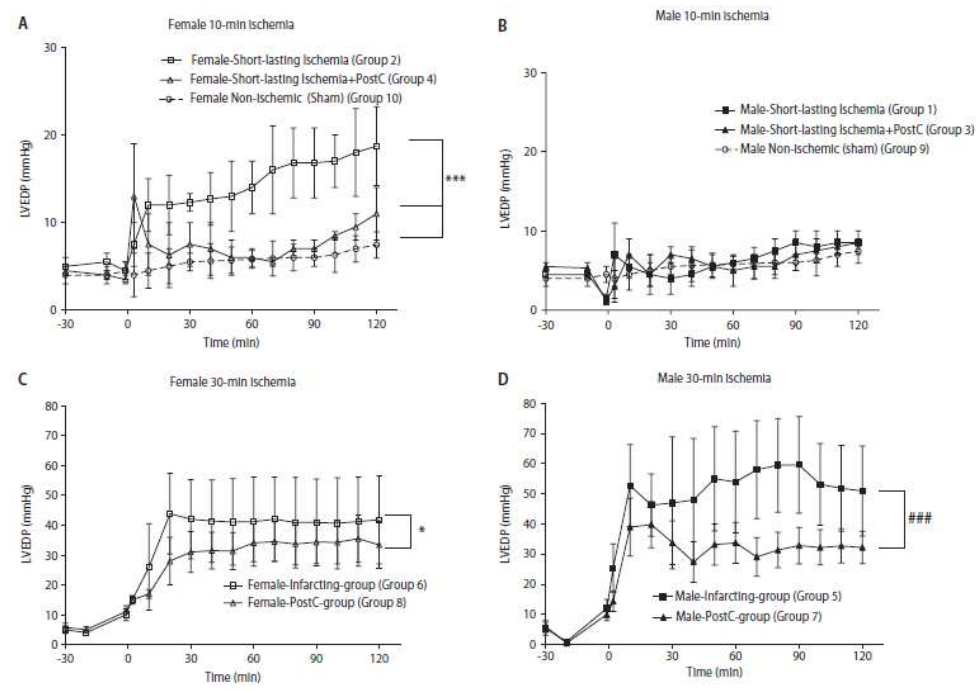


Fig.4

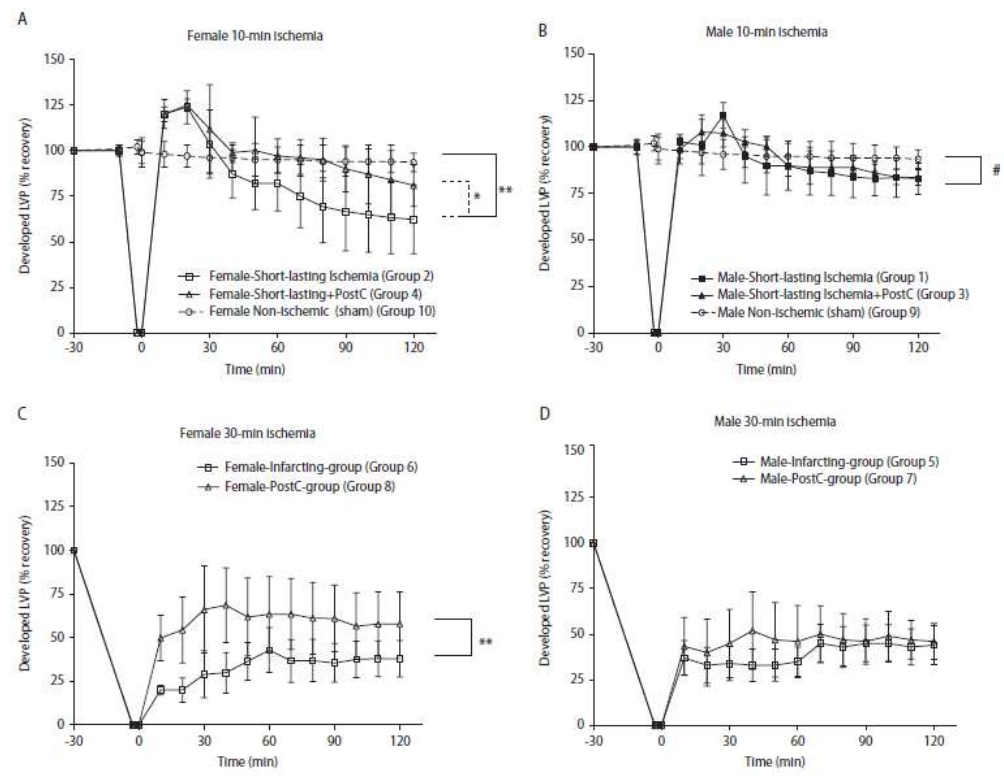


Fig.5

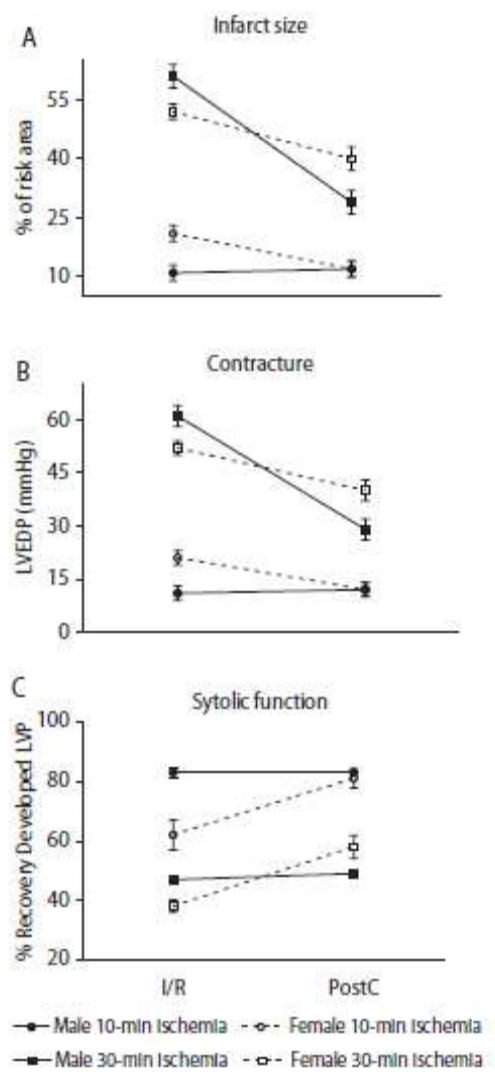


Fig.6

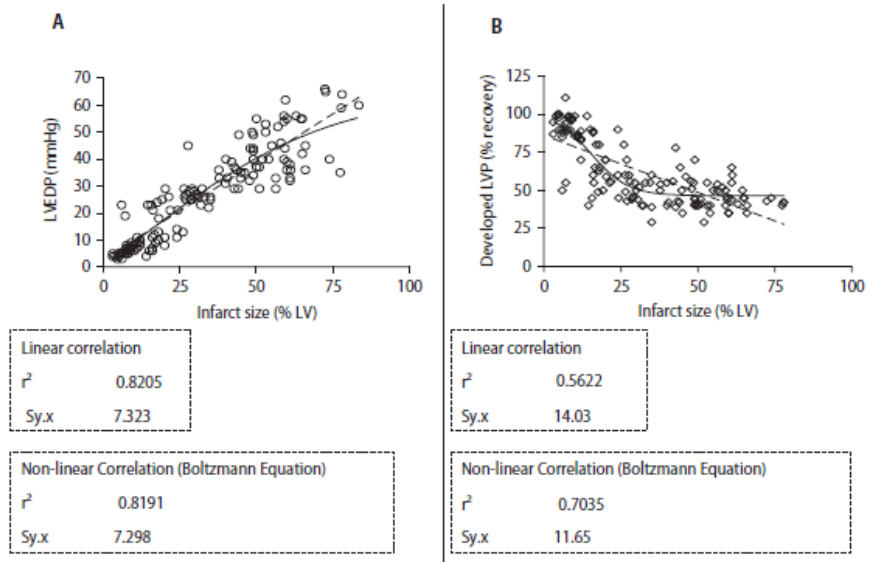


Fig.7

**Table 1****Comparison between male and female organ and body weight**

	Body weight (g)	Cardiac weight (mg)	LV weight (mg)	Cardiac to body weight ratio
Males	499±9	1482±21	905±17	2.67 ±0.012
Females	327±6*	1117±33*	717±12*	3.66 ±0.010*

\*= p< 0.01 vs Female.

**Table 2****Functional parameters before ischemia**

<i>Group</i>	<b>Developed LVP mmHg</b>	<b>LVEDP mmHg</b>	<b>Perfusion Pressure mmHg</b>
<i>Male Short-lasting Ischemia</i>	83±5	5±1	85±3
<i>Female Short-lasting Ischemia</i>	82±3	5±1	83±3
<i>Male Short-lasting Ischemia +PostC</i>	81±4	6±1	82±4
<i>Female Short-lasting Ischemia +PostC</i>	79±2	5±1	84±4
<i>Male-Infarcting</i>	83±1	5±2	85±1
<i>Female-Infarcting</i>	81±2	5±1	86±1
<i>Male-Infarcting+PostC</i>	81±4	6±2	86±4
<i>Female-Infarcting+PostC</i>	80±3	6±1	83±5

Acronyms as in figures.