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## ESTRADIOL TREATED MESENCHYMAL STEM CELLS IMPROVE MYOCARDIAL RECOVERY AFTER ISCHEMIA

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### Abstract

**Background.**—Stem cell therapy is a promising treatment modality for injured cardiac tissue. A novel mechanism for this cardioprotection may include paracrine actions. Our lab has recently shown that gender differences exist in mesenchymal stem cell (MSC) paracrine function. Estrogen is implicated in the cardioprotection found in females. It remains unknown whether 17 $\beta$ -estradiol (E2) affects MSC paracrine function and whether E2 treated MSCs may better protect injured cardiac tissue. We hypothesize that E2 exposed MSCs infused into hearts prior to ischemia may demonstrate increased VEGF production and greater protection of myocardial function compared to untreated MSCs.

**Methods.**—Untreated and E2 treated MSCs were isolated, cultured, plated, and supernatants were harvested for VEGF assay (ELISA). Adult male Sprague-Dawley rat hearts (n=13) were isolated and perfused via Langendorff model, and subjected to 15min equilibration, 25min warm global ischemia, and 40min reperfusion. Hearts were randomly assigned to perfusate vehicle, untreated male MSC, or E2 treated male MSC. Transcoronary delivery of 1 million MSCs was performed immediately prior to ischemia in experimental hearts.

**Results.**—E2 treated MSCs provoked significantly more VEGF production than untreated MSCs (933.2 $\pm$ 64.9 vs. 595.8 $\pm$ 10.7pg/mL). Post-ischemic recovery of left ventricular developed pressure was significantly greater in hearts infused with E2 treated MSCs (66.9 $\pm$ 3.3%) than untreated MSCs (48.7 $\pm$ 3.7%) and vehicle (28.9 $\pm$ 4.6%) at end reperfusion. There was also greater recovery of the end diastolic pressure with E2 treated MSCs than untreated MSCs and vehicle.

**Conclusions.**—Pre-ischemic infusion of MSCs protects myocardial function and viability. E2 treated MSCs may enhance this paracrine protection, which suggests that ex-vivo modification of MSCs may improve therapeutic outcome.

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## Keywords

sex hormones; cardioprotection; estrogen; paracrine; VEGF

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## INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States. However, treatment of this prevalent disease presents interesting challenges over other tissues because myocardium has limited regenerative abilities (1), and reperfusion therapy, the treatment of choice for infarction, is accompanied by the deleterious effects of ischemia / reperfusion (I/R) injury (2, 3). Considering the irreversible nature of cardiac tissue myopathies, remodeling of injured tissue may be a promising treatment modality (4).

Stem cells have generated ongoing interest from researchers and clinicians for their ability to specialize into mature cell types (5). Ferrari et al initially found that bone marrow derived stem cells (BMSCs) transplanted into injured muscle tissue participated in the regeneration process (6). However, it now appears that the cardioprotection afforded by stem cells may not result from differentiation (7, 8). Paracrine actions may also be responsible for the cardioprotective effects of stem cells (9, 10). Indeed, our lab has previously demonstrated that acute pretreatment of myocardium subjected to I/R with human BMSCs increased growth factor production, improved functional recovery, decreased proinflammatory cytokine production and decreased activation of proapoptotic caspases without evidence of differentiation (11).

Gender differences have been observed in myocardial function following I/R injury. Clinical studies have shown that pre-menopausal women have a lower incidence of CVD and increased survival (12). Interestingly, our lab has previously shown that gender differences also exist in MSC growth factor and cytokine production (13). Indeed, female MSCs express greater vascular endothelial growth factor (VEGF) production and less tumor necrosis factor alpha (TNF) production, a proinflammatory cytokine that causes myocardial dysfunction and cardiomyocyte death (1), following insult than male MSCs (14). Hiasa and colleagues found that VEGF plays an important role in BMSC mediated cardioprotection from ischemia (15). Previous studies have found that estrogen is attributable to the cardioprotection found in females (16, 17). MSC sex differences may also reflect chronic/genomic effects of estrogen (14, 18). No study has addressed the role of estrogen on stem cell activation and protection. The main objectives of this study are to investigate the cardioprotective effects of male MSCs treated with 17 $\beta$ -estradiol (E2) on I/R injury and to compare them to the cardioprotective effects of untreated male MSCs on I/R injury. Therefore, we hypothesize that E2 treated MSCs infused into hearts prior to ischemia may demonstrate increased VEGF production and greater protection of myocardial function compared to untreated MSCs.

## MATERIALS AND METHODS

### Animals

Age-matched (250–300 g, 9- to 10-week) normal male Sprague–Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 1 week before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85–23, revised 1985).

### Preparation of mouse bone marrow stromal cells

A single-step purification method using adhesion to cell culture plastic is used as previously described (19) with the following modifications: After killing 8-week old mice, mouse bone marrow stromal cells were collected from bilateral femurs and tibias by removing the epiphyses and flushing the shaft with complete media (Iscove modified Dulbecco medium [GIBCO Invitrogen, Carlsbad, Calif] and 10% fetal bovine serum [GIBCO Invitrogen]) using a syringe with a 23-G needle. Cells were disaggregated by vigorously pipetting several times. Cells were passed through a 30- $\mu$ m nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete media, centrifuging for 5 min at 300 rpm at 24°C, and removing supernatant. The cell pellet was then resuspended and cultured in 75 cm<sup>2</sup> culture flasks with complete media at 37°C. Mesenchymal stem cells were preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete medium was added and replaced every 3 or 4 days thereafter. Mesenchymal stem cell cultures were maintained at 37°C in 5% CO<sub>2</sub> in air. When the cultures reached 90% of confluence, the MSC culture was passaged; cells were recovered by the addition of a solution of 0.25% trypsi-EDTA (GIBCO Invitrogen) and replated in 75 cm<sup>2</sup> culture flasks.

### Measurement of MSC VEGF production

MSCs were plated in 12-well plates in a concentration of  $1 \times 10^6$  cells/well/mL, and divided into four groups: 1) untreated MSCs; 2) 10 nM E2 treated MSCs; 3) 100 nM E2 treated MSCs; and 4) 1000 nM E2 treated MSCs. The 17 $\beta$ -estradiol solution was prepared with water soluble  $\beta$ -Estradiol (Sigma-Aldrich, St. Louis, MO) and PBS. After 24 h, supernatants were harvested for VEGF assay (enzyme-linked immunosorbent assay [ELISA]).

To determine the dilution of estradiol for ex vivo isolated heart experiments, the latter dose response curve of estradiol measuring VEGF production was used. Treated MSCs were subjected to 1  $\mu$ M 17 $\beta$ -estradiol for 24h.

### Preparation of E2 treated MSCs for isolated heart

E2 treated MSCs were subjected to 1  $\mu$ M estradiol for 24 h. After 24 h, the estradiol treated supernatant was removed, and the cells were washed with PBS to remove any residual E2 from the incubation medium. The cells were then recovered by the addition of a solution of 0.25% trypsi-EDTA and resuspended in perfusate vehicle to further eliminate any residual E2.

### Experimental isolated heart groups

All isolated rat hearts were subjected to the same I/R protocol: 15 min equilibration period, 25 min of global index ischemia (37°C), and 40 min of total reperfusion. Male rats were randomly assigned to three experimental groups: 1) perfusate vehicle (n = 4); 2) untreated male mesenchymal stem cell (n = 5); and 3) 17β-estradiol treated male mesenchymal stem cell (n = 4). Experimental hearts were infused with 1 million mesenchymal stem cells in 1 mL over 1 minute immediately prior to ischemia. Vehicle and MSC solutions were infused through a port above the aortic root one minute prior to ischemia.

### Isolated heart preparation (Langendorff)

Rats were anesthetized (sodium pentobarbital, 60 mg/kg intraperitoneal) and heparinized (500 U intraperitoneal), and hearts were rapidly excised via median sternotomy and placed in 4°C Krebs–Henseleit solution. The aorta was cannulated and the heart was perfused (70 mmHg) with oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) Krebs–Henseleit solution (37 °C). A left atrial resection was performed prior to insertion of a water-filled latex balloon through the left atrium into the left ventricle. The preload volume (balloon volume) was held constant during the entire experiment to allow continuous recording of the LVDP and EDP. The balloon was adjusted to a mean EDP of 5 mmHg (range 4–8 mmHg) during the initial equilibration. Pacing wires were fixed to the right atrium and hearts were paced at approximately 6 Hz, 3 V, 2 ms (350 bpm) throughout perfusion. A three-way stopcock above the aortic root was used to create global ischemia, during which the heart was placed in a 37°C degassed organ bath. Coronary flow was measured by collecting pulmonary artery effluent. Data were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and an Apple G4 PowerPC computer (Apple Computer Inc., Cupertino, CA). The +dP/dt and –dP/dt were calculated using PowerLab software. After reperfusion, the heart was removed from the apparatus, immediately sectioned, and snap frozen in liquid nitrogen.

### Presentation of data and statistical analysis

All reported values are mean ± S.E.M. Data were compared using two-way analysis of variance (ANOVA) with post-hoc Bonferroni test or Student's *t*-test. A two-tailed probability value of less than 0.05 was considered statistically significant.

## RESULTS

### MSC Activation

E2 exposure at 1000 nM, but not 10 nM or 100 nM, resulted in significantly increased production of VEGF. 1000 nM E2 treated MSCs provoked significantly more (*p*<.05) VEGF production (pg/mL) than untreated MSCs (1667 ± 44.4 vs. 1037 ± 165), as shown in Figure 1.

### Myocardial function

Ischemia / reperfusion resulted in markedly decreased left ventricular developed pressure (LVDP) in all groups (Fig 2). Postischemic recovery of LVDP (expressed as percentage of

preischemic function) was significantly greater ( $p < .001$ ) in the E2 treated group ( $66.9 \pm 3.3\%$ ) than the untreated MSC group ( $48.7 \pm 3.7\%$ ) and vehicle ( $28.9 \pm 4.6\%$ ) at end reperfusion (Fig 2).

Left ventricular end diastolic pressure (mmHg) was elevated in response to I/R in all groups (Figure 3). However, isolated hearts in the E2 treated group ( $45.0 \pm 4.2$ mmHg) had significantly decreased EDP after forty minutes of reperfusion compared to those who received untreated MSCs ( $55.0 \pm 6.1$ mmHg,  $p < .05$ ) or vehicle ( $71.9 \pm 4.4$ mmHg,  $p < .001$ ) as shown in Fig 3.

Postischemic recovery of the maximal positive and negative values of the first derivative of pressure (+dP/dt and -dP/dt) declined in response to I/R. Interestingly, recovery of +dP/dt and -dP/dt was very similar with E2 treated MSC ( $1645.4 \pm 180.9$ mmHg/s and  $-854.9 \pm 100.2$ mmHg/s, respectively, Fig. 4 and 5) and untreated MSC ( $1334.9 \pm 132.3$ mmHg/s and  $-784.4 \pm 94.0$ mmHg/s, respectively). Both E2 treated MSC and untreated MSC groups showed better recovery than vehicle ( $589.7 \pm 79.8$ mmHg/s and  $-403.6 \pm 43.5$ mmHg/s, respectively, Fig. 4 and 5) at end reperfusion.

No significant differences in coronary flow were observed between the control ( $11.0 \pm 0.4$  to  $9.8 \pm 0.9$ ml/min) and the untreated MSC ( $11.0 \pm 1.9$  to  $11.8 \pm 4.2$ ml/min) and E2 treated MSC groups ( $9.5 \pm 0.5$  to  $10.0 \pm 0.6$ ml/min, Fig. 6).

## DISCUSSION

The results of this study indicate that acute exposure of MSCs to 24h of 17 $\beta$ -estradiol increases MSC VEGF production. Furthermore, isolated hearts infused with these E2 treated MSCs via transc coronary delivery prior to ischemia demonstrate: 1) greater post-ischemic end reperfusion LVDP; and 2) decreased end reperfusion EDP compared to untreated MSCs and vehicle. This study is the first demonstration that suggests that estrogen may have a positive influence on mesenchymal stem cell VEGF production as well as mesenchymal stem cell induced myocardial protection during I/R injury.

These findings give further evidence that untreated MSCs are cardioprotective (20–22). We found that untreated MSCs significantly increased left ventricular developed pressure, contractility (+dP/dt) and rate of relaxation (-dP/dt) at the end of reperfusion. Additionally, left ventricular end diastolic pressure was significantly decreased at end reperfusion compared to control, indicating perhaps a reduction in the unstressed volume of the ventricle, possibly due to myocardial contracture or edema. Previous studies by our lab (9, 11) have also demonstrated that untreated MSCs are myocardial protective. Wang et al (11) demonstrated that pretreatment with human MSCs improved functional recovery, decreased myocardial proinflammatory cytokine production, and decreased myocardial pro-apoptotic caspases following I/R.

Paracrine effects may help explain why stem cell delivery into injured tissue confers cardioprotection in an acute setting (< 24 hrs), without evidence of differentiation. Macrophages and cardiomyocytes release proinflammatory cytokines following injury (1). In response to the inflammatory environment, stem cells may release growth factors such as

VEGF (9), hepatocyte growth factor (HGF) (23), and insulin-like growth factor 1 (IGF-1) (23) that interact with neighboring cardiomyocytes. These growth factors may promote angiogenesis (24, 25), decrease proinflammatory cytokine production (26, 27), and reduce apoptosis (28, 29).

Recently, some researchers have focused on the modification of stem cells in order to maximize therapeutic benefit. Transduction of MSCs to overexpress VEGF has been studied. Matsumoto et al infused MSCs transduced with VEGF into hearts following coronary ligation and found decreased infarct size and improved functional recovery (30). Similarly, Wang et al determined that over-expression of VEGF by bone marrow mesenchymal stem cells improved recovery after experimental coronary LAD ligation (31). Enhancing the survival of transplanted stem cells may also improve stem cell efficacy. Li et al infused MSCs that overexpressed an anti-apoptotic Bcl-2 gene into hearts and reported decreased apoptosis and increased functional recovery following myocardial infarction compared to normal MSCs (32). Similar to Bcl-2, Uemura and colleagues found that MSC proliferation and survival may depend on Akt, a prosurvival gene (8). Accordingly, transduction of Akt1 into MSCs followed by an infusion of these Akt1 overexpressing stem cells conferred increased functional recovery and decreased inflammation and apoptosis (33). Further study of these Akt1 overexpressing MSCs revealed that the cardioprotective effects observed may be from paracrine actions (34, 35). The modification of stem cells to increase potency and cell survival may lead to improved outcomes.

Estrogen has been widely implicated in the increased myocardial protection found in females via direct interactions with the heart. Interestingly, the findings of this study suggest that estrogen may also have other indirect cardioprotective effects via enhancement of MSC paracrine pathways. When E2 binds with estrogen receptor alpha, it may cause MSCs to release growth factors. Indeed, in this study, E2 treated MSCs showed an increased production of VEGF in cell culture as compared to untreated MSCs (Fig. 1). Further, estrogen may inhibit part of the inflammatory cascade, which produces proinflammatory cytokines and increases apoptosis. Xu et al showed that exogenous estrogen mediates protection from I/R injury by decreasing TNF production (36). Estrogen may be a novel adjunct to improve MSC paracrine mediated protection. This study demonstrates that isolated hearts infused with E2 treated MSCs infused prior to ischemia exhibit greater recovery of myocardial function (LVDP and EDP) than untreated MSCs. Therefore, ex vivo modification of MSCs prior to therapeutic implementation may confer increased benefit.

This study suggests that estrogen enhances a stem cell's ability to improve myocardial functional recovery following I/R injury. Future studies are necessary to understand the gender differences associated with estrogen treated stem cells as well as to elucidate the mechanism by which E2 mediates this enhancement.

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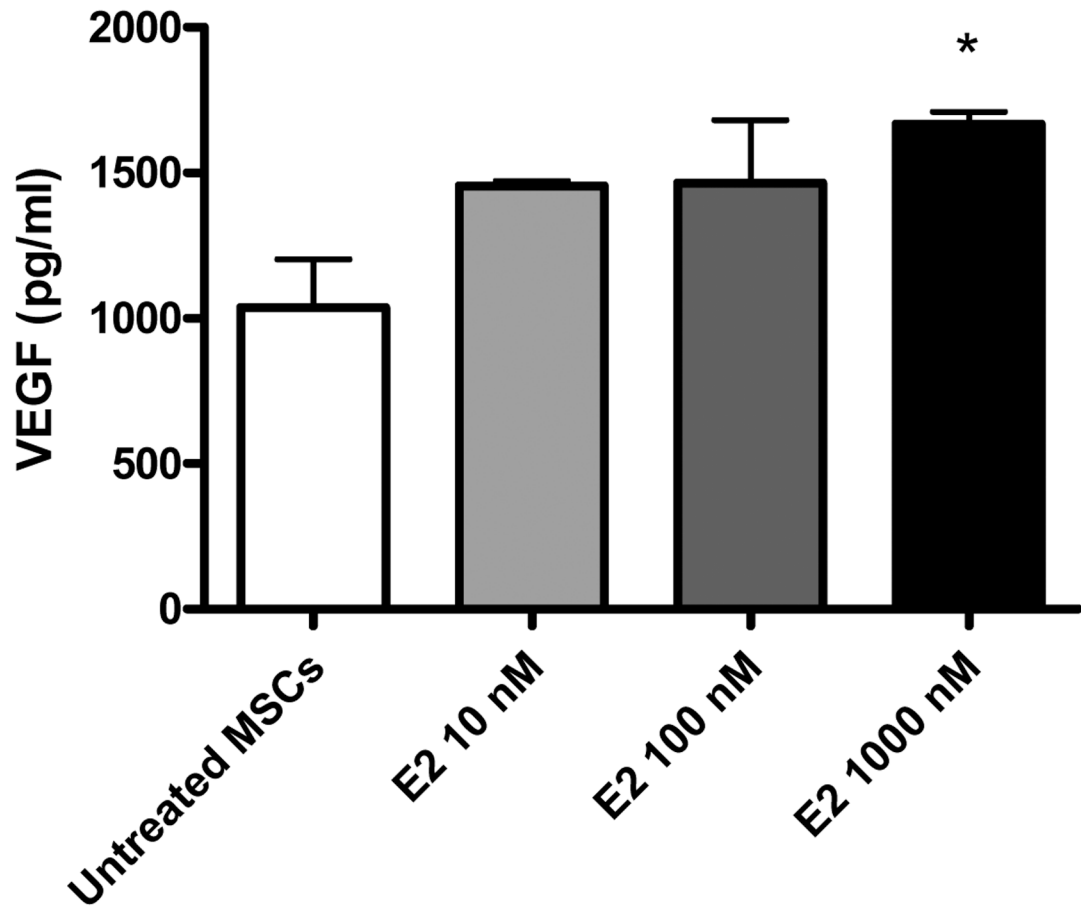
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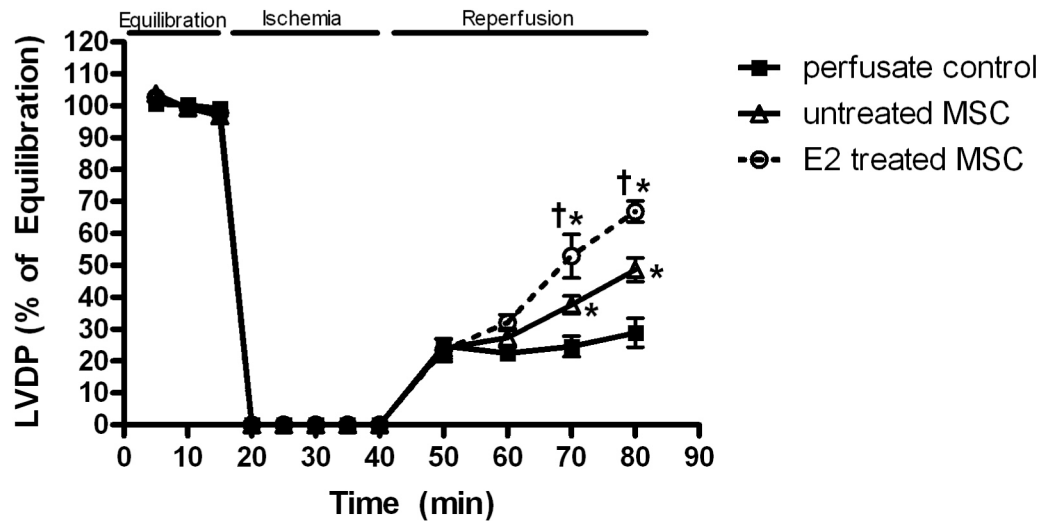
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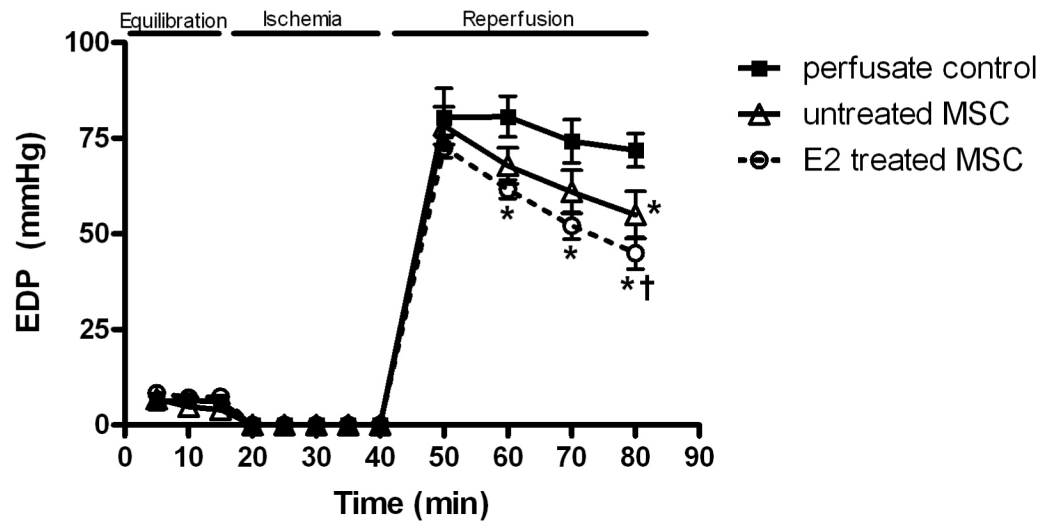
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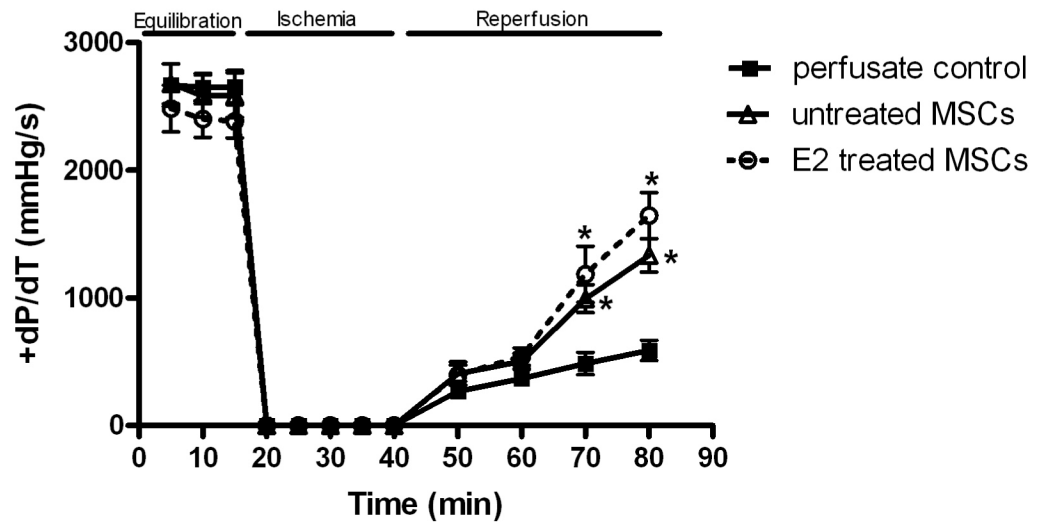
**FIG. 1.** Expression of VEGF in untreated male MSCs and MSCs exposed to increasing doses of 17 $\beta$  Estradiol (E2). \* $p < 0.05$  vs. untreated MSC; results are expressed as pg/mL, mean  $\pm$  SEM.



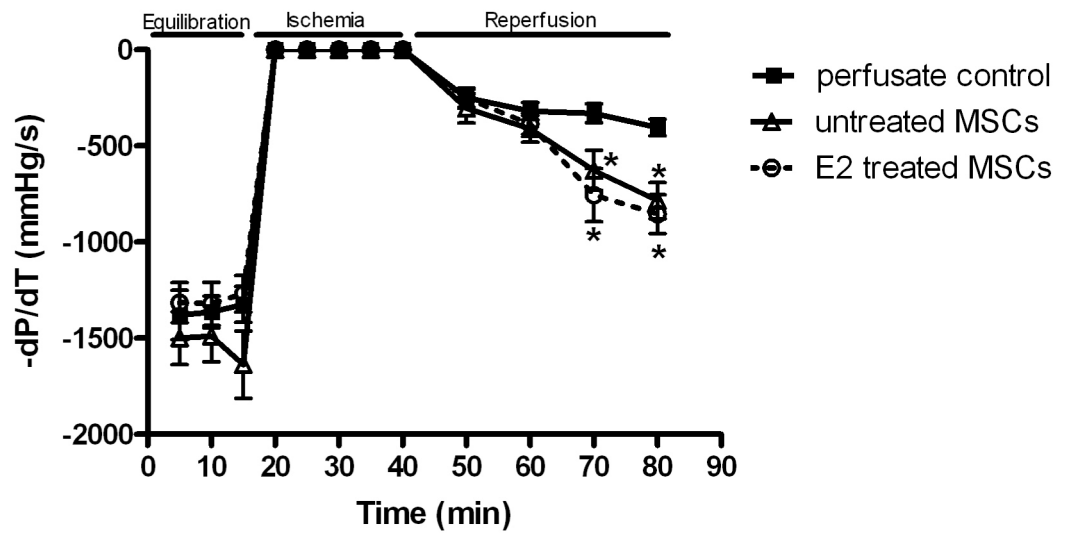
**FIG. 2.** Changes in left ventricular developed pressure (LVDP % of equilibration) during I/R. E2 treated MSC group compared to untreated MSC group and control. Results are mean  $\pm$  SEM, \* $p$ <0.001 vs control, † $p$ <0.01 vs untreated MSCs.



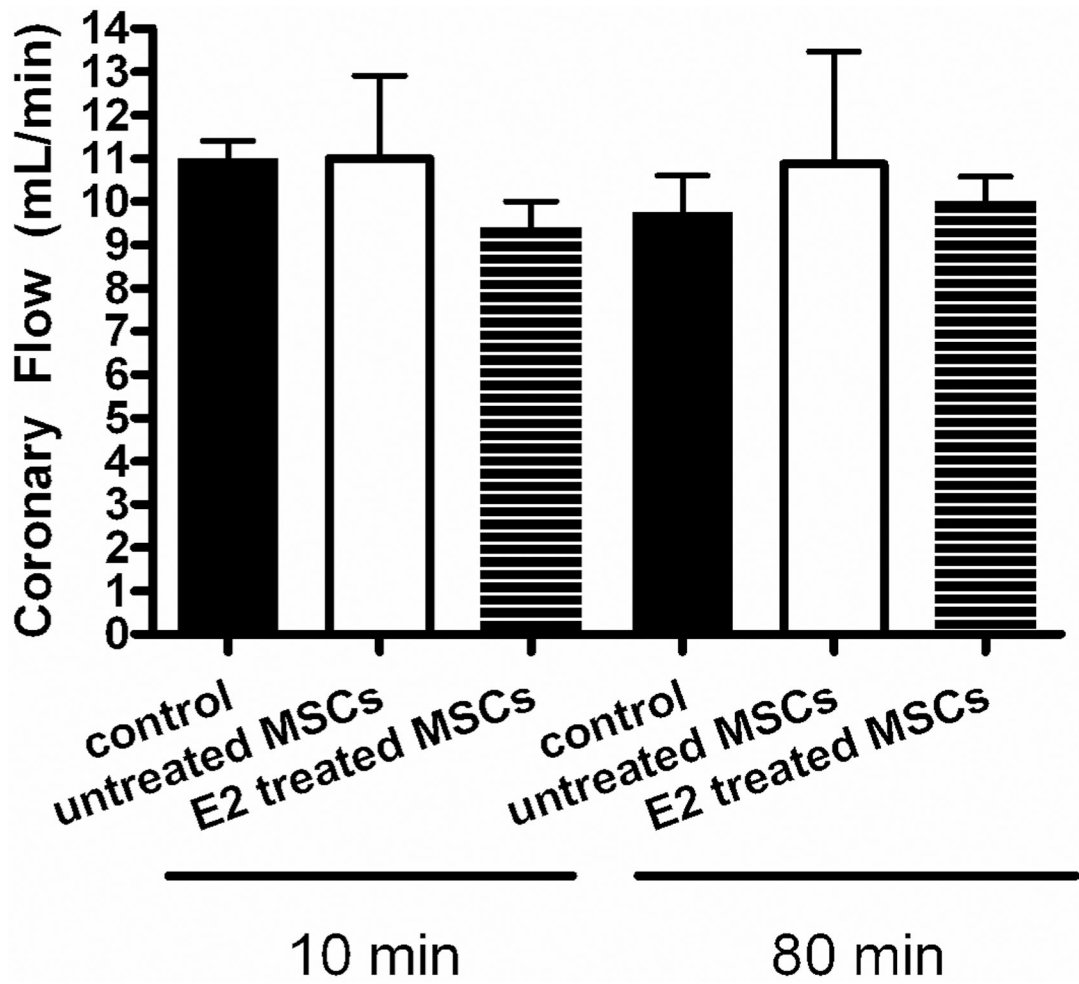
**FIG. 3.** Changes in left ventricular end diastolic pressure (EDP) during I/R. E2 treated MSC group compared to untreated MSC group and control. Results are mean  $\pm$  SEM, \* $p$ <0.01 vs control, † $p$ <0.05 vs untreated MSCs.



**FIG. 4.** Changes in the maximal positive values of the first derivative of pressure (+dP/dt). E2 treated MSC group and untreated MSC group compared to control. Results are mean  $\pm$  SEM, \* $p < 0.01$  vs control.



**FIG. 5.** Changes in the maximal negative values of the first derivative of pressure (-dP/dt). E2 treated MSC group and untreated MSC group compared to control. Results are mean  $\pm$  SEM, \* $p < 0.01$  vs control.



**FIG. 6.** Coronary flow at 10 minutes (during equilibration and before ischemia) and 80 minutes (at the end of reperfusion). E2 treated MSC group and untreated MSC group compared to control.