Original Article

Evaluation the effect of testosterone on the number of endothelial progenitor cells and amount of SDF-1α, PDGF, bFGF, and NO

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Abstract

Background: Recent therapeutic advances in cardiovascular disease, thanks to the discovery of endothelial progenitor cells (EPCs). Stromal cell-derived factor-1α (SDF-1α), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and nitric oxide (NO) play a role in migration, homing, and differentiation of EPCs into mature endothelial cells. The incidence of cardiovascular disease is higher in men than in women. This fact suggests the influence of sex hormones on incidence of cardiovascular disease. **Methods:** Twenty-four female wistar rats weighing 160–180 g were randomly divided into four groups ($N = 6$): 1. sham-treated by sesame oil, 2. ovariectomized (OVX)-treated by sesame oil, 3. OVX-treated by 10 μg/kg/day testosterone, and 4. OVX-treated by 100 μg/kg/day testosterone. After 21 days, the animals were euthanized and blood samples were saved for determination of EPC count and serum levels of SDF-1α, PDGF, bFGF, and NO production. **Results:** High-dose testosterone induced significant increase in EPC count in OVX rats ($P < 0.05$). Also 100 μg/kg/day testosterone increased serum level of SDF-1α more than OVX-treated by 10 μg/kg/day testosterone ($P < 0.05$). But 10 μg/kg/day testosterone increased significantly the serum level of PDGF >100 μg/kg/day testosterone-treated group ($P < 0.05$). The serum level of bFGF in sham-treated by sesame oil was equal with its concentration in OVX-treated by 100 μg/kg/day testosterone. And the serum concentration of NO production in testosterone-treated groups were significantly less than other groups ($P < 0.05$). **Conclusions:** This study suggests that testosterone might be effective on cardiovascular disease in females by increasing EPC count through SDF-1α and PDGF mechanisms which are some of the vascular healing factors.

Keywords: *Basic fibroblast growth factor, endothelial progenitor cell, nitric oxide, platelet-derived growth factor, stromal cell-derived factor-1α, testosterone*

Introduction

Androgen production in women declines gradually throughout the reproductive years.[1] A woman of 40 years has approximately half the testosterone of a 21-year-old one.[2] Recent studies have focused on testosterone therapy in both pre- and post-menopausal women with symptoms of relative androgen deficiency including; diminished sense of well-being, dysphoric mood (sadness, depression, anxiety, and irritability), fatigue, decreased libido, insomnia, hot flashes, bone loss, decreased muscle strength, changes in cognition and memory, pain, vaginal dryness, and incontinence. Continuous testosterone delivered by subcutaneous implant has been safely used in women since 1938 and until recently was the only licensed form of testosterone for women in England.[3,4]

Cardiovascular risk is markedly higher in men compared to age-matched women throughout premenopausal years. This difference is abrogated after menopause, suggesting an association with sex steroid hormones and proposes that cardiovascular events are gender driven.[5,6]

Endothelial progenitor cells (EPCs) are thought to stimulate repairing damaged endothelial cells (ECs) in injured vessels leading to improvement of vessel function, and play an important role in the process of neoangiogenesis and re-endotheliazation.[7] Several studies have shown that EPCs have the potential to differentiate into cardiomyocytes and smooth muscle cells, therefore enhancing myocardial neovascularization and improvement of myocardial function and it has shown that patients with lower level of circulating EPCs are in a higher risk of cardiovascular diseases (CVD).[8-10] EPCs

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are originally derived from bone marrow and migrated to peripheral blood, expressing specific surface membrane markers such as the hematopoietic progenitor cell markers CD34, CD45, and the vascular endothelial growth factor receptor (VEGFR) KDR. Levels of circulating EPCs, determined as CD34 positive, CD45 positive, and VEGFR-2 positive cells in peripheral blood.[11,12]

Sex hormones play an important role in stimulation, migration, homing, and differentiation of circulating EPCs into mature cells.[5,13] With regard to more incidence of CVD among men versus women, a positive correlation between estrogen levels and the number and function of circulating EPCs has been shown. Despite the fact that extensive studies have shown the impress of 17β-estradiol (E2) on angiogenesis and vasculogenesis, but the exact effect of sex steroid hormones and especially androgen is still unclear.[14,15] We do not know exactly whether the reason of less incidence of CVD among women in reproductive years is the high level of estrogen or low testosterone levels.

Sex hormones have various effects on vascular cells, and that many of these effects are achieved through rapid, membrane-initiated receptor-dependent signaling responses, which are different from the classical genomic actions.

Estrogen moderates reproduction of ECs via endothelial receptors (ER)α and also supports ischemic vessels via ERβ and decrease apoptosis.[16] Androgen receptor (AR) is expressed in vascular ECs, EPCs, mesenchymal stem cells, and muscular cells. Testosterone rapidly activates endothelial nitric oxide synthase (eNOS) and enhances nitric oxide (NO) production via activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt cascade through interaction between AR and the p85a subunit of PI3-kinase.[17] A number of reports have indicated that testosterone appears to have very rapid effects on the vascular system, including vasodilatation. It has been shown that physiological concentrations of testosterone causes actually (in minutes) NO-dependent vasodilatation via AR-mediated eNOS activation, which is consistent with the non-genomic nature of the response in arteries.[18]

NO is a radical that affect migration and homing of EPCs. NO have widespread physiological and pathophysiological effects result in vascular tone adjustment, angiogenesis, wound, and inflammation healing. There are several studies that established sex hormones affect EC by changing production and bioavailability of NO.[19]

In this study, we aimed to determine the effect of testosterone on CD34+ EPCs by measuring the number of circulating EPC and stromal level of NO production in wistar rats.

Methods

Animals, this study was approved by the local Ethics Committee of the Isfahan University of Medical Sciences. Twenty-four adult female wistar rats weighing about 160–180 g were purchased from the Pasteur Institute of Iran were randomly divided into four groups (*N* = 6/group), separately. All animals were in equal condition and housed in plastic cages with metal doors (three animals/cage). All conditions including temperature, humidity, light (12 h light/12 h darkness), and enough access to food and water were in control. For rats' adaptation, all experiments were done after 1 week of complete staying of animals in their home.

Gonadectomy, rats were gonadectomized at 4 weeks of age basically under the WaynForth method.[20] For gonadectomy, rats were anesthetized with ketamin 10%, 60–70 mg/kg BW (Alfason, Holland) and xylazine 2%, 5–13 mg/kg BW (Alfason, Holland) intraperitoneal (IP) and then operation started. Briefly, skin incision was approximately 10 mm. The ovary and oviduct were removed, and allowed uterus to return to the abdomen. Muscle layer was closed with absorbable suture and skin was closed with 6.0 silk (Ethicon LTD, Edinburgh, Scotland, UK) suture.

Sham operation, an incision was made in the location of ovary for female rats, and then the incision was closed without any specific manipulation.

Rats received 0.2 ml penicillin 80,0000 U/ml (IP) post-operatively for prevention from infection and they were sent back to their cages for recovery after anesthesia. The animals were evaluated at least daily for 3 days and additional analgesics given as needed. For deletion the whole endogenous androgen and estrogen, this androgen replacement study, began 2 weeks after surgery.

All injections, including sesame oil and testosterone (sigma, Germany) performed daily subcutaneously (SC) in rats' back for 21 days.

Groups

First group was sham-operative female rats received only 0.1 ml sesame oil as a vehicle (sham-vehicle) and OVX female rats (*N* = 18) were subdivided into three groups (*N* = 6/group). Second group received 0.1 ml subcutaneous injection of sesame oil (OVX-vehicle), third group treated by 10 μg/kg/day testosterone dissolved in sesame oil (OVX-10 μg/kg/day tes.), and fourth group also treated by 100 μg/kg/day testosterone (OVX-100 μg/kg/day tes.).

Flow cytometry

After 24 h from the last injection, blood depletion was performed from orbital sinus of rats' eyes. Circulating progenitor cells were analyzed by flow cytometry on blood samples. In this way, we used the markers presenting by EPCs such as VEGFR-2, CD34+, and CD45+.[21] Briefly, blood samples were collected in tubes with ethylenediaminetetraacetic acid (EDTA) and incubated by FcR blocking for 10 min. Then we incubated 50 μl of whole blood with 4 μl of anti-KDR (R&D system, USA), 5 µl of each anti-CD34+ (FITC eBioscience, San Diego, California), and anti-CD45+ (eBioscience, Santa Cruz, California). Control samples that considered as negative controls were incubated with control isotype antibody. After red cell lysis, suspension was analyzed by the FACS Caliber. After gating, the number of lymphocytes and EPCs, VEGFR-2, CD34+, and CD45+ were determined.

Enzyme-linked immunosorbent assay

Finally, the animals were euthanized under standard method and 5 ml blood collected from their hearts. Blood samples were saved in tubes with 0.1 ml EDTA (1 mM) and maintained at room temperature for 40 min, then the samples were centrifuged by 3000 rpm for 15 min and their supernatant collected and reserved in −70°C for measuring the serum level of SDF-1α, PDGF, bFGF, and NO production. The concentration of SDF-1α, PDGF, and bFGF was measured by the enzyme-linked immunosorbent assay (ELISA) kit (R and D system, USA). NO production was measured by the Griess reaction as previously described.[22]

Statistical analysis

Values expressed as means ± standard error unless where specified. Progenitor cell count is expressed as cell number/106 cytometric events. Comparison between two or more groups was assessed using the Student's t-test and analysis of variance (ANOVA), respectively. The least significance difference (LSD) test was used for multiple testing. A multiple regression analysis was built with the EPC level as the dependent variable and sex hormone concentrations as explanatory variables to determine any independent associations. SPSS version 20.0 (version 20, SPSS Inc., Chicago, IL) was used, and statistical significance was set at $P = 0.05$.

Results

Changes in CD34+ cells in peripheral blood

The number of circulating CD34+ EC in rats 21 days after high-dose testosterone injection was significantly higher than count measured in other groups of animals (all $P < 0.05$, Figure 1). The CD34+ cells count in peripheral blood in sham-vehicle, OVX-vehicle, OVX-10 µg/kg/day testosterone, and OVX-100 µg/kg/day testosterone groups were 11.4 ± 1.89, 7.6 ± 2.25, 10.4 ± 6.02, and 29 ± 18.64, respectively.

Serum level of SDF-1α, PDGF, bFGF, and NO production

About 100 µg/kg/day testosterone increased serum level of SDF-1α more than OVX-treated by 10 µg/kg/day testosterone. The corresponding values for serum level of SDF-1α were 125 ± 42.4, 70.6 ± 10.3, 69.4 ± 15.2, and 166 ± 62 pg/ml of blood serum level of PDGF in sham-vehicle, OVX-vehicle, OVX-10 µg/kg/day

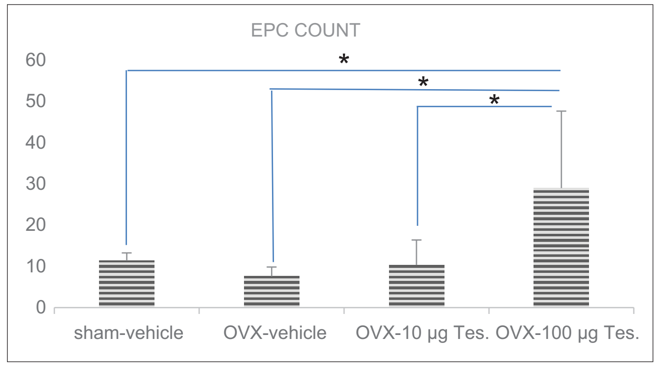

Figure 1: Effect of exogenous testosterone one the EPC count. 1. Sham-vehicle 2. Ovariectomized-sesame oil 3. Ovariectomized rats treated by 10 µg/kg/day testosterone 4. Ovariectomized rats treated by 100 µg/kg/day testosterone

testosterone, and OVX-100 µg/kg/day testosterone groups were 350 ± 70.34, 334.7 ± 93.34, 622.2 ± 231.9, and 381.9 ± 207.7 ng/ml of blood, respectively ($P < 0.05$, Figure 2).

There were significant differences in the serum level of PDGF between the group OVX-100 µg/kg/day and other groups ($P < 0.05$, Figure 3). Serum level of PDGF in sham-vehicle, OVX-vehicle, OVX-10 µg/kg/day testosterone, and OVX-100 µg/kg/day testosterone groups were 350 ± 70.34, 334.7 ± 93.34, 622.2 ± 231.9, and 381.9 ± 207.7 ng/ml of blood, respectively. Also the serum level of bFGF in group sham-vehicle was significantly higher than animals treated by low-dose testosterone but its concentration was exactly equal to concentration of bFGF in animals treated by high-dose testosterone ($P < 0.05$, Figure 4). Serum level of bFGF in sham-vehicle, OVX-vehicle, OVX-10 µg/kg/day testosterone, and OVX-100 µg/kg/day testosterone groups were 61.4 ± 7.25, 55.1 ± 6.39, 49.1 ± 5.42, and 61.4 ± 2.98 ng/ml of blood, respectively. Moreover, the corresponding values for serum level of NO production were 30.7 ± 1.4, 22.4 ± 0.3, 18.3 ± 1.7, and 19.2 ± 0.4 µmol/dl of blood. The serum concentration of NO production in testosterone-treated groups was significantly less than other groups ($P < 0.05$, Figure 5).

Neovascularization plays a critical role in tissue repair, wound healing, and tumor growth. CD34+ cells have been shown to induce therapeutic angiogenesis in animal models of myocardial, peripheral, and cerebral ischemia.[23,24] The present study demonstrated that 3-week administration of testosterone in gonadectomized rats increased EPC count in groups treated by 10 µg/kg/day and 100 µg/kg/day testosterone, but high-dose testosterone is more effective in increasing EPC count which seems to be useful in vascular healing.

Foresta *et al*., reported that hypogonadal men have a low number of circulating EPCs as a subset of CD34+ cells which increase significantly after testosterone treatment.[25]

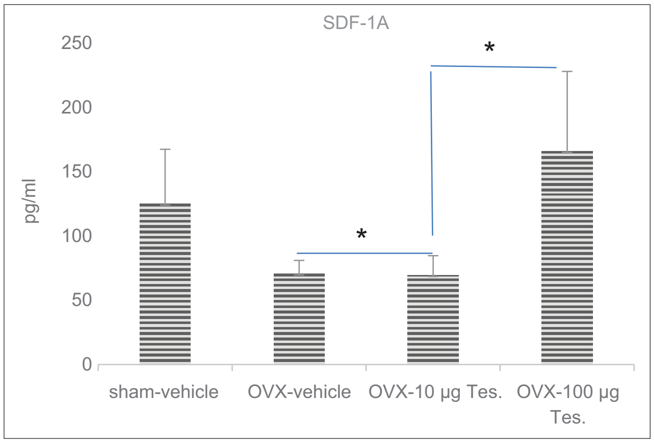

Figure 2: Effect of exogenous testosterone one the SDF-1α concentration. 1. Sham-vehicle 2. Ovariectomized-sesame oil 3. Ovariectomized rats treated by 10 µg/kg/day testosterone 4. Ovariectomized rats treated by 100 µg/kg/day testosterone

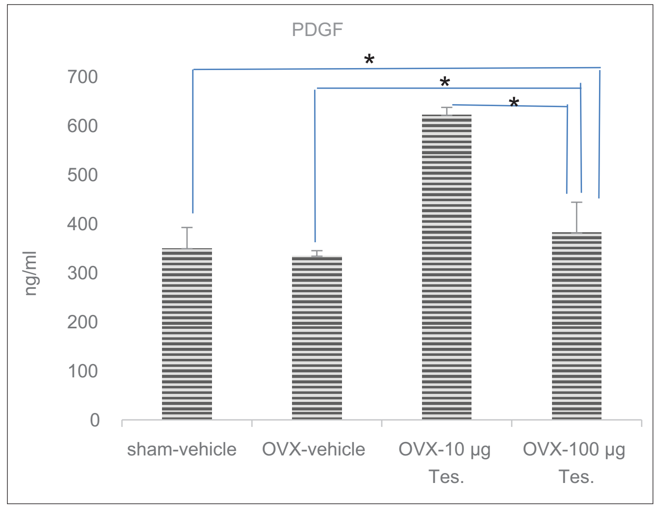

Figure 3: Effect of exogenous testosterone one the PDGF concentration. 1. Sham-vehicle 2. Ovariectomized-sesame oil 3. Ovariectomized rats treated by 10 µg/kg/day testosterone 4. Ovariectomized rats treated by 100 µg/kg/day testosterone

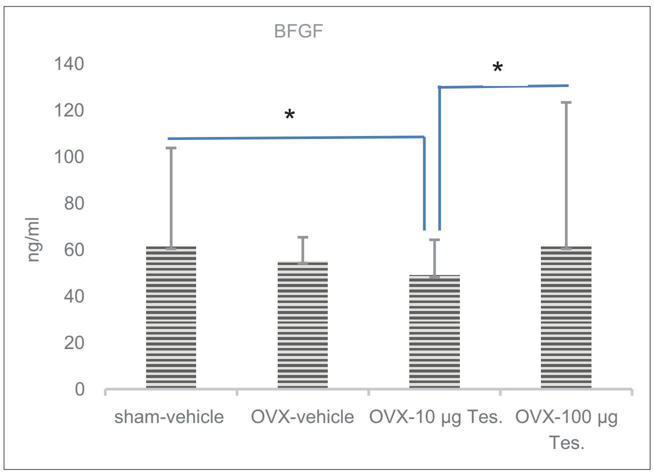

Figure 4: Effect of exogenous testosterone on the bFGF concentration. 1. Sham-vehicle 2. Ovariectomized-sesame oil 3. Ovariectomized rats treated by 10 µg/kg/day testosterone 4. Ovariectomized rats treated by 100 µg/kg/day testosterone

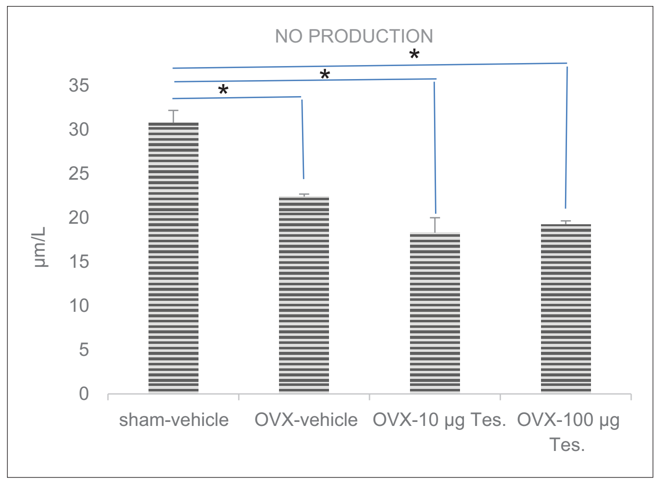

Figure 5: Effect of exogenous testosterone on the NO production level. 1. Sham-vehicle 2. Ovariectomized-sesame oil 3. Ovariectomized rats treated by 10 µg/kg/day testosterone 4. Ovariectomized rats treated by 100 µg/kg/day testosterone

Similarly, the findings of a recent study showed that in a murine model of hind limb ischemia castration impaired the number of early EPC in the bone marrow and the spleen.[26] In contrast, Fadini *et al*. demonstrated that castration in rats was followed by advance in circulating EPC, and testosterone and dehydrotestosterone replacement failed to restore these cells toward normal levels.[27]

Hypogonadal hypogonadotropic patients also have low circulating level of EPCs that significantly increase by testosterone treatment.[28] *In vitro* study indicates that EPCs may directly response to testosterone through a dose-dependent increase in profile migration and colony-forming ability.[29-31]

The present data showed the serum concentration of SDF-1α increased in both groups: sham-vehicle and OVX-treated by 100 µg/kg/day testosterone that presents both estrogen and testosterone increase the serum level of SDF-1α but high-dose testosterone is more effective. These changes of SDF-1α concentration have positive correlation with changes of EPC count suggesting this idea that testosterone can increase EPC count through the SDF-1α mechanism.

Chen *et al*. found in an animal study that castration significantly decreased the number of CD34+ cells in the peripheral blood of rats and castration also impaired the early expression of SDF-1α. Endogenous testosterone deprivation in rats significantly worsened cardiac function, increased infarct size and cardiomyocyte apoptosis, and reduced the capillary density. Interestingly, testosterone replacement therapy reversed the castration-related impairment of angiogenesis.[32] SDF-1α is the crucial cytokine in CD34+ hematopoietic progenitor cells and it can induce directional migration of CD34+ hematopoietic progenitor cells.[33] SDF-1α plays a central role in the homing of circulatory CD34+ cells in peripheral tissue such as ischemic myocardium,[28] but the mechanism of its action remain obscure. SDF-1α is also involved in recruitment of stem cells to the liver and to the site of vascular injury.[30,34,35]

In addition, female rats received 10 μg/kg/day testosterone showed significant increase in PDGF concentration than the group treated by 100 μg/kg/day. Our data present the concentration of PDGF was significantly higher in groups treated by exogenous testosterone than fertile female rats with endogenous estrogen.

Study of the role of PDGF in angiogenesis and EPC migration *in vivo* has been limited. One study reported that PDGF has no effect on EC outgrowth from the aorta.[36] Li *et al*. showed that PDGF stimulates the recruitment of endothelial progenitors from the bone marrow. Numerous studies have documented that adult bone marrow-derived progenitor cells can contribute to the revascularization, they found that PDGF mobilize EPC within the first 2–5 days after tissue ischemia, this is precisely the time window within which new blood vessels start to grow in this ischemic tissues.[37]

Angiogenesis, the process of new blood vessel formation from pre-existing ones, plays a key role in various physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth. The local, uncontrolled release of angiogenic growth factors, and/or alterations of the production of natural angiogenic inhibitors, with a consequent alteration of the angiogenic balance, are responsible for the uncontrolled EC proliferation that takes place during tumor neovascularization and in angiogenesis-dependent diseases.[38]

Fibroblast growth factors (FGF) are important modulators of cellular proliferation, migration, and differentiation depending on cell type and tissue context. FGF ligands expressed by the vascular cells. FGF induces a wide range of effects on EC including cell proliferation, production of extracellular membrane, modifying proteases, and cell migration. Many *in vivo* and *in vitro* systems have proved its effectiveness to induce neovascularization and ECs sprouting.[38,39] FGF may exert their effects on ECs via an endocrine mode as same as our study showed that endogenous estrogen has the same effect of high-dose exogenous testosterone in female rats because the serum concentration of bFGF significantly increased in group 1 (sham-sesame oil) that only have endogenous estrogen more than group 3 (OVX-10 μg/kg/day tes.) that received 10 μg/kg/day testosterone without endogenous estrogen. We found that estrogen is more effective on serum level of bFGF.

And finally, our data showed the serum level of NO production was significantly higher in group 1 (sham-sesame oil) that only used endogenous estrogen than all other groups. Reversely, EPC count promoted in group 4 with high-dose exogenous testosterone (OVX-100 μg/kg/day tes.). These data demonstrated that the rising in EPC count is probably not through NO mechanism.

Some studies suggest that the estrogen-induced augmentation of NO production by vascular endothelium may contribute to its vasculoprotective effects.[40] In rabbits, rats, and guinea pigs NO was reported to be enhanced in OVX 17β-estradiol (E2)-treated female compared with OVX control. Several studies recently investigated the short-term effect of E2 on NO production in cultured EC. Accumulating evidence indicates that abuse anabolic steroids may cause cardiovascular adverse side effects including endothelial dysfunction.

In one study, *in vivo* results showed that NO level significantly decreased after testosterone administration and a supraphysiological dose of testosterone decreases the expression of eNOs and consequently the formation of NO. Furthermore, recent results indicate that supraphysiological doses of testosterone may induce endothelial dysfunction, which is of interest in relation to the cardiovascular adverse side effects observed in anabolic androgenic steroid abusers.[41]

Conclusions

Testosterone administration induces a meaningful positive effect on the circulatory EPCs count in a basal condition that appears to be dose-dependent and this effect becomes particularly evident in the OVX female rats treated by 100 μg/kg/day testosterone. This positive effect seems to be due to stromal cell-derived factor and platelet cell-derived factor mechanisms. However, our study has some limitations. It would be better to study further doses of testosterone with larger sample size. Further studies especially well-designed clinical trials are required to confirm this finding.

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Conflicts of interest

There are no conflicts of interest.

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