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Testosterone stimulates proliferation and preserves stemness of human adult mesenchymal stem cells and endothelial progenitor cells

MARIA CRISTINA COROTCHI, MIREL ADRIAN POPA, MAYA SIMIONESCU

Angiogenesis and Cardiovascular Remodeling Group, Department of Regenerative Medicine, "Nicolae Simionescu" Institute of Cellular Biology and Pathology of the Romanian Academy, Bucharest, Romania

Abstract

Human adult stem and progenitor cells are promising cell types widely studied for their clinical benefits. A reduced number of stem cells present in the human body are associated with numerous dysfunctions. Since androgens have a profound effect on different cell types, we questioned whether testosterone (T), one of the main androgens, influence and are involved in the proliferation of stem cells and/or affect their stemness potential. Isolated mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) were cultured and then stimulated with different concentration of testosterone (10–100 nM). The cellular proliferation rate, adhesion, and viability were measured in real-time using xCELLigence system and DNA-cell proliferation assay. The immunophenotype of the stimulated cells *versus* non-stimulated cells was determined by flow cytometry. The maximal effect on MSCs and EPCs proliferation was obtained at 40 nM testosterone; this concentration was used in further experiments. The cellular index measured in real-time by impedance-based dynamic measurements revealed that 40 nM testosterone had a proliferative effect on both MSCs and EPCs, having a proliferative index of ~50% above the control (non-stimulated) cells. Furthermore, flow cytometry assay indicated that testosterone stimulation did not alter the phenotype of MSCs and EPCs, both cell types preserving the expression of the characteristic surface markers. Testosterone stimulation increases the proliferation and preserves stemness of MSCs and EPCs suggesting that, besides other factors, the hormone may engineer these cells and increase their therapeutic potential.

Keywords: mesenchymal stem cells, endothelial progenitor cells, androgens, testosterone, proliferation.

Introduction

The therapeutic potential of bone marrow-derived mesenchymal stem cells (MSCs) was reported several years ago [1]. In the past decade, MSCs derived from Wharton's jelly (WJ, the matrix of the human umbilical cord) were proposed as a novel potential source of cells to be employed for the treatment of cardiovascular disease (CVD) [2–4] and for cancer therapies [5, 6]. This is because of their rapid expansion *ex vivo*, self-renewal potential and the capacity to differentiate into multiple mesodermal lineages [7] such as fat, cartilage, bone and heart muscle.

Stem cells were employed in several clinical trials [1, 4, 8] given the fact that a connection between their reduced number in the circulation and the cardiovascular events and risk factors for coronary artery disease was reported [9, 10]. Furthermore, the decreased number of circulating endothelial cells is associated with cardiovascular complications and endothelial dysfunction.

Although with age, all individuals, men and women, are affected by CVD, several sex differences suggest a key role for the sex steroid hormones in the developmental process of the disease [11–14]. The main androgens, testosterone (T) and dihydrotestosterone (DHT), are implicated in male gender development [14], whereas in women the enzyme aromatase converts testosterone into the primary female sex hormone, 17β-estradiol (E2) [15].

In men, during aging, there is an annual decline rate of 1–2% of the physiological level of testosterone [16]. Several reports revealed that the concentration of circulating testosterone is low in hypertensive and coronary artery disease men [13, 17] and there are contradictory data for the circulating testosterone level in man with myocardial infarction [18].

Moreover, *in vivo* and *in vitro* studies on testosterone and other hormones from the androgens' group raised a major interest due to their influences on stem cells [12, 19, 20].

In vitro and *in vivo* models suggest that MSCs enhance post-ischemia angiogenesis and stabilize blood vessel formation [21–23]. Recently, studies on patients brought into light the impact of testosterone on stem cells, either MSCs or circulating EPCs disclosing that the number of these cells increases significantly in a testosterone-dependent manner by modulation of NO synthetase in an androgen receptor (AR)-mediated pathway [8, 12, 24, 25]. Moreover, testosterone administration in castrated mice restores the decreased platelet production by increasing thrombocytopoiesis [26].

Thus, we questioned whether testosterone has a direct effect on human stem/progenitor cells. To this aim, umbilical cord matrix and umbilical cord blood were used as rapid and non-invasive sources of MSCs and EPCs, respectively.

The effect of testosterone on stem cells was tested by investigating their proliferation *in vitro* using real-time measurements and their stemness potential.

We report here that *in vitro* stimulation of MSCs and EPCs with optimal concentration of testosterone increases the cellular adhesion and proliferation and does not affect the stemness potential of both cell types.

Materials and Methods

Isolation of MSCs and EPCs

The collection of samples used in these experiments complies with the conditions required to respect human samples collection, personal data, manipulation, and protection in conformity with European Union and national legislation. MSCs were isolated by enzymatic digestion of Wharton's jelly and characterized as previously described [7]. EPCs were obtained from human umbilical cord blood samples. Briefly, after collection, the blood was diluted 1:1 with phosphate-buffered saline (PBS), placed onto Histopaque 1.077 (Sigma-Aldrich, St. Louis, USA), and centrifuged at 400×g. The fraction of mononuclear cells (MNCs) was collected, washed three times with EBM-2 basal medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Gibco®, Life Technologies, Thermo Fisher Scientific, California, USA), and antibiotic–antimycotic solution (ZellShield, Minerva Biolabs, GmbH). Thereafter, MNCs were plated on tissue culture flasks coated with collagen I (rat tail; BD Biosciences, MA, USA) and maintained in EGM-2 BulletKit (Lonza, Basel, Switzerland) medium in normoxic conditions. After 24 hours, the culture medium was replaced and changed again after three days.

Characterization of MSCs and EPCs

Wharton's jelly-derived MSCs were characterized as previously described [7]. Umbilical cord blood (UCB)-isolated EPCs (at ~85% confluence) were characterized morphologically by light microscopy using a phase-contrast microscope (Eclipse TE300, Nikon, Tokyo, Japan) and a digital camera system for imaging (Digital Net Camera DN100, Nikon). To determine whether the cells express the EPCs specific surface markers, flow cytometry analysis was employed (FACSCalibur instrument, BD Biosciences, Franklin Lakes, NJ, USA).

In addition, to test whether EPCs form tube-like structure in a three-dimensional (3D) system the Matrigel assay was used.

Cell culture, adherence and proliferation assays. Experimental design to test the effect of testosterone on MSCs and EPCs

MSCs and EPCs were plated at a density of 25×10^4 cells per 75 cm^2 flasks in the presence of various concentrations (10–100 nM) of testosterone. Each day, the morphology of the cells was analyzed by phase-contrast microscopy. The adherence and proliferation of MSCs and EPCs stimulated with testosterone was determined in real-time using the xCELLigence and E-plate 16 system. After the cells were seeded onto the E-plate, various concentrations of testosterone (AppliChem, GmbH) were added and automatically, the impedance value of each well was monitored by the xCELLigence system for 150 hours (MSCs) and 96 hours (EPCs) and expressed as a cell index (CI) value. These experiments were done three times in duplicates. In parallel with the E-plate experiment, we determined the DNA content in order to control the number of cells and the behavior of stimulated cells during the 150 hours and 96 hours of real-time monitoring. The DNA analysis was achieved using the CyQuant® Cell Proliferation Kit from Life Technologies (Invitrogen), a method that is based on DNA content and offers an accurate measure of cell number.

Flow cytometry

The effect of testosterone on the specific surface markers was ascertain by immunophenotyping analysis of stimulated and non-stimulated MSCs and EPCs as previously described [7]. The flow cytometry data were evaluated using the Summit 4.0 (Dako) software.

Statistical analysis

The data obtained were analyzed by one-way analysis of variance (ANOVA) with QtiPlot-0.9.8.9 (ProIndep Serv S.R.L., Romania). The results were presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant when p-value <0.05.

Results

Testosterone stimulates adhesion and proliferation of MSCs and EPCs

The effect of the hormone was assessed on MSCs grown in Dulbecco's modified Eagle's medium (DMEM) and EPCs cultures in EGM-2 medium. The cell adhesion and proliferation was determined by phase-contrast microscopy and xCELLigence real-time cell analyzer with continuous registration of the cellular impedance. All the experiments were done in duplicate and repeated three times.

A comparative analysis by phase contrast microscopy revealed that after four days in culture, both testosterone-stimulated MSCs and EPCs had a higher proliferation rate than the non-stimulated cells (Figure 1) indicating a response of the cells to testosterone.

To reinforce these data, the adherence and proliferation of the cells were assayed employing xCELLigence real-time cell analyzer. The results showed that in the case of EPCs, during the first 38 hours, the proliferation rate was higher than for the control cells that is EPCs cultured only in their special EGM-2 medium. After 50 hours, a difference between non-stimulated and testosterone-stimulated EPCs was evident; the latter presented an increased CI value (~3.5) than the non-stimulated cells (Figure 2A).

The effect of testosterone on MSCs was tested for 150 hours because at 96 hours of incubation with the hormone, there was no significant change in cell proliferation. As shown in Figure 2B, the proliferation rate, *i.e.*, the CI value of the stimulated MSCs was significantly increased after 130 hours of incubation with the 40 mM testosterone as compared to the cells grown in DMEM medium in the presence or absence of dimethyl sulfoxide (DMSO), the hormone's vehicle. After 130 hours, the increase in the CI index attained a plateau that was maintain until 150 hours of the experiments as revealed by the statistical analysis of the CI values done with real-time cell analysis (RTCA) software.

These results indicate that testosterone affects significantly the adhesion and the proliferation rate of EPCs and MSCs; that the effect is be time-dependent, and simultaneously the hormone increases their life span. To confirm the above-mentioned data, we employed the

DNA-based proliferation assay using CyQuant® from Life Technologies (Invitrogen).

The results obtained (Figure 3) showed that in the case of the stimulated MSCs, the number of cells at day 6 was higher (~27 000 cells/well) than at day 1 (7000 cells/well).

In the case of EPCs, the DNA-based proliferation assay revealed that at day 4 the number of stimulated cells was ~22 000 cells/well, in comparison with day 1 when 8000 cells/well were seeded.

Testosterone exposure does not affect the specific immunophenotype of MSCs and EPCs

To investigate whether the testosterone stimulation affects the identity of Wharton's jelly-derived MSCs and UCB-derived EPCs, we employed flow cytometry assay using the following panel of markers: CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD144, and CD309. Immunophenotyping analyses of testosterone-stimulated MSCs showed that these cells express CD44, CD73, CD90, and CD105 and were negative for CD31, CD34, CD45, CD144, and CD309.

Furthermore, the flow cytometry experiments revealed that EPCs were positive for CD31, CD44, CD144, CD309, and negative for CD45, CD73, CD90, and CD105 surface markers (Figure 4). These results denoted that testosterone does not affect the specific immunophenotype of MSCs and EPCs and that their stemness is preserved.

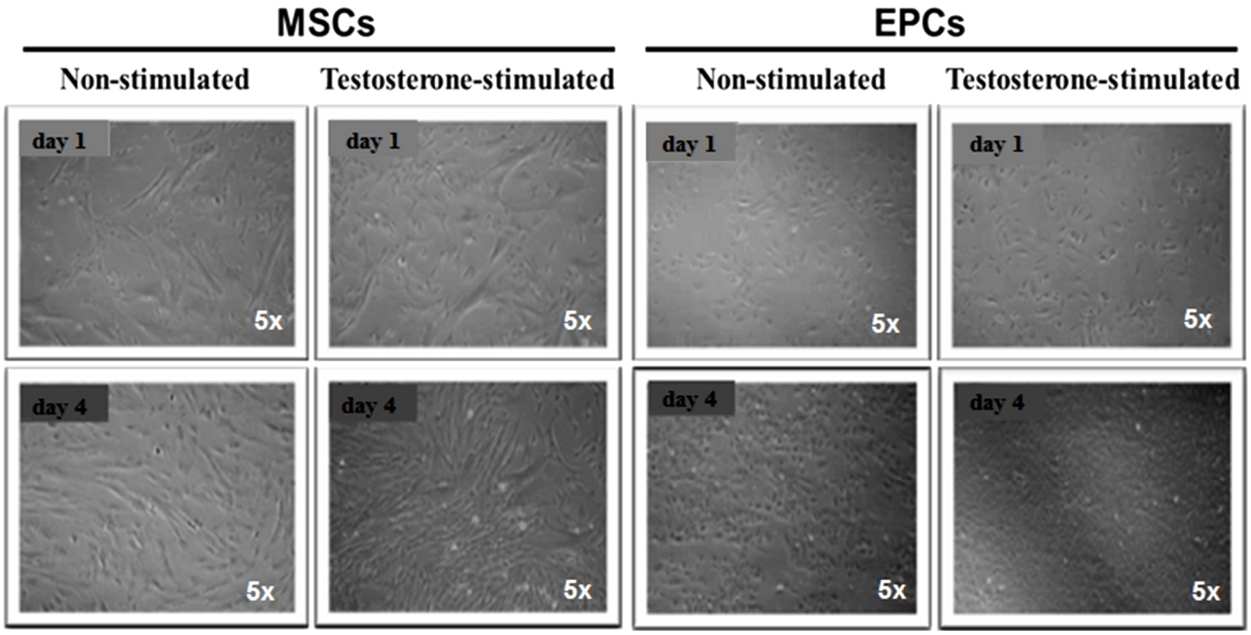

Figure 1 – ***Phase-contrast microscopy illustrating the morphology and proliferation of MSCs (left) and EPCs (right) cultured in the absence (non-stimulated) or presence of 40 nM T for one to four days. Note that both MSCs and EPCs, cultured in the presence of testosterone, exhibit an increased proliferation as compared to the non-stimulated (control) cells.***

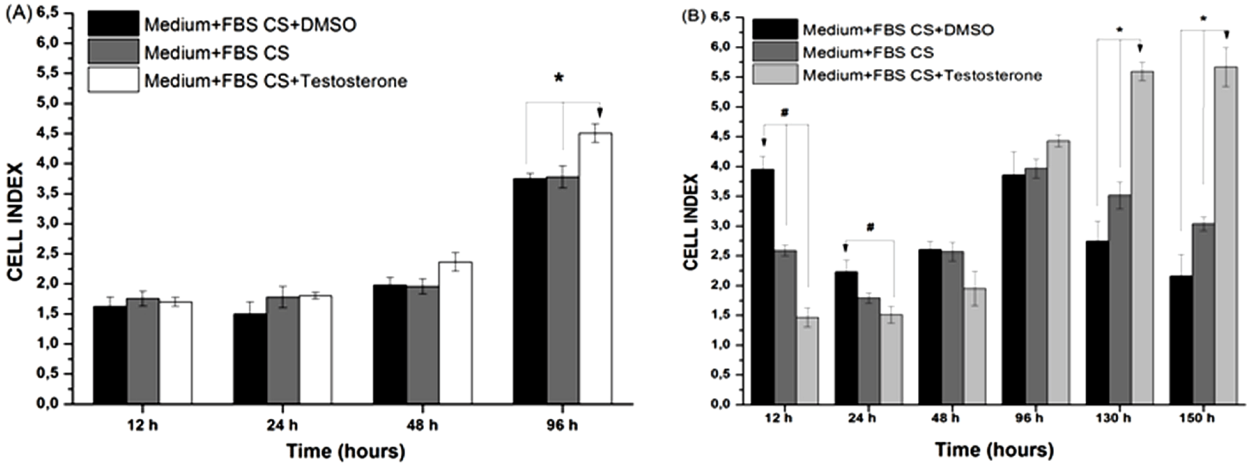

Figure 2 – ***Effect of testosterone on the adherence and proliferation of EPCs and MSCs assessed by xCELLigence system: (A) Statistical analysis of cell index (CI, n=3) of EPCs (8000 cells/well) after 96 hours of impedance measurements showing the time-dependent proliferation in time of stimulated EPCs. The cells were grown in EGM-2 + 10% FBS C.S. (Fetal Bovine Serum – Charcoal Stripped) + 1% Ab + T (40 nM) and kept for 96 hours. Controls consisted in cultured EPCs in EGM-2 + FBS C.S. + DMSO vehicle; (B) Statistical analysis of CI (n=3) indicating the proliferation of MSCs (7000 cells/well) incubated for 150 hours with 40 nM testosterone. MSCs grown in DMEM 1‰ + 10% FBS C.S. + 1% Ab for 150 hours exposed to 40 nM testosterone. As controls, MSCs were grown in DMEM 1‰ + 10% FBS C.S. + DMSO vehicle.***

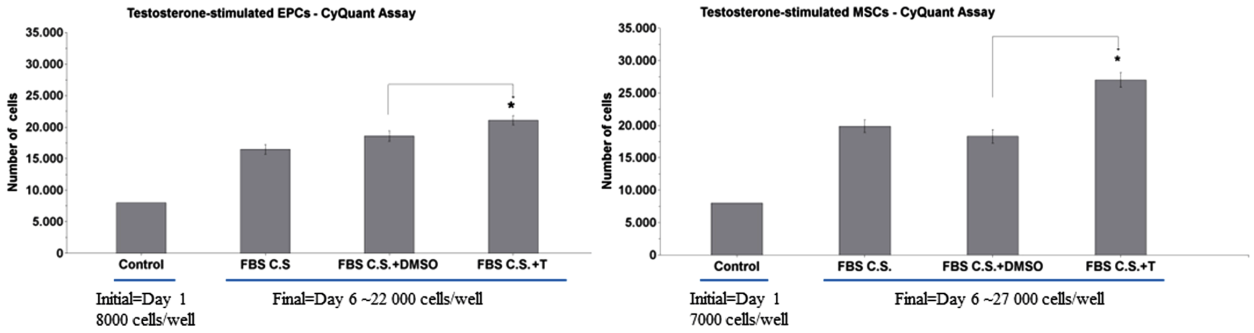

Figure 3 – ***DNA-based proliferation assay (CyQuant) of MSCs and EPCs. Cultured MSCs were exposed to 40 nM of testosterone for six days (150 hours), whereas cultured EPCs for four days (96 hours), the times when a significant change by xCELLigence system was detected. Note that as compared to controls (cells grown in DMEM 1‰ + 10% FBS C.S. + DMSO vehicle) a significant difference is evident at 150 hours in testosterone-stimulated cultured MSCs, whereas for EPCs, the proliferation rate attained a significant value after 96 hours of exposure to the hormone (*p<0.05).***

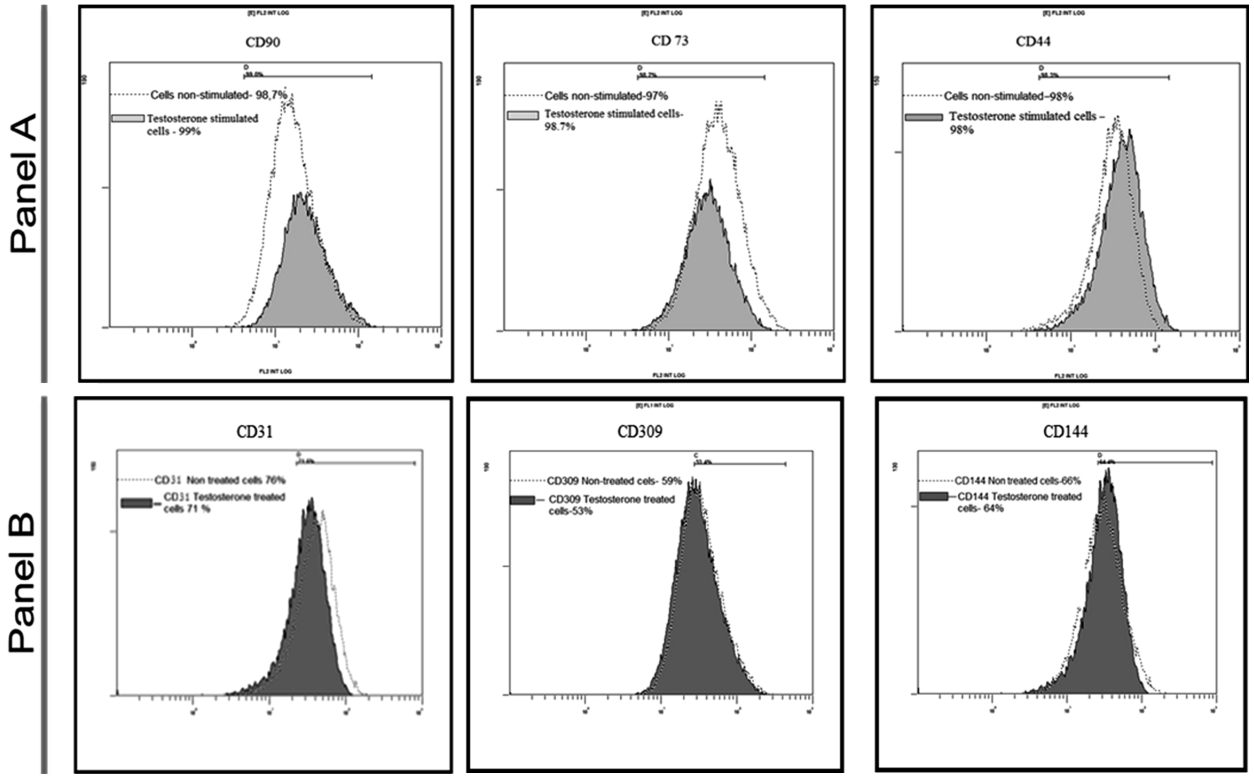

Figure 4 – ***Analysis of testosterone-stimulated MSCs (Panel A) or EPCs (Panel B) by fluorescent-activated cell sorting (FACS). The hormone-stimulated MSCs are positive for CD44, CD73, CD90, and negative for CD31, CD45, CD144, CD309 (not shown); EPCs exposed to testosterone are positive for CD31, CD144, CD309 and negative for CD45, CD73, CD90, CD105; Control: non-stimulated MSCs and EPCs cultured in the same condition.***

Discussion

Since over a decade, human adult stem and progenitor cells, such as bone-marrow derived stem cells were widely used due to their therapeutically benefits in several diseases [1, 20, 21]. Recently, stem cells obtained from fetal tissue, namely Wharton's jelly, or from umbilical cord blood, are promising candidates for multiple diseases [6].

Considering their important characteristics, rapid expansion *ex vivo*, self-renewal potential and the capacity to differentiate into multiple mesodermal lineages, these stem cells were used in clinical trials for cancer therapies and for the treatment of CVDs [2–5, 8]. Bone marrow-derived stem cells and other types of adult stem cells were used in clinical trials [8–10], but due to the reduced number in the circulation or the low number that are attaining the main organs [9, 10, 20], were not considered highly successful.

Gender differences are considered a major interest when studying CVDs at men and women. It is thought that males in comparison with female present a greater survival rate after they have suffered a myocardial infarction [20]. Women until menopause are protected by estrogens – the most important is 17β-estradiol –, and men are protected approximately until 60 years by androgens.

Hence, estrogens and androgens have a robust impact on different cell types; our aim was to study if one of the main androgens – testosterone – influences the proliferation of stem cells and/or affect their stemness potential.

First, we tested the effect of different concentrations of testosterone (10–100 nM) on cultured MSCs and EPCs employing impedance-based real-time measurements. The

xCELLigence system that we have used in our experiments is real-time cell analysis (RTCA) dual plate (DP) instrument [27]. The RTCA DP instrument possesses three 16-well E-plates, which can be individualistically managed. We have employed the special plates named E-Plate VIEW 16 (ACEA Biosciences Inc., San Diego, CA, USA).

In comparison with E-Plate 16, the advantage of E-Plate VIEW 16 is that allows visualizing the cells through a microscope or other cell-imaging systems. E-Plate VIEW 16 was modified by eliminating four rows of microelectrodes sensors in each well enabling image acquisition using microscopes. Therefore, the cell index profile of the stimulated and non-stimulated cells with testosterone was attained with the xCELLigence system. Due to this system, we were able to obtain results in real-time; the variation of the cell index was time-dependent.

The experiments revealed that from all the concentrations used, 40 nM testosterone was the optimal dose for each cell line. Thus, this concentration of testosterone was employed in all further experiments. The cultured cells were stimulated with 40 nM testosterone, visualized daily, and the viability assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (data not shown).

The real-time measurements and the MTT assays revealed that testosterone stimulates adhesion and proliferation of MSCs and EPCs *in vitro*. Moreover, the optimal dose of 40 nM testosterone is non-toxic for these types of cells: MSCs and EPCs obtained from Wharton's jelly and umbilical cord samples.

Furthermore, the flow cytometry analysis showed that in comparison with the non-stimulated cells, the phenotype of the MSCs and EPCs that received hormone treatment is not affected.

Conclusions

The novel data obtained from our experiments indicate that *in vitro* stimulation with testosterone of human adult stem (MSCs) and progenitor cells (EPCs) preserves stemness and increases significantly the cells' adhesive and proliferative capacity. Notwithstanding, additional studies are needed to determine the mechanisms involved in the testosterone effect on MSCs and EPCs survival, mobilization and homing. Amplification of the proliferation potential of testosterone-stimulated MSCs and EPCs without affecting their stemness potential could be useful in the therapeutic interventions in which these cells are not only candidates but also an excellent option for vascular regenerative medicine in general and for ischemic diseases, in particular.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Corresponding author
Maria Cristina Corotchi, Research Assistant, PhD Student, Angiogenesis and Cardiovascular Remodeling Group, Department of Regenerative Medicine, "Nicolae Simionescu" Institute of Cellular Biology and Pathology of the Romanian Academy, 8 Bogdan Petriceicu Hasdeu Street, PO-Box 35–14, 050568 Bucharest, Romania; Phone +4021–319 45 18, Fax +4021–319 45 19, e-mail: cristina.corotchi@icbp.ro

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