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Testosterone Propionate Promotes Proliferation and Viability of Bone Marrow Mesenchymal Stem Cells while Preserving Their Characteristics and Inducing Their Anti-Cancer Efficacy

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Background: Various studies have reported the effects of testosterone on different cell types, yet bone marrow-derived mesenchymal stem cells' cellular responses to testosterone remain unknown.

Aims: To investigate the effects of testosterone propionate, an oil-soluble short-acting form of testosterone, on human bone marrow-derived mesenchymal stem cells' proliferation and viability after 24 hours of incubation. We also investigated the impact of testosterone propionate on bone marrow-derived mesenchymal stem cell's polarization and cytotoxicity on K562 leukemia cell line

Study Design: In vitro study.

Methods: We expanded commercially available bone marrow derived mesenchymal stem cells in vitro and treated them with testosterone propionate at concentrations ranging from 10^{-6}-10^{-10} M for 24 hours. Ideal concentration was determined by evaluating cellular viability and proliferation with Annexin V/Propidium Iodide assay and carboxyfluorescein succinimidyl ester staining. The characteristic features of bone marrow-derived mesenchymal stem cells were evaluated by immunophenotyping and investigating their differentiation capacities. Bone marrow-derived mesenchymal stem cells' cytotoxic properties upon testosterone propionate treatment were determined by co-culturing the cells with K562 cells and with confocal imaging investigating polarization.

Results: Testosterone propionate promoted proliferation and maintained the viability of bone marrow-derived mesenchymal stem at 10^{-8} M concentration. Further evaluations were conducted with the determined dose. The results showed that, apart from promoting mesenchymal stem cells' polarization and increasing their cytotoxicity on K562 cells, testosterone propionate did not alter differentiation capacities of bone marrow-derived mesenchymal stem cells and certain cell surface markers, but led to a significant increase in HLA-DR expression.

Conclusion: The findings reveal that testosterone propionate promotes the proliferation and survival of bone marrow-derived mesenchymal stem cells in a dose-dependent manner without hampering their differentiation capacities, induces their polarization to the pro-inflammatory phenotype, and increases their cytotoxicity on the K562 cell line.

INTRODUCTION

The concept of mesenchymal stem cells (MSCs) were first introduced after experiments of bone marrow (BM) transplantation to heterotopic anatomical sites resulted in de novo ectopic bone and marrow formation.[1] Friedenstein et al.[2] were the first to demonstrate that the osteogenic potential of the BM is associated with a minor subpopulation of BM cells distinguished from most of the hematopoietic cells with their fibroblast-like morphologies, their ability to attach to tissue culture plate surface rapidly and form small aggregates resembling bone and cartilage, thereby revealing their differentiation capacity. The International Society for Cellular Therapy provided the following criteria for defining human MSCs: adherence to plastic surfaces in standard cell culture conditions, expressing $CD73^{+}$, $CD90^{+}$, $CD105^{+}$, $CD34^{neg}$, $CD45^{neg}$, $HLA\text{-}DR^{neg}$, $CD14^{neg}$ or $CD11b^{neg}$, $CD79a^{neg}$, or $CD19^{neg}$ cell surface markers as assessed by immunophenotyping and ability to differentiate into osteoblasts, adipocytes and chondroblasts.[3] MSCs have also been shown to

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form bone and cartilage following ectopic implantation and promote bone regeneration in in vivo models with genetic bone defects.

MSCs have homing potential into injured tissues after being by inflammatory cytokines, toll like receptor ligands and hypoxia, and can release certain factors that facilitate tissue renewal.[4,5] They can prevent allogenic immune responses and act as immune system regulators. Furthermore, MSCs exhibit low immunogenicity,[6] are easy to obtain,[7] and have no ethical issues related to their research and application, making these cells remarkable options for regenerative medicine. However, primary MSC cultures are heterogenous populations until 3rd passage, and these cells have limited proliferation and differentiation capacities.[8-11] Thus, stimulating MSCs' division in vitro may be helpful for obtaining higher number of cells in lower passages.

Administration of MSCs for cancer treatment is a novel concept.[12,13] It has been shown that using engineered MSCs encoding anti-proliferative mediators is a promising strategy for targeted anti-cancer treatments with lower side effects. These cells also have the potential to be used in leukemia treatment and were suggested as an adjunctive therapy. However, there are currently no studies evaluating BM-MSCs' anti-cancer efficacy on leukemia cell lines along with their polarization states in vitro.[14] Similar to macrophages, MSCs can polarize into either pro-inflammatory (MSC-1) or anti-inflammatory (MSC-2) phenotypes, where the former inhibits tumor growth and the latter promotes carcinogenesis.[15,16] MSCs possessing pro- or anti-inflammatory properties can be determined by measuring the levels of chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL5, respectively instead of;.[17,18]

Testosterone, an androgenic steroid hormone, is secreted by Leydig cells located in testicles in men[19] and in the ovaries, adrenal gland, and peripheral tissues in women.[20] Its production is stimulated by gonadotropin releasing hormone secreted from the hypothalamus under the control of luteinizing hormone and follicle stimulating hormone.[21] Once internalized, testosterone interacts with the androgen receptor and forms a hormone-receptor dimer that then binds to hormone response elements to regulate the expression of various genes.[22]

Herein, we aimed to evaluate effects of testosterone propionate (TP), an injectable, oil-soluble, short-acting form of testosterone, at various doses on BM-MSCs in terms of viability, proliferation, stemness features and their cytotoxic capacity on leukemic cells.

MATERIALS AND METHODS

Cell Culture

Healthy human bone marrow-derived mesenchymal stem cells (BM-MSCs, passage 3) were purchased (Gibco, #A15652) and cultured in low-glucose DMEM (Thermo Fisher Scientific, #11054020) supplemented with 10% Fetal Bovine Serum (FBS - Pan Biotech, #P30-3306), 1% L-glutamine (2 mM, Sigma Aldrich, #G7513) and penicillin/streptomycin antibiotic solution (100 U/0.1 mg/ml, Sigma Aldrich, #P4333) at 37 °C degrees containing 5% CO_2 in humid environment. For the evaluation of cell surface markers, cells were detached with trypsin-EDTA (Thermo Fisher Scientific, #25300054), collected by centrifuging at 300 X G for 5 minutes, and then suspended in Dulbecco's phosphate buffered saline (DPBS) containing 0.1% sodium azide. Next, the cells were labeled with anti-CD73 FITC (Clone:AD2, Biolegend, #344016), anti-CD90 PE (Clone: 5E10, Biolegend, #328110), anti-CD105 PerCP/Cy5.5 (Clone:43A3, Biolegend, #323216), anti-CD34 PE/Cy7 (Clone:581, Biolegend, #343515), anti-CD11b Alexa Fluor 700 (Clone:ICRF44, Biolegend, #301355), anti-HLA-DR Pacific Blue (Clone:L243, Biolegend, #307633), and anti-CD45 (Clone: J33, Beckman Coulter, #A96416) antibodies by incubating under dark conditions for 15 minutes at room temperature. The cells were immediately acquired on Beckman Coulter DxFLEX flow cytometry system. Analyses were performed on CytExpert software.

Preparation of TP

TP (Sigma Aldrich, T-#1875) was dissolved in an acetone-water mixture (1:3 v/v) according to the manufacturer's instructions by heating at 70 °C for 15 minutes. The solution was cooled and mixed with complete culture media to obtain TP at 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M at final concentrations. The final volume of acetone in cell culture was 0.3%, and culture medium with 0.3% acetone addition was used as vehicle. BM-MSCs were incubated with either TP at concentrations between 10^{-6} M and 10^{-10} M or vehicle for 24 hours while untreated cells were used as control.

Cell Proliferation Measurements for MB-MSCs

The proliferation rates of BM-MSCs were determined by carboxyfluorescein succinimidyl ester (CFSE) staining, which relied on the equal sharing between two daughter cells upon cell division. For this study, $2x10^5$ BM-MSC were seeded into 6-well tissue culture plates as triplicates and incubated overnight to allow attachment. For labeling cells, the complete medium was discarded, and cells were incubated in 1 ml DMEM containing CFSE at the final concentration of 5 µM at 37 °C for 20 minutes. Media containing CFSE were discarded, and cells were either treated with TP at concentrations between 10^{-6} M and 10^{-10} M or vehicle. At the end of the 24-hour incubation period, cells were detached by trypsinization, washed once with DPBS, and read by Beckman Coulter FC500 flow cytometry system. Analyses were performed on CXP software.

Cell Viability and Apoptosis Measurements for MB-MSCs

Effects of TP at various concentrations on MSCs' viability and apoptosis rates were determined by Annexin V/Propidium Iodide (PI) staining by ApopNexin Annexin V FITC Apoptosis Kit (Merck Millipore, #APT750). This procedure relies on the simultaneous analysis of membrane asymmetry and integrity that are gradually lost during apoptosis. For this purpose, $2x10^5$ BM-MSCs were seeded into 6-well tissue culture plates as triplicates and treated with TP at concentrations between 10^{-6} M and 10^{-10} M or vehicle for 24 hours, while untreated cells were used as control. At the end of incubation, cells were detached by trypsinization, washed once with DPBS, and labeled with Annexin V-FITC and PI

according to the manufacturer's instructions. Briefly, cells were suspended in 1 ml Annexin V binding buffer, and then incubated with 3 µl Annexin V FITC and 2 µl PI provided by the kit at room temperature under dark conditions for 15 minutes. The cells were placed on ice at the end of incubation and immediately analyzed with Beckman Coulter FC500 flow cytometry system. Analysis was performed on CXP software.

Characterization of BM-MSCs Upon TP Treatment

Impact of TP at 10^{-8} M concentration on the characteristic features of BM-MSCs were determined by investigating cell surface markers and by evaluating BM-MSCs' differentiation capacities. For immunophenotyping, $2x10^5$ BM-MSCs were seeded into 6-well tissue culture plates as triplicates, incubated in the presence of 10^{-8} M TP for 24 hours followed by collecting cells with trypsinization and labeling with antibodies abovementioned. Cells were immediately read with Beckman Coulter DxFLEX flow cytometry system and analysis was performed on CytExpert software. For evaluation of BM-MSCs' differentiation capacities, $1x10^4$ BM-MSCs were seeded into 96-well cell culture plates and once they reached 80% confluency, differentiation was initiated by commercial chondrogenesis (Thermo Fisher Scientific, #A1007101), osteogenesis (Thermo Fisher Scientific #A1007201), and adipogenesis (Thermo Fisher Scientific, #A1007001) kits, either supplemented with 10^{-8} M TP or not. Differentiation media were replaced twice a week. On the 21st day of differentiation, cells were fixed with 10% neutral-buffered formalin solution. Chondrogenesis, osteogenesis, and adipogenesis were evaluated by Alcian Blue, Alizarin Red, and Oil Red-O staining, respectively.

Co-culture of BM-MSCs with K562 Cell Line

K562 cell line (ATCC #CCL-243) was maintained in RPMI medium (Gibco, #11875093) supplemented with 10% FBS (Pan Biotech, #P30-3306) and 1% penicillin/streptomycin antibiotic solution (Sigma Aldrich, #P4333). For evaluating cytotoxic effects of BM-MSCs on the K562 cell line, BM-MSCs were seeded into 35-mm cell culture dishes ($1x10^4$ cells/dish) and cultured overnight to allow attachment. Then, the medium was discarded, and BM-MSCs were either treated with 10^{-8} M TP for 24 hours followed by co-culturing with K562 cells ($1x10^5$ cells/dish) for an additional 24 hours; otherwise, K562 cells were added on BM-MSCs in the presence of 10^{-8} M TP and incubated for 24 hours. The effects of TP on the K562 cells were determined by incubating them with medium supplemented with 10^{-8} M TP for 24 hours, whereas the effects of BM-MSCs on K562 cells were evaluated by co-culturing cells for 24 hours. K562 cells' viability was determined by labeling them with 4′,6-diamidino-2-phenylindole (DAPI, Biolegend, #422801) by incubating the cells for 15 minutes under dark conditions at room temperature. The cells were read with Beckman Coulter DxFLEX flow cytometry system, and analysis was performed with CytExpert software.

Confocal Microscopy

For the evaluation of BM-MSC polarization and the cellular morphology, upon TP treatment, the cells were seeded in 8-well chamber slides (SPL Life Sciences, #30108) at a density of $2x10^4$ cells per well and incubated overnight to allow attachment, followed by treatment with 10^{-8} M TP for 24 hours. The slides were fixed and permeabilized as previously described[23] and labeled either with anti-CXCL9 PE (Clone: J1015E10, Biolegend, #357903) and anti-CXCL5 APC (Clone: J111B7, Biolegend, #524105) antibodies or with F-actin probe Phalloidin (Alexa Fluor 555 conjugated, Thermo Fisher Scientific, #A34055) by incubating overnight at 4 °C. Cell nuclei were counterstained with DAPI, and the slides were mounted with FluoroShield medium (Sigma Aldrich, #F6182). Micrographs were taken with a Zeiss LSM 780 confocal microscope.

Statistical Analyses

All experiments were performed in triplicates. Statistical analysis was done with GraphPad Prism 8 software. Normality was evaluated with Shapiro-Wilk test. One-way ANOVA followed by Tukey's multiple comparisons test was applied for data with normal distribution; otherwise, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was employed. Unpaired t-test was used for comparing cell surface markers between the control and the TP group. *P* values lower than 0.05 were considered statistically significant.

RESULTS

TP Promotes BM-MSCs' Proliferation and Alters Cell Viability in a Dose-Dependent Manner

TP, at all concentrations tested, significantly promoted cell division in comparison to the vehicle and control groups ($p < 0.05$), confirming the proliferative effect of the hormone. Interestingly, proliferation was also increased in the vehicle group in comparison with the control group ($p < 0.05$) (Figure 1a). Among all groups, 10^{-8} M TP group had the highest division rate ($p < 0.05$), followed by the groups treated with 10^{-10}, 10^{-9}, and 10^{-7} M TP, although no differences among the last three were observed ($p > 0.05$). TP treatment did not alter early apoptosis (Figure 2b) or necrosis (Figure 2d) levels, yet the acetone (vehicle) group had significantly lower viability rates compared to the control as well as 10^{-10} and 10^{-9} M groups ($p < 0.05$) (Figure 2a, Table S1). Furthermore, viability was found to be significantly decreased in the groups treated with 10^{-10}, 10^{-9}, 10^{-7}, and 10^{-6} M TP ($p > 0.05$), yet it should be noted that all TP groups, except 10^{-6} M, had significantly higher viability rates compared to the vehicle group, suggesting that TP can conceal the cytotoxic impact of the solvent to a certain extent ($p < 0.05$) (Figure 1a, Table S1). This finding is also supported by the decreased late apoptosis rates in both 10^{-7} and 10^{-8} M TP groups compared to the vehicle ($p < 0.05$), while no difference between these two treatment groups was observed ($p > 0.05$) (Table S1). In addition to decreased viability, 10^{-6} M group had significantly higher late apoptotic cell populations in comparison to both vehicle and control groups ($p < 0.05$) (Table S1), implying toxicity of TP at high doses on BM-MSCs. According to the results obtained, further studies were conducted with TP at 10^{-8} M concentration.

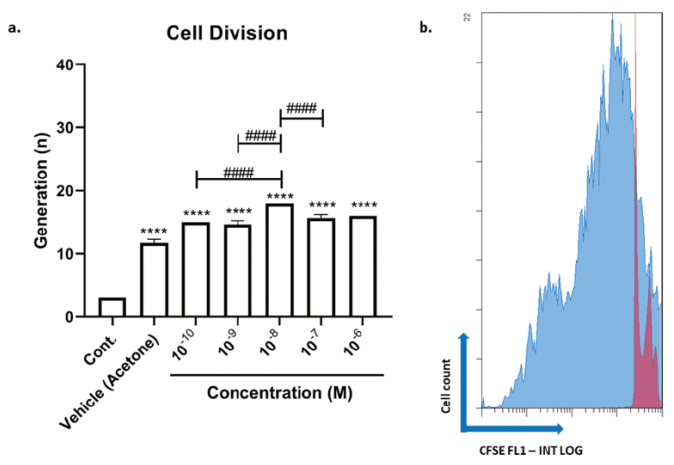

FIG. 1. Testosterone propionate promoted cell division on BM-MSCs while the highest increase was observed in the 10^{-8} M TP group. (a) Bar graphic indicating differences regarding cell division. (b) Representative overlay histogram plot comparing the control (red) and the 10^{-8} M TP (blue) groups in terms of cell division.
*denotes significant difference between the treatment and control groups; #denotes significant difference between the treatment groups. P values lower than 0.05 were considered statistically significant. **p < 0.01, ***p < 0.001, and ****p < 0.0001

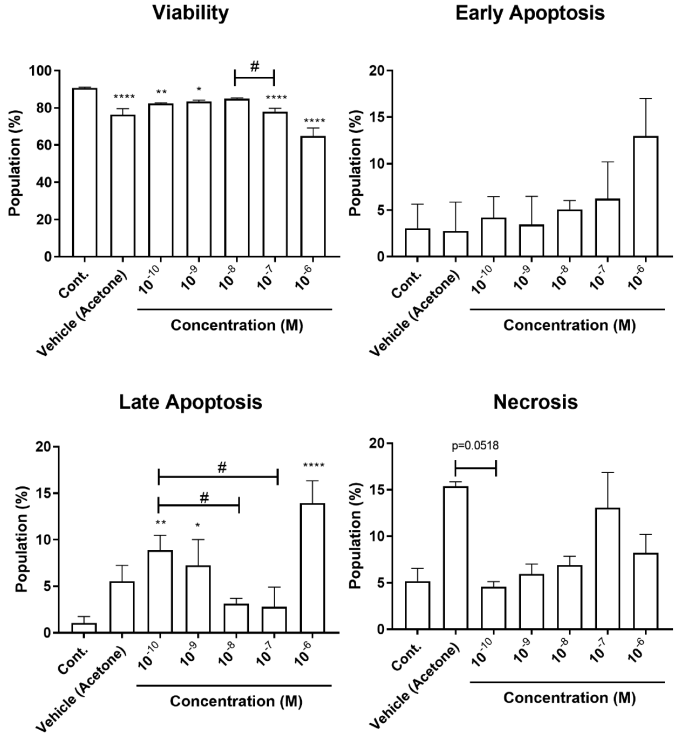

FIG. 2. Testosterone propionate promoted apoptosis in a dose-dependent manner in BM-MSCs. Bar graphics indicates (a) apoptosis, (b) early apoptosis, (c) late apoptosis, and (d) necrosis. * denotes significant difference between the treatment and control groups; # denotes significant difference between the treatment groups. P values lower than 0.05 were considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.000.1

TP does not Alter the Characteristic Features of BM-MSCs

Immunophenotyping revealed that over 95% of BM-MSCs used in this study were $CD11b^{neg}/CD34^{neg}CD45^{neg}/HLA\text{-}DR^{neg}/CD73^{+}/CD90^{+}/CD105^{+}$, thereby confirming pure MSC population. However, 10^{-8} M TP treatment led to a significant increase in HLA-DR expression ($p < 0.0001$) without altering CD11b, CD34, CD45, CD73, CD90, and CD105 levels ($p > 0.05$) (Figures 3 a-g). In addition, 10^{-8} M TP did not alter in vitro differentiation capacities on BM-MSCs, as both groups were able to differentiate into chondroblasts, osteoblasts, and adipocytes (Figure 3i). HLA-DR expression on MSCs was previously reported and suggested as an informative criterion instead of a definitive characteristic feature.[24,25]

TP Promotes Anti-cancer Potential of BM-MSCs

In the presence of 10^{-8} M TP, BM-MSCs significantly decreased the viability of K562 cells in comparison with the control group ($p < 0.01$) and BM-MSCs pre-treated with TP before co-culturing with the K562 cells ($p < 0.05$)(Figure 4a) While BM-MSCs without TP as well as BM-MSCs pre-treated with TP before co-culture promoted cytotoxicity on K562 cells, these increases were not statistically significant ($p > 0.05$). Interestingly, TP alone also exerted cytotoxicity on K562 cells in comparison to the control group, even though this difference was not significant ($p > 0.05$) Polarization towards the inflammatory phenotype of BM-MSCs was confirmed with confocal microscopy, where more prominent CXCL9 staining along with decreased CXCL5 expression in 10^{-8} M TP group was observed compared to the control group. Moreover, TP treatment also increased the cell surface area and slightly altered cellular morphology, as the cell bodies were broader in the 10^{-8} M TP group in comparison to the control group, where the cells maintained their spindle shapes (Figure 4b).

DISCUSSION

Various studies have investigated the impact of testosterone derivatives in vitro on both healthy and cancer cells. For instance, a study performed on dermal papilla cells indicated that 5α-dihydroxy testosterone promoted cell proliferation at 10^{-7} M concentration while suppressing cell growth at higher doses in a time-dependent manner.[26] Dihydroxy testosterone also promoted proliferation and viability in primary aggressive neurofibromatosis cells when administered up to 100 μg/ml.[27] In human visceral adipocytes, 10^{-7} M testosterone supplement significantly promoted proliferation.[28] On the other hand, testosterone at 10^{-6} M and 10^{-5} M concentrations promoted cell death in SH-SY5Y neuroblastoma cell line in 24 hours, though at 10^{-7} M it did not decrease viability.[28] Corotchi et al.[29] reported that testosterone treatment at $4x10^{-8}$ M for 150 hours promoted cell proliferation without exerting cytotoxicity while preserving their stemness features and did not alter surface markers of Wharton Jelly derived MSCs. Consistent with this study, our results also revealed that TP at 10^{-8} M concentration did not alter the characteristic features of BM-MSCs while promoting their proliferation and preserving their

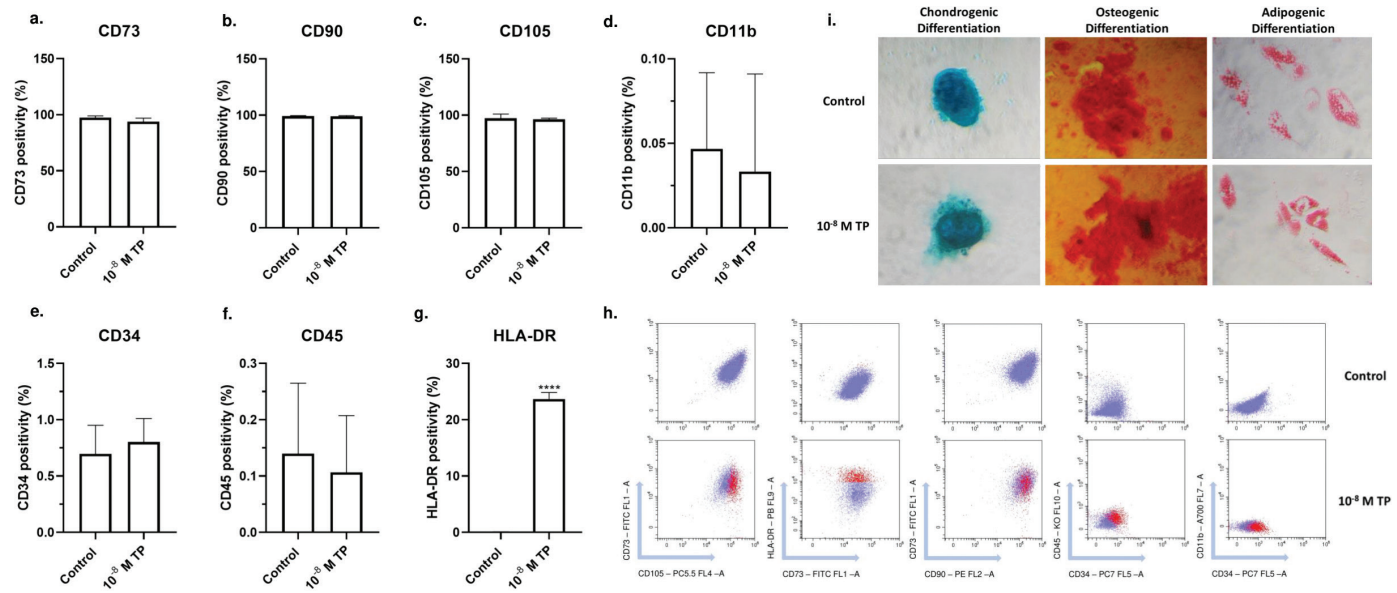

FIG. 3. Testosterone propionate does not alter cell surface markers' expressions on BM-MSCs. Bar graphics indicating (a) CD73, (b) CD90, (c) CD105, (d) CD11b, (e) CD34, (f) CD45, and (g) HLA-DR levels; (i) Representative micrographs indicating chondrogenic, osteogenic, and adipogenic differentiation on the 21st day of control (upper row) or 10^{-8} M TP (lower row) groups; (h) Representative flow cytometry dot plots regarding immunophenotyping of control (upper row) or 10^{-8} M TP (lower row) groups. P values lower than 0.05 were considered statistically significant; ****$p < 0.0001$.

differentiation capacities. However, the increased HLA-DR expression may suggest that the TP supplementation promotes class II-restricted antigen presentation of BM-MSCs, which has been previously proven under different experimental conditions.[25,30] However, as suggested by van Megen et al.,[25] increased HLA-DR expression in BM-MSCs after glutamic acid decarboxylase 65-kilodalton isoform (GAD65) exposure did not increase the levels of activating co-stimulatory molecules CD86 and CD80, or chemokine receptors CXCR3 and CCR7, but promoted immunosuppressive mediators, including programmed death-ligand 1 (PD-L1) and indoleamine 2,3, dioxygenase (IDO). Herein, even though the authors revealed enhanced immunosuppressive effects of BM-MSCs followed by incubation with HLA-mismatched CD4+ T-cells, these results may indicate that anti-cancer properties on TP-treated BM-MSCs should be evaluated in a more comprehensive manner. In addition, the synergistic effects of TP with BM-MSCs on the K562 cell line should be considered, as TP alone also led to cytotoxicity on these cells.

Our studies also revealed that 24-hour TP incubation promoted MSC-1 polarization and exerted cytotoxic effect on the leukemic cell line K562. However, this effect was reported to last for a limited duration as TP pre-treatment had significantly lower impact on leukemic cells, yet, enhanced effects may be observed by treating cells with longer-acting isoforms of testosterone such as testosterone undecanoate.[31] Moreover, it should be noted that the presence of cancer-associated MSCs (CA-MSCs) with pro-tumorigenic features have been reported in certain tumors, and MSCs can facilitate carcinogenesis by promoting epithelial to mesenchymal transition, migration and invasion.[32-36] Thus, the pro-carcinogenic features of these cells should be considered when MSCs are used in cancer treatment.

MSCs hold a great potential for regenerative medical applications,[4,7] but their usage is limited in vitro as they lose their differentiation and proliferation capacities and undergo epigenetic alterations at later passages.[9,11,37] As mentioned earlier, BM is a heterogenous population, and pure MSC culture cannot be obtained until 3rd passage. Addition of certain factors to increase cell proliferation rate may be a suitable option to increase their growth rate. Some examples of these types of applications are already present in the literature, including insulin treatment in MCF-7 breast cancer cell line culture,[38] fibroblast growth factor-2 supplement for human stromal cell culture,[39] or triiodothyronine supplementation in rat pancreatic islet cell culture.[40] The results of our study revealed that 10^{-8} M TP supplementation increased the survival and proliferation rates of BM-MSCs in vitro without altering their characteristics. This finding may enable to obtain more and healthier BM-MSCs at lower passages, though ideal doses of TP may differ among individuals. However, the solvent for dissolving TP should be carefully determined, because even if it was added at low concentrations, acetone promoted cell proliferation and necrosis of BM-MSCs. Moreover, our results should be verified with primary cultures.

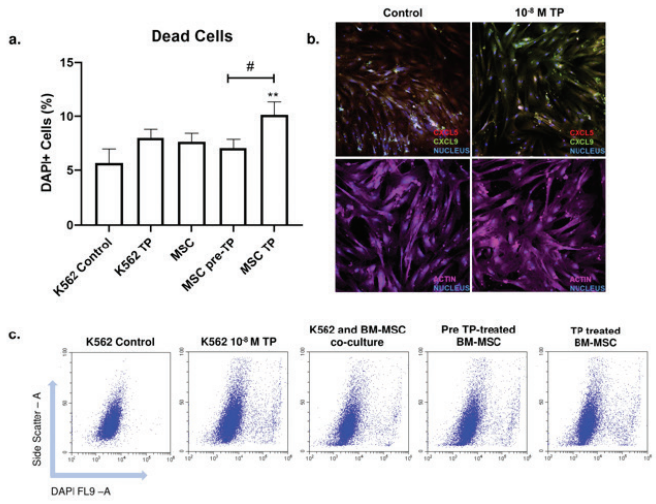

FIG. 4. Testosterone propionate treatment promoted MSC-1 polarization and enhanced cytotoxic features of BM-MSCs. (a) Bar graphics indicating dead cell ratios upon co-culturing BM-MSCs with K562 cells for 24 hours. * denotes significance between treatment and the control group, while # denotes significance between treatment groups. (b) Confocal microscopy images indicating either CXCL5 (red) and CXCL9 (green) protein expression or cellular morphology indicated with F-actin labeling, where TP at 10^{-8} M concentration led to increased CXCL9 protein expression and broadened cell surface area. (c) Representative flow cytometry dot plots indicating cell death upon co-culturing BM-MSCs with K562 cells at 1:10 ratio.

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Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Concept- B.A., T.A., G.Y.D.; Design- B.A., T.A., G.Y.D.; Data Collection or Processing- B.A., T.A., H.D., G.G.; Analysis or Interpretation- B.A.,T.A.,G.Y.D.; Literature Search- B.A., T.A., G.Y.D.; Writing- B.A., G.Y.D.

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Supplementary: http://balkanmedicaljournal.org/uploads/pdf/2022-10-21-supplementarymaterials.pdf

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