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Original Article

Monophosphoryl Lipid A and Retinoic Acid Combinations Increased Germ Cell Differentiation Markers Expression in Human Umbilical Cord-derived Mesenchymal Stromal Cells in an *In vitro* Ovine Acellular Testis Scaffold

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Infertility is known as one of the most common problems among couples. In this regard, generation of male germ cells from adult stem ones are among the current promising priorities of researchers .Mesenchymal stromal cells (MSCs) were previously induced to differentiate into germ-like progenitors *in vitro*. Monophosphoryl lipid A (MPLA) is a detoxified derivative of lipopolysaccharides (LPS) that lacks many of the endotoxic properties of LPS. Our present study aimed to investigate the expression of migration genes (*CXCR4*, *VCAM1*, *VEGF*, *MMP2*, and *VLA4*), and differentiation markers during human umbilical mesenchymal stromal cells (hUMSCs) culture in the presence of retinoic acid (RA) and MPLA-treated acellular testis. Accordingly, the high expression levels of deleted in azoospermia-like (DAZL), piwi-like RNA-mediated gene silencing 2 (PIWIL2) transcripts as well as protein were consequently observed in treated hUMSCs. It was concluded that combination treatment (i.e., MPLA/RA) had more prominent results than each of the treatments alone, even though MPLA and RA could be regarded as inducer of migration and differentiation, respectively. Ultimately, it was suggested to introduce the use of combination treatment as a more effective strategy to improve therapies in regenerative medicine.

Key words: Infertility, monophosphoryl lipid A, retinoic acid, DAZL, PIWIL2

It is widely accepted that infertility is one of the common clinical conditions among couples. Infertility affects about 10-15% of both males and females worldwide, with almost half of them being related to males and occuring due to oligospermia or azoospermia (1). Regenerative medicine-based therapies, including tissue engineering and cell therapy techniques, have been introduced as potential methods for the treatment of many diseases, and serious efforts have been made to

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enhance their effectiveness. These methods have attracted a great deal of attention in helping to treat infertility. The utilization of appropriate cells and natural scaffolds in the generation of male germ cells with specialized functions is one of the main research areas in this field (2). All therapeutic interventions in this context are based on the potential of stem cells to migrate, proliferate, differentiate, and survive in the natural scaffolds. If successful, cell-containing scaffolds have the potential to regenerate damaged tissues (3). The differentiation potential of human umbilical mesenchymal stromal cells (hUMSCs) is far higher than other adult mesenchymal stromal cells (MSCs), including bone marrow- derived mesenchymal stromal cells (BMSCs) (4). The application of non-embryonic stem cells to differentiate cells is a priority for researchers due to the lack of ethical paradox and their immunogenicity (5). Different gram-negative and gram-positive bacteria as well as their products, such as lipopolysaccharide (LPS) and monophos-phoryl lipid A (MPLA), were reported to contribute to the differentiation of stem cells into adipose and bone cells (6). Numerous studies have shown that the mesenchymal cells carry toll-like receptors (TLR) on their surface, which are involved in the identification of bacterial structures such as LPS (7). The receptor activated by bacterial exposure mediates the process of mesenchymal cell migration toward the bacterium. The MPLA is a chemically modified non-toxic derivative of LPS, which has immunomodulatory properties. The TLR ligand is 100 times less pyrogenic than the LPS. The administration of this substance has been proven to be completely safe for human beings (8). Accordingly, the present study utilized this non-toxic derivative to prevent any damage and cytotoxicity. The differentiation of BMSCs to male germ cells in the presence of retinoic acid (RA) was first reported in 2006. The RA is an irreversibly oxidized form of retinol that can differentiate embryonic stem cells towards primordial germ cells (9, 10). The availability of sheep testis and their histological similarity with human testicular tissue led to the use of sheep acellular testis as a natural scaffold in the present study. In this study, the human umbilical cord-derived mesenchymal stromal cells (hUCMSCs) were cultured in the presence of natural scaffolds of sheep acellular testis. The purpose of the present study was to investigate the migration and differentiation potential of hUMSCs into male germ cell co-cultured with the sheep acellular testis in the presence of RA and MPLA.

Materials and methods

Derivation and expansion of MSCs from human umbilical cord

Isolation and expansion of hUMSCs was performed according to the method previously published (11). Briefly, following the steps for obtaining informed consent and ethical permission, fresh umbilical cords were conveyed to sterile phosphate-buffered saline and then rinsed to remove any vestige of blood. After preparation stages such as cutting of tissue (10 cm), the vessels were subsequently detached and shut in the terminal region with a sterile plastic clamp. Vascular loops together with tissue fragments were partially digested via modulated explant/enzyme method (MEEM). Upon incubation of explants in collagenase type I (for 3 h) as well as inactivation of collagenase by Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, Life Technologies, USA) containing 10% fetal bovine serum (FBS, Life Technologies, USA), vascular loops and partially digested explants were cultured in DMEM-LG. Once the confluency of cells reached 80-90%, they were spilt at 1:3 ratios for the subsequent experiments. All experiments were approved by the Ethics and Clinical Studies Research Committee of SKUMS according to Helsinki declaration, and informed consents were

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obtained from all mothers before surgery.

Multilineage differentiation

In the next step, the hUMSCs were subjected to differentiation into adipogenic and osteogenic cell lineages based on different induction conditions as previously described (12).

Flow cytometry

To identify surface antigens, the hUMSCs (1×10^6) were stained with anti-CD105, anti-CD90, anti-CD73, anti-CD44, anti-CD45, anti-CD29, anti-CD34, anti-CD31, and human leukocyte antigen-DR isotype (HLA-DR) (Abcam, UK). The related isotype controls including fluorescein isothiocyanate (FITC)- and PE-conjugated mouse IgG isotype antibodies were also utilized for negative staining. Following incubation at room temperature in the dark (20 min), stained cells were re-suspended in 500 µL PBS and analyzed through CyFlow® Space flow cytometer (Partec, Germany). The histograms were then generated based on computed results using Windows™-based flow cytometry software.

Acellular testis preparation

First, 0.5 mm sections of sheep testis were immersed in 5% SDS solution for three days, and then the solution was renewed every hour in the early hours until complete acellularity.

Treatment of acellular testis with MPLA

At first, the acellular testes were placed in MPLA (10^{-3} µg/mL) solution overnight. One day before putting the acellular testis on monolayer hUMSCs, approximately 2.5×10^5 hUMSCs per well were cultured in 6-well plates containing DMEM-LG supplemented with 10% FBS and 1% penicillin-streptomycin (Pen/Strep); then, the next day, two pieces of the treated acellular testis were put on hUMSCs in each well. After 5 days, RNA and protein extraction were performed for gene expression analyses.

Scanning electron microscopy (SEM)

The treated acellular testis and controls (untreated acellular testis) were placed on hUMSCs and then evaluated using SEM. To prepare the samples for SEM, the tissue pieces were incubated with glutaraldehyde 2.5% and then dehydrated by alcohol for 20 min and transferred to the freeze dryer.

Gene expression analyses

Total RNA was isolated from the hUMSCs using TRIzol reagent (Sigma-Aldrich Corp., MI, USA) according to the manufacturer's protocol and measured by NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, MA, USA). The 260/280 and 260/230 values were higher than 1.9. As well, 1 µg total RNA of each sample was synthesized using a cDNA synthesis kit (Yekta-Tajhiz-Azma [YTA], Tehran, Iran) and transferred into the qRT-PCR reaction. The primer sequences used were as follows: *VCAM1*, (F) 5'CGAACCCAAACAAAGGCAGA3' and (R) 5'ACAGGATTTTCGGAGCAGGA3'; *MMP2*, (F) 5'ACCACAGCCAACTACGATGA3'; and (R) 5'GCTCCTGAATGCCCTTGATG3'; *VLA4*, (F) 5'TCCAACCTGATCCTGTGTC3' and (R) 5'TCGTTGTTCCCATTCACT3'; *CXCR4*, (F) 5'ACCATCTACTCCATCATCTTC3' and (R) 5'TGATGACAAAGAGGAGGTC3'; *VEGF*, (F) 5'ATCAAACCTCACCAAAGCC3' and (R) 5'TCTTTGGTCTGCATTCACATC3'; *DDX4*, (F) 5'TCATACTTGCAGGACGAGATTTG3' and (R) 5'AACGACTGGCAGTTATTCCATC3';*DAZL*, (F) 5'ATGTTGTACCTCCGGCTTATTCA3' and (R) 5'CCATTTCCAGAGGGTGGAGTA3'; *PIWIL 2*, (F) 5'CTGAAACGGGAAATGCTTCCA3' and (R) 5'CCTGTCCTTGCGTACCAGATTA3'; *GAPDH*, (F) 5'GAGTCCACTGGCGTCTTCAC3' and (R) 5'ATGACGAACATGGGGGCA3'. Besides, the transcription level of GAPDH was used as an endogenous control. The qRT-PCR reactions were performed using a Rotor-Gene 3000™ System (Corbett Research, Sydney, Australia).

Western Blotting

Cells (2×10^6) were harvested, rinsed with PBS, and the extract was then prepared with lysis

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buffer (10% glycerol, 10% sodium dodecyl sulfate (SDS), 25% ß-mercaptoethanol, Tris-HCl 0.5 M pH 6.8, and 0.5% bromophenol blue) containing 1% protease inhibitors. The samples were subsequently placed on ice for 60 min. Protein concentration of the cell lysates were also quantified by the Bradford protein assay. Afterwards, the proteins were transferred from SDS- polyacrylamide gel electrophoresis (PAGE) to a polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane (16 h/86 mA and 2 h/200 mA) in a transfer buffer (Tris 25 mM, glycine 192 mM, 20% methanol). The DAZL, and PIWIL2 proteins were also detected using primary polyclonal anti-DAZL antibody, and anti-PIWIL2 antibody (Abcam, USA) at 1:2000 dilution in 5% BSA blocking solution in wash buffer (TBST) at 4 ºC overnight followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody at 37°C for 60 min, and developed with a Western blotting Luminol Reagent (ThermoFisher Scientific, USA). The bands were finally visualized by imaging using the LI-COR Odyssey® scanner and densitometry was also accomplished via software (LI-COR Biosciences).

Statistical analysis

Statistical analyses were performed using GraphPad statistical software (GraphPad Software, CA). Data were analyzed using one-way ANOVA and compared with the control.

Results

Characterization of human umbilical cord-derived mesenchymal stromal cells

Flow cytometric analysis revealed that the studied cells were positive for CD29, CD105, and CD90, and negative for CD34 and CD45. These multipotent cells were able to differentiate into osteogenic and adipogenic lineage.

SEM and ophthalmic microscope images

Scanning electron microscope (SEM) images for three treated groups (MPLA-RA, MPLA, RA) indicated the cell migration to acellular tissue, which was more frequent in the MPLA-treated tissues. There was a significant difference in the pore-filling of the treated tissues in comparison with control tissues. In fact, the number of mesenchymal cells that migrated to the acellular tissue was higher in the MPLA/RA and MPLA treatments in comparison with the untreated tissue (Figure 1). Also, clear tissue tropism of hUMSCs was present in MPLA/RA-treated testis on day 5 (Figure 2).

Expression of migration related genes

The analysis of the expression of migration-related genes (*VCAM1*, *VLA4*, *VEGF*, *MMP2*, and *CXCR4*) in the RA-treated cells in the presence of MPLA-treated acellular testis showed an increased expression for all the studied genes. The increased expression was seen for the *CXCR4*, *MMP2* and *VEGF* genes in the RA-treated cells and the cells

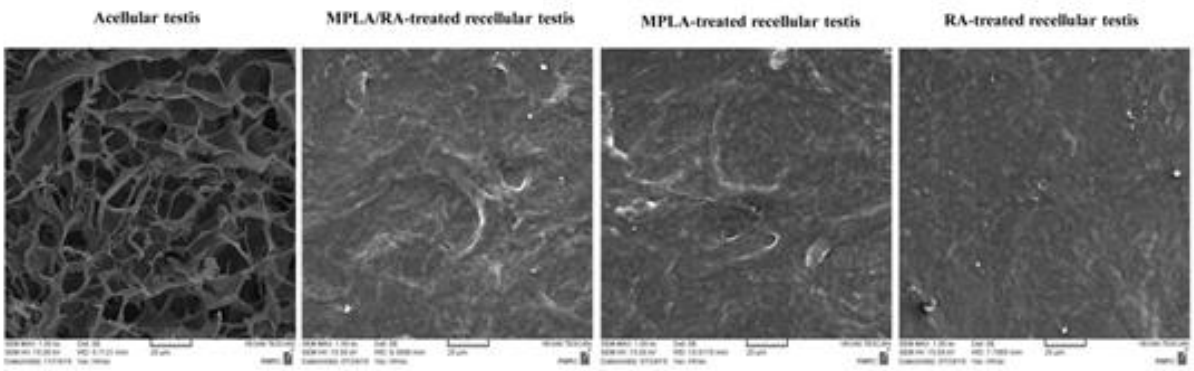

Fig. 1. SEM images of recellular testis after 5 days treatment. Accordingly, the number of migrated MSCs remarkably increased after MPLA/RA combination treatment. Also, acellular testis was considered as control. All SEM images are represented at 1000 × magnification.

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Fig. 2. Tropism of hUMSCs for acellular testis with MPLA and MPLA/RA treatments. Clear tissue tropism of hUMSCs can be seen in the presence of MPLA/RA-treated testis after 3 days in comparison with MPLA-treated testis.

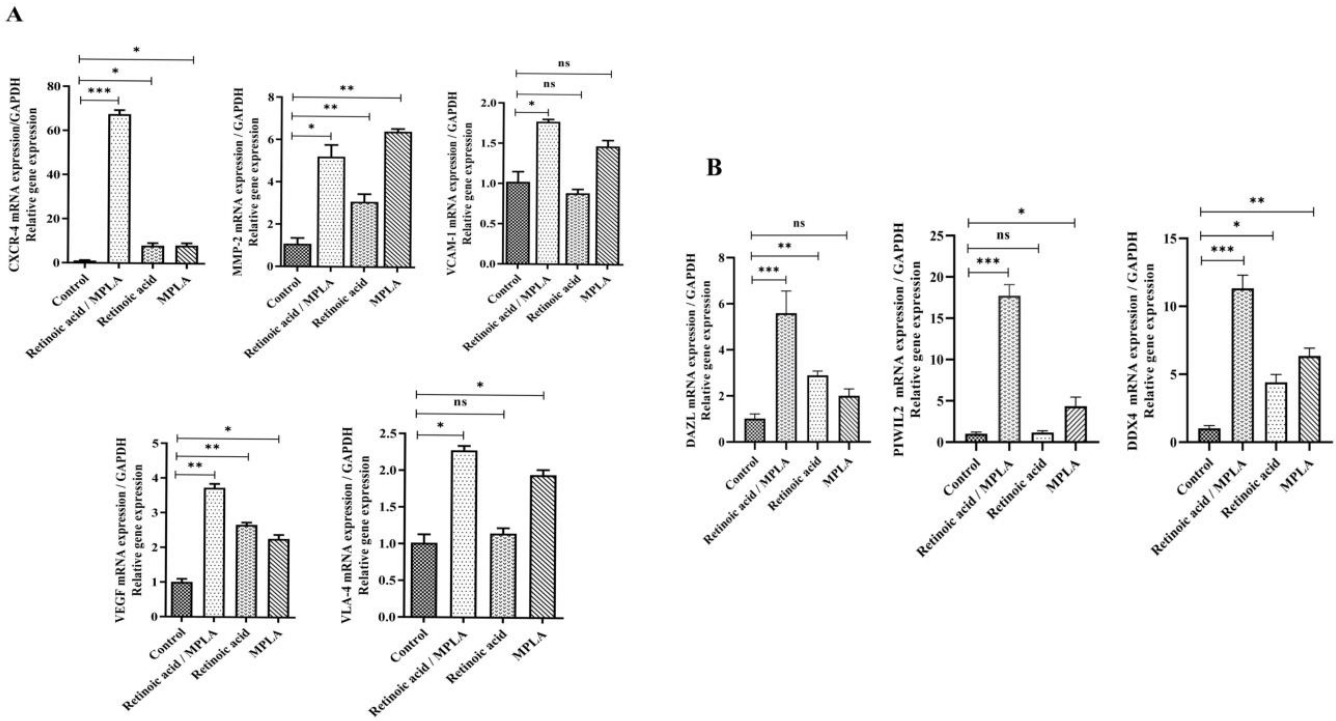

Fig. 3. Expression of genes involved in cell migration and spermatogenesis. A) qRT-PCR analyzes of VEGF, CXCR4, VLA4, VCAM1, and MMP2 expression in hUMSCs after treatement with MPLA/RA, RA and MPLA in comparison with control. The data are normalized to the expression levels of GAPDH in untreated controls; B) qRT-PCR analyses of DAZL, PIWIL2, and DDX4 in hUMSCs after treatment with MPLA/RA, RA and MPLA in comparison with control. All values are expressed as mean ± standard deviation (SD) in each group; qRT-PCR results are representative of two independent samples in triplicate.

exposed to MPLA-treated testes, but not for the *VLA4* gene in the RA-treated cells. No change in the *VCAM1* expression was observed in the RA-treated cells or the cells exposed to MPLA-treated testes (Figure 3A).

Analysis of genes involved in spermatogenesis.

The treatment of hUMSCs with RA in the presence of MPLA-treated testes could result in increased expression of genes involved in spermatogenesis (*DAZL*, *DDX4*, and *PIWIL2*). On the other hand, the treatment of hUMSCs with RA as an inducer of stem cell differentiation increased the expression of *DAZL* and *DDX4* genes, but not the expression of *PIWIL2*. In the hUMSCs in the presence of MPLA-treated testes, the expression was increased only for *DDX4* and *PIWIL2* genes, but no significant change was observed for *DAZL* (Figure 3B). In addition, the results in the protein

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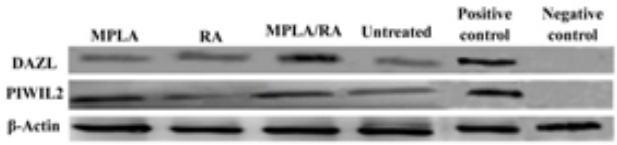

Fig. 4. PIWIL2 and DAZL expression after 5 days. Positive control: human sperm; negative control: hUMSCs. hUMSCs were treated during 5 days. β-actin was used as a housekeeping control.

level assessment showed the increased expression of PIWIL2 in the RA and MPLA/RA treatment groups in comparison with the control group, but no significant change was observed in the MPLA treated group. On the other hand, the *DAZL* gene had an increased expression in the RA, MPLA and MPLA/RA treatment groups in comparison with the control group. Interestingly, the increase in the DAZL protein expression was significantly greater in the MPLA/RA treated group in comparison with other groups (Figure 4).

Discussion

In this present study, we provided an *in vitro* co-culture model to evaluate the ability of hUMSCs in migration and differentiation into male germ cells. This approach is based on our previous findings that demonstrated the MPLA could be effective in cell migration into the acellular tissue (13). On the other hand, because RA was identified as a significant modulator for spermatogonial differentiation (14-17), we co-cultured the RA-treated hUMSCs with MPLA-treated acellular testis. Our hypothesis was that the hUMSCs employ the acellular tissue as an ecological niche, and the MPLA as an adsorbent is involved in further migration of hUMSCs into the acellular tissue while the RA may assist the differentiation process. There is no study to date on whether increased stem cell tropism toward acellular tissue as a natural scaffold in the presence of RA has an incremental effect on the expression of genes involved in differentiation. Indeed, the novelty of the present study is the co-culture of sheep acellular testis with hUMSCs in the presence of MPLA and RA. In this study, we utilized 10^{-6}M RA in accordance with previous studies (3, 18). The results of this study showed an increased expression of migration-related genes in the presence of MPLA-treated testis. Since the expression profile of many genes is related to male germ cell differentiation, we investigated the expression of some genes involved in spermatogenesis including *DAZL*, *DDX4*, and *PIWIL2*. Analysis of the expression of these genes in the male germ cells in MPLA/RA and RA treatments revealed that the migration of hUMSCs to the acellular tissue played an important role in the expression of male germ cell differentiation markers because the upregulation of these markers was lower in the MPLA-untreated acellular testes. Researchers have been recently interested in using MSCs in the regenerative medicine as a new approach to the management of infertility (19). Regarding the use of treatments in this study, it can be stated that the MPLA has been used here to accelerate the migration of hUMSCs into the tissue, and possibly improve the expression of germ cell-related differentiation genes. Studies have shown that the incubation of MSCs in conditions equivalent to ecological niche could affect the expression of genes involved in cell proliferation and differentiation (20). RA is a small polar molecule capable of diffusing across the tissue, and exerts its function through nuclear retinoid receptors. The binding of RA to nuclear retinoid and retinoid receptors on nuclei of spermatogonia enhances the expression of SALL4A transcription factor, thereby resulting in higher expression of receptor tyrosine kinase that is essential for spermatogonial differentiation (21, 22).

DAZL is an RNA-binding protein in the male germ cell. The absence of DAZL is associated with

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failure to differentiate into the male germ cell in the process of spermatogenesis, as well as apoptosis induction in the primordial germ cells (23, 24). PIWIL2, also known as MILI, is expressed in the male germ cells, and plays a role in self-renewal of spermatogonial stem cells. As well, the protein encoded by the gene belonging to the argonaute protein family is involved in the development and survival of germline stem cells (25). In a study, human amniotic epithelial cells were cultured in a medium containing serum substitute supplement for differentiation into cells expressing germ cell specific markers (DAZL and DDX4). The results showed that these cells have the potential to differentiate into germ cells (26-28). Studies have shown that increased expression of certain genes, including *DAZL* and *PIWIL2*, is associated with induction of *in vitro* differentiation of BMSCs into germ-like cells (29, 30).

In summary, this research is the first report about using RA-treated hUMSCs in the presence of MPLA-treated acellular testis in male germ cells differentiation process. We also demonstrated the capacity of hUMSCs for expressing genes characteristic of germ cells under presented *in vitro* treatments. However, future studies should focus on further investigation of mitotic and meiosis genes and proteins for planning more effective culture conditions promoting male germ cells gene expression, and providing a compatible pattern with developmental stages of spermatogenesis.

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Conflict of interest

The authors declare that they have no conflict of interest

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