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Comparison of the effects of progesterone and 17 β-estradiol on Schwann cell markers expression in rat adipose-derived stem cells

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Abstract

Steroids promote the myelination and regeneration in the peripheral nervous system. Whereas, little is known about the inducing effects by which the hormones exert their effects on Schwann cells differentiation. This could be revealed by the expression of Schwann cell markers in adipose-derived stem cells (ADSCs). The purpose of this study was to present the effects of progesterone and 17 β-estradiol on the Schwann cell markers in rat ADSCs. The mesenchymal stem cell markers (CD73, and CD90) were assayed by flow cytometry. Rat ADSCs were sequentially treated with β-mercaptoethanol, and all-trans-retinoic acid, followed by a mixture of basic fibrobroblast growth factor, platelet-derived growth factor, forskolin and heregulin. In experimental groups, forskolin and heregulin were substituted by progesterone and 17 β-estradiol. After induction, the expression of Schwann cell markers P0, and S-100 and the cellular immunocytochemical staining positive rate of anti-S100 and anti-glial fibrillary acidic protein (GFAP) antibodies were compared in the experimental and control groups. Progesterone and 17 β-estradiol triggered P0 and S-100 genes expression and induced a cellular immunocytochemical staining positive rate of S-100 and GFAP in rats ADSCs. Progesterone induced these changes stronger than 17 β-estradiol. Thus, progesterone may induce rat ADSCs toward Schwann-like cells by expression of Schwann cell markers and is more potent than 17 β-estradiol in the expression of these markers.

مقایسه آثار پروژسترون و ۱۷ بتا – استرادیول بر بیان شاخص های سلول شوان در سلولهای بنیادی مشتق از چربی موش صحرایی

چکیده

استروئید ها موجب بهبود میلینه شدن و بازسازی در دستگاه عصبی محیطی می شوند. اما آثار القایی این هورمونها جهت تاثیر بر تمایز سلولهای شوان کمتر شناخته شده اند. این امر می تواند به واسطه بیان شاخص های سلول های شوان در سلولهای بنیادی مشتق از چربی آشکار گردد. هدف این مطالعه نشان دادن آثار پروژسترون و ۱۷ بتا- استرادیول بر بیان شاخص های سلول شوان در سلولهای بنیادی مشتق از چربی موش صحرایی می باشد. شاخص های سلول بنیادی مزانشیمی (CD73 و CD90) با روش فلوسیتومتری مورد ارزیابی قرار گرفتند. سلولهای بنیادی مشتق از چربی موش صحرایی ابتدا توسط بتا- مرکاپتواتانول و اسید رتینوئیک و در ادامه توسط مخلوطی از فاکتور رشد فیروبلاستی بازی، فاکتور رشد مشتق شده از پلاکت، فورسکولین و هرگولین تیمار شدند. در گروههای تجربی، پروژسترون و ۱۷بتا – استرادیول جایگزین فورسکولین و هرگولین شدند. بعد از القاء، بیان شاخص های سلول شوان P0 و S-100 و میزان مثبت شدن آنتی بادی های آنتی پروتئین اسیدی فیبریلی گلیال (GFAP) و آنتی S-100 به روش رنگ آمیزی ایمونوسیتوشیمیایی در گروههای تجربی و شاهد مورد مقایسه قرار گرفتند. پروژسترون و ۱۷ بتا—استرادیول باعث تحریک بیان ژنهای P0 و S-100 و القاء میزان مثبت شدن رنگ آمیزی ایمونوسیتوشیمیایی پروتئین های S-100 و GFAP در سلولهای بنیادی مشتق از چربی موش صحرایی شدند. پروژسترون بیش از ۱۷ بتا —استرادیول این تغییرات را القاء کرد. بنابراین، احتمالاً پروژسترون با بیان شاخص های سلول شوان باعث القای سلولهای بنیادی مشتق از چربی موش صحرایی به سمت سلولهای شبه شوان می شود و در بیان این شاخص ها نسبت به ۱۷ بتا —استرادیول تواناتراست.

واژه های کلیدی: ۱۷ بتا استرادیول، بافت چربی، پروژسترون، سلولهای بنیادی مزانشیمی، سلولهای شوان

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Introduction

Schwann cell is a principle glia and plays a pivotal role in the development, regeneration and maintaining of the peripheral nervous system.[1,2] Schwann cells release neurotrophic factors and involve in peripheral axons myelination, directional guidance of neurons, and cellular fragments removal.[2] Various studies have shown that implanted Schwann cells in nerve guidance channels improve the advantageous endogenous responses to nerve injury and allow nerve regeneration.[3-5] However, the clinical application of Schwann cells is debatable because it is complicated to find enough numbers of Schwann cells.

Adipose-derived stem cells (ADSCs) are useful in regenerative medicine.[6] Several recent studies have introduced ADSCs as a source of multipotent stem cells which can be differentiated into several cell lines.[7,8] Obtaining of ADSCs is easy, and efficient; it shows very low donor site morbidity.[9] In addition, adipose tissue contains more than a thousand-fold more mesenchymal stem cell (MSC) compared to bone marrow.[10,11]

The MSCs and glial cells are targets for progesterone and estrogen.[12-15] Schwann cells synthesize and metabolize neurosteriods such as progesterone and 17 β-estradiol and express steroid receptors.[16] Neurosteroids regulate the expression of myelin proteins and transcription factors with a key role in Schwann cells physiology and in their myelinating program.[16,17] Progesterone plays a significant function in peripheral nerves myelination.[17,18] It could improve the formation of new myelin sheaths subsequent to the mouse sciatic nerve damage.[19] In addition, progesterone has promoted axons myelination when added to explanted cultures of rat dorsal root ganglia,[12,19] accelerated the time of myelination nitiation and increased the amount of myelin synthesis in co-cultures of Schwann cells and sensory neurons.[20] Estrogens regulate re-myelination and promote neuronal survival via expression of growth factors by glial cells.[21,22] Interestingly, previous study has indicated that progesterone stimulates the expression of Schwann cell markers in bone marrow MSCs, [13] but the stimulating effect of neuroesteroids on the expression of Schwann cell markers in ADSCs has not been investigated. In the current study, a comparative study of the inducing effects of progesterone and 17 β-estradiol with a focus on the expression of Schwann cell markers in the ADSCs culture was carried out.

Materials and Methods

Isolation and culture of ADSCs. The ADSCs were isolated from adult 4-6 weeks old male Wistar rats (n =6). Animal experimental procedures were approved by the Research Committee of Kashan University of Medical Sciences (N:5219, 2010). The rats were euthanized and fat pads were removed carefully from inguinal region. The fat pieces were washed with phosphate buffered saline (PBS) and mechanically dissociated using sterile scalpel blades. After removal of debris, the adipose tissue was enzymatically digested for 60 min at 37.00 ˚C using 0.50 mg mL^{-1} collagenase type I (Sigma-Aldrich, Saint Louis USA). The solution was centrifuged to separate the floating population of mature adipocytes from the pelleted cells of stromal vascular fractions. The suspension containing dissociated tissue cells was neutralized by the addition of ADSC medium containing Dulbecco's modified Eagle medium (DMEM/F12; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin solution and centrifuged at 1000 *g* for 7 min, re-suspended and filtered. The cells were centrifuged and the pellet was re-suspended in ADSC medium and transferred to a T75 flask centrifuged at density of 1.00×10^6 cells. The culture medium was changed every other day until the cells became 80.00% confluent. The cells were incubated at 37.00 ˚C with 5.00% CO_2 and passaged up to three times using trypsin- ethylene-diaminetetraacetic acid (EDTA; Gibco). [23] Cell viability of the cultured cells in each passage was frequently about 95.00%. Cell count was determined using a manual hemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany).

Characterization of ADSCs properties. At passages 3-5, cells were harvested by the addition of 0.20% trypsin-EDTA and washed by PBS 3 times. Aliquots were incubated with different rat antibodies (CD90, CD45, CD73 or VEGFR2)[8,23,24] conjugated with fluorescein isothiocyanate (Chemicon, Temecula, USA) in dark, at room temperature for 45 min. The phenotype of cultured was analyzed by flow cytometry using a FACscan cytofluorometry (Becton Dickinson, San Jose, USA).

Differentiation to a Schwann cell-like phenotype. The ADSCs medium was removed from the cultures at passage 3 and replaced with medium containing 1 mM β-mercaptoethanol (Sigma) for 24 hr. Then, cells were washed and fresh medium supplemented with 35 ng mL^{-1} all-trans-retinoic acid (Sigma) was added. After 72 hr, cells were washed and the medium was replaced with induction medium in 6 different groups: In the first group, the cell differentiation medium supplemented with 5 ng mL^{-1} platelet-derived growth factor (PDGF; R&D), 10 mL^{-1} basic fibroblast growth factor (bFGF; Gibco), 5.00 µM forskolin (Sigma) and 200 ng mL^{-1} heregulin-β (Milipore) was added (positive control group); forskolin and heregulin were replaced with progesterone (10^{-6} M; Sigma) respectively in the second and third groups and 17 β-estradiol (10^{-6} M; Sigma) respectively in the fourth and fifth groups. In the sixth group (negative control group), the medium contained only 5 ng mL^{-1} PDGF and 10 ng mL^{-1} bFGF. The cells cultures were followed by incubation for eight days.[13]

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Following the differentiation period, total RNA was extracted with RNX plus (Ceinna-Gen, Tehran, Iran). Complementary DNA (cDNA) synthesis was carried out from total RNA using Fermentas kit (Fermentase, Waltham, USA) based on the manufacturer's instructions. The cDNA was amplified using the primers designed for S-100 and P0 genes (Table 1). Each 12.50 µL PCR reaction mixture included 0.25 µL of each primer, 0.25µL dNTP, 0.38 µL $MgCl_2$, 0.08 µL taq DNA polymerase, 1.25 µL 10 × PCR buffer (Fermentas) and 9.30 µL ultrapure H_2O. BioRad thermocycler was used for PCR with the following program: 94 ˚C for 30 sec, 35 cycles at 94 ˚C for 45 sec, 55 ˚C for 45 sec 72 ˚C for 45 sec, and a final extension at 72 ˚C for 10 min. 10 µL of the PCR product was separated, in a 1.50% agarose gel and stained with ethidiumbromide. The results were expressed relative to the housekeeping gene control glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[23]

Table 1. Primer sequences used for the RT-PCR.

Gene	Primers	Size (bp)
P0	Forward: CTTCCAAAGGCTCTCAGGTG Reverse: ACGGTCACTTGTTCG AGTCC	153
S100	Forward: ATAGCACCTCCGTTGGACAG Reverse: TCGTTTGCACAGAGGACAAG	169
GAPDH	Forward: TTAGCACCCCTGGCCAAGG Reverse: CTTACTCCTTGGAGGCCATG	531

Immunocytochemistry. The ADSCs of different groups were digested by trypsin-EDTA, and seeded into 24 cell plates following centrifugation and collection. Then, the cells were incubated at 37 ˚C, washed with PBS, dried and fixed in 4.00% paraformaldehyde for 20 min. Following permeablization using 0.40% Triton X-100 and blocking by 5.00% normal goat serum, the cells were incubated with primary antibodies against S-100 (1:100; Sigma), or GFAP (1:100; Sigma) at 4 ˚C for 1 hr. After washing, the cells incubated with FITC-conjugated anti-rabbit IgG (1:100; Sigma) for 45 min at room temperature. The 4'6'-diamidino-2-phenylondole dihydro-chloride (DAPI) was used to determine the nuclei as a counterstain. Finally, the cells were examined using an inverted fluorescence microscope (Eclipse Ti-S; Nikon, Tolyo, Japan) and the percentage of immuno-positive cells was calculated in five randomly selected fields at 40× magnification.

Statistical analysis. The data were analyzed using one-way ANOVA and Tukey's post-hoc test and the results were expressed as mean ± standard deviation. A *p*-values less than 0.05 was considered statistically significant.

Results

Adipose tissue was enzymatically digested and stromal cells were isolated. The cells were cultured and after 4 to 5 days, they proliferated rapidly and their condensation reached to approximately 80.00 to 90.00%. The cultured cells exhibited fibroblast-like morphology in microscopic observations.

Most of MSCs were negative for the expression of CD45 and VEGFR2 but they were positive for CD90 and CD73 expression. These data demonstrated the mesenchymal nature of the isolated cells.

Differentiation. The ADSCs expressed low levels of S-100, and P0 mRNAs. The $ADSC_s$ at passage three were treated with a mixture of glial growth factors containing FGF, PDGF, forskolin, and heregulin for a period of one week. This treatment increased the expression of Schwann cell markers (S-100 and P0) intensely at mRNA level. Progesterone (groups 2 and 3) or 17 β-estradiol (groups 4 and 5) increased these markers compared to the negative control group (group 6). The expression of S-100 mRNA in ADSCs in the differentiation media containing progesterone and heregulin (groups of 1-3) was significantly higher than other groups ($p < 0.0001$), (Fig. 1).

The expression level of P0 in the presence of progesterone and heregulin was near to positive control group (Fig. 2).

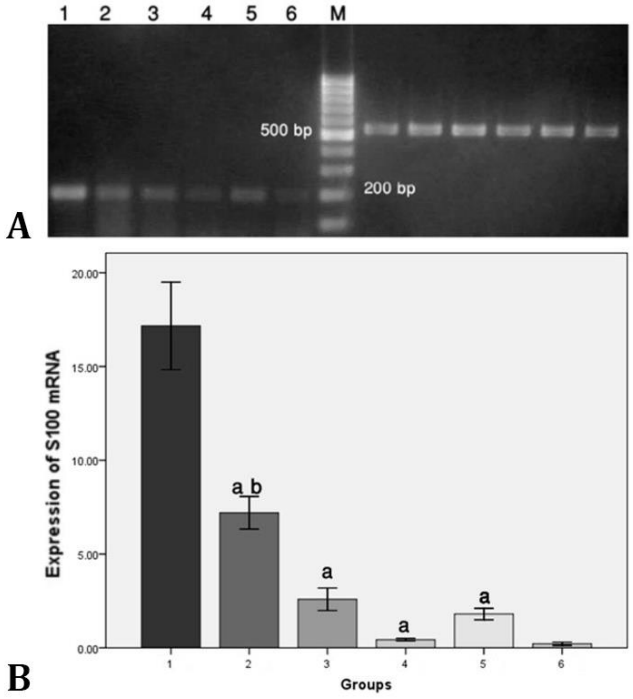

Fig. 1. A) A representative result of S100 mRNA expression in differentiation groups by RT-PCR. Lane 1: Group 1 (bFGF + PDGF + Forskoin + Heregulin); Lane 2: Group 2 (bFGF + PDGF + Heregulin + Progesterone); Lane 3: Group 3 (bFGF + PDGF + Forskoin + Progesterone); Lane 4: Group 4 (bFGF + PDGF + Heregulin + Estradiol); Lane 5: Group 5 (bFGF + PDGF + Forskoin + Estradiol); Lane 6: Group 6 (bFGF + PDGF); M: Marker. **B)** a: Significant difference with 1st group (positive control group), ($p < 0.0001$); b: Significant difference with other experimental groups ($p < 0.0001$).

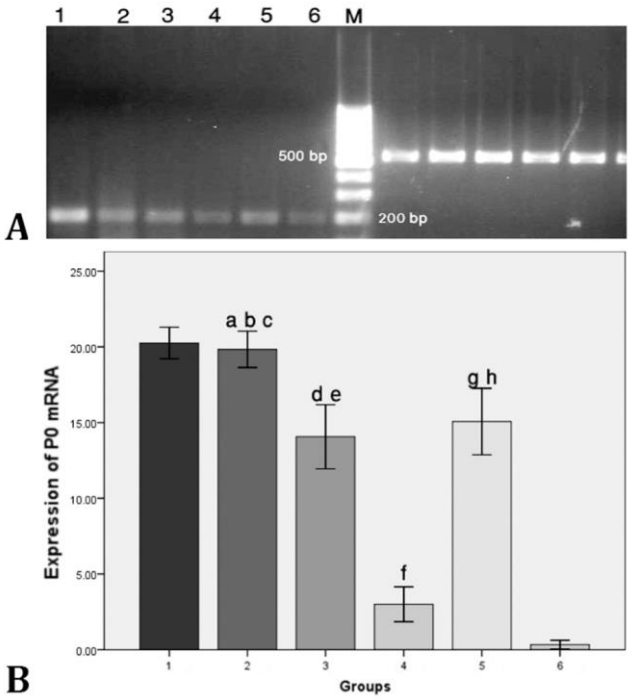

Fig. 2. A) A representative RT-PCR analysis and data of P0 mRNA expression in differentiation groups. Lane 1: Group 1 (bFGF + PDGF + Forskoin + Heregulin); Lane 2: Group 2 (bFGF + PDGF + Heregulin + Progesterone); Lane 3: Group 3 (bFGF + PDGF + Forskoin + Progesterone); Lane 4: Group 4 (bFGF + PDGF + H Lane eregulin + Estradiol); 5: Group 5 (bFGF + PDGF + Forskoin + Estradiol); Lane 6: Group 6 (bFGF + PDGF); M: Marker. **B)** a: Significant difference with 3rd group, ($p < 0.001$); b: Significant difference with 5th group, ($p < 0.007$); c: Significant difference with 4th and 6th groups ($p < 0.0001$); d: Significant difference with 1st group, ($p < 0.001$); e: Significant difference with 4th and 6th groups, ($p < 0.0001$); f: Significant difference with 1st group, ($p < 0.0001$); g: Significant difference with 4th and 6th groups, ($p < 0.0001$); h: Significant difference with 1st group, ($p < 0.004$).

In immunocytochemistry experiment, S-100 and GFAP positive cells were counted in five fields under fluorescent microscope. The undifferentiated cells of the control group had no expression of S-100 and GFAP. The staining intensity of S-100 and GFAP of cells in groups 2 to 5 (experimental groups) was more strongly positive than that of cells in group 6 (negative control group). In group 2 (in the presence of progesterone and heregulin), mean number of the S-100 and GFAP staining positive cells was 20.00 ± 5.29 and 18.30 ± 1.15, respectively. In group 3 (in the presence of progesterone and forskolin) mean number of the S-100 and GFAP staining positive cells was 21.30 ± 6.50 and 18.30 ± 1.15, respectively. In group 4 (in the presence of 17 β-estradiol and heregulin), mean number of the S-100 and GFAP staining positive cells was 20.30 ± 2.80 and 20.30 ± 1.15, respectively. In group 5 (in the presence of 17 β-estradiol and forskolin), mean number of the S-100 and GFAP staining positive cells was 25.30 ± 4.70 and 11.60 ± 3.50, respectively.

Immunocytochemistry showed that the expressions of S-100 and GFAP in the experimental groups were significantly superior to those of the negative control group (group 6), ($p < 0.0001$). Also, the GFAP proteins were significantly expressed higher in the cytoplasm of group 4 cells than group 5 cells ($p < 0.001$), (Figs. 3 and 4).

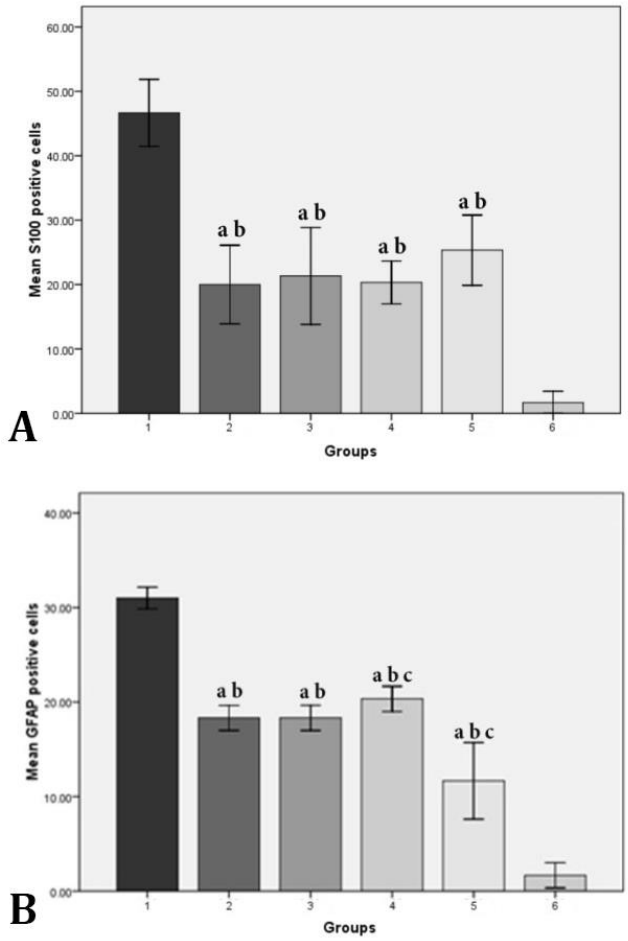

Fig. 3. A, and B) Quantitative analysis of morphology and expression of S100 (I), and GFAP (II) proteins. a: Significant difference with 1st group, ($p < 0.0001$); b: Significant difference with 6th group, ($p < 0.0001$); c: Significant difference between 4th and 5th groups ($p < 0.001$).

Discussion

The purpose of this study was to examine the inducing effects of progesterone and 17 β-estradiol on expression of Schwann cell markers in ADSCs. According to the results of present study, treatment of ADSCs with progesterone or 17 β-estradiol could induce the expression of Schwann cell markers.

Adipose tissue contains a high number of multipotent MSCs which are able to differentiate into different cells. The ADSCs are useful for a range of therapeutic applications such as treatment of peripheral nerve injuries.[25-27] Several experiments have indicated that

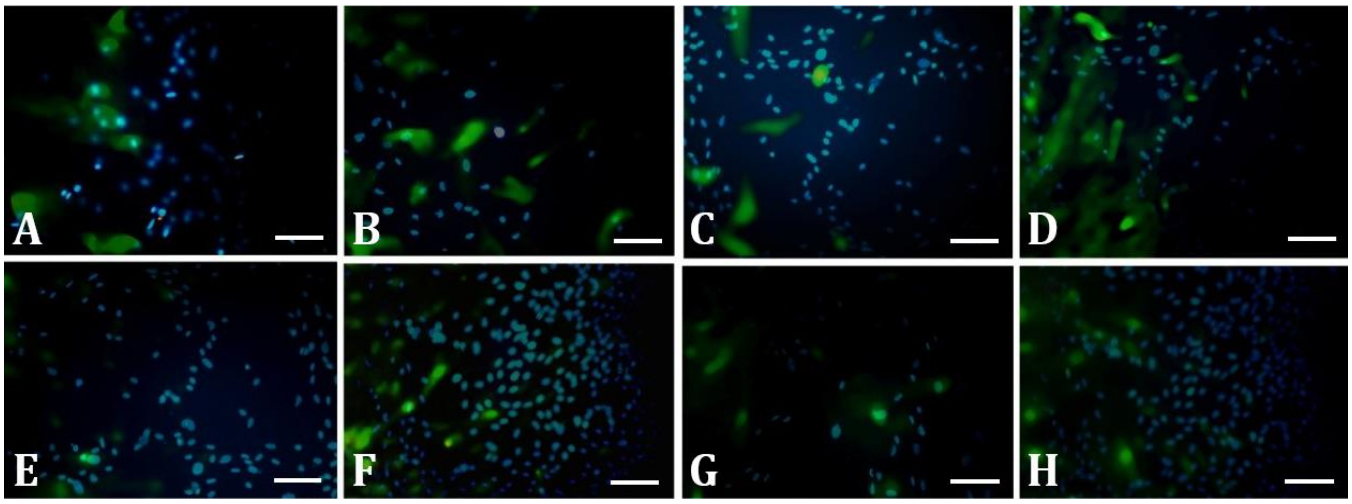

Fig. 4. A - D: Anti-GFAP immunostaining in different groups; E- H: Anti-S100 immunostaining in different groups. A and E: Group 2 (bFGF + PDGF + Heregulin + Progesterone); B and F: Group 3 (bFGF + PDGF + Forskoin + Progesterone); C and G: Group 4 (bFGF + PDGF + Heregulin + Estradiol); D and H: Group 5 (bFGF + PDGF + Forskoin + Estradiol), Bar = 200 μm.

ADSCs can be differentiated into a Schwann cell phenotype as well.[28-30] Dezawa *et al*. differentiated bone marrow separated MSCs into myelinating cells with the ability to induce nerve regeneration.[31] This method is based on the effect of a variety of inducers that used one by one.[32] Our experiment showed that the sequential administration of various factors β-mercaptoethanol and retinoic acid, followed by a mixture of bFGF, PDGF, forskolin and hereguiln is more efficient than other inducer mixtures and confirmed Dezawa method. The combination of β-mercaptoethanol and retinoic acid in culture media is an appropriate pre-inducer[21] and a mixture of bFGF, PDGF, forskolin and hereguiln has a synergistic effect on Schwann cell differentiation.[31] In Schwann cell development process, forskolin can increase the level of intracellular cyclic adenosine monophosphate (cAMP) causing cells response to the trophic factors leading to myelin marker of P0 mRNA expression.[33,34] Hereguiln, a subtype of neuregulin, is one of the crucial axon-derived signals influencing Schwann cells development.[35]

Neurosteriods involve in the regulation of Schwann cells proliferation and their cellular products.[36-38] Progestins have the ability to activate heregulin receptors of ErbB2 and ErbB3.[39] In the present study, forskolin and heregulin were replaced by progesterone and 17 β-estradiol. Among the various Schwann markers, we particularly focused on P0, S-100, and GFAP. In our experiment, P0, a representation marker of myelinating cells, was expressed in ADSCs in the presence of progesterone. Melcangi *et al*. have showed that the expression of P0 is under the control of progesterone receptor and progesterone increases its expression in rat Schwann cell culture.[40,41] Our data support those of Roglio *et al*. that suggesting that progesterone may be necessary for inducing P0 synthesis.[16]

Due to estrogen function in tissue and organ development through regulating cell proliferation and differentiation, we hypothesized that 17 β-estradiol induces expression of Schwann cell markers in ADSCs. Based on our results, S-100, P0, and GFAP were expressed significantly in 17 β-estradiol groups (the fourth and fifth groups). The MSCs are anabolic targets of estrogen action, via estrogen receptor α activation.[42] 17 β-estradiol induces an earlier onset of axonal regeneration and myelination.[43] The estradiol alone had no effect on cell multiplication, but in the presence of forskolin it became a potent mitogen.[36] In the present study, in the fifth group, 17 β-estradiol in the presence of forskolin could induce a significantly higher expression of P0 mRNA compared to the forth group. It seems that estradiol becomes an inducer for ADSCs when levels of cAMP are elevated.[44] However, in our study, 17 β-estradiol was a weaker inducer compared to progesterone for expression of Schwann cell markers in ADSCs. Higher expression of GFAP protein positive cells in the presence of 17 β-estradiol and heregulin than combination of 17 β-estradiol and forskolin is evidence for the role of heregulin in induction of ADSCs. Dezawa *et al*. believed that no growth factors can be a substitute for heregulin in Schwann cell differentiation.[31]

Our study showed that progesterone has an inducing effect similar to that of heregulin and forskolin in expression of Schwann cell markers. This is consistent with the study of Movaghar *et al*. that showing that progesterone could induce the transdifferentiation of bone marrow stem cells (BMSCs) into Schwann cell phenotype.[13] It seems that the combinations of heregulin and forskolin,[31] forskolin and progesterone[13] or heregulin and progesterone (present study) have cumulative effects in the differentiation of MSCs into Schwann-like cell phenotype. However, in contrast to Movaghar *et al*., the strongest expression of Schwann cell markers was not

seen in groups treated with progesterone, but it was seen in group 1 treated with heregulin and forskolin. Owing to the differences in the immunophenotype, transcriptome, proteome, and immunomodulatory activity, BMSCs and ADSCs have diverse potential in differentiation into variety of cells.[6,45,46] Although it has been shown that heregulin is able to induce progesterone receptor activation[47] it seems that the combination of heregulin and progesterone induces BMSCs and ADSCs through different pathways.

In conclusion, we induced the rat ADSCs by progesterone and 17 β-estradiol *in vitro* and demonstrated the expression of Schwann cell markers. There are still some limitations in identification of the Schwann cell markers, hence further studies on the induction mechanism of progesterone and estradiol and differentiation of ADSCs into Schwann-like cell phenotype are recommended.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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