Stem Cell Reviews and Reports
https://doi.org/10.1007/s12015-019-09892-5

Effects of VEGF^{+} Mesenchymal Stem Cells and Platelet-Rich Plasma on Inbred Rat Ovarian Functions in Cyclophosphamide-Induced Premature Ovarian Insufficiency Model

Birol Vural[1] · Gokhan Duruksu[2,3] · Fisun Vural[4] · Merve Gorguc[2] · Erdal Karaoz[5]

Abstract
Premature ovarian insufficiency (POI), a fertility disorder affecting women under 40 years of age, is characterized by early loss of ovarian function. This study was aimed to maintain ovarian function in POI animal models by mesenchymal stem cells (MSCs) transplantation with/without the supplementation of platelet-rich plasma (PRP). Adipose tissue-derived MSCs were isolated from inbred rats (Fisher-344), and constitutive expression of both VEGF and GFP were maintained by transfection with plasmids, pVEGF and pGFP-N. PRP was derived from the blood of healthy untreated rats. A total of 60 rats were divided into 5 groups of 12 rats in each. First group was kept as untreated-control (Control), and POI model was induced in Fisher-344 rats by cyclophosphamide in the next four groups. Second group was kept as sham-operated-control (Sham). MSC, PRP and MSC+ PRP-treated groups were transplanted following the validation of POI model in rats. After 2 months following the transplantation, anti-mullerian-hormone (AMH) and oestradiol (E_2) blood levels were measured. Follicles were evaluated after hematoxylin-eosin staining, and the immunofluorescence staining and gene expression analyses were performed to show the ovarian regeneration. The follicular count was improved after MSC- and MSC + PRP-treatment to 63% of Control-group and significantly higher than that in Sham-group, but a significant increase was not observed in PRP-group. Higher AMH and E_2 levels were measured in MSC + PRP than in Sham-group, and CXCL12, BMP-4, TGF-β and IGF-1 expressions were also increased. This study showed MSCs +/-PRP transplantation after POI supports recovery of the follicular count and function. For ovarian recovery, a single administration of PRP was found not sufficient. Although MSC treatment increased follicular regeneration, better results were obtained in the co-transplantation of MSCs and PRP. These results might be promising for follicular regeneration in POI patients.

Keywords Cyclophosphamide · Granulosa cells · Mesenchymal stem cells · Oocyte · Platelet-rich plasma · Premature ovarian insufficiency · Theca cells · Vascular endothelial growth factor

✉ Birol Vural
vuralbirol@yahoo.com.tr; birol.vural@kocaeli.edu.tr

Gokhan Duruksu
gokhan.duruksu@kocaeli.edu.tr

Merve Gorguc
merve.gorguc@tubitak.gov.tr

Erdal Karaoz
ekaraoz@istinye.edu.tr

[1] School of Medicine, Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility and Assisted Reproductive Technology (ART), Kocaeli University, Unit. Izmit, 41380 Kocaeli, Turkey

[2] Graduate School of Health Sciences, Department of Stem Cell, Center for Stem Cells and Gene Therapies Research and Practice, Kocaeli University, Izmit, 41380 Kocaeli, Turkey

[3] Center for Stem Cells and Gene Therapies Research and Practice, Kocaeli University, Izmit, 41380 Kocaeli, Turkey

[4] Department of Obstetrics and Gynecology, University of Health Sciences- Haydarpasa Numune Training and Research Hospital, 34668 Istanbul, Turkey

[5] Department of Histology and Embryology, Istinye University, 34460 Istanbul, Turkey

Published online: 29 April 2019

Abbreviations

AMH	Anti-mullerian-hormone
AT-MSCs	Adipose tissue-derived mesenchymal stem cells
bFGF	Basic fibroblast growth factor
BMP-4	Bone morphogenetic protein 4
CNTF	Ciliary neurotropic factor
CXCL12	C-X-C motif chemokine ligand 12
CYP19A	Cytochrome P450 aromatase
R	Pearson product-moment correlation coefficient
E_2	Oestradiol
FBS	Foetal bovine serum
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF9	Growth Differentiation factor 9
GFP	Green fluorescent protein
GV	Germinal vesicle
H&E	Hematoxylin and Eosin
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL1b	Interleukin 1b
KGF	Keratinocyte growth factor
KL	Kit-ligand
LIF	Leukemia inhibitory factor
MSCs	Mesenchymal stem cells
OSCs	Oogonial stem cells
p	Probability
PCNA	Proliferating cell nuclear antigen
POI	Premature ovarian insufficiency
PRP	Platelet-rich plasma
TGF-β	Transforming growth factor beta 1
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
VSEL	Very small embryonic-like stem cells

Introduction

Premature ovarian insufficiency causes infertility in 1–2% of women younger than 40 years of age. The majority of cases are idiopathic [1, 2], but in some cases, the follicular pool and oocytes are affected by mitochondrial dysfunction, chemotherapeutic treatments and genetic, endocrine, autoimmune, inflammatory, psychological, paracrine or metabolic factors [3, 4]. Despite the relatively high prevalence of POI, there are no proven treatment methods; therefore, we investigated the healing power of mesenchymal stem cells and PRP.

Today, 5% of cancer patients are under 50 years old and the female survivors of heavy treatment protocols will likely suffer from ovarian infertility problems. The degree of ovarian damage and risk of infertility depends on the dose and type of chemotherapeutic agent and is related to the woman's age at the time of treatment [5]. One of the most commonly used chemotherapeutic treatments for malignancies, the alkylating agent, cyclophosphamide, has a high risk of ovarian failure. The active metabolites form cross-links with DNA, resulting in inhibition of DNA synthesis and function; the DNA double-strand breaks resulting in p63-mediated apoptotic death in human primordial follicles are the proposed mechanisms of alkylating agents [6]. Ovarian pathological examination usually reveals diminishing primordial follicle oocyte and granulosa cells, ovarian blood vasculature damage and ovarian atrophy in these patients [7, 8]. To offset these risks, women can be offered several options for fertility preservation, including conservative cancer management or cryopreservation of oocytes, embryos or ovarian tissue [5].

Mesenchymal stem cells are one of the cell sources for using the repair and regeneration of damaged tissues for regenerative medicine and the treatment of many serious diseases, such as degenerative and inflammatory diseases. Mesenchymal stem cells, which have come to the forefront of research due to their differentiation characteristics, are characterized with respect to differentiation potency [9]. Although MSCs are involved in the transformation of mesenchymal tissues such as adipogenic, osteogenic, and chondrogenic cell lineages, they also have the potential to differentiate into cells of other germ layer tissues, such as neuronal tissue cells. However, the mechanism of differentiation into functional germ cells is not clear [10, 11]. Mesenchymal stem cells differ in their ability to differentiate and regulate tissue functions in the presence of cytokines and growth factors [12]. The protein matrix structure of the damaged tissue can be reorganized by secretomes of MSCs, and apoptosis can be repressed by MSCs. Meanwhile, immunomodulatory activities in the tissue are regulated by MSCs [13]. PRP treatments are administered with different clinical indications in regenerative medicine. PRP exerts its effects by releasing growth factors and cytokines, through which angiogenesis and stem cell stimulation are achieved [14]. It has been shown that ovarian function improved by the administration of PRP into the ovaries of the patients with diminished ovarian reserve [15].

MSCs derived from human placenta [16, 17], bone marrow [18–20] and menstrual blood [21] have been used in experimental POI therapy trials. Alternatively, Sun et al., (2013) and Takehara et al. (2013) used MSCs obtained from adipose tissue in POI animal models [22, 23]. The success of adipose tissue-derived MSCs in initiating folliculogenesis has been shown in ovarian stroma and granulosa cells, but these results have not yet been reflected in human clinical trials. In this model, we used $VEGF^+$ AT-MSCs and/or PRP treatments to reactivate ovarian function and follicle production by using stem cell-related and other regenerative mechanisms. Along with the effects of AT-MSCs effects, the expression of VEGF in AT-MSCs was expected to stimulate tissue cell mitosis, angiogenesis, and regenerative processes. On the other hand, PRP was thought to support the tissue with a number of bioactive molecules, such as growth factors and cytokines, which modulate inflammatory reactions, angiogenesis, and stem cell activation.

Materials and Methods

Ethical Approval and Animals

The use of animals and animal protocols were approved by the local animal care committee according to the institutional guidelines and national animal welfare with the principles of the Declaration of Helsinki (Approval No. KOU-HADYEK 3/2–2014). Female Fisher F344 inbreed rats (6 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany; exported by Kobay AS, Ankara, Turkey) and housed in the Experimental Animal Care Center at Kocaeli University. Firstly, the POI model was optimized by cyclophosphamide dose and duration. Then, the animals were categorized into five groups as untreated control (1), sham operated control (2), treatment with AT-MSCs (3), PRP (4) and AT-MSCs+PRP (5).

Optimization of Cyclophosphamide Dose

The animals were injected with 100 or 200 mg/kg cyclophosphamide (Endoxan, Eczacibasi-Baxter, Istanbul, Turkey) intraperitoneally to develop the POI model. The animals were injected with either 200 mg cyclophosphamide/kg ($n = 48$) or 100 mg cyclophosphamide/kg (n = 48). The control group, animals were kept under the same conditions without receiving an injection ($n = 12$). The animals were housed under aseptic conditions for 30 days and the efficiency of the cyclophosphamide treatment doses on ovarian function was evaluated. Blood obtained from the tail of the rats was used for the AMH and E_2 analyses. The ovarian tissue sections were first analyzed with H&E stain for the folliculogenesis and follicle counts. The functionality and proliferating potential of ovarian sections were evaluated by immunostaining against PCNA.

Isolation of AT-MSCs

Fifteen rats were used for the isolation of AT-MSCs and PRP. Female F344 rats were anaesthetized by injecting 10 mg/kg of xylazine and 80 mg/kg of ketamine. A total of 1–2 cm^3 of clean peritoneal adipose tissue was removed. The isolation procedure used was described previously [24]. The cells were cultured in DMEM: F12 culture medium (Gibco, Paisley, UK) supplemented with 10% FBS (Gibco) and 1% Pen-Strep (Gibco). Cells were detached from tissue culture flasks with 0.25% (*w*/*v*) trypsin-EDTA (Gibco) and washed with PBS (Gibco). The characterization of the isolated stem cells was performed by flow cytometry, and differentiation studies were performed as previously defined [25]. For the adipogenic and osteogenic differentiation, the previously published protocols were used [24]. Alizarin Red Staining (red) was performed for the osteogenic differentiation, and Oil Red O staining for the adipogenic differentiation. For the chondrogenic differentiation, cells were centrifuged at 1300 g for 5 min to form pellet micromass. Then, the pellet was cultured with chondrogenic medium for 2 weeks. Medium was refreshed twice a week. Chondrogenic medium consists of high-glucose DMEM (Gibco) supplemented with 10 ng/ml transforming growth factor-β1 (TGF-β1; Biosource), 50 μg/ml ascorbate-2-phosphate (Wako Chemicals, Richmond, VA, USA), 0.1 μM dexamethazone (Sigma-Aldich, St. Louis, MO, USA), 100 μg/ml sodium pyruvate (Sigma-Aldich), 40 μg/ml proline (Merck, Darmstadt, Germany), 50 mg/ml ITS premix (BD Biosciences, Bedford, MA, USA), 1% antibiotic/antimycotic (Gibco). The chondrogenic differentiation was shown by Alcian Blue staining according to the manufacturer's protocol (Alcian Blue Stain Kit, Atom Scientific, Manchester, UK).

VEGF Gene Transfer into AT-MSCs

The MSCs were transfected with the plasmids, pVEGF and pGFP-N (Clontech, Palo Alto, CA) by electroporation (Neon Transfection System, Invitrogen, Carlsbad, CA) as described previously [24]. The transformed cells were selected with G418 (200 μg/ml) under the standard culture conditions. The co-expression of VEGF and GFP genes in MSCs has been achieved by the same method in the early studies by our group [26–28].

PRP Isolation from Blood Samples

PRP was obtained from the blood of female F344 inbreed rats by the two-step centrifugation method [29]. The blood samples were collected in a sodium citrate buffer (0.1 M) containing vacuum tube (BD Vacutainer; BD, Plymouth, UK). The blood cell components were removed from the medium by centrifugation at 160 g for 20 min at room temperature. The upper fraction was transferred into a new tube, where it was centrifuged at 400 g for 15 min at 4 °C to separate PRP from the serum component. The PRP was activated by calcium chloride prior to the injection. The final fraction contained 6.2×10^6 platelets/ml.

Transplantation of MSCs and PRP

The animals were divided into five groups to analyse the effect of $VEGF^+$AT-MSCs and PRP:

1. Control group ($n = 12$): the normal group without POI (untreated),
2. Sham group (n = 12): POI model rats without treatment (sham-operated),
3. PRP group (n = 12): POI model with PRP treatment,
4. MSCs group (n = 12): POI model with $VEGF^+$AT-MSCs treatment,

5. PRP + MSCs group (n = 12): POI model with both PRP and VEGF$^+$AT-MSCs treatments.

After validation of the POI model in experimental rats, VEGF$^+$AT-MSCs and/or PRP were transplanted to the subcortical region of rat ovaries. After the culture of VEGF$^+$AT-MSCs in serum-free medium for 24 h, the cells were washed twice with PBS. Each ovary in the MSCs group was treated with 1×10^6 cells/ 30 μl physiological serum. Both ovaries in the same animal received an equal number of VEGF$^+$AT-MSCs. PRP injections were prepared to contain 6.2×10^5 particles/ 30 μl physiological serum. In MSC + PRP group, VEGF$^+$AT-MSCs (1×10^6 cells) and PRP (6.2×10^5 particles) were mixed in 30 μl physiological serum and injected into the subcortical region of each ovary. The animals in the Sham group were treated by the same method but no MSC or PRP was added into the physiological serum in this case.

All laboratory assessments were performed two months after transplantation. After 8 weeks, rats were sacrificed, ovaries were removed from the animals, and intracardiac blood was collected under anaesthesia.

Enzyme-Linked Immunosorbent Assay

The blood AMH and E_2 levels were studied by ELISA according to the manufacturer's instructions (CUSABIO, Wuhan Huamei Biotech, Wuhan, PRC). Briefly, a 50 μL sample was added per well in duplicate. After addition of the antibody provided within the kit, the plate was incubated at 37 °C for 1 h. The 100 μL substrate solutions were added to each well and the plates were incubated for 15 min at 37 °C. The reaction was stopped and the absorbance was measured at 450 nm.

H&E and Immunofluorescence-Histochemical Staining

The ovarian samples were removed and embedded in paraffin, and sections were taken according to the previous method [30]. First, the tissue sections were analysed by H&E for folliculogenesis and follicle count and immunostaining against PCNA (Santa Cruz;sc-56) under the microscope for the study to optimize the Cyclophosphamide dose [28]. After two months of treatment, the ovarian tissue sections of the groups were analysed by H&E for folliculogenesis and follicle count. After excision, ovaries were fixed in 10% buffered-formalin for at least12 h, embedded in paraffin block. Serial sections at a thickness of 5 mm were taken. Following H&E staining, ovarian follicles were counted using the fractionator and nucleator principles [31, 32]. On every fifth section, the follicles with a visible nucleus were counted, and the follicle numbers was multiplied by 5 to estimate the total number of follicles per ovary [31]. The follicles were classified according to Pederson's system [33]. According to this classification, oocytes surrounded by a single, flat layer of granulose cell were named as primordial follicles; Pederson class 3 follicles were named as primary follicles; Pederson class 4–5 follicles were named as secondary follicles; Pederson class 6 follicles were named as pre-antral follicles and Pederson 7–8 follicles were named as antral follicles [31].

The immunostaining procedure was performed as described previously [28]. The antibodies used in the analysis were transforming growth factor β (TGF-β) (Santa Cruz; sc-146), GFP (Santa Cruz; sc-9996) and VEGF (Santa Cruz; sc-507). Counting of cells was performed by double-blind counting method by two independent researchers (G.D., M.G.). The staining was evaluated by scoring the ratio of positive (stained) cells from 1 to 6 (1 = 0–4%; 2 = 5–19%; 3 = 20–39%; 4 = 40–59%; 5 = 60–79%; 6 = 80–100%) [34].

Gene Expression Analysis

The expression analysis of AMH, GFP, CYP19A (Cytochrome P450 aromatase), CXCL12 (C-X-C motif chemokine ligand-12), BMP-4 (bone morphogenetic protein-4), TGF-β, IGF-1 (insulin-like growth factor-1), TRAIL (TNF-related apoptosis-inducing ligand), IL1b (interleukin-1b), IL-10 (interleukin-10), CNTF (ciliary neurotropic factor) and VEGF was performed with the Power SYBR Green Master Mix (Thermo, Applied Biosystems, Warrington, UK) by using a LightCycler 480-II (Roche Diagnostic, Basel, Switzerland). After excision, the ovarian tissue samples were divided into two parts. One part was saved for the Western blot analysis for the protein analysis and the second part was used for the gene expression analysis, which was described previously [30]. Shortly, homogenization of the excised tissue was performed with a rotor blade homogenizer, and total RNA was purified using the GeneJET RNA purification kit (ThermoScientific, Waltham, MA, USA) following manufacturer's instructions exactly. cDNA was derived by Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) following manufacturer's instructions. The amplification of target gene was performed by Power SYBR Green Master Mix (Thermo, Applied Biosystems Life Technologies, Carlsbad, CA, USA). The primer sequences are indicated in Table 1. G3PDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as the reference gene in the calculations. Cp values were calculated by LightCycler 480 Software (release 1.5), and the relative expression levels were normalized with the ActB Cp value derived for each sample.

Western Blot Analysis

The protein level of BMP-4, TGFβ and IGF-1, which are are key regulatory proteins in folliculogenesis secreted by granulosa and other somatic cells, were analyzed by Western blot. After excision, the ovarian tissue samples were washed with PBS and then protein was isolated from cells using M-PER

Table 1 Primers used in Real-Time PCR analysis

	Forward (5′-3′)	Reverse (5′-3′)
AMH	cacacctctctctgctgctg	gactcttggacagcctccag
BMP4	tcgttacctcaagggagtgg	agtccacgtagagcgaatgg
CNTF	caccccaactgaaggtgact	accttcaagccccatagctt
CXCL12	tgtgcattgacccgaaatta	ctttgtgctggcaaatctca
CYP19A	cagagtatccggaggtggaa	catgaccaagtccacgacag
GFP	cttgttgaattagatggtgatg	ctgttacaaactcaagaaggacc
IGF-1	cagttcgtgtgtggaccaag	tcagcggagcacagtacatc
IL-10	cctgctcttactggctggag	tgtccagctggtccttcttt
IL1b	aggcttccttgtgcaagtgt	tgagtgacactgccttcctg
TGFβ	ctccacatatgccagtggtg	ctaaagcaataggcggcatc
TRAIL	atggctatgatggacgtcca	ttagccaactaaaaaggcccc
VEGF	cacataggagagatgagcttc	ccgcctcggcttgtcacat
G3PDH	agagagaggccctcagttgct	tggaattgtgagggagatgct

Mammalian Protein Extraction Buffer (Thermo Scientific). For Western Blotting 10 μg of protein sample was mixed with Bolt LDS Sample Buffer (dye solution) and Bolt Sample Reducing Agent (Thermo Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. The protein mix was centrifuged and denatured at 70 °C for 10 min. Then, the mixture was loaded on ready-to-use Bolt 4–12% Bis-Tris Plus mini-gels (Thermo Scientific), and run at constant 200 V for 30 min. iBlot dry blotting system was used to blot the proteins on the nitrocellulose membrane. iBind western-processing device (Thermo Scientific) was used to stain the membrane. After incubation of membrane with primary and secondary antibodies, the detection was performed with LumiGLO Reagent (Cell Signaling Technology, Danvers, MA, USA) by MF-ChemiBIS3.2 (DNR Bioimagining Systems, Jerusalem, Israel), according to the protocol's instruction. The primary antibodies used in the analysis were supplied from Santa Cruz Biotechnology (Heidelberg, Germany): BMP4 (Santa Cruz; sc-137,087), TGF-β (Santa Cruz; sc-130,348), GFP (Santa Cruz; sc-9996), IGF-1 (Santa Cruz; sc-518,040), VEGF (Santa Cruz; sc-507) and ActB (Santa Cruz; sc-8432). Anti-GFP antibody was utilized for the transgene expression of GFP in the MSC implanted tissues. Anti-VEGF antibody was used for the detection of both transgenic and endogenous VEGF protein expressions in the ovarian tissues.

Statistical Analyses

All experiments were repeated a minimum of three times. Data are reported as the mean ± SD. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). In addition to descriptive statistics, the Wilcoxon rank-sum test was used to analyse the significance differences between the two groups. A p value of less than 0.05 was accepted as statistically significant. The Pearson linear correlation coefficient (R) has been used to compare the gene expression data sets to assess the similarities. An R-value closer to 1 indicates a stronger positive relationship, and a zero value indicates no relationship at all.

Results

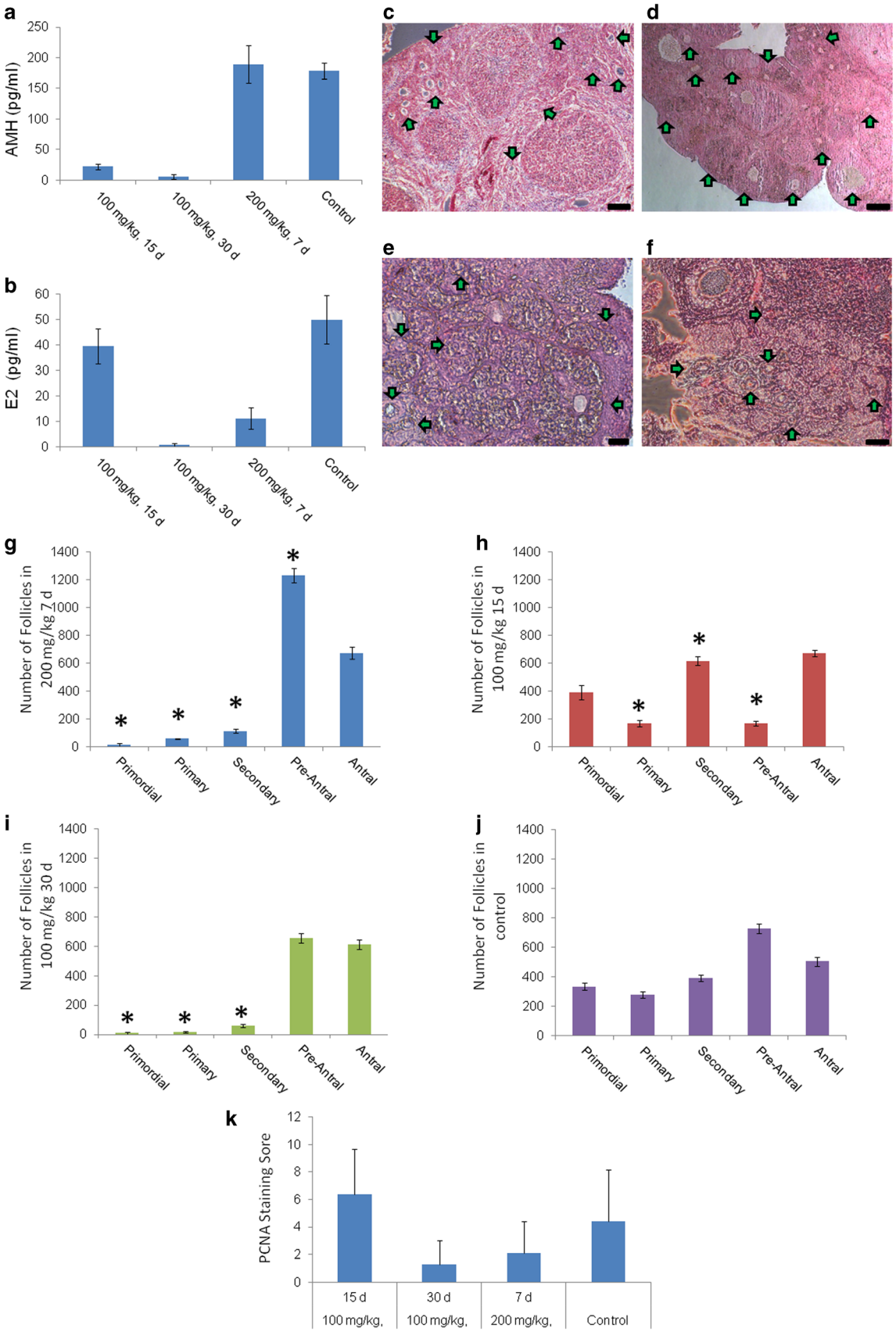
Optimization of Cyclophosphamide Dose

The cyclophosphamide dose of 200 mg/kg decreased the E_2 level in the blood rapidly within 7 days, while the AMH level was still preserved (Fig. 1). In the days following the experiment, all animals ($n = 48$) were lost within the first week. The 100 mg/kg dose was not as lethal, and the dose-effect could be observed after 15 and 30 days. After 15 days, E_2 and AMH levels were calculated at 75.9% and 12.4% of the control, respectively. The 100 mg/kg dose significantly decreased the AMH and E_2 levels within 30 days with a total loss of 2 out of 48 animals. The experimental model was optimized at the 100 mg/kg cyclophosphamide dose after 30 days: E_2 and AMH levels were estimated as 1.19% and 3.21%, respectively, compared to those in the control group (Fig. 1a, b).

The ovarian sections stained by H&E were analysed for follicles. The majority of the follicles, including the primordial and primary follicles, were degenerated (Fig. 1c-f). The total follicle numbers were estimated to be 2072, 2016 and 1343 for the applications of 200 mg/kg (7 days), 100 mg/kg (15 days) and 100 mg/kg (30 days), respectively. These numbers correspond to 92.5%, 90% and 59.9% of the number of follicles in the control groups (Fig. 1g-j). The functionality and proliferation potential of ovarian sections was evaluated by immunostaining against PCNA. The number of proliferating ($PCNA^+$) cells was reduced in the 200 mg/kg (7 days) and 100 mg/kg (30 days) groups, but the period of 15 days was found to be insufficient to cause a significant effect (Fig. 1k). Therefore, the most effective dose and duration were found at 100 mg/kg for 30 days.

Characterization of AT-MSCs and Constitutive VEGF Expression

Cells were isolated and cultured for three passages. The cells were characterized by flow cytometry (Fig. 2a) and were differentiated into osteogenic (Fig. 2b,c), adipogenic (Fig. 2d,e) and chondrogenic (Fig. 2f,g) cell lines. The cells showed typical mesenchymal stem cell characteristics and differentiated into the three mesodermal cell lineages. The cells were then transfected with VEGF and GFP, and their stable co-expressions were maintained. Following selection, constitutive VEGF


a
AMH (pg/ml)
250
200
150
100
50
0
100 mg/kg, 15 d
100 mg/kg, 30 d
200 mg/kg, 7 d
Control
b
E2 (pg/ml)
60
50
40
30
20
10
0
100 mg/kg, 15 d
100 mg/kg, 30 d
200 mg/kg, 7 d
Control
c
d
e
f
g
Number of Follicles in 200 mg/kg 7 d
1400
1200
1000
800
600
400
200
0
*
Primordial
Primary
Secondary
Pre-Antral
Antral
h
Number of Follicles in 100 mg/kg 15 d
1400
1200
1000
800
600
400
200
0
*
Primordial
Primary
Secondary
Pre-Antral
Antral
i
Number of Follicles in 100 mg/kg 30 d
1400
1200
1000
800
600
400
200
0
*
Primordial
Primary
Secondary
Pre-Antral
Antral
j
Number of Follicles in control
1400
1200
1000
800
600
400
200
0
Primordial
Primary
Secondary
Pre-Antral
Antral
k
PCNA Staining Sore
12
10
8
6
4
2
0
15 d
100 mg/kg,
30 d
100 mg/kg,
7 d
200 mg/kg,
Control

◀ **Fig. 1** Optimization of cyclophosphamide dose. The AMH and E_2 levels were estimated (**a**, **b**). H&E staining of follicles was performed for 200 mg/kg for 7 days (**c**), 100 mg/kg for 15 days (**d**), 100 mg/kg for 30 days (**e**), and control group (**f**). The follicle (green arrows) count is provided in (**g**-**j**). The PCNA staining and scoring are in (**k**). Scale bar, 200 μm for C&F, 500 μm for D and 50 μm for E

gene expression was achieved in the cells. The expression was demonstrated by immunostaining for GFP (Fig. 2h), which showed constitutive expression, and by immunostaining for VEGF (Fig. 2i), which indicated VEGF expression in the cells.

Evaluation of Ovaries after MSCs and PRP Treatment

Total follicular numbers after treatment: Two months following treatment with MSCs and/or PRP, the total follicle numbers in the MSC (1418 ± 459) and MSC + PRP (1419 ± 162) groups improved compared to those in the sham group (471 ± 177) ($p = 0.003$ and $p = 0.001$, respectively). However, the total number of recovered follicles from either treatment group was lower than that from the control group (2240 ± 150) (63% of control group) (Fig. 3a). In the PRP group, there was

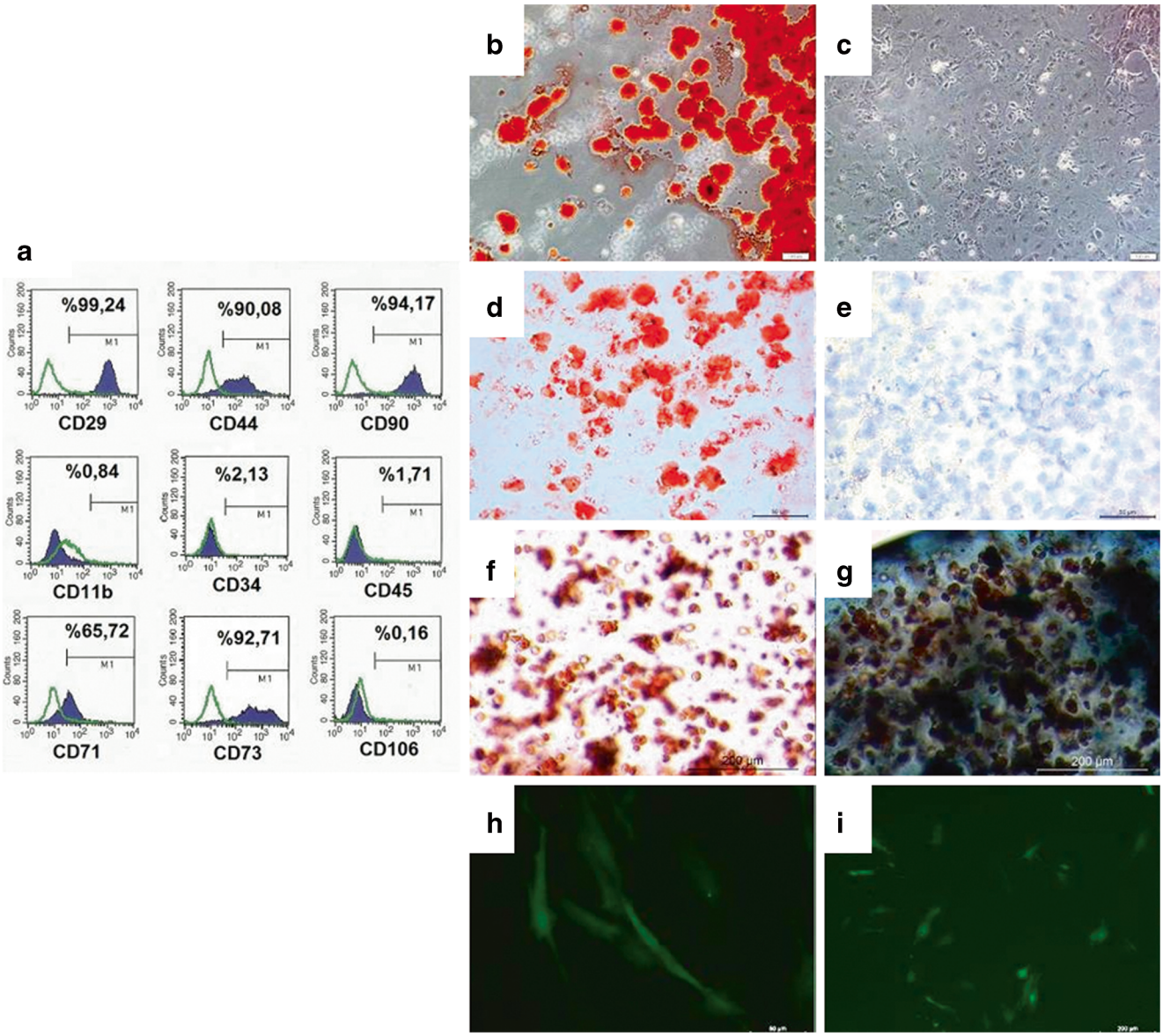


Fig. 2 Characterization of AT-MSCs by flow cytometry. The positive (CD29, CD44, CD90, CD71, CD73) and negative markers (CD11b, CD34, CD45) for MSCs are shown (**a**). The cells were differentiated into osteogenic (**b**), adipogenic (**d**) and chondrogenic (**g**) cell lines in comparison with the differentiation controls (**c**, **e**, **f**). Alizarin Red Staining (red) was performed for calcium deposits formed during the osteogenic differentiation (**b**, **c**), Oil Red O staining (red) to demonstrate the lipid accumulation in adipogenic differentiation (**d**, **e**), and glycoproteins formed during the chondrogenic differentiation by Alcian Blue staining (blue) (**f**, **g**). The expression of GFP (H) and VEGF (I) were demonstrated. Scale bar, 50 μm for B-E and H, and 200 μm for F,G and I

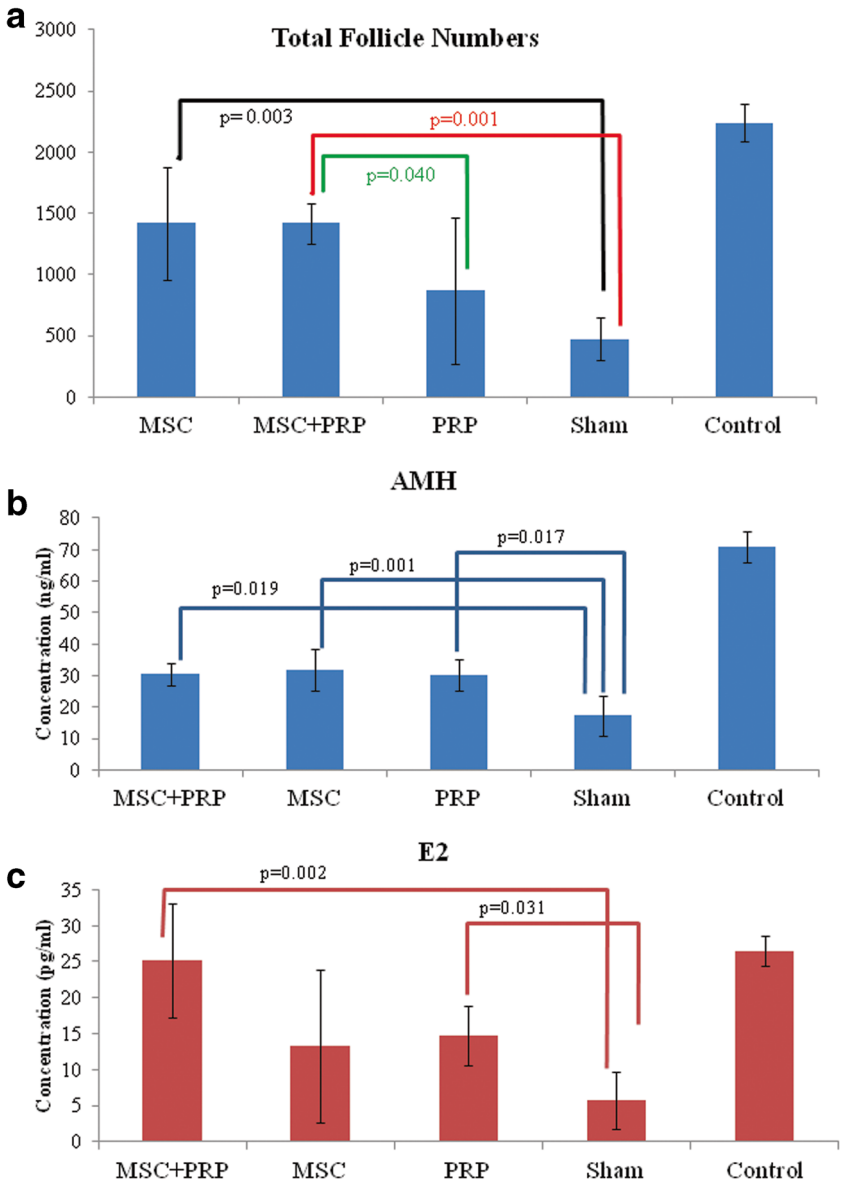


Fig. 3 Total follicular count (**a**) in ovaries and estimation of AMH (**b**) and E_2 (**c**) levels in the blood after 2 months of treatments with MSCs and PRP

no significant difference compared to the sham group. Remarkably, MSC and MSC + PRP application significantly improved follicle numbers (Fig. 3a).

AMH and E_2 Levels after Treatment and the Recovery of Folliculogenesis

MSCs, PRP and MSC + PRP- treated groups had significantly higher AMH levels than the sham group (Fig. 3b). E_2 levels were significantly higher in the MSC + PRP and PRP groups than in the sham group (Fig. 3c). MSC group had higher, but not significantly higher E_2 levels than the sham group. The addition of PRP to MSCs significantly increased E_2 levels.

H&E staining showed the atrophic tissue morphology in the ovaries of the sham group compared to that in ovaries of the control group, in which the regular folliculogenesis process was observed (Fig. 4a-f). Although the number of recovered follicles in the MSCs and MSC + PRP groups was limited compared to that in the control group, the distribution of the follicle types was similar across groups (Fig. 4g). The ratio of primordial follicles in MSC+/-PRP group was similar to that in the control group and higher than that in the sham group, but this difference did not reach statistical significance.

The Effect of MSCs and VEGF Gene Expression

VEGF and GFP co-expression between the MSCs and MSC + PRP groups was compared. The GFP^+ cells (i.e., MSCs groups) showed VEGF expression (Fig. 5b). But,

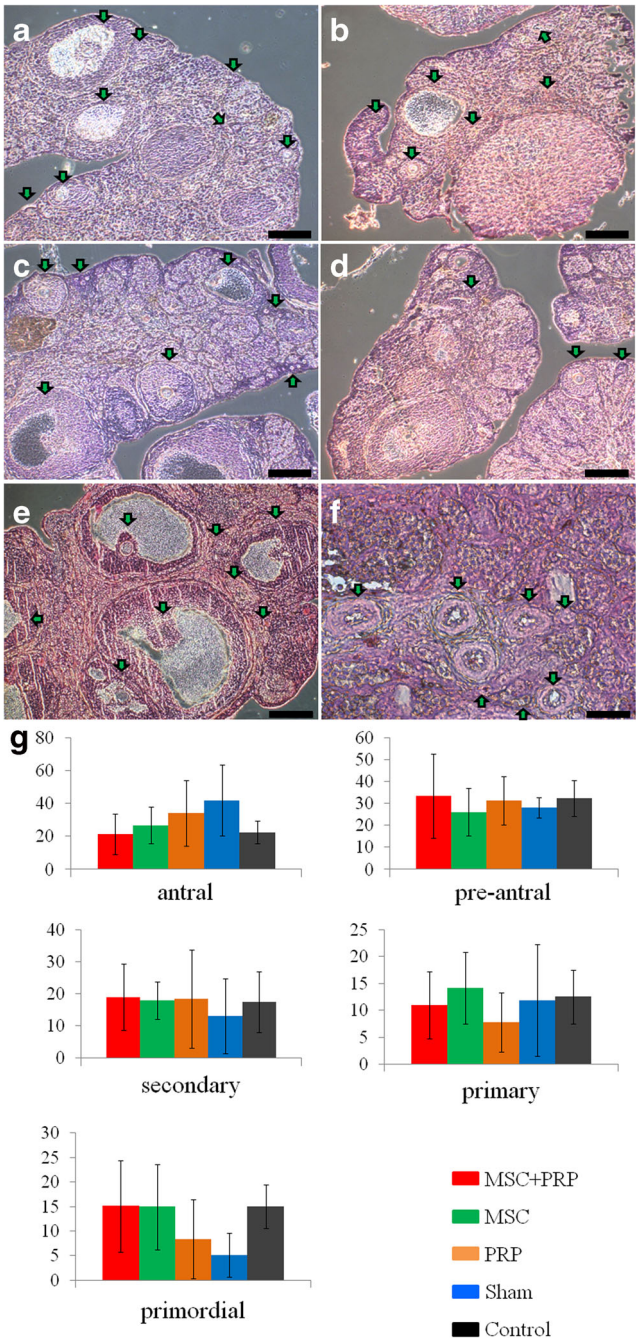


Fig. 4 The change in the number of follicle structures after the treatment with MSC + PRP (**a**), MSC (**b**), PRP (**c**) Sham (**d**), and control (**e**, **f**). The follicle structures (green arrows) are categorized and represented as a percentage (**g**). Scale bar, 200 μm for A-E and 50 μm for F

the transplantation of VEGF$^+$AT-MSCs did not change total VEGF (endogenous or recombinant VEGF) gene expression in the tissue (Fig. 5a). After transplantation, the increase in the expression of AMH (C = 0.61) and CYP19a (C = 0.69) genes paralleled the increase in GFP gene expression in the cells (Fig. 5d,f), but the correlation of VEGF to AMH (C = 0.12) and CYP19a (C = 0.48) gene expression was weak (Fig. 5c,e). The AMH-GFP and CYP19A-GFP expression levels showed high correlation factors (R = 0.89 and R = 0.69). However, the correlation factors for AMH-VEGF and CYP19A-VEGF were low, which indicates a weaker interaction of VEGF level and the regenerative effect observed in the tissue compared to the effect of GFP$^+$ stem cells.

The Expression of Factors Related to Apoptosis and Inflammation

CXCL12 gene, an anti-apoptosis marker, was highly expressed in the ovaries transplanted with MSC + PRP compared to the expression in the PRP group (Fig. 6a). TRAIL (apoptosis), IL1b (inflammation), CNTF (anti-inflammation) and VEGF (inflammation, angiogenesis) expressions were similar across groups (Fig. 6a, b).

The Expression of Bioactive Molecules Important for Regeneration

BMP4, IL-10, TGF-β, and IGF-1 gene expression was assessed. The IL-10 expression was similar among groups. BMP4 and TGF-β were significantly increased in the MSC + PRP group compared to that in the PRP group. IGF-1 expression in the MSC + PRP group was significantly higher than in the sham ($p = 0.014$) and MSCs ($p = 0.044$) groups (Fig. 6c). The alteration of BMP4, IGF-1, GFP, VEGF and TGF-β protein levels in tissue were confirmed by Western blot analysis (Fig. 6d). In the MSC + PRP group, the BMP4 and IGF-1 levels are the highest compared to other groups. The GFP protein can only be detected in the groups with AT-MSCs, and the IGF-1 protein level positively correlated with the presence of GFP in the tissue. BMP4 and TGFβ were also expressed endogenously, as it could be seen in Sham group, but highest expressions can be observed in MSC + PRP group. No significant difference in VEGF level can be detected in the groups.

The Interaction between AT-MSCs and the Mesenchymal Tissue in Ovaries

A significant increase in the intensity of GFP and TGF-β immunostaining was observed in the MSC + PRP group. The distribution of GFP$^+$ cells was mainly localized around the primordial, primary, secondary, pre-antral and antral follicles, and was indicative of mesenchymal stem cell activation in the tissue. GFP staining was observed in the theca and granulosa cells. The staining was not only observed in the external theca, but also in the internal theca cells and granulosa cells.

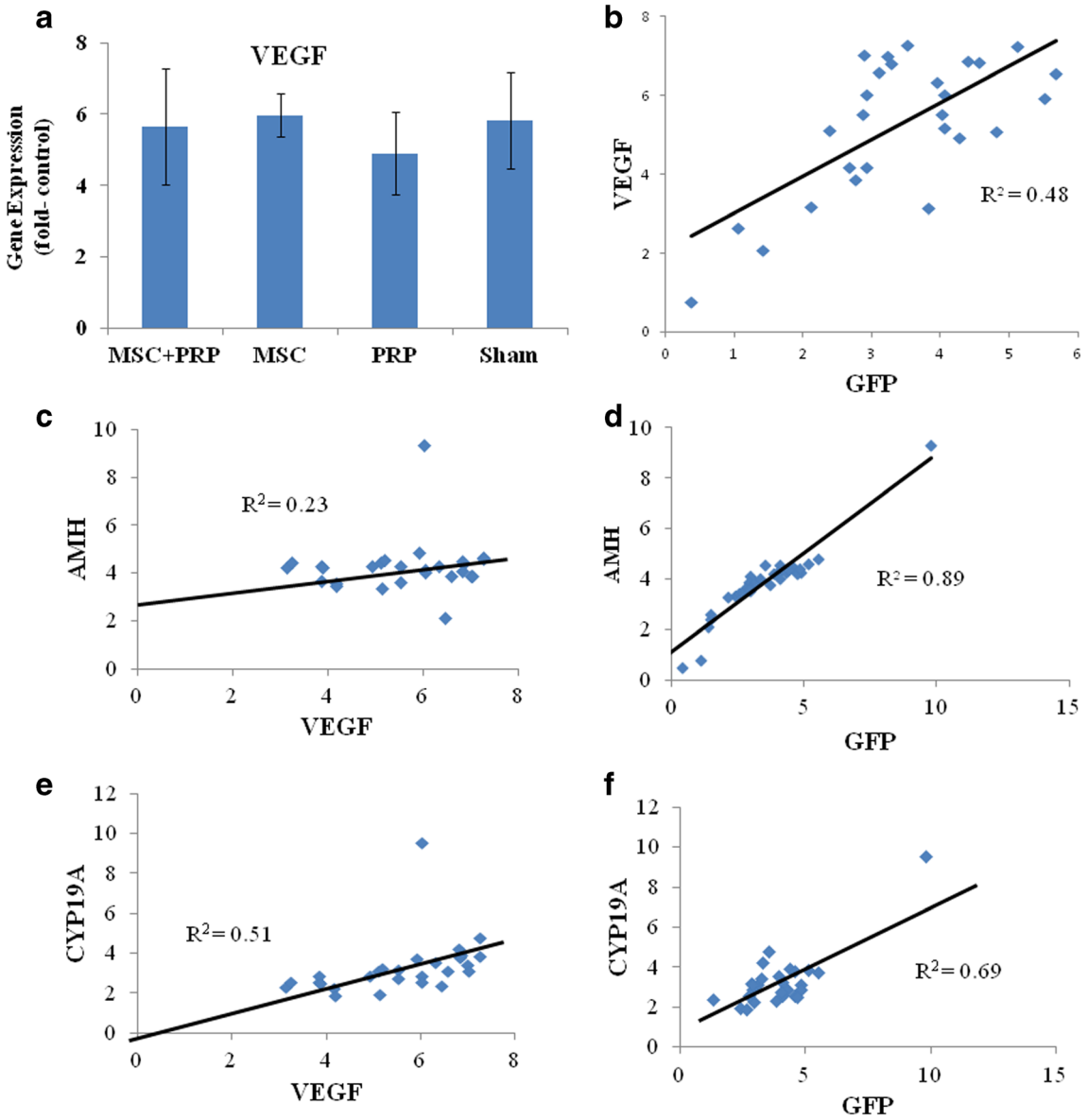


Fig. 5 The effect of VEGF expression in the regeneration of the folliculogenesis process. The gene expression analysis in the ovarian tissue shows that the induction of VEGF in the injury-site without MSCs treatment is the same as in the groups with MSCs treatments (**a**). The GFP$^+$ cells (i.e., MSCs groups) show VEGF expression (**b**), but the effect of VEGF expression on AMH and CYP19A (aromatase) expressions remains limited (**c**, **e**) compared to the effect of MSCs (GFP$^+$) on those parameters (**d**, **f**). The Pearson linear correlation coefficients (R) for the log ratio ($n = 38$) was calculated

GFP staining was not observed in the oocytes. Furthermore, the observed TGF-β staining, a bioactive molecule in this region, may be evidence of regeneration (Fig. 7). The surrounding area was infiltrated by GFP$^+$ MSCs, which also attenuated the expression of TGF-β in the tissue and leads to accelerate the regeneration process. The TGF-ß expression was significantly increased in the MSC + PRP group compared to the MSC, PRP and Sham groups (Figs. 6d and 7d) with the p value of 0.0046, 0.0004, and 0.011, respectively. The increase in the TGF-ß staining was also in parallel with the GFP$^+$ cells (Fig. 7e). The PRP application together with the MSCs supported the number of stem cells, which is shown by GFP staining (Fig. 7a-c).

Discussion

This study investigated the effect of MSCs, PRP, MSCs + PRP treatments on follicular count and function in a cyclophosphamide-induced POI rat model. MSC-treatment increased regeneration of follicles, and co-transplantation of MSCs and PRP further improved regeneration and functional

recovery. On the other hand, PRP-treatment alone and the addition of VEGF to MSCs added no further benefit.

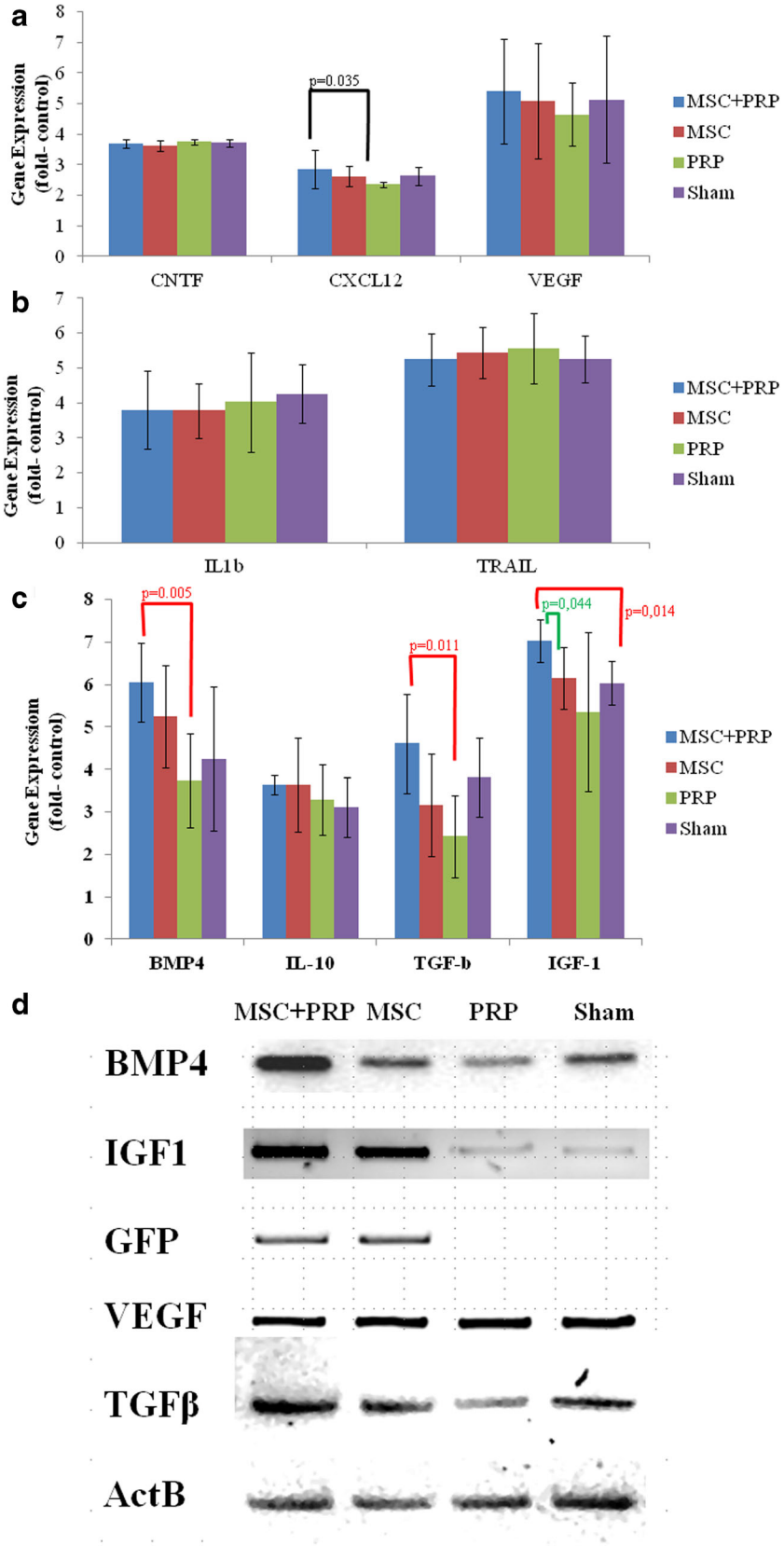
In the induction of POI, alkylating drugs, such as cyclophosphamide, can theoretically affect both resting cells, such as oocytes, and dividing cell and therefore, they are most potent at inducing ovarian failure [35]. In studies using a similar method, a single dose of 200 mg/kg –cyclophosphamide is frequently used [36, 37]. These studies proposed that 200 mg/kg cyclophosphamide is effective and non-lethal in Wistar albino rats, but the F344 rats were found to be highly susceptible to this dose according to our observation. The 200 mg/kg dose was quite effective at decreasing blood E_2 levels by 21.4% compared to that in control rats after 7 days of treatment. The AMH values decreased to 12.4% and 3.21% and E_2 values to 75.9% and 1.19% of the control group at 15 and 30 days after 100 mg/kg cyclophosphamide, respectively. Cyclophosphamide induces its effects by depleting the stock of mitotically active cells, but quiescent stem cells might survive. These quiescent stem cells may be the reason for the partial recovery of ovarian tissue in the sham group.

Although there are scientific studies that offer the opposite view about the existence of oogonial stem cells (OSCs) [38–40], the re-initiation of follicle generation might be associated with the differentiation of these stem cells in ovarian surface epithelium and/or bone marrow-derived stem cells as previously proposed [41–43]. The recovery of folliculogenesis without treatment might evidence of ovarian germ-line stem cells, which was first suggested by Johnson et al. (2004) and later Bukovsky et al. (2005) [43, 44]. According to the study by Bukovsky et al. (2011, 2015), dormant stem cells and stem cells located beneath the ovarian surface epithelium and respond to stimuli by promoting ovarian function and folliculogenesis [45, 46]. In 2014, mouse functional oocytes and offspring were generated in the rat by xeno-ectopic transplantation of primordial germ cells [47]. Although the results of this study showed that the fertility of the POI inbred rat model can be recovered without any treatment, MSCs +/-PRP application further improved folliculogenesis. Cyclophosphamide might not affect dormant stem cells, but theca, granulosa and other mesenchymal cells in the ovary are potential targets of this drug. The effects of cyclophosphamide administration may be associated with age-related dysfunction, particularly in the theca and granulosa cells [48]. We showed that MSCs supported the mesenchymal tissue in the ovary by promoting the viability and the functionality of stromal and theca/granulosa cells and subsequently oocyte formation from primordial germ cells or oogonial stem cells, thereby resulting in the development of functional follicles. For an optimum folliculogenesis process, the structural integrity of theca internal and theca external should be preserved and connect with granulosa cells [49, 50]. In this study, cyclophosphamide-induced POI was improved with MSCs +/-PRP treatments.

Interestingly, the increase in primordial follicle number in the MSC + PRP group might also indicate an increase in oocyte reserve. Although the total number of follicles in the ovary in this group was lower than in the control, the proportion of follicle types were the same. The total follicle numbers in MSC and MSC + PRP groups significantly increased compared to Sham group. The somatic cells supporting oocyte and oocytes coordinately control the development and maturation of the follicle as well as obtaining of a meiotically adequate oocyte [51]. This ovarian morphostasis has been suggested to be under the coordination of a tissue control systems consisting of immune system cells, vascular pericytes and autonomic innervations [52]. MSCs, including AT-MSCs, show immune modulation, and the probable effects of MSCs on the immune system are thought to be very important in ovarian regeneration control, which should be proven by other studies in the future [53].

AMH levels are related to follicular reserve, especially the reserve of primordial follicles and pre-antral follicles. The MSC-, PRP- and MSC + PRP-treated groups had significantly higher AMH levels than the sham group. Despite the reported transformation of primordial follicles into primary follicles by AMH, the increase in AMH also supports cell proliferation and folliculogenesis [54]. On the other hand, the increase in aromatase activity was paralleled by an increase in E_2 levels, indicating E_2 synthesis in granulosa cells, and E_2 levels were higher in the PRP + MSC group than in the sham and MSC only groups.

Granulosa cells were reported to involve in the development of ovarian somatic tissues during embryogenesis, or the ovarian surface epithelium [55]. Theca cells were thought to generate from the ovarian stromal cells in response to granulosa cells, or theca cell precursor in the ovarian stroma [49]. A significant increase in the intensity of GFP and TGF-β immunostaining was observed in the MSC + PRP groups. GFP staining is observed not only in the stroma-theca cells but also in the granulosa cells. The MSCs support the stroma and became a part of the tissue. The differentiation of MSCs to theca or granulosa cells is one possibility, and cell fusion is another. In some cases, GFP expression might continue in the cells as a marker even after the cells were separated [56]. GFP^+ oocytes were not observed in any of the MSC-treated groups; therefore, the trans-differentiation of AT- MSCs into germ line cells was not considered in this study. After MSC transplantation, the increase in the expression of AMH and CYP19a genes paralleled the change in GFP gene expression in the cells; these results might indicate active folliculogenesis in treated follicles. Restoration of the stromal cells and theca/granulosa cells with MSC (+/-PRP) therapy is speculated to allow paracrine-endocrine functions of an ovary and to return to



normal. Primordial follicle formation may be performed the interactive regulation of factors secreted by granulosa and theca cells [57] and the differentiation of the stem cell remaining after ovarian injury [58]. We might explain the formation

◀ **Fig. 6** Expression analysis of genes related to apoptosis-inflammation (**a**, **b**) and genes of folliculogenesis regulatory proteins (**c**) in the tissue after 2 months of treatment. The CTNF, CXCL12 and VEGF (**a**), and IL1b and TRAIL (**b**) gene expressions have minor differences. However, gene expressions of BMP-4, TGF-β, and IGF-1 are significantly higher in the MSC + PRP group (**c**). Western blot analysis for BMP4, IGF-1, TGF-β, VEGF and GFP showed the expression of their respective proteins in the tissue (**d**). Beta-actin (ActB) was utilized as control. The transgene expression of GFP was only documented in MSC and MSC + PRP groups. On the other hand, the Western blot analysis for VEGF showed both the transgene and endogenous expressions respectively

of the primordial follicle and secondary follicle after MSC (+/- PRP) treatment as follows: Primordial follicle formation starts from the early stages of folliculogenesis. The germ-line oogonial stem cells, VSEL cells and/or primordial germ cells are probably surrounded by pre-granulosa and granulosa cells. Then, already existing PGS in the ovarian tissue or the development of PGS from OSC or VSELs followed by Germinal vesicle (GV) oocyte development from PGSs and, consequently, primordial follicle formation is proposed to occur. Oocytes are surrounded by theca cells after the secondary follicle stage.

The TGF-β family of proteins, i.e., TGF-βs, activins, BMPs, and growth differentiation factor 9 (GDF-9), are key regulatory proteins in cellular growth and differentiation, and they are expressed in oocytes, granulosa and theca cells in stage-dependent specificity [59, 60]. TGF-β and BMPs play important roles in early stage folliculogenesis and ovulation, in which the cell-to-cell communication between the granulosa cells are provided by Connexin43 expression [61]. TGF-β family proteins have been reported to be secreted by ovarian stromal and /or granulosa-theca cells. As reciprocal response, the number of granulosa cells and primary follicles improve together [60, 62] and consequently support the survival of oocytes [63]. In the study by Park et al. (2013), the oocyte formation of OSCs is improved by BMP-4 [64]. In recent studies by Bayne et al. (2016) and Tan et al. (2017), BMP-4 was shown to regulate the development of both germ and somatic cells in the ovary to modulate early ovarian follicle development [65, 66]. In this study, both BMP-4 and TGF-β were highly expressed in the MSC + PRP group. This finding might indicate a very close relationship between the MSCs and the somatic cells within the ovarian regeneration period, especially in early folliculogenesis.

The expression of the IGF-1gene in the tissues also increased, suggesting that a close interaction is established between the granulosa and the theca cells, to support the folliculogenesis process. Prior studies have shown that the addition of IGF-1 to in vitro culture medium both prevents apoptosis [67] and determines oocyte quality and follicle maturation [68]. In this study, the expression of IGF-1 in the MSC + PRP group was significantly higher than that in the sham group.

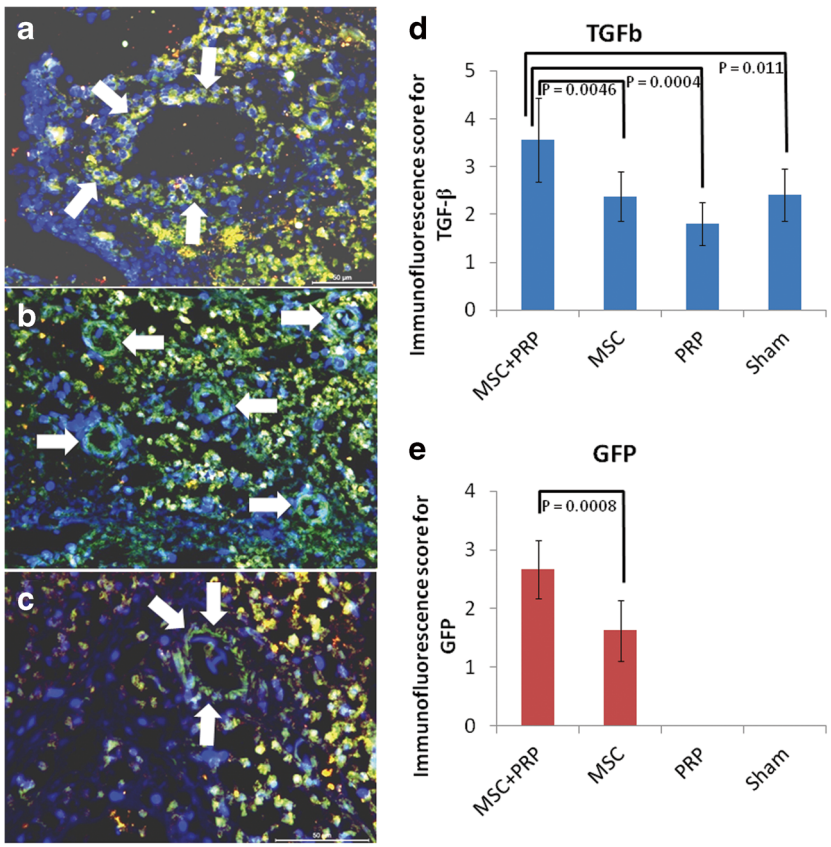


Fig. 7 The accumulation of GFP$^+$ cells (green) around the antral (**a**), primordial and primary (**b**) pre-antral (**c**) follicles (white arrow) indicated MSCs' effect on granulosa, theca cells, and the surrounding cells. The TGF-ß expression (red) in the ovarian tissue and perifollicular area of MSC + PRP treated group is significant. The staining was scored for TGF-ß (**d**) and GFP (**E**). The TGF-ß expression was significantly increased in the MSC + PRP compared to other groups. The number of GFP$^+$ cells was also significantly higher in the ovarian tissue of MSC + PRP than in MSCs groups. Scale bar, 50 μm

The microenvironment of OSCs is formed of granulosa cells, vascular endothelial cells, ovarian monocyte-derived cells, T cells and other molecules, and in an isolated in vitro environment, ovarian functions are fulfilled by OSCs separated from the ovaries. Any systemic immune response due to aging or genetic disorders can damage the germ line oogonial stem cells' microenvironment [69, 70]. The decline in ovarian function correlates with aging or damage of the ovarian germ cell microenvironments, but not with the OSCs themselves [71]. CXCL12 gene (anti-apoptosis factor) expression was higher in the MSC + PRP group than in the PRP group. Furthermore, there was a decrease in the expression of TRAIL (apoptosis) and IL1b (inflammation) in the MSC + PRP group, but this decline did not reach statistical significance. When we consider our findings of, initiation of granulosa cell growth and function, vital growth factors (like BMP-4, TGFβ, IGF-1) of folliculogenesis secreted by granulosa and other somatic cells, apoptosis reduction (CXCL12 increase), angiogenesis, immune system cell and autonomic innervations regulation by MSC and other unknown mechanisms, we conclude that those may provide ovarian germ stem cell nesting. Consequently, the relationship between ovarian germ stem cell homing and somatic and stromal cells may initiate folliculogenesis.

In conclusion, the results of this study were generated from an in vivo animal experimental model, which might limit the outputs. Further studies on POI or chemotherapy-induced ovarian failure are needed to assess the safety of ovarian stem cell transplantation in the female reproductive system. A single dose of 100 mg/kg cyclophosphamide was determined to be the ideal dose to generate a POI model in Fisher 344 rats. GFP staining was positive in the tissues of the MSC + PRP group, particularly in ovarian stromal, theca cells and granulosa cells. Oocytes were not stained with GFP. MSC treatment increased CXCL12, IGF-1, BMP4, and TGF-β gene expression, and blood AMH level, potentially indicating functional recovery. The addition of PRP to MSCs further increased CXCL12 and IGF-1 gene expression and blood oestradiol levels but not BMP-4 or TGF-β expression. MSC treatment increased the regeneration of follicles, and the addition of PRP to MSCs further supported functional recovery. However, transfection of VEGF to mesenchymal stem cells did not provide any extra benefit in overcoming ovarian insufficiency.

Acknowledgements We thank Experimental Medicine Research and Application Unit (DETAB) at Kocaeli University for their assistance in our experimental work. This work was supported by the Scientific and Research Council of Turkey (TUBITAK) [grant number 114S398].

Compliance with Ethical Standards

Disclosure of Interest The authors have no conflict of interest.

References

1. Woad, K. J., Watkins, W. J., Prendergast, D., & Shelling, A. N. (2006). The genetic basis of premature ovarian failure. *Australian and New Zealand Journal of Obstetrics and Gynaecology, 46*, 242–244.
2. Shelling, A. N. (2010). Premature ovarian failure. *Reproduction, 140*, 633–641.
3. Sheikhansari, G., Aghebati-Maleki, L., Nouri, M., Jadidi-Niaragh, F., & Yousefi, M. (2018). Current approaches for the treatment of premature ovarian failure with stem cell therapy. *Biomedicine & Pharmacotherapy, 102*, 254–262.
4. Sukur, Y. E., Kivancli, I. B., & Ozmen, B. (2014). Ovarian aging and premature ovarian failure. *Journal of The Turkish-German Gynecological Association, 15*, 190–196.
5. Bedoschi, G., Navarro, P. A., & Oktay, K. (2016). Chemotherapy-induced damage to ovary: Mechanisms and clinical impact. *Future Oncology, 2*, 2333–2344.
6. Soleimani, R., Heytens, E., Darzynkiewicz, Z., & Oktay, K. (2011). Mechanisms of chemotherapy-induced human ovarian aging: Double strand DNA breaks and microvascular compromise. *Aging, 3*, 782–793.
7. Zhou, L., Xie, Y., Li, S., et al. (2017). Rapamycin prevents cyclophosphamide-induced over-activation of primordial follicle pool through PI3K/Akt/mTOR signaling pathway in vivo. *Journal of Ovarian Research, 10*(1), 56.
8. Yuksel, A., Bildik, G., Senbabaoglu, F., et al. (2015). The magnitude of gonadotoxicity of chemotherapy drugs on ovarian follicles and granulosa cells varies depending upon the category of the drugs and the type of granulosa cells. *Human Reproduction, 30*, 2926–2935.
9. Galvez-Martin, P., Sabata, R., Verges, J., Zugaza, J. L., Ruiz, A., & Clares, B. (2016). Mesenchymal stem cells as therapeutics agents: Quality and environmental regulatory aspects. *Stem Cells International, 2016*, 9783408.
10. Qiu, P., Bai, Y., Pan, S., Li, W., Liu, W., & Hua, J. (2013). Gender depended potentiality of differentiation of human umbilical cord mesenchymal stem cells into oocyte-like cells in vitro. *Cell Biochemistry and Function, 31*, 365–373.
11. Bukovsky, A. (2011). Immune maintenance of self in morphostasis of distinct tissues, tumor growth, and regenerative medicine. *Scandinavian Journal of Immunology, 73*, 159–189.
12. Pourgholaminejad, A., Aghdami, N., Baharvand, H., & Moazzeni, S. M. (2016). The effect of pro-inflammatory cytokines on immunophenotype, differentiation capacity and immunomodulatory functions of human mesenchymal stem cells. *Cytokine, 85*, 51–60.
13. Caplan, A. I., & Correa, D. (2011). The MSC: An injury drugstore. *Cell Stem Cell, 9*, 11–15.
14. Gobbi, A., & Fishman, M. (2016). Platelet-rich plasma and bone marrow-derived mesenchymal stem cells in sports medicine. *Sports Medicine and Arthroscopy Review, 24*, 69–73.
15. Sills, E. S., Rickers, N. S., Li, X., & Palermo, G. D. (2018). First data on in vitro fertilization and blastocyst formation after intraovarian injection of calcium gluconate-activated autologous platelet rich plasma. *Gynecological Endocrinology, 2018*, 1–5.
16. Wang, S., Yu, L., Sun, M., et al. (2013). The therapeutic potential of umbilical cord mesenchymal stem cells in mice premature ovarian failure. *BioMed Research International, 2013*, 690491.
17. Liu, T., Huang, Y., Guo, L., Cheng, W., & Zou, G. (2012). CD44+/CD105+ human amniotic fluid mesenchymal stem cells survive and proliferate in the ovary long-term in a mouse model of chemotherapy-induced premature ovarian failure. *International Journal of Medical Sciences, 9*, 592–602.

18. Johnson, J., Bagley, J., Skaznik-Wikiel, M., et al. (2005). Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell, 122*, 303–315.
19. Lee, H. J., Selesniemi, K., Niikura, Y., et al. (2007). Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *Journal of Clinical Oncology, 25*, 3198–3204.
20. Santiquet, N., Vallières, L., Pothier, F., Sirard, M. A., Robert, C., & Richard, F. (2012). Transplanted bone marrow cells do not provide new oocytes but rescue fertility in female mice following treatment with chemotherapeutic agents. *Cellular Reprogramming, 14*, 123–129.
21. Wang, Z., Wang, Y., Yang, T., Li, J., & Yang, X. (2017). Study of the reparative effects of menstrual-derived stem cells on premature ovarian failure in mice. *Stem Cell Research & Therapy, 8*, 11.
22. Sun, M., Wang, S., Li, Y., et al. (2013). Adipose-derived stem cells improved mouse ovary function after hemotherapy-induced ovary failure. *Stem Cell Research & Therapy, 4*, 80.
23. Takehara, Y., Yabuuchi, A., Ezoe, K., et al. (2013). The restorative effects of adipose-derived mesenchymal stem cells on damaged ovarian function. *Laboratory Investigation, 93*, 181–193.
24. Demirayak, B., Yüksel, N., Çelik, O. S., et al. (2016). Effect of bone marrow and adipose tissue-derived mesenchymal stem cells on the natural course of corneal scarring after penetrating injury. *Experimental Eye Research, 151*, 227–235.
25. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Kortenbach, I., Marini, F., & Krause, D. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy, 8*, 315–317.
26. Ozbek, E., Adas, G., Otunctemur, A., et al. (2015). Role of mesenchymal stem cells transfected with vascular endothelial growth factor in maintaining renal structure and function in rats with unilateral ureteral obstruction. *Experimental and Clinical Transplantation, 13*, 262–272.
27. Öksüz, S., Alagöz, M. Ş., Karagöz, H., et al. (2015). Comparison of treatments with local mesenchymal stem cells and mesenchymal stem cells with increased vascular endothelial growth factor expression on irradiation injury of expanded skin. *Annals of Plastic Surgery, 75*, 219–230.
28. Adas, G., Koc, B., Adas, M., et al. (2016). Effects of mesenchymal stem cells and VEGF on liver regeneration following major resection. *Langenbeck's Archives of Surgery, 401*, 725–740.
29. Nagata, M. J., Messora, M. R., Furlaneto, F. A., et al. (2010). Effectiveness of two methods for preparation of autologous platelet-rich plasma: An experimental study in rabbits. *European Journal of Dentistry, 4*, 395–402.
30. Cakici, C., Buyrukcu, B., Duruksu, G., et al. (2013). Recovery of fertility in azoospermia rats after injection of adipose-tissue-derived mesenchymal stem cells: The sperm generation. *BioMed Research International, 2013*, 529589.
31. Sun, X., Su, Y., He, Y., et al. (2015). New strategy for in vitro activation of primordial follicles with mTOR and PI3K stimulators. *Cell Cycle, 14*, 721–731.
32. Flaws, J. A., Abbud, R., Mann, R. J., et al. (1997). Chronically elevated luteinizing hormone depletes primordial follicles in the mouse ovary. *Biological Reproduction, 57*, 1233–1237.
33. Pedersen, T. (1970). Determination of follicle growth rate in the ovary of the immature mouse. *Journal of Reproduction and Fertility, 21*, 81–93.
34. Detre, S., Saclani Jotti, G., & Dowsett, M. A. J. (1995). A "quickscore" method for immunohistochemical semiquantitation: Validation for oestrogen receptor in breast carcinomas. *Clinical Pathology, 48*, 876–878.
35. Epstein, R. J. (1990). Drug-induced DNA damage and tumor chemosensitivity. *Journal of Clinical Oncology, 8*, 2062–2084.
36. Kilic, S., Pinarli, F., Ozogul, C., Tasdemir, N., Naz Sarac, G., & Delibasi, T. (2014). Protection from cyclophosphamide-induced ovarian damage with bone marrow-derived mesenchymal stem cells during puberty. *Gynecological Endocrinology, 30*, 135–140.
37. Song, D., Zhong, Y., Qian, C., et al. (2016). Human umbilical cord mesenchymal stem cells therapy in cyclophosphamide-induced premature ovarian failure rat model. *BioMed Research International, 2016*, 2517514.
38. Kerr, J. B., Brogan, L., Myers, M., et al. (2012). The primordial follicle reserve is not renewed after chemical or gamma-irradiation mediated depletion. *Reproduction, 143*, 469–476.
39. Lei, L., & Spradling, A. C. (2013). Female mice lack adult germline stem cells but sustain oogenesis using stable primordial follicles. *Proceedings of the National Academy of Sciences, 110*, 8585–8590.
40. Yuan, J., Zhang, D., Wang, L., et al. (2013). No evidence for neo-oogenesis may link to ovarian senescence in adult monkey. *Stem Cells, 31*, 2538–2550.
41. Virant-Klun, I., Skutella, T., Bhartiya, D., & Jin, X. (2013). Stem cells in reproductive tissues: From the basics to clinics. *BioMed Research International, 2013*, 357102.
42. White, Y. A., Woods, D. C., Takai, Y., Ishihara, O., Seki, H., & Tilly, J. L. (2012). Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nature Medicine, 18*, 413–421.
43. Johnson, J., Canning, J., Kaneko, T., Pru, J. K., & Tilly, J. L. (2004). Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature, 428*, 145–150.
44. Bukovsky, A., Svetlikova, M., & Caudle, M. R. (2005). Oogenesis in cultures derived from adult human ovaries. *Reproductive Biology and Endocrinology, 3*, 17.
45. Bukovsky, A. (2011). Ovarian stem cell niche and follicular renewal in mammals. *The Anatomical Record, 294*, 1284–1306.
46. Bukovsky, A. (2015). Novel methods of treating ovarian infertility in older and POF women, testicular infertility, and other human functional diseases. *Reproductive Biology and Endocrinology, 13*, 10.
47. Hayama, T., Yamaguchi, T., Kato-Itoh, M., et al. (2014). Generation of mouse functional oocytes in rat by xeno-ectopic transplantation of primordial germ cells. *Biology of Reproduction, 91*, 89.
48. Morgan, S., Anderson, R. A., Gourley, C., Wallace, W. H., & Spears, N. (2012). How do chemotherapeutic agents damage the ovary? *Human Reproduction Update, 18*, 525–535.
49. Young, J. M., & McNeilly, A. S. (2010). Theca: The forgotten cell of the ovarian follicle. *Reproduction, 140*, 489–504.
50. Virant-Klun, I. (2015). Postnatal oogenesis in humans: A review of recent findings. *Stem Cells Cloning, 8*, 49–60.
51. Eppig, J. J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction, 122*, 829–838.
52. Bukovsky, A., & Caudle, M. R. (2012). Immunoregulation of follicular renewal, selection, POF, and menopause in vivo, vs. neo-oogenesis in vitro, POF and ovarian infertility treatment and a clinical trial. *Reproductive Biology and Endocrinology, 10*, 97.
53. Frese, L., Dijkman, P. E., & Hoerstrup, S. P. (2016). Adipose tissue-derived stem cells in regenerative medicine. *Transfusion Medicine and Hemotherapy, 43*, 268–274.
54. Dewailly, D., Robin, G., Peigne, M., Decanter, C., Pigny, P., & Catteau-Jonard, S. (2016). Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary. *Human Reproduction Update, 22*, 709–724.
55. Virant-Klun, I., Skutella, T., Stimpfel, M., & Sinkovec, J. (2011). Ovarian surface epithelium in patients with severe ovarian infertility: A potential source of cells expressing markers of pluripotent/multipotent stem cells. *Journal of Biomedicine and Biotechnology, 2011*, 381928.
56. Huda, F., Fan, Y., Suzuki, M., et al. (2016). Fusion of human fetal mesenchymal stem cells with "degenerating" cerebellar neurons in

spinocerebellar Ataxia type 1 model mice. *PLoS One, 11*, e0164202.
57. Binelli, M., & Murphy, B. D. (2010). Coordinated regulation of follicle development by germ and somatic cells. *Reproduction Fertility and Development, 22*(1), 12.
58. Shaikh, A., Anand, S., Kapoor, S., Ganguly, R., & Bhartiya, D. (2017). Mouse bone marrow VSELs exhibit differentiation into three embryonic germ lineages and Germ & Hematopoietic Cells in culture. *Stem Cell Reviews and Reports, 13*, 202–216.
59. Xia, X., Wang, T., Yin, T., Yan, L., Yan, J., & Lu, C. (2015). Mesenchymal stem cells facilitate in vitro development of human Preantral follicle. *Reproductive Sciences, 22*, 1367–1376.
60. Pangas, S. A. (2012). Regulation of the ovarian reserve by members of the transforming growth factor beta family. *Molecular Reproduction and Development, 79*, 666–679.
61. Chen, Y. C., Chang, H. M., Cheng, J. C., Tsai, H. D., Wu, C. H., & Leung, P. C. (2015). Transforming growth factor-β1 up-regulates connexin43 expression in human granulosa cells. *Human Reproduction, 30*, 2190–2201.
62. Al-Samerria, S., Al-Ali, I., McFarlane, J. R., & Almahbobi, G. (2015). The impact of passive immunisation against BMPRIB and BMP4 on follicle development and ovulation in mice. *Reproduction, 149*, 403–411.
63. Dunlop, C. E., & Anderson, R. A. (2014). The regulation and assessment of follicular growth. *Scandinavian Journal of Clinical and Laboratory Investigation, 244*, 13–17.
64. Park, E. S., Woods, D. C., & Tilly, J. L. (2013). Bone morphogenetic protein 4 promotes mammalian oogonial stem cell differentiation via Smad1/5/8 signaling. *Fertility and Sterility, 100*, 1468–1475.
65. Bayne, R. A., Donnachie, D. J., Kinnell, H. L., Childs, A. J., & Anderson, R. A. (2016). BMP signalling in human fetal ovary somatic cells is modulated in a gene-specific fashion by GREM1 and GREM2. *Molecular Human Reproduction, 22*, 622–633.
66. Tan, S., Feng, B., Yin, M., et al. (2017). Stromal Senp1promotes mouse early folliculogenesis by regulating BMP4 expression. *Cell & Bioscience, 7*, 36.
67. Polat, I. M., Alçiğir, E., Pekcan, M., et al. (2015). Characterization of transforming growth factor beta superfamily, growth factors, transcriptional factors, and lipopolysaccharide in bovine cystic ovarian follicles. *Theriogenology, 84*, 1043–1052.
68. Vural, F., Vural, B., Doğer, E., Çakıroğlu, Y., & Çekmen, M. (2016). Perifollicular blood flow and its relationship with endometrial vascularity, follicular fluid EG-VEGF, IGF-1, and inhibin-a levels and IVF outcomes. *Journal of Assisted Reproduction and Genetics, 33*, 1355–1362.
69. Bukovsky, A., Keenan, J. A., Caudle, M. R., Wimalasena, J., Upadhyaya, N. B., & Van Meter, S. E. (1995). Immunohistochemical studies of the adult human ovary: Possible contribution of immune and epithelial factors to folliculogenesis. *American Journal of Reproductive Immunology, 33*, 323–340.
70. Jasti, S., Warren, B. D., McGinnis, L. K., Kinsey, W. H., Petroff, B. K., & Petroff, M. G. (2012). The autoimmune regulator prevents premature reproductive senescence in female mice. *Biology of Reproduction, 86*, 110.
71. Ye, H., Zheng, T., Li, W., et al. (2017). Ovarian stem cell nests in reproduction and ovarian aging. *Cellular Physiology and Biochemistry, 43*, 1917–1925.