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Original Article

Determination of the effects of bone marrow derived mesenchymal stem cells and ovarian stromal stem cells on follicular maturation in cyclophosphamide induced ovarian failure in rats

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ABSTRACT

Objective: Chemotherapy causes depletion of primordial follicles that leads to premature ovarian failure in female cancer survivals. We investigated the effect of bone marrow derived mesenchymal (BMMSCs) and ovarian stromal stem cells (OSSCs) on follicle maturation in chemotherapy induced ovarian failure.
Material and methods: Thirty six Wistar Albino female rats were divided into three groups. Cyclophosphamide at a dose of 200 mg/kg was intraperitoneally (IP) given to the rats in all groups two times. 4×10^6 BMMSCs (IP) was injected to the group-2 and 4×10^6 OSSCs (IP) was injected to the group-3. Serum Anti-Müllerian Hormone (AMH) levels was determined with ELISA and primordial follicles were counted for investigation of primordial follicle reserve. The ovarian structure were evaluated histomorphologically. Localization of BrdU labeled stem cells, the expression of the cell cycle regulator p34Cdc2, gap junction protein p-connexin43 and intraovarian regulators of folliculogenesis Bone Morphogenic Protein 6 and 15 (BMP-6 and BMP-15) were investigated by immunohistochemistry.
Results: The immunstaining of BMP-6 was higher in oocytes of group-3 more than group-1 and group-2. The immunpositivity of p34cdc2 and BMP-15 were also higher in follicular cells of group-3 than the other groups. The presence of p-connexin43 in group-3 was determined more than group-1 and group-2. The ovarian follicles with normal histological structure were observed just in group-3. Although, The AMH levels were decreased in rats from all groups at the end of experimental procedure the primordial follicle counts in group-3 was significantly higher than group-1.
Conclusion: Our findings suggest that OSSCs have more protective effect on follicle maturation than BMMSCs in cyclophosphamide induced ovarian damage.

Introduction

Although early diagnosis and chemotherapy treatments extend the life of cancer patients, chemotherapy causes early menopause and infertility which depends on depletion of primordial follicles in female cancer survivors [1,2]. Chemotherapeutic drugs effect on germ cells and follicular cells which proliferate during ovarian cycle [3]. Effects of stem cells on fertility preservation have been investigated [4–6]. Also our previous study shows that BMMSC therapy is protective from germ cell apoptosis and DNA damage [4]. Furthermore, recent studies propose that ovarian stem cells can be the source of new oocytes and granulosa cells in adult life [7–11].

Members of bone morphogenic protein (BMP) subfamily act as intraovarian regulators of folliculogenesis. Bone morphogenic protein-6 and 15 (BMP-6 and BMP-15), which are expressed in ovary, maintain the follicle development by inhibiting the early luteinisation. The autocrine and paracrine effects of BMP-6, which is secreted from granulosa cells, and autocrine effects of BMP-15

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and BMP-6, which are secreted from the oocyte, promote granulosa cell proliferation and regulate follicle stimulating hormone (FSH) dependent follicle functions [12,13].

Gap junctions have important roles in the female reproductive system. Connexin43 is a gap junction protein and the amount of connexin43 increases with granulosa cell populations from the beginning to the latter stages of follicular development. In addition, follicular development is arrested in pre-antral stage in the absence of connexin43 [14,15].

p34cdc2 is also known as CDK1 which is a member of the cyclin-dependent kinases subfamily. p34cdc2 is necessary for progression of cell cycle and completion of M-phase [16,17].

The aim of this study was to compare the protective effects of BMMSCs and OSSCs on ovarian follicle maturation, which was administered with alkylating agents. We investigated immunohistochemically BMP-6, BMP-15, p-connexin43 expressions for evaluation of follicle development and p34cdc2 expression for evaluation of continuity of cell cycle. In addition, we evaluated histomorphology of ovarian follicles, primordial follicle counts and serum Anti-Müllerian hormone (AMH) levels which is the indicator of the amount of primordial follicle pool.

Material & methods

The present study was performed with the local ethics committee approval at Stem Cell and Genetic Diagnostic Center, Yıldırım Beyazıt Training and Research Hospital in Ankara, Turkey. All animals were handled in accordance with the national ethics guidelines.

Thirty six Wistar Albino female rats were used for this study. The rats were eight weeks old and weighted between 150 and 180 gr. The rats were caged in a controlled environment with 12-h light/dark cycles and were fed ad libitum. Animals were randomly divided into three groups with 12 animals in each group.

Cyclophosphamide at a dose of 200 mg/kg was injected to group-1, group-2 and group-3 via intraperitoneally in phosphate buffered saline on the first and eighth days of the study. We labeled the stem cells with BrdU for determination of their localization. Before the injection, 2×10^6 stem cells were incubated with 1 ml pbs/10 μl BrdU solution in 37 °C %5 CO_2 for 2 h. BMMSCs and OSSCs injected intraperitoneally as 2×10^6 cell/ml per injection on the second and ninth days of the study. Total 4×10^6 BrdU labeled BMMSCs for group-2 and Total 4×10^6 BrdU labeled OSSCs for group-3 were injected. Blood samples were collected at the beginning and in the end of the study to analyze and compare the serum AMH levels. All rats were sacrificed on the 10th day of the experiment. We planned to three cycle of cyclophosphamide and stem cell application in the original study design. However, we had to end study earlier than planned due to start the loss of rats and healths of rats went bad.

BMMSCs were obtained by density gradient method and OSSCs were obtained from fetal (day 16) ovarian tissue by explant method. Cell growth was monitored, and medium was changed every 3 days. The obtained cells were identified by flow cytometry (FACS ARIA III, Becton, Dickinson and Company, Vancouver, Canada) device with surface markers [BMMSCs; CD45(−), CD11b/c(−) CD90(+), CD44(+) and OSSCs; CD11b/c(−), CD45(−), CD49(+), CD90(+)] at the end of the second passage. After the second passage, the cells were differentiated in the adipocyte, osteocytes and chondrocytes. Both the number and viability of cells were evaluated with Countess® Automated Cell Counter device (Invitrogen,USA).

Standart histochemical procedure applied on the ovarian tissue samples and stained with H&E for histomorphological assessment.

For IHC assessments; Samples were heated in a high-temperature microwave oven in sodium citrate solution for antigen retrieval after deparaffinization and rehydration. After the treatment of serum blocking solution, p34Cdc2 (1:250, Cat:sc-954, Santa-Cruz), BMP-6 (1:100, Cat:ab15640, Santa-Cruz), anti-BMP-15 (1:100,Cat:ab198226, Abcam) primary antibodies exposed to samples for overnight at +4 °C and p-connexin43 (1:100,Cat:sc-101660, Santa-Cruz) and BrdU (1:250,Cat:550803, BD Pharmingen) primary antibodies exposed for 2 h at room temperature. After incubation, mause+rabbit HRP kit (Cat:54-003, Acustain, Genemed) was applied according to the manufacturer procedures. Chromogen-containing diaminobenzidine substrate (Cat:DABS-125, Thermo Fisher) was applied to the samples until a visible immunoreaction occur. All of the samples were photographed with computer-assisted imaging system (Leica DM4000, LeicaQVin3 Software).

Cross sections of entire ovary were used for counting primordial follicles and immunohistochemical assesment. The samples were serially sectioned at 4 μm thickness. Every fifth section was used for counting primordial follicles and sixth section was used for immunohistochemical assesment. Counting of the primordial follicles and evaluation of the immun-positivity were performed by two independent researchers.

The primordial, primary and antral follicles were used for immunohistochemical assesment. BMP-6 positive oocytes and p34cdc2 and BMP-15 positive cells for every 100 follicular cells were calculated. The gradation of staining was evaluated in a semi-quantitative method (0"negative"≤5%, +1"weak" = 6–35%, +2"moderate" = 36–65%, +3"strong" = 66–100%). Additionally, the presence of p-connexin43 and BrdU was determined.

Serum AMH levels were determined by ELISA method with Rat AMH ELISA Kit (Sunred Biological Technology). The samples were examined according to the manufacturer procedures.

Statistical analysis and calculations were performed with SPSS 15.0 (SPSS Inc.). Mean ± SD was used for parametric data and median was used for non-parametric data. Ln transformation was performed to primordial follicle counts for providing of normality. ANOVA and post-hoc Bonferroni test were used for comparison of follicle counts. The Mann–Whitney test with Bonferroni's adjustment was applied for comparing the AMH levels' difference between the groups and Wilcoxon test was used to compare the beginning and the end AMH levels. $p \leq 0.05$ was considered as statistically significant.

Results

Flowcytometry results confirm the characterization of BMMSCs and OSSCs. BMMSCs were negative for CD45(−) and CD11b/c(−) and positive for CD44(+) and CD90(+) (Fig. 1A). OSSCs are also negative for CD45(−) and CD11b/c(−) and positive for CD90(+) and CD49(+) (Fig. 2A). Differentiation of the cells in adipocytes, osteocytes and chondrocytes was observed both BMMSCs (Fig. 1B–D) and OSSCs (Fig. 2B–D). Presence of the stem cells was determined in both follicle wall and stroma of group-2 and group-3 (Fig. 3A).

A decrement of serum AMH levels was determined for all groups on 10th day. However, the difference between the groups was not statistically significant. Also the difference between the 1st and the 10th day serum AMH levels wasn't statistically significant respectively for each group (Table 1A). The difference between the means of primordial follicle numbers of the group-1 and the group-2; and between the group-2 and group-3 was not statistically significant but the difference between the mean primordial follicle numbers of group-1 and group-3 was statistically significant (Table 1B).

Cytoplasmic loss and vacuolization were determined in oocytes from primordial and primary follicles in both group-1 and group-2. Also junctional degenerations were observed between the follicular cells and follicle cell-oocytes junctions of these two groups. These junctional degenerations were observed less in group-2 than

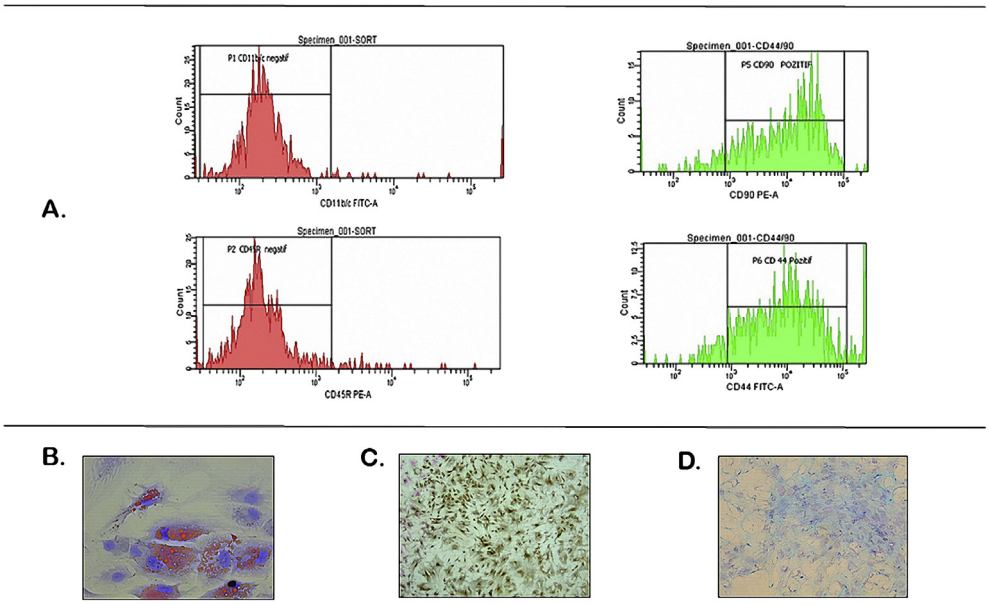

Fig. 1. (A) BMMSCs characterization by flow cytometry [a; CD 45 (−), CD 11b/c (−), CD 90 (+) CD 44 (+)]. BMMSCs differentiation to three germ layers (B): Adiposit differentiation (oil red), (C): Osteosit differentiation (Von kossa), (D): Chondrosit differentiation (Alcian blue). (×100).

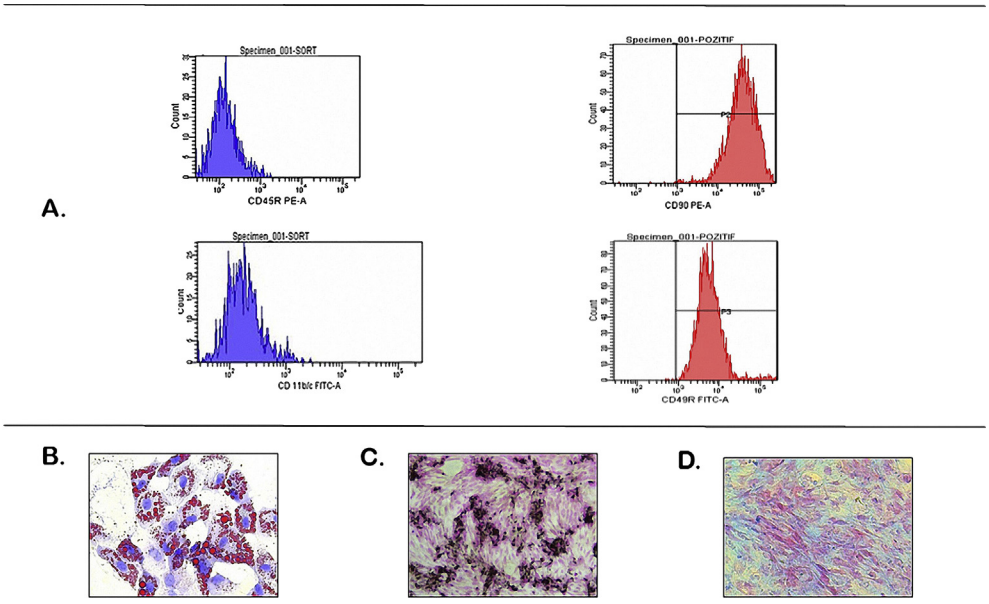

Fig. 2. (A) OSSCs characterization by flow cytometry [CD 45 (−), CD 11b/c (−), CD 90 (+) CD, CD49 (+)]. OSSCs differentiation to three germ layers (B): Adiposit differentiation (oil red), (C): Osteosit differentiation (Von kossa), (D): Chondrosit differentiation (Alcian blue). (×100).

group-1. Group-3 was determined as quite normal structure but some oocytes had vacuolizations which were less than other groups (Fig. 3B).

The presence of p-connexin43 wasn't observed primary and antral follicles in group-1 and primary and early antral follicles group-2. However, graafian follicles from group-1, late antral and graafian follicles from group-2 had p-connexin43 immunpositivity. Primary, antral and graafian follicles of group-3 had prominently p-connexin43 immunstaining (Fig. 3C).

The immunstaining of BMP-6 was negative in oocytes of group-1. BMP-6 was weakly positive in oocytes from primordial and primary follicles and moderately positive in oocytes from antral follicles in group-2. BMP-6 was determined moderately as positive in oocytes from primordial, primary and antral follicles in group-3 (Fig. 4A, Table 2A).

BMP-15 was negative for follicular cells of primordial and primary follicles and weakly positive for follicular cells of antral follicles in group-1. BMP-15 was observed as negative for primordial follicles and moderately positive primary and antral follicles in group-2. In group-3, BMP-15 was moderately positive in primordial follicles and strongly positive in primary and antral follicles (Fig. 4B, Table 2B).

p34Cdc2 was negative for follicular cells of primordial follicle in both group-1 and group-2, weakly positive for follicular cells of primary follicle in group-1 and moderately positive for follicular cells of primary follicle in group-2. Follicular cells of antral follicles

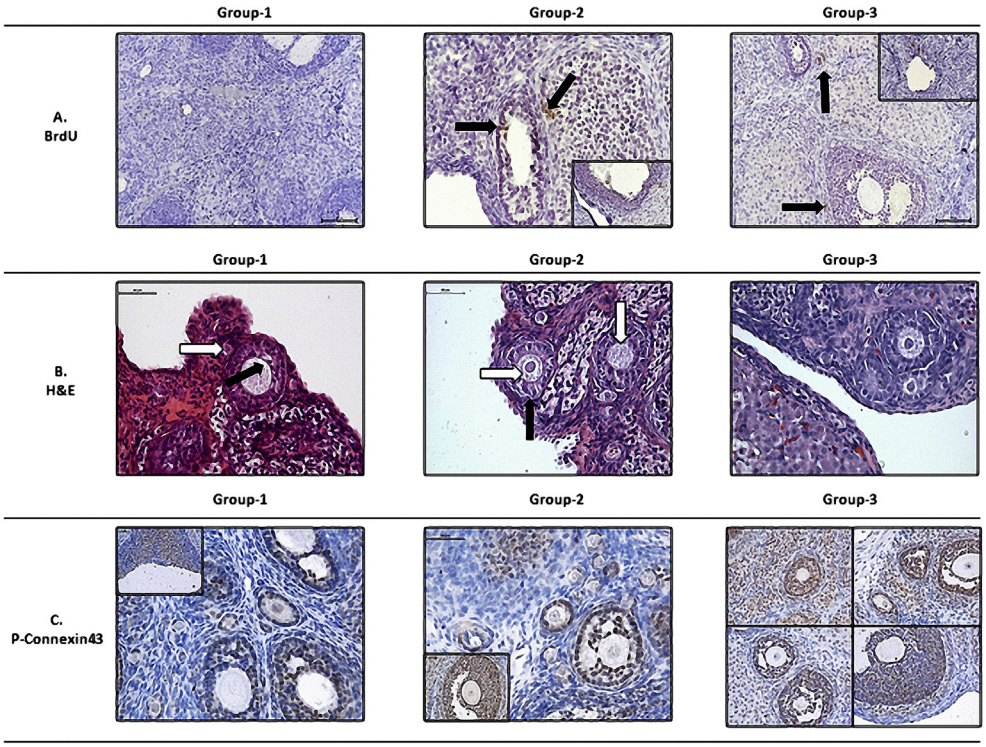

Fig. 3. (A): Localization of BrdU labeled stem cells for groups (→) (Group-1 and small picture of group-3, DAB,×200, the magnification of other images, DAB, ×400). (B): Cytoplasmic loss and vacuolization in oocytes (⇨), junctional degeneration between the follicular cells and follicle cell-oocytes junctions (→) in Group-1 and Group-2; connection between the granulosa cells and integrity between oocyte and follicle cells of Group-3 (H&E, ×400). (C): Negative rosette shaped immunstaining of p-connexin43 between the follicular cells of primary and early antral follicles in Group-1 and Group-2 and immunpositivity of p-connexin43 between the follicular cells of graaf follicle in Group-1 and between the follicular cells of late antral follicle in Group-2 (small pictures, DAB, ×400); rosette shaped p-connexin43 immunstaining between the follicular cells of primary, antral and graaf follicles in Group-3 (DAB, ×400).

Table 1
(A): Median values of serum AMH levels, 1st Day vs. 10th Day comparisons (Wilcoxon Test) and binary comparisons of decrements of the groups (Mann–Whitney test with Bonferroni's adjustment). (B): Average primordial follicle counts and binary comparisons of the groups (ANOVA and post-hoc Bonferroni test).

A. Serum AMH Levels				
	1st day serum AMH levels (median IQR)	10th day serum AMH levels (median IQR)	Serum AMH levels 1st day vs. 10th Day	Decrement of serum AMH levels
Group-1	0,91 (0,23)	0,82 (0,24)	$p = 0{,}248$ Z = −1156	Gr-1 vs. Gr-2 $p = 0{,}478$
Group-2	0,95 (0,93)	0,77 (0,32)	$p = 0{,}306$ Z = −1023	Gr-1 vs. Gr-3 $p = 0{,}101$
Group-3	1,65 (5,72)	1,08 (0,47)	$p = 0{,}075$ Z = −1779	Gr-2 vs. Gr-3 $p = 0{,}332$
B. Primordial Follicle Counts				
		(mean ± SD)		p values
Group-1		19,91 ± 5,24		Gr-1 vs. Gr-2 $p = 0{,}591$
Group-2		26,18 ± 5,38		Gr-1 vs. Gr-3 $p = 0{,}025$
Group-3		33,09 ± 3,31		Gr-2 vs. Gr-3 $p = 0{,}433$

of group-1 and group-2 were determined moderately positive for p34Cdc2. We observed in group-3 that p34Cdc2 was weakly positive for follicular cells of primordial follicles and strongly positive in follicular cells of primary and antral follicles (Fig. 4C, Table 2C).

Discussion

Although clinical and experimental studies are promising to prolongation of the life span of the cancer patients, gonadotoxic effects of chemotherapy cause infertility and sexual disfunction due to depletion of follicle pool. Chemotherapeutic drugs damage germ cells and growing follicles [3].

Recent studies present the protective effects of the stem cells [4–6,18]. MSCs can reach to the target area and show regulatory and therapeutic effects via their differentiation and secretion capacity [19,20]. It is indicated that ovarian stem cells can be transformed into oocyte and granulosa cells in adults [7–11]. However, none of these studies have investigated the regenerative function of ovarian stromal stem cells on injured ovarian tissue. Our study is the first study that compares the effects of OSSCs and BMMSCs on chemotherapy induced ovarian damage.

In this study, we observed that the in-vivo transplanted BMMSCs and OSSCs can migrate and locate in ovarian stroma, theca cell layer and granulosa cell layer like the previous studies [4–6]. Although, the count of transplanted stem cells is few, we observed their protective effects. Mesenchymal stem cells effects on damaged tissue not only directly but also with paracrine and autocrine activity. The recuperation of damaged tissue is partially provided by growth factors, immune modulators, proteins and chemokines which are secreted from stem cells [21]. Although,

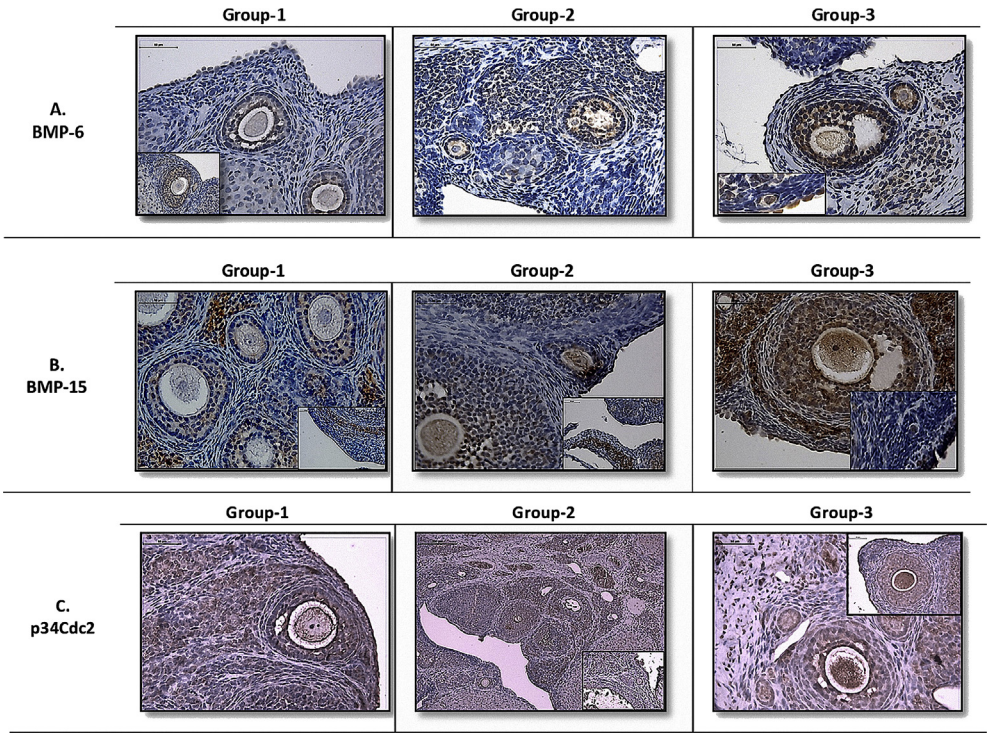

Fig. 4. (A): Immunpositivity of BMP-6 in oocytes for each follicle type (Small picture of group-3, DAB, ×100, Big pictures and small picture of group-1, DAB, ×400). (B): Immunpositivity of BMP-15 in follicular cells and the for each follicle type (DAB, ×400). (C):Immunpositivity of p34Cdc2 in follicular cells for each follicle type (Big picture of group-2, DAB,×100, the chromogen and magnification of other images, DAB, ×400).

Table 2
(A): The gradation of BMP-6 immunopositivity for each follicle type. (B): The gradation of BMP-15 immunopositivity for each follicle type. (C): The gradation of p34Cdc2 immunopositivity for each follicle type.

A. BMP-6		3+	2+	1+	0
Primordial	Group 1	0/70(%0)	2/70(%2,9)	15/70(%21,4)	53/70(%75,7)
	Group 2	2/57(%3,5)	0/57(%0)	33/57(%57,9)	22/57(%38,6)
	Group 3	4/44(%9,1)	20/44(%45,5)	7/44(%15,9)	13/44(%29,5)
Primary	Group 1	0/86(%0)	2/86(%2,3)	33/86(%38,4)	51/86(%59,3)
	Group 2	0/86(%0)	40/86(%46,5)	42/86(%48,8)	4/86(%4,7)
	Group 3	33/101(%32,7)	53/101(%52,5)	15/101(%14,8)	0/101(%0)
Antral	Group 1	0/51(%0)	2/51(%3,9)	13/51(%25,5)	36/51(%70,6)
	Group 2	2/59(%3,4)	33/59(%55,9)	20/59(%33,9)	4/59(%6,8)
	Group 3	20/70(%28,6)	37/70(%52,8)	13/70(%18,6)	0/70(%0)
B. BMP-15		3+	2+	1+	0
Primordial	Group 1	0/68(%0)	0/68(%0)	9/68(%13,2)	59/68(%86,8)
	Group 2	2/64(%3,1)	2/64(%3,1)	27/64(%42,2)	33/64(%51,6)
	Group 3	7/77(%9,1)	46/77(%59,7)	15/77(%19,5)	9/77(%11,7)
Primary	Group 1	0/86(%0)	2/86(%2,3)	40/86(%46,5)	44/86(%51,2)
	Group 2	7/136(%5,1)	79/136(%58,1)	48/136(%35,3)	2/136(%1,5)
	Group 3	81/169(%47,9)	42/169(%24,9)	44/169(%26)	2/169(%1,2)
Antral	Group 1	0/99(%0)	9/99(%9,1)	55/99(55,5)	35/99(%35,4)
	Group 2	7/107(%6,6)	70/107(%65,4)	26/107(%24,3)	4/107(%3,7)
	Group 3	60/106(%56,6)	24/106(%22,6)	18/106(%17)	4/106(%3,8)
C. p34Cdc2		3+	2+	1+	0
Primordial	Group 1	0/35(%0)	0/35(%0)	9/35(%26)	26/35(%74)
	Group 2	7/37(%19)	2/37(%5,4)	9/37(%24,3)	19/37(%51,3)
	Group 3	11/37(%29,7)	7/37(%19)	17/37(%45,9)	2/37(%5,4)
Primary	Group 1	9/123(%7,3)	42/123(%34,1)	59/123(%48)	13/123(%10,6)
	Group 2	39/134(%29,1)	53/134(%39,6)	42/134(%31,3)	0/134(%0)
	Group 3	75/132(%56,8)	46/132(%34,8)	9/132(%6,8)	2/132(%1,6)
Antral	Group 1	7/84(%8,3)	46/84(%54,8)	13/84(%15,5)	18/84(%21,4)
	Group 2	26/68(%38,2)	35/68(%51,5)	7/68(%10,3)	0/68(%0)
	Group 3	55/79(%69,6)	24/79(%30,4)	0/79(%0)	0/79(%0)

stem cells can not maintain their existence in target tissue long term, they shows paracrine and autocrine effects to contribute healing and activating local stem cells that are in the sleeping phase. In this reason, the count of transplanted stem cells in the damaged tissue and healing rate can not be always correlative.

Total number of primordial follicles is reduced by a proportional increase in the cyclophosphamide application dose [1]. It is showed that the number of primordial follicles reduce 48 h after cyclophosphamide administration [22]. We have determined that in-vivo transplanted BMMSCs and OSSCs partially inhibit primordial follicles reduction caused by cyclophosphamide injection. But this protective effect is statistically significant only in group-3. Serum AMH level is a useful indicator that shows the amount of primordial follicle pool and cyclophosphamide administration causes decrement on follicle counts and serum AMH level [23,24]. We have also determined the reduction of serum AMH levels in all groups at the end of the study.

Cyclophosphamide and the other alkylating agents prevent cell division and BMMSCs have a protective effect on proliferation [3,25]. p34Cdc2 is a regulator protein of cell cycle and we have determined that BMMSCs administration partially protected the expression of p34Cdc2. Additionally, the immunoassay of p34Cdc2 was determined as more positive in OSSCs administrated group. We thought that OSSCs are much more protective on continuity of cell division.

BMP-6 mRNA levels increase during follicle development and BMP-6 can be effective on the increment of oocyte quality [26,27]. In this study, we observed that cyclophosphamide administration prevents BMP-6 expression in oocytes from group-1. BMP-6 is consistently expressed by BMMSCs [28]. We have also determined BMP-6 expression in oocytes from stem cell injected groups and OSSCs injected group had more immunstaining.

BMP-15 is expressed by all follicle types in goat ovary [29]. BMP-15 expression increase during folliculogenesis and can have a regulatory role on granulosa cell proliferation and differentiation [30]. An increment of BMP-15 expression is showed when ovarian follicles are in-vitro cultured with BMMSCs [31]. We have also determined that stem cell administration maintained the expression of BMP-15 in-vivo. Furthermore, OSSCs were observed more effective than BMMSCs for this maintaining.

Gap junctions have important roles in female reproductive system such as ovarian functions and oocyte maturation [32,33]. Connexin43 expression increases during folliculogenesis [15]. Because of this, we have observed p-connexin43 expression only in graafian and late antral follicles of group-1 and group-2. Due to OSSCs administration, p-connexin43 expression was obviously determined in primary, antral and graafian follicles of group-3. The less developed zona pellusida, vacuolization in oocytes and granulosa cells is determined in connexin43 knockout mice's ovary [14]. Similarly, we observed vacuolizations and junctional degenerations in group-1 and group-2.

In conclusion, our results suggested that cyclophosphamide administration caused damage to the ovary and this damage may give rise to primary ovarian insufficiency. The in-vivo transplantation of OSSCs can be more effective protectors than BMMSCs for follicle maturation after chemotherapy. Unfortunately, the source of OSSCs is still a limitation for clinical applications. The use of abortion materials that do not have any genetic and microbiological problems as an allogeneic stem cell source has been a frequently discussed approach among stem cell researchers in recent years [34]. We also used a fetal rat ovarian tissue that obtained from terminated pregnancy on the 16th day as an abortion-material simulation to demonstrate the efficacy of OSSCs in our study. However, the usage of abortion material is still contradictive due to ethical reasons. The adult ovarian tissue may use as an alternative source of OSSCs. The ovarian tissue resection before cancer treatment is already used for ovarian tissue cryopreservation (OTC) [35]. OSSCs can be isolated from resectioned tissue before cryopreservation and can be used before, during and after chemotherapy to protect ovarian function. In addition, OSSCs may show regenerative effects when use with cryopreserved ovarian tissue transplantation. This study an futrher studies on OSSCs can help to prevent primary ovarian insufficiency and early menopause which are important problems of chemotherapy patients.

Because of the reason described above (in material and methods section), we had to end the study earlier than planned and could not observe the transplanted rats for long-term. Different chemical agents and their concentrations are commonly used for POF generation in animals and there has not been any standardization yet [36]. Furthermore, these models mostly reduce life times of animals depends on the cytotoxic effects of the agents [37]. We applied the same cyclophosphamide induced POF model according to our previous study [4]. We planned to administrate cyclophosphamide and stem cell injection 7 days interval for 21 days in the original study design but we had to end study earlier than planned due to the death of rats at a rate that will affect statistical results. An effective and standardized method should be generated for further studies.

Conflicts of interest

The authors declare no conflict of interest.

Declaration of interest

This study was funded by the Gazi University Scientific Research Projects Unit, Turkey (Project-Code: 01/2015-07).

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