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Adipose tissue–derived mesenchymal stem cells restorates damaged ovary and activates primordial follicles via pten/akt/foxo3a pathway.

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Abstract
18 Wistar albino female rats were divided into three groups. Control group; C, Cyclophosphamide group; CTX, Cyclophosphamide + Mesenchymal stem cell; CTX+SC. CTX and CTX+SC were injected intraperitoneally with 50 mg / kg cyclophosphamide on the first day and 8 mg / kg cyclophosphamide was injected daily for the following 13 days .15 days after the first day of cyclophosphamide injection, we injected adipose tissue–derived mesenchymal stem cells(ADMSC) (50,000 mesenchymal stem cells in 0.05ml FBS per ovary) in CTX+SC group directly into both ovaries After 8 weeks from the stem cell injection, we sacrificed the rats and removed the ovaries of the rats. Primordial follicles were evaluated for PTEN, p-PTEN, AKT, p-AKT, FOXO3a, p-FOXO3a proteins by using immunohistochemistry, andsubsequently H-score analyses were performed.
Expression of PTEN and AKT was higher in control than in CTX and CTX+SC ($p < 0.05$). The positive reaction of FOXO3a was observed in the nucleus in the control group, in the cytoplasm in the CTX group, and in the nucleus and cytoplasm in the CTX + SC group.The mean primordal follicle count was the highest in Control. The number of secondary and antral follicles in CTX+SC were higher than C and CTX.
The results of PTEN expression suggest that the effect of CTX on the PTEN pathway is acute. However, when CTX is applied, it inflictsserious damage on the ovarian tissue, which turns out to be irreversible. ADMSCs restorate ovarium tissue and activate primordial follicles.
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Keywords: PTEN/Akt/FOXO3a, premature ovarian failure, cyclophosphamide, mesenchymal stem cell

Introduction

Avital problem that cannot be worked out for premenopausal women exposed to chemotherapy is infertility due to the loss of non-renewable ovarian primordial follicles [1]. For women with cancer, chemotherapy and radiotherapy result in early and massive loss of primordial follicles representing ovarian reserve in the ovary, leading to the formation of amenorrhea and Premature Ovarian Failure (POF) [2-7]. POF is associated with decreased fertility as well as hypergonadotropic hypogonadism and amenorrhea in women under 40 years of age.Further, Cyclophosphamide (CTX) induced POF is clinically irreversible. Following the treatment with CTX, the incidence of amenorrhea among young women turns out to be 84%, and eventually POFbegins to develop in half of the patients [8]. The majority of ovarian primordial follicles should be preserved in a stagnant condition to ensure regular production of gametes over the course of female reproductive life. Nevertheless, the molecular mechanism that enables primordial follicles to remain dormant for a long time is not well-established yet.Under certain pathological conditions, the entire pool of primordial follicles matures at the same time, contributing to the rapid loss of primordial follicles and premature ovarian failure (POF).In recent years, some research has begun to shed light on molecular mechanisms underlying the dormancy and activation of primordial follicles [9,10].Some previous studies have reported that chemotherapy triggers apoptosis of dormant primordial follicles, causing POF and infertility [11,12]. However, Kalich-Philosoph et al. reported that cyclophosphamide, a chemotherapeutic agent, did not induce apoptosis of

primordial follicles. Instead, cyclophosphamide treatment induces the apoptosis of actively growing follicles and triggers the transition from primordial follicles to primary follicles, leading to the destruction of the primordial follicle pool [10,13]. In addition, chemotherapy drugs dynamically target dividing cells, suggesting that chemotherapy is less toxic to dormant follicles [10].

The foremost indicator of ovarian reserve is the primordial follicles.Recent studies have revealed that the PTEN / AKT / FOXA3a signaling pathway is associated with the prevention of primordial follicle loss and the preservation of the ovarian reserve.When PI3K is increased or PTEN is suppressed, the path moves towards AKT / FOXO3a. The transition of FOXO3a from cell nucleus to the cytoplasm initiates the activation of primordial follicle [10,14,15]. Besides, the cyclophosphamide is known to cause the induction of the PTEN / AKT / FOXO3a signaling pathway, leading to the activation of an excessive amount of primordial follicle.Activated primordial follicles turn into primary follicles and then into secondary and tertiary follicles, thereby causing cyclophosphamide to destroy the ovarian reservoir [13].

Stem cell therapies, which are of great importance in regenerative medicine today, are thought to replace pharmacological agents and surgical methods in the treatment of numerous diseases in the near future.Even now, stem cell therapy is practised as the primary mode of treatment for some diseases, and thus the literature abounds with the studies carried out in this field. Previous studies have reported that mesenchymal stem cells might create a positive effect upon the restoration and function of ovarian tissue [8,16-20].

In this study, we tried to gain an insight into the effect of mesenchymal stem cells on ovarian degeneration caused by cyclophosphamide as well as their role on PTEN / AKT / FOXO3a signaling pathways.

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study was approved by Pamukkale University Animal Experiments Ethics Committee with the number PAUHADYEK-2016/21on November 8, 2016. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The animals required for our study were obtained from Pamukkale University Experimental Surgery Application Center. Being 8 weeks old and weighing 150-160 gr on average,18 healthy Wistar-Albino rats were randomly assigned into 3 equal groups. Group 1 was the control group (group C; n = 6), cyclophosphamide was administered to Group 2 (CTX group; n = 6), and Group 3 was given cyclophosphamide and mesenchymal stem cells (CTX + SC group; n = 6).

Formation of experimental ovarian toxicity with cyclophosphamide in rats

Cyclophosphamide (CTX) was administered to create experimental ovarian toxicity in rats.The predominant method used to produce experimental ovarian toxicity in the relevant literature is that CTX is injectedintraperitoneally as 50 mg/kg on Day 1 and as 8 mg/kg daily for the following 13 days.To this end, the dose required for each rat in the CTX and CTX + SC group was calculated.Afterwards CTX (ENDOXAN 1g Baxter) was removed, and the required amounts were placed in 14 separate tubes for 14 days after being measured on the precision scale.CTX was dissolved in distilled water at 20 ml each 1 ml (because it could remain intact for 1-2 hours in the dissolved state, only the dose to be administered was melted before each injection.) The CTX injection was performed for 14 days in accordance with the literature [16].

Cyclophosphamide + the group given stem cells obtained from adipose tissue (ctx+sc group)

The rats in this group were also injected intraperitoneally at a daily dose of 50 mg/kg CTX and 8 mg/kg CTX daily for the following 13 days. Then each rat was injected with 50000 mesenchymal stem cells obtained from adipose tissue, directly into the ovary within 0.05 ml Fetal Bovine Serum (FBS).

After 8 weeks from the stem cell injection, all rats were decapitated and both ovaries of them were removed. These ovariums were fixed in 10% formaldehyde for 24 hours. Following the fixation, the ovaries were embedded in the paraffin blocks. Then, 5 µm–thick sections from the paraffin blocks were stained with hematoxylin and eosin for ovarian histology and follicle counting. Moreover, the 5^{th} sections were evaluated in an attempt to avoid the sequential assessment of the same folliclesin the counting process.

Mesenchymal stem cell

What was used in our study isthe stem cells that were previously obtained, characterized, and then frozen in the second passage.

Follicle counting method

After the tissueswere monitored andthe ovarian tissues were embedded in the paraffin blocks, 5–micron sections were taken with the microtome device. The 1^{st}, 5^{th}, and 10^{th}sections were mounted on the slides and stained with Hematoxylin-Eosin. Then, primordial, primary, secondary and tertiary follicles were counted in the stained sections. In the end, the results in the 1^{st}, 5^{th}, and 10^{th} sections taken from both ovaries of each rat were collected. (The follicle evaluation criteria are explained in the findings section.

Immunohistochemistry

Immunostaining was performed using the avidin-biotin peroxidase complex method. After deparafinisation and rehydration, citrate buffer (pH 6.0) was used for antigen retrievalthrough microwave. Later, endogenous peroxidase activity was blocked with 3% hydrogen peroxide inside methanol at room temperaturefor 15 minutes. The sections were then incubated at 4°C overnight with primary antibodies [PTEN, p-PTEN, AKT, p-AKT, FOXA3a, p-FOXA3a].

After washing the primary antibodies, the sections were incubated with secondary antibody (Vector, catalog no. Ba-1000, 1:1000) at room temperature for 60 minutes, followed by its incubation with the avidin-biotin complex (Vectastain elite kit, catalog no. pk-6100) for 30 minutes.

Immunohistochemical reactions were visualized with diaminobenzidine chromogen (Sigma-Aldrich #D4168) under a light microscope. For opposite staining, Mayer's hematoxylin (Merck, Darmstadt, Germany) was administered for 15 hours.

The expressions of PTEN (LOT#AK00017JUN10090,Elabscience), pPTEN (LOT#AK00017JUN10090,Elabscience), AKT (LOT#AK00017JUN10092,Elabscience), pAKT (LOT#AK00017JUN10093,Elabscience), FOXA3a (LOT#AK00017JUN10094Elabscience), pFOXA3a (LOT#AK00017JUN10095Elabscience), were visualized under the OLYMPUS CX51 light microscope.Subsequently, the immunohistochemical staining was examined in primordial follicles. The staining intensity was scored as such: (/) no structure to examine, (–) no staining, (+/–) very weak staining, (+)weak staining, (++) medium staining, (+++) strong staining. The intensity of the staining and the number of stained cells were calculated using the H score [∑Pi(i+1)].

Statistical analysis

The primordial, primary, secondary and tertiary follicle counts were performed in the ovarian sections of all the subjects. The difference between the groups were analyzed by Kruskal–Wallis, while the difference between the two groups were calculated by Mann-Whithey U. SPSS 21 package program was used for statistical analysis, and the p valuewas set as <0.05.

Results

Ovarian Histology and Follicle counts

The appearance of the ovary was normal with follicles at all stages of development in control group (Figure1)

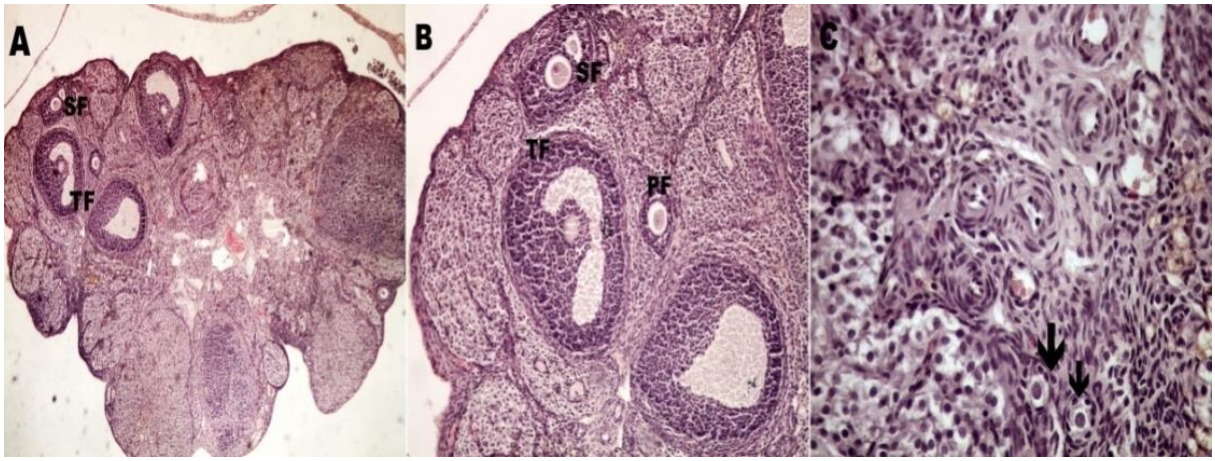

Figure 1: Ovarian tissue in the control group (A,B,C), Arrow primordial follicle, PF; Primary follicle, SF; Secondary follicle, TF; Tertiary follicle, star; Area where separations occur in the integrity of the tissue. Hematoxylin & eosin.

It was observed that the integrity of the ovary impaired in the group that is treated CTX. The majority of follicles had lost its normal structure, the granulosa cell cytoplasm had lossses and the nuclei of some granulosa cells appeared to be picnotic.It was evident that the normal structure of the primordial follicles disappeared and there was damage in the follicular cells.Separations were also observed in stromal tissue (Figure 2 A,B,C).

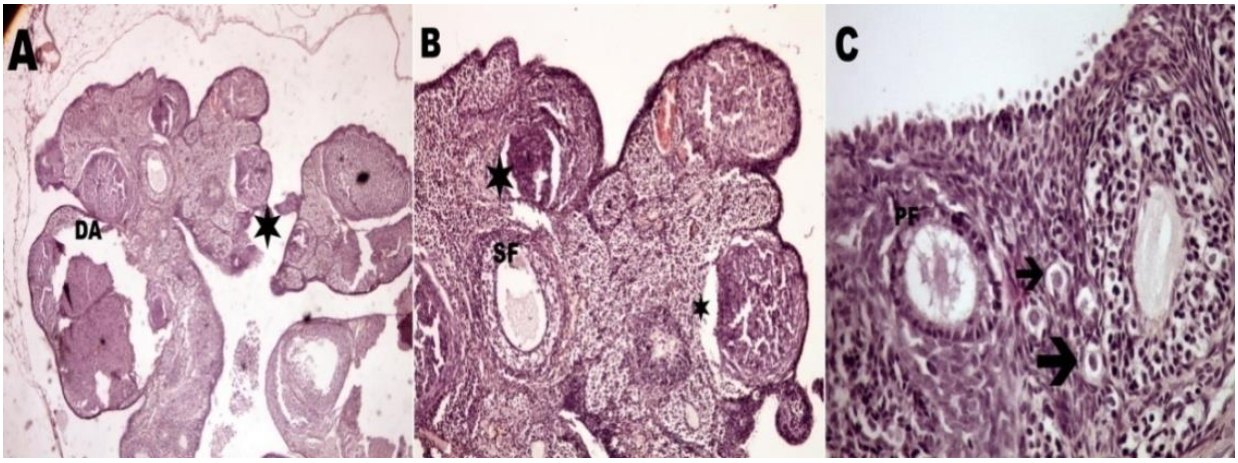

Figure 2: Ovarian tissue taken from CTX group (A,B,C), Arrow primordial follicle, PF; Primary follicle, SF; Secondary follicle, TF; Tertiary follicle, star; Area where separations occur in the integrity of the tissue. Hematoxylin & eosin. A:X4,B:X10,C:X40

In the group that is treated with CTX +SC group, it was observed that the number of follicles with degenerative appearance was decreased while the integrity of the ovary was preserved. The normal structure of follicular cells was maintained in the primordial follicles and appearence of granulosa cells near to normal.However, it was observed the separations the stromal tissue continued even a little (Figure 1g,h,ı).

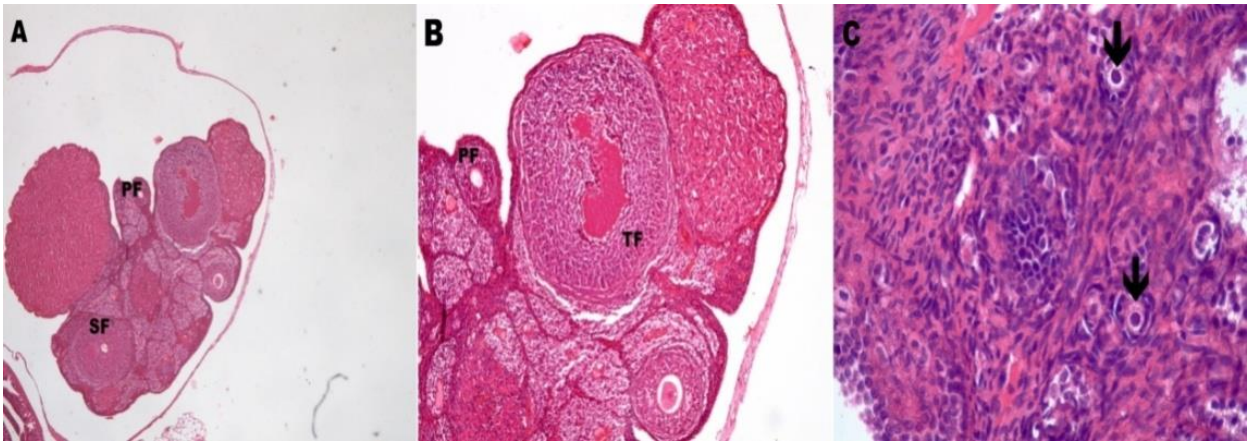

Figure 3:Ovarian tissue taken from CTX+SC group (A,B,C). Arrow primordial follicle, PF; Primary follicle, SF; Secondary follicle, TF; Tertiary follicle, star; Area where separations occur in the integrity of the tissue. Hematoxylin & eosin. A:X4,B:X10,C:X40

The mean primordial follicles in the control group was significantly higher than in the CTX and CTX + SC groups. (p=0,02). There was no significant difference between the CTX and CTX+SC groups, regarding the primordial folicles numbers.(p=0,936).

The mean primary follicles in the CTX+SC and control group was significantly higher than the CTX group. (p = 0.035) and between the CTX and control group (p = 0.010)

A significant lower secondary (p=0,002) and tertiary (p=0,007) follicles was observed in the CTX group when compared to the control and CTX+SC groups (Figure 4).

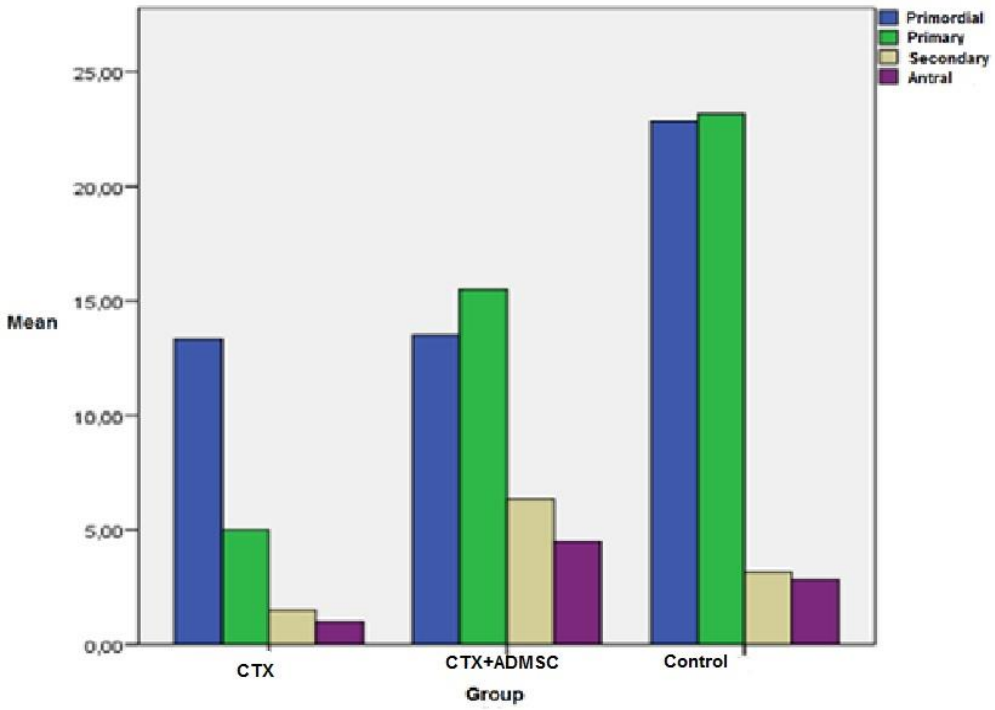

Figure 4: Mean number of total follicles

Immunohistochemical findings

PTEN expression

PTEN expression was strongly positive in both the cytoplasm and nucleus in the control group, whereas it was found to be cytoplasmic and weak positive in CTX and CTX + SC groups. Granulosa cells were negative in all three groups (Figure 5 a,b,c).

p-PTEN expression

There was no significant difference between the CTX, CTX + SC and control group in terms of p-PTEN reaction. In all three groupsoocyte cytoplasm showed weak positive expression while follicular cells and nucleus were negative (Figure 5 d,e,f).

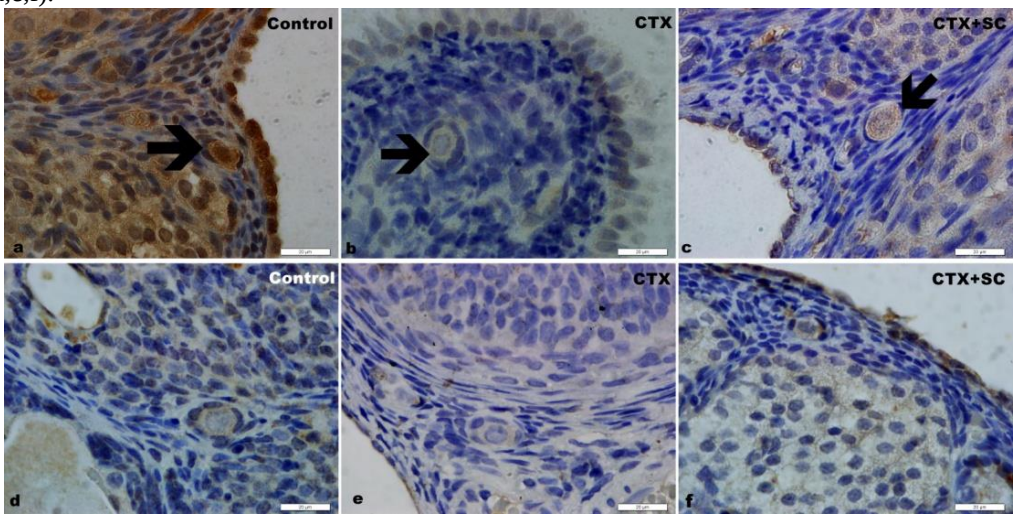

Figure 5: Expressions of PTEN (a,b,c), pPTEN (d,e,f) in primordialfollicles. Hematoxylin & immunperoxidase, scalebars = 50 μm.

FOXA3a expression

FOXA3a showed positive expression in all three groups. However the localization of the expression was different. Positive expression was observed in the nucleus in the control group whereas it was observed in the oocyte cytoplasm in CTX group and both in the nucleus and cytoplasm in the CTX + SC group. Follicle epithelial cells that demonstrated positive expression were found in all three groups (Figure 6 a,b,c).

p-FOXA3a expression

p-FOXA3a expression was similar to FOXA3a expression. But the CTX and CTX + SC groups had lessdegree of expression (Figure 6 d,e,f).

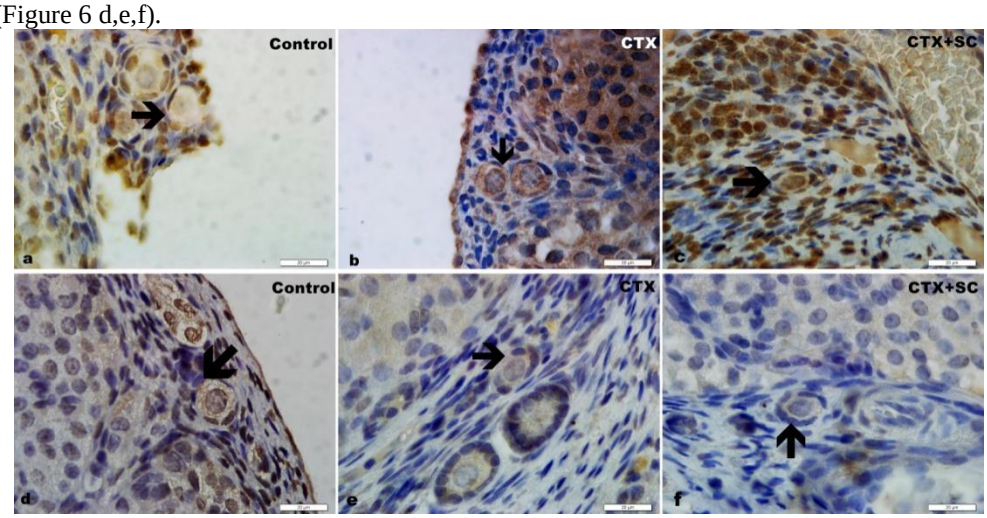

Figure 4: Expressions of FOXA3a (a,b,c) and pFOXA3a (d,e,f) in primor dialfollicles. . Hematoxylin&immunperoxidase,scalebars = 50 µm.

AKT expression

AKT expression was strongly positive in both oocyte cytoplasm and nucleus in the control group primordial follicles. In the CTX and CTX + SC groups, weak positive akt expression was observed only in the oocyte cytoplasm, whereas follicle epithelial cells demonstrated negative expression in all three groups (Figure 7 a,b,c).

p-AKT expression

Negative expression was detected in control group while weak positive expression was observedin the oocyte cytoplasm, in CTX and CTX+SC. No expression was observed in follicle epithelial cells in all three groups, too (Figure 7 d,e,f).

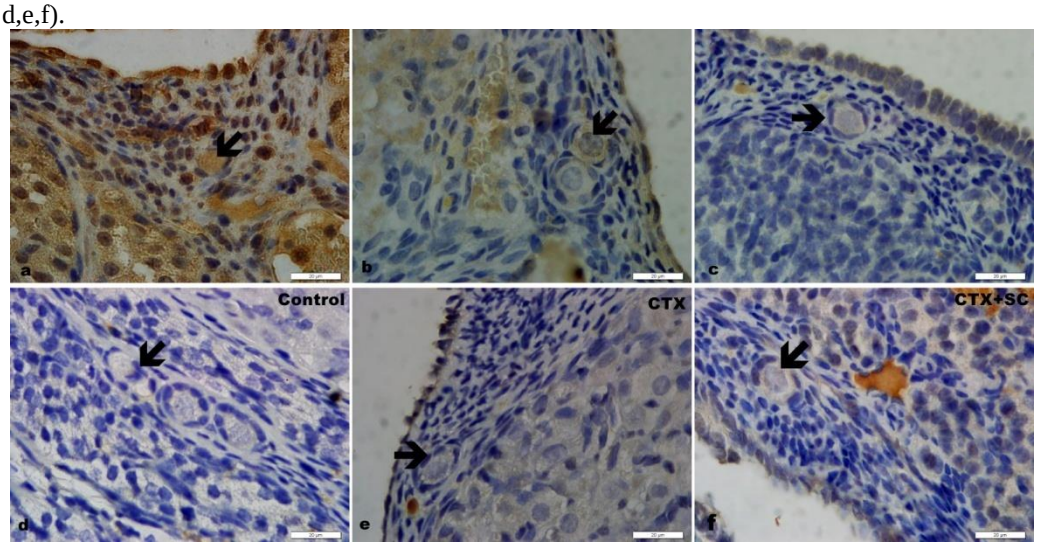

Figure 7: Expressions of AKT (a,b,c), pAKT (d,e,f) in primordial follicles. Hematoxylin&immunperoxidase scalebars = 50 µm.

Expression levels of PTEN, pPTEN, FOXA3a, pFOXA3a, AKT, pAKT in primordial follicles between the groups were semiquantitatively evaluated with H-score. H-score results summarized in Table 1 and 2.
AKT expression was significantly higher in the control group than in the CTX group (0,001). There was no difference in pAKT, pPTEN, FOXA3a, p-FOXA 3a expression between the groups. A significant increase in PTEN expression was observed in control groups when compared to CTX and CTX-SC groups.

Table 1. H-score results (mean_SD).

	Mean± SD	**P Values**
PTEN	0,01006±0,037	0,002
p-PTEN	0,08560±0,012	0,161
FOXA3a	0,48900±0,014	0,686
PFOXA3a	0,11500±0,012	0,328
AKT	0,10560±0,042	0,001
p-AKT	0,07729±0,009	0,092

Table 2. H-score results between groups

	PTEN	**p-PTEN**	**FOXA3a**	**p-FOXA3a**	**AKT**	**p-AKT**
C-CTX	0,004	0,676	0,510	0,162	0,001	0,096
C-CTX+SC	0,004	0,1	0,414	0,682	0,566	0,698
CTX-CTX+SC	0,192	0,114	0,935	0,284	0,064	0,98

Discussion

Infertility and premature ovarian failure (POF) are some of the most critical and common side effects of chemotherapy [21]. Chemotherapy drugs lead to abnormal activation of the dormant follicles in the ovary, triggering the development of POF [10,13]. Therefore, there has been a growing body of research into the prevention of POF and the protection of ovarian follicle pool in cancer patients in recent years.Chemotherapy-induced POF is clinically persistent and also persists after treatment. The incidence of amenorrhea after treatment with cyclophosphamide (CTX) among young women is 84%, and 50% of the patients end up developing POF [8].
CTX is known to bring about the activation of primordial follicles rather than apoptosis in oocytes or granulosa cells. Further, PTEN / AKT / FOXO3a is an important signaling pathway involved in the activation of dormant primordial follicles. PTEN is the negative regulator in the AKT / FOXO3a pathway,and its suppression results in an increase either in PI3K or in AKT and FOXA3a. In addition, primordial follicle is activated by the release of FOXA3a from nucleus to cytoplasm [10,13,22,23]. The research on both experimental animals and humanshas shown that a good number of primordial follicles are activated and converted into primary follicles by the suppression of PTEN in ovaries [24-26].
In our study, PTEN expression signalled a weak reaction in CTX and CTX + SC groups but strong positive reaction in the primordial follicules of the control group. P-PTEN did not differ between the groups (p = 0.157), and AKT expression was similar to PTEN expression. While AKT expression was most frequently observed in the control group, it was remarkable that there was no difference between the groups in p-AKT expression. As a matter of fact, we would expect p-AKT expressionto be intensivein the CTX group. Kalich-Philosoph et al. reported that the phosphorylation of AKT was the highest following the first 24 hours after the cyclophosphamide treatment, which was gradually decreased, and that one week later p-AKT / AKT was almost equaled in the ovaries of the mice in the group that had not received the cyclophosphamide [13].Our immunohistochemical analysis was performed approximately 8 weeks after the CTX administration. The reason why p-AKT expression did not differ in the CTX group may be that CTX had lost its effect on the PTEN / AKT / FOXA3a pathway during this time, or that CTX might not have activated the AKT pathway.
The positive reaction of FOXA3a occured in the nucleus in the control group, cytoplasm in the CTX group and both the nucleus and cytoplasm in the CTX

+ SC group. In the CTX group, the existence of FOXO3ain the cytoplasm was expected,because when FOXA3a is phosphorylated and crossesfrom the nucleus to the cytoplasm, the primordial follicles are activated. In fact, the cytoplasmic expression in the CTX group is indicative of the activation of primordial follicles. While p-AKT did not differ between the groups, p-FOXO3a was slightly stronger in the CTX and CTX+SC group than the control group and the reaction occured in the cytoplasm, suggesting that the effect of CTX might be by means of FOXA3a.

Providing tissue repair with paracrine effect by forming micro-environment with secreted cytokines,stem cells have recently been tried out to repair the destroyed ovarian tissue and follicles. Moreover, they help to regenerate the tissue through their ability to transform into any other cells in the tissue [8,16-20]. It has been revealed that stem cells derived from adipose tissue, umbilical cord, mens blood and amniotic membrane augment follicles in ovaries, reduce apoptosis in fibrosis and granulosa cells, and return sex hormones to normal levels [16-20].

Our immunohistochemical results indicate that mesenchymal stem cells activate the PTEN / AKT / FOXA3a pathway. However, in the preparations stained with hematoxylin eosin, there were histologicallymarked differences between only CTX-administered groups and the control and CTX+SC treated groups. In the CTX group, noticeabledeterioration was observed in follicles whose integrity was mostly lost in the ovary. The antral follicle ratio was markedly decreased in this group ($p = 0.002$). When it comes to the stem cell-administered group,not only did the CTXdrivendamage seemto disappear to a large extent andapproximate to the normal appearance of the follicles, but the antral follicle ratioalso increased considerably ($p = 0.007$).

Conclusion

The results of PTEN expression suggest that the effect of CTX on the PTEN pathway is acute. However, when CTX is administered, it inflicts serious damage on the ovarian tissue, which then turns out to be irreversible.

The mesenchymal stem cells not only activate primordial follicles but also repair CTX-induced damage, which is probably due to the paracrine effect of the secreted factors. Additionally, it also augments antral follicles leading to ovulation.

Although mesenchymal stem cell administration triggers the primordial pool, they seem to maintain the usual structure of the activated folliclesand then reach ovulation.

Acknowledgments

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