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## Original Article

# The therapeutic effects of ovarian stromal stem cells in the natural ovarian aging process in rats

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## Abstract

**Background:** Natural ovarian aging (NOA) is characterized by the age-dependent, gradual depletion of the primordial follicle reserve in the ovarian cortex, a decline in oocyte quality, and the potential to lead to infertility. **Aims:** This study investigated the effects of stromal stem cells (OSCs) isolated from the ovary on ovarian tissue and folliculogenesis by transplanting these cells into the ovaries of rats undergoing ovarian aging. **Methods:** Twelve 12–14-month-old Sprague Dawley rats with regular oestrous cycles and reduced oocyte counts were divided into control (n=6) and OSC transplantation (n=6) groups. Stromal stem cells isolated from 23–25-day-old female rat ovaries were injected into the OSC group. Follicle counts and ovarian morphology were assessed via hematoxylin-eosin (H&E) staining. Insulin-like Growth Factor 1 (IGF-1), Follicle-Stimulating Hormone Receptor (FSHR), and Anti-Müllerian Hormone (AMH) expression in ovarian tissue were analyzed by immunohistochemistry, while serum AMH, FSH, and estradiol (E2) levels were measured using enzyme-linked immunosorbent assay (ELISA). **Results:** After 30 days, ovarian function improved with increased ovarian reserve and tissue architecture showed marked improvement. IGF-1 and AMH expression were significantly higher in the OSC group than in controls (P=0.02), there was no statistically significant difference in FSHR expression between groups (P=0.09). Unlike AMH levels (P=0.5129), Serum E2 levels rose significantly in the OSC group (P=0.0001). Serum FSH levels were similar between the groups (P=0.444). **Conclusion:** These findings suggest that OSC transplantation may improve ovarian function and reserve in aged rats, presenting a potential regenerative treatment for natural ovarian aging.

**Key words:** Infertility, Natural ovarian aging, Ovarian reserve, Ovarian stromal stem cells

## Introduction

With the impact of modern life, women are postponing pregnancy, and as maternal age increases, infertility rates are rising. This situation negatively affects women who wish to have children (Amanak *et al.*, 2014). Age is one of the factors contributing to infertility (Olooto *et al.*, 2012). Fertility rates in women are observed to be high below the age of thirty, but a decline is noted after thirty (Amanak *et al.*, 2014). As age increases, the number and quality of oocytes decrease. This leads to natural ovarian aging, causing a reduction in fertility in women (Meczekalski *et al.*, 2016). The likelihood of follicle and oocyte damage also increases, elevating the risk of genetic malformations in the fetus (Cruz *et al.*, 2017).

At puberty, the ovary contains approximately 400,000 primordial follicles, each housing a primary oocyte. Throughout a woman's reproductive lifespan, approximately 300 to 400 follicles undergo the ovulation

process (Amanvermez and Tosun, 2016). In older women, ovarian reserve begins to decline due to the apoptotic loss of primordial follicles. Ovarian aging plays a significant role in the age-related decline of ovarian reserves. With ovarian aging, oocyte quality deteriorates, the number of primordial follicles and implantation rates decrease, and the incidence of chromosomal anomalies in embryos and consequently miscarriage rates increase (Durdağ and Berker, 2008).

Hormone replacement therapy is primarily used to alleviate menopausal symptoms and prevent long-term degenerative changes associated with natural ovarian aging. However, it is not primarily aimed at restoring fertility. As a result, mesenchymal stem cells (MSCs) obtained from various tissues, which show therapeutic potential, are being extensively studied in the context of natural ovarian aging (Ding *et al.*, 2018). Mesenchymal stem cells can be isolated from many tissues or organs and differentiate into various tissues or organs. Therefore, depending on the tissue, they exhibit different

differentiation capacities and CD markers. Today, stem cell therapies hold a crucial place in regenerative medicine (Altinbasak *et al.*, 2024). Furthermore, it has been reported that with stem cell therapy, folliculogenesis resumes in the ovaries, ovarian functions improve, and the expression of genes and proteins related to folliculogenesis increases (Ding *et al.*, 2017; Huang *et al.*, 2020).

The effects of stromal stem cells isolated from their microenvironment during the natural ovarian aging process are not yet fully understood. This study aimed to comprehensively evaluate the restorative and functional effects of ovarian-derived stromal stem cells (OSSCs) on ovarian tissue in rats undergoing natural ovarian aging.

## Materials and Methods

### Establishment of Natural Ovarian Aging Rat Models

This study used 12 healthy female Sprague Dawley rats aged 12 to 14 months with an average weight range of 306 to 381 g. The animals were obtained from the Experimental Surgery Application Center of Pamukkale University. They were provided unrestricted access to standard rat chow and tap water and housed individually in cages in a controlled environment with a temperature of  $21 \pm 1^\circ\text{C}$ , a ventilation rate of 65-70%, and a 12 h light-dark cycle throughout the experiment. The rats were randomly assigned to groups 1, the control group (n=6), and group 2, the group receiving ovarian-derived stromal cells (OSC group, n=6). The study was approved as a research project by the Animal Experiments Ethics Committee of Pamukkale University on 03.02.2021, with the project number PAUHDEK-2021/04.

### Isolation of OSSCs

In this study, stromal stem cells (SSCs) were isolated from the ovarian tissue of three healthy rats, aged  $\leq 4$  weeks, using the explant method. After the rats were sacrificed under sterile conditions and anesthesia, their ovaries were washed with Dulbecco's phosphate-buffered saline (DPBS, Capricorn Scientific GmbH,

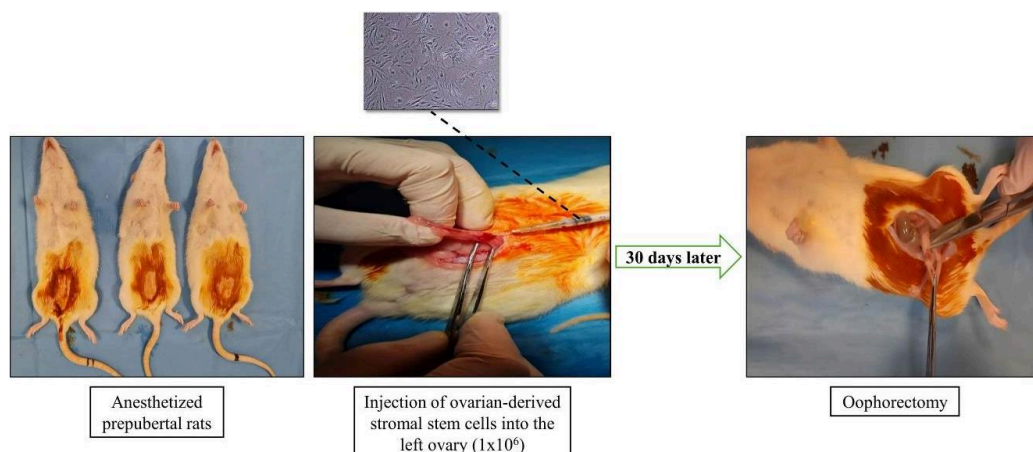
Germany, Cat. No. PBS-1A), and transported to the laboratory in falcon tubes containing DMEM (Dulbecco's Modified Eagle's Medium) (Capricorn Scientific GmbH, Germany, Cat. No. DMEM-HA) supplemented with penicillin/streptomycin. The complete medium was prepared by mixing 450 ml of DMEM with 10% FBS (Fetal Bovine Serum) (Capricorn Scientific GmbH, Germany, Batch No.: FBS-11B – FBS Advanced, collected in South America) and 5 ml of penicillin/streptomycin (50 U/ml penicillin and 50  $\mu\text{g}/\text{ml}$ ). The adipose tissue surrounding the ovarian tissue was carefully removed, and the cleaned tissues were dissected and transferred to new petri dishes containing 8 ml of complete medium. These petri dishes were then incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Cell migration, proliferation, and potential contamination were evaluated 24 h post-incubation. The medium was changed daily for the first five days and subsequently every other day. Once cell confluence reached 70-80%, the cells were detached using 0.25% trypsin/1 mM EDTA (0.05% in DPBS, 1 $\times$ - Capricorn Scientific GmbH, Germany, Cat. No.: TRY-1B), centrifuged, and re-seeded into flasks at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> based on cell count using a hemocytometer. Throughout this process, cell growth was monitored using an inverted microscope, and once the cells proliferated, they were stained with trypan blue for viability assessment and counted.

### Characterization of OSSCs

In passage 3, the quantification of surface markers CD90, CD54, CD45, CD29, and RT1D for the characterization of stromal stem cells was performed through flow cytometry analysis using the Sysmex Cube 8 Model device at the Kocaeli University Stem Cell and Gene Therapy Research and Application Center.

### Administration of OSSCs to the rats in the second group

A total of  $1 \times 10^6$  OSSCs obtained from the ovarian tissue of young rats were injected into the left ovary of the rats in this group using a surgical method with 0.01 ml of PBS. Thirty days after the stem cell injection, the



**Fig. 1:** Surgical procedure demonstrating the injection of OSSCs into the left ovary of anaesthetized prepubertal rats, followed by an oophorectomy procedure performed 30 days post-injection

ovaries of all the rats were removed, and approximately 1 ml of blood was aspirated by entering through the horizontal axis of the heart for the ELISA assay (Fig. 1). Subsequently, all the rats were sacrificed. The ovaries extracted from the rats were histopathologically examined under a routine light microscope.

### Measurement of serum Anti-Müllerian Hormone, Follicle-Stimulating Hormone, and Estradiol levels

For the analysis of serum AMH, FSH, and E2 levels, blood samples were collected from rats in each group and transferred to anticoagulant tubes. The samples were centrifuged twice—first at 500 xg for 10 min and then at 1400 xg for another 10 min—to obtain clear serum. Hormone concentrations were determined using commercially available ELISA kits (Human AMH: E4968Hu, Human FSH: E1001Hu, Human Estradiol: E1034Hu, BT LAB, China), and absorbance was measured at 450 nm with a microplate reader, following the manufacturers' instructions.

The technical characteristics of the kits were as follows:

AMH (E4968Hu): Sensitivity: 0.1 ng/ml; detection range: 1-25 ng/ml; intra-assay CV: <10%; inter-assay CV: <10%; high specificity with no significant cross-reactivity reported.

FSH (E1001Hu): Sensitivity: 0.938 mIU/ml; detection range: 1.563-100 mIU/ml; intra-assay CV: <8%; inter-assay CV: <10%; high specificity.

Estradiol (E2, E1034Hu): Sensitivity: 7.54 ng/L; detection range: 15-3000 ng/L; intra-assay CV: <10%; inter-assay CV: <12%; high specificity.

### Histopathological Analysis and Follicle Counting Method

Ovarian tissues from all groups were fixed in formaldehyde solution for 24 h, and paraffin blocks were prepared. For follicle counting, 5 µm-thick sections were obtained from the paraffin blocks at the 1st, 5th, and 10th cuts. Primordial, primary, secondary, and tertiary follicles were counted in (H&E) stained sections.

### Immunohistochemical staining method

Anti-rat FSHR (Elabscience, USA), anti-rat IGF-1 (Finetest, China), and anti-human AMH (Finetest, China) primary antibodies were used to characterize ovarian tissues. Serial sections of 5 µm thickness were deparaffinized and dehydrated by incubating in xylene three times for 10 min each. Antigen retrieval was performed using sodium citrate buffer. The sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 15 min. Immunoblocking was carried out on the sections, bordered with Pappen, using Ultra V Block for 30 min. After diluting the primary antibodies (AMH: 1/1000, FSHR: 1/100, IGF-1: 1/100), they were applied to the tissues and incubated at +4°C for 16 h. The sections were treated with a biotinylated secondary antibody for 30 min, followed by incubation with Streptavidin Peroxidase (HRP-SA) for 30 min. Subsequently, the sections underwent a series of steps

including 3, 3'-diaminobenzidin (DAB) staining, counterstaining with Hematoxylin, dehydration, clearing in xylene (Merck, Germany, Cat. No. M108297), and mounting. The expression of AMH, FSHR, and IGF-1 was evaluated in a semiquantitative manner using the H-score,

$$[H\text{ Score}=\sum P_i (I+1)]$$

Where,

I: Represents staining intensity (0: no expression, 1: weak, 2: moderate, 3: strong)

The evaluation of the H-score was performed using ImageJ software.

### Statistical analysis

Descriptive statistics and quantitative variables are presented as median (minimum-maximum) values. The Kruskal-Wallis test was used to compare the levels of AMH, FSH, E2, and the H-score in immunohistochemistry between the groups. The Kruskal-Wallis test was performed using SPSS (Statistical Package for the Social Sciences, Version 15.0, Chicago, IL). The analysis was conducted in RStudio (Version 1.2.5033, RStudio, Inc., Boston, MA) with the "clusrank" package (Datta-Setten method). A P-value of <0.05 was considered statistically significant for all analyses.

## Results

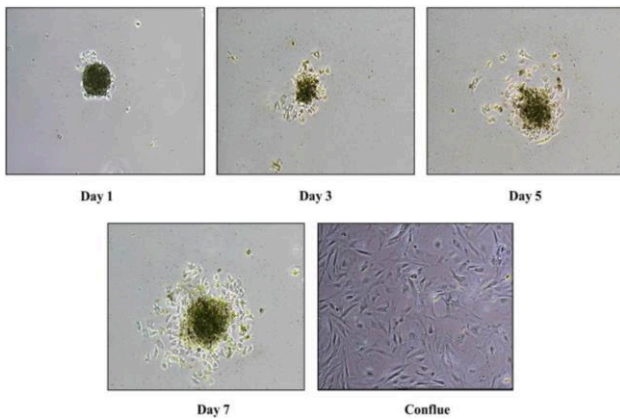
### OSSCs isolation and flow cytometry results

It was observed that stromal stem cells isolated from ovarian tissue using the explant culture method began adhering to the culture dish and migrating by the third day. The cell density increased over the following days, and by the ninth day, it reached approximately 70-80% confluence. According to microscopic counts performed at passage 3, approximately 2×10<sup>6</sup> cells had proliferated in each culture dish. The cells exhibited a fibroblast-like morphology and spindle-shaped structure (Fig. 2).

According to the flow cytometry analysis results, OSSCs were found to express CD90 at 82.48%, CD54 at 82.76%, and CD45 at 82.92%. Additionally, 81.39% of the cells co-expressed the CD90 and CD54 markers. However, a low population of cells expressed CD29 or RT1D (MHCII) was detected. The overall cell population was evaluated by flow cytometry using the parameters of cell granularity (side scatter, SSC) and cell size (forward scatter, FSC) (Figs. 3a and b).

### Hematoxylin and eosin (H&E) staining results

In the control group, degenerative changes in the morphological structures of some follicles and pyknotic nuclei in certain granulosa cells were observed due to ovarian aging. In secondary and tertiary follicles, it was determined that the antra, surrounded by granulosa cells, exhibited a wavy appearance, with pyknotic cells dispersed within the antra. Additionally, separations, cell reductions, and localized voids were observed in some granulosa cells and stromal tissue. Degeneration of

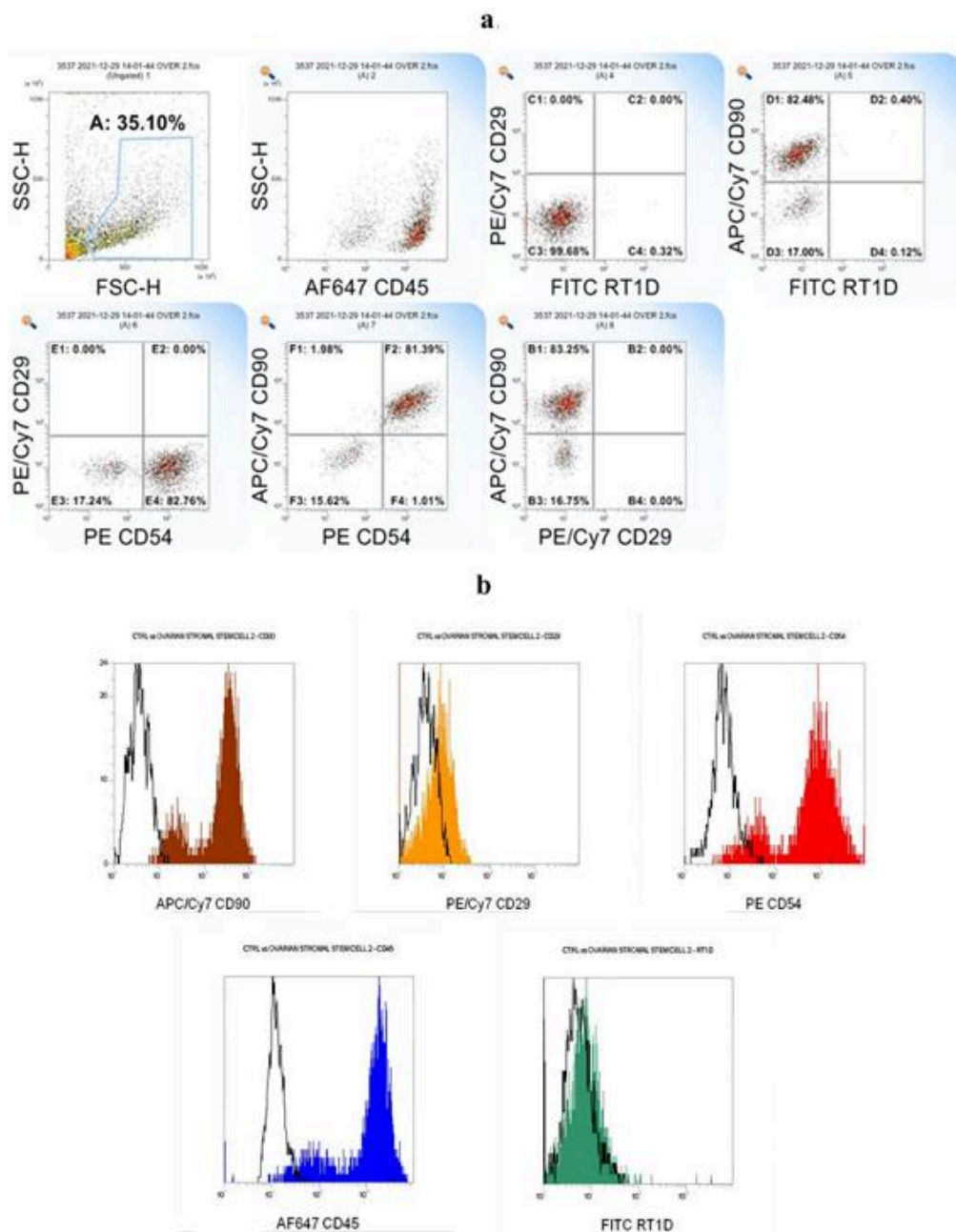


**Fig. 2:** Phenotypic and morphological features of OSSCs. The

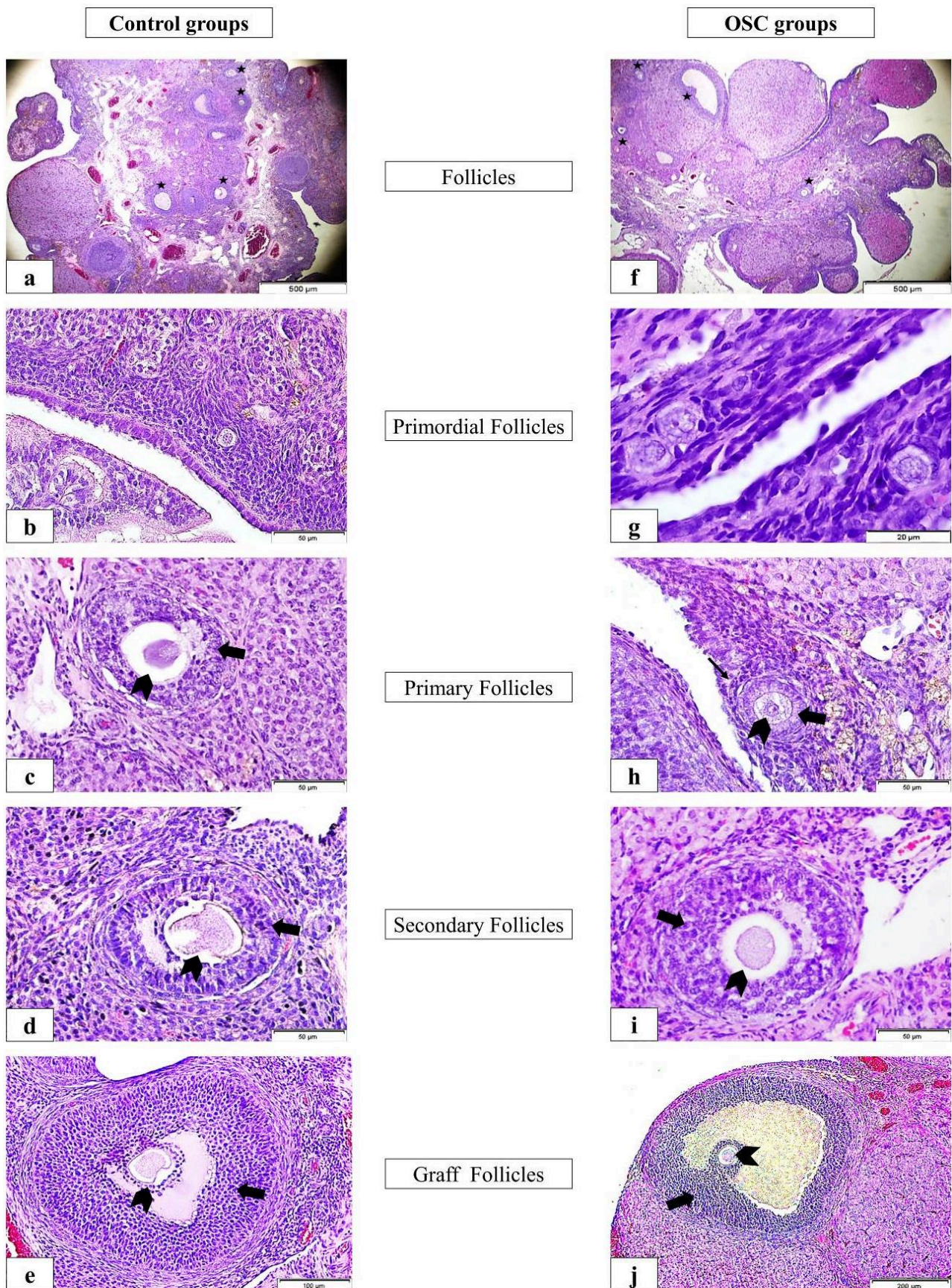
cells migrated from ovarian tissue by the third day and reached confluence by the ninth day, displaying a fibroblast-like morphology (magnification  $\times 20$ , inverted microscope)

oocytes and the zona pellucida was noted in atretic follicles.

In the OSC group, a noticeable improvement in overall morphology was observed. Granulosa cells, oocytes, and the zona pellucida appeared close to normal, with no signs of degeneration. A reduction in the number of atretic follicles was noted, and the number of healthy follicles was greater than in the control group. While the separations in the stromal tissue largely improved, some areas of persistence were still observed (Figs. 4a-j).



**Fig. 3:** Characterization of undifferentiated OSSCs, (a) Immunophenotyping of OSSCs by flow cytometry and (b) Graphical representations of the results



**Fig. 4:** H&E photomicrographs of ovarian sections. Panels (a-e) belong to the control group, while panels (f-j) belong to the OSC group. Follicles are indicated by stars (★), theca cells by thin arrows (→), granulosa cells by thick arrows (→), and oocytes by arrowheads (▶). Scale bars represent 500 μm (a, f), 50 μm (b, c, d, h, i), 20 μm (g), 100 μm (e), and 200 μm (j)

**Follicle counting**

Follicle counting was conducted using sections obtained from a total of six specimens from both groups. The right ovaries of the six rats in the treatment group that did not receive stem cell injections were evaluated as positive control. Based on these criteria, it was determined that the number of primordial follicles (Mean=9.50, SD=5.87, Min=2.33, Max=22.00) showed a statistically significant increase in the stromal stem cell group compared with the control and positive control groups ( $P<0.05$ ).

Although the number of primary follicles (Mean=13.12, SD=6.51, Min=6.00, Max=34.33) and preantral follicles increased in the OSC group as compared with the control and positive control groups, this difference was not statistically significant ( $P>0.05$ ). The numbers of secondary follicles (Mean=1.38, SD=0.70, Min=0.00, Max=2.67) and Graafian follicles (Mean=0.82, SD=0.47, Min=0.00, Max=1.50) in this group remained at the same level as the control group and did not show statistical significance ( $P>0.05$ ).

When comparing the positive control group with the OSC group, an increase in the number of secondary follicles was observed in the latter; however, this increase was not statistically significant ( $P>0.05$ ). Conversely, the number of atretic follicles (Mean=1.18, SD=1.05, Min=0.00, Max=3.33) in the OSC group significantly decreased compared with the control and positive control groups ( $P<0.05$ ) (Table 1 and Fig. 5).

**Immunohistochemical results**

In the OSC group, strong cytoplasmic and moderate nuclear expression of AMH was observed in the granulosa cells of primary, secondary, and Graaf follicles. The theca layer of secondary and Graaf follicles exhibited strong cytoplasmic staining. AMH expression in the oocytes of primary, secondary, and Graaf follicles was moderate in the cytoplasm and weak in the nucleus ( $P<0.05$ ) (Fig. 6, Fig. 7a, and Table 2).

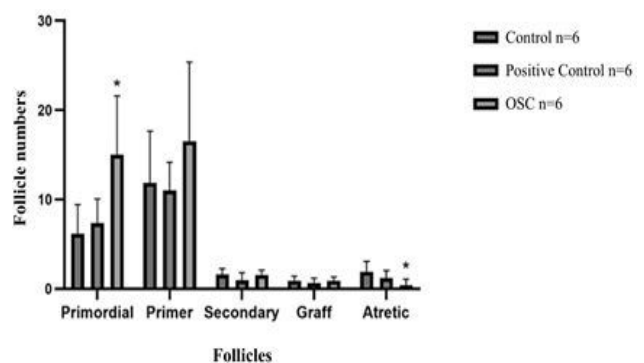
Additionally, in the OSC group, strong cytoplasmic and nuclear expression of FSHR was detected in the

granulosa cells of primary, secondary, and Graaf follicles, as well as in the theca layer of secondary and Graaf follicles and the stromal cells surrounding primary follicles. FSHR expression was also strong in the cytoplasm and nucleus of oocytes in primary, secondary, and Graaf follicles ( $P>0.05$ ) (Fig. 6, Fig. 7a, and Table 2).

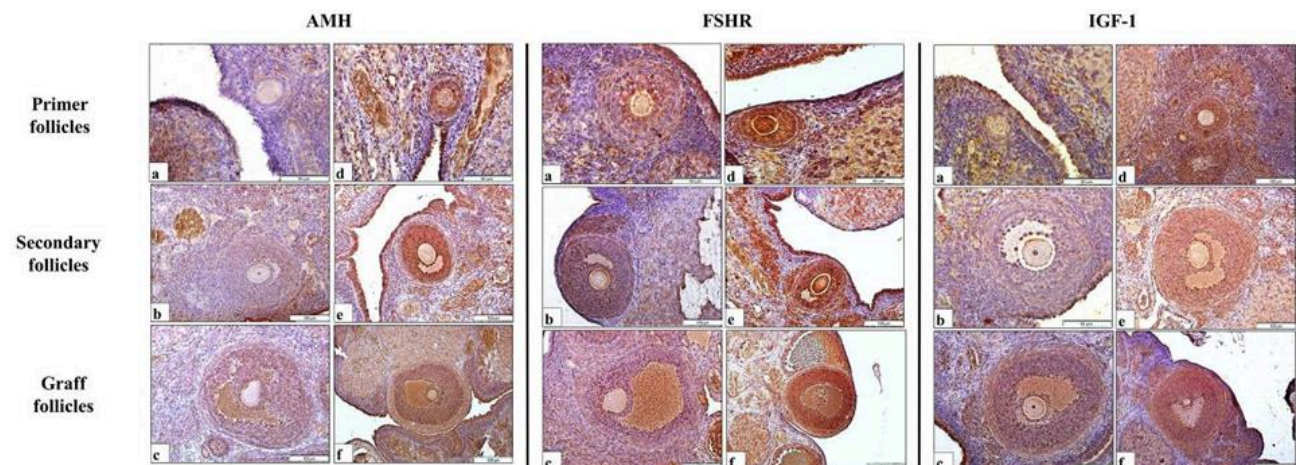
For IGF-1 expression, the OSC group showed strong cytoplasmic and moderate nuclear expression in the granulosa cells of primary, secondary, and Graaf follicles, while oocytes in these follicles exhibited moderate cytoplasmic and weak nuclear expression. In the stromal cells surrounding primary follicles and in the

**Table 1:** P-values for the statistical comparison of follicle counts (primordial, primary, secondary, Graafian, atretic, and total follicles) among the experimental groups.  $P<0.05$  was considered statistically significant

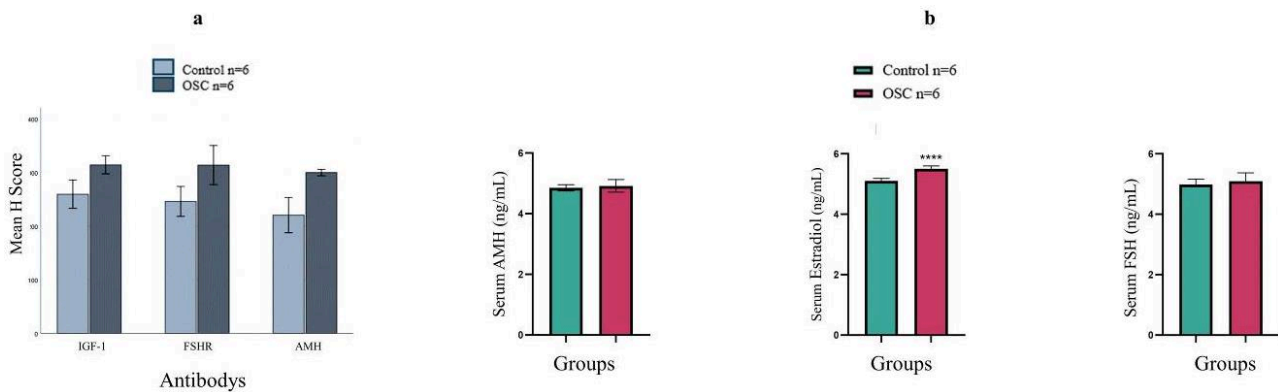
Follicle type	P-value
Primordial	0.008
Primer	0.3008
Secondary	0.227
Graff	0.6326
Atretic	0.036
Total follicle	0.0623



**Fig. 5:** Statistical analysis of follicle counts determined by hematoxylin and eosin (H&E) staining in the control, positive control, and OSC groups



**Fig. 6:** Images of ovarian photomicrographs immunostained for AMH, FSHR, and IGF-1. Control group (a-c) and OSC group (d-f). Scale bars: AMH, 50  $\mu$ m (a, d), 100  $\mu$ m (b, c, e), and 200  $\mu$ m (f); FSHR, 50  $\mu$ m (a, d), 100  $\mu$ m (b, c, e), and 200  $\mu$ m (f); IGF-1, 50  $\mu$ m (a, b), and 100  $\mu$ m (c, d, e, f)



**Fig. 7:** (a) Graphical representation of the H-score for IGF-1, FSHR, and AMH expression among groups, (b) The mean levels of AMH, FSH, and E2 among groups

**Table 2:** Comparison of the H-score and serum AMH, FSHR, and IGF-1 between the groups

Measured parameters	Control (n=6)	OSC (n=6)	P-value
<b>H SCORE</b>			
AMH	220.31±31.34	299.64±5.71	<0.02
FSHR	245.88±26.54	313.57±34.69	<0.09
IGF-1	259.41±25.22	314.08±16.12	<0.02
<b>ELISA</b>			
Serum AMH (ng/ml)	4.85±0.09	4.91±0.18	<0.5
Serum E2 (ng/ml)	5.10±0.07	5.02±0.20	<0.0001
Serum FSH (ng/ml)	4.98±0.16	5.09±0.25	<0.44

theca layer of secondary and Graaf follicles, IGF-1 expression was moderate both cytoplasmically and nuclearly ( $P < 0.05$ ) (Fig. 6, Fig. 7a, and Table 2).

### Comparison of serum AMH, FSHR, and IGF-1 levels

The ELISA results revealed that AMH levels increased in the OSC group compared with the control group; however, this increase did not reach statistical significance ( $4.919 \pm 0.18$ ) ( $P > 0.05$ ). Regarding FSH levels, no statistically significant decrease was observed in the OSC group, with values remaining like those of the control group ( $5.092 \pm 0.25$ ) ( $P > 0.05$ ). In contrast, a statistically significant increase in E2 levels was observed in the OSC group ( $5.502 \pm 0.08$ ) ( $P < 0.05$ ) (Table 2 and Fig. 7b).

### Discussion

One of the organs that shows the first signs of aging in a woman's life is the ovary (Zhang *et al.*, 2019a). In the last twenty years, several therapeutic strategies have emerged to enhance women's fertility and health, as well as to slow down, reverse, or improve ovarian aging. Hormone replacement therapy, which helps alleviate menopause symptoms in women, has been regarded as a universal treatment method for ovarian aging; however, it has been a subject of debate for many years due to its adverse side effects (Lobo, 2017). Various treatment methods are also used, such as mitochondrial therapy, antioxidants, epigenetic regulators, telomerase activators,

and traditional Chinese medicine. Still, most of these treatments have yet to undergo clinical trials (Wu *et al.*, 2022). Furthermore, there has been a significant increase in studies investigating the therapeutic potential of stem cells in improving ovarian function, preserving ovarian reserve, and addressing ovarian aging (Volarevic *et al.*, 2014). It has been reported that numerous groups of stem cells, such as embryonic stem cells, mesenchymal stem cells, extra-embryonic stem cells, induced pluripotent stem cells, and ovarian stem cells, have a therapeutic effect on ovarian damage (Filip *et al.*, 2008; Zhang *et al.*, 2019). For instance, Yan *et al.* (2024) reported that stem cell transplantation extended the reproductive lifespan in naturally aging cynomolgus monkeys. Their findings indicated improvements in hormone levels, follicular health, and ovarian morphology, highlighting the regenerative potential of stem cell therapy.

Moreover, recent evidence suggests that stem cell therapies may exert their effects not only through direct differentiation but also via paracrine mechanisms. Li *et al.* (2023) demonstrated that exosomes derived from human umbilical cord mesenchymal stem cells (UCMSCs) improved ovarian function in a model of natural ovarian aging. This was achieved through the delivery of miR-21-5p, which downregulated PTEN expression and inhibited apoptosis. Similarly, administration of adipose-derived mesenchymal stem cells (ASCs) significantly increased oocyte numbers and anti-Müllerian hormone (AMH) levels, as well as improved blastocyst formation rates in aged mice, further supporting their potential role in ovarian rejuvenation (Hirakawa *et al.*, 2024). In another study, Shin *et al.*

(2024) investigated whether mesenchymal progenitor cells derived from human embryonic stem cells (hESC-MPCs) could provide functional protection in aging ovarian tissue. Their results showed that multiple administrations of these cells enhanced ovarian reserve, regulated the estrous cycle, and improved fertility. Additionally, hESC-MPCs were found to reduce ovarian fibrosis by suppressing inflammation-related genes and inhibiting bone marrow-derived myeloid-derived suppressor cells (MDSCs) (Shin *et al.*, 2024).

In this study, we investigated the effects of stromal stem cells isolated from ovarian tissue on their own microenvironment. Specifically, our observations on the regulation of the ovarian extracellular matrix, support for follicular development, and improvement in overall morphology by stromal stem cells align with existing findings in the literature (Zhang *et al.*, 2021). Our flow cytometry analysis identified these cells as ovarian-derived stromal stem cells expressing CD90, CD54, and CD45 surface molecules, while exhibiting low levels of CD29 expression. Furthermore, the characterization of these cells revealed properties similar to those of MSCs. Previous research has shown that the CD surface molecules expressed by MSCs vary widely, making molecular characterization of the MSC phenotype ambiguous. However, some subpopulations of MSCs isolated from the same or different tissues have demonstrated non-uniform cell differentiation potential. In this context, the stromal stem cells used in our study are similar to findings from other studies (Roson-Burgo *et al.*, 2014). Similarly, Besikcioglu *et al.* (2019) characterized ovarian stromal stem cells isolated from rats and reported that these cells were negative for CD45 and CD11b/c, while positive for CD90 and CD49, further supporting the phenotypic heterogeneity and tissue-specific expression profiles observed in MSC populations (Besikcioglu *et al.*, 2019). Nevertheless, it is important to note that variations exist in the literature regarding the specific markers used to characterize and identify these cells.

In this study, the levels of serum AMH, FSH, and E2 hormones, as well as the expression levels of genes such as FSHR, IGF-1, and AMH in ovarian tissue, are among the markers that help us understand this process (Elvin and Matzuk, 1998; Maheshwari *et al.*, 2006). Although the effect of AMH on ovarian activity is quite complex, it is widely accepted that AMH regulates the number of developing follicles and the selection of follicles for ovulation. The cessation or reduction of AMH gene expression leads to the depletion of the primordial follicle pool. In our study, the increase in AMH expression levels led to characterizing and identifying primordial follicles and an increase in the ovarian reserve. This finding is consistent with the results of other studies by Durlinger *et al.* (1999) and Peluso *et al.* (2014) while increased serum AMH levels contributed to preserving the follicular reserve, it did not reach statistical significance. This outcome supports the regulatory role of AMH in ovarian function while indicating the need for further research.

The ovarian aging process has been associated with elevated serum FSH levels, low serum E2 levels, and decreased FSHR expression in ovarian tissue (Mehalko *et al.*, 2023). In one study, insufficient FSHR levels resulting from ovarian hypo-stimulation led to oocyte loss in mice (Danilovich and Sairam, 2002). However, while FSHR deficiency inhibits follicular development, high FSHR levels have been shown to slow ovarian aging (Casarini and Crépieux, 2019). Similarly, in our study, an increase in FSHR levels was observed along with improvements in follicular development and quality, while the rates of pre-ovulatory atresia significantly decreased.

IGF-1, which regulates lifespan and longevity, induces E2 production in granulosa cells in the ovary and enhances oocyte quality. It also plays a key role in dominant follicles selection, growth, and maturation (Kwintkiewicz *et al.*, 2010; Melnik *et al.*, 2011). Similarly, in our study, increased IGF-1 levels were accompanied by an increase in serum E2 levels, consistent with the findings of Kwintkiewicz *et al.* (2010). Additionally, it has been reported that elevated serum E2 levels improve ovarian function by enhancing estrogen biosynthesis in the body. In this context, the rise in E2 levels observed in our study aligns with the improvement in ovarian function (Rahayu *et al.*, 2018). *In vitro* studies have shown that IGF-1 added to ovarian tissue culture media supports primordial follicle activation and reduces DNA fragmentation. These findings support the contribution of increased IGF-1 levels to the rise in primordial follicles observed in our study (Louhio *et al.*, 2000; Bezerra *et al.*, 2018). Furthermore, we observed a significant reduction in morphological abnormalities in granulosa cells and the rate of atresia, consistent with the studies of Guthrie *et al.* (1998) and Demeestere *et al.* (2005). Studies by Baker *et al.* (1996) and Nakae *et al.* (2001) have demonstrated that mice deficient in IGF-1 exhibited decreased FSHR expression in granulosa cells, impaired ovarian steroidogenesis, and subsequent infertility. These findings support the simultaneous increase in IGF-1 and FSHR levels in our study. However, this increase did not reach statistically significant levels.

FSH, which plays a fundamental role in the reproductive system, is essential for the survival and growth of preantral follicles in the ovary (Randolph Jr *et al.*, 2004). A reduction in FSH levels in a model of premature ovarian insufficiency has been shown to improve ovarian function (Zhu *et al.*, 2015). In a study by Parte *et al.* (2013) and Patel *et al.* (2013) investigating ovarian stem cells from humans, sheep, marmosets, rabbits, and mice, it was reported that ovarian stem cells from sheep and humans express FSHR and are activated by FSH. However, in our study, despite improvements in ovarian function, the increase in FSHR levels was not statistically significant, and no changes were observed in serum FSH levels.

Despite notable histological improvements and increased expression of AMH and FSHR at the tissue level, serum levels of these hormones did not show

statistically significant changes in our study. However, it is important to note that these differences, although not being statistically significant, may still be biologically meaningful, especially when considered alongside the observed improvements in ovarian tissue structure and marker expression. This discrepancy may be attributed to several factors. Firstly, ovarian-derived changes in gene and protein expression often manifest locally and may not be immediately reflected in the systemic circulation, particularly within short experimental windows such as the 30 day period used in our model. Secondly, AMH and FSHR primarily function through paracrine and autocrine mechanisms within the ovarian microenvironment. Their local upregulation can support folliculogenesis without necessarily altering serum concentrations, especially when the endocrine feedback mechanisms within the hypothalamic-pituitary-gonadal axis remain intact. Thirdly, the relatively small sample size and physiological variability among subjects may have limited the statistical power to detect minor systemic changes, despite clear biological trends. Similar findings have been reported in previous ovarian rejuvenation studies, where histological and molecular benefits preceded detectable endocrine shifts (Li *et al.*, 2023; Hirakawa *et al.*, 2024; Shin *et al.*, 2024). Therefore, while serum AMH and FSH levels remained statistically unchanged, the observed histological restoration and enhanced intratissue expression of key regulatory markers suggest a favorable local response to OSSC therapy, warranting further investigation in larger cohorts and over extended observation periods.

While our study primarily focused on the roles of IGF-1 and AMH in the regulation of ovarian function, emerging evidence suggests that mesenchymal stem cells exert their therapeutic effects through a broader range of mechanisms. In particular, paracrine signaling has been highlighted as a key pathway through which stem cells promote tissue repair without differentiating into ovarian-specific cell types. For example, transplantation of human amnion-derived mesenchymal stem cells (hAD-MSCs) has been shown to improve ovarian function in a chemotherapy-induced premature ovarian insufficiency (POI) model by secreting regenerative factors such as VEGF, HGF, and IGF-1. These factors enhanced follicular survival, reduced granulosa cell apoptosis, and improved the ovarian microenvironment (Ling *et al.*, 2019). Likewise, angiogenin—secreted by MSCs—was identified as a critical factor promoting angiogenesis and follicle preservation in human ovarian tissue transplants (Zhang *et al.*, 2017). In a natural aging model, repeated administrations of human embryonic stem cell-derived mesenchymal progenitor cells (hESC-MPCs) increased primordial follicle counts, restored estradiol levels, and reduced ovarian fibrosis by downregulating inflammation-related pathways and modulating immune responses (Shin *et al.*, 2024). Taken together, these findings support the concept that ovarian-derived stromal stem cells (OSSCs) may exert regenerative effects not only through endocrine modulation, but also via paracrine, angiogenic, and

immunoregulatory mechanisms. Further studies are warranted to elucidate these pathways in greater detail.

Our research demonstrates that stromal stem cells (SSCs) transplanted into ovarian tissue significantly improve ovarian function and support folliculogenesis. OSSCs have been observed to increase the number of follicles in ovarian tissue and contribute to preserving primordial follicles. The findings from this study indicate that SSCs in ovarian tissue exhibit therapeutic effects, including regulating hormone levels, enhancing oocyte quality, and supporting follicle health. Notably, the increased expression of factors such as IGF-1 and AMH, which play crucial roles in folliculogenesis and cell growth, confirms the revitalizing effects of OSSCs on ovarian function.

These data suggest that SSCs therapy could emerge as an important treatment option for patients facing fertility challenges, particularly in pathophysiological conditions such as ovarian aging. This study provides valuable insights for the clinical transition to infertility treatments, representing a promising step toward broader applications of stem cell-based therapies.

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## Conflict of interest

The authors declare no conflict of interest.

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