

Comparison of ovarian striation and ovarian fragmentation in a rat model of ovarian insufficiency

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Ethics Committee Approval

Ethics committee approval was obtained from the University of Health Sciences Experimental Application and Research Center (HSEARC) Ethics Committee (Date:5.3.2019/No:4).

All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

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Conflict of Interest

No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Primary ovarian insufficiency (POI) is defined as the depletion of the primordial follicle pool in women under the age of 40. New methods for stimulating ovarian follicle cells are being investigated in order to ensure the continuity of the menstrual cycle and fertility. The present study aimed to compare follicle reserves after ovarian striation or ovarian fragmentation in rats with ovarian insufficiency.

Methods: Thirty adult female rats in the estrus phase were randomized into three groups. Group 1 and Group 2 were medicated with intraperitoneal 7.5 mg/kg paclitaxel to create ovarian insufficiency. Group 3 was the control group, and intraperitoneal 3 mL 0.9% sterile saline solution was administered. The first laparotomy was performed to evaluate ovarian insufficiency 1 week after chemotherapy. In Group 1, the right ovarian cortex was striated using an insulin injector. In Group 2, the right ovary was divided into five parts. These five pieces were transferred to the pocket created under the right pelvic peritoneum. In Group 3, only laparotomy was performed. After 1 month, all rats underwent a second laparotomy, and the number of ovarian follicles (primordial, primary, secondary, antral) were compared, as were their serum follicle-stimulating hormone (FSH) and estradiol (E2) levels.

Results: There was a significant difference in the number of follicles among all three groups ($P<0.05$). The number of follicles (primordial, primary, secondary, antral) was significantly higher in the striated group than in the fragmented group ($P<0.001$). There were no statistically significant differences between the three groups in terms of mean serum FSH and E2 values measured at the second laparotomy ($P>0.05$).

Conclusion: Ovarian striation on the ovary cortex may be a new method for the treatment of ovarian insufficiency.

Keywords: Follicle count, Fragmentation, Ovarian insufficiency, Ovarian striation, Rats

Introduction

Primary ovarian insufficiency (POI) is defined as the depletion of the primordial follicle pool and the permanent seizure of menstruation in women under the age of 40 years [1]. Usually, when the number of follicles declines below 1000 (considered the threshold value) follicular development and/or activation do not occur [2]. The prevalence of POI is reported to be approximately 1–3.7% and chemotherapy has been shown to cause increased risk [3, 4]. Patients with POI show symptoms of estrogen deficiency at an early age and they experience infertility problems due to impaired follicular development and ovulation. The probability of spontaneous pregnancy in patients after the diagnosis of POI can be 5–10% [5]. In patients with POI whose oocytes cannot be obtained, oocyte donation is recommended as an effective treatment [3].

Ovarian transplantation has been the subject of research for more than half a century. The first orthotopic transplantation of ovaries and cryopreservation in mice was reported by Parrott in 1960 [6]. The first live birth with ovarian cryopreservation in a human was reported by Donnez et al [7]. However, other methods of follicle stimulation have been explored in other studies. Recently, Kawamura et al. reported that follicle development can be stimulated by fragmentation and in vitro activation (IVA) [8].

In addition to ovarian fragmentation, new methods for stimulating ovarian follicle cells are being investigated. Several authors have explored the possibility of activating dormant cells via physical manipulation on the ovary, such as causing damage with an oocyte pick up needle, laparoscopic grasper, or scissor [9, 10]. These studies have reported promising results, but it is apparent that current evidence is insufficient to determine the comparative efficacy of such methods.

In the current study, we used a model of ovarian insufficiency in rats and applied striation and fragmentation to compare these two methods in terms of the changes in ovarian follicle reserves and the levels of follicle stimulating hormone (FSH) and estradiol (E2).

Materials and methods

Animals

Ethics committee approval was obtained from the University of Health Sciences Experimental Application and Research Center (HSEARC) Ethics Committee (Date:5.3.2019/No:4). Thirty adult female Wistar-Albino rats (16 weeks old, 200–250 grams) in the estrus phase were included in the study. The study was conducted at the HSEARC in accordance with the National Institutes of Health guidelines (NIH Publications No. 8023, revised 1978) for the care and use of animals. The rats were kept in a wire cage with a maximum of four rats per cage, under controlled light (12 hours light/12 hours dark), at 22–24°C, with a humidity level of 45–55%. Food (standard rodent chow) and water (tap water) were provided ad libitum.

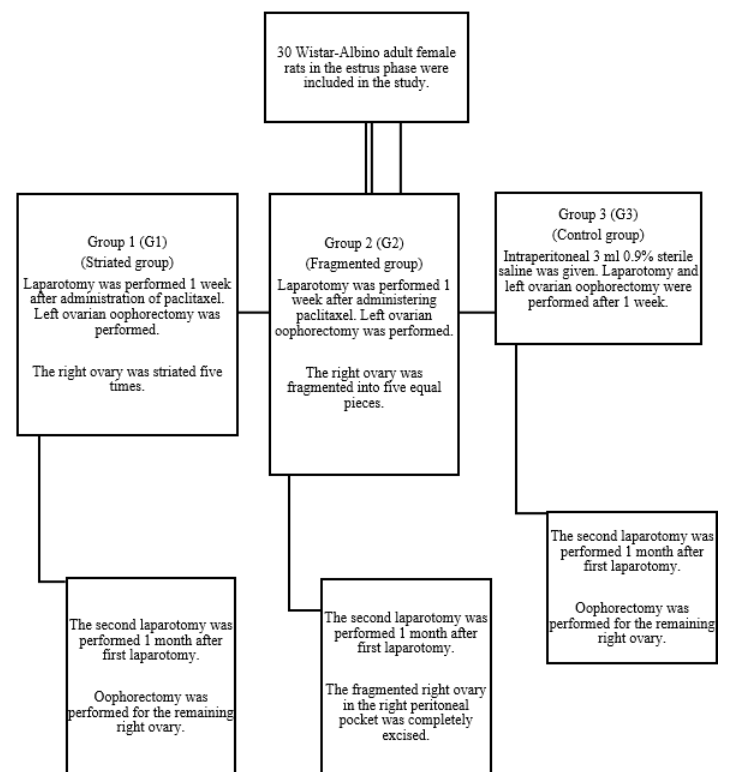
All animals were followed daily by research staff for pain-related behavioral changes in addition to the routine controls performed by veterinary staff at the research center. Weight was measured on a weekly basis and recorded. Any need

for urgent interventions were immediately reported by veterinary staff or researchers after daily checks, and the principle investigator responsible for animals was contacted to decide a course of action (intervening or euthanizing for humane purposes).

Experimental Design

The 30 adult female Wistar-Albino rats (in the estrus phase) were divided into three groups. Group 1 (G1) (n=10) and Group 2 (G2) (n=10) were administered intraperitoneal 7.5 mg/kg paclitaxel (Taxol, Bristol Myers Squibb, New York, USA) in the estrus phase to create ovarian insufficiency [11]. Group 3 (G3) (n=10) was the control group and received intraperitoneal 3 mL 0.9% sterile saline solution in the estrus phase. One rat from each of the G1 and G2 groups died on the third day after chemotherapy administration. Ovarian insufficiency was evaluated after 1 week, since the average estrous cycle is 4 days in rats. Before laparotomy was performed under anesthesia, all rats in the G1 (n=9), G2 (n=9), and G3 (n=10) groups were administered intraperitoneal ketamine (40 mg/kg; Ketalar, Pfizer, New York, USA) and xylazine (10 mg/kg; Rompun, Bayer, Leverkusen, Germany). The schematic representation of the study design is shown in Figure 1.

Figure 1: Flow chart of the study design



The primary outcome assessed in this study was follicle count after the fragmentation and striation procedures in rats with ovarian insufficiency, and the comparison of groups in terms of this result. Secondly, we also aimed to determine whether there were hormonal changes associated with these procedures.

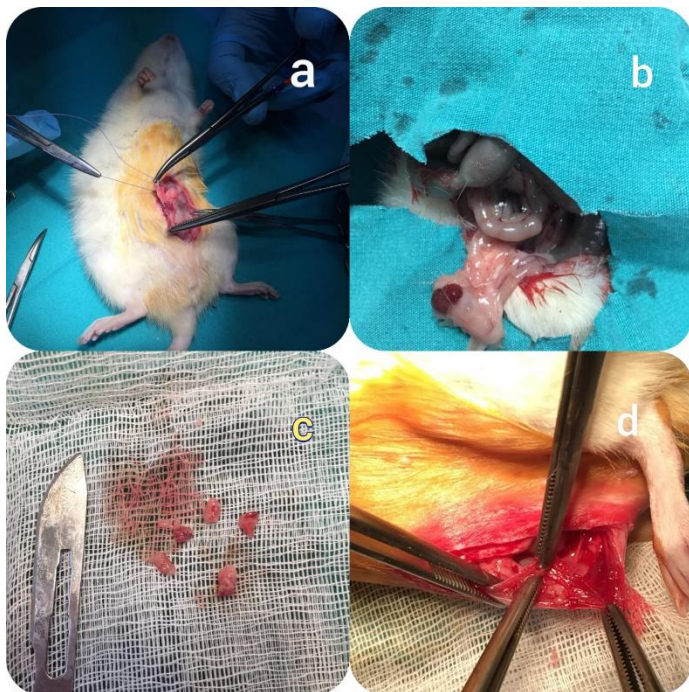
First laparotomy

The abdomen was aseptically prepared and a 3-cm vertical incision was made with a no. 11 scalpel, providing access through the midline. The surgical field was irrigated at regular intervals to reduce fluid loss. Left ovarian oophorectomy was performed in all rats. In the G1 (striated) group (n=9), the right ovarian cortex was striated five times with an insulin

injector at a width and depth of 1 mm. To increase consistency, all procedures were performed by the same operator. In the G2 (fragmented) group (n=9), the same operator performed all fragmentation procedures using a sterile ruler to divide the right ovary into five equal parts. These were kept in 0.9% sterile saline solution for about 15 minutes and were then transferred to the pocket created under the right pelvic peritoneum with the help of a cannula (Figure 2). In the G3 (control) group (n=10), laparotomy was performed, and the abdomen was closed after the completion of oophorectomy (left ovary). However, one rat in this group died after administration of anesthesia, reducing the number of rats in the control group to 9. A blood sample (1.5 ml) was taken from the tails of all rats to study serum levels of follicle stimulating hormone (FSH) and estradiol (E2).

After procedures and blood withdrawal, the rats were administered 3-5 ml of saline (0.9% NaCl) to compensate for fluid loss.

Figure 2: Images of the procedures; a) The right ovary after the striation procedure; b) The aspect of the striated right ovary during the second laparotomy; c) A fragmented ovary; d) The pocket created under the right pelvic peritoneum to transfer the fragmented ovary.



Second laparotomy

The second laparotomy of each animal was performed 1 month after the first via the same approach. However, during the second procedures, one rat from the G3 group died after the administration of anesthesia. At this point, there were a total of 26 rats left (G1=9, G2=9, G3=8). In the striated group (G1), oophorectomy was performed in the remaining right ovary. In the fragmented group (G2), the pocket containing the fragmented ovary (right pelvic peritoneum) was completely excised. In the control group (G3), oophorectomy was performed in the remaining right ovary. Following the respective procedures, the abdomen was closed. In all groups, 1.5-ml blood samples were obtained from the vena cava inferior to study serum FSH and E2 levels.

At the end of the experimental procedures, all 26 rats were euthanized. Cervical dislocation was performed under anesthesia using ketamine (75 mg/kg) and xylazine (10 mg/kg). The results of any animals that had died prior to intended sacrifice were excluded from the analyses.

Hormone analysis

All blood samples taken from the tail veins of rats were drawn into Becton Dickinson vacutainer gel tubes. Centrifugation was performed at 2000g for 8 minutes and sera were obtained. The serum FSH and E2 levels were measured with ELISA kits according to the manufacturer's guidelines (Wuhan USCN Business Co., Wuhan, China). Briefly, the procedure was as follows: 50 μ L of samples and standards were added to each well, followed by the immediate addition of 50 μ L of prepared Detection Reagent A. The plate was mixed by shaking, and incubated for 1 hour at 37°C. After aspirating and washing three times, 100 μ L of the prepared Detection Reagent B was added to the wells. The tubes were then incubated 30 minutes at 37°C. After aspirating and washing five times, 90 μ L of Substrate Solution was added. The final incubation was performed for 10–20 minutes at 37°C, followed by the addition of Stop Solution (50 μ L) and immediate measurement at 450 nm wavelength on a Biotech ELx800 device. In the second laparotomy, the serum FSH and E2 levels were studied twice, from samples obtained after the first and second laparotomy procedures.

Follicle count and morphological analysis

The G1, G2, and G3 oophorectomy materials and the fragmented ovary in G2 were fixed with 10% formaldehyde for pathological examination. The tissues were embedded in paraffin blocks for 24 hours, and serial sections of 4 μ m thickness were obtained. The entire ovarian surface was sectioned for follicle count and morphological analysis. The sections were stained with hematoxylin and eosin (H&E). All preparations were evaluated at x400 magnification under light microscopy (Leica, Wetzlar, Germany) by the same histopathologist (Ö.K.), who was blinded to the study. The follicles were classified as primordial, primary, secondary, early antral, antral, and atretic according to the definitions in the study by Myer et al., as follows:

1. Primordial follicle: Including an oocyte encircled by a partial or full squamous layer.
2. Primary follicle: Including a single layer of cuboidal granulosa cells.
3. Secondary follicle: Including multiple layers of granulosa cells and no detectable antrum.
4. Early antral follicle: Including one or two small follicular fluid sites; Antral follicle: including one large antral space.
5. Atretic follicle: Including a degenerate oocyte because of apoptosis [12].

Follicles containing prominent nuclei were counted. After the first laparotomy, the oophorectomy materials were used to demonstrate whether ovarian insufficiency occurred in G1 and G2. The oophorectomy performed in the second laparotomy was used to investigate whether there was a difference in the number of ovarian primordial, primary, secondary, and antral follicles between the three groups (G1, G2, and G3).

Statistical analysis

SPSS 20 (SPSS Inc, Chicago, IL, USA) software was used for the statistical analysis. The nonparametric Kruskal-Wallis test was used to assess the differences between the continuous dependent variables and the Bonferroni correction

was utilized to determine pairwise differences. The results are expressed as median and minimum-maximum values, if not stated otherwise. Box-plot graphical presentation was used to visualize group comparisons. Statistical significance was defined as P -value <0.05 .

Results

Ovarian hormone profile

The mean serum FSH value from the samples taken at the first laparotomy (G1, G2, G3) was significantly higher in G3 (the controls) than in G1 and G2 ($P=0.002$) (Table 1). There were no significant differences between the groups (G1, G2, G3) in terms of first laparotomy serum E2 values, and the serum FSH and E2 values from samples taken at the second laparotomy ($P>0.05$) (Table 1).

Table 1: Mean serum FSH and estradiol levels

Initial samples	Control (n=9) Median (Min-Max)	Striated (n=9) Median (Min-Max)	Fragmented (n=8) Median (Min-Max)	P-value
FSH (mIU/mL)†	7.25 (3.7-8.6)	3.96 (2.9-5.4) ^a	4.91 (3.9-5.7) ^a	0.002
E2 (pg/mL)†	107.9 (58-311)	84.84 (51-176)	78.30 (44-163)	0.599
Final samples				
FSH (mIU/mL)†	7.04 (4.1-10.0)	5.15 (3.8-6.7)	5.12 (3.8-5.5)	0.379
E2 (pg/mL)†	119.8 (44-310)	89.69 (47-435)	80.20 (52-138)	0.296

Same letter denotes the lack of statistically significant difference in pairwise comparison of the two groups (Bonferroni correction after >2 -group comparison reveals significance). FSH: follicle stimulating hormone; E2: estradiol.

Evaluation of the number of follicles and histological analysis

When the left oophorectomy materials (initial samples obtained 1 week after chemotherapy) were examined, the number of primordial follicles, secondary follicles, antral follicles, and total follicle count were significantly higher in G3 (the control group) ($P<0.005$) compared to the other groups. Therefore, the chemotherapy treatment was successful in creating ovarian insufficiency in the G1 and G2 groups.

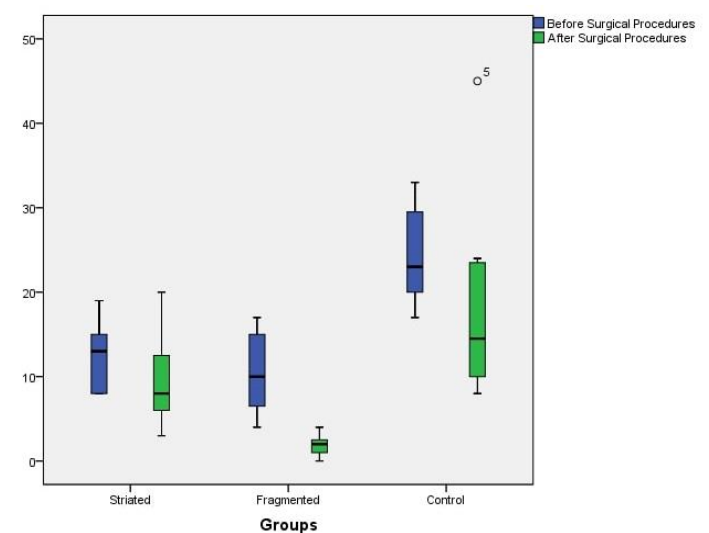
Evaluation of ovarian tissues after the second laparotomy (laparotomy + striation in G1, laparotomy + fragmentation in G2, and laparotomy in G3) demonstrated that there was a statistically significant difference in the number of follicles (primordial, primary, secondary, antral, and total follicle number) in the comparison of the three groups ($P<0.05$) (Table 2). The number of follicles (primordial, primary, secondary, antral follicles, and total follicle count) were significantly higher in G1 (Striated group) compared to G2 (Fragmented group) ($P<0.001$) (Table 2). The number of follicles after the striation and fragmentation procedures are depicted in box-plot graphical analysis (Figure 3).

Table 2: Follicle counts in the studied groups

Initial specimens	Control (n=9) Median (Min-Max)	Striated (n=9) Median (Min-Max)	Fragmented (n=8) Median (Min-Max)	P-value
Primordial follicles†	7 (3-10) ^a	5 (1-8) ^{a,b}	2 (1-6) ^b	0.003
Primary follicles†	3 (1-8)	3 (1-4)	2.5 (2-5)	0.477
Secondary follicles†	9 (5-13)	4 (2-9) ^a	3 (1-9) ^a	0.001
Antral follicles†	4 (3-10)	2 (0-5) ^a	1 (0-3) ^a	<0.001
Total number of follicles†	22* (17-33)	14 (8-21) ^a	10 (4-17) ^a	<0.001
Final specimens				
Primordial follicles†	4 (1-16) ^a	2 (1-5) ^{a,b}	1 (0-1) ^b	0.025
Primary follicles†	3 (1-7) ^a	1.5 (0-4) ^{a,b}	0.5 (0-2) ^b	0.004
Secondary follicles†	6.5 (3-17) ^a	3 (1-10) ^{a,b}	0.5 (0-1) ^b	0.003
Antral follicles†	2.5 (0-7)	2 (0-3)	0	0.002
Total number of follicles†	14.5 (8-45) ^a	8 (3-20) ^{a,b}	2 (0-4) ^b	<0.001

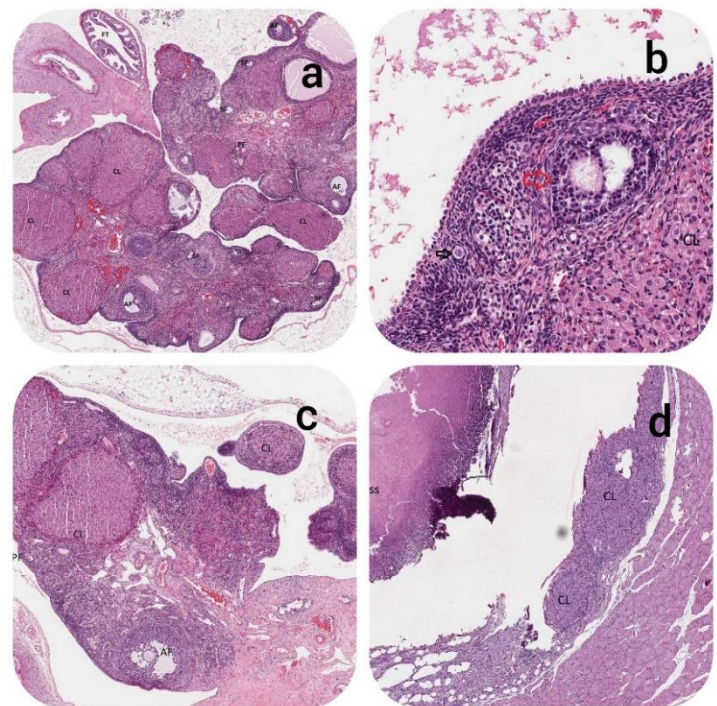
Same letter denotes the lack of statistically significant difference in pairwise comparison (Bonferroni correction after >2 -group comparisons have revealed significant difference). FSH: follicle stimulating hormone; E2: estradiol.

Figure 3: Box-plot graphical analysis of the total number of follicles for the three groups.



Cellular degeneration and follicular atresia were more frequent after chemotherapy (G1 and G2) compared to controls (G3). Abscess and fibrosis had developed in the ovarian tissue of three rats in G2 which had undergone fragmentation (Figure 4). An increase in the size of the right ovary was observed in the striated group (G1), as determined by macroscopic examination during the second laparotomy (Figure 2).

Figure 4: Ovarian tissue samples; a) General ovarian morphology in control ovary [H&E staining, x20]; b) Primordial follicle and secondary follicle in control group [H&E staining, x200]; c) Minimal fibrosis secondary to paclitaxel administration in striated group [H&E staining, x40]; d) Abscess areas and corpus luteum in the fragmented ovary group [H&E staining, x40] CL: Corpora lutea; PF: Primary follicle; SF: Secondary follicle; AF: Antral follicle; FT: Fallopian tube



Apart from the deaths that were detailed in the previous section, no adverse events were recorded throughout the study.

Discussion

Chemotherapy agents used for cancer treatment in women can impair ovarian function, which may result in reversible or irreversible amenorrhea and infertility [13]. Permanent ovarian insufficiency after chemotherapy is more common in women >35 -years-of-age. This is thought to occur due to age-related deterioration of the primordial follicle reserve [14]. Paclitaxel is a chemotherapeutic agent commonly used in the reproductive period for the treatment of epithelial cancer

[15]. In their study with mice, Gucer et al. [16] reported that paclitaxel reduced the number of primordial follicles in a dose dependent manner. In the study by Yucebilgin et al. [11], which utilized 7.5 mg/kg paclitaxel and 5 mg/kg cisplatin to create a model of ovarian insufficiency, it was reported that both agents were equally effective in causing ovarian insufficiency. Thus, we used paclitaxel to create ovarian insufficiency. Our results show that a 50% reduction was found in the number of follicles (primordial, secondary, antral, total) 1 week after chemotherapy.

Kawamura et al. reported that ovary fragmentation stimulated follicle development and mature oocyte formation via the Hippo signal mechanism [8]. They also noted stimulation of follicles and mature oocyte development with the use of AKT stimulators. In a remarkable study on mice by Li et al., the ovaries of animals were resected and placebo and activating treatments were administered. After the administration of treatments, both ovaries were placed under the kidney capsule of a host mice to stimulate the release of gonadotropin [17]. With this *in vitro* activation protocol, ovarian weight was reported to increase 3.4-times, and the number of antral follicles increased between 1.8- and 6-times in comparison to controls.

Polycystic ovary syndrome is a gynecological disease that causes anovulation. Ovary wedge resection or ovarian drilling methods have been used as surgical approaches to stimulate follicular development and achieve ovulation [18,19]. The mechanism of action of the ovarian drilling method is unclear [20]. In the oocyte pick-up procedure applied during *in vitro* fertilization, follicular aspiration, leads to follicle collapse. The remaining follicle cells are thought to undergo atresia after aspiration, but there are not enough studies on this subject to confirm this finding [10]. Ginther et al. hypothesized that the follicle content is filled after aspiration and that the follicle active components may not be completely damaged [21]. The appearance of the remaining ovarian tissue on ultrasonography 12–24 hours after follicle aspiration was suggested to demonstrate that steroidogenic activity continues in the residual follicle cells [22]. Furthermore, Viana et al. questioned whether residual follicular cells have a stimulating effect on follicular development before undergoing complete atresia [10]. Drawing from these studies, we investigated whether forming striation on the ovary cortex would be more effective than ovarian fragmentation in terms of stimulating follicles in the presence of ovarian insufficiency. In our study, there was no significant increase in the number of follicles after the striation procedure. However, the decrease in the number of follicles in the ovarian fragmentation group was significantly higher than in the striation group. Moreover, we observed that abscess and fibrosis developed in three rats whose ovarian pieces were placed under the parietal peritoneum. This finding indicates that blood supply may be impaired after relocation of ovaries. Interestingly, in a case report by Stern et al., it was found that ovarian pieces placed in the right pelvic peritoneum, left pelvic side wall, and in the atrophic left ovary showed activity in all three regions after cryopreservation [23]. However, in another study, it was reported that ischemia/reperfusion injuries may occur during cryopreservation, which may reduce the number of follicles [24].

C

Considering the varying results and conflicting findings, it is apparent that many factors could have an impact on the outcome, including technique, operator experience, and the blood supply potential of the inserted tissue. In addition, it should be noted that fragmentation is more invasive than striation. Furthermore, we did not apply stimulator therapy or vitrification in our study. Therefore, the effect of stimulator therapy on the ovaries must be investigated in further research.

We used paclitaxel chemotherapy to successfully induce ovarian insufficiency within one week. As such, the possibility of ongoing effects (of chemotherapy) on ovaries in the G1 and G2 groups should be considered as a cause of continued reduction in follicle count. The findings reported in previous studies support this view. In a study in which patients underwent chemotherapy for cancer (mean age: 34), the researchers found that mean anti-Mullerian hormone levels were 0.4 ng/mL 1 year after chemotherapy [14]. In agreement, a study by Decanter et al. reported that AMH levels continued to decline until 6 months after seizing chemotherapy [13]. Moreover, ovarian surgery itself may also have a negative effect on ovarian reserve. In a meta-analysis investigating the effect of oophorectomy on ovarian reserve, it was reported that ovarian reserve decreased after unilateral oophorectomy [25]. This may have affected the lower number of follicles observed after the second laparotomy in the control group.

We believe that utilizing the same operator in all interventions prevented inter-operator differences, which is an important advantage. We also attempted to ensure standardization by using a sterile ruler when applying treatments. To the best of our knowledge, this is the first study to compare ovary striation and ovarian fragmentation methods for the stimulation of follicles in the presence of ovarian insufficiency.

Limitations

Since rat ovaries are small, an insulin injector was used to striate the cortex of the ovaries; thus, the manual creation of striation and fragmentation methods can be considered the limitations of the current study. Additionally, the species-related differences may also cause significant differences in the results of these procedures; thus, future studies should investigate whether the results would be similar when these procedures are performed on humans.

Conclusion

Trying to stimulate the follicles by creating striation on the ovary cortex may be a new method for the treatment of ovarian insufficiency. There is a need for additional studies to elucidate whether performing striation on the ovary capsule and using stimulating agents can be utilized to achieve follicle stimulation in the presence of ovarian insufficiency.

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