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ORIGINAL ARTICLE

Evaluation effects of allopurinol and FSH on reduction of ischemia–reperfusion injury and on preservation of follicle after heterotopic auto-transplantation of ovarian tissue in mouse

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Abstract
Purpose Allopurinol and FSH injection are applied to reduce ischemia–reperfusion injury and to increase survival rate for ovarian follicles after ovarian heterotopic autotransplantation in mice.
Methods Ovarian tissues from 6-week-old mice were grafted into back muscle then collected after 3 weeks. A total of five groups were included in this experiment as follows: control group ($n = 5$), sham-operated group ($n = 5$), allopurinol treatment group (AP) ($n = 5$), follicle stimulating hormone (FSH) treatment group ($n = 5$), as well as, allopurinol and FSH treatment group (APF) ($n = 5$). We investigated survival, number and development of follicles, vaginal cytology along with plasma malondialdehyde (MDA) concentration in grafted ovary.
Results Total follicles count significantly increased in APF group compared with other treatment groups ($p < 0.05$). MDA concentration significantly decreased in AP group and APF treatment group compared with sham-operated group. In AP group, vaginal smears showed presence of cornified epithelial cells three–five day after surgery.
Conclusions We demonstrated that allopurinol, as a XO inhibitor, plays an important role in order to decrease ischemia injury and to increase survival rate for follicles. Also, FSH, as a folliculogenesis and angiogenesis factor, increases development of follicles. It seems that allopurinol can cause re-establishing hypothalamus–pituitary axis and finally can restore estrous cycle earlier than for the sham operated group, so it explains the increasing survival rate for follicles.

Keywords Allopurinol · Follicle survival · FSH · Ischemia/reperfusion · Ovarian transplantation

Introduction

Cancer is usually treated using chemotherapy, radiotherapy or surgery. These treatments lead to infertility or ovarian failure [1, 2]. One of the only options for fertility preservation in these patients is ovarian cryopreservation and followed by grafting of frozen-thawed ovarian tissue. It is noted that chemotherapy can reduce dramatically in vitro fertilization (IVF); therefore, before starting chemotherapy, cryopreservation of ovarian and ovarian transplantation should be applied [3]. However, ischemia and reperfusion after transplantation is the main problem which decreases follicular density in the grafted ovarian. Many sources of free radicals, such as xanthine oxidase, cyclooxygenase, lipoxygenase, mitochondrial enzymes, and activated neutrophils were generated during ischemia [4–9]. ATP synthesis is suppressed during ischemia [10–12] while ATP breakdown is increased during reperfusion (no neosynthesis), resulting in accumulation of

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hypoxanthine inside the cell and leading to transformation of xanthine dehydrogenase to xanthine oxidase (XO). During reperfusion, oxygen increases, and, XO metabolizes hypoxanthine to xanthine and uric acid, as well as reactive oxygen species (ROS). It is noted that XO has an important role in the generation of superoxide anions (such as generation of two O_2^- and two H_2O_2) [6, 13]. As regards, xanthine oxidase, as an important sources of free radical [14, 15] and allopurinol, and as an inhibitor of xanthine oxidase, it can block the synthesis of xanthine from hypoxanthine and uric acid from xanthine, therefore, the formation of the free radical superoxide, O_2^- and H_2O_2, are prevented [8, 13]. Recent studies have revealed the role of allopurinol on ischemia/reperfusion-induced liver injury [16–20]. Also, other studies have demonstrated that allopurinol has a protective effect on cerebral ischemia in rats [4]. Therefore, it seems that preservation of ovarian tissue and follicular density depends on reduction of ischemic injury and re-establishment of blood vessels in the grafted ovary [21]. One of the important angiogenic factors is vascular endothelial growth factor (*VEGF*) [22]. Several factors, such as hypoxia, cytokines and growth factors regulate *VEGF* gene expression [22]; in addition, it is reported that FSH and LH/hCG regulate the expression of *VEGF* in ovary [23–25]. So after ovarian transplantation, gonadotropins, such as FSH, increase the level of neovascularization and decrease ischemia–reperfusion injury [26]. A study has showed that treatment of a mouse with FSH for 4 or 7 days after ovarian transplantation causes the production of greater numbers of normal oocytes [27]. Also, mice treated with FSH for over 20 weeks show an increase in the number of antral follicles within human ovarian xenografts [28]. Gonadotropin treatment for 7 days has effects on porcine primordial follicles following xenografting to nude mice, so in treatment group, FSH (for 7-day) increases the level of follicular development compared with control mice [29]. The aim of this study was to evaluate the protective effects of allopurinol, as an xanthine oxidase inhibitor, on ischemia and reperfusion; and in addition, to assess the effects of FSH, as a folliculogenesis and angiogenesis factor, on increasing vascular formation and resulting in survival and development of follicles.

Materials and methods

Animals

Six-week-old (NMRI) mice were used in this study. The animals were housed in a 12:12-h light/dark cycle and 18–25 °C with the free access to food and water. The protocol was approved by the Ethics Committee of Royan Institute. Female mice were randomly assigned to one of five following groups (1) Control group was with intact ovary without treatment factors ($n = 5$), (2) Second group was grafted group without treatment factors ($n = 5$), (3) Third group received allopurinol ($n = 5$) (SIGMA-ALDRICH, Co., ST. Louis, USA), (4) Fourth group was treated with FSH ($n = 5$) (GONAL-F, MERCK SERONO, USA) and (5) Fifth group was treated with allopurinol and FSH ($n = 5$).

Transplantation procedure

Six-week-old female mice were anesthetized by intraperitoneal injection of ketamine and xylazine diluted with phosphate-buffered saline (PBS). After shaving and sterilizing skin, an incision of about one cm long was made on the skin, and then muscle was scratched. It was followed by immediate transplantation of fresh ovaries isolated from the fallopian tube and oviduct to back muscle of the same mice ovariectomized. In each mouse, the left ovary was transplanted to back muscle and the right ovary was removed. The grafting site was carefully stitched under sterile conditions.

Experimental design

In a preliminary study, various doses of allopurinol were injected into mice and the optimal doses were selected (data not shown).

A total of five groups were included in this experiment as follows: (1) Control group was with intact ovary without treatment factors, (2) sham-operated group received 100 µl/day PBS during 2 weeks after grafting intraperitoneally. (3) Allopurinol treatment group received 5 mg/kg/day allopurinol from 30 min before grafting to 1 week after grafting intraperitoneally. (5) FSH treatment group, received 1 IU/day FSH intraperitoneally from 1 week after grafting, according to a report by Soleimani et al. [30], starting FSH stimulation from the day or 1 week after grafting does not make a significant difference, so the FSH treatment group received a FSH injection 1 week after grafting. Allopurinol treatment group received 5 mg/kg/day allopurinol from 30 min before grafting to 1 week after grafting intraperitoneally. (5)Allopurinol and FSH treatment group received 5 mg/kg/day allopurinol from 30 min before grafting to 1 week after operation, as well as, 1 IU/day FSH from 1 week after grafting for 1 week. Three weeks after transplantation, all groups received 7.5 IU FSH, followed by 7.5 IU human chorionic gonadotropin (hCG) in, 48 h later. Also, grafts from back muscle sites were recovered 10–12 h after hCG injection.

Histological analysis

Three weeks after ovarian transplantation, all grafted ovary tissues were fixed in Bouin's solution followed by formaldehyde in, 48 h later. Then, they were embedded in paraffin wax; serial sections of 5 μm in thickness were made and they were stained by hematoxylin and eosin (H&E) staining. The follicles were examined and counted, under a microscope. To prevent recounting of the same follicle, follicles with dark-staining nucleolus of oocyte were counted, only the healthy follicles were counted among all groups. Follicles are considered atretic when more than 1 % pyknotic nuclei are found in the granulosa cells, or when the oocyte begins to degenerate [31]. Follicles were classified as primordial, primary, preantral, and antral follicles. Primordial follicles were confirmed when oocytes were surrounded by one layer of flattened pregranulosa cells. Primary follicles were confirmed when an oocyte was seen with one layer of cuboidal granulosa cells. Preantral follicles were confirmed with two or more layers of granulosa cells without antrum, and antral follicles were identified with an antral cavity [32].

Vaginal smear

Vaginal cytology is used as a method for evaluating ovarian activity after transplantation [33]. A vaginal smear were taken daily after transplantation, from all mice in the treatment group. This method consists of inserting a moistened cotton bud swab into the vagina, gently removing the cells from the vaginal walls and the cells were transferred to a glass slide. Vaginal cells were left to air dry after being smeared on a lumen, and then the stage of the estrous cycle was determined from the cell types (presence or absence of leukocytes, cornified epithelial, and nucleated epithelial), observed in the smear [34].

Measurement of malondialdehyde content

Two week after transplantation, some animals in each group were anesthetized and bled by cardiac puncture. The blood was centrifuged with 3000 rpm for 5 min, serum was separated and it was stored at −30 °C for stress oxidative assay.

Concentration of total malondialdehyde in serum, as a marker of oxidative stress, was determined by NWLSS™ malondialdehyde assay (Northwest Life Science Specialties, NWK-MDA01, USA). The lipid peroxidation product, such as MDA, is usually used to measure the level of the oxidative stress.

In this test, butylated hydroxyl toluene (BHT) was added to a microcentrifuge vial to minimize oxidation of lipids that contribute artifactually during sample processing and the Tio-barbituric acid (TBA) reaction [35]. The MDA assay was estimated by assessing the level of TBARS, so calibrator or sample, acid reagent and thio-barbituric acid reactive substances (TBARS), were added to a vial and were incubated for 60 min at 60 °C. Then, microcentrifuge vials were centrifuged at $10000 \times g$ for 2–3 min and the absorbance was recorded at 532 nm.

Statistical analysis

Data are present as the mean ± SEM and were analyzed by one way ANOVA and Kruskal–Wallis test. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Estrus cycle stage determination

All animals demonstrated cyclic changes in vaginal cytology characteristic of estrous cycles. When cornified cells are in high numbers, they usually form clumps and sheets as observed in all groups. But, vaginal smears in mice receiving allopurinol (AP and APF groups) showed the presence of cornified epithelial cells within 5–7 days after transplantation. But, the sham-operated group and the FSH treatment group showed an estrus cycle in 7–10 days after transplantation. So, the treatment group with allopurinol showed the estrus cycle earlier than the sham-operated group.

Follicle morphology in grafted ovary

Ovarian grafts were collected within 3 weeks after transplantation. Establishment of well-organized blood vessels was apparent in tissue and neovascularization of the grafted ovaries was visible. Among different groups, re-vascularization was more prominent in the APF group. Figure 1 demonstrates re-vascularization, providing a specific blood stream around the grafted ovary site. All type of follicles (primary, primordial, pre antral and antral) were observed in the grafted ovary among all groups, but the number of follicles was significantly more in the APF group compared with other treatment groups (Fig. 2).

Follicular survival and development

The results of the follicular count per ovary are shown in Table 1. The number of total follicles showed a significant increase (433.60 ± 5.38) in mice receiving allopurinol and FSH in comparison with other groups. Also, the population of primordial, primary, preantral and antral follicles revealed a significant increase in the APF group

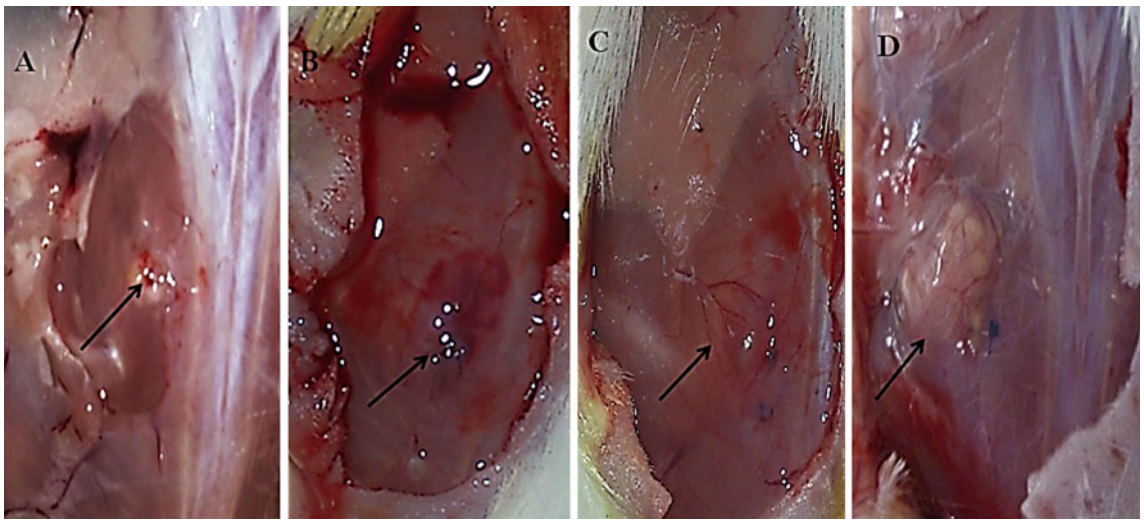

Fig. 1 **A–D** Reestablishment of blood vessels: **A** sham-operated group, **B** FSH treatment group, **C** allopurinol treatment group, and **D** allopurinol and FSH treatment group

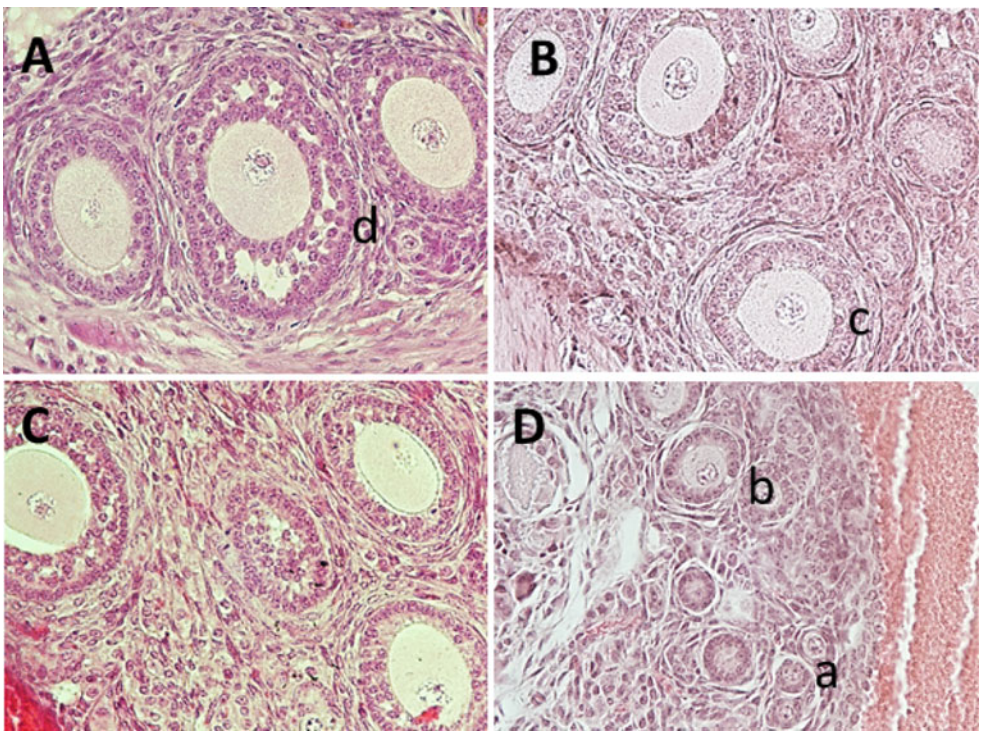

Fig. 2 Follicular density in all groups. **A** Allopurinol and FSH treatment group, **B** allopurinol treatment group, **C** FSH treatment group, and **D** sham operated group. *a* primordial follicle, *b* primary follicle, *c* preantral follicle and *d* antral follicle

compared with other groups. Approximately 49 % of the total follicle population was made up of primary follicles, whereas the percentage of primordial follicles in the APF group was dropped to 36 % among the follicle population. A similar result could be seen in the FSH treatment group, the percentage of primary follicles is more than primordial follicles that were recorded as 58 and 43 %, respectively. In the absence of hormonal treatment (FSH), the percentage of primordial follicles was more than primary follicles, such as in the AP group. The obtained data of the APF group showed the mean numbers of preantral follicles and antral follicles are 48.80 ± 2.20 and 12.20 ± 1.46, respectively, so these treatment factors caused a significant increase in all follicular stages. Stimulating with gonadotropin (FSH) had no significant effect in increasing the number of

Table 1 Number of follicles in autotrasplanted mouse ovary to the back muscle

Treatment	Primordial	Primary	Preantral	Antral	Total	%
Control ($n = 5$)	262.27 ± 12.84^{a}	149.60 ± 9.17^{b}	76.80 ± 3.80^{a}	17.40 ± 1.88^{a}	506.60 ± 15.90^{a}	100
FSH ($n = 5$)	109.20 ± 2.45^{d}	$144.40 \pm 11.09^{b,c}$	14.20 ± 2.51^{d}	4.80 ± 1.15^{b}	253.60 ± 12.13^{d}	50
AP ($n = 5$)	159.20 ± 5.48^{b}	117.40 ± 5.83^{c}	27.20 ± 3.30^{c}	5.20 ± 1.35^{b}	309.01 ± 7.59^{c}	61
APF ($n = 5$)	157.80 ± 3.58^{b}	213.80 ± 5.91^{a}	48.80 ± 2.20^{b}	12.20 ± 1.46^{a}	433.60 ± 5.38^{b}	85
Sham ($n = 5$)	99.40 ± 6.31^{d}	32.20 ± 2.67^{d}	4.20 ± 0.73^{d}	1.40 ± 0.40^{b}	137.20 ± 7.76^{e}	27

Values (mean ± SEM) with the different superscript letter within the same column are significantly different ($p < 0.05$)

APF allopurinol and FSH treatments group, *AP* allopurinol treatment group, *FSH* FSH treatment group

Table 2 Concentration of MDA in serum in treatment groups

Treatment	Control	FSH group	AP group	APF group	Sham group
Mean ± SEM	2.75 ± 0.32^{c}	$4.74 \pm 0.18^{b,c}$	3.57 ± 0.27^{c}	3.19 ± 0.16^{c}	7.35 ± 0.45^{a}

Values (mean ± SEM) with the different superscript letter are significantly different ($p < 0.05$)

APF allopurinol and FSH treatments group, *AP* allopurinol treatment group, *FSH* FSH treatment group

primordial, primary, preantral and antral follicles, compared with other treatment groups. However, the primary follicles count had a significant increase (144.40 ± 11.09) compared with the sham-operated group (32.20 ± 2.67). Total follicles count was significantly higher in the FSH treatment group compared to the sham-operated group (253.60 ± 12.13 vs. 137.20 ± 7.76, respectively). Total follicles count was significantly higher in the AP group compared to the FSH treatment and the sham-operated groups (309.01 ± 7.59 vs. 253.60 ± 12.13 and 137.20 ± 7.76, respectively). There was no significant difference between APF and AP groups in the number of primordial follicles(159.20 ± 5.48 and 157.80 ± 3.58, respectively, $p < 0.05$). AP and FSH treatment groups showed significant differences in the number of primordial follicles (159.20 ± 5.48 vs. 109.20 ± 2.45) and preantral follicles (27.20 ± 3.30 vs. 14.20 ± 2.51). The AP group and sham-operated group showed significant differences in the number of primordial follicles (159.20 ± 5.48 vs. 99.40 ± 6.31) and preantral follicles (27.20 ± 3.30 vs. 4.20 ± 0.73). The number of primordial, primary, preantral and antral follicles was significantly lower in the sham-operated group compared with treatment groups.

Serum MDA level

Concentration of MDA was measured with the NWLSS™ malondialdehyde assay kit. The results are summarized in Table 2. There was no significant difference in concentration of MDA between the control group with the APF group (2.75 ± 0.32 and 3.19 ± 0.16, respectively) and the AP group (2.75 ± 0.32 and 3.57 ± 0.27, respectively). The animals receiving allopurinol and FSH showed a significant decrease in MDA concentration in comparison with the sham-operated group (3.19 ± 0.16 vs. 7.35 ± 0.45). Concentration of MDA in the FSH treatment group revealed a significant increase compared with the control group (4.74 ± 0.18 vs. 2.75 ± 0.32), but it was significantly reduced compared with the sham-operated group (4.74 ± 0.18 vs. 7.35 ± 0.45).

Discussion

Suppression of blood supply, during ischemia, is the critical factor that can cause production of ROS, releasing of free radicals, tissue injury, and finally activation of the endogenous defense. Ischemic–reperfusion injury occurs after ovarian transplantation, while it is an important reason for follicular loss rather than the cryopreservation in the grafted ovary [36, 37]. Different studies have demonstrated that free radicals play an important role in ischemia–reperfusion injury in several organ systems [38–40].It takes approximately 48 h to establish revascularization; however, ischemia–reperfusion injury occurs before revascularization on a graft site [26]. In this condition, some follicles may be lost due to lipid peroxidation, releasing free radicals and ROS production after surgery and before re-establishing new blood vessels. The antioxidant defense mechanism regulates the amount of ROS production. Allopurinol is capable of reducing free radicals due to its antioxidant properties and its ability of acting as a XO inhibitor [12]. In this regard, Xo plays an important role in generating superoxide anions, so allopurinol, as a XO inhibitor, reduces superoxide anions production, such as O_2^- and H_2O_2. Some studies have reported that ROS production during organ transplantation causes irreparable

injury to the tissue, whereas allopurinol is capable of reducing the damage though decreasing the production of free radicals [12, 19]. A lot of studies have demonstrated that ischemia injury causes dramatic reduction of follicle density after ovarian transplantation. Some studies have demonstrated that 25 % of the primordial follicles [37] and 60–95 % of growing follicles [41] are reduced after transplantation. A study by Liu et al. [42] has reported that only 58 % of total follicles remain after heterotopic transplantation of ovarian tissue. In another study, follicle density dramatically decreases in intact and non-intact intramuscular auto-grafted mouse ovaries in comparison with a control group [43]. In our study, we demonstrated that the survival rate for folliculars is approximately 85 % after allopurinol along with FSH injection, compared with the control group.

Another major factor for functional preservation of a grafted ovary is to re-establish neovascularization after transplantation [26]. FSH modulates the expression of *VEGF*, as a critical angiogenic factor, in an ovary [23–25]. In this regard, FSH can't directly scavenge free radicals (such as allopurinol), so its angiogenesis properties may increase follicular survival. In our study, new blood vessels formation around the grafted ovary site was clearly visible after 3 weeks. Israely et al. [44] showed that grafts remained avascular on days 1–3 after transplantation but showed functional vessels on day 7. Another study has demonstrated that murine angiogenesis initiated from day 5 and completed in human ovaries transplanted into naked mice by day 10 [45]. Therefore, FSH injection from 7 day after transplantation can increase functional vessels and decrease ischemia–reperfusion injury and MDA concentration. Kou et al. [46] have reported that exogenous XO reduce vascular tube formation, but endogenous XO increased the level of *VEGF* and angiogenesis. Also, another study has demonstrated that H_2O_2 in low concentration induces new vessel formation, while it suppresses the process at high concentration [47, 48]. In this respect, allopurinol inhibits XO, and consequently reduces H_2O_2 production and FSH is an angiogenesis factor, so we can expect an increasing level of angiogenesis.

Reducing plasma MDA concentration can be due to a decrease in free radicals through inhibition of xanthine oxidase in the treatment groups. Our results demonstrate a beneficial effect of allopurinol on free radical reduction. Therefore, it seems that allopurinol increases blood supply to tissue, causes re-establishment of the hypothalamus–pituitary axis, and finally restores the estrous cycle earlier than that in the sham operated group.

Reducing MDA levels in the FSH treatment group can be because of its angiogenesis property, re-establishment of blood vessels, decreases in the level of ischemia–reperfusion injury and free radicals, and MDA concentration.

Another study has reported that allopurinol can reduce the level of apoptotic cell death, MDA concentration and serum tumor necrosis factor-alpha (TNF-α) during a hepatic ischemia–reperfusion procedure [20]. Our study suggests that the protective effect of allopurinol prevents tissue injury and causes survival of follicles. We demonstrated that allopurinol, as a reducer factor in the formation of ROS, decreases ischemia–reperfusion injury and FSH, as angiogenesis and folliculogenesis factors, increases neovascularization and development of follicles toward antral follicles.

We observed resumption of meiosis in follicles. The resumption of nuclear maturation in oocytes within the antral follicles indicates that preservation of follicular and oocyte function occurs during 3 weeks after transplantation using allopurinol and FSH. These observations confirm that after grafting, follicles at the primordial stage or later stage start growing; furthermore, after transplantation, it starts developing rather than remaining static.

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