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Combination of FSH and testosterone could enhance activation of primordial follicles and growth of activated follicles in 1-day-old mice ovaries *in vitro* cultured for 12 days

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Research Article

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Abstract

Treatment with follicle-stimulating hormone (FSH) and testosterone (T2) and their combination have been observed to be influential on ovarian follicles of 1-day-old mice ovaries cultured for 8 days. Given that extension of the culture period could positively impact the development of follicles in cultured ovaries, the present study was conducted to evaluate the main and interaction effects of FSH by T2 on the development of ovarian follicles in 1-day-old mice ovaries cultured for 12 days. One-day-old mice ovaries were initially cultured with base medium for 4 days; thereafter, different hormonal treatments were added to the culture media, and the culture was continued for 8 additional days until day 12. Ovaries were collected for histological and molecular assessments on day 12. The greatest activation of primordial follicles and progression of activated follicles to the preantral stage was detected in ovaries treated with the combination of FSH and T2 ($P < 0.05$). This positive effect on the morphology of ovarian follicles was accompanied by upregulation of *Pi3k*, *Gdf9*, *Bmp15*, *Cx37* and *Fshr* in the ovaries cultured with the combination of FSH and T2 ($P < 0.05$). Nonetheless, treatment with FSH and T2 led to a diminished proportion of intact follicles ($P < 0.05$), even though *Bax*/*Bcl2* gene expression ratio, as an apoptotic index, was less in hormone-treated ovaries ($P < 0.05$). In conclusion, the combination of FSH and T2 could improve the activation of primordial follicles and the growth of activated follicles towards the preantral stage. This positive effect of FSH plus T2 appeared to be at least partly mediated through the upregulation of *Pi3k* and oocyte-derived growth factors including *Gdf9* and *Bmp15*.

Introduction

Development of the ovary in mouse has a unique biology since the formation of primordial follicles initiates from the late fetal period and continues during the early neonatal period (Eppig and O'Brien, 1996; Findlay *et al*., 2015; Kerr *et al*., 2006; Pepling, 2006). Due to this characteristic, a 1-day-old mouse ovary only contains germ cells and primordial follicles, and in turn, it can be used as a model for studying the formation and activation of primordial follicles under *in vitro* condition (Atrabi *et al*., 2019; Eppig and O'Brien, 1996; Khodadi *et al*., 2022; Morohaku *et al*., 2016; O'Brien *et al*., 2003). This animal model could assist to refine the approaches for *in vitro* activation of primordial follicles and development of activated follicles towards the production of MII-stage oocyte in cancer patients having experienced excision of the ovaries owing to oncotherapy (Atrabi *et al*., 2019; Ford *et al*., 2020; Sarma *et al*., 2019). This technique has some advantages as compared with other contemporary techniques because it does not postpone the commencement of oncotherapy as do cryopreservation of oocyte and *in vitro* production of the embryo and it does not potentiate the risk for re-introduction of malignant cells in the body as does transplantation of ovarian tissue (Amorim and Shikanov, 2016; Bockstaele *et al*., 2012; Fathi *et al*., 2017; Jeruss and Woodruff, 2009). Nonetheless, this technique currently has some limitations since it has not been as successful in prepubertal girls as it has been in pubertal women (Luyckx *et al*., 2013; Telfer *et al*., 2008; Telfer and Zelinski, 2013). In addition, *in vitro* activation of primordial follicles could serve as a potential method for the preservation of endangered animal species, in which lack of mature and fertilizable oocytes is

a serious restricting concern for the application of assisted reproductive techniques (ARTs) (Jewgenow and Paris, 2006; Khodadi *et al.*, 2022; Nagashima *et al.*, 2019).

In this context, a number of studies have investigated the impact of various components and biological materials, including granulosa cell conditioned medium, amino acids, pyruvate, testosterone and insulin-transferrin-selenium, on the development of follicles in 1-day-old mice ovaries (Alborzi *et al.*, 2020; Atrabi *et al.*, 2019; Atrabi *et al.*, 2021; Khodadi *et al.*, 2022). More recently, the main and interaction effects of follicle-stimulating hormone (FSH) and testosterone were investigated on 1-day-old mice ovaries under *in vitro* conditions for 8 days, and FSH was found to increase the activation of primordial follicles, development of primary and preantral follicles and expression of some genes related to development of ovarian follicles (Torkzadeh *et al.*, 2023). The combination of FSH and testosterone could also upregulate the expression of some factors contributing to the development of ovarian follicles; however, it did not significantly affect the morphological development of ovarian follicles based on histological analyses (Torkzadeh *et al.*, 2023). Given that there is a time lag between molecular alterations and cellular changes (Fan *et al.*, 2019; Kranc *et al.*, 2019; Mukherjee *et al.*, 2012), we hypothesized that culturing for a longer period of time might lead to the manifestation of the positive effects of FSH plus testosterone combination in the morphology of ovarian follicles as well. In corroboration with this hypothesis, the extension of culture duration from 5 to 11 days has been reported to improve the growth of follicles to the preantral stage (Alborzi *et al.*, 2020; Atrabi *et al.*, 2021).

Accordingly, the present study was conducted to evaluate the main and interaction effects of FSH by testosterone on the development of ovarian follicles in a 1-day-old mouse ovary cultured for 12 days.

Materials and methods

Animals

Female 1-day-old Naval Medical Research Institute mice were maintained under stable conditions (temperature: 20–25°C, 40–60% humidity) in a 12 h light-dark cycle. All animal procedures were conducted in accordance with the guidelines of the Royan Ethics Committee (IR.ACECR.ROYAN.REC.1398.248). In the present study, 36 1-day-old female mice were used for histological and molecular examinations. Herein, it is worth noting that the left and right ovaries from the same mouse were used for a single experimental group to avoid any potential confounding factors concerning the side of the ovary.

Isolation of 1-day-old mice ovaries

The ovaries were isolated from newborn 1-day-old mice under a stereomicroscope (Nikon, Tokyo, Japan). Immediately after removal, ovaries were placed in α-minimum essential medium (α-MEM; Gibco, Paisley, UK) containing 10% knockout serum replacement (KSR; Gibco, Paisley, UK), supplemented with 100 IU/ml penicillin-streptomycin under mineral oil (Sigma-Aldrich, St. Louis, USA), and subsequently, the surrounding tissues were meticulously removed.

Culture of isolated ovaries and experimental design

Isolated 1-day-old mice ovaries were cultured in 96-well plates containing 300 µl of base medium containing α-MEM supplemented with 10% KSR and 100 IU/ml penicillin-streptomycin for the initial 4 days. Given that the assembly of primordial follicles continues up until post-natal day 5 (Eppig and O'Brien, 1996; Findlay *et al.*, 2015; Kerr *et al.*, 2006; Pepling, 2006) and we intended to evaluate the effect of various hormones on primordial follicles, the hormonal treatments were added to the culture media after implementation of a preliminary 4-day culture. After day 4 of culture, the ovaries were randomly allocated to four experimental groups, including (1) control group (CON; base medium), (2) FSH group (FSH; base medium plus 1.36 IU/ml FSH), (3) testosterone group (T2; base medium plus 0.1 mM testosterone) and (4) combination of FSH and testosterone group (FSH+T2; base medium plus 1.36 IU/ml FSH and 0.1 mM testosterone). Next, the ovaries were cultured for 8 additional days, and the ovarian samples were collected on day 12 for histological and molecular assessments. The ovaries allocated to various experimental groups were cultured in a single plate in each culture replicate, and each culture replicate for all experimental groups began on the same day to avoid any potential confounding factor concerning the culture plate or date for the commencement of culture. The plates were incubated at 37°C with 5% CO2, and approximately 75% of the culture medium was replaced by fresh medium every 4 days in all experimental groups.

Histological analysis

To examine follicular development in the experimental groups, the cultured ovaries (n = 10 ovaries belonging to 5 mice for each experimental group) were fixed in Bouin's solution for 45 min and then in formalin at 4°C for at least 48 h, dehydrated with an alcohol gradient procedure, embedded in paraffin, serially sectioned at a thickness of 6 µm and mounted on glass slides. After drying at 42°C for 12 h, the sections were deparaffinized in xylene, hydrated by a gradient alcohol protocol and stained by haematoxylin and eosin. In order to avoid counting a follicle more than once, only one out of four consecutive sections was assessed under a light microscope (Nikon, Tokyo, Japan), and merely data of follicles with a clear oocyte nucleus were recorded for further analysis. The follicles were classified into primordial follicles (an oocyte surrounded by a few squamous granulosa cells; Figure 1.A), transitional follicles (an oocyte surrounded by both squamous and cuboidal granulosa cells; Figure 1.B), primary follicles (an oocyte surrounded by a single layer of cuboidal granulosa cells; Figure 1.C), preantral follicles (an oocyte surrounded by two or more layers of cuboidal granulosa cells; Figure 1.D) and atretic follicles (a degenerating oocyte with shrunken cytoplasm and deteriorated nucleus; Figure 1.E). The percentage of non-atretic follicles was considered as the proportion of intact follicles. Activation of follicles was the cumulative proportion of all activated follicles including transitional, primary and preantral follicles.

Real-time PCR (RT-PCR)

Real-time polymerase chain reaction (RT-PCR) was applied to evaluate the gene expression of factors regulating the development of ovarian follicles (Table 1). Initially, cultured ovaries in various experimental groups (n = 4 mice for each experimental group) were stored in RNA later (Qiagen, Hilden, Germany) at −80°C. Total RNA was extracted using RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Then, cDNA was synthesized from RNA using the standard oligo (dT) reverse transcription protocol considering the manufacturer's guidelines. qRT-PCR was conducted using Power SYBR Green PCR Master Mix on an ABI Step One Plus

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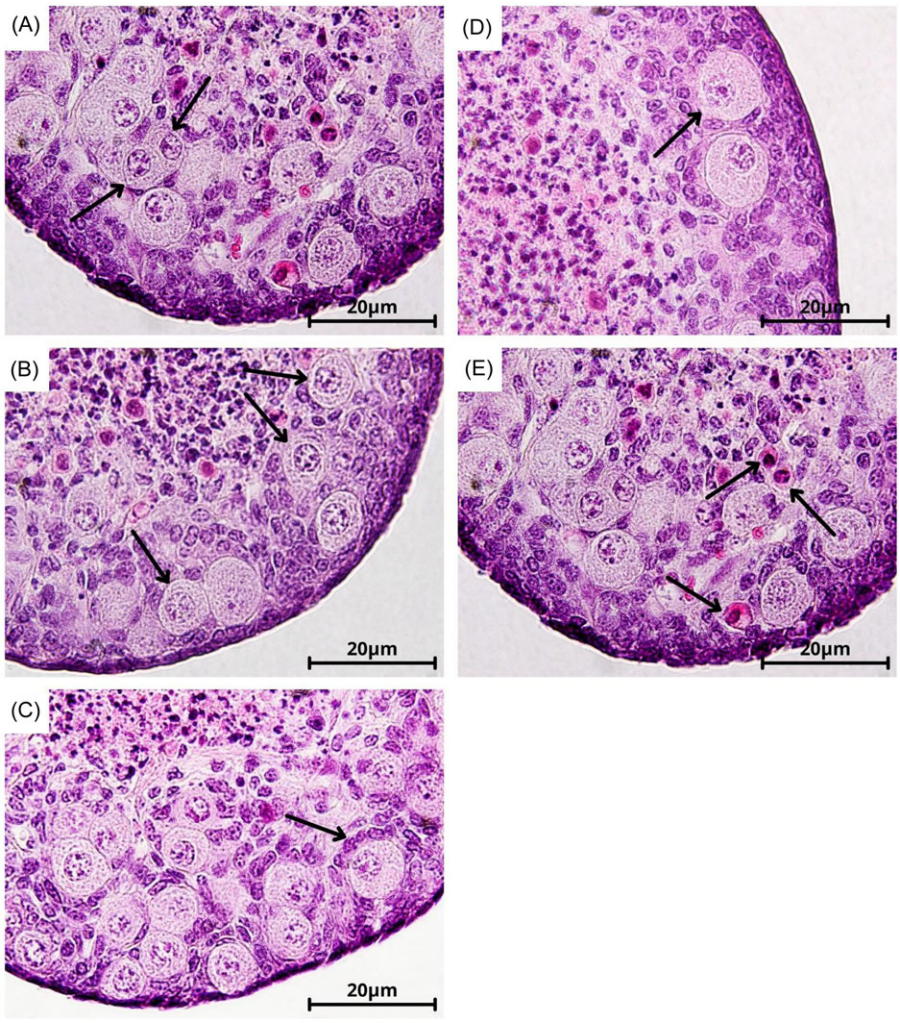

Figure 1. Various stages of ovarian follicles in 1-day-old mouse ovaries stained with haematoxylin and eosin staining. (A) Primordial follicles with one layer of squamous granulosa cells. (B) Transitional follicles with both squamous and cuboidal granulosa cells. (C) Primary follicles with a single layer of cuboidal granulosa cells. (D) Preantral follicles with two or more layers of cuboidal granulosa cells. (E) Atretic follicles with a degenerating oocyte. Black arrows denote the specified follicle.

thermocycler (Applied Biosystems, Warrington, UK). Reactions were run for 40 cycles at 95°C for 10 min, 95°C for 15 s and 61°C for 61 s, and each sample was run in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used to normalize the data as a housekeeping gene. The sequence of primers used in the present study is presented in Table 1. Primer efficiency analysis was carried out by running a standard curve and calculating the slope. The primers were diluted to the concentration recommended by the kit supplier (1:10), and qPCR reactions were performed using various concentrations of the template cDNA. All the primers used in our experiment showed efficiency ranging between 90 and 110%. To confirm the success of qPCR amplifications, we conducted gel electrophoresis using the qPCR products. For this purpose, 1% agarose gel was prepared with Tris-acetate-EDTA buffer, which included a DNA ladder as a standard beside the qPCR products to assess the presence and size of DNA fragments. Electrophoresis was carried out at 150 V for 1 h. Following this, a UV transilluminator was utilized to visualize the DNA bands, which verified the success of qPCR amplifications by confirming that the bands matched the expected sizes.

Statistical analysis

Initially, data were tested for normal distribution using the *Kolmogorov–Smirnov* test (UNIVARIATE procedure). This study had a factorial experimental design, in which the main and interaction effects of two factors (i.e. FSH and testosterone) on the development of follicles and expression of genes were analyzed (two-way model). Therefore, data of histological and molecular assessments were analyzed using the generalized linear model procedure. Multiple comparisons were conducted using the LSMEANS statement. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± SEM and differences were considered statistically significant at $P < 0.05$.

Results

Histological analysis

The absolute number of primordial follicles was negatively affected by the main effect of T2 ($P = 0.033$); however, it was not influenced by the interaction effect of FSH by T2 and the main effect of FSH ($P > 0.05$; Table 2). The proportion of primordial follicles was less in FSH+T2 than FSH group ($P = 0.033$). The proportion of primordial follicles was not affected by the main effect of FSH ($P > 0.05$); however, it was negatively affected by the main effect of T2 ($P = 0.015$; Table 2). The absolute number of transitional follicles was less in the FSH than in the CON group ($P = 0.001$; Table 2). The proportion of transitional follicles was not affected by

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Table 1. Sequences of primers for real-time polymerase chain reaction test

Gene	Primer sequence	Primer length (bp)
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	F: GACTTCAACAGCAACTCCCAC	125
	R: TCCACCACCCTGTTGCTGTA	
Phosphatase and tensin homolog (*Pten*)	F: TGGATTCGACTTAGACTTGACC	180
	R: GCGGTGTCATAATGTCTCTCAG	
Phosphoinositide 3-kinase (*Pi3k*)	F: GGAGACCAGTCACCCTATC	214
	R: CCGAGGTAGTGAGAGGATGTC	
KIT ligand (*Kitl*)	F: CTAAAGATGAACCCTCAGCCT	142
	R: GCATAACACATGAACACTCCA	
Anti-Müllerian hormone (*Amh*)	F: CTAGCCACCTTCGGAGTCTG	162
	R: AGGCTCTTGGAACTTCAGCA	
Growth differentiation factor 9 (*Gdf9*)	F: TGAACAACTCTGCCTCTTCC	250
	R: ATGCTAAACACTCCGTCCTC	
Bone morphogenetic protein 15 (*Bmp15*)	F: AAATGGTGAGGCTGGTAA	148
	R: TGAAGTTGATGGCGGTAA	
Connexin-37 (*Cx37*)	F: GTTCCTCTTCGTCAGCAC	254
	R: CCAGCACACTCTTACACAG	
Connexin-43 (*Cx43*)	F: TAAGTGAAAGAGAGGTGCCCAGA	200
	R: GGTTGTTGAGTGTTACAGCGAAAG	
Bcl-2-associated X protein (*Bax*)	F: TTGCTACAGGGTTTCATCCAG	246
	R: CCAGTTGAAGTTGCCATCAG	
B-cell lymphoma 2 (*Bcl2*)	F: GAGACTTCCCTGCTGAAAGAC	214
	R: TCCAGAAGCCTTTGTTTCCTC	
Ki67	F: ATCATTGACCGCTCCTTTAGGT	104
	R: GCTCGCCTTGATGGTTCCT	
Follicle-stimulating hormone receptor (*Fshr*)	F: ACGCCATTGAACTGAGATTTG	134
	R: GAACACATCTGCCTCTATTACC	

the interaction effect of FSH by T2 and the main effect of FSH ($P > 0.05$); however, it was positively influenced by the main effect of T2 ($P = 0.022$; Table 2). The absolute number of primary follicles was less in the FSH and T2 groups than in the CON group ($P < 0.05$; Table 2). The proportion of primary follicles was not affected by the interaction effect of FSH by T2 and the main effects of FSH and T2 ($P > 0.05$; Table 2). The absolute number of preantral follicles was less in the FSH and T2 groups than in the CON and FSH+T2 groups ($P < 0.01$; Table 2). The proportion of preantral follicles was greater in the FSH+T2 group than in the FSH and T2 groups ($P < 0.05$). Yet neither the main effect of FSH nor the main effect of T2 affected the proportion of preantral follicles ($P > 0.05$; Table 2). The absolute number of activated follicles was less in the FSH and T2 groups than CON group ($P < 0.05$; Table 2). Activation of primordial follicles was greater in FSH+T2 than FSH group ($P = 0.030$). Activation of primordial follicles was not affected by the main effect of FSH ($P > 0.05$); however, it was positively affected by the main effect of T2 ($P = 0.017$; Table 2). The absolute number of intact follicles was less in the FSH, T2 and FSH+T2 groups than the CON group ($P < 0.01$; Table 2). The proportion of intact follicles was less in the FSH and FSH+T2 groups than the CON group ($P < 0.01$). The proportion of intact follicles was negatively affected by the main effect of FSH ($P < 0.001$); however, it was not impacted by the main effect of T2 ($P > 0.05$; Table 2). The total number of follicles was fewer in the FSH and T2 groups than the CON group ($P < 0.05$; Table 2).

Gene expression

Factors associated with development of ovarian follicles

The absolute and relative expression of *Pten* was not affected by the interaction effect of FSH by T2 and the main effects of FSH and T2 ($P > 0.05$; Table 3). The absolute and relative expression of *Pi3k* was greater in the FSH+T2 group than in CON, FSH and T2 groups ($P < 0.05$); moreover, it was greater in T2 than in the CON group ($P < 0.05$; Table 3). The absolute and relative expression of *Gdf9* was greater in the FSH and FSH+T2 groups than in the CON group ($P < 0.05$; Table 3). The absolute and relative expression of *Bmp15* was greater in the FSH+T2 group than CON and FSH groups ($P < 0.05$; Table 3). The absolute and relative expression of *Gdf9* and *Bmp15* was positively influenced by the main effects of FSH and T2 ($P < 0.05$). The absolute and relative expression of *Amh* and *Kitl* was not affected by the interaction effect of FSH by T2 and the main effects of FSH and T2 ($P > 0.05$; Table 3). The absolute and relative expression of *Cx37* was greater in FSH+T2 than in the CON group ($P = 0.017$; Table 3). The absolute and relative expression of *Cx37* was positively influenced by the main effect of FSH ($P < 0.05$); moreover, the absolute expression of *Cx37* was positively impacted by the main effect of T2 ($P = 0.022$). The absolute and relative expression of *Cx43* was not affected by the interaction effect of FSH by T2 and the main effects of FSH and T2 ($P > 0.05$; Table 3). The absolute and relative expression of *Fshr* was greater in T2 and FSH+T2 groups than in CON and FSH groups ($P < 0.01$; Table 3). Moreover, the expression of *Fshr* was positively influenced by the main effect of T2 ($P < 0.0001$).

Factors associated with cellular proliferation and apoptosis

The absolute and relative expression of *Ki67*, *Bax* and *Bcl2* was not affected by the interaction effect of FSH by T2 and the main effects of FSH and T2 ($P > 0.05$; Table 4). However, the *Bax*/*Bcl2* gene expression ratio based on either absolute or relative expression of *Bax* and *Bcl2*, as an apoptotic index (Atrabi *et al.*, 2019), was less in FSH, T2 and FSH+T2 groups than CON group ($P < 0.05$; Table 4). *Bax*/*Bcl2* gene expression ratio was negatively influenced by the main effects of FSH and T2 ($P < 0.05$).

Discussion

The present study was conducted to evaluate the main and interaction effects of FSH and T2 on follicular development of 1-day-old mice ovaries cultured for 12 days. To begin with,

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Table 2. The number and proportion of various stages of follicles, activated follicles and intact follicles as well as total number of follicles of the ovaries in CON, FSH, T2 and FSH + T2 groups (n = 10 ovaries belonging to 5 mice for each experimental group). Data are presented as mean ± SEM

		Experimental group			
Variable		CON	FSH	T2	FSH + T2
Primordial follicles	Number	47.25 ± 8.95	34.88 ± 2.09	29.75 ± 3.24	28.63 ± 4.17
	Percentage	8.28 ± 1.07^{ab}	9.84 ± 0.75^{a}	7.36 ± 0.75^{ab}	6.19 ± 0.92^{b}
Transitional follicles	Number	210.88 ± 17.35^{a}	137.13 ± 5.87^{b}	174.13 ± 14.02^{ab}	183.38 ± 9.18^{ab}
	Percentage	37.55 ± 1.04	38.37 ± 1.88	42.58 ± 1.41	40.40 ± 1.34
Primary follicles	Number	227.13 ± 27.51^{a}	142.75 ± 12.01^{b}	158.75 ± 12.98^{b}	167.13 ± 13.59^{ab}
	Percentage	39.33 ± 1.34	39.19 ± 1.70	38.77 ± 1.30	36.19 ± 0.99
Preantral follicles	Number	82.63 ± 6.03^{a}	45.75 ± 5.22^{b}	47.25 ± 6.44^{b}	81.13 ± 9.41^{a}
	Percentage	14.83 ± 0.64^{ab}	12.60 ± 1.22^{a}	11.28 ± 0.90^{a}	17.22 ± 1.11^{b}
Activated follicles	Number	520.63 ± 48.87^{a}	325.63 ± 16.57^{b}	380.38 ± 29.88^{b}	431.63 ± 30.51^{ab}
	Percentage	91.72 ± 1.07^{ab}	90.16 ± 0.75^{a}	92.64 ± 0.75^{ab}	93.81 ± 0.92^{b}
Intact follicles	Number	248.13 ± 28.97^{a}	110.75 ± 8.09^{b}	149.88 ± 15.72^{b}	136.50 ± 12.67^{b}
	Percentage	43.46 ± 3.37^{a}	30.85 ± 1.93^{b}	36.32 ± 1.95^{ab}	29.72 ± 1.83^{b}
Total number of follicles	Number	567.88 ± 54.19^{a}	360.50 ± 15.96^{b}	410.13 ± 30.69^{b}	460.25 ± 31.91^{ab}

^{a,b}Different letters denote significant difference among various experimental groups ($P < 0.05$). Differences presented by superscripts here in this table are based on multiple comparisons by the post-hoc test LSMEANS.

Table 3. Absolute and relative gene expression of factors associated with development of ovarian follicles in CON, FSH, T2 and FSH + T2 groups (n = 4 mice for each experimental group). Data are presented as mean ± SEM

		Experimental group			
Gene		CON	FSH	T2	FSH + T2
Pten	Absolute expression	$(4.16 \pm 0.28) \times 10^{-2}$	$(3.60 \pm 0.50) \times 10^{-2}$	$(3.41 \pm 0.36) \times 10^{-2}$	$(2.94 \pm 0.30) \times 10^{-2}$
	Relative expression	1.00 ± 0.07	0.86 ± 0.12	0.82 ± 0.09	0.71 ± 0.07
Pi3k	Absolute expression	$(1.35 \pm 0.05) \times 10^{-4a}$	$(1.49 \pm 0.13) \times 10^{-4ab}$	$(2.10 \pm 0.22) \times 10^{-4b}$	$(3.10 \pm 0.22) \times 10^{-4c}$
	Relative expression	1.00 ± 0.04^{a}	1.11 ± 0.09^{ab}	1.56 ± 0.16^{b}	2.30 ± 0.17^{c}
Gdf9	Absolute expression	$(1.34 \pm 0.16) \times 10^{-2a}$	$(2.23 \pm 0.26) \times 10^{-2b}$	$(2.13 \pm 0.20) \times 10^{-2ab}$	$(2.90 \pm 2.03) \times 10^{-2b}$
	Relative expression	1.00 ± 0.12^{a}	1.67 ± 0.19^{b}	1.59 ± 0.15^{ab}	2.17 ± 0.19^{b}
Bmp15	Absolute expression	$(3.28 \pm 0.44) \times 10^{-3a}$	$(4.36 \pm 1.27) \times 10^{-3a}$	$(5.59 \pm 0.66) \times 10^{-3ab}$	$(8.49 \pm 0.65) \times 10^{-3b}$
	Relative expression	1.00 ± 0.13^{a}	1.33 ± 0.39^{a}	1.71 ± 0.20^{ab}	2.59 ± 0.20^{b}
Amh	Absolute expression	$(3.63 \pm 0.44) \times 10^{-4}$	$(5.86 \pm 1.22) \times 10^{-4}$	$(4.98 \pm 0.95) \times 10^{-4}$	$(6.63 \pm 1.03) \times 10^{-4}$
	Relative expression	1.00 ± 0.12	1.61 ± 0.34	1.37 ± 0.26	1.83 ± 0.28
Kitl	Absolute expression	$(3.32 \pm 0.48) \times 10^{-3}$	$(4.58 \pm 0.81) \times 10^{-3}$	$(4.28 \pm 1.35) \times 10^{-3}$	$(4.75 \pm 1.10) \times 10^{-3}$
	Relative expression	1.00 ± 0.14	1.38 ± 0.25	1.29 ± 0.41	1.43 ± 0.33
Cx37	Absolute expression	$(2.34 \pm 0.69) \times 10^{-3a}$	$(7.67 \pm 3.30) \times 10^{-3ab}$	$(8.61 \pm 2.92) \times 10^{-3ab}$	$(13.28 \pm 2.08) \times 10^{-3b}$
	Relative expression	1.00 ± 0.29^{a}	4.35 ± 1.44^{ab}	3.68 ± 1.25^{ab}	5.68 ± 0.89^{b}
Cx43	Absolute expression	$(4.03 \pm 1.28) \times 10^{-3}$	$(10.92 \pm 2.74) \times 10^{-3}$	$(8.17 \pm 1.17) \times 10^{-3}$	$(6.29 \pm 1.26) \times 10^{-3}$
	Relative expression	1.00 ± 0.21	2.04 ± 0.51	1.52 ± 0.22	1.17 ± 0.24
Fshr	Absolute expression	$(1.10 \pm 0.27) \times 10^{-4a}$	$(0.98 \pm 0.19) \times 10^{-4a}$	$(2.51 \pm 0.41) \times 10^{-4b}$	$(3.32 \pm 0.16) \times 10^{-4b}$
	Relative expression	1.00 ± 0.24^{a}	0.89 ± 0.17^{a}	2.28 ± 0.38^{b}	3.02 ± 0.15^{b}

^{a,b,c}Different letters denote significant difference among various experimental groups ($P < 0.05$). Differences presented by superscripts here in this table are based on multiple comparisons by the post-hoc test LSMEANS.

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Table 4. Absolute and relative gene expression of factors associated with cellular proliferation and apoptosis in CON, FSH, T2 and FSH + T2 groups (n = 4 mice for each experimental group). Data are presented as mean ± SEM

Gene		Experimental group			
		CON	FSH	T2	FSH + T2
Ki67	Absolute expression	$(3.11 \pm 0.64) \times 10^{-3}$	$(2.91 \pm 0.60) \times 10^{-3}$	$(4.00 \pm 0.84) \times 10^{-3}$	$(3.80 \pm 0.71) \times 10^{-3}$
	Relative expression	1.00 ± 0.21	0.93 ± 0.19	1.28 ± 0.27	(1.22 ± 0.23)
Bcl2	Absolute expression	$(2.37 \pm 0.46) \times 10^{-3}$	$(3.69 \pm 0.91) \times 10^{-3}$	$(3.21 \pm 0.26) \times 10^{-3}$	$(3.45 \pm 0.83) \times 10^{-3}$
	Relative expression	1.00 ± 0.19	1.56 ± 0.38	1.36 ± 0.11	1.46 ± 0.35
Bax	Absolute expression	$(8.63 \pm 0.72) \times 10^{-2}$	$(5.87 \pm 1.96) \times 10^{-2}$	$(4.74 \pm 1.04) \times 10^{-2}$	$(6.00 \pm 1.26) \times 10^{-2}$
	Relative expression	1.00 ± 0.08	0.68 ± 0.23	0.55 ± 0.12	0.70 ± 0.15
Bax/Bcl2	Absolute expression	53.85 ± 14.05^{a}	14.68 ± 4.85^{b}	13.93 ± 2.43^{b}	20.41 ± 3.45^{b}
	Relative expression	1.48 ± 0.39^{a}	0.40 ± 0.13^{b}	0.38 ± 0.07^{b}	0.56 ± 0.09^{b}

[a,b]Different letters denote significant difference among various experimental groups ($P < 0.05$). Differences presented by superscripts here in this table are based on multiple comparisons by the post-hoc test LSMEANS.

it was shown that T2 promoted the activation of primordial follicles in ovaries cultured for 12 days, which agreed with the results of previous studies in ovine (Qureshi *et al.*, 2008), porcine (Magamage *et al.*, 2011) and human (Bailie *et al.*, 2023). Conversely, treatment with T2 in 1-day-old mice ovaries cultured for 8 days did not impact the activation of primordial follicles in our recent study (Torkzadeh *et al.*, 2023). In this context, it is possible that the impact of T2 on 1-day-old mice ovaries required more time to materialize and its effect on ovarian follicles could be less pronounced in short-term culture systems. The positive effect of T2 on follicular activation was probably mediated through upregulation of *Pi3k* in the cultured ovaries because PI3K plays a key role in the activation of primordial follicles (Kim and Kurita, 2018; Li *et al.*, 2021a; Makker *et al.*, 2014). Likewise, T2 treatment has been reported to augment the transcription of *Pi3k* and activate PI3K signalling in various tissues (Li *et al.*, 2020, 2021b; White *et al.*, 2013; Zhao *et al.*, 2016).

Unlike T2, FSH treatment did not influence the activation of primordial follicles in 1-day-old mice ovaries cultured for 12 days. By contrast, we found a positive effect of FSH on follicular activation in 1-day-old mice ovaries cultured for 8 days in our previous study (Torkzadeh *et al.*, 2023). Comparison of the findings between the present and previous studies implicate that the positive effect of FSH may be more instantaneous than T2 but could not last for a culture system longer than 8 days. It was the same case with the effect of FSH on *Pi3k* expression because FSH did not influence gene expression of *Pi3k* in the ovaries cultured for 12 days, but FSH upregulated *Pi3k* in the ovaries cultured for 8 days (Torkzadeh *et al.*, 2023). One potential explanation for such phenomena could be the dynamics in the expression of *Fshr* considering the length of exposure to the ligand since prolonged exposure to FSH could downregulate FSH receptor and desensitize the cells to FSH (Casarini and Crépieux, 2019; Griswold *et al.*, 2001; Simoni *et al.*, 1997). In this sense, FSH could upregulate gene expression of its receptor in the ovaries cultured for 8 days in the previous study (Torkzadeh *et al.*, 2023); however, it failed to affect the expression of its receptor in the ovaries cultured for 12 days in the present study. Herein, it is worth noting that the neutral impact of FSH on *Fshr* was abrogated when FSH was used in combination with T2; furthermore, expression of *Pi3k* and activation of primordial follicles were the greatest in the ovaries simultaneously treated with FSH as well as T2. The upregulatory influence of T2 on FSH receptor is well-established (Fujibe *et al.*, 2019; Laird *et al.*, 2017; Sen *et al.*, 2014; Torkzadeh *et al.*, 2023), yet the present study implicated that T2 can also override the desensitization of ovarian follicles to FSH due to prolonged exposure and potentiate the beneficial effects of FSH treatment on the cultured ovaries.

Neither FSH nor T2 could solely improve the transition of follicles to the preantral stage, but the combination of FSH and T2 successfully increased the proportion of preantral follicles. Enhancement in the development of activated ovarian follicles towards the preantral stage is the main target in culturing dormant primordial follicles since progression to this stage is a prerequisite before isolation of follicles for *in vitro* production of MII-stage oocytes, which could be used for ART purposes (Amorim and Shikanov, 2016; Khajedehi *et al.*, 2024; Khodadi *et al.*, 2022). The synergistic effect of FSH and T2 was also observed on the expression of oocyte-derived growth factors (i.e. *Gdf9* and *Bmp15*) which was in accord with previous findings (Gilchrist *et al.*, 2008; Otsuka *et al.*, 2011). GDF9 contributes to not only the activation of primordial follicles but also the development of activated follicles (Gilchrist *et al.*, 2008; Hreinsson *et al.*, 2002; Kedem *et al.*, 2011; Otsuka *et al.*, 2011; Vitt *et al.*, 2000), and BMP15 contributes to proliferation of granulosa cells, growth of follicle and sensitization of follicle to FSH by upregulation of FSH receptor (Otsuka *et al.*, 2011; Persani *et al.*, 2014). Therefore, the positive effect of FSH+T2 on the development of follicles could be attributed to their upregulatory effects on *Gdf9* and *Bmp15*.

Regardless, no significant effect of FSH, T2 or FSH+T2 was found on the expression of factors originating from granulosa cells (i.e. *Amh* and *Kitl*), which indicates the hormonal treatments were not as influential on the granulosa cells as they were on the oocytes, at least within a 12-day culture period. One explanation for such a phenomenon may be the fact that alterations in the oocyte precede changes in granulosa cells in newly activated follicles (Chen *et al.*, 2020; Zhang *et al.*, 2023; Zhao *et al.*, 2021), and this system of *in vitro* culture could merely help activated follicles develop up to early steps of the preantral stage, at which limited progression in growth of granulosa cell layer occurred. In corroboration with this notion, the hormonal treatments in this study augmented gene expression of *Cx37*, which codes a connexin protein present at the interface between oocyte and granulosa cells and facilitates reciprocal communication between the germ cell and surrounding somatic cells (Firestone and Kapadia, 2012; Simon *et al.*, 2006;

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Simon *et al*., 1997; Teilmann, 2005). However, these hormonal treatments did not significantly impact gene expression of *Cx43*, which codes another protein from the connexin family localized in inter-granulosa cells gap junctions and regulates the coordinated function of granulosa cells (Firestone and Kapadia, 2012; Juneja *et al*., 1999; Santiquet *et al*., 2013; Teilmann, 2005). Interestingly, there is evidence indicating that alterations in the expression of CX37 precede altercations in the expression of CX43 in ovarian follicles (Teilmann, 2005). Nevertheless, whether the continuation of the culture of ovaries for longer than 12 days would lead to significant positive morphologic and molecular changes in granulosa cells requires further research to be addressed.

Despite the aforementioned positive effects of hormonal treatments on the development of ovarian follicles, they adversely influenced the viability of follicles in cultured ovaries. The negative effects of hormonal treatment were also evident in fewer absolute number of follicles, particularly in ovaries treated solely with FSH. In this regard, both FSH and testosterone have been reported to protect ovarian follicles from apoptosis (Shen *et al*., 2014; Shi *et al*., 2020; Torkzadeh *et al*., 2023; Yoo *et al*., 2020). In addition, although degeneration of follicles could occur at a normal rate in the process of ovarian development (Hussein, 2005), the rate of degeneration in small activated follicles is believed to be commonly negligible under a normal condition (Tingen *et al*., 2009). Nevertheless, hyperactivation of primordial follicles through PI3K signalling has been observed to dramatically increase the rate of degeneration in the growing follicles (Chen *et al*., 2016). As a result, one potential reason for the decreased proportion of intact follicles in ovaries treated with hormones could be the augmented rate of follicular activation and development in the corresponding ovaries rather than the direct apoptotic effect of FSH or testosterone on the ovarian follicles. As aforementioned, the effect of FSH on the progression of follicular activation and development initiates earlier than the effect of T2 on these biological processes (Torkzadeh *et al*., 2023), which may partly explain why the negative effect of hormonal treatments on the number of follicles was more pronounced in FSH-treated than T2-treated ovaries. Interestingly, the *Bax*/*Bcl2* gene expression ratio as an apoptotic index was lower in all hormone-treated ovaries than in untreated ones. This finding implicates that mechanisms other than apoptosis might have been involved in the degeneration of ovarian follicles in the hormone-treated ovaries. Hence, further studies are required to unravel the mechanisms that contributed to the follicular loss following treatment with FSH and T2, and in this regard, autophagy could be one of the potential mechanisms (Bhardwaj *et al*., 2022; Pagotto *et al*., 2017; Zhou *et al*., 2019).

Conclusion

In conclusion, the present study showed that treatment with the combination of FSH and T2 improved not only the activation of primordial follicles but also the growth of activated follicles towards preantral stage. This positive effect of FSH+T2 appeared to be at least partly mediated through the upregulation of *Pi3k* and oocyte-derived growth factors including *Gdf9* and *Bmp15*.

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Author contributions. **T.T.:** data curation, methodology, investigation, project administration, writing – original draft. **Z.A.:** data curation, methodology, investigation, project administration, writing – original draft. **M.J.A.:** methodology, investigation. **M.K.:** methodology, investigation. **F.E.:** methodology, investigation. **S.H.:** methodology, investigation. **V.A.:** conceptualization, methodology, investigation, resources, supervision, formal analysis, writing – original draft, writing – review and editing. **R.F.:** conceptualization, methodology, investigation, resources, supervision, writing – review and editing.

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