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Protective effects of hydro-alcoholic extract of *foeniculum vulgare* and *linum usitatissimum* on ovarian follicle reserve in the first-generation mouse pups

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ABSTRACT

Objective: The present study was conducted to investigate the protective effects of fennel and flaxseed during pre- and post-natal period until puberty and menopause on ovarian follicular reserve (OFR).
Methods: Pregnant NMRI mice received fennel (FV, 500 mg/kg/day), flaxseed (LU, 500 mg/kg/day), LU + FV (500 mg/kg/day) and no treatment was given to the controls. Female pups were studied on post-natal-days 1, 56 and 240 (PND1, 56, 240). Ovary weight and diameters, the number of primordial (PF), atretic (AF) and apoptotic (APF) follicles were determined. The expression of Bcl2 and STAT3 (apoptosis-related-genes), micoRNA-125a-5p, and also serum levels of sex hormones were measured. Data were analyzed using one-way ANOVA test.
Results: FV and FV + LU groups showed a marked rise in body and ovary weights and diameters as compared to the control group. The number of PF at PND1, PND56, and PND240 increased significantly in the FV and FV + LU groups but decreased in the LU group compared to the control mice. There was a significant reduction in the mean of AF in the FV and FV + LU group and a marked increase in the LU group compared to the controls. Also, more APF were observed in the LU group, whereas less apoptotic follicles were present in the FV group. FSH and estradiol serum levels increased significantly while LH decreased in the FV group. The anti-apoptotic-genes expression and pro-apoptotic microRNA, respectively, increased and decreased in the FV group versus control group.
Conclusions: It can be concluded that fennel alone and in combination with flaxseed could improve OFR during pregnancy, lactation, and afterwards until puberty and menopause.

1. Introduction

Women are the key members of any society with undeniable roles in maintaining the population. The health and fertility status of women depend on the amount of ovarian follicular reserve (OFR). OFR represents the stock of quiescent primordial follicles in the ovaries which is gradually depleted during a woman's reproductive lifespan leading to menopause, defined as permanent cessation of bleeding due to the lack of ovarian follicular activity that occurs 12 months after the last menstruation. Most of the woman, experience menopause at 45–55 years old while some experience it sooner or later [1]. Menopause causes several symptoms such as hot flushes and sweating, along with hormonal changes [2]. After menopause, systemic prescription of estrogen-containing products is a standard therapeutic method for alleviating the menopause symptoms; however, it may increase the risk of breast cancers [3].

Folliculogenesis involves follicular growth which finally leads to ovulatory follicle, including a fully mature oocyte. During this process, different factors including gonadotropins and estrogens could inhibit ovarian follicles atresia [4, 5]. In contrast, several factors such as androgens and Fas-ligand could induce this phenomenon [5, 6, 7, 8]. Most of the ovarian follicles are destroyed in the embryonic period and many follicles undergo atresia from birth up to the menopause [1].

Nowadays, medicinal herbs are being more popular due to

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complications of chemical drugs. Some medicinal plants like fennel (*Foeniculum vulgare*) and flaxseed (*Linum usitatissimum,* flax) have high antioxidant content and reduce expression of apoptosis related genes [9, 10]. Flaxseed, which is known as linseed, contains lignan that, as active oxygen species scavenger, is involved in the antioxidant activity of this plant [10]. The lignans is chemically similar to 17 beta-estradiol [11]. Previous studies have shown that the use of flaxseed, and an Iranian traditional mixture of flaxseed (Ghavoot), could improve the estrous cycle [12]. Also, anti-apoptotic properties of flaxseed have been reported on the ovarian follicles [13]. In females, the use of flaxseed in a dose-dependent manner is reported to change the body weight, ovary and uterus volume, puberty age, and the level of sex hormones [14].

Foeniculum vulgare, known as fennel, is an indigenous plant in different regions such as Iran, and especially Kerman province [15, 16]. Fennel is a phytoestrogenic plant, which increases estrogen and progesterone levels [9]. It plays a role in libido improvement, menstrual cycle regulation, folliculogenesis induction, fertility, delivery enhancement, and milk production rise [9]. It also has antioxidant compounds that can decrease the levels of oxidative factors [9]. A recent study reported that fennel extract (100–200 mg/kg) can increase the number of ovarian follicles and level of sex hormones [17].

Formation and development of follicular ovarian pool begins in the embryonic stage, which is highly altered by mother's diet during pregnancy and lactation. Also, offspring's diet might affect the amount of OFR. Some of the medicinal plants with estrogenic and anti-apoptotic properties can affect OFR via decrease in apoptosis. Therefore, the aim of the present study was to investigate the effects of fennel and flaxseeds, in combination and alone, on the OFR of the first-generation mice pups.

2. Materials and methods

2.1. Preparation of hydroalcoholic extracts

Fennel (*Foeniculum vulgare*) and flaxseeds (*Linum usitatissimum*, flax or linseed) were collected from Kerman province, Iran, in July 2017. Plants were authenticated by the Department of Pharmacognosy, Kerman University of Medical Sciences, Kerman, Iran (KMU). A voucher specimen of fennel (Voucher number: KF1466) and flaxseed (Voucher number: KF1628) were deposited at the Herbarium Center of the KMU. Dried seeds were milled and passed through a sieve (mesh 300). About 100 grams of powdered seeds were extracted using warm maceration method with 80% ethanol for 72 h. The extracts were filtered every 24 h and were replaced with fresh solvent. The extracts were pooled and concentrated under vacuum condition, dried in oven at 40 °C for 48 h, and kept at -20 °C until use [18].

2.2. Determination of total flavonoid contents of fennel and flaxseed extracts

Total flavonoid content of the plants were determined using aluminium chloride colorimetric assay as described elsewhere [19]. Briefly, the plant extracts were spotted on thin layer chromatography (TLC) plated (silica gel GF254, Germany) along with different flavonoid standards and then were chromatographed. The results indicated rutin as the main flavonoid of both plants, so it was used to for the rest of the study. At first rutin (with 100 ppm concentration) was provided in methanol. Then, it was added to an equal volume of ALC13 2% and then incubated for 30 min at room temperature. The mixture was scanned at 200–400 nm, absorption spectrum was prepared, and the maximum wave length of rutin was determined (ʎmax 275nm). Different concentrations of rutin were prepared and, by plotting the absorbance versus concentration, calibration curve of rutin was prepared. Flavonoid content of the plant was determined on the basis of rutin equivalent [20].

2.3. Animals

A total of 60 female and 30 male NMRI (Naval Medical Research Institute) mice, aged 6–8 weeks (25–30 g weight), were used for breeding. They were maintained in a 12/12 h light/dark cycle at 21 ± 2 °C with free access to rodent pellets food and water in the animal house affiliated with Afzalipour School of Medicine, Kerman, Iran. All protocols were approved by the institutional Ethics Committee of Kerman University of Medical Sciences (approval number: IR.KMU.REC.1397.148).

2.4. Experimental design

In each cage, five mature female mice and two mature male mice were randomly placed for mating. Female mice were examined the next morning for vaginal plague (Day zero of pregnancy). Next, pregnant animals were randomly divided into four groups (n = 6) (one pregnant animal in each cage): 1) the control group had free access to rodent pellets food, 2) the FV group: the animals which received hydroalcoholic extract of fennel (500 mg/kg body weight/day) [21], 3) the LU group: the animals which received hydroalcoholic extract of flaxseed (500 mg/kg body weight/day) [22], and 4) the LU + FV group: the animals which received hydroalcoholic extract of flaxseed and fennel seed (500 mg/kg body weight/day). The extracts were dissolved in distilled water, added to rodent feed on a weekly basis. The pregnant mice received different treatments from day one to the end of lactation period. The female pups, which were randomly distributed in the different groups (n = 24) (after weaning, two pups in each cage), received the same treatment as mothers did to the end of day 240 (8 months old) [23]. The female pups in each group were examined at different intervals (post-natal day 1, PND56 [24], and PND240).

2.5. Evaluation of ovarian morphology and histology

The female pups were weighed on 3 days of PND1, PND56, and PND240, and euthanized via cervical dislocation. The right ovary was removed, weighed, and its large and small diameters were measured using a digital caliper. The right ovaries were fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Five μm thick sections were prepared using a rotary microtome (cat no. DS4055, Did sabz, Iran). From every 10 serial sections, one was selected and stained using Hematoxylin and Eosin (eight sections from each mouse pup, n = 8). Follicles were counted and classified under light microscope (Olympus IX51, Japan), as follows:

1. Primordial follicle, with an oocyte surrounded by a layer of flatted granulosa cells, and
2. Atretic follicle, with a fragmented oocyte, ruptured plasma membrane, and irregularly shaped granulosa cells dropped into the antrum.

2.6. Hormone assessment

ON PND56 and PND240 days, blood was collected from the heart of anesthetized animals at a specific time of 8–9a.m. in the diestrus stage [25]. The samples were centrifuged at 2000–2500 rpm for 10 min. The serums were extracted and kept at -20 °C until hormone analysis. FSH (Follicle-stimulating hormone) (EAETBIOPHARMA, cat.no.CK-E20419, China), 17-β estradiol (EAETBIOPHARMA, cat.no.CK-E20381, China) and LH (Luteinizing hormone) (EAETBIOPHARMA, cat.no.CK-E20 343) hormones were measured using ELISA method.

2.7. TUNEL assay

TUNEL staining was performed to evaluate the rate of apoptosis in the ovary follicles using the In-Situ Cell Detection Kit, Fluorescein (cat.no.11684795910 Roche, Germany), according to the manufacturer's

instructions. Briefly, five μm thick paraffin sections were rehydrated in descending concentrations of ethanol, incubated in proteinase K solution (cat.no. P2308, Sigma, Germany) (10 μg/10mTris/HCL) in a humidified chamber at room temperature for 15 min. After washing with PBS, the slides were incubated with TUNEL reaction mixture, including fluorescent-dUTP (450μl label solution) and terminal deoxynucleotidyl transferase (TdT) (50μl enzyme solution) for 1 h. After washings in PBS, the slides were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride ((DAPI) (cat.no. D9542, Sigma, USA)) for 5 min and observed under fluorescent microscope (Olympus IX71, Tokyo, Japan). For negative control staining, TdT solution in reaction mixture was omitted. Eight sections were selected (n = 8) in each group. The number of positively stained antral and pre-antral follicles (x) were recorded and scored as follows: 0 (score 1), $0 < x \leq 2$ (score 2), $2 < x \leq 3$ (score3), $3 < x \leq 5$ (score4), and $x > 5$ (score 5). For different TUNEL signal intensities, different scores were used to refer to different intensities: 2 for weak, 3 for moderate, 4 for strong, 5 for very strong.

Finally, apoptosis was recorded using apoptotic index (AI) according to the formula: $AI = 0n_1 + 1n_2 + 2n_3 + 3n_4 + 4n_5$, where n_1, n_2, n_3, n_4 and n_5 are the numbers of cells exhibiting scores1, 2, 3,4, and 5, respectively. Then, the apoptotic score indicated the mean number of apoptotic index for each follicle type [26].

2.8. RNA extraction and complementary DNA synthesis

To assess the expression level of the apoptotic (microRAN125a-5p) and anti-apoptotic (Bcl2, STAT3) related genes in the left ovary tissue of pups, total RNA was isolated as follows. The ovary samples were homogenized using TRIZOL reagent (1ml) and then chloroform (200μl) was added to each tube and centrifuged at 12,000 rpm for 20 min at 4 °C. The aqueous phase was removed and absolute ethanol was added and kept at –20 °C overnight. The next day, the samples were centrifuged for 1h at 4 °C, and the supernatant was removed, 70% ethanol was added and then centrifuged for 20 min at 4 °C. The RNA pellet dried at room temperature and 50μl RNase-free water was added. The quantity of extracted RNA was determined using spectrometry. The cDNA synthesis was carried out from the total RNA (1μg), according to the manufacturer's protocol (cat.no.YT4500, YTA).

2.9. Quantitative real-time PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was done using Rotore-Gene 6000 (Corbett Research, Australia). Three replicates of qRT-PCR reactions were used on a mixture consisting of 5 ml cybergreen Master Mix, 1 μl from each of the two specific primers, and 2 μl of cDNA to reach the final volume of 10 μl. Real-time PCR procedure was run, as follows: initial denaturation at 95 °C for 40s, followed by 40 cycles of denaturation at 95 °C for 20s, primer annealing at 60 °C for 18s, and final extension at 72 °C for 30s. Beta actin and U6 were considered as reference for genes and microRNA, respectively. Data were subsequently analyzed using $2^{-\Delta\Delta CT}$method. A specific reverse primer, TagMan probe, and RT stem-loops set were provided by Dr. Afgar from Hydatid Disease Research Center at Kerman, Iran. The sequences of the primers are shown in Table 1.

2.10. Statistical analysis

Data analyses were performed using Statistical Package for the Social Sciences (SPSS software, version 22). First, the distribution pattern was investigated using the One-sample Kolmogorov-Smirnov test. If the study parameters had a normal distribution, the one-way ANOVA test was followed by post hoc Tukey, otherwise the nonparametric Kruskal-Wallis test was run. Data are expressed as Mean ± SEM and p values ≤ 0.05 were considered statistically significant.

Table 1
Details of primers used in real time PCR and quantitative real time PCR.

Genes	Primer sequence (5′-3′ orientation)	Annealing temp (°C)	Gene Bank Accession no.
Bcl2	F: GCTA CCG TCG TGA CTT CGC R: CCC CAC CGA ACT CAA AGA AGG	60	NM_000633.2
STAT3	F: CAC CTT GGA TTG AGA GTC AAG AC R: AGG AAT CGG CTA TAT TGC TGG T	60	NM_139276.2
miRNA 125a-5p	F: TCC CTG AGA CCC TTT AAC CTG TGA R: GCT GCT ACC TCG GAC CCT	60	NC_000083.6
β-actin	F: GTCCACACCCGCCACCAGTT R: GAGCCGTTGTCGACGACCAG	60	NM_001101.5
U6	F: CTC GCT TCG GCA GCA CA R: TGC GTT TAA GCA CTT CGC AA	60	NR_004394

3. Results

3.1. Flavonoids content of fennel and flaxseed extract

Calibration curve of rutin was provided with regression equation (y = 0.0191x-0.0968) and correction coefficient (r = 0.993). Total phenolic contents of the plants were determined at 14.42% and 4.76% rutin equivalent/g dried extract for fennel and flaxseed, respectively (Fig. 1).

3.2. Body weight, ovarian weight, and diameter

Daily intake of hydroalcoholic extract of fennel significantly increased body weight at PND56 ($p \leq 0.001$, $p = 0.021$) and PND240 ($p = 0.005$, $p = 0.012$) when compared to those of the LU and control groups, respectively. Also, a significant rise in body weight was observed in the FV-treated pups compared with the LU group at PND1 ($p = 0.004$). The body weight in the FV + LU group increased at PND1 ($P = 0.048$) and PND56 ($P = 0.035$) versus the LU group, whereas at PND240, a marked decrease was observed in the FV + LU group as compared to the FV group ($p \leq 0.05$). Treatment of the animals with FV and FV + LU significantly increased ovary weight at PND56 and PND240 as compared with that in control and LU groups ($P < 0.05$). The large diameters of ovaries were significantly greater ($P < 0.05$) in the FV and FV + LU groups when compared to those of the control and LU groups at PND56. At PND240, they were also significantly greater ($P < 0.05$) in FV and FV + LU groups compared with that in the LU group. Moreover, the mean of small diameter in the FV + LU group was also greater than those of other groups with no significant difference (Table 2).

3.3. Histological study of ovary

The mean number of primordial follicles in the FV and FV + LU groups was significantly higher than those of LU group at PND1 ($p = 0.003$, $p = 0.016$, respectively), PND56 ($p = 0.016$, $p = 0.021$, respectively), and PND240 ($p = 0.011$, $p = 0.04$, respectively). In contrast, there was a marked reduction of the primordial follicles in the LU group compared to that of the CTL group at PND1 and PND240 ($p = 0.06$). Also, atretic follicles in the LU group were found to be significantly more abundant than those in the FV and FV + LU groups at PND56 ($p \leq 0.001$) and PND240 ($p = 0.005$) (Table 3).

3.4. Hormone assays

A significant difference in the serum FSH level was detected between the FV group versus the control and LU groups ($p \leq 0.001$) at PND56 and PND240. Likewise, a significant difference was observed in the FV + LU group versus the control and LU groups ($p \leq 0.001$) at PND240. Also, a

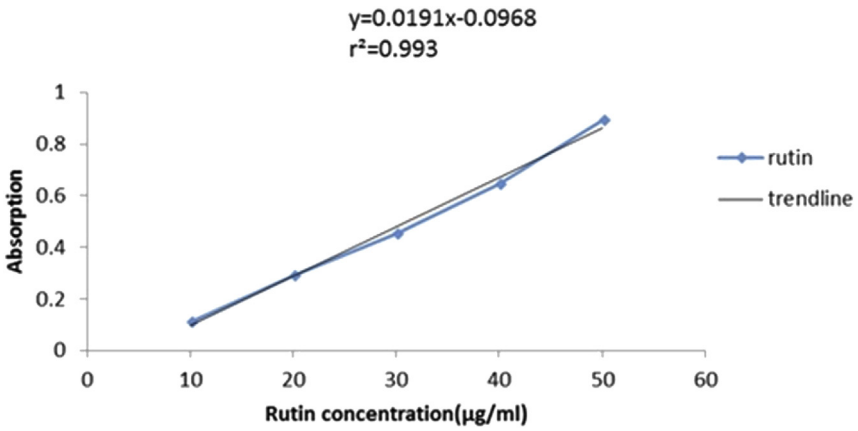

Fig. 1. The standard curve of rutin, the major flavonoid of flax and fennel at maximum wave length of absorption (λmax 275nm**).**

Table 2
Mice body and ovary weight and diameters in day1 (PND1), 56 (PND56) and 240 (PND240) in the different groups (n = 8).

Groups	Ages	Variables			
		Mice weight (g)	Ovarian weight (mg)	Large ovaries diameter (mm)	Small ovaries diameter (mm)
CTL	1D	1.41 ± 0.04	-	-	-
	8W	23.53 ± 0.61	3 ± 0.06	2.06 ± 0.12	1.36 ± 0.09
	8M	29.62 ± 0.71	6.6 ± 0.04	2.93 ± 0.07	1.79 ± 0.05
LU	1D	1.32 ± 0.03	-	-	-
	8W	23.11 ± 0.4	2.8 ± 0.05	1.99 ± 0.19	1.36 ± 0.13
	8M	28.33 ± 0.78	6.5 ± 0.04	2.6 ± 0.07^{a*}	1.64 ± 0.07
FV	1D	1.53 ± 0.03^{b**}	-	-	-
	8W	25.78 ± 0.28^{a*} ^{b***}	5.6 ± 0.05^{a*} ^{b**}	2.61 ± 0.14^{a*} ^{b*}	1.62 ± 0.08
	8M	32.88 ± 0.86^{a*} ^{b**}	8.1 ± 0.04^{a*} ^{b*}	3.07 ± 0.12^{b**}	1.91 ± 0.07
FV + LU	1D	1.45 ± 0.05^{b*}	-	-	-
	8W	24.61 ± 0.43^{b*} ^{c*}	5.5 ± 0.06^{a*} ^{b*}	2.75 ± 0.10^{a**} ^{b*}	1.46 ± 0.04
	8M	31.33 ± 1.06	8.1 ± 0.04^{a*} ^{b*}	3 ± 0.12^{b*}	2.02 ± 0.11

The values are expressed as (mean ± SEM). The values are comparable in the same column, **a**: significant difference versus control group (CTL; b: significant difference versus hydroalcoholic extract of *linum usitatissimum* treated group (LU); c: significant difference versus hydroalcoholic extract of *foeniculum vulgare* treated group (FV); ($p < 0.05$*), ($p < 0.01$**), ($p \leq 0.001$***).

marked decrease was observed in the FSH level in the FV + LU group in comparison with the control and FV groups ($p \leq 0.001$) at PND56 (Fig. 2a).

At PND56 and PND240, the levels of serum LH were significantly lower in all of the experimental groups compared with those of the control group ($p = 0.002$). Furthermore, the animals that simultaneously consumed both extracts showed an obvious lowest level of serum LH when compared to those of the other treatment groups that consumed each of the two extracts alone at PND56 (FV; $p = 0.018$, LU; $p = 0.004$) and PND240 (FV; $p = 0.007$, LU; $p = 0.019$) (Fig. 2b).

As shown in Fig. 2c, serum E2 levels in the FV and FV + LU groups were higher than those of other experimental groups at PND56 and PND240. Co-administration of FV and LU extracts and also FV extract

Table 3
Distribution of primordial and atretic follicles in the ovary of different groups at PND1(1D), PND56 (8W) and PND240 (8M).

Variables	Ages	Groups			
		CTL	LU	FV	FV + LU
Numbers of primordia f	1D	1043.75 ± 49.27	882.87 ± 63.68^{a**}	1280.25 ± 68.15^{a*} ^{b**}	1170.62 ± 48.35^{b*}
Numbers of primordial f	8W	49.75 ± 12.64	41 ± 4.39	58.62 ± 4.02^{b*}	56.87 ± 4.33^{b*}
Numbers of atretic f		47.5 ± 3.66	48.5 ± 3	19.62 ± 1.4^{ab***}	20.62 ± 2.49^{ab***}
Numbers of primordial f	8M	19.87 ± 1.05	13.12 ± 2.23^{a*}	25.87 ± 3.93^{b**}	20.75 ± 2.21^{b*}
Numbers of atretic f		533.62 ± 72.03	644.75 ± 55.35	381.25 ± 33.13^{b**}	410.25 ± 40.6^{b**}

The values are expressed as (mean ± SEM), a: a significant difference versus control group and b: a significant difference versus LU group were shown. *, ** and *** were $p \leq 0.05$,$p \leq 0.01$ and $p \leq 0.001$, respectively.

alone caused a significant elevation of serum levels of 17β estradiol (E2) as compared with that in the LU group at PND56 ($p \leq 0.001$). Also, intake of fennel extract induced a significant improvement in the serum levels of E2 versus the control group at PND56 ($p \leq 0.001$), whereas the pups that were treated with flaxseed extract had a marked decrease in E2 concentration versus the control group at PND56 ($P \leq 0.001$). A significant reduction of E2 level was also observed in the animals treated with FV + LU compared to that of the FV group at PND56 ($P \leq 0.001$).

3.5. Follicular cell apoptosis

Analysis of TUNEL staining data (Fig. 3) showed that animals that consumed FV had the least number of TUNEL positive cells which was significantly different versus the LU group at PND1 ($p \leq 0.05$) (Fig. 4a).

The numbers of the TUNEL positive cells in the pre-antral and antral follicles were recorded as weak, intermediate, strong, and very strong at PND56 and PND240.

At PND56, the LU group had the highest level of very strong apoptotic signals, whereas no significant change was observed in the number of pre-antral and antral follicles with moderate, strong, and very strong apoptotic signals among the different groups. The FV and FV + LU groups showed a significant decrease of the apoptotic weak signal in pre-antral follicles ($p = 0.063$). The mean number of antral follicles with weak signal in the FV + LU group reduced significantly as compared with the control and FV groups ($p = 0.025$ and $p = 0.063$, respectively). Also, the mean apoptotic index of pre-antral follicles in the FV group was low, while this parameter was high in the LU group, although this difference did not reach a significant level among the different groups. Moreover,

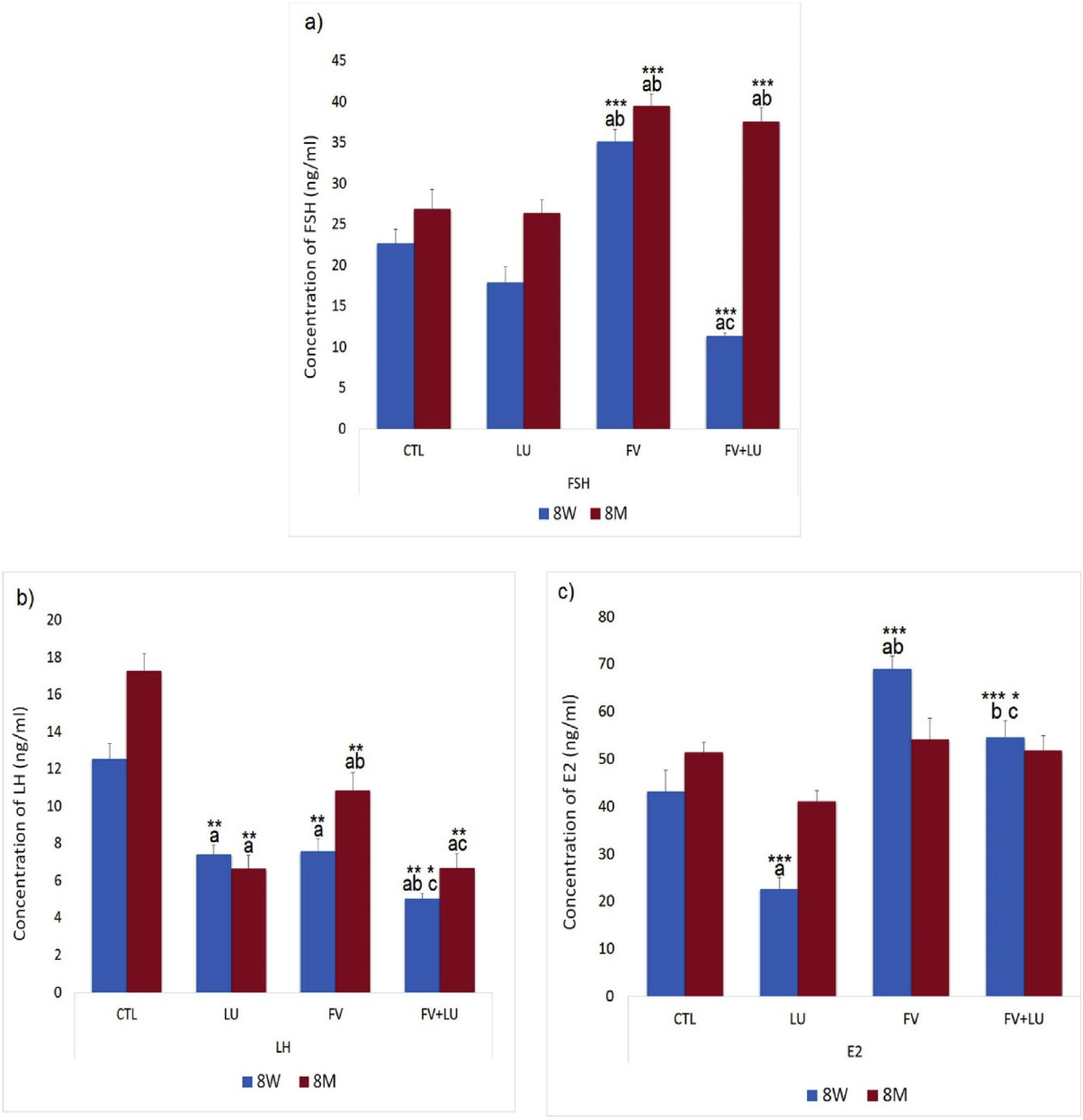

Fig. 2. Serum concentration of FSH (a), LH (b), E2 (c) hormones in F1 female mice at PND56 (8W) and PND240 (8M). Values are mean ± SEM, a: significant difference versus the control group; b: a significant difference versus the LU group; c: a significant difference versus the FV group. *, **, *** were $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

the mean apoptotic index of antral follicles had a considerable decrease in the FV + LU group versus the control group ($P = 0.02$) (Fig. 4b).

At PND240, no significant difference was observed among the different groups compared with the control group. There was a significant increase in apoptotic weak signal of pre-antral follicles in the FV + LU group versus the LU group ($p = 0.02$). However, a marked reduction of strong signals of antral follicles in the FV group was observed when compared to the LU-treated animals ($P = 0.03$). In addition, the mean apoptotic indices of pre-antral and antral follicles in the FV group were lower than those of the other groups.

3.6. *Bcl2 and STAT3 gene expression*

The relative transcript abundances of the anti-apoptotic genes (Bcl2 and STAT3) in the left ovary of the offspring at PND1, PND56, and PND240 were measured via qRT-PCR. The expression of Bcl2 gene was significantly enhanced in the FV group compared with those of the control and LU groups at PND1 and PND56 ($P \leq 0.05$). At PND240, the Bcl2 expression in the FV group was higher than those of the other experimental groups but it did not make statistically significant difference. The expression level of Bcl2 in the FV + LU group reduced markedly compared to that of the FV group at PND1 ($p \leq 0.05$) (Fig. 5a).

As shown in Fig. 5b, quantitative RT-PCR analysis showed that the expressions of STAT3 significantly upregulated in the ovarian tissue of animals that consumed fennel extract alone compared to those in the LU group at PND1 ($p = 0.046$), PND56 ($p \leq 0.05$), and PND240 ($p \leq 0.05$), whereas simultaneous consumption of fennel with flaxseed extract could significantly reduce the expression of STAT3 when compared to the FV group ($p \leq 0.05$). It was noteworthy that STAT3 expression significantly elevated in the FV group compared with that of the control group at PND240 ($p \leq 0.05$). Moreover, a significant decrease in the expression of this anti-apoptotic gene (STAT3) was

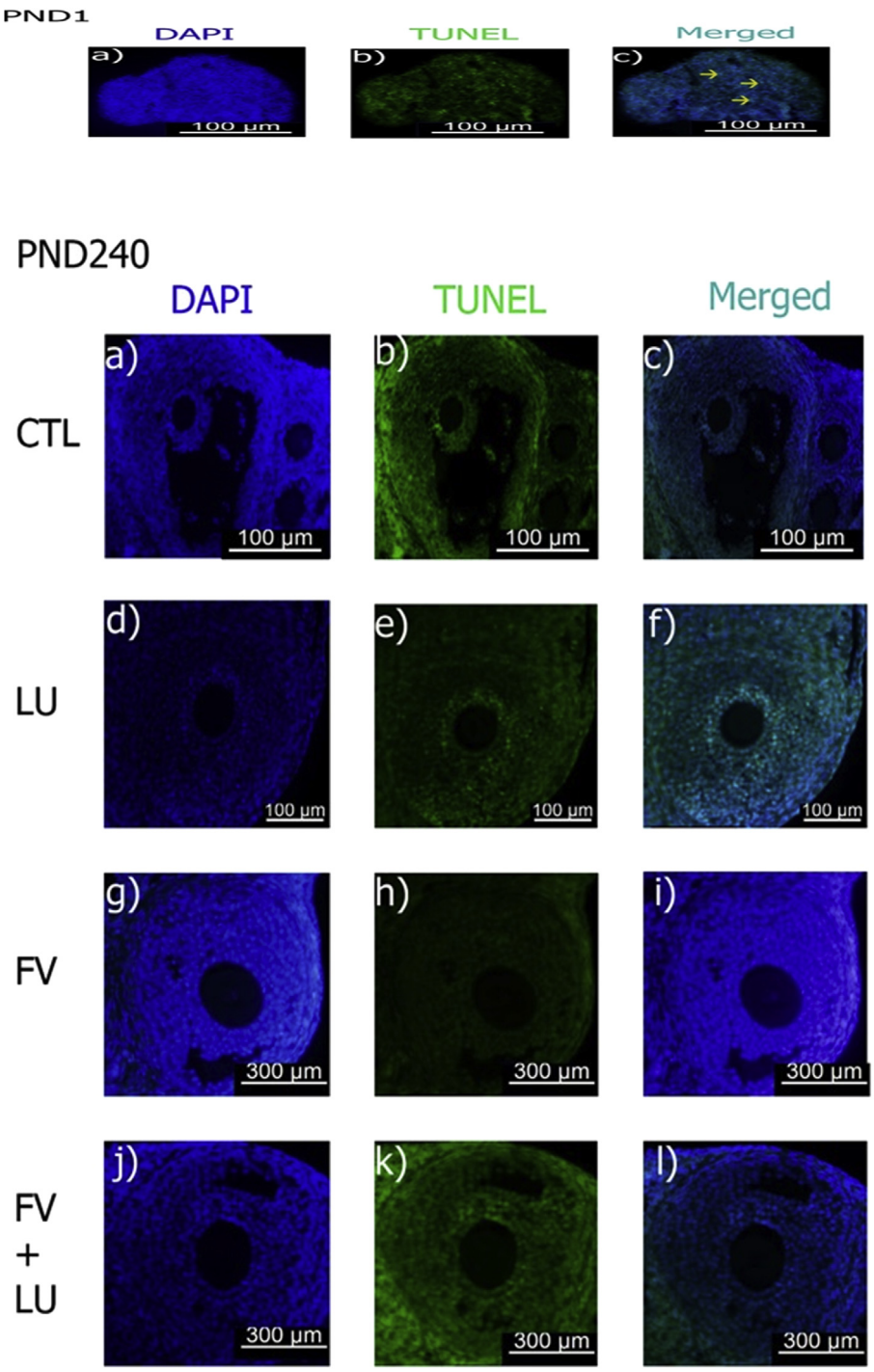

Fig. 3. Photographs are representative of immunofluorescence staining of ovarian section at PND1, PND56 and PND240. a) Counterstained with DAPI (blue), b) TUNEL positive granulosa cell (shiny green) and c) merged picture. Light yellow arrowheads points TUNEL positive granulosa cells at PND1.

detected in the LU group, versus the control group, at PND56 ($p \leq 0.05$) (Fig. 5b).

3.7. Expression of miRNA125a-5p

As shown in Fig. 5c, LU extract significantly increased the expression of pro apoptotic micro RNA 125a-5p in comparison with the control group at PND240 ($p \leq 0.05$). However, it decreased significantly ($p = 0.046$ and $p \leq 0.05$, respectively) at PND1 and PND240 after the treatment with FV extract versus the LU group. Daily intake of fennel plus flax seed extract had a significant impact on miRNA125a-5p expression compared with the FV group at PND1 ($p = 0.046$) and PND240 ($p \leq 0.05$). Significant low expression of miRNA125a-5p level was observed in the FV, LU, and FV + LU groups compared with that of the control group at PND1 ($p = 0.046$, $p \leq 0.05$, $p \leq 0.05$, respectively). In contrast, at PND56, treatment of animals with FV, LU and FV + LU significantly increased the expression of miRNA125a-5p in comparison with the FV, LU, and FV + LU groups ($p \leq 0.05$, $p \leq 0.05$, $p = 0.046$, respectively). Also, at PND240, miRNA125A-5P level in the FV + LU group significantly decreased as compared with the LU and CTL groups ($p \leq 0.05$).

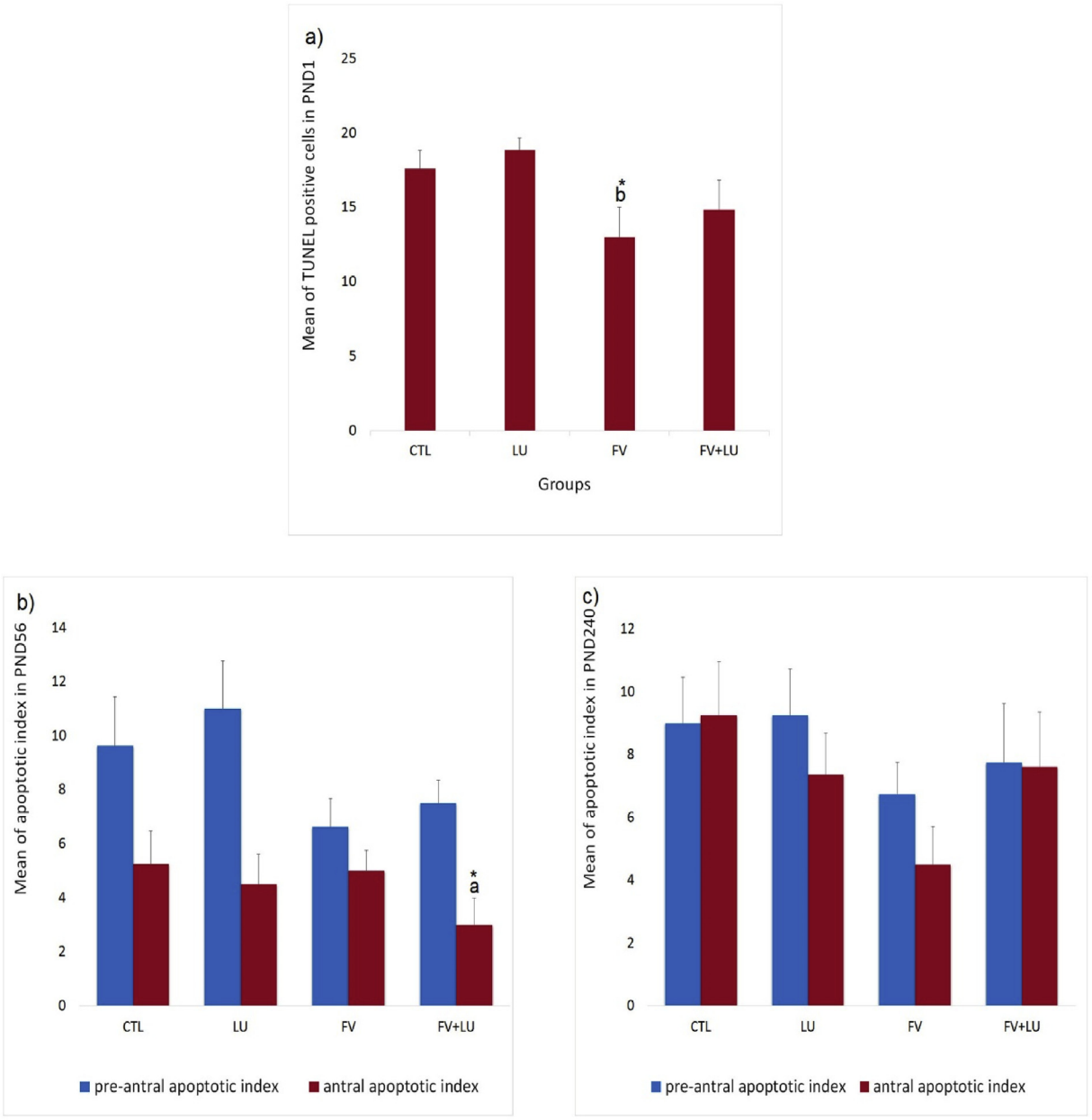

Fig. 4. The effects of hydroalcoholic extract of fennel and flaxseed on apoptosis incidence of granulosa cells at PND1, PND56 and PND240. a) the mean number of TUNEL positive cells at PND1. b) mean apoptotic index in the different groups at PND56. c) mean apoptotic index in the different groups at PND240. Values are expressed as mean ± SEM, a: Significant difference versus CTL group; b: Significant difference versus LU group; c: Significant difference versus FV group. *, shows $p \leq 0.05$.

4. Discussion

Death of millions of follicles during the three critical phases (intrauterine development, birth to puberty and fertility period) seems to influence women's normal physical and mental health. The main goal of the present study was to evaluate the rescue power of flaxseed (LU) and fennel (FV) as herbal medicines to prevent or reduce the death of ovarian follicular reserve (OFR) as a real support for women's health based on animal model. In brief, the results confirmed the hypothesis posed at the beginning of the present study. Treatment of the animals with FV, and in combination with LU, has increased the ovarian weight and diameters and also the total body weight at different time points (from birth to middle age) in the first-generation female pups. However, the FV consumption alone exhibited better impacts compared with when it was combined with LU. In contrast, treatment of animals with LU decreased these parameters. Our data suggests the estrogenic and anti-estrogenic effects of these herbs. Flavonoids, a type of phytoestrogens, are abundant in fennel [9]. The estrogenic properties of phytoestrogens, both *in vivo* and *in vitro*, have been reported in many researches [27, 28]. This plant could increase the weight and the diameters of ovary due to its estrogenic property [17]. Dietary supplementation of polyherbal such as fennel improved ovary characteristics [29]. Several studies indicated that flaxseed have estrogenic and non-estrogenic properties [14]. This herb could have different impacts on the genital tracts and sex hormone levels as well as duration of consumption dependently [30] so that flax seed at 200 or 300 mg/kg doses can increase the weight of body, ovary, and also uterus [31], while at higher doses, it could induce a negative feedback [32]. Flaxseed contains lignan compound [14], which have weak estrogenic effects and shows an anti-estrogenic property in the presence of a stronger estrogen compound [14]. We have shown that treatment of

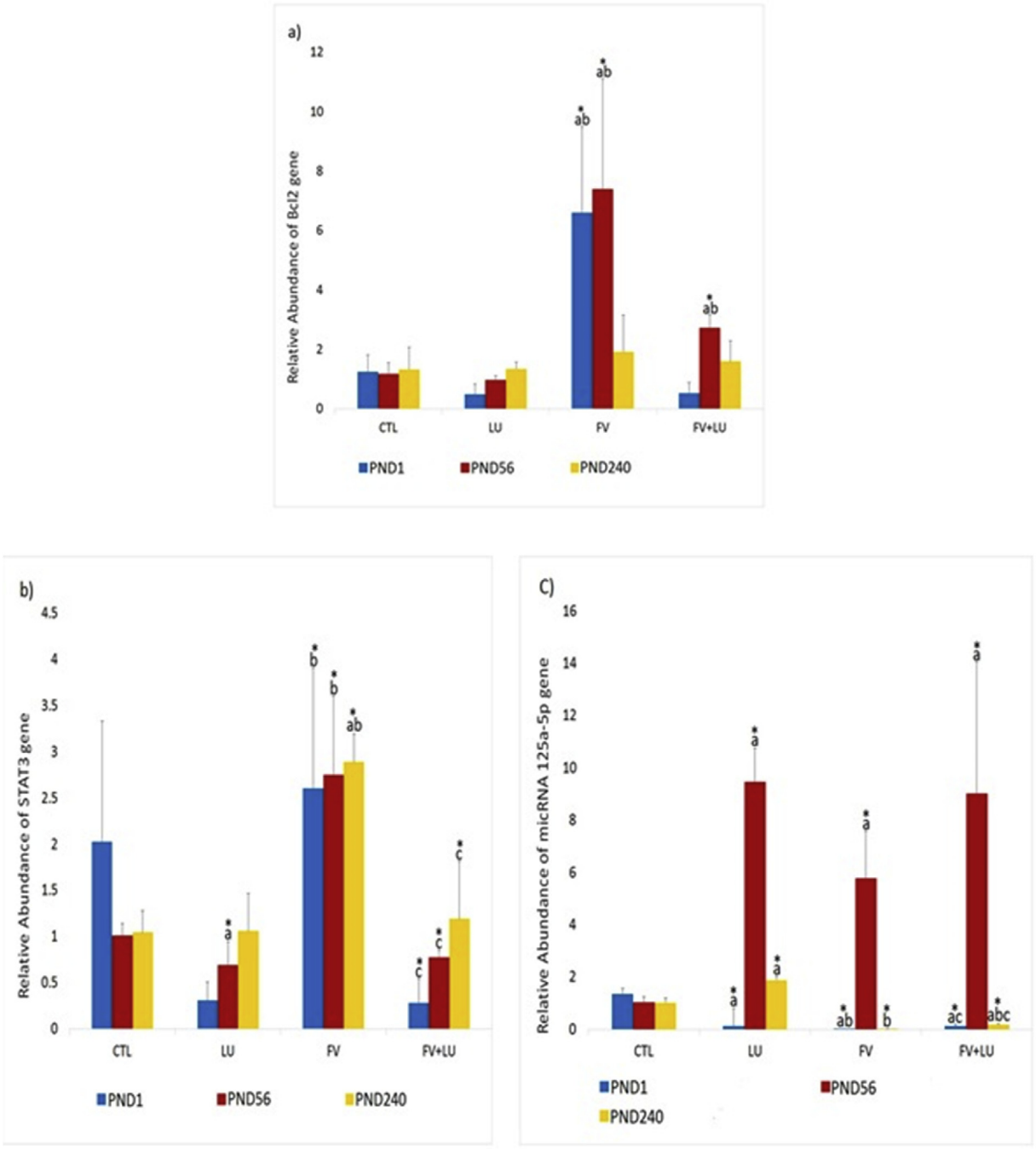

Fig. 5. Relative abundance of Bcl2, STAT3, micRNA125a-5p genes in ovarian tissue of pups at PND1, PND56 and PND240. The mRNA level of each sample was normalized against B actin and U6 mRNA levels. Data are presented as mean ± SEM, a: Significant difference versus CTL group; b: Significant difference versus LU group; c: Significant difference versus FV group. * shows $p \leq 0.05$.

animals with high doses of flaxseed has reduced the pup's body weight at PND1 and PND56, which could suggest its toxic properties at higher doses.

Our study has revealed for the first time that, FV and FV + LU but not LU alone, had protective effects on F1 offspring OFR at the studied time points. Prescribed herbal medicine (FV & LU) have increased the number of primordial follicles while they markedly decreased the number of atretic follicles. Based on the previous evidence, fennel has an antioxidant effect to prevent ovarian follicle atresia by reducing oxidative stress [33]. Besides, the estrogenic properties of fennel could improve folliculogenesis and increase the number of growing follicles and ovulation [27]. Recently, an emerging body of interest has focused on the human health benefits of phytoestrogen consumption, particularly in the treatment of hormone dependent cancers, menopausal symptoms, and cardiovascular disease [34]. In males, the use of phytoestrogen compounds such as fennel, reduces serum levels of FSH and LH as well as the quantity of sperm cells, leading to infertility [35]. Previous studies have indicated that consumption of fennel (500 mg/kg body weight/day) during lactation period improved rat offspring ovary development via exerting estrogenic effects. Neonate exposure to phytoestrogens delays primordial to primary follicle transition and reduces the number of growing follicles but exposure in pre-puberty stage promotes follicular development of rat offspring ovary [20]. Also, previous reports demonstrated that estrogen hormone and anti-oxidant factors could enhance ovarian follicles

viability by increasing the expression of Bcl2 gene (an anti-apoptotic gene), so it reduced apoptosis in growing follicles and protected ovarian pool [36]. Another anti-apoptotic gene that excretes a protective impact on ovarian follicles is STAT3. This gene is expressed in various ovarian follicles, improves follicle differentiation, and suppresses the apoptosis pathway [37]. This gene plays an important role in the improvement of human ovarian cancer [38]. Previous studies have also shown that decrease in the STAT3 expression could induce apoptosis by raising the miRNA125a-5p expression. This mechanism plays an important role in the pathogenesis of POF (premature ovarian failure). The expression of miRNA125a-5p is effective to prevent the differentiation and metastasis of ovarian cancer [37]. Our results indicated that the relative abundance of anti-apoptotic gene (Bcl2 and STAT3) increased in the FV group at different time points, while it decreased in LU-treated mice. Intake of hydroalcoholic extract of fennel also caused a decrease in the expression of pro-apoptotic gene (miRNA125a-5p) in contrast to flaxseed extract which resulted in the enhancement of pro-apoptotic gene expression. Interestingly, these data confirm that long term traditional consumption of fennel might improve ovarian follicle reserve even in the first generation via suppression of apoptosis pathway factors.

The flax seed contains a large amount of mammalian lignan precursor secoisolariciresinol-di-glycoside (SDG) [10]. Colonic bacterial flora could convert SDG to mammalian lignans enterodiol, which has estrogenic and anti-estrogenic properties [10]. The lignans, with anti-estrogenic properties, could prevent aromatase enzyme activity, a regulator in the synthesis of estrogen and conversion of testosterone to 17 beta-estradiol, which can itself lead to increase in the estrogen level due to its estrogenic activity [10]. Following decrease in the level of estrogen, the effective hormones in folliculogenesis, follicles cannot grow well and eventually atresia of growing follicles occurs. In post-menopausal women reports, consumption of flaxseed can reduce risk of breast cancer due to reduction of estrogen levels, while post-menopausal estrogen therapy can increase the risk of breast cancer [39]. As described in many studies, foods that contain high levels of lignans, have protective effects on breast cancer by reducing the body's endogenous estrogen [39]. It has been shown that a diet containing 5% flaxseed could delay puberty, whereas using a diet containing 1% flax seed could result in early maturity and increase ovarian weight as well as serum estrogen level [40]. Vlckova R et al showed that supplemental flaxseed modulates ovarian functions of weanling gilts via the action of selected fatty acids [41]. The previous studies have indicated that phytoestrogens, such as soy, red clover, and flaxseed, are effective in reducing serum estrogen levels and thus could decrease the severity of menopausal symptoms, such as flushing [42] Previous studies demonstrated that excessive consumption of phytoestrogens, such as soy, could negatively affect the genital system, destroy ovarian follicles, and decrease body weight and ovary [43]. The results of the present study on the first generation pups are in agreement with those of the previous reports on the adults. However, we observed a reduction of the number of primordial follicles and a rise of atretic follicles when the mice were treated with flaxseed.

Conclusively, consumption of FV and LU has increasing and decreasing effects on the levels of serum sex hormones, respectively. This finding is in agreement with those previously published for phytoestrogenic effect of fennel [27]. An increase in the serum estrogen level via exogenous consumption has a positive feedback on the hypothalamus so that stimulation of the releasing FSH hormone can lead to improvement in the ovarian folliculogenesis [44]. FSH and estrogen are two inhibitor agents of follicular atresia [27]. FSH stimulates the expression of aromatase in granulosa cells that lead to increases in the estrogen levels [43]. The current study showed that the levels of serum estrogen and FSH hormones in mouse pups that consumed LU are reduced. This can be related to the anti-estrogenic property of flaxseed considering the duration and dosage of the herbs. A look into the data revealed that, serum LH levels of treated- animals decreased as compared with those of control group. The greatest decrease was found in FV + LU group and the lowest reduction was related to the FV group. Consistent with the results of the current study, the previous studies confirmed that fennel is important in the treatment of polycystic ovarian syndrome by increasing the levels of FSH and estrogen hormones and also reducing LH and testosterone levels [45].

5. Conclusion

Based on the results of the present study, we may conclude that hydroalcoholic extract of fennel can reserve the follicle pool in the ovary of F1 offspring, enhance sex hormones level in the serum, and increase the expression level of anti-apoptotic gene in the ovary by controlling follicle atresia in embryonic period and childhood. These events collectively protect ovarian reserve which can affect the sexual cycles in the life. Whether or not these findings could be transferred to human reproductive life span requires further investigation, whereas consumption of hydroalcoholic extract of flaxseed might negatively affect reproduction of first generation offsprings.

Declarations

Author contribution statement

Fahimeh Pourjafari: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tahereh Haghpanah: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fariba Sharififar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Seyed Noreddin Nematollahi-Mahani: Conceived and designed the experiments; Analyzed and interpreted the data.

Ali Afgar, Gholamreza Asadi Karam: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Massood Ezzatabadipour: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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