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Effect of dietary supplementation with nettle or fenugreek on folliculogenesis and steroidogenesis in the rabbit ovary – An *in vivo* study

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ABSTRACT

The objective of the study was to investigate the effect of dietary supplementation with nettle or fenugreek on folliculogenesis and steroidogenesis in the juvenile rabbit ovary. To gain insight into the mechanism of action of these herbs, we examined follicle formation, ovarian cell proliferation and apoptosis, steroidogenic enzyme abundance and steroid concentrations in ovarian tissue and plasma. Animals were fed with control, 1% nettle- or 1% fenugreek-supplemented pellets from 5 to 12 weeks of age ($n = 10$ per each group), when animals were slaughtered for ovary and blood collection. The addition of nettle decreased the numbers of primordial ($P = 0.015$) and early antral ($P = 0.02$) follicles and increased the number of primary ($P = 0.04$) ones when compared with the control group. Following fenugreek supplementation, the numbers of primary ($P = 0.008$) and antral ($P = 0.027$) follicles were greater, while the number of early antral ($P = 0.003$) follicles was lower in comparison with the control group. Nettle revealed apoptotic activity through activation of caspases 9 ($P = 0.047$), 8 ($P = 0.022$) and 3 ($P = 0.004$), whereas fenugreek increased ($P = 0.042$) follicular cell proliferation marked by PCNA protein abundance. Furthermore, only fenugreek targeted steroidogenic enzymes, decreasing CYP17A1 ($P = 0.043$) and increasing CYP19A1 ($P = 0.048$) protein abundances that resulted in enhanced estradiol biosynthesis and its elevated ($P = 0.006$) plasma concentration. In conclusion, both herbs affected follicle development in the rabbit ovary in a stage specific manner. Additionally, fenugreek altered ovarian steroidogenesis in a way that might affect sexual maturation in rabbits.

1. Introduction

In recent years, there has been growing interest in the use of herbs as a part of complementary medicine in the treatment of reproductive disorders in women [1]. Among a plethora of medical plants, special attention has been paid to nettle and fenugreek, which were found to have anti-androgenic properties, regulate menstrual cycle and improve the metabolic symptoms, such as insulin resistance, glucose and lipid metabolism, in women with polycystic ovary syndrome [1–4].

Nettle is a herbaceous plant belonging to the family Urticaceae. Numerous *in vitro* and *in vivo* studies have shown its anti-proliferative, anti-inflammatory, antioxidant and antidiabetic effects [5], caused by flavonoids, phenolic acids, carotenoids, vitamins and minerals contained in leaves as well as lectins, polysaccharides, sterols and lignans present in leaves and roots [6]. Fenugreek is an annual herb belonging to the family Leguminosae. Its seeds contain biologically active alkaloids, saponins (*e.g.* fenugreekine, fenugrin B), steroidal diosgenin, flavonoids (*e.g.* quercetin), polyphenols, coumarin, and soluble fiber [7]. Fenugreek exerts estrogen- and/or progesterone-like effects in the female reproductive tract [8]. Furthermore, it has anti-inflammatory, antioxidant, antidiabetic, hepatoprotective and antitumor actions [9]. Although clinical studies suggest beneficial effects of nettle and fenugreek supplementation on reproductive disturbances in women, the cellular and molecular mechanism of their actions within female reproductive

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organs, including the ovary, is still unclear and requires further elucidation.

The ovary is a multicompartmental organ with two key functions required for the maintenance of female fertility. First, the ovary produces fertilizable oocytes, which are released during ovulation [10]. Second, the ovary synthesizes steroid hormones necessary for the regulation of female reproductive function, including the establishment of pregnancy [11]. Ovulatory and steroidogenic processes both take place within the functional units that are ovarian follicles [10]. Follicular development and growth, called folliculogenesis, begins with the assembly of primordial follicles, which are further recruited to growth and develop into primary, secondary or antral ones [12,13]. As the follicles grow, granulosa cells differentiate from a squamous to cuboidal shape and proliferate, while theca cells are recruited around the granulosa-oocyte structure [14]. Notably, the overwhelming majority of ovarian follicles are destined to degenerate through atresia [15]. During this process both oocytes and granulosa cells undergo apoptosis that can be activated through two routes: a mitochondria-mediated (intrinsic) pathway involves the release of cytochrome *c* to the cytoplasm followed by the activation of caspase 9, and a death receptor-mediated (extrinsic) pathway activates caspase 8 [15]. Cleaved caspases 9 and 8 downstream stimulate apoptosis by caspase 3 activation, which is a key protease leading to biochemical and morphological changes associated with apoptosis [16]. Taken together, normal follicle growth and development are ensured by a balance between proliferation and apoptosis.

In the rabbit, the ovarian endowment of follicles starts after birth. The ovary contains primordial and developing follicles up to weeks 3 of age, whereas antral ones appear at week 12 [17]. Therefore, this species seems to be a useful model for studying follicular dynamics under experimental conditions, as we have recently shown [18]. In the current study, we hypothesized that nettle and fenugreek influence folliculogenesis and steroidogenesis in 12-week-old rabbit ovaries. To test this hypothesis, we performed histological examination of ovarian structures, analysis of ovarian cell proliferation and apoptosis, assessment of steroidogenic enzyme abundance and steroid concentrations in ovarian tissue and plasma samples from control rabbits and those fed a diet supplemented with nettle or fenugreek.

2. Materials and methods

2.1. Animals, experiment design and tissue collection

The experiment was performed on Termond White female rabbits ($n = 30$) housed in a heated hall with water available *ad libitum*, lighting (14 L:10D) and exhaust ventilation (from day 35). On postnatal day 35, animals were weaned, randomly assigned into three experimental groups ($n = 10$ per group) and housed in metal cages arranged in batteries (4 rabbits per cage). From weaning to 12 weeks of age, the rabbits were fed *ad libitum* with pellets, supplied by Barbara Sp. z o. o. (Turza, Poland), containing min. 16.5% crude protein, max. 14% crude fiber and 2–4% crude fat according to rabbit nutritional standards. Control animals received non-supplemented pellets, while in the other groups, the pellets contained 1% nettle (*Urtica dioica* L.) leaves or 1% fenugreek (*Trigonella foenum-graecum L.*) seeds. The composition of the diets is presented in Table 1. Rabbits were slaughtered at 12 weeks of age ($n = 10$ per group; average body weight 2.6 kg; sexually immature). All procedures were performed following guidelines for animal experiments in EU Directive 2010/63/EU regarding the protection of animals used for experiments and Council Regulation (EC) n. 1099/2009 on the protection of animals at the time of killing. Just after slaughter, one ovary of each rabbit was fixed in 10% buffered formalin for histology, immunohistochemistry and TUNEL assay, while contralateral ovaries were snap frozen for Western blot and steroid concentration analyses. Blood samples were collected from each animal, placed in heparinized test tubes, centrifuged (2000×*g*, 10 min, 4 °C) and stored (−20 °C) for the assessment of steroid concentrations (Fig. 1).

Table 1
Composition and nutritional value of rabbit diets (%).

Ingredients	Treatment		
	Control	Nettle	Fenugreek
Wheat	29.58	28.58	28.58
Corn	24.50	24.50	24.50
Bran	15.00	15.00	15.00
Sunflower meal	11.00	11.00	11.00
Dehydrated alfalfa	10.00	10.00	10.00
Soybean meal	7.00	7.00	7.00
Premix	1.50	1.50	1.50
Fodder chalk	0.80	0.80	0.80
Feed phosphate	0.62	0.62	0.62
Nettle	–	1.00	–
Fenugreek	–	–	1.00

Each treatment (diet) contains (amount per kilogram): vitamin A 10.000 IU; vitamin D_3 1.500 IU; vitamin E 30 mg; Cu 7.5 mg; Fe 50 mg; Mn 75 mg; Zn 50 mg; I 1.0 mg; Se 0.2 mg. Estimated digestible energy is 10.11 MJ/kg of pellet.

2.2. Ovarian histology

To investigate the development of ovarian follicles, ovaries were embedded in paraffin, sectioned (5 μm) and stained with hematoxylin-eosin (H&E). The number of follicles in each class were counted blindly in five separate sections (the central section and two lateral sections on each side of the ovary) per ovary. The total number of follicles was counted across the entire section. Follicles were classified as: (1) primordial - surrounded partially or completely by squamous pregranulosa cells, (2) primary - surrounded by a complete layer of cuboidal granulosa cells, (3) preantral - having two or more complete layers of granulosa cells, a theca layer and no antrum, (4) early antral - having five or more granulosa layers and a fluid-filled antrum, (5) antral - having less than five granulosa layers and a large antrum (6) atretic – antral follicles with granulosa cells with pyknotic nuclei [18,19].

2.3. Immunohistochemistry

Immunohistochemistry was conducted routinely as previously described [18]. Briefly, dewaxed and rehydrated sections were treated using an unmasking procedure with microwave heating in 0.01 M citrate buffer (pH 6.0) followed by 30 min in 0.3% H_2O_2 in Tris-buffered saline (TBS, pH 7.4) to quench endogenous peroxidase activity. Blocking of non-specific binding sites was performed with 5% normal horse serum before incubation with monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Table 2). After overnight incubation at 4 °C in a humidified chamber, sections were washed with TBS containing 0.1% Tween 20 (TBST). Subsequently, antigens were visualized using secondary biotinylated horse anti-mouse IgG (1:300; 1.5 h; room temperature (RT); Vector Laboratories, Burlingame, CA, USA), avidin-biotin-peroxidase complex (40 min at RT; Vectastain Elite ABC Reagent, Vector Laboratories) and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) as chromogen staining substrate. Sections were counterstained with Hematoxylin QS (Vector Laboratories), dehydrated and mounted in dibutylphthalate polystyrene xylene (DPX; Sigma-Aldrich). Negative controls were incubated with non-immune mouse IgG (cat. # NI03; Sigma-Aldrich) instead of primary antibody. Selected

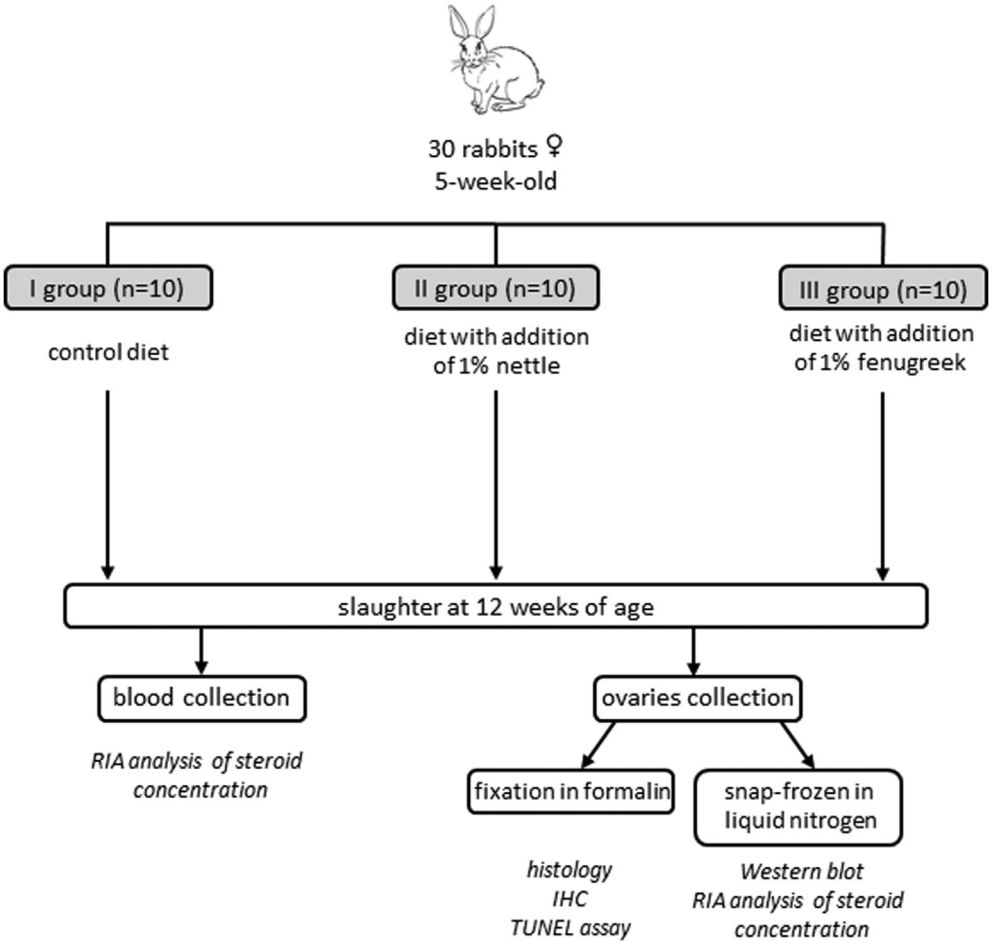

Fig. 1. Schematic representation of the experimental protocol. RIA, radioimmunoassay; IHC, immunohistochemistry.

sections were photographed using a Nikon Eclipse Ni–U microscope and a Nikon Digital DS-Fi1-U3 camera (Nikon, Tokyo, Japan) with corresponding software.

2.4. TUNEL assay

Apoptotic cells were detected in ovarian sections using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Melbourne, Australia) following the manufacturer's protocol and our previous experiments [18,20]. In brief, slides were pre-treated with proteinase K solution (10 μg/mL, 15 min at RT), immersed in 3% H_2O_2 (10 min at RT) and incubated in the equilibration buffer (10 min at RT) in a humidified chamber. Next, working strength TdT enzyme was applied to sections and incubated for 1 h at 37 °C in a dark humidified chamber. Slides were then rinsed with wash buffer and phosphate buffered saline (PBS; pH 7.4). Anti-digoxigenin conjugate was added directly to the sections and incubated (30 min at RT in a humidified chamber). Apoptotic cells were visualized by addition of DAB solution. All slides were counterstained with Hematoxylin QS, dehydrated through an increasing series of ethanol concentrations and mounted with DPX. A minimum of three randomly selected ovarian sections were evaluated for each animal and follicles with apoptotic cells were counted across the entire section under light microscopy.

Table 2
Primary antibodies used for Western blot (WB) and immunohistochemistry (IHC).

Antibody	Serum	Host species	Supplier	WB dilution	IHC dilution	Secondary antibody
Anti-PCNA	5% NHS	Mouse	Novocastra, Newcastle, UK cat. no. NCL-L-PCNA	1:500	1:150	Horse anti-mouse IgG
Anti-caspase 9	–	Goat	Santa Cruz Biotechnology, CA, USA Cat. no. sc-8297	1:200	–	Mouse anti-goat IgG
Anti-caspase 8	–	Rabbit	Biorbyt Ltd., Cambridge, UK cat. no. orb 10664	1:1000	–	Goat anti-rabbit IgG
Anti-caspase 3	–	Rabbit	Cell Signaling, MA, USA cat. no. 9662	1:1000	–	Goat anti-rabbit IgG
Anti-3β-HSD	–	Mouse	Abcam, Cambridge, UK cat. no. ab55268	1:1000	–	Horse anti-mouse IgG
Anti-CYP17A1	–	Mouse	Novus Biologicals, LLC, CO, USA Cat. No. NBP2-01151	1:2000	–	Horse anti-mouse IgG
Anti-CYP19A1	–	Mouse	AbD Serotec, Milan, Italy cat. no. MCA2077S	1:250	–	Horse anti-mouse IgG
Anti-β-actin	–	Mouse	Sigma-Aldrich, St. Louis, MO, USA cat. no. A2228	1:4000	–	Horse anti-mouse IgG

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; CYP17A1, cytochrome P450 17α-hydroxylase/17,20-lyase; CYP19A1, cytochrome P450 aromatase; NHS, normal horse serum; PCNA, proliferating cell nuclear antigen.

A negative control was performed without the active TdT enzyme. Representative sections were photographed using a Nikon Eclipse Ni–U microscope and a Nikon Digital DS-Fi1-U3 camera with corresponding software.

2.5. Western blot

Total protein extraction and Western blot analysis was carried out as previously described [20,21]. The protein content was assayed using the Bradford method with a Pierce Detergent Compatible Bradford Assay Reagent (Thermo Scientific, Rockford, IL, USA), using bovine serum albumin as the standard. Samples were separated by 10% Mini-PROTEAN TGX Gels (Bio-Rad Labs, GmbH, Munchen, Germany) under reducing conditions and electroblotted using *Trans*-Blot Turbo Mini 0.2 μm PVDF Transfer Packs and *Trans*-Blot® Turbo™ Transfer System (Bio-Rad Labs). The blotted membranes were blocked in 5% non-fat dried milk in TBST (1 h at RT, with shaking) followed by an overnight incubation (4 °C) with primary antibodies against PCNA, caspase 9, caspase 8, caspase 3, 3βHSD, CYP17A1 and CYP19A1 (see Table 2). After washing in TBST, they were incubated with respective secondary antibodies linked to horseradish peroxidase (1:4000 for each; Vector Laboratories) for 1 h (RT). Chemiluminescent signal was developed using Clarity™ Western ECL Substrate (Bio-Rad Labs) and detected on a ChemiDoc XRS + System (Bio-Rad Labs). Next, each membrane was stripped and reprobed with monoclonal mouse anti-β-actin antibody (see Table 2) followed by horseradish peroxidase-conjugated anti-mouse IgG (1:4000; Bio-Rad Labs). The densitometric intensity of PCNA, caspase 9, caspase 8, caspase 3, 3βHSD, CYP17A1 and CYP19A1 abundance was quantified and normalized against β-actin using Image Lab. 4.0 software (Bio-Rad Labs).

2.6. Radioimmunoassay (RIA) of steroid hormones

Prior to RIA analysis, fragments of ovarian tissue from the control and herb-supplemented groups were weighed, homogenized in liquid nitrogen and dissolved in 0.1 M PBS [22]. Concentrations of progesterone (P4), testosterone (T) and estradiol (E2) in homogenates were determined using commercial RIA kits (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium) following the manufacturer's protocol. Detection limits were: 0.05 ng/mL for P4, 0.005 ng/mL for T and 6.4 pg/mL for E2, while mean recoveries were 97%, 94% and 96%, respectively. The intra- and inter-assay coefficients of variation for P4, T and E2 were 4% and 6.5%, 3.3% and 4.8%, 5.6% and 10.4%, respectively. Steroid concentrations were expressed as ng or pg per mg of ovarian tissue. All analyses were performed in duplicate.

2.7. Statistical analysis

Data, expressed as mean ± standard error of the mean (SEM), were analyzed using Statistica *v.*13.1 software (StatSoft, Inc, Tulsa, OK, USA). To verify the normal distribution of data the Shapiro-Wilk and the Lilliefors tests were applied. Due to the lack of normal distribution of data, the nonparametric Kruskal-Wallis test was used and the differences between control and dietary supplemented groups were determined by *post hoc* Dunn's multiple comparison test. All results were considered statistically significant at the 95% confidence level ($P < 0.05$).

3. Results

3.1. Effect of dietary supplementation with nettle or fenugreek on ovarian histology

Ovarian structures including primordial (Fig. 2A), primary (Fig. 2A), secondary (Fig. 2B), early antral (Fig. 2C), antral (Fig. 2D and E) and atretic follicles (Fig. 2F) were found in the ovaries of 12-week-old rabbits from all examined groups. The addition of nettle to the diet resulted in decreased numbers of primordial ($P = 0.015$) and early antral ($P = 0.02$) follicles, and an increased number of primary follicles ($P = 0.04$) when compared with the control group. Following fenugreek supplementation, the numbers of primary ($P = 0.008$) and antral ($P = 0.027$) follicles were greater, while the number of early antral follicles was lower ($P = 0.003$) in comparison with the control group (Table 3).

3.2. Effect of dietary supplementation with nettle or fenugreek on ovarian cell proliferation

In the control group (Fig. 3A–C) and those fed with the addition of nettle (Fig. 3D–F) or fenugreek (Fig. 3G–I), immunohistochemical analysis showed the presence of proliferating cells (PCNA-positive) in follicles at different developmental stages (Fig. 3A–I) as well in stroma cells (Fig. 3A, E, G). Positive PCNA-nuclear staining was found in oocytes within primordial, primary and secondary follicles (Fig. 3A, D, G), in granulosa cells of primary and secondary (Fig. 3A, D, G), early antral (Fig. 3B, E, H) and antral (Fig. 3C, F, I) follicles, and in theca interna cells of early antral (Fig. 3B, E, H) and antral (Fig. 3C, F, I) follicles of all examined sections. Negative control sections incubated with non-immune mouse IgG showed no PCNA staining (Fig. 3C inset).

Western blot analysis revealed that dietary addition of fenugreek resulted in a significantly higher ($P = 0.042$) PCNA protein abundance compared with the control group, whereas a diet supplemented with nettle had no effect (Fig. 5A, E).

3.3. Effect of dietary supplementation with nettle or fenugreek on ovarian cell apoptosis

In situ detection of DNA fragmentation demonstrated the presence of single apoptotic cells displaying brown nuclear staining in ovaries from the control (Fig. 4A and B), nettle (Fig. 4C and D) and fenugreek (Fig. 4E and F) supplemented groups. Apoptotic signs were found in oocytes of primordial follicles (Fig. 4A, C, E) as well as in granulosa cells of secondary (Fig. 4A inset, D, E inset) and antral (Fig. 4B, C, F) follicles. Dietary supplementation with nettle resulted in a significant increase ($P = 0.026$) in the number of atretic follicles among the secondary follicles class in comparison with the control group (Table 4).

Western blot analysis revealed two forms of caspase 9, 8 and 3 in all examined groups (Fig. 5E). The 46, 52 and 32 kDa forms correspond to the inactive proenzymes, while 10, 12 and 17 kDa forms correspond to the active enzymes (cleaved caspases). Both nettle and fenugreek significantly increased ($P = 0.047$) the abundance of the active caspase 9 form compared with the control group (Fig. 5B). Furthermore, the addition of nettle resulted in greater abundance of cleaved caspase 8 ($P = 0.022$; Fig. 5C) and caspase 3 ($P = 0.004$; Fig. 5D) forms in comparison with the control group.

3.4. Effect of dietary supplementation with nettle or fenugreek on ovarian steroidogenesis

Ovaries obtained from rabbits fed with fenugreek had a markedly lower T content ($P = 0.047$) and higher E2 content ($P = 0.01$),

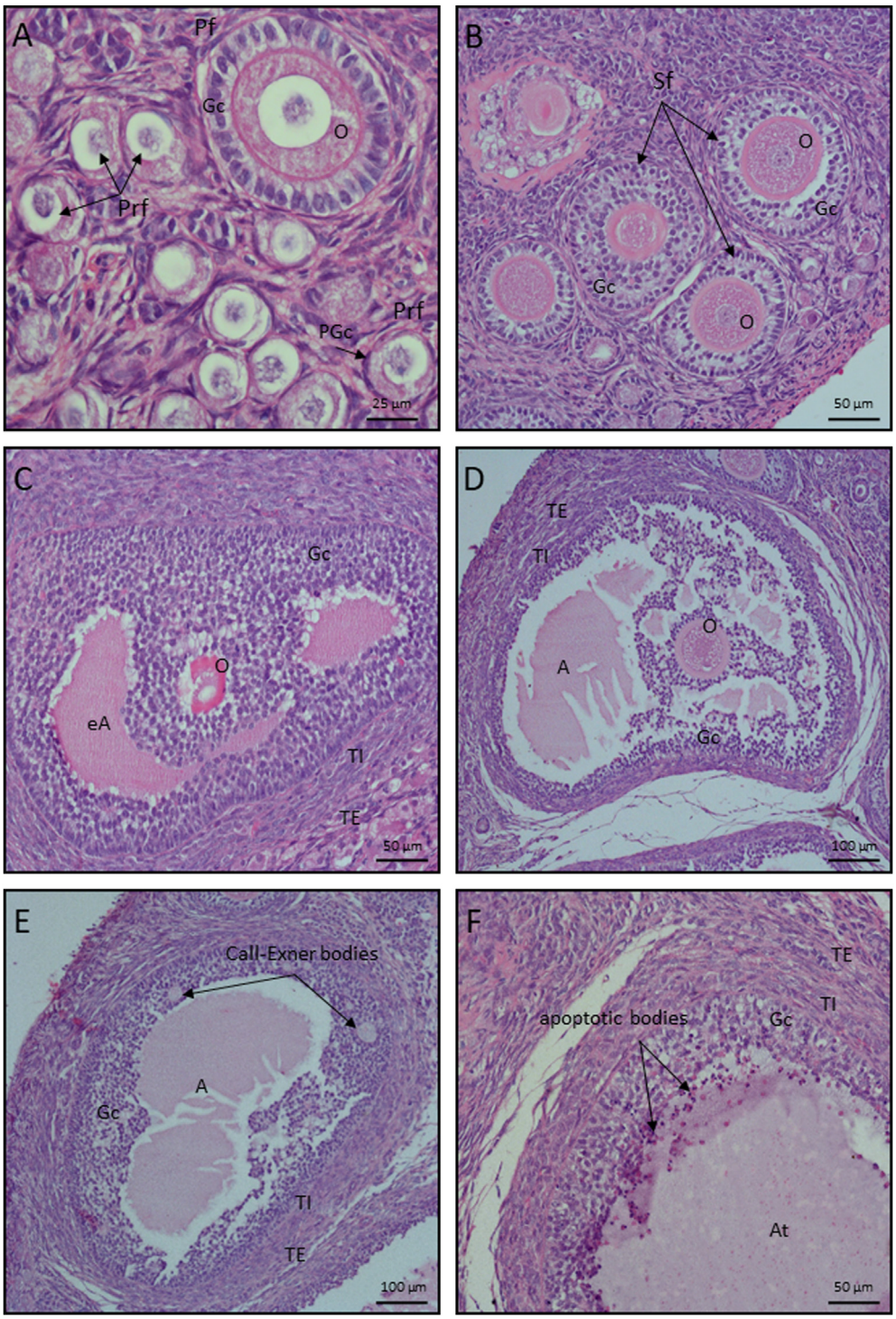

Fig. 2. Stages of follicular development in 12-week-old rabbit ovary (A–F). Prf, primordial follicle; Pf, primary follicle; Sf, secondary follicle; eA, early antral follicle; A, antral follicle; At, atretic follicle; Gc, granulosa cells; TI, Theca interna; TE, theca externa; O, oocyte.

while the P4 content was unchanged in comparison with the control group. Dietary supplementation with nettle did not influence steroid concentrations in rabbit ovarian tissue (Table 5).

Regarding the level of proteins for steroidogenic enzymes, CYP17A1 abundance was decreased ($P = 0.043$; Fig. 6B, D), CYP19A1 abundance was increased ($P = 0.048$, Fig. 6C and D), and 3βHSD (Fig. 6A, D) abundance was unchanged following fenugreek addition when compared with the control group. Steroidogenic enzyme protein abundance was unaffected by supplementation with nettle (Fig. 6A–D).

3.5. Effect of dietary supplementation with nettle or fenugreek on plasma steroid concentrations

Dietary supplementation with fenugreek significantly increased ($P = 0.006$) plasma E2 concentration in juvenile rabbits, while P4

Table 3
Effects of dietary supplementation with nettle or fenugreek on the follicle number in the rabbit ovary ($n = 10$ per each group).

Treatment	Follicle number (mean ± SEM)				
	Primordial	Primary	Secondary	Early antral	Antral
Control	$148.44^{a} \pm 52.09$	$5.7^{a} \pm 2.83$	$18.5^{a} \pm 8.62$	$4.0^{a} \pm 2.67$	$4.6^{a} \pm 1.12$
Nettle	$101.5^{b} \pm 22.82$	$10.83^{b} \pm 3.19$	$17.33^{a} \pm 5.92$	$0.83^{b} \pm 1.17$	$6.17^{ab} \pm 1.94$
Fenugreek	$115.17^{ab} \pm 27.35$	$11.67^{b} \pm 3.27$	$14.17^{a} \pm 8.23$	$0.33^{b} \pm 0.52$	$7.83^{b} \pm 1.40$

Different letter superscripts indicate statistically significant differences between groups ($P < 0.05$; Dunn's multiple comparison test).
Treatment (Diet): Control, rabbits fed with pellet without any supplementation; Nettle, rabbits fed with addition of 1% nettle in pellet; Fenugreek, rabbits fed with addition of 1% fenugreek in pellet.

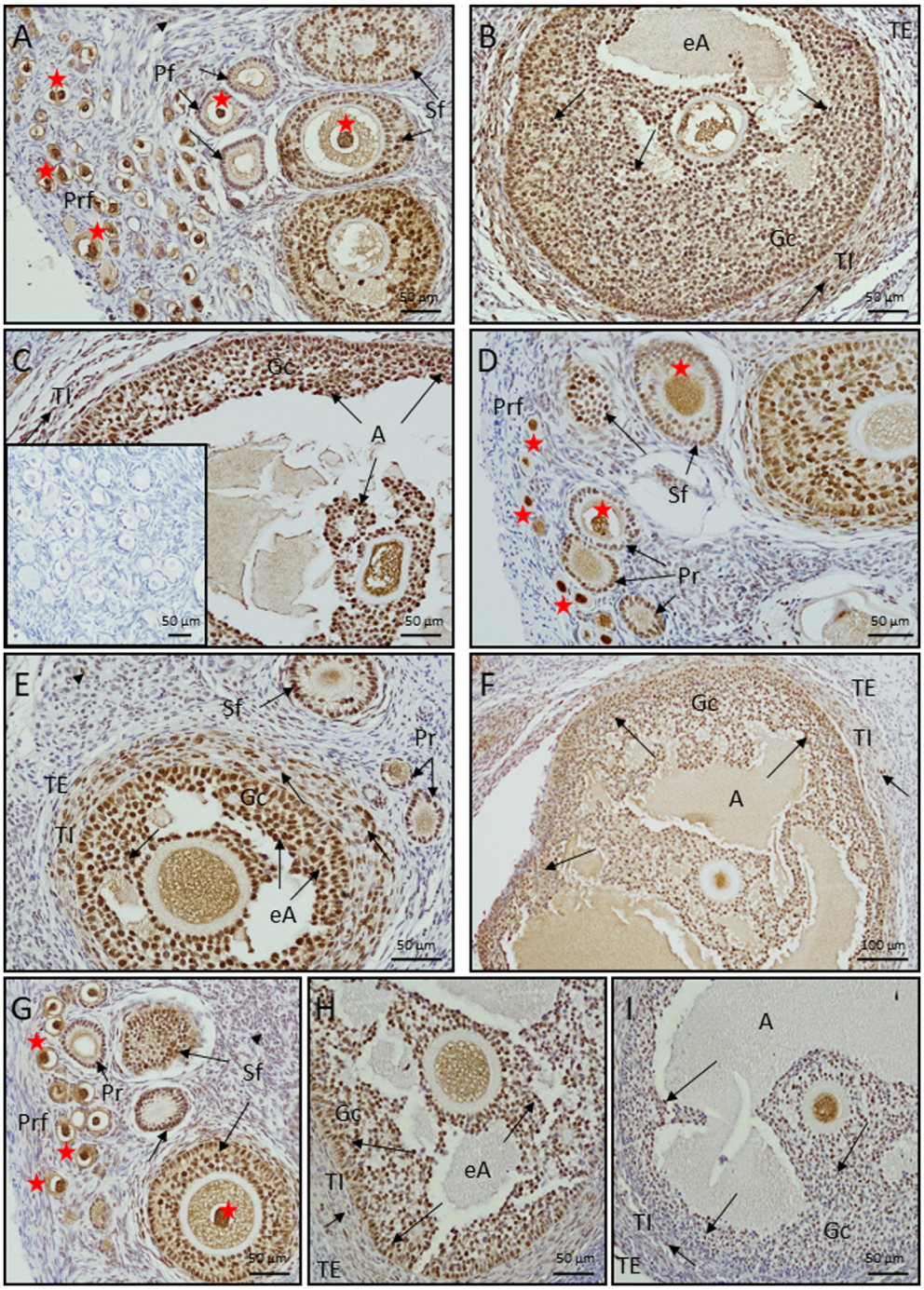

Fig. 3. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA)-positive cells in ovaries obtained from 12-week-old rabbits of the control group (A–C) and those fed with addition of nettle (D–F) or fenugreek (G–I). Negative control section did not exhibit positive staining (C inset). Nuclear PCNA staining was observed in stroma cells (A, E, G; arrowheads), oocytes (red asterisks) of primordial (Pfr), primary (Pf) and secondary (Sf) follicles (A, D, G), in the granulosa cells (black arrows) of Pf, Sf, early antral (eA) and antral (A) follicles as well as theca interna cells (black arrows) of eA and A. Gc, granulosa cells; Tl, Theca interna; TE, theca externa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

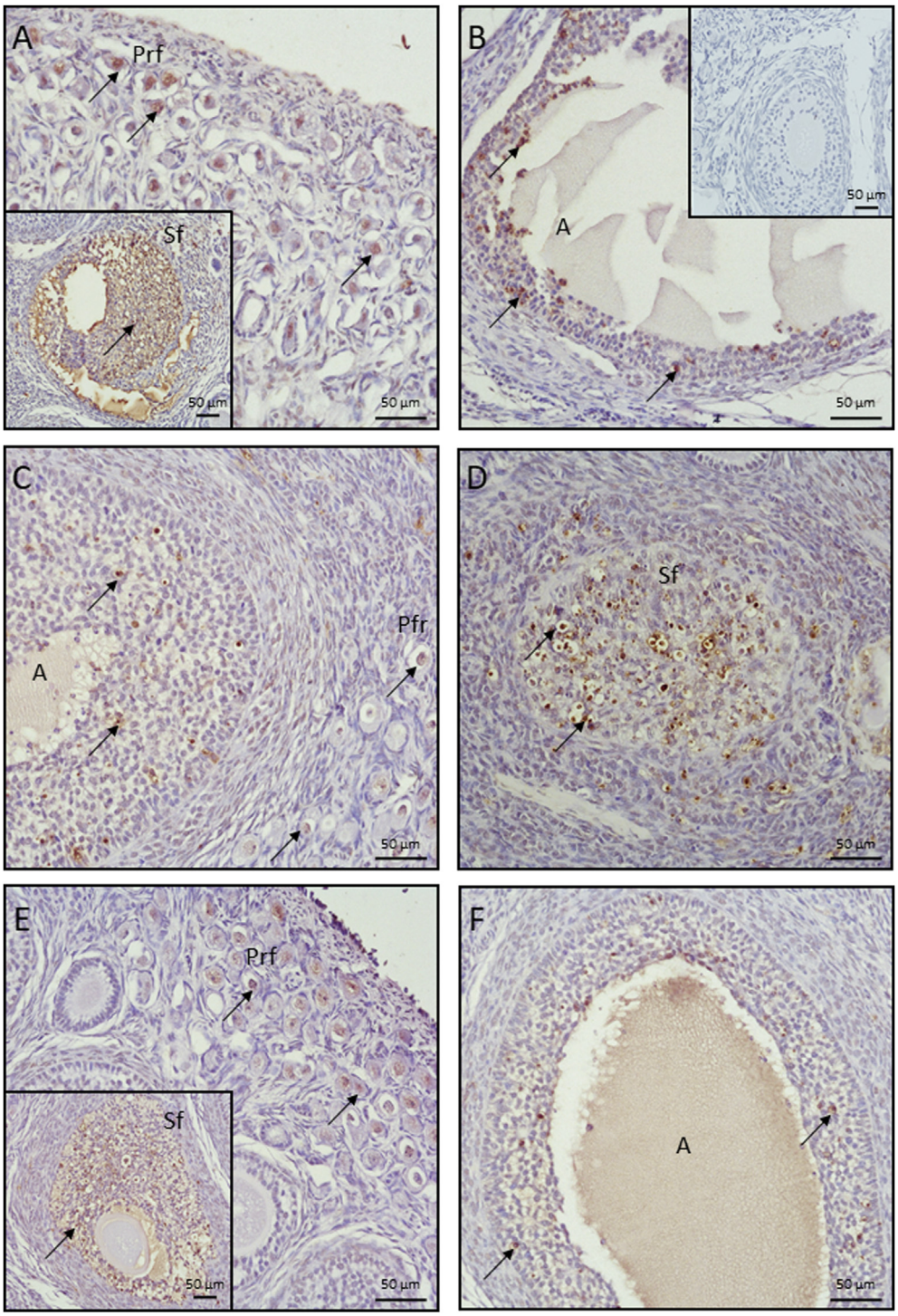

Fig. 4. *In situ* detection of apoptotic cells in ovaries obtained from 12-week-old rabbits of the control group (A, B) and those fed with addition of nettle (C–D) or fenugreek (E–F). Apoptotic cells were identified using TUNEL assay. Arrows indicate apoptotic cells that are oocytes within primordial follicles (PrF) or granulosa cells in secondary (Sf) and antral (A) follicles. A negative control was performed without active TdT enzyme (B, inset).

and T plasma levels were unaffected. The addition of nettle to the diet did not change plasma steroid concentrations (Table 5).

4. Discussion

Proper folliculogenesis and effective steroidogenesis within the whole ovarian life cycle determine female fertility. They both remain under the control of pituitary gonadotropins, intraovarian factors and environmental compounds that may interfere with endogenous hormone action [23,24]. Keeping in mind the growing importance of nettle and fenugreek in the improvement of reproductive function in women, it is necessary to determine the mechanisms underlying their influence on follicle development and ovarian steroid biosynthesis. The rabbit provides a model for

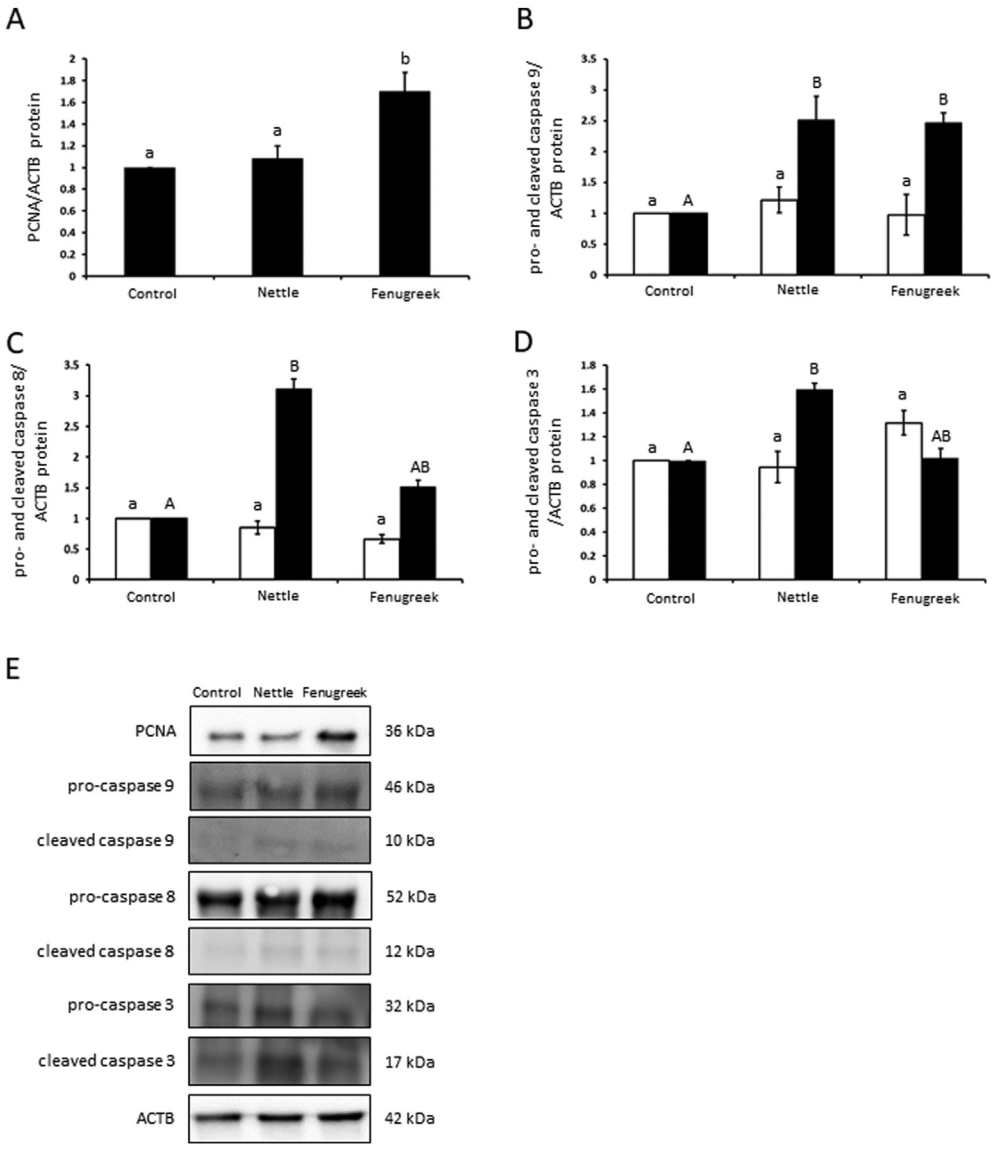

Fig. 5. Western blot analysis of proliferating cell nuclear antigen (PCNA) (A), pro- and cleaved caspase 9 (B), 8 (C) and 3 (D) protein abundance in ovaries obtained from 12-week-old rabbits of the control group and supplemented with nettle or fenugreek. Representative Western blots are shown (E). The relative level of studied protein was normalized to β-actin (ACTB). The protein levels within the control group were arbitrarily set at 1. Each value represents the mean ± SEM. Different letter superscripts indicate statistically significant differences between groups ($P < 0.05$; Dunn's multiple comparison test). $n = 10$ per each group.

this.

Folliculogenesis starts with the assembly of primordial follicles and takes place either prenatally or neonatally in rabbits [17]. The size of the primordial follicle cohort and the rate of their activation to growth (called initial recruitment) determine the reproductive potential of females [25]. We have shown for the first time that addition of nettle markedly decreased the number of primordial and increased the number of primary follicles, whereas supplementation with fenugreek resulted only in an elevated number of primary ones. These results indicate an effect of both herbs on initial recruitment of follicles from the resting pool. Furthermore, nettle and fenugreek decreased the number of early antral follicles, whereas only fenugreek significantly increased the number of antral ones. Development of preantral follicles to the antral stage depends on their responsiveness to circulating follicle-stimulating hormone (FSH) released by the pituitary, which is responsible for cyclic recruitment [26]. It appears that dietary enrichment with nettle and fenugreek influence cyclic recruitment of early antral follicles, but the effect of fenugreek is more pronounced. There are scarce data regarding the impact of nettle and fenugreek on ovarian follicle development. To date, Jahromi and Jashni [27] showed

Table 4
Effects of dietary supplementation with nettle or fenugreek on the number of atretic follicles in the rabbit ovary ($n = 10$ per each group).

Treatment	Atretic follicle number (mean ± SEM)		
	Primordial	Secondary	Antral
Control	$59^{a} \pm 5.96$	$2.25^{a} \pm 0.63$	$1.25^{a} \pm 0.25$
Nettle	$54.75^{a} \pm 4.82$	$6.67^{b} \pm 0.88$	$0.75^{a} \pm 0.48$
Fenugreek	$66.67^{a} \pm 5.04$	$4.5^{ab} \pm 0.65$	$1.67^{a} \pm 0.33$

Different letter superscripts indicate statistically significant differences between groups ($P < 0.05$; Dunn's multiple comparison test).
Treatment (Diet): Control, rabbits fed with pellet without any supplementation; Nettle, rabbits fed with addition of 1% nettle in pellet; Fenugreek, rabbits fed with addition of 1% fenugreek in pellet.

Table 5
The concentration of progesterone (P4), testosterone (T), and estradiol-17β (E2) in plasma and ovarian tissue (mean ± SEM) of rabbits fed with control diet or diet supplemented with nettle or fenugreek (n = 10 per each group).

Treatment	Plasma			Ovary		
	P4 (ng/mL)	T (ng/mL)	E2 (pg/mL)	P4 (ng/mg tissue)	T (ng/mg tissue)	E2 (pg/mg tissue)
Control	$3.46^{a} \pm 0.28$	$0.16^{a} \pm 0.01$	$25.04^{a} \pm 0.53$	$0.22^{a} \pm 0.05$	$0.34^{a} \pm 0.02$	$2.46^{a} \pm 0.68$
Nettle	$2.82^{a} \pm 0.18$	$0.20^{a} \pm 0.08$	$47.33^{a} \pm 1.22$	$0.22^{a} \pm 0.04$	$0.22^{a} \pm 0.06$	$4.86^{a} \pm 1.77$
Fenugreek	$2.86^{a} \pm 0.15$	$0.22^{a} \pm 0.02$	$62.80^{b} \pm 4.90$	$0.15^{a} \pm 0.03$	$0.11^{b} \pm 0.04$	$5.60^{b} \pm 0.95$

Different letter superscripts indicate statistically significant differences between groups (P < 0.05; Dunn's multiple comparison test).
Treatment (Diet): Control, rabbits fed with pellet without any supplementation; Nettle, rabbits fed with addition of 1% nettle in pellet; Fenugreek, rabbits fed with addition of 1% fenugreek in pellet.

enhanced folliculogenesis in rats following nettle root extract treatment, marked by increased primary, secondary and tertiary follicles number. Fenugreek was found to increase the total number of growing follicles in rat ovaries, but with no difference in their number at individual developmental stages [7]. Taken together, our present results suggest accelerated folliculogenesis in the juvenile rabbit ovary following dietary supplementation with either herb. However, it seems that nettle influences the early stages of folliculogenesis, while fenugreek affects rather antral follicle development.

The growth of ovarian follicles involves the proliferation of granulosa cells [11]. Therefore, the present study evaluated PCNA localization and protein abundance in the ovaries of juvenile rabbits fed with nettle or fenugreek. In each group, PCNA immunolocalization gave a positive signal in oocytes of primordial, primary and secondary follicles, granulosa cells of follicles at all developmental stages and theca interna cells of early antral and antral follicles, in accordance with previous research [17,18]. Our data showed that PCNA protein abundance in the rabbit ovary was significantly increased following fenugreek supplementation, while nettle had no effect. A methanol extract of fenugreek seeds produced an up-regulation of PCNA expression in hepatocellular carcinoma cell line HepG2 [28]. Additionally, a positive impact of fenugreek on cell proliferation was observed in rat uterus epithelium *in vivo* [29] and the MCF-7 cell line *in vitro* [30]. This suggests that greater PCNA protein abundance observed in the ovaries after fenugreek supplementation is attributed to accelerated folliculogenesis and results in the increased number of antral follicles in this group.

Throughout ovarian development, most follicles become atretic and do not reach the ovulatory stage [16]. During atresia, follicular cells undergo apoptosis, which is mainly scattered within oocytes and the granulosa layer [15]. In the current study, we have observed an increased number of atretic follicles at the secondary stage following nettle supplementation, in comparison with the control group. Although the total number of secondary follicles was unchanged, the increased atresia among them might impair the promotion to the antral stage manifested by decreased number of early antral follicles in this group. Notably, nettle increased the abundance of the active forms of initiator caspases (9 and 8) as well as executioner caspase 3, indicating the activation of apoptosis. Apoptotic activity of nettle has been shown in cancer cells and

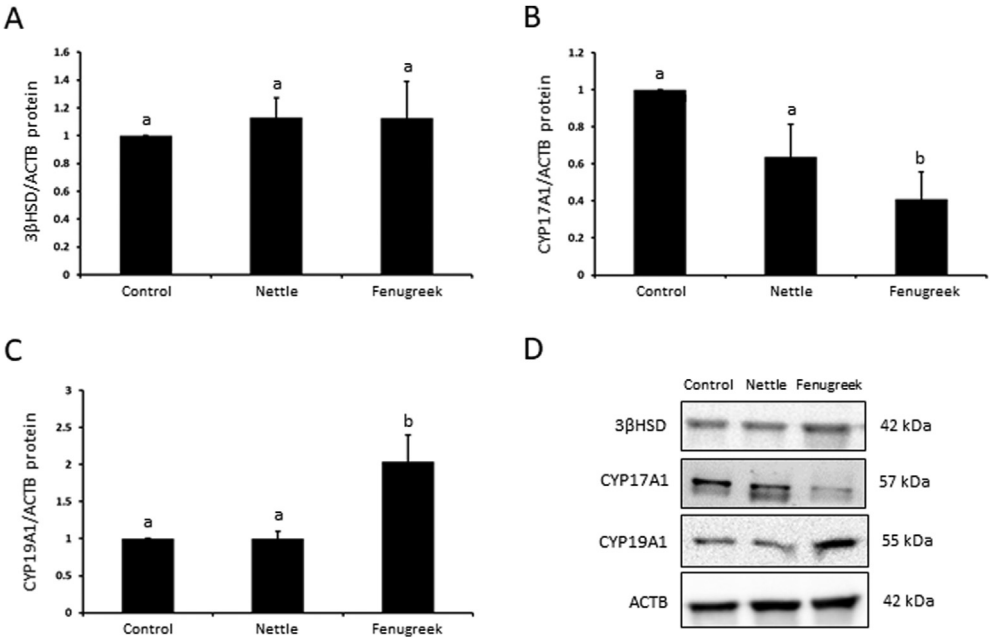

Fig. 6. Western blot analysis of 3β-HSD (A), CYP17A1 (B) and CYP19A1 (C) protein abundance in ovaries obtained from 12-week-old rabbits of the control group and supplemented with nettle or fenugreek. Representative Western blots are shown (D). The relative level of studied protein was normalized to β-actin (ACTB). The protein levels within the control group were arbitrarily set at 1. Each value represents the mean ± SEM. Different letter superscripts indicate statistically significant differences between groups (P < 0.05; Dunn's multiple comparison test). In B, C and D charts, differences among groups related to pro-caspases are marked by lowercase letters and among groups related to cleaved caspases by uppercase letters. *n* = 10 per each group.
3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP19A1, cytochrome P450 aromatase.

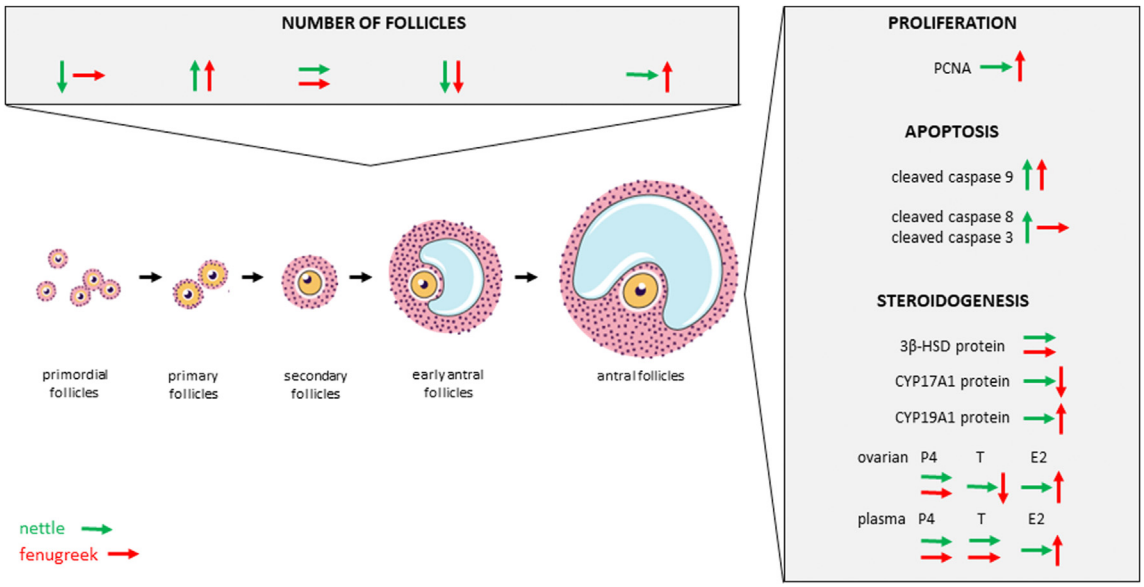

Fig. 7. The effects of diet supplemented with nettle or fenugreek on folliculogenesis and steroidogenesis in the ovary of juvenile rabbits.
PCNA, proliferating cell nuclear antigen; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; CYP17A1, 17α-hydroxylase/17,20-lyase; CYP19A1, cytochrome P450 aromatase; P, progesterone; T, testosterone; E2, 17β-estradiol; → no changes; ↑ increase; ↓ decrease.

tissues, and might be caused by polyunsaturated oxidised fatty acids (rutin and oxylipins) [31]. Nettle-mediated induction of apoptosis through activation of caspase 9 and caspase 3 has been demonstrated in a colorectal cancer cell line [32] and prostate cancer cells [33], while both caspase 8 and caspase 9-dependent apoptosis was showed in Jurkat cells [34]. Our findings suggest that induction of apoptosis in the juvenile rabbit ovary following nettle supplementation might take place through either extrinsic or intrinsic pathways.

Besides folliculogenesis, the ovary is also responsible for the biosynthesis of steroid hormones. Estradiol plays a crucial role in follicle development, maturation and ovulation, whereas progesterone is essential for maintaining pregnancy [35]. The initial step of ovarian steroidogenesis involves the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1). Next, pregnenolone is metabolized by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) to progesterone, which is further converted to androgens by 17α-hydroxylase/17,20-lyase (CYP17A1) [36]. Those steps take place in theca interna cells, but final aromatization of androgens to estrogens occurs in granulosa cells by cytochrome P450 aromatase (CYP19A1) [11]. In the present study, we examined for the first time the abundance of crucial steroidogenic enzyme proteins in the rabbit ovary in response to nettle or fenugreek supplementation. Only fenugreek resulted in decreased CYP17A1 and increased CYP19A1 protein abundance, followed by the expected reduction in tissue testosterone concentration and elevated estradiol concentration that presumably contributes to the increased plasma estradiol concentration in this group. Although there is no information on the direct effect of fenugreek on CYP17A1 and CYP19A1 expression or activity, we propose that observed changes might be attributed to contained phytoestrogens known as inhibitors or inducers of steroidogenic enzymes [37–40].

Herein, a diet enriched with nettle or fenugreek enhanced plasma concentration of estradiol in juvenile rabbits, but only fenugreek caused a statistically significant increase. It is known that nettle and fenugreek are sources of phytoestrogens, which can mimic estrogenic effects [9,41]. Fenugreek seeds contain diosgenin, which binds to estrogen receptors (ERs) mimicking estrogens action [42]. An *in vivo* study using ovariectomized rats found that feeding with fenugreek seeds produced estrogen-like effects such as increased uterine weight, epithelial cell proliferation, vaginal cornification and opening [29]. Dietary supplementation with fenugreek seeds also improved laying performance in hens by enhancing plasma estradiol concentration [43]. *In vitro* study of the MCF-7 breast cancer cell line confirmed the estrogenic activity of fenugreek seed extract: it stimulated cell proliferation, bound to ERs and induced the expression of estrogen regulated genes [30]. Phytoestrogens such as lignans, sterols and β-sitosterol have been identified in nettle [44] and beneficial estrogenic effects of nettle have been found in rats with ovariectomy-induced osteoporosis [45].

5. Conclusions

In conclusion, our study demonstrates the influence of nettle and fenugreek on the central ovarian functions of folliculogenesis and steroidogenesis in the juvenile rabbit ovary (Fig. 7). Both herbs affected follicle development in a stage specific manner and these effects are probably related to changes at the cellular level: nettle had apoptotic effects through caspase-mediated routes, while fenugreek increased follicular cell proliferation. Interestingly, fenugreek influenced the local androgen-estrogen ratio in the ovary by targeting steroidogenic enzyme abundance. That led to elevated circulating estradiol concentration, which in turn might influence ovarian follicle development. Based on both increased plasma estradiol concentration and the number of antral follicles, we propose that a fenugreek-enriched diet might accelerate sexual maturation in juvenile rabbits.

CRediT authorship contribution statement

Malgorzata Grzesiak: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Klaudia Kapusta:** Investigation, Formal analysis. **Kinga Kaminska:** Investigation, Formal analysis.

Sylwia Palka: Conceptualization, Methodology, Resources, Writing – review & editing. **Michal Kmiecik:** Investigation, Methodology. **Joanna Zubel-Lojek:** Investigation.

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