

Fennel Affects Ovarian Cell Proliferation, Apoptosis, and Response to Ghrelin

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Summary

The objective of this study was to examine the direct effects of the medicinal plant fennel (*Foeniculum vulgare* Mill.) on basic functions of ovarian cells, including proliferation, apoptosis, and response to the physiological hormonal stimulator, ghrelin. In the first series of experiments, porcine ovarian granulosa cells were cultured with (1, 10, 100 µg/ml) or without fennel extract. In the second series of experiments, cells were cultured with (1, 10, 100 ng/ml) or without ghrelin, alone or in combination with fennel extract (10 µg/ml). Expression of the proliferation marker, PCNA, and the apoptosis marker, *bax*, were analyzed via quantitative immunocytochemical methods. Fennel stimulated the accumulation of the proliferation marker, and suppressed the expression of the apoptosis marker. Ghrelin alone promoted proliferation and apoptosis of ovarian cells. The presence of fennel inhibited these ghrelin effects. These observations provide the first demonstration of (1) effects of fennel on farm animal reproduction, (2) direct effects of fennel on ovarian cells, (3) the ability of fennel to promote ovarian cell proliferation, to inhibit ovarian cell apoptosis, and to enhance the ovarian cell proliferation:apoptosis ratio. Furthermore, our results (4) confirm the involvement of ghrelin in the control of ovarian cell apoptosis and proliferation, and (5) demonstrate the ability of fennel to affect not only ovarian cell proliferation and apoptosis, but also to suppress the responses of ovarian cells to the upstream hormonal regulator ghrelin. Our results indicate the potential applicability of fennel as a bio-stimulator of farm animal reproduction.

Key words

Fennel (*Foeniculum vulgare* Mill.) • Ovary • Proliferation • Apoptosis • Ghrelin

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Introduction

The study and application of natural/plant substances have become a popular alternative to regular medicines, hormones, and antibiotics in human and veterinary medicine and animal production. One plant used extensively in Oriental medicine is fennel (*Foeniculum vulgare* Mill.). Fennel contains numerous biological active substances, and has antioxidant, cytotoxic, anti-inflammatory, anti-microbial, bronchodilatory, estrogenic, diuretic, lithontriptic, galactagogue, emmenagogue, antithrombotic, hypotensive, gastroprotective, hepatoprotective, memory enhancing, and anti-mutagenic activities. Due to these activities, fennel is used as a functional food and treatment for more than 43 disorders (Rahimi and Ardekani 2013, Badgujar *et al.* 2014, Sadati *et al.* 2016). There is already some evidence for the utility of fennel in animal production. Fennel can be used to reduce methane production by ruminant gastric microflora (Patra *et al.* 2010, Abdalla *et al.* 2012), to improve feed conversion and growth in calves (Saeedi *et al.* 2017), to increase food conversion and performance in quails (Cabuk *et al.* 2014) and to prevent coccidiosis in chicken (Dragan *et al.* 2014).

There are indications that fennel phytoestrogens can influence female reproductive processes. Fennel can promote menstruation and prevent the signs of menopause in women (Albert-Puleo 1980, Badgular *et al.* 2014, Cirmanova *et al.* 2017). Fennel can also increase ovulation/egg laying rate in quails (Cabuk *et al.* 2014), promote ovarian growth and follicle development in mice (Khazaei *et al.* 2011, Hassanpour *et al.* 2017) and improve fertility in rats (Abdel-Wahab *et al.* 2017). Moreover, fennel can prevent the ovarian response to the toxic effects of cyclophosphamide in mice (Hassanpour *et al.* 2017) and cadmium in rats (Abdel-Wahab *et al.* 2017). Nevertheless, the effects of fennel on farm animal reproduction, as well as the mechanisms of fennel action on reproductive processes, have not been previously studied. It remains unknown whether fennel molecules affect ovarian cells directly, or via upstream central (hypothalamo-hypophysial) regulators. Furthermore, ovarian folliculogenesis and fertility, which could be affected by fennel, are determined by the equilibrium between two antagonistic processes – ovarian cell proliferation and apoptosis, as well as the response of these processes to upstream hormonal regulators. The effects of fennel on these processes have not been previously studied.

The objective of this study was to examine the direct effects of fennel on the basic functions of ovarian cells, including proliferation, apoptosis, as well as their responses to the physiological hormonal stimulator, ghrelin (Sirotkin and Meszarosova 2010, Sirotkin *et al.* 2011, Rak-Mardyla 2013, Sirotkin and Harrath 2014). For this purpose, we analyzed proliferation and apoptosis of porcine ovarian granulosa cells cultured with or without fennel extract, with or without ghrelin, and with a combination of fennel extract and ghrelin.

Materials and Methods

Isolation and culture of granulosa cells

Granulosa cells were isolated from the ovaries of non-cycling pre-pubertal gilts aged approximately 180 d, that were slaughtered at a local abattoir. The ovaries were processed, and granulosa cells were isolated and collected as previously described (Sirotkin and Meszarosova 2010, Sirotkin *et al.* 2011) in 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µl/well). After creating cell monolayers, cells were cultured either without fennel extract (controls), or with 90 % extract of fennel (Changsha Sunfull Bio-tech. Co, Hunan China) at

concentrations of 1, 10, and 100 µg/ml. Other cells were cultured either without (control) or with biological-grade human ghrelin (Sigma-Aldrich Inc., St. Louis, MO, USA) at concentrations of 1, 10, and 100 ng/ml, alone. A third group of cells were cultured with ghrelin (1, 10, and 100 ng/ml), in combination with 1 µg/ml extract of fennel. The molecules were dissolved in the culture medium immediately prior to their addition to cells. The cells in the control treatments were cultured in the absence of both fennel and ghrelin, while the blank control consisted of the cell-free medium. The cells remaining at the bottom of the wells were rinsed with ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4 % in PBS, pH 7.2–7.4) for 60 min, and kept at 4 °C in preparation for immunocytochemical analysis. The number and viability of the cells were determined using Trypan blue staining and a hemocytometer. Cell viability ranged between 70 and 80 %, and no statistically significant differences were observed in these indices between the control and experimental groups.

Quantitative immunocytochemistry

Granulosa cells were processed as previously described (Sirotkin and Meszarosova 2010, Sirotkin *et al.* 2011), and incubated in the presence of primary monoclonal antibodies, at dilutions of 1:500 in PBS, against PCNA (marker of proliferation; (Shiomi and Nishitani 2017), and bax (marker of cytoplasmic apoptosis; (Pena-Blanco and Garcia-Saez 2018) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, USA). Thereafter, cells were incubated with secondary swine antibody against mouse IgG, labeled with horseradish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) or porcine secondary antibody labeled with fluorescein isothiocyanate (FITC, Sevac, dilution 1: 500), for 1 hr. Cells labelled with FITC were mounted into VECTASHIELD Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI), a selective stain for cell nuclear DNA (Vector Laboratories, Burlingame, CA, USA). DAPI and the FITC-labeled secondary antibody were detected by fluorescence microscopy. Cells labelled with horseradish peroxidase were stained with 3,3'-diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH, Mannheim, Germany), mounted into glycergel histologic mounting medium (DAKO Denmark A/S, Glostrup, Denmark), and then visualized under a light microscope. The number of stained cells and the location of intracellular molecules were determined based on the brown coloration of DAB peroxidase or the green

fluorescence emitted by the FITC label, and the ratio of stained cells to the total number of cells was calculated.

Statistical analysis

The results represent the data summarized from three experiments performed on separate days with distinct groups of granulosa cells that were obtained from 10–20 pigs. All the experimental groups were represented by three wells containing granulosa cells. The proportion of cells containing the specific immunoactivity were calculated from at least 1,000 cells per well, and the percentage of cells containing the antigens was determined after estimating the cell numbers. Significant

differences between the experimental and control groups were evaluated with one- or two-way analysis of variance (ANOVA), followed by Duncan's test, using the software Sigma Plot 11.0 (Systat Software, GmbH, Erkrath, Germany); results were considered to be statistically significant at $P < 0.05$.

Results

Cultured porcine ovarian cells contained both PCNA and bax. PCNA was located primarily in the nuclei, and, to a lesser extent, in the cytoplasm, while bax was predominantly located in the cytoplasm (Fig. 1).

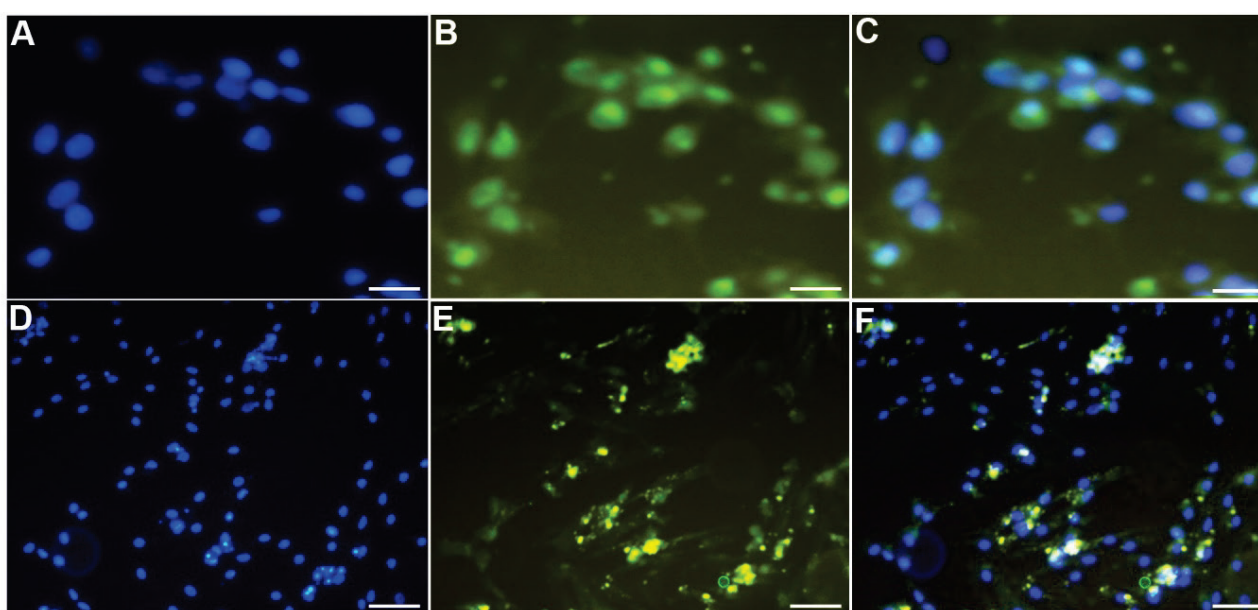


Fig. 1. Immunofluorescent detection of PCNA (A–C) and bax (D–F) proteins in porcine granulosa cells. Immunofluorescence was performed using specific PCNA and BAX primary antibodies. The Secondary antibody was labelled with FITC (green), cell nuclei were stained with DAPI (blue). Scale bar= 20 μm .

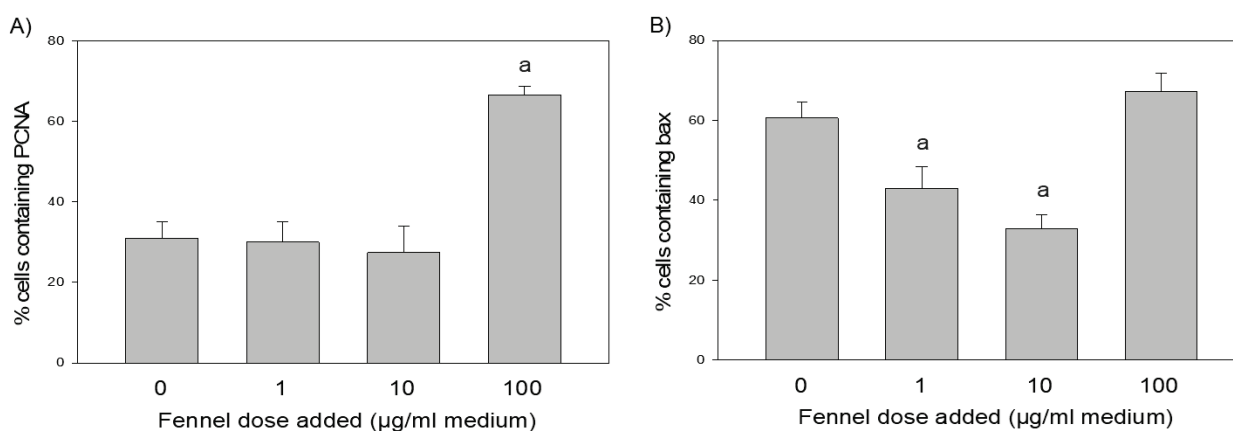


Fig. 2. The effect of the addition of fennel (*Foeniculum vulgare* Mill.) extract at concentrations of 0 (control), 1, 10, and 100 $\mu\text{g/ml}$, on the expression of the proliferation marker PCNA (A) and the apoptosis marker Bax (B) in cultured porcine ovarian granulosa cells. Data were derived from quantitative immunocytochemical analysis. a: significant differences ($P < 0.05$) were observed between cells cultured with and without (0 $\mu\text{g/ml}$) fennel extract.

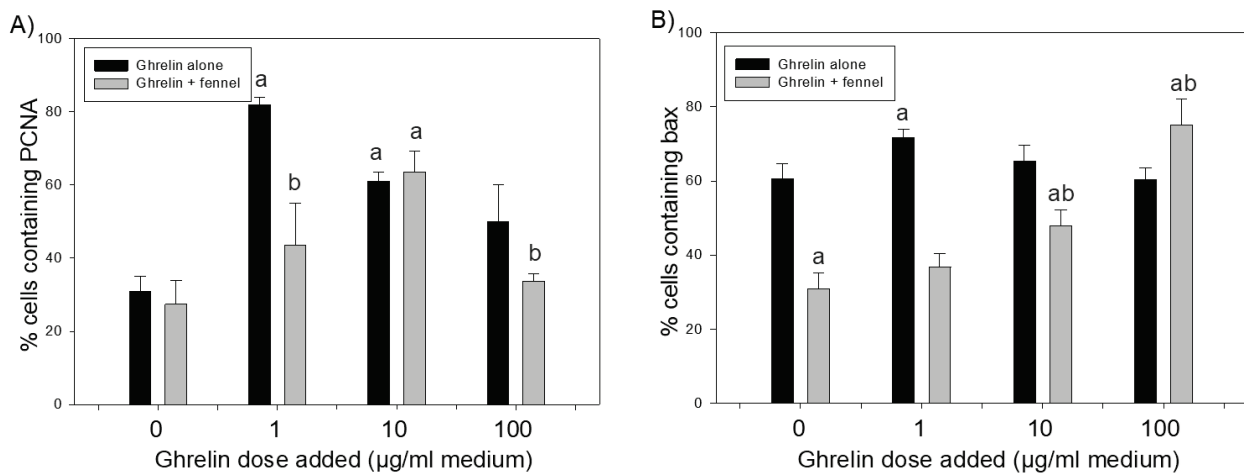


Fig. 3. The effect of the addition of ghrelin, at concentrations of 0 (control), 1, 10 and 100 ng/ml, either alone or in combination with 10 µg/ml fennel (*Foeniculum vulgare* Mill.) extract on the expression of the proliferation marker PCNA (A) and the apoptosis marker bax (B) in cultured porcine ovarian granulosa cells. Data were derived from quantitative immunocytochemical analysis. a: effect of fennel: significant differences ($P < 0.05$) were observed between the corresponding groups of cells cultured with and without fennel. b: effect of ghrelin: significant differences ($P < 0.05$) were observed between cells cultured with and without ghrelin.

The first series of experiments demonstrated that the addition of the fennel extract increased the percentage of cells containing PCNA (after addition at dose of 100 µg/ml, Fig. 2A). Furthermore, addition of fennel extract (at doses of 1 or 10 µg/ml) reduced the percentage of apoptotic (bax-positive) cells (Fig. 2B).

In the second series of experiments, similar to the first series, fennel, when added alone at dose 10 µg/ml (i.e. a ghrelin dose of 0 µg/ml) did not affect the percentage of proliferating (PCNA-positive) cells (Fig. 3A), but reduced the percentage of apoptotic (bax-positive) cells (Fig. 3B).

In these experiments, ghrelin, when added alone, increased the proportion of PCNA-positive cells at all doses (Fig. 3A), and ghrelin increased the proportion of bax-positive cells at a dose of 1 ng/ml. In presence of fennel, ghrelin (at a dose of 10 µg/ml) promoted PCNA accumulation (Fig. 3A), and ghrelin promoted bax accumulation at the doses 10 and 100 µg/ml (Fig. 3B).

Discussion

Trypan blue staining and cell counting did not reveal differences in the number and viability of cells among the different groups of cells after two days of treatment (see Materials and Methods). Nevertheless, the presence of both PCNA (a marker of the S-phase of the cell cycle, (Shiomi and Nishitani 2017) and bax (a marker of cytoplasmic apoptosis, (Pena-Blanco and Garcia-Saez 2018), was observed in the cultured cells, which indicates that the cells used in experiments were viable, and that

both proliferation and apoptosis had taken place in these cells. Furthermore, the results demonstrate that the addition of both fennel and ghrelin affected these processes.

The ability of fennel to promote proliferation (PCNA accumulation) and to suppress apoptosis (bax accumulation) suggests that this plant can change ovarian cell proliferation:apoptosis ratios in favor of proliferation, and therefore to stimulate ovarian cell functions, and probably ovarian folliculogenesis. These observations represent the first evidence for the influence of fennel on reproduction in pigs, and indeed any farm animal species. Furthermore, our results are the first demonstration that fennel can affect ovaries directly, i.e. not via the upstream central nervous system (CNS). These observations are in agreement with previous studies on the ability of fennel to stimulate ovarian folliculogenesis and fertility in other species: human women: (Albert-Puleo 1980, Badgujar *et al.* 2014) quails: (Cabuk *et al.* 2014) mice: (Khazaei *et al.* 2011, Hassanpour *et al.* 2017) rats: (Abdel-Wahab *et al.* 2017). Furthermore, our results provide a description of possible mechanisms of the stimulatory action of fennel on reproduction, i.e., via promotion of ovarian cell proliferation, reduction in ovarian cell apoptosis, and increases in the proliferation:apoptosis ratio.

Another possible mechanism by which fennel affects ovarian cells could be the fennel-induced change in the response of the ovary to ghrelin, an upstream hormonal regulator. In our experiment, when cells were cultured with only ghrelin, it promoted both proliferation

and apoptosis. Increases in both these processes suggest that ghrelin could promote ovarian cell turnover, which defines the growth, development, selection, and finally the fecundity of ovarian follicles (Sirotkin 2014). This confirms the results of previous reports regarding the importance of this hormone in the direct control of basic ovarian functions in pigs and other species (Sirotkin and Meszarosova 2010, Sirotkin *et al.* 2011, Rak-Mardyla 2013, Sirotkin 2014, Fontana *et al.* 2020). Furthermore, in the present study, fennel suppressed ghrelin action on both proliferation and apoptosis - in the presence of fennel, the effective doses of ghrelin were significantly higher than in the absence of fennel. The ability of fennel to inhibit the response of ovarian cells to some exogenous drugs that suppress reproduction has been previously reported. Fennel has been found to increase the response of mouse ovaries to cyclophosphamide (Hassanpour *et al.* 2017) and the response of rat ovaries to cadmium (Abdel-Wahab *et al.* 2017). To the best of our knowledge, the present study is the first to demonstrate the ability of fennel to reduce ovarian responses to an upstream hormonal stimulator.

The mechanisms of fennel action on all these processes require further elucidation. Nevertheless, previous studies indicate that the reproduction-promoting effect of fennel could be due to the presence of phytoestrogens (Rahimi and Ardekani 2013, Badgajar *et al.* 2014, Sadati *et al.* 2016). Plant and animal estrogens can induce various reproductive and non-reproductive processes (Sirotkin and Harrath 2014), including promotion of ovarian cell proliferation, inhibition of ovarian cell apoptosis, and plant and animal estrogens can affect hormonal reception and response to upstream hormonal regulators (Sirotkin 2014, Sirotkin *et al.* 2020). In addition, fennel can inhibit the nuclear transcription factor-kappaB pathway involved in the control of a wide array of physiological processes (Aggarwal and Shishodia 2004), including porcine ovarian cell proliferation and apoptosis (Pavlova *et al.* 2011, Pavlova *et al.* 2013, Sirotkin *et al.* 2014).

The results of the present study also suggest the potential applicability of fennel in animal production, not

only as an anti-bacterial and digestion-promoting food additive (Patra *et al.* 2010, Abdalla *et al.* 2012, Cabuk *et al.* 2014, Dragan *et al.* 2014, Saeedi *et al.* 2017), but also as a potential stimulator of reproduction. To validate this hypothesis, in-vivo experiments in farm animals are needed.

Although the mechanisms and areas of application of fennel require further elucidation, our observations provide the first demonstration of (1) effects of fennel on farm animal reproduction, (2) direct effects of fennel on ovarian cells, (3) the ability of fennel to promote ovarian cell proliferation, to inhibit ovarian cell apoptosis, and to increase the ovarian cell proliferation:apoptosis ratio. Furthermore, our results (4) confirm the involvement of ghrelin in control of ovarian cell apoptosis and proliferation, and (5) demonstrate the ability of fennel to affect not only ovarian cell proliferation and apoptosis, but also to suppress the responses of ovarian cells to the upstream hormonal regulator, ghrelin. Our results indicate the potential applicability of fennel as a bio-stimulator of farm animal reproduction.

Conflict of Interest

There is no conflict of interest.

Ethics statement

This research did not include any human subjects and animal experiments.

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