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Original Article

Ginger (zingiber officinale) might improve female fertility: A rat model

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Abstract

Background: Ginger (*Zingiber officinale*) is a well known and extensively used antioxidant in traditional remedies. In this study, we aimed to investigate the effects of ginger powder on ovarian folliculogenesis and implantation in rats.
Methods: There were two study groups. In the 5-day treatment group (one estrous cycle), 100 mg ginger powder, 200 mg ginger powder or distilled water was given for 5 days to the three subgroups each containing seven rats. In the 10-day treatment group, same doses were given for 10 days (two estrous cycle) to the three subgroups each containing seven rats. At the end of the 5th and 10th days, ovarian volumes, ovarian weights, primordial follicles, antral follicles, atretic follicles, and corpus luteum counts were assessed. To evaluate the angiogenic effects of ginger, vascular endothelial growth factor (VEGF) and for the antioxidant effects of ginger endothelial nitric oxide synthase (eNOS) were examined in the ovaries and in the endometrium immunohistochemically.
Results: In the 5-day treatment group, antral follicle count and ovarian stromal VEGF were significantly high in the 100 mg ginger subgroup in comparison to the control group ($p < 0.05$). In the 10-day treatment group, endometrial VEGF and ovarian stromal eNOS were significantly high in the 100 mg ginger subgroup in comparison to the control group ($p < 0.05$). There was no statistically significant difference at 200 mg ginger dose both in 5-day and 10-day treatment groups.
Conclusion: The increases in the antral follicle count and ovarian stromal VEGF in the 100 mg/5-day treatment subgroup indicate that ginger have positive effects on folliculogenesis in short term with low dose. Additionally, ginger may enhance implantation in rats in long term with low dose.

Keywords: eNOS; Folliculogenesis; Ginger; Implantation; Oxidative stress; VEGF

1. Introduction

Herbal medicine is very popular and gains much attention nowadays. It has been believed that it is much more safer than synthetic drugs. Ginger (*Zingiber officinale*) has a long historical medicine use dating back 2500 years in China and India.[1] Its pharmacological properties are varied including antioxidant, anti-inflammatory, anticancer and antimicrobial activities.[2–5]

More than 60 active constituents are known to be present in ginger, which have been broadly divided into volatile and nonvolatile compounds. Hydrocarbons mostly monoterpenoid hydrocarbons and sesquiterpene include the volatile component of ginger and impart distinct aroma and taste to ginger. On the other hand, nonvolatile compounds include gingerols, shogaols, paradols, and also zingerone. The active ingredients

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like gingerols, shogaols, zingerone, and so forth present in ginger exhibit antioxidant activity. It inhibits an enzyme xanthine oxidase, which is mainly involved in the generation of reactive oxygen species.[6]

Antioxidant applications are important for protecting the human body from various sources of oxidative damage and are used extensively for prevention of a variety of diseases. In order to protect the human body from various forms of oxidative damage, recently there has been a noticeable increase in the search and identification of natural and safe antioxidants.

Oxidative stress can significantly negatively impact cellular survival and longevity and lead to programmed cell death.[7] The generations of reactive oxygen species (ROS) that result in oxidative stress include nitrogen based free radical species such as nitric oxide and peroxynitrite as well as superoxide free radicals, hydrogen peroxide, and singlet oxygen.[8] Physiological levels of ROS are required for proper functioning of different biological pathways and in maintaining homeostasis within the human body. Low levels of free radicals act as modulators in female reproductive pathways such as oocyte maturation, physiological follicular atresia, ovulation, fertilization, luteal regression, and corpus luteum formation during pregnancy.[9] ROS is also believed to play a role in the different phases of the endometrial cycle. Disruption in physiological levels of ROS leads to female reproductive dysfunction.[10]

Nitric oxide (NO) is known to mediate physiological functions, such as vasodilation, regulation of angiogenesis, and blood flow in many tissues, including the ovary.[11] Endothelial NO synthase (eNOS) was detected in ovarian follicles and in the corpus luteum during the estrous cycle in several species. It has been demonstrated that NO plays a role in the regulation of angiogenesis, steroidogenesis, apoptosis, and luteolysis.[12]

Defects in ovarian angiogenesis may contribute to a variety of disorders including anovulation and infertility, pregnancy loss, ovarian hyperstimulation syndrome, and ovarian neoplasms.[13] Vascular endothelial growth factor (VEGF), during gonadotropin surge, controls the crucial follicles transition from preovulatory to periovulatory stage that precedes ovulation.[13] Besides, VEGF is known to play an essential role in the regulation of angiogenesis in the endometrium. Its expression increases during the proliferative phase and has a second expression peak later during the mid-secretory phase, being responsible for maturation of spiral arteries during the "implantation window".[14]

The effects of ginger on male infertility and sperm parameters were investigated in a few studies.[15–19] The results showed favorable outcomes on sperm indices.[15–18] However, the effects of ginger on ovarian functions have not been studied so far. In this study, we aimed to investigate the effects of ginger powder on ovarian folliculogenesis and implantation in rats. We evaluate the effects of ginger in the ovaries and in the endometrium by VEGF and eNOS levels. This is the first study in the literature that investigates this topic.

2. Methods

The experiments were approved by the Experimental Animal Ethics Committee of Ankara Training and Research Hospital (protocol no: 0019/23.10.2014). There were 42 female albino rats in estrous cycle, each weighing approximately 200 gr (28 in the study groups and 14 in the control groups). The animals were housed in standard propylene cages in the same animal facility under conventional conditions (12:12-h light:dark; room temperature: 22 ± 2 °C). Specific pelleted food and filtered bottled tap water were supplied *ad libitum*. The animals were allowed to acclimatize for 2 weeks. Three days before the beginning of the experiments, the female rats were exposed to soiled bedding of a mature male rat to synchronize their estrous cycles.[20] Estrous phase was confirmed by vaginal smear examinations. Organic ginger roots were rinsed with distilled water. After drying, the roots were grated into small pieces and dried again using a dehydrator. Then, a mixer was used to grind the small ginger pieces until a powder was obtained. There were two study groups, each with a different length of treatment.

Group 1 (5-day treatment group): 100 mg ginger powder, 200 mg ginger powder or 2 cc distilled water (control group) was given to three subgroups, each containing seven rats, daily for 5 days (one estrous cycle). Ginger powder was mixed with 2 cc distilled water and administered by gavage. The control group also received 2 cc distilled water by gavage.

Group 2 (10-day treatment group): 100 mg ginger powder, 200 mg ginger powder or 2 cc distilled water (control group) were given to three subgroups, each containing seven rats, daily for 10 days (two estrous cycle). Like the 5-day treatment group, the ginger powder was mixed with 2 cc distilled water and administered by gavage. The control group also received 2 cc distilled water by gavage.

At the end of the 5th and 10th days, the rats were sacrificed and the inner genital organs were removed. All surgeries were performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering. The ovarian volumes and ovarian weights were measured. The primordial, antral, and atretic follicles and the corpus luteums were counted using histopathological examination stained with hematoxylin eosin in the entire cross-sectional area of both ovaries for each rat. To evaluate the angiogenic effects of ginger, immunohistochemical assessment of ovarian cortical, ovarian stromal, and endometrial VEGF were done by anti-VEGF receptor 2-antibody kit (catalog number ab15292, Abcam, Cambridge, UK). For both groups, eNOS was immunohistochemically examined in the ovaries (cortical and stromal) and in the endometrium by eNOS antibody kit (catalog number ab66127, Abcam, Cambridge, UK). For each rat, the entire cross-sectional area of the ovaries and endometrium were scanned consecutively and the stained cells were counted at ×200 magnification. All pathological and immunohistochemical examinations were done by the same pathologist who was blinded to the codes given to the rats.

Statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 (SPSS, Chicago, IL, USA). The variables are expressed as mean ± standard

deviation (SD). Shapiro–Wilk tests were used to determine the normality of the distributions of the continuous variables. The normally distributed variables were examined using one-way analyses of variance, followed by Tukey's post-hoc tests. The non-normally distributed variables were analyzed with Kruskal–Wallis tests and Mann–Whitney U tests, with post hoc Bonferroni corrections. p values < 0.05 were considered to be statistically significant.

3. Results

First, we analyzed Group 1 (5-day treatment group), which was divided into three subgroups and given 100 mg ginger, 200 mg ginger, or 2 cc distilled water for 5 days (Table 1). In the 100 mg subgroup, the ovarian volume, ovarian weight, primordial follicle count, atretic follicle count, corpus luteum count, ovarian cortical VEGF, endometrial VEGF, ovarian cortical eNOS, ovarian stromal eNOS, and endometrial eNOS were not statistically different in comparison to the control group ($p > 0.05$). The antral follicle count and ovarian stromal VEGF were significantly high in the 100 mg group ($p < 0.05$) (Fig. 1-A, B). The comparison of the 200 mg subgroup and the control group revealed no statistically significant differences between the variables.

We then analyzed Group 2 (10-day treatment group), which was divided into three subgroups and given 100 mg ginger, 200 mg ginger or 2 cc distilled water for ten days (Table 2). Ovarian volume, ovarian weight, primordial follicle count, antral follicle count, atretic follicle count, corpus luteum count, ovarian cortical and stromal VEGF, ovarian cortical eNOS, and endometrial eNOS were not statistically different between the three subgroups ($p > 0.05$). Endometrial VEGF and ovarian stromal eNOS were significantly high in the 100 mg ginger subgroup in comparison to the control group ($p < 0.05$) (Fig. 1C and D). No statistically significant differences in the variables were found between the 200 mg subgroup and the control group.

Figs. 2 and 3 show the immunohistochemical staining images of two proteins in the three subgroups for the 5-day and 10-day treatment groups, respectively.

4. Discussion

The present study aimed to investigate the effects of ginger powder on ovarian folliculogenesis and implantation in rats. Many studies have described the antioxidant, anti-inflammatory, anticancer, and antimicrobial activities of ginger. But, this is the first study in the literature, which investigates the optimal dose and duration of the ginger powder on the female reproductive system. In our study, we found statistically high antral follicle count and ovarian stromal VEGF in the 100 mg/5-day treatment subgroup. Antral follicle count is one of the most reliable tools that show ovarian reserve. These two findings indicate the favorable effects of ginger in ovarian folliculogenesis.

The control of ovarian stromal cells and germ cell function is a diverse paradigm and oxidative stress may be one of the modulators of ovarian germ cell and stromal cell physiology. A number of autocrine and paracrine factors affect the modulation of various ovarian functions and steroidogenesis.[21] Mammalian ovulation or follicular rupture was proposed to result from the vascular changes and the proteolytic cascade.[22] The cross talk between these two cascades is mediated by cytokines, VEGF and reactive nitrogen and oxygen radicals.[21] Oxidative stress and cytokines are proposed to be interlinked and act as intercellular and intracellular messengers in the ovary.

The importance of the follicular vasculature for maintaining follicular health has been emphasized in several studies.[23] VEGF is expressed and secreted in the human ovary in a manner that suggests a role for this growth factor in both cyclic angiogenesis and regulation of vascular permeability, both of which are critical for ovarian folliculogenesis and normal reproductive function.[13] Follicle selection success is strictly related to the development of a widespread blood vessel network required to sustain the enhanced proliferative and endocrine function of follicles. Blood vessels allow growing follicles to acquire an increasing amount of nutrients, precursors and hormones, as to release steroids and other regulating ovarian hormonal molecules to the systemic circulation. In addition, reduced follicular vascularity is one of the earliest signs of atresia marked by a smaller vascular network and increased apoptosis in thecal capillaries.[24]

Table 1
Comparison of the variables between the Group 1 subgroups with ± standard deviations.

Group 1 (5-day treatment)	Control	100 mg ginger	200 mg ginger	p
Ovarian volume (mm^3)	4.35 ± 1.02	5.14 ± 0.55	5.07 ± 1.09	0.241
Ovarian weight (mg)	45 ± 8.1	53.5 ± 6.2	54.2 ± 16.1	0.245
Primordial follicle count	11.14 ± 8.33	6.86 ± 3.53	8.86 ± 4.05	0.695
Antral follicle count	18.57 ± 5.47	28.29 ± 7.91	18.71 ± 6.34	0.020[a]
Atretic follicle count	5.00 ± 1.91	5.29 ± 2.98	4.14 ± 1.34	0.605
Corpus luteum count	13.43 ± 4.99	11.00 ± 3.74	11.43 ± 3.20	0.502
Ovarian cortical VEGF	116.71 ± 45.24	163.86 ± 58.80	96.00 ± 45.72	0.058
Ovarian stromal VEGF	47.14 ± 23.21	105.71 ± 61.37	50.43 ± 15.64	0.019[a]
Endometrial VEGF	79.86 ± 51.91	80.86 ± 40.34	44.86 ± 22.07	0.188
Ovarian cortical eNOS	70.14 ± 22.01	75.17 ± 56.09	66.14 ± 21.13	0.896
Ovarian stromal eNOS	14.71 ± 9.53	12.67 ± 11.29	19.57 ± 10.37	0.226
Endometrial eNOS	45.71 ± 18.08	51.00 ± 21.61	40.43 ± 19.33	0.709

VEGF = vascular endothelial growth factor; eNOS = endothelial nitric oxide synthase.
[a] The difference between the control and the 100 mg ginger subgroup is statistically significant.

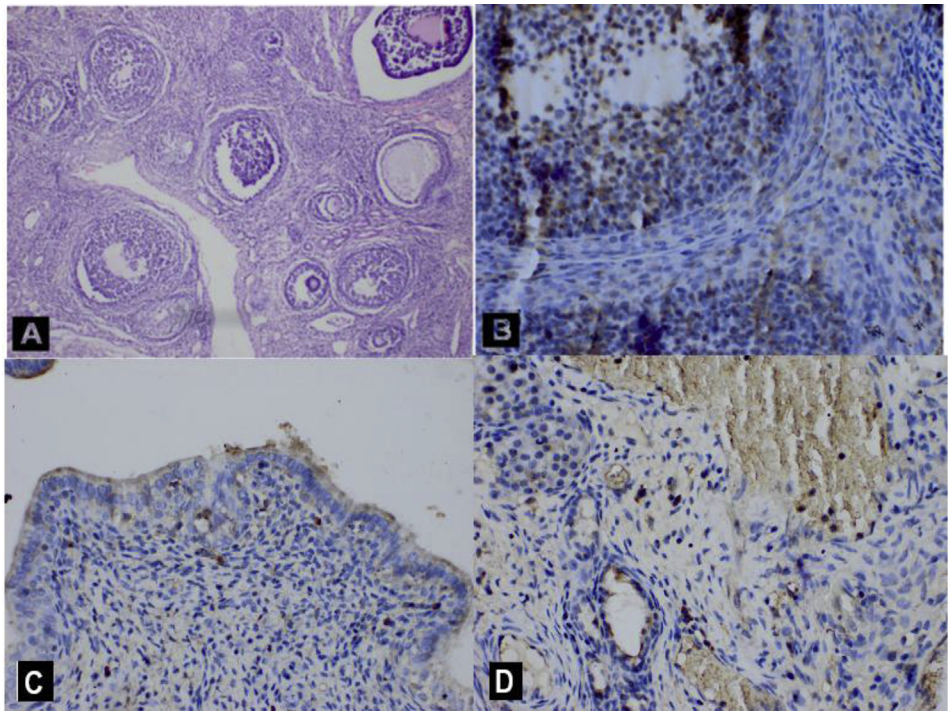

Fig. 1. AHistopathological image of the increased antral follicle count in the 100 mg/5-day treatment subgroup; B Immunohistochemical staining of the ovarian stromal VEGF in the 100 mg/5-day treatment subgroup; C Immunohistochemical staining of the endometrial VEGF in 100 mg/10-day treatment subgroup; D Immunohistochemical staining of the ovarian stromal eNOS in 100 mg/10-day treatment subgroup.

Table 2
Comparison of the variables between the Group 2 subgroups with ± standard deviations.

Group 2 (10-day treatment)	Control	100 mg ginger	200 mg ginger	*p*
Ovarian volume (mm^3)	4.71 ± 0.85	5.75 ± 2.11	5.00 ± 0.50	0.570
Ovarian weight (mg)	67.1 ± 20.7	62.5 ± 20.6	59.2 ± 8.8	0.705
Primordial follicle count	4.71 ± 4.68	8.33 ± 3.93	6.86 ± 4.84	0.214
Antral follicle count	8.86 ± 3.62	11.67 ± 7.73	7.14 ± 4.33	0.338
Atretic follicle count	39.43 ± 6.13	40.17 ± 14.55	52.29 ± 20.15	0.225
Corpus luteum count	11.29 ± 3.98	11.83 ± 4.35	8.00 ± 2.38	0.142
Ovarian cortical VEGF	98.43 ± 21.90	103.33 ± 30.57	76.43 ± 22.50	0.140
Ovarian stromal VEGF	27.00 ± 10.56	35.83 ± 10.88	24.00 ± 6.53	0.073
Endometrial VEGF	29.71 ± 8.51	52.00 ± 13.94	39.29 ± 11.28	0.009[a]
Ovarian cortical eNOS	157.71 ± 59.36	138.71 ± 50.96	117.86 ± 52.48	0.409
Ovarian stromal eNOS	39.29 ± 20.04	76.14 ± 19.57	53.29 ± 9.75	0.003[a]
Endometrial eNOS	85.43 ± 29.43	99.29 ± 48.07	79.29 ± 23.40	0.564

VEGF = vascular endothelial growth factor; eNOS = endothelial nitric oxide synthase.
[a] The difference between the control and the 100 mg ginger group is statistically significant.

Angiogenesis is important for cyclical regeneration of endometrium in the menstrual cycle. Any imbalance between the cytokines and angiogenic factors could result in implantation failure and pregnancy loss.[25] Estrogens promote angiogenesis in the endometrium by controlling the expression of factors such as VEGF.[26] Reactive oxygen species generated from NADP (H) oxidase is critical for VEGF signaling in vitro and angiogenesis in vivo.[27] Recent studies suggest that early pregnancy loss and implantation failure may be caused by an impaired VEGF expression, although the data are controversial.[28] Luteal phase defect was found to be accompanied by an increased impedance of blood flow in the corpus luteum and spiral arteries in infertile patients.[29] It was recently shown that recurrent miscarriage and failed IVF attempts may be associated with an impaired expression of VEGF, the main angiogenic factor in the endometrium.[30]

Another our finding was significantly high endometrial VEGF in the subgroup, which was given 100 mg ginger for 10 days. This finding may indicate the positive effects of ginger in implantation. Though we did not observe any changes in endometrial eNOS levels, both iNOS and eNOS proteins are shown to be up-regulated in the implantation sites.[31] NOS inhibitors block decidualization and establishment of pregnancy and additionally NOS inhibitors act synergistically with antiprogestins. However, besides progesterone, additional embryonic signals are shown to be involved in NOS

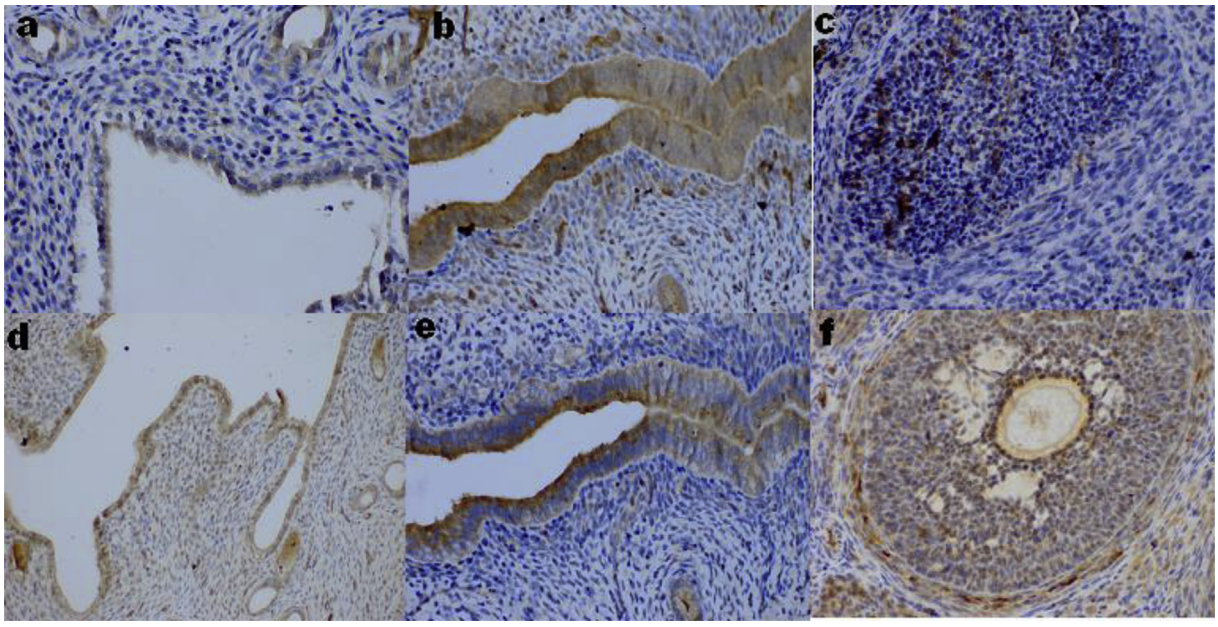

Fig. 2. **a** Immunohistochemical staining of the endometrial stromal VEGFx400 in the 5-day control group; **b** Immunohistochemical staining of the endometrial stromal VEGFx400 in the 100 mg/5-day treatment subgroup; **c** Immunohistochemical staining of the ovarian antral follicle wall VEGF ×400 in the 200 mg/5-day treatment subgroup; **d** Immunohistochemical staining of the endometrial stromal eNOS ×200 in 5-day control group; **e** Immunohistochemical staining of the endometrial stromal eNOS ×400 in the 100 mg/5-day treatment subgroup; **f** Immunohistochemical staining of the ovarian antral follicle wall eNOS ×400 in the 200 mg/5-day treatment subgroup.

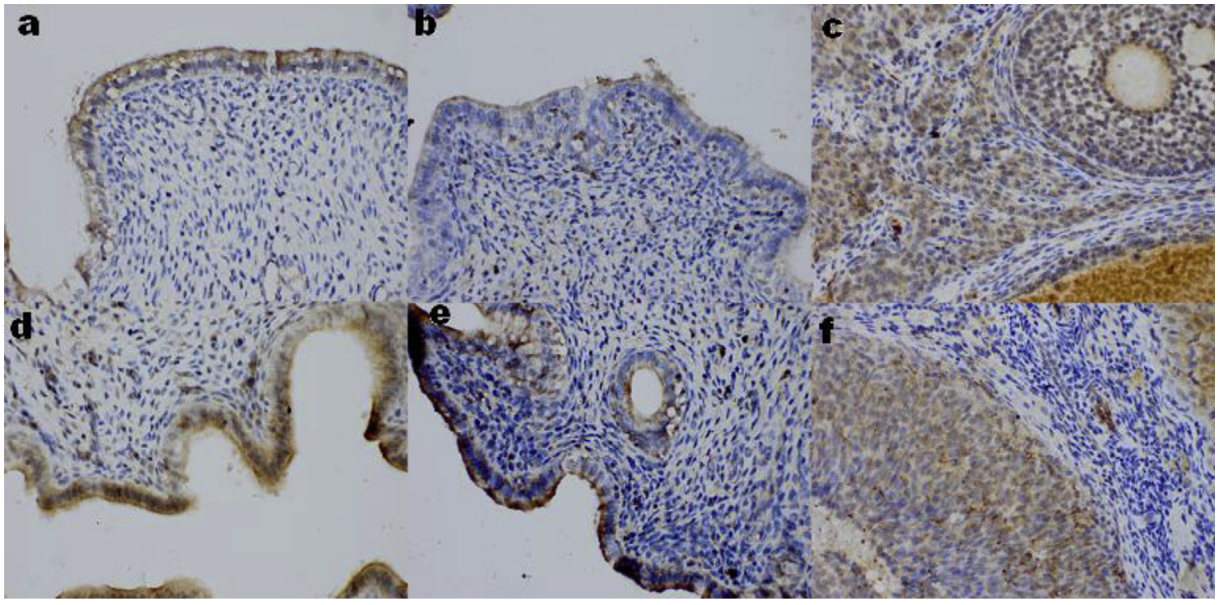

Fig. 3. **a** Immunohistochemical staining of the endometrial stromal VEGF ×400 in the 10-day control group; **b** Immunohistochemical staining of the endometrial stromal VEGF ×400 in the 100 mg/10-day treatment subgroup; **c** Immunohistochemical staining of the ovarian antral follicle wall VEGF ×400 in the 200 mg/10-day treatment subgroup; **d** Immunohistochemical staining of the endometrial stromal eNOS ×200 in the 10-day control group; **e** Immunohistochemical staining of the endometrial stromal eNOS ×400 in the 100 mg/10-day treatment subgroup; **f** Immunohistochemical staining of the ovarian antral follicle wall eNOS ×400 in 200 mg/5-day treatment subgroup.

regulation.[31] As we did not mate the rats and look for pregnancy outcome, perhaps lack of embryonic signals may the reason for unchanged levels of endometrial eNOS.

Ovarian folliculogenesis not only involves gonadotropins and the steroids, but it also involves local autocrine and paracrine factors. Nitric oxide radical is one of the local factors involved in ovarian folliculogenesis and steroidogenesis.[21] The major regulator of NO production is the enzyme, NO synthase (NOS), which appears in three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both eNOS and iNOS have been detected in ovarian tissues of several species.[32] A regulatory role for NO

in ovulation is supported in most species, with sources thought to be both iNOS and eNOS, with iNOS activity increasing with the LH surge.[33] eNOS was detected in ovarian follicles and corpus luteum during the estrous cycle in several species. It has been demonstrated that NO plays a role in the regulation of angiogenesis, steroidogenesis, apoptosis, and luteolysis.[12]

NO is one of several intraovarian mediators that have been shown to influence ovarian functions, including follicular development and atresia, ovulation, steroidogenesis, oocyte quality, apoptosis, and luteal function.[34] In addition, NO may positively regulate the expression of angiogenic factors, including VEGF and the angiogenesis in the ovaries and other tissues.[35]

Folliculogenesis involves the participation of both growth and programmed cell death and NO regulates both. We found statistically high ovarian stromal eNOS in the 100mg/10-day subgroup. The interpretation of our result is somewhat difficult but a negative relationship between VEGF and NO levels were observed in porcine granulosa cells.[36] Although low concentrations of NO may prevent apoptosis, pathologically high concentrations of NO may promote cell death.[37] On the other hand, in the 100 mg/5-day subgroup, we found high levels of antral follicle count and high levels of ovarian stromal VEGF with normal levels of ovarian stromal eNOS. Under these findings, we can only speculate that the optimal duration of ginger powder on folliculogenesis may be for short term. We postulate that with the increase of ovarian stromal eNOS in the long protocol, the positive effects of ginger displayed in the short protocol are lost. Very recently, a study showing similar detrimental effects of increased levels of eNOS and iNOS in rat spermatogenesis has been published.[38] In this study, impaired spermatogenesis could not been improved by long usage of antioxidants (12 weeks) and especially overexpression of iNOS was shown to be responsible for destructive effects. In fact, the studies indicating beneficial effects on sperm indices have used ginger for 4−8 weeks, which is the average duration of spermatogenesis in rats.[39]

Physiological levels of ROS are required for proper functioning of different biological pathways and in maintaining homeostasis within the human body. Any disruption in the antioxidant/ROS balance leads to a state of oxidative stress in the cell with damaging consequences In our study, positive findings were only observed in 100 mg ginger powder given group, instead of 200 mg ginger powder given group. This shows that high dose antioxidant disturbs physiologic balance in folliculogenesis and implantation. This is in close correlation with the statement that follicle maturation is a classic example of the delicate balance that exists between ROS and antioxidants in the maintenance of the regulated sequence of events that culminate in ovulation as mentioned by Gupta et al.[10]

In conclusion, our results showed that ginger powder increases antral follicle count and ovarian stromal VEGF at low dose with short duration and endometrial VEGF at low dose with long duration. Our results suggest positive effects of ginger in folliculogenesis and implantation. Essentially, the results of this study stress the importance of ginger as an antioxidant to suppress ROS buildup and maintain physiological levels of free radicals for proper cell functioning and homeostasis. Newer studies should be designed with different doses, intervals and parameters to show the exact effects of ginger in female reproductive system before routine recommendation for infertile women to achieve pregnancy.

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