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Original article

Iodine supplementation activates folliculogenesis in rabbit ovary

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Abstract

The aim of this study was to assess the biological effect of the product Jodis Concentrate (JC) on the rabbit ovaries by evaluating the folliculogenesis and expression of oocyte-specific growth differentiation factor 9 (GDF9).

The experiment was conducted with 30 female two month old New Zealand rabbits that were the F1 offspring born to mothers differently treated with Jodis concentrate. The control group (n=10), consisted of F1 offspring born to mothers without iodine treatment, and was not supplemented with JC. The first experimental group (n=10), consisted of F1 offspring born to mothers treated with JC during pregnancy and the suckling period, and was supplemented with JC daily at a dose of 2 ml/L drinking. The second experimental group (n=10), consisted of F1 offspring born to mothers without iodine treatment, and was also supplemented daily with the same dose of JC - 2 ml/L drinking. All groups were fed with total mixed ration for growing rabbits. The trial lasted 48 days. The ovaries were weighed and prepared for histological examination. The GDF9 protein expression in the ovary was determined by immunohistochemical analysis. The addition of JC to the drinking water of female rabbits led to more active development of the ovarian follicles from primordial to tertiary stage in both experimental groups. More intensive GDF9 protein expression in the oocytes and cumulus cells of rabbits, supplemented with JC was observed.

Key words: ovary, growing rabbits, growth-differentiation factor 9, iodine supplement

Introduction

Iodine is recognized as an essential constituent of the thyroid hormones in mammals. Normally it is provided in the diet as iodized salt. Minerals in drinking water are more readily absorbed than food, because water does not usually contain chelating agents, which might prevent absorption of the elements (Porter et al. 1988). Triiodothyronine (T3) and thyroxine (T4) have a pronounced physiological effect in the control of respiration and energy metabolism as well as in the biogenesis of the mitochondria. Their production is important in conditions such as disease, starvation or hibernation that lead to altered metabolic status (Hussein and Azab

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1999). Inadequate iodine intake leads to inadequate thyroid hormone production, and all the consequences of iodine deficiency stem from the associated hypothyroidism. It has been reported, that dietary iodine is efficiently absorbed from the gastrointestinal tract and has several benefits for the body: it affects the thyroid gland and other tissues; it alters the gut micro flora, which leads to an increase in the availability of nutrients, and plays the role of water sanitizer (Michalaki et al. 2014). Both insufficient and excessive iodine intake can have a negative effect. Many authors have reported the negative effects of a high dose of iodine in the diet (>500 ppm): in laying hens it reduced or ceased egg production, increased embryonic mortality and delayed hatching (Saki et al. 2012); in rats lactation fails and leads to mortality of offspring (Ammerman et al. 1964). Pregnant rabbits, after consumption of a high dose of iodine, produced offspring that died within 48 hours after birth (Halls 2010). Iodine added to drinking tap water at a rate of 5 ppm improved growth performance and thyroid functions through a significant increase in serum thyroxine (T4) and triiodothyronine (T3) hormones (Hussein and Azab 1999). Thyroid physiology has also been known to alter ovarian function as well as female fertility. Iodine accumulation is higher in the ovary than all other organs except the thyroid gland. Although numerous reports have been published on the use of iodine as a growth stimulator, no sufficient attempts have been yet made to determine the effect of iodine addition to drinking water on reproductive performance, especially on folliculogenesis in livestock animals. It is well known that one of the members of the TGFb superfamily - GDF9 - plays a key role in folliculogenesis. GDF9 induces the proliferation and differentiation of the follicular cells during follicular development from the primordial stage. It is involved in the final events of maturation and ovulation, such as cumulus cell expansion and yellow body formation (Erickson and Shimasaki 2001). Lack of GDF9 in *Gdf9* null mice exhibits a block in follicle growth at the primary stage (Dong et al. 1996). For efficient female fertility, a precisely balanced level of GDF9 expression in oocytes is required (Abadjieva and Kistanova 2016). However, there is a lack of knowledge on how external factors affect the expression of this oocyte-derived growth factor, particularly in the rabbit ovary.

Furthermore, Jodis Concentrate (JC), which is a new and more stable source of iodine, has been examinted in poultry, pigs and cattle (Petkova 2009), but not in rabbits. The aim of our investigation was to examine the biological effect of JC on the rabbit ovary by morphometric evaluation of folliculogenesis and expression of the oocyte-specific protein - GDF9 - which has great importance for the whole process of follicular growth.

Materials and Methods

The additive

The tested additive, Jodis Concentrate (Patent No. PCT/ UA, 990020/22.08.2001, Geneva, Switzerland) is a product certificated by the Ministry of Health of Bulgaria (Sanitary permission for import of "Raw material for the production of iodine products "Jodis concentrate"). This product is a mineral water enriched with biologically active iodine. It contains: 80 mg/dm^3 iodine in biological active form, 10-100 mg/dm^3 /Na + K, 5-150 mg/dm^3 Ca, 10-100 mg/dm^3 Mn, 50 mg/dm^3 chlorides, 50 mg/dm^3 sulphates and 300-600 mg/dm^3 hydrocarbons (Maksin et al. 2015). The product is harmless for humans and animals. There are no side effects (www.jodiscentr-bg.com/index).

Study design

The current study was conducted at the Experimental Base of the Institute of Animal Sciences – Kostinbrod, Bulgaria, with 30 two month old White New Zealand weaned female rabbits. They were the F1 offspring born to mothers differently treated with Jodis Concentrate. The animals were divided into three groups - a control (without additive) and two experimental groups with 10 animals in each. The control group consisted of F1 offspring born to mothers without iodine treatment. The first experimental group consisted of F1 offspring born to mothers treated with JC during pregnancy and the suckling period. The secnd experimental group consisted of F1 offspring born to mothers without iodine treatment. Both experimental groups received Jodis Concentrate as an additive. The rabbits were raised in wire cages, laid in a single layer, with 5 rabbits in each cage. All groups received ad libitum standard granulated total mixed ration (TMR) for growing rabbits with the following nutritional characteristic: 16.9% crude protein, 13.2% crude fiber, 3% crude fat, 0.96% Ca and 0.64% P (Grigorova et al. 2013). We measured the dose of Jodis Concentrate for the rabbits of both experimental groups as 2 ml/l of drinking water. Although the exact requirements are not known, this dose was chosen bearing in mind the content of iodine in the supplement and also the explanation for the iodine level given in Nutrient Requirements of Rabbits (1977). Each morning the dose of supplement and the amount of water were measured. The day after, in the morning, the remainder of the water plus supplement was measured and a new dose of both the supplement and water were given. By using this method of determining the additive and the absorbed water we found the following average daily intake of Jodis Concentrate of a rabbit: 0.46 and

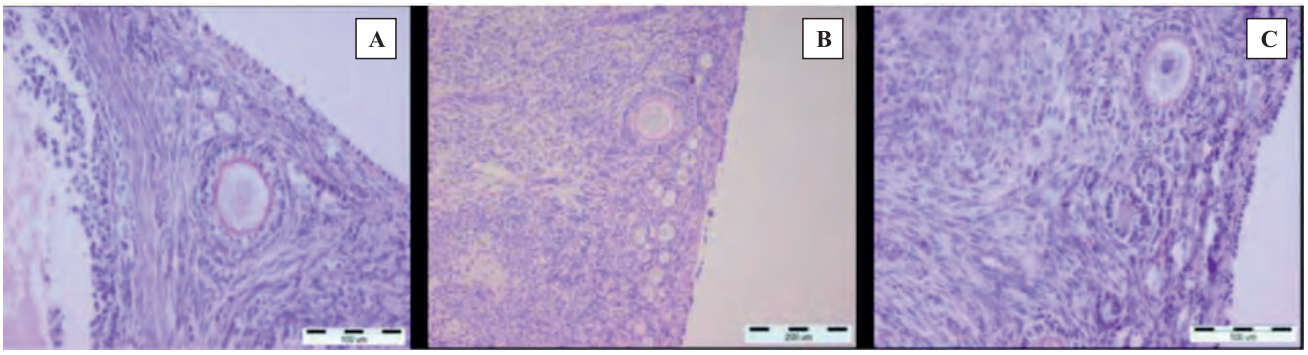

Fig. 1. Microphotographs of rabbit's ovary with primordial and primary follicles (PAS): A. control (x 40); B. first experimental group (x20); C. second experimental group (x40).

0.50 ml JC/head/day for the first and second experimental group respectively. The feed and the water plus supplement intake from the animals were calculated as an average for the whole group of 5 animals. The access to water with supplement was ad libitum and they were supplied via a nipple watering trough. The duration of the JC administration was 48 days. The animals were kept and handled in accordance with the provisions of the Bulgarian Veterinary Law of 25 January 2011 on the protection of animals used for experimental and other scientific purposes, which conform to the relevant provisions of Council Directive 86/609/EEC.

Histological estimation

At the end of the experiment the ovaries were removed from the slaughtered animals and fixed in 10% formaldehyde, and were then embedded in paraffin. Serial sections of 5μm thickness were cut by using a microtome type 2125RT (Leica, Germany). Each tenth section of ovarian tissue fragment was mounted and stained with Periodic Acid Schiff (PAS) and hematoxylin (PAS-Hematoxylin). The count of ovarian follicles was done according to Pedersen's classification in rodents (Pedersen and Peters 1968). Follicles were divided into 3 main categories: small (Primordial and Primary), medium (Secondary and Preantral) and large (Antral or Graafian) follicles respectively; the larger follicles were about 40% atretic, but they were not the subject of this observation. The number of follicles was determined by the fractional method according to Gundersen et al. (1988). This method consists in the application of a dissector with clear size, where each object is counted in only one field. It is thought that this approach provides an accurate assessment of the number of follicles.

Immunohistochemistry

For the detection and analysis of GDF9 protein an immunohistochemical technique was applied using commercial antibodies – goat polyclonal anti-human, in a working dilution of GDF9 1:100 (Santa cruz Biotech., USA) as described by Ramos-Vara (2005). The primary antibody reaction was achieved using donkey anti-goat biotinylated secondary antibodies (1:200), followed by avidin-biotin complex (Vector, USA) for 1 h each. The binding was visualized by the addition of 3,3′-diaminobenzidine (DAB) chromogen solution (Vector, USA).

Immunohistochemically negative controls were carried out using an irrelevant IgG. The average score was taken from slides of animals in each experimental group and photomicrographs from one of three slides is presented here. Photomicrographs of representative fields of immunohistochemistry were evaluated using an Olympus BX51 (Japan) microscope and images were captured with a Powershot G6, 7.1 mp digital camera.

Statistical analysis

All data are presented as means with their standard errors. Statistical examination of obtained results was determined by SPSS, single factor, ANOVA program. A t-test was used to compare the results between control and experimental groups. Statistical significance was set at $p<0.05$.

Results

The examination of PAS stained sections of rabbit's ovary in all groups revealed that the ovary was covered with a single layer of cuboidal epithelium. The ovarian parenchyma was formed of cortex and medulla, but there was no sharp demarcation between these two components. In the ovarian cortex, the primordial follicles were seen underneath the tunica albuginea (Fig. 1). No destructive changes were observed in any group.

All observed animals had started folliculogenesis, which is normal for rabbits at the age of four months. The secondary follicles had appeared and they con-

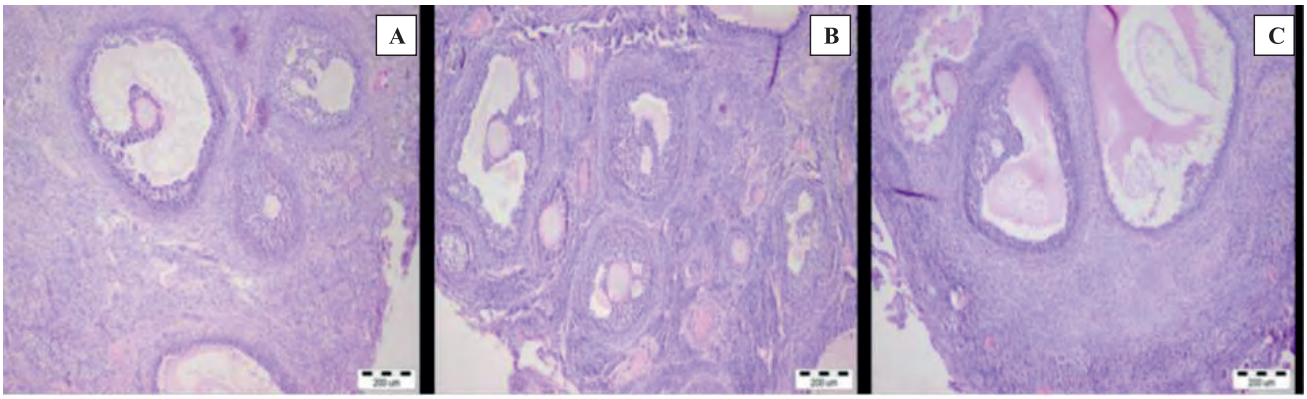

Fig. 2. Microphotographs of rabbit's ovary with preantral and antral follicles (PAS; x 10): A. control; B. first experimental group; C. second experimental group.

Table 1. Morphometric evaluation of rabbit ovaries.

Follicles Group	Primordial and Primary follicles	Secondary and Preantral follicles	Antral follicles
Control * 10.5 slides per animal	12.20 ± 0.60 AB	3.75 ± 0.45 A	2.50 ± 0.20 A
First experim. * 10.5 slides per animal	17.00 ± 0.40 A	5.75 ± 0.47 A	3.75 ± 0.50 A
Second experim. * 10.5 slides per animal	14.75 ± 0.60 B	4.00 ± 0.40	3.20 ± 0.50

Values represent mean ±SE. The mean values along the same colons, marked with same letters (A or B), differ significantly ($p<0.05$).

sisted of an ovum in the center surrounded by shiny eosinophilic structure zona pellucide. One row of low cumulus cells surrounded the zona pellucide that represented the corona radiate, characterized by acidophilic cytoplasm and basely situated basophilic nuclei. Small, round cells with centrally located basophilic nuclei surrounded the corona radiate that represented granulosa cells. The tertiary follicles had appeared and were characterized by well-organized corona radiate cells and an increased number of granulosa and theca cells. The antral cavity had formed and was filled with antral fluid. Antral follicles were characterized by well organized, granulosa cells, theca cells and the appearance of cumulus oophorous, which supported the oocyte inside the antral cavity (Fig. 2).

According to the morphometric analyses (Table 1), the numbers of follicles in ovaries decreases from the primordial and primary to antral stages in all groups in conformity with regular folliculogenesis. However, a significant increase in primordial, primary, secondary and preantral follicles was observed after using iodine in drinking water. Also, more antral follicles were observed in the ovaries of rabbits born to mothers treated with JC compared to the control. All these parameter tended to be higher in the second experimental group compared to the first one.

The immunohistochemical analysis of ovaries showed well expression and typical distribution of the GDF9 protein in the ovarian structures of rabbits: intensity of the immunostaining in the oocytes decreases during the development from primordial and primary to antral stages (Fig. 3 A, D). A more intensive level of signals was defined in the primary and secondary oocytes from the ovaries of the animals treated with JC (Fig. 3 B, C). Moreover, the intensity of signals in the experimental ovaries was high also in the antral follicles (Fig. 3 E, F) in contrast to the control group. It should be noted, that in the ovaries of animals supplemented with JC many ovary structures such as cumulus, theca and luteal cells showed high positive immunostaining to GDF9 (Fig. 3 B-F).

Discussion

Since the beginning of the 19th century the iodine concentration in the ovary has been known to be higher than in any other organ except the thyroid gland, but the physiological role of such high ovarian iodine uptake and accumulation has not been fully elucidated until now (Slebodzinski 2005). Iodine consumption in excess of its recommended levels over a prolonged

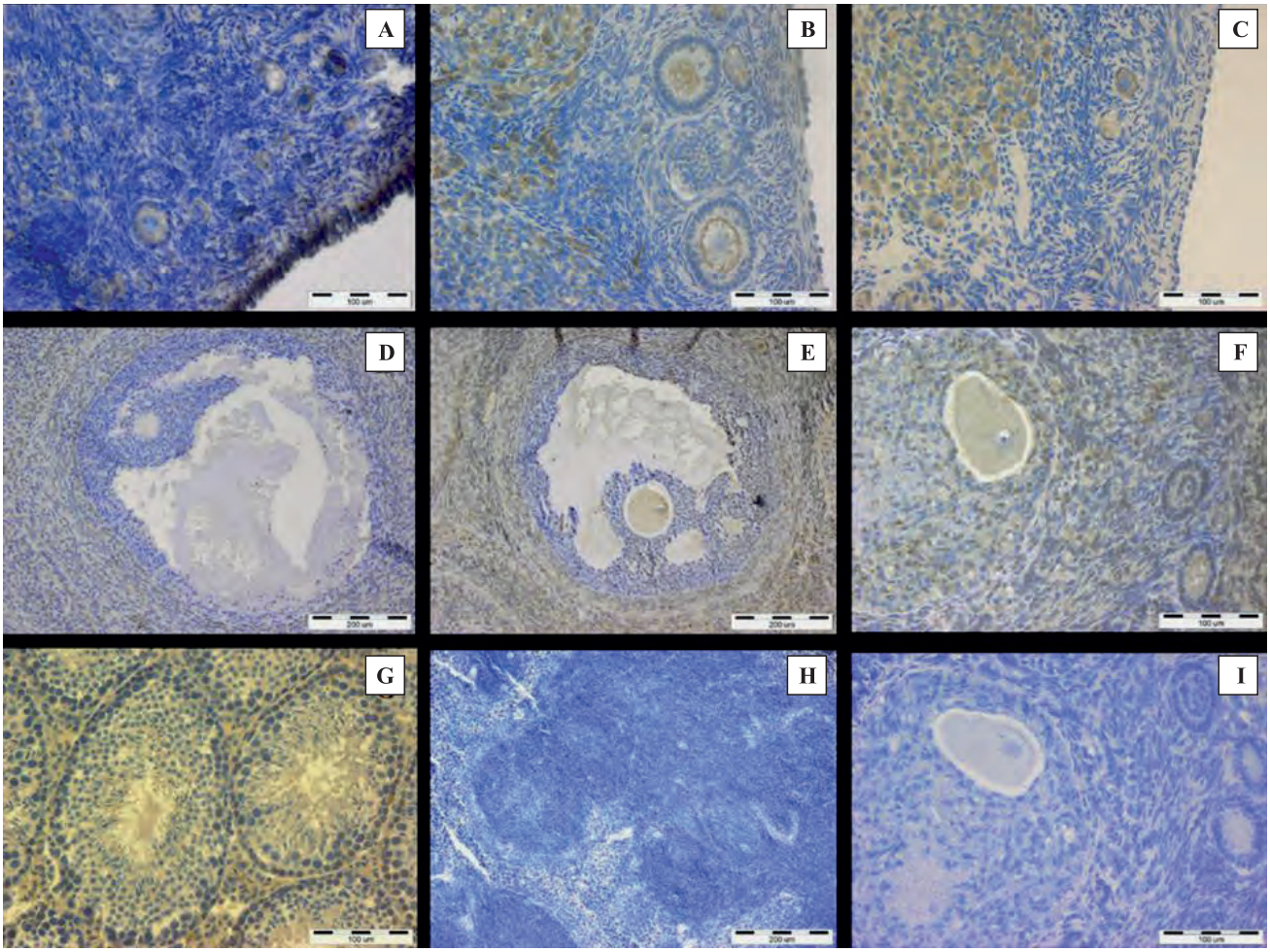

Fig. 3. Immunolocalization of the protein GDF9 in: A. oocytes of primordial and primary follicles in control animals (x40); B. oocytes and granulosa cells of the secondary follicles and luteal cells of animals from the first experimental group (x40); C. oocytes of primordial follicles and luteal cells of animal from second experimental group (x40); D. theca cells of antral follicle of control animal (x20); E. oocytes and theca cells of antral follicles, and ovary cells in first group (x20); F. oocytes and cumulus cells of antral follicles, and ovary cells in second group (x40); G. mouse testis - positive control of antibody (x40); H. negative control of tissue – spleen (x20); I. antral follicle - negative control of antibody (x40).

period of time is well known to cause thyroid disorders. The effect of iodine on gonads may vary with animal species, the age of the animals, and the duration of the treatment (Chan and Ng 1995). In rats, obese chicken strain, and non-obese diabetic mice, excess iodine is associated with autoimmunity. Excess iodine induces rapid apoptosis of pretreated Wistar rats, possibly connected with inhibition of polyamine synthesis, which are inhibitors of DNA fragmentation. Chronic iodine excess induces apoptosis and necrosis of thyroid follicular and endothelial cells, leading to thyroglobulin accumulation in connective tissue. Iodide excess requires peroxidase enzymatic activity to induce apoptosis. Ionic iodide is not directly toxic, whereas its molecular form, I_2 mediates the apoptotic effect of KI (Markovic 2017). Mahapatra and Chandra (2017) revealed a biphasic action of excess iodine on the ovarian function that depends on its dose. At a dose of 100EI iodine, excess iodine did not alter thyroid physiology but lead to the development of a hypoestrogenic state. There was an increased accumulation of iodine in the ovary with decreased activity of ovarian steroidogenic enzymes and lowered serum estradiol levels. Conversely, an excessive dose of 500EI iodine is intolerable to the thyroid and a hyperthyroid condition develops that leads to a hyperestrogenic state. Therefore, prolonged exposure to iodine in excess exerts a biphasic mode of action depending on the dose in female reproductive physiology and both doses destroyed fertility. The effect of iodide uptake resulted in disturbance of the folliculogenesis in treated animals, particularly the follicular structure and communication between oocyte and granulose cells (Mahapatra and Chandra 2017). Results of our investigation demonstrated a clear positive effect of low iodine supplementation with drinking water for 48 days on folliculogenesis in rabbit ovaries,

resulting in a higher number of developing follicles and a more active expression of GDF9 protein. The number of primary and primordial as well as antral follicles was higher in the ovaries of F1females born to mothers supplemented with JC during pregnancy and suckling. The specific response in the iodine supplemented group suggests that the iodine supplementation has addressed the thyroid expression in animals. The discovery of a sodium iodide symporter (NIS) in ovaries has offered a possible mechanism for ovarian iodide intake and other functional similarities to its thyroid counterpart (Furnaletto et al. 1999, Perron et al. 2001). Nevertheless, the physiological significance of ovarian iodine uptake and accumulation remains unknown (Slebodzinski 2005). Pasupathi et al. (2014) reported that the iodine supplementation of rabbit diet at a dose of 100 μg per animal per day from 6 weeks of age did not affect reproduction or fetal performance, nor the subsequent performance in the pre-weaning period. Improper iodine intake is a major concern in the health of humans and animals. Chronic deficiency as well as an excess of iodine affect gonadal functions in women and animals due to the disturbance of the T3/T4 hormone production that is dependent on the iodine intake. In the ovaries of humans and mice, thyroid hormone receptors (TRα-1, TRα-2 and TRβ-2) are present in the oocytes, cumulus, granulosa and luteal cells (Zhang et al. 1997). Moreover, not only the presence of thyroid hormones in follicular fluid, but also the presence of the ovarian 5′-monodeiodinase system capable of generating an ovary-born T3 has been revealed recently (Slebodzinski 2005). It is well established that adequate levels of circulating T3 are important for normal female reproductive functions. Women suffering from thyroid disorders frequently experience impaired fertility, a condition readily improved with the restoration of the euthyroid state (Poppe et al. 2003). Chronic hypothyroidism initiated in the fetal period through a diet low in iodine also results in a decreased ovulation rate associated with a disturbance of the antioxidant defense system in the ovary (Meng et al. 2016). Persistent generation of reactive oxygen species (ROS) in the testis as a result of prolonged iodine exposure affects the hypothalamus-pituitary-adrenal axis that stimulates the synthesis and secretion of corticosterone, which inhibits FSH and LH release (Chakraborty et al. 2016).

The transition of the developing follicle from the preantral to early antral stage is primarily controlled by intraovarian regulators such as GDF9, but is also responsive to FSH and LH. While FSH promotes growth of mouse cultured follicles, this response is markedly enhanced by the presence of T3 hormone (Cheng et al. 2013). In vitro studies on rats have shown that thyroid hormone regulates ovarian follicular development through up-regulation of FSHR, a mechanism dependent on the expression and action of oocyte-derived GDF9 (Kobayashi et al. 2009), which is in line with our investigation. Results of our experiment are the first evidence supporting this finding in vivo in rabbits. Using histological and immunohistochemical methods we established that the high level of GDF9 protein expression associated with active folliculogenesis and higher number of follicles reached the antral stage in the ovaries of animals supplemented by JC. The relationship between T3 action and the action of GDF9, established by Kobayashi et al. (2009), may provide a significant clue to the understanding of the possible mechanisms of iodine action on folliculogenesis in the ovary. Although many follicles were observed in the ovaries of animals treated with JC, there was no presence of cystic formations. Polycystic ovary syndrome (PCOS), which is the most frequently seen endocrine disorder, caused subfertility or infertility in women of reproductive age and often develops as a result of the decreased level of GDF9 and BMP15 since the beginning of follicle formation (Karagül et al. 2018). Our results showed enhanced expression of GDF9, and suggest that treatment with iodine could have a positive effect in overcoming PCOS.

Additionally our research evidenced a positive effect of the mothers' supplementation during the pregnancy and suckling periods the folliculogenesis in the offspring ovaries. This study is in agreement with others that provided further evidence that early malnutrition can program the function of the ovary (da Silva et al. 2010). The alteration in the expression of GDF 9 as a result of the supplementation of JC to rabbits indicated their high sensitivity to metabolic changes in the ovaries, which is in support of the research data of Daoud et al. (2012). Previous studies have shown that growth factor GDF9 plays an important role in the follicular development of female rabbits and is very sensitive to dietary treatment with a biologically active additive, confirmed in this study (Abadjieva and Kistanova 2016). The more intensive expression of the GDF9 protein in the follicles at all stages of development, as well as in the theca and luteal cells in the ovaries of the experimental animals, established by us in the present study, not only confirm its pivotal role in the whole process of folliculogenesis in rabbits, but show a clear response to JC supplementation. An interesting finding is a high positive staining to GDF9 in the luteal cells of treated animals. The nucleus of luteal cells contains thyroid hormone receptors, but how thyroid hormone affects growth factor in luteal cells is not clear (Dupont et al. 2014). Poppe et al. (2004) underlined that thyroid hormones could interact with reproductive hormones

and growth factors to preserve the normal function of the ovaries and maturation of the oocytes. Whether these downstream signaling pathways are involved in the regulation of GDF9 expression in mammalian oocytes requires further study. Clarification of this problem may have important clinical implications for women of child-bearing age. Our findings suggest that female receiving iodide treatment during the follicular phase would minimize negative side effects and would provide a significant benefit to folliculogenesis.

In conclusion, the present research demonstrated that iodine addition to drinking water at a dose of 2 ml/l has a clear positive effect on folliculogenesis in rabbits and should be used for reproductive stimulation. The results provide the first in vivo evidence of the stimulating effect of iodine on GDF9 protein expression in the oocytes and cumulus cells of female rabbits as a marker of proper folliculogenesis. These data support the concept based on in vitro research that thyroid hormone promotes FSH-induced preantral follicular growth through a pathway, which includes GDF9 action.

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