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CHANGES IN THE EXPRESSION OF THE AROMATASE AND SUPEROVULATORY RESPONSE IN MICE TREATED WITH THE FEED ADDITIVE PROVIT E10% SUPER

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Abstract

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The success rate of superovulation procedure is crucial for the embryo transfer biotechnology, applied to the preservation of the genetic resources. *The aim* of the present study was to track the changes in superovulatory response and the expression of the aromatase in the ovaries of mice treated with the supplement Provit E10% Super. The experiment was conducted in vivarium of the Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences with 30 female laboratory mice, separated and leveled by weight into three groups (n = 10): control group, first experimental group, stimulated by standard protocol for superovulation and second experimental group, in which an effect of superovulation was combined with the individual intake of the supplement for 30 days before hormonal treatment. *The methodology* included an evaluation of success rate of superovulation, through extraction of the ovulated oocytes from the ampoule of the oviduct. Histological examination of an ovary was carried out on 5 μm paraffin slices stained with H & E by routine method. Aromatase expression was estimated by immunochistochemical method using antibody agains CYP19. Preparations were examined and documented by microscope system Olympus BX 51 (Japan). *The results* showed that treatment of mice with supplement before superovulation exerts an effect on the folliculogenesis and improves cumulus expansion that reflects on the number of the ovulated oocytes. A more active expression of the enzyme aromatase in the theca cells of preantral and antral follicles in both treated groups compared to the control was observed. For the first time the positive immunostaining of the aromatase in the mice oocytes was found. Stronger immunochemical reaction of aromatase was observed in the corpora lutea of the mice, received the feed additive. *In conclusion*, supplementing of the Provit E10% Super before hormonal treatment affects the folliculogenesis activity and could be considered as an improvement of the superovulation protocol for the laboratory animals as well as for other species.

Key words: mice, feed additive, superovulation, immunohistochemistry, aromatase

Introduction

Superovulation is an essential step for the embrio biotechnologies, which are the effective methods for increasing the success rate of breeding programs. The wide distribution and application of the different protocols are not yet guarantee of the high success rate of superovulation and quality of received and transferred embryos. It is known that a number of factors influence the response of superovulation, such a season (Mitchell, et al., 2002), temperature (Kamimura et al., 2003) and condition of the ovaries or hormonal preparation (Gonzalez-Bulnes et al., 2000). Smith (1991) reports that nutrition affects the ovulation rate in mammals, by reflection on the hypothalamic–pituitary–gonadal - axis. Nutrition modulates the reproductive function mainly by circulating the metabolic hormones such as insulin, growth hormone, leptin, and ghrelin (Scaramuzzi et al., 2015). These hormones, probably, are mediators between the food and the

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follicular growth and ovulation. However, data on the impact of diet on the success of superovulation are contradictory. Additional supplement of fatty acids and proteins to a well-balanced feed does not lead to an increased production of embryos from superovulated animals (Velazquez, 2011). On the other hand, studies of Sales et al. (2008) and Evangelista et al. (2011) demonstrate that supplements containing vitamins, macro- or micro elements increase the success rate of the superovulation in farm animals. However there is no data on the influence of the protein aromatase expression by dietary factors. Also it is unclear how the exogenous hormones, used in superovulation protocol, modulate the activity of aromatase, while it is known that a major inducer of aromatase activity is endogenous FSH. To get a better understanding of the metabolism of estrogens, it is very important to study the protein aromatase (P450), which catalyzes the conversion of androgens into estrogensthat are necessary for creating an conducive environment to develop the healthy follicles (Fitzpatrick and Richards, 1991).

The aim of the present study was to track the changes in the superovulatory response and the expression of aromatase in the ovaries of mice treated with the feed additive Provit E10% Super.

Materials and Methods

The experiment was conducted with Swiss white female line CD1 mice, breed in the vivarium of the Institute of Biology and Immunology of Reproduction of Bulgarian Academy of Sciences. 30 female animals equaled by weight, were divided into three groups (n = 10): control group, first experimental group, stimulated by protocol for the superovulation and second experimental group, in which the effect of superovulation was combined with individual intake of the additive Provit E10%Super in a dose of 1.5 μg/g for 30 days prior hormonal treatment (Table 1).

All animals received standard food for the laboratory mice and water without limits. The healthy status was checked daily. Provit E10%Super is a nutritional supplement Company Profeed – Animals, Poland containing a vitamin-mineral complex (vitamin E, Ca, Mg) and extract of the plant artichoke. The animals were stimulated by the standard protocol for the superovulation, which included an initial injection of FSH (follicle stimulating hormone) in a dose of 6.00 IU, and an injection of LH (luteinizing hormone) in a dose of 6.00 IU after 48 hours. After completion of the experiment (14 hours after the second step of the superovulation protocol) mice were killed humanly, in accordance with the requirements of the ethic Committee (report no: 2009-4-12/40). Isolated ovaries were cleaned from the fat. For evaluating the success rate of the superovulation, the oocytes were extracting from the oviduct ampoule of the superovulated animals and analyzed under stereomicroscope. In control group the oocytes were recovered from the ovulatory follicles by ovary dissection. One ovary of each animal was fixed in 10% formalin for 48 hours. After dehydration in an ascending alcohol series, tissues were included in paraffin blocks. Subsequently, serial slices with thickness from 5μm were obtained by microtome (Leica, Germany), mounted on slides and used for histological and immunohistochemical study. For this aim, the first preparations were stained with hematoxylin-eosin (H & E) according to a routine protocol. Morphometric analysis of the ovarian structures was documented by microscopic system Olympus BX51 (Tokyo, Japan). In the current study an indirect method of a visualization of the aromatase enzyme was used. It includes two steps, in which the enzyme-marked secondary antibody (peroxidase conjugated polymer, HRP-anti-polyvalent, ScyTek Inc., USA) reacts with the antigen-related primary antibody, incubated for overnight at 4°C with antibody against CYP19 (H-300, Santa Cruz Biotech., Germany). The data was processed with statistical method Tukey HSD test, the differences were considered significant at $p < 0.05$.

Results and Discussion

Supplementation of the Provit E10% Super (1.5 μg/g), to the food of animals shown a trend to decrease the live weight (g) the mice of the second experimental group, compared to the first one and control ($P > 0.05$). No statistically differences between groups were observed by the total weight of the reproductive tract (g) and by the ovary weight (g) which is

Table 1

Tukey HSD test indicator the number of obtained oocytes

Groups statistical	statistical critically valuue Q	Tukey HSD p-value	significant
I experimental. vs. II experimental	2.9011	0.1194032	n. significant
I experimental vs Control group	2.4233	0.2187038	n. significant
II experimental. vs Control group	5.3244	0.0022914	** - p<0.01

indication that the hormonal treatment does not influence on these parameters. During the estrus cycle, the development of ovarian follicles and luteinisation are the main processes that are closely related to the lipid metabolism. Precisely the fatty acids provide energy for oocyte maturation and early embryo development. The gonadotropins may regulate the steroidogenesis and the newly synthesized fatty acids, but are not reflected on the weight of the gonads (Liu et al., 2009). In contrast, Wang et al (2015) reported the statistically heavy ovaries of mice, stimulated with 10 IU PMSG and hCG. This was due to the higher dose of gonadotropins, which affects cholesterol-dependent genes to activate the newly synthesis of the cholesterol or lipoproteins during luteinisation. An interesting question is there any factors allowed to predict the total number of mature oocytes in protocols with application of gonadotropins? It is known that the level of estradiol, progesterone and increasing secretion of LH could predict the answer to this question. However, there are many inter-species differences in response, the use of various hormones and other factors which may explain the lower success of assisted biotechnology than theoretically expected (Nagy, 2003). The number of recovered oocytes is presented on Figure 1.

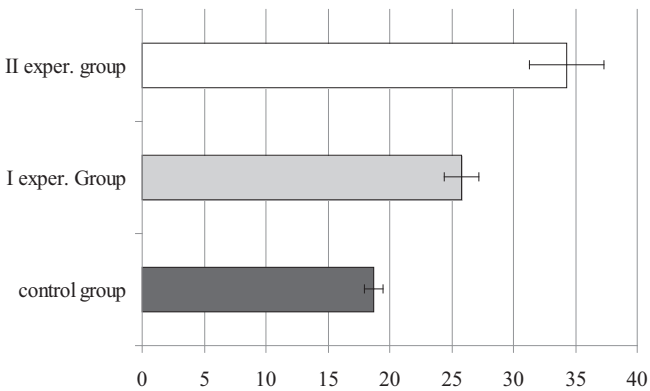

Figure 1. The number of oocytes recovered (n) from the control, I and II treated groups

The data show the drastically increase of the oocytes number in the second experimental group. The *Tukey HSD* test was applied to determine significance in inter-groups differences. It was establish significant (P = 0.002) higher number of obtained oocytes from animals in IInd experimental group in comparison with control group. The results allow to suppose that both factors supplement and hormonal treatment given additionally value to the ovulation success. In our experience, probably, the supplementation of feed additive has caused an increased blood flow to the follicles and more active proliferation of the granulosa cells, so that they can adequately produce the estrogens. Regarding to the morphology of the oocytes, the ovarian stimulation and the intake of supplement in the treatment groups lead to the recovery of a significant number of mature oocytes and only single cases of the fragmented oocytes compared to the control (Figure 2).

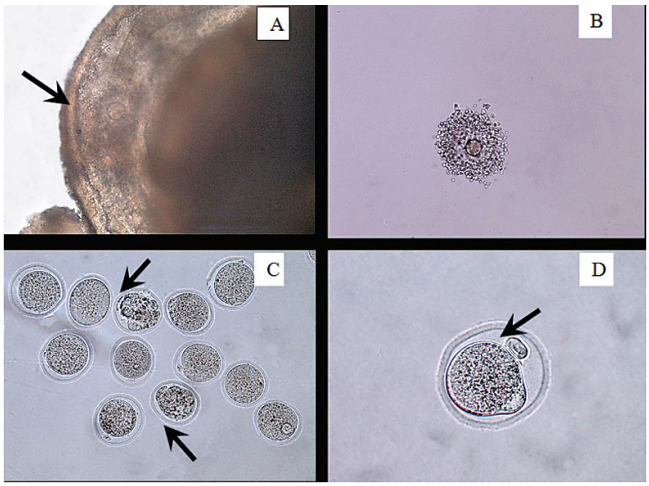

Fig. 2. Microphotographs of mice oocyte from stereo microscope

A. Ampoule, bulging of the oocytes (arrow) in the experimental animal (x10); **B.** Cumulus-oocyte complex from the control animal (x10); **C.** Multiple oocytes and single fragmented oocytes (arrows) in experimental animals (x20); **D.** Mature oocyte (MII) with polar body (arrow; x20)

Expansion of the cumulus is a morphological marker to predict competence (capacity) of the oocyte used in assisted reproductive technologies (Russell and Robker, 2007). Our study has shown that in the control group were prevalent oocytes, densely surrounded by cumulus cells. In the case of dense cumulus mass, it is difficult to remove even with the enzyme hyaluronidase, which is not typical for the mature oocytes and is not compatible with a further embryonic development (Mandelbaum, 2000). All mature oocytes without stimulation had complete cumulus expansion, but in most case it was characterized by fragmentation, which may be due to the activity of LH. There is a clinical data reported that the concentration of LH influences the development of the oocyte in a dose dependent manner (Hillier, 1994). In vitro experiments have illustrated that low doses of LH enhances steroid genesis, while higher doses enhance the secretion of progesterone, but inhibits the activity of aromatase and growth of granulosa cells (Shoham, 2002). Increased concentrations of LH are also associated with poor fertilization and implantation, as well as adverse effects on pregnancy (Hillier, 1994). The evidence t supports the theory of LH threshold, above which occur the adverse effects on the

folliculogenesis (Tesarik and Mendoza, 2002). On histological preparations was made evaluation morphometric on the number of follicles and corpora lutea (Corpus luteum), (Figure 3) on ten consecutive slices from the ovaries of all 30 female mice. Morphometric evaluation of the ovaries was done by the counting of the follicles and corpora lutea (CL) numbers on ten consecutive slices (Figure 3).

The results showed a statistically greater number of CL in second experimental group (P = 0.02) compared to the control, which correlates positively with the number of obtained oocytes from the same group (r = 0.504). Our results coincide with those of Swann (2014), which reported a significant increase in the number of CL (P <0.001) and up to almost double diameter of the corpus lutea in mice, stimulated with 5 IU hMG than in nonstimulated control ovaries. Preparations from protocol to superovulation, for example PMSG, contain long acting LH bioactivity. LH in combination with FSH stimulates estrogen production. Estrogen is produced by granulosa cells in response to FSH and LH gonadotropins throughout the hormone depended stage of the folliculogenesis by the participation of the P450 aromatase (McNatty et al., 1999). One of the most remarkable actions of the ovaries is the expression of the aromatase, an enzyme encoded by the gene Cyp19. Aromatase converts androstenedione to estron, testosterone and 17-β estradiol. In our study we found that in the primordial and primary follicles aromatase does not occur (Figure 4). It should be noted that the immune reaction to this protein was observed in the stromal cells of ovary, encircling primordial and primary follicles. When follicles become sensitive to gonadotropins, it was noticed a more intensive aromatase expression in follicular cells, particularly, in the theca cells (Figure 4, C). Those confirm that the theca cells are the main site of the estrogen synthesis in the ovulated follicles. The positive immunohistochemical reaction to aromatase was observed in the CL of the mouse ovaries (Figure 4, F).

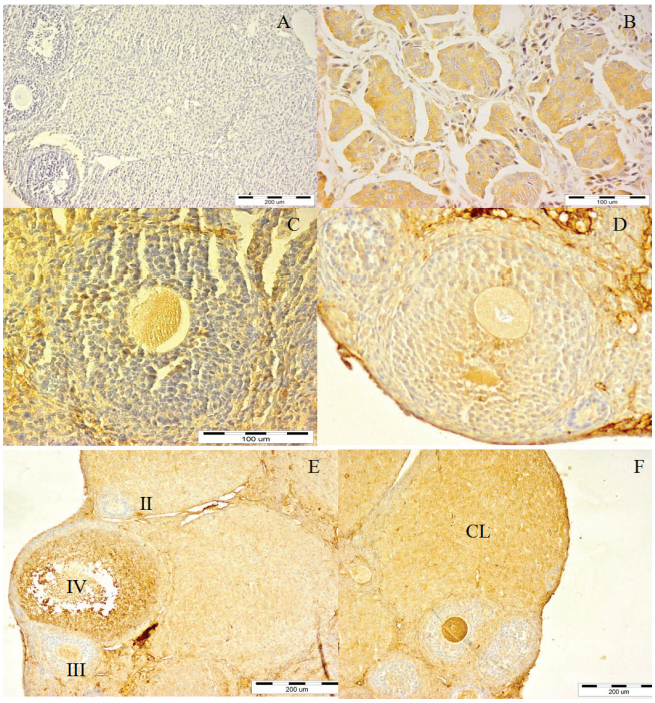

Fig. 4. Immunohistochemical staining of the aromatase
A negative control on reaction (20X); **B**. Positive control on reaction – tissue from mammary gland (40X); **C**. in the oocyte and theca cells of the preantral follicle (40X); **D**. in follicular cells and oocyte of the preantral follicle (20X); **E**. Panoramic photo showing the difference in the localization of aromatase between secondary (II), tertiary (III) and antral follicle (IV), (20X); F. in corpus luteum (CL) in mouse ovary from the second experimental group (20X)

Laurincik et al. (1996) reported the similar results in terms of primary follicular phases. Even before the peak of LH all layers of granulosa cells were not stained until 5-7 hours after the LH peak, only granulosa cell layers located

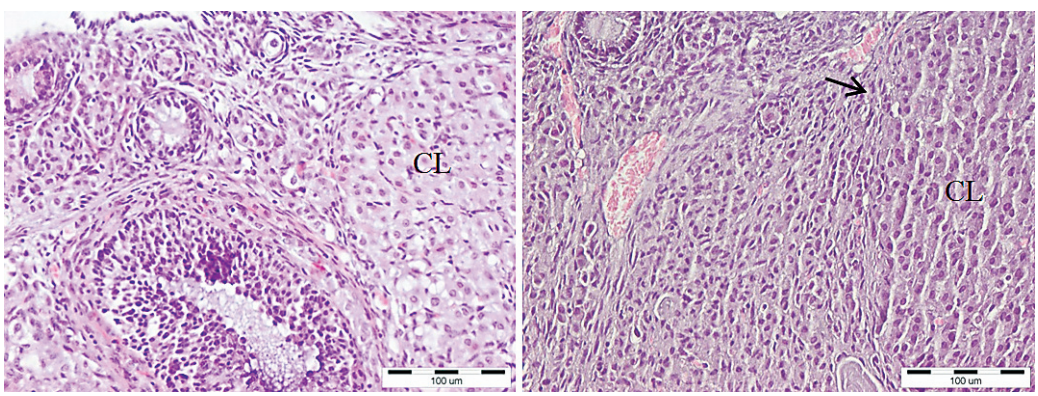

Fig. 3. Microphotographs of Corpus luteum in mouse ovaries (H & E; 40X)

near the basal lamina had a positive immune reactivity. However, contrary to our results, Laurincik et at. (1996) did not find staining in the cells of the gland. A study of ovarian biopsies synchronized cows on different days of the estrus cycle showed different concentrations of specific proteins in the CL. The highest concentration of progesterone receptors is observed at 6 day of estrus cycle, and the most intensive immunostaining for the enzyme aromatase at 10 day of the cycle. The highest score of the immunostaining for LH receptors was observed on the 15th day of the estrous cycle (Martin et al, 2013). This finding suggests that these receptors and enzymes and their interactions are important for the regulation of the viability of CL. In our study the results clearly shown that the intensity of the immunoreaction to the aromatase in the CL cells in both experimental groups was stronger compared to the control. The most literature data have focused on the role of the aromatase in steroidogenesis in follicular cells and paid less attention to the oocytes. Our research for the first time identified aromatase activity in oocytes of the mouse ovaries. Those suggest that the mouse oocytes should produce androgens, especially during the late oogenesis and during the maturation of the oocytes. There are few references in the scientific literature confirming our data, but in other species. Localization of the aromatase in oocytes was reported in the gonads of pig fetuses on the different days of development (Wei Xu, 2009). Their results shown that aromatase is immune active in the cytoplasm and nucleus of oogonia and cytoplasm of the surface epithelium at 33 day of the fetal ovaries. At 54 day of fetal development the aromatase observed in the cytoplasm of the cells from the ovary cortex, and at 61 day reaction occurs in the medulla of the ovary.

Conclusion

Hormonal treatment for superovulation and supplementing a food additive ProvitE10% Super impacts the activity of folliculogenesis that reflected on the expression of aromatase and the number of recovering oocytes. Our results proven that aromatase enzyme, which converts androgens into estrogens, is localized in the cytoplasm of the different structures in mouse ovary and it is important for the processes of folliculogenesis. The observed more strongly expression of the aromatase in the theca cells of the preantral and antral follicles confirmed their important role in the estradiol secretion. For the first time in the mouse study was found positive immunoreactivity of aromatase in the oocyte. Application of the bioactive feed additive before hormonal treatment could be considered as an improvement of the superovulation protocol for the laboratory animals as well as for other species.

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References

Evangelista, J. J. F., C. E. A. Souza, M. E. A. Moraes and A. A. A. Moura, 2011. Treatment with vitamins A and E improve oocyte quality and in vitro embryo development in Bos indicus cows. *Reprod. Fertil. Dev*., **23**: 175.

Fitzpatrick, S. L. and J. S. Richards, 1991. Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. *Endocrinology*, **129**: 1452-1462.

Gonzalez-Bulnes, A., J. Santiago-Moreno, M. J. Cocero and A. Lopez-Sebastian, 2000. Effects of FSH commercialpreparation and follicular status on follicular growth and superovulatory response in Spanish Merino ewes. *Theriogenol.,* **54**: 1055-1064.

Hillier, S. G., 1994. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum Reprod*, **9**: 188-191.

Kamimura, E., T. Nakashima, M. Ogawa, K. Ohwada and N. Nakagata, 2003. Study of low-temperature (4 degrees C) transport of mouse two-cell embryos enclosed in oviducts. *Comp. Med*., **53** (4): 393-396.

Laurincik, J., L. Kolodzieyski, P. Hyttel, Y. Osawa, H. Niemann, F. Schmoll, G. Brem and K. Schellander, 1996. Granulosa-cumulus-corona expansion and aromatase localization in preovulatory follicles in superovulated heifers. Research Institute of Animal Production, Nitra, Slovak Republic. *Acta Veterinaria Scandinavica*, **37** (1): 99-107.

Liu, Z., M. D. Rudd, I. Hernandez-Gonzalez, I. Gonzalez-Robayna, H. Y. Fan, A. J. Zeleznik and J. S. Richards, 2009. FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells. *Mol. Endocrinol.,* **23**: 649-661. doi: 10.1210/me.2008-0412.

Mandelbaum, J., 2000. Oocytes. *Hum Reprod.,* **15** (Suppl. l4): 11-18.

Martin, I., M. M. Rodrigues, C. J. Fujihara, W. Filho, E. Obaa, R.Laufer-Amorimb, J. C. P. Ferreira, 2013. Localization patterns of steroid and luteinizing hormone receptors in the corpus luteum of Nelore (*Bostaurus indicus*) cows throughout the estrous cycle*. Livestock Science.*, **155** (2-3): 442-453.

McNatty, K. P., D. A. Heath, T. Lundy, A. E. Fidler, L. Quirke, A. O'Connell, P. Smith, N. Groome and D. J. Tisdall, 1999. Control of early ovarian follicular development. *J. Reprod Fertil Suppl*, **54**: 3-16.

Mitchell, L. M., W. S. Dingwall, M. J. Mylne, J. Hunton, K. Matthews, F. E. Gebbie, G. J. McCallum and T. G. McEvoy, 2002. Season affects characteristics of the preovulatory LHsurge and embryo viability in superovulated ewes. *Anim. Reprod. Sci*., **74**:163-174.

Nagy, A., M. Gersenstein, K. Vintersten and R. Behringer, 2003. Manipulating the mouse embryo: a laboratory manual. 3rd ed.

Cold Spring Harbor, NY: Cold Spring Harbor Press., p. 359-397, 453-506, 687-690.

Russell, D. L. and R. L. Robker, 2007. Molecular mechanisms of ovulation: coordination through the cumulus complex. *Hum Reprod Update,* **13**: 289-312.

Sales, J. N. S., L. M. K. Dias, A. T. M. Viveiros, M. N. Pereira and J. C. Souza, 2008. Embryo production and quality of Holstein heifers and cows supplemented with scaroteneand tocopherol. *Anim. Reprod. Sci*., **106**: 77-89.

Scaramuzzi, R. J., N. Zouaidi, J. P. Menassol and J. Dupont, 2015. The effects of intravenous, glucose versus saline on ovarian follicles and their levels of some mediators of insulin signalling. *Reproductive Biology and Endocrinology*, **13**, p. 6, doi:10.1186/1477-7827-13-6.

Shoham, Z., 2002. The clinical therapeutic window for luteinizing hormone in controlled ovarian stimulation. *Fertil Steril*, **77**: 1170-1177.

Smith, J. F., 1991. A review of recent developments on the effect ofnutrition on ovulation rate (the flushing effect) with particular reference to research at Ruakura. *Proceed. ew Zeal. Soc. Anim. Prod*., **51**: 15-23.

Swann, K., 2014. Effects of ovarian stimulation on oocyte development and embryo quality. Thesis, *University of Nottingham.*

Tesarik, J. and C. Mendoza, 2002. Effects of exogenous LH administration during ovarian stimulation of pituitary down-regulated young oocyte donors on oocyte yield and developmental competence. *Hum. Reprod*, **17**: 3129-3137.

Velazquez, M. A., 2011. The role of nutritional supplementation on the outcome of superovulation in cattle. *Animal Reproduction Science,* **126**: 1-10.

Wang, Li-Ya, N. Wang, F. Le, L. Li, H.-Y. Lou, X.-Z. Liu, Y.-M. Zheng, Y.-Q. Qian, Y. L. Chen, X.-H. Jiang, H.-F. Huang and F. Jin, 2015. Superovulation Induced changes of lipid metabolism in ovaries and embryos and its probable mechanism. *PlosOne,* DOI: 10.1371/journal.pone.0132638.

Wei Xu, 2009. Immunohistochemical Localization of P450 Aromatase on Gonads in Fetal Pig. Master thesis, *Anhui Agricultural University.*

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