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THE EFFECT OF PROLACTIN ON THE QUALITY OF HEIFER OOCYTES RETRIEVED BY TRANSVAGINAL PUNCTURE OF FOLLICLES

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Abstract

In both dairy and beef cattle breeding, producing the larger number of the offspring from the best mothers to increase the degree of genetic progress through the generations is of particular interests. One of the attractive ways to resolve this problem is the development and implementation of the technology for obtaining embryos in vitro (in vitro embryo production, IVP) using oocytes derived from live animals by transvaginal puncture of follicles — Ovum-Pick-Up (OPU). The extracorporeal maturation of oocytes is an important element of this technology that may significantly affect its efficiency. In this paper, for the first time, we evaluated the advantage of the pituitary hormone prolactin (PRL) — a potential regulator of the quality of mammalian oocytes, during the maturation of OPU-oocytes. The effect of this hormone on the completion of nuclear maturation of the OPU-oocytes, as well as on the development and quality of IVP embryos, was studied. The mature Simmental heifers at the age from 19 to 25 months ($n = 4$) with a natural sexual cycle were used as the oocyte donors. Transvaginal aspiration of the follicles was performed every 4 days using the OPU system for cattle (Minitube, Germany). A total of 28 OPU sessions were carried out. The derived cumulus-oocyte complexes (COCs) were cultured in TS-199 medium supplemented with 10 % bovine fetal serum, 10 μg/ml of follicle-stimulating (FSH) and 10 μg/ml of luteinizing (LH) hormones in the absence (control) or presence of PRL. After 24 hours, the mature oocytes were subjected to fertilization to assess their developmental competence. Morphological analysis did not reveal the effect of culture conditions on the completion of nuclear maturation. The rate of mature oocytes was similar in both groups and was 82.8 and 88.9 %, respectively, in the control and the PRL groups. However, the oocyte cleavage rate after in vitro fertilization in the control group was lower comparing to the PRL group (69.7±2.4 % vs. 81.7±4.9 %, $p < 0.05$). A positive effect of the PRL on the development of mature oocytes to the blastocyst stage was observed. When COCs were cultured in the control medium, the yield of blastocysts was 11.0±1.8 %, while the adding of PRL into the IVM medium increased this indicator to 17.2±2.0 % ($p < 0.05$). However, we did not find significant differences among compared groups in the relation to the total number of nuclei in blastocysts. Thus, prolactin hormone in the maturation environment has a stimulating effect on the developmental competence of donor oocytes. The positive effect is observed at the stage of the first cleavage and maintained during the development of embryos to the blastocyst stage. Our data indicate the positive effect of prolactin on the quality of OPU-oocytes, that makes it reasonable the using this hormone at the maturation stage to increase the effectiveness of IVP technology.

Keywords: cattle, transvaginal aspiration of follicles, in vitro oocyte maturation, prolactin, embryonic development

Obtaining the largest number of calves from the best mothers for a more complete realization of their genetic potential in generations remains a challenge for both dairy and beef animal husbandry. The development and practical application

of in vitro embryo production (IVP) using eggs from live animals through transvaginal follicle puncture (ovum-pick-up, OPU) can address this urgent problem [1, 2]. It has been shown that OPU is the most flexible and reproducible method for obtaining embryos from living donors. Unlike multiple ovulation and embryo transfer, OPU does not interfere with the normal reproduction and production cycle of the donor. Any female between the ages of 6 months and the 3rd month of pregnancy and soon after calving (in 2-3 weeks) can be a suitable donor [2]. OPU has been now recognized as good alternative to the traditional in vivo embryo production program [3, 4] and is increasingly used commercially worldwide [5-7].

As is known, the efficiency of the IVP technology depends not only on the quality of the initial population of gametes isolated from the ovaries [8, 9], but also on the environmental impact that oocytes are exposed to in vitro [10, 11]. The maturation of oocytes is the most important stage of culture. By means of its modeling, it is possible to significantly increase both quantitative (the proportion of embryos at the blastocyst stage) and qualitative (the usefulness of blastocysts) indicators of the effectiveness of the IVP method [12]. The vast majority of modern research is focused primarily on the search for physiologically relevant substances (growth factors, hormones, steroids, fatty acids, amino acids, metabolites) that can specifically affect oocytes, increasing or maintaining their viability and developmental competence, as well as identifying mechanisms underlying such influence [13-16].

To date, it has been established that the pituitary hormone prolactin (PRL) affects the ovarian function of females and can positively modulate the maturation of oocytes and their ability for embryonic development [17-20]. Receptors for this hormone or its mRNA have been found in oocytes and associated cumulus cells of various mammalian species, including cows [20-23]. In in vitro conditions, the addition of PRL to the medium for maturation of post mortem bovine oocytes positively affects their nuclear maturation and quality and increases competence for further embryonic development [20, 24, 25]. In prolonging oocyte in vitro culture it has been shown that PRL inhibits destructive changes in the morphology of metaphase chromosomes and reduces the frequency of apoptotic degeneration of senescent oocytes of this species [26, 27]. In addition, prolactin increases the competence of mature oocytes for further embryonic development, which decreases with aging [27]. In general, prolactin is deemed a potential regulator of the female germ cells capable of increasing their quality under in vitro conditions.

In this work, we have for the first time revealed the positive effect of the pituitary hormone prolactin on the quality of donor OPU oocytes of cows during their maturation in vitro.

The aim of the work was to assess the effect of prolactin on the completion of nuclear maturation by oocytes obtained by transvaginal follicle puncture and on the development and quality of embryos after in vitro fertilization of donor oocytes.

Materials and methods. In all experiments, except for separately indicated cases, reagents from Sigma-Aldrich (USA) were used.

Oocytes were collected from mature heifers (*Bos taurus taurus*) of the Simmental breed aged from 19 to 25 months (n = 4) with a natural sexual cycle. Follicle puncture was performed every 4 days using an OPU system for cattle (Minitube, Germany), which included an SSD Pro Sound 2 ultrasound scanner, a convection sector probe, a vacuum pump, and a probe holder. Aspiration of all visible follicles was performed with a needle (1.2 mm in diameter and 75 mm long) connected with a silicone hose to a 50 ml vial. Phosphate buffered saline (PBS) supplemented with 10% bovine fetal serum (BFS), 18 IU/ml heparin, and 50 mg/ml gentamicin was used as aspiration fluid. Aspirates from each donor were

filtered individually, washed with PSB supplemented with 1% BFSPBS. Cumulus-oocyte complexes (COCs) were searched and evaluated under a stereomicroscope (Nikon, Japan). The isolated COCs were divided into those suitable for in vitro culture, including oocytes lacking cumulus cells, and those with obvious cytoplasmic abnormalities not suitable for in vitro culture. Selected COCs were incubated for 24 h to mature in TS-199 medium supplemented with 10% bovine fetal serum (BFS), 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml luteinizing hormone (LH) in the absence (control) or presence of PRL (50 ng /ml) (experiment).

Mature oocytes were fertilized to assess competence for embryonic development. COCs were washed once in BO-IVF fertilization medium (IVF Bioscience, UK) and placed in drops of the same medium 30 min before contact with spermatozoa.

Oocytes were fertilized using frozen-thawed semen from one Simmental bull. Straws with frozen sperm were thawed 1.5 hours before fertilization, and active spermatozoa were obtained by the swim-up method [28] using Sperm-TALP medium containing 1 mM sodium pyruvate, 6 mg/ml BSA [27]. The contents of the straws were layered with 220 μl in 1.8 ml tubes (Nunc, Denmark) containing 1 ml of Sperm-TALP medium and placed in an incubator (MCO-18AIC, Sanyo, Japan) for 50 min. At the end of the incubation, 750 μl of the upper layer was taken from the tubes, diluted with fresh medium and centrifuged (a centrifuge 3-30KS, Sigma, Germany) at 300 g for 7 min. The resulting sediment containing motile spermatozoa was introduced into the fertilization medium (BO-IVF) with previously transferred COCs to a final concentration of 1.5×10^6 spermatozoa per 1 ml.

COCs were maturated and fertilized in 4-well plates (Biomedical, Russia) in drops of 90 μl medium completely covered with light mineral oil.

After 16-18 h co-incubation with sperm, oocytes were carefully pipetted and washed in CR1aa medium [29] to remove cumulus cells and adhering spermatozoa. Simultaneously, morphology of isolated oocytes was assessed, the oocytes with target bodies (first or first and second) were counted and the percentage of maturation was determed. Putative zygotes (regardless of the presence or absence of polar bodies) were transferred to the CR1aa medium and cultured for 4.5 days. The developing embryos were placed in the same medium containing 5% BFS.

Embryos were developed in 4-well plates (Nunc, Denmark) in 90 μl of medium completely covered with light mineral oil. On day 2 after the fertilization of the oocytes, the morphological assessment of the fragmented zygotes was carried out; on day 7, the number of embryos that had developed to the blastocyst stage was determined. The evaluation was performed under an SMZ stereomicroscope (Nikon, Japan) at a magnification of ×40-60.

The oocyte maturation and fertilization, as well as the embryo culture occurred in a 5% CO_2 atmosphere at 38.5 °C and 90% humidity.

Embryos obtained on day 7 were fixed with 4% paraformaldehyde solution (60 min), permeabilized in 0.1% sodium citrate solution containing 0.5% Triton X-100 (30 min) and stained with DAPI to localize the nuclei (20 min). Embryos treated in this way were transferred to a glass slide and placed in Vectashield medium (Vector Laboratories, UK). Microphotography and evaluation of preparations were performed under an Axio Imager.M2 microscope (Carl Zeiss, Germany) using the ZEN 2 pro program (Carl Zeiss, Germany).

Statistical processing was performed by one-way analysis of variance using the SigmaStat program (Systat Software, Inc., USA). The data were expressed as means (*M*) and standard errors of the means (±SEM). The significance of differences between the compared mean values was assessed using Tukey's test.

Results. To date, significant progress has been made in the development of IVP technology in cattle using donor oocytes, however, the usefulness of embryos developed in vitro from OPU oocytes still remains significantly lower than those developed in vivo [5-8]. The identification of biologically relevant factors responsible for the regulation of oocyte quality during their in vitro maturation will contribute to solving this problem [12-13, 16].

Since PRL positively modulates post mortem maturation of bovine oocytes and their ability for embryonic development [20, 24, 25], it is likely that this hormone may similarly affect donor oocytes. In the present work, oocytes obtained by transvaginal follicle aspiration were cultured in the presence or absence of PRL (50 ng/ml), followed by fertilization in vitro and cultured to the blastocyst stage. The effect of PRL on the completion of nuclear maturation by OPU oocytes, as well as on the development and quality of IVP embryos, was evaluated.

A total of 360 follicles were aspirated from four Simmental heifers during 28 OPU sessions, of which 166 COCs were isolated. The number of oocytes isolated from individual donors (1 OPU session) averaged 5.9. COCs (Fig. 1, a) obtained in the OPU session, except for oocytes with obvious cytoplasmic abnormalities (total 140 COCs, 5.0 per 1 OPU session), were cultured in IVM medium until maturation was completed (see Fig. 1, b) without (control) or with PRL.

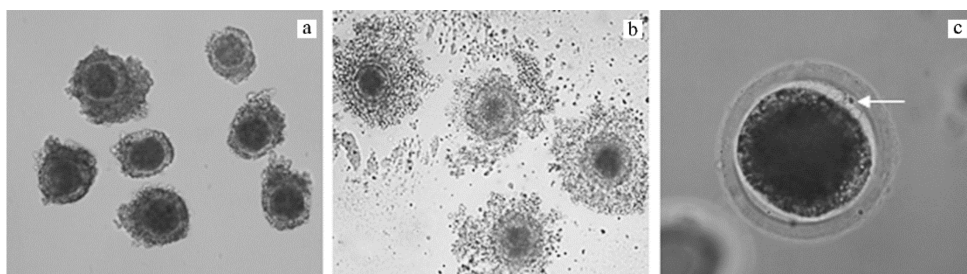

Fig. 1. Micrographs of oocytes of Simmental heifers collected by transvaginal follicle puncture: a — immature cumulus-oocyte complexes (magnification ×100), b — cumulus-oocyte complexes after 24 hours of maturation in vitro (magnification ×100), c — mature oocytes after the fertilization procedure in vitro (white arrow indicates polar bodies, magnification ×400) (microscope Eclipse Ti-U, Nikon, Japan).

Morphological analysis did not reveal the effect of prolactin on the completion of nuclear maturation. The proportion of mature oocytes as the ratio of oocytes with polar bodies (see Fig. 1, c) to the initial oocyte number determined after the IVF procedure when oocytes were separated from cumulus cells and spermatozoa, was high and did not differ significantly between the control and experimental groups (Table 1).

1. The competence of oocytes collected by transvaginal puncture of follicles of Simmental heifers and maturing in the presence of prolactin to embryonic development **after in vitro fertilization** (*M*±SEM)

Group	Oocytes, *n*	Mature oocytes, %	Cleaved oocytes, %	Oocytes developed to the blastocyst stage, %
Control	80	82.8±3.8	69.7±2.4	11.0±1.8
Experimental	60	88.9±3.9	81.7±4.9*	17.2±2.0*

N o t e. For a description of the groups, see the Materials and methods section.
* Differences between groups are statistically significant at $p < 0.05$.

The competence of mature oocytes to development after in vitro fertilization was assessed by their ability to enter the first cleavage division (Fig. 2, a) and reach the blastocyst stage (Fig. 2, b, Table 1). On day 2, the proportion of cleaved oocytes after in vitro fertilization in the control was lower than in the experiment ($p < 0.05$). A positive effect of the hormone on the development of

mature oocytes up to the blastocyst stage also occurred (see Table 1). In general, the number of blastocysts per OPU session in the experimental group was 1.5 times higher than in the control (Table 2).

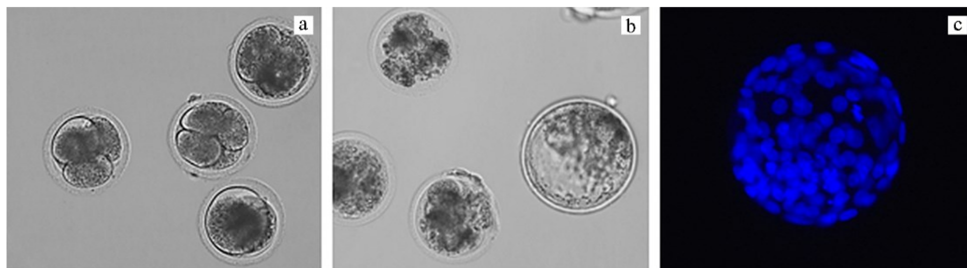

Fig. 2. Micrographs of embryos developed after in vitro fertilization of donor Simmental heifer oocytes collected by transvaginal follicle aspiration: a — cleaved oocytes (×200 magnification), b — embryos developed to the blastocyst stage (×100 magnification) (Eclipse Ti- U microscope, Nikon, Japan); c — staining of nuclei in the blastocyst with DAPI (blue color, cytological preparation, magnification ×400; microscope Axio Imager.M2, Carl Zeiss, Germany).

Prolactin did not significantly change the quality of the IVP embryos, which was assessed by the number of nuclei on day 7 after fertilization (see Fig. 2, c), however, this parameter tended to increase upon maturation of OPU oocytes in the presence of PRL (see Table 2).

2. Efficiency of IVP (in vitro embryo production) **technology and quality of IVP embryos from oocytes collected by transvaginal aspiration of follicles of Simmental heifers and matured in the presence of prolactin (*M*±SEM)**

Group	OPU sessions, *n*	Embryos at the blastocyst stage, *n*	Blastocysts per OPU session, *n*	Nuclei per blastocyst, *n*
Control	16	9	0.59±0.11	67.3±3.0
Experimental	12	10	0.86±0.09	78.6±5.2

N o t e. OPU — ovum-pick-up. For a description of the groups, see the Materials and methods section.

The stimulatory effect of PRL during IVM on the development of fertilized oocytes up to the blastocyst stage was previously reported for rabbit and mouse [30, 19]. A similar effect of PRL was also observed in the co-culture of post mortem bovine COCs with granulosa cells [25] and in the presence of gonadotropic hormones [20]. In the latter case, the addition of prolactin to the COCs culture medium containing FSH and LH led to an increase in the yield of embryos from the total number of oocytes fertilized in vitro, a 2-fold increase in the yield of blastocysts, and an increase in the average number of nuclei per blastocyst. In our study, the addition of PRL to the INM medium with FSH and LH, although it had a similar effect on the oocyte ability to develop in vitro, did not provide such a significant yield of blastocysts and a statistically significant change in the number of their nuclear material.

As is known, cumulus cells are involved in maintaining normal maturation and fertilization of mammalian oocytes [31]. In addition, the presence of cumulus cells ensures the positive effect of PRL on the embryonic development of post mortem oocytes described above [20]. Post mortem oocytes, unlike donor oocytes, are carefully selected prior to in vitro culture by morphological features, in particular, by the presence of a compact multilayer cumulus. In this work, we used for culture not only morphologically normal COCs but also oocytes partially enclosed by cumulus cells, as well as practically devoid of cumulus cells. Selection criteria may have influenced the nature of the revealed positive effect of the studied hormone.

Thus, the pituitary hormone prolactin added to the maturation medium stimulates the competence of donor bovine oocytes collected by transvaginal

follicle puncture to further embryonic development. The beneficial effect occurs at the first cleavage division and persists during the development of embryos up to the blastocyst stage. This indicates a positive influence of the hormone on the oocyte quality and the possibility to use prolactin to increase the effectiveness of the IVP technology at the stage of extracorporeal oocyte maturation.

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