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RESISTANCE OF BOVINE OOCYTES TO AGE-RELATED CHANGES AFTER EXPOSURE TO LUTEOTROPIC FACTORS DURING THE SECOND PHASE OF CULTURE TILL THE METAPHASE II STAGE

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Abstract

After the completion of the first division of meiosis in oocytes of various mammalian species, including cattle, the aging processes are acutely activated, which adversely affects the quality of mature ova and their competence to embryonic development after fertilization. Endogenous progesterone (P4) is known to play an important role in maintaining the viability of bovine oocytes, with its production by surrounding cumulus cells increasing significantly in the course of the final maturation period of female gametes from metaphase I to metaphase II (MII). However, effects of P4 and its two main stimulators, prolactin (PRL) and luteinizing hormone (LH), during maturation of oocytes on their resistance to age-related transformations are still poorly studied. We performed for the first time a comparative investigation of abnormal changes of MII chromosomes and apoptotic degeneration of oocytes, ripened by exposure to P4, PRL, and LH in the absence and in the presence of granulosa cells, during the subsequent prolonged culture of the ova. The aim of the present work was to study effects of luteotropic factors, P4, PRL and LH, during the second phase of in vitro maturation of bovine oocytes on the resistance of these latter to age-related changes. Oocytes surrounded by cumulus matured for the first 12 hours in the medium TCM 199 containing 10 % fetal bovine serum (FBS), 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml LH. Then the oocytes were transferred to a new medium, i.e. TCM 199 containing 10 % FBS (control) or the same medium supplemented with 50 ng/ml P4, 50 ng/ml PRL or 5 μg/ml LH, and cultured for the next 12 h in the presence and in the absence of granulosa cells. After 24 h of maturation in the two-phase system, the oocytes were transferred to an aging medium (TCM 199 containing 10 % FBS) and further cultured for 24 h. In media collected after oocyte aging, the P4 content was determined by enzyme immunoassay. The state of the nuclear material in oocytes was assessed using cytogenetic analysis, the presence of apoptosis in oocytes was determined by the TUNEL method. The rate of oocytes being at the MII stage of meiosis after 24 h of aging was similar in all groups and amounted 78.2-88.4 %. In the absence of granulosa cells, the effect of P4 on oocytes during the second phase of maturation led to a subsequent decrease in the frequency of destructive changes in MII chromosomes, from 67.1±2.0 (control) to 51.2±2.9 % ($p < 0.01$), whereas the introduction of these cells into the culture system eliminated the positive effect of the hormone ($p < 0.001$). On the contrary, a similar effect of PRL, reducing the rate of aging ova with the abnormal morphology of MII chromosomes, from 67.7±1.6 (control) to 46.5±5.0 % ($p < 0.001$), we detected only in the presence of granulosa cells. In addition, after ripening in the system not containing granulosa cells, the frequency of the apoptotic degeneration is lowest in the group of aging oocytes exposed to P4 (17.6 % vs. 23.5-25.2 %, $p < 0.05$). In the presence of granulosa cells, this anti-apoptotic effect of P4 is less pronounced. Meanwhile, there is no difference between the compared groups in the content of P4 in the aging medium. The results of the study suggest that effects of P4 and PRL on bovine oocytes during the second phase of maturation may increase their resistance to subsequent age-related changes associated with a decline in the quality. Thus, these hormones can be used to optimize the

maturation conditions of cattle oocytes in the two-phase system.

Keywords: cattle, oocyte, two-phase system of in vitro maturation, age-related changes of oocytes, progesterone, prolactin, luteinizing hormone, granulosa cells

Auxiliary reproductive technologies are one way to maintain stock reproduction in dairy farming. In vitro embryo transfer to recipient animals is widely used in animal breeding practice. Oocyte in vitro maturation used for production of native, cloned or genetically modified embryo is a technology underlying this method [1]. It is known that quality of matured oocytes determining their ability for further development is a key limiting factor at getting of embryo suitable for transfer [2]. Culture systems render critical effect on quality of oocytes, making them the subjects of specific treatments [3, 4].

Regardless of improvement of the methods of in vitro maturation of oocytes in cattle, quality of embryos got in vitro remains lower than those naturally developed, which results in higher frequency of their abnormalities and the offspring with low viability [5]. In standard practice, modernization of culture systems is mainly aimed at modeling conditions inside of ovarian follicles [6-8]. However, applied approaches usually solely account for changes occurring in functional state of oocytes during their maturity. That being said, ageing processes, which negatively influence the quality of matured oocytes and their competence to embryo development after conception, are sharply activated in vivo and in vitro after the first meiotic division in oocytes of different mammal species, including cows [9, 10]. Besides, somatic cumulus cells surrounding oocytes are subjected to apoptotic degeneration at termination of maturation of female gametes and may accelerate negative changes in the later due to ageing [10, 11].

Ageing of matured oocytes, which is called post-ovulatory, is initiated very fast at molecular level [9, 12]. This process, evidently, also occurs during conception, which should be accounted for at modernization of in vitro maturation (IVM) systems for oocytes. Any delay in conception (or artificial activation) may cause low viability of embryos, and, in case of offspring birth, may lead to weakening of its fertility and shorter life [13, 14]. Creation of a culture system promoting increased oocyte resistance to age-related changes may be a way to improve embryo quality in vitro.

With ageing, molecular changes of oocytes are accompanied by morpho-functional changes, which are observed significantly later and studied to a great extent [9, 14]. We have established that such changes in cattle involve destructive transformation of metaphase chromosomes, as well as apoptotic degeneration of oocytes [10, 15]. Pituitary prolactin hormone (PRL) fulfilling luteotropic function in mammal females [16], may slow down such age-related changes and maintain potential to development of cow oocytes matured in vitro during their further prolonged culture [15]. Identified IVM effect could be possibly related to progesterone (P4) production by cumulus cells surrounding ageing oocytes.

It is known that endogenous P4 plays an important role in acquisition of bovine oocytes of the ability to further develop, whereas its production of cells significantly grows during the final stage of maturation of female gametes from metaphase I to metaphase II (MII) [17, 18]. F. Nuttinck et al. [19] found 2-time growth of P4 secretion by cumulus cells associated with matured bovine oocytes 24 hours after their in vitro fertilization, which evidences on possible positive role of such ovarian steroid hormone in preservation of oocyte quality after maturing. The issue on influence of IVM and P4 during maturation of oocytes on their further resistance to ageing transformations remains open. Moreover, information on the role of luteinizing hormone (LH), one more luteotropic factor, in regulation of oocyte ageing is also absent.

The research of regulatory influence of luteotropic factor on anti-ageing resistance of oocytes during final maturation stage is of the greatest interest, since main molecular cytoplasmic transformations occur in this particular period, due to which oocytes acquire competence to further embryo development [2]. Previously, we had developed a two-phase system of in vitro maturation of cattle oocytes and had shown that it could be used as an alternative for the commonly accepted IVM protocol upon getting embryos at blastocyst stage [20].

In present paper we had for the first time conducted comparative research of abnormal changes in chromosomes at stage MII, as well as apoptotic degeneration of oocytes matured under P4, PRL and LH effect at absence and in the presence of granulosa cells during further prolonged culturing of oocytes.

Purpose of present paper is to study the influence of luteotropic factors (progesterone, prolactin, and luteinizing hormone) during the second phase of in vitro maturation on resistance of bovine oocytes to age-related changes.

Techniques. Oocyte-cumulus complexes (OCC) of cows and adult heifers were extracted from antral follicles of 2-8 mm in diameter. Ovaries without any pathology got after slaughter and delivered to the laboratory in 3-4 hours in physiological solution at 30-35 °C were used. Except for specially agreed cases, Sigma-Aldrich (USA) reagents were used.

Prior to extraction of oocytes, ovaries were repeatedly washed in sterile physiological solution with antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin) and placed in Petri dish with manipulation medium TC-199 containing 5 % fetal bovine serum (FBS), 10 μg/ml heparin, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin. Follicle walls were cut by sterile blade; released oocyte-cumulus cells were collected from manipulation medium and washed 3 times by fresh manipulation medium. Oocytes were collected as described [20]. Oocytes were extracted and collected at 37 °C with the use of a stereomicroscope SMZ (Nikon Corporation, Japan) and heating table MATS-OZ (Tokai Hit, Japan).

OCC (30-35 per 500 μl medium) were cultured in 4-well plates at 38.5 °C, 90 % moisture and 5 % CO_2 in the air. Two-phase culture system was used for getting the matured oocytes. During the first 12 hours, oocytes matured in medium TC-199 with 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml pig follicle stimulating hormone (FSH) and 10 μg/ml sheep LH. Afterwards, OCC were transferred to fresh medium and incubated during the next 12 hours in the presence or at absence of monolayer granulosa cell culture. At the second stage of two-phase culture, TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin (control) was used, or the same medium added with P4 (50 μg/ml), bovine PRL (50 μg/ml) (Endocrinology Research Center RAS, Moscow) or sheep LH (5 μg/ml) was used (as per our preliminary research, such concentrations of hormones cause the least frequency of chromosome abnormalities in matured cow oocytes).

Extraction and preparation of granulosa cells was done subject to our previously described methodology [20]. Cells washed from blood and follicle liquids (1×10^6/ml) were pre-cultured in 4-well plates in 500 μl TC-199 medium with 10 % FBS, 1 mM sodium pyruvate and 50 μg/ml gentamicin. In 12 hours 250 μl of the medium was replaced by fresh medium with one of the studied hormones in the above concentrations. Afterwards, OCC were placed in wells for co-culture with granulosa cells during 12 hours.

In 24 hours of maturing in two-phase system, OCC were placed in ageing medium (TC-199 containing 10 % serum and 50 μg/ml gentamicin) and additionally cultured during 24 hours. At the end of culture, mediums were collected and kept at of –20 °C. Progesterone concentration in media conditioned

by OCC and granulosa cells was determined by immune-enzyme analysis (EIA) using microplate reader Uniplan (Pikon, Russia) and commercial reagent kits (Innovative Society Immunotech, Russia) at sensitivity 0.4 μmol/l. All analysis were conducted in two replicates, variation coefficient between measurements had not exceeded 13 %.

In 24 hours of prolonged culture, oocytes were released from cumulus cells by OCC incubation in 0.1 % hyaluronidase (in fresh ageing medium) during 1 min at 37 °C and further disaggregation by pipetting (130 μm micropipette hole in diameter). Isolated oocytes were used for cytogenetic analysis or identification of apoptosis by immune fluorescent method.

Oocyte nuclear material was prepared as described [10] and examined at magnification ×1000 (microscope Axio Imager.M2 (Carl Zeiss, Germany). Meiosis stages were determined by morphological criteria [21]. Destructive chromosome changes at MII was assessed as decondensation (loss of clear morphological contours, increase in volume of chromosomes, uneven morphological contours), partial agglomeration, and formation of dense lumpy structures [10].

For analysis of apoptosis, oocytes were fixed in 4 % paraformaldehyde solution in sodium-phosphate buffer (pH 7.2) for 1 hour at room temperature. After fixation, oocytes were incubated during 1 hour in 0.1 % sodium citrate containing 0.5 % Triton X-100. Sings of nuclear material apoptosis in oocytes was assessed by TUNEL method with In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) subject to manufacturer's instructions. Afterwards, oocytes were stained for 20 min with DAPI solution (1 μg/ml) to localize chromosomes, transferred on slide SuperFrostPlus (Thermo Scientific, USA) and embedded into Vectashield medium (Vector Laboratories, United Kingdom). Microscope Axio Imager.M2 equipped with fluorescent attachment and ZEN 2 pro software (Carl Zeiss, Germany) were used for photo documentation and assessment of preparations (magnification ×400). Apoptosis rate was determined by a share of TUNEL-positive oocytes (green stain of MII chromosomes) of the total number of oocytes at stage MII.

Oocyte culture tests were conducted in 4-5 independent repeats in each group, there were at least 75 OCC. Data were processed by one-way ANOVA and two-way ANOVA with SigmaStat software (Systat Software, Inc., USA). Results are presented as means (*M*) and standard error of means (±SEM). Tukey's test was used to assess statistical significance between the compared mean values.

Results. Previously we had developed two-phase system for in vitro maturation of cattle oocytes allowing us to improve in vitro quality of late morulas/blastocysts [20]. Such improvement may be due to increase in resistance of matured oocytes to age-related changes, reducing their ability to embryonic development after fertilization [14]. Besides, maturation of oocytes in two-phase system resulted in optimization of estradiol-17β profile in culture medium, whereas P4 concentration by the end of the second phase of maturation remained insufficiently high [20]. At the same time, role of endogenous P4 as positive regulator of oocyte viability, evidently, grows at final stage of oocyte maturation [17, 18].

In this paper, we added luteotropic factors (P4 and two potential stimulators of its production by cumulus cells, PRL and LH) to the medium to normalize P4 level at the second stage of oocyte maturing. To identify possible ways of hormone influence on oocyte competence to embryo development under long culturing, we studied age-related changes in cow oocytes maturing in the two-phase system.

Cytogenetic analysis did not reveal the effect of luteotropic factors on termination of nuclear maturation of oocytes or on meiosis blockage during fur-

ther prolonged culture (Fig. 1, A). Portion of oocytes at stage MII of meiosis in 24 hours of ageing (Fig. 2) was similar in all groups and reached 78.2-88.4 % (see Fig. 1, A). Nevertheless, we revealed influence of P4 and PRL on further state of MII chromosomes during oocyte maturation (see Fig. 1, B).

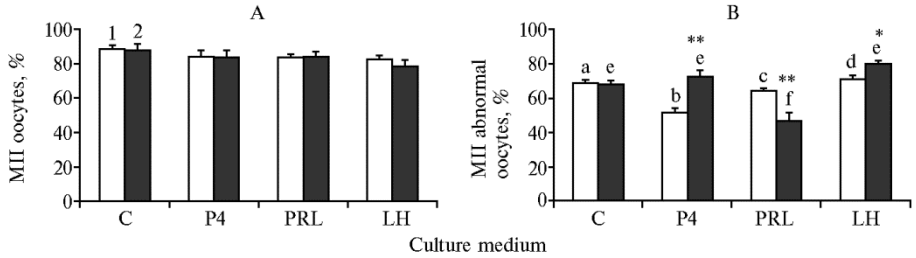

Fig. 1. Chromosomes of cow oocytes surrounded by cumulus cells after 12-hour maturing under the effect of luteotropic factors at absence (1) **and in presence** (2) **of granulosa cells and further 24-hour ageing:** A — nuclear maturation of oocytes, B — abnormalities in chromosomes at metaphase II. C — control, P4 — progesterone, PRL — prolactin, LH — luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 4$, independent tests).

a, b; b, c; b, d; e, f Differences between the compared groups are statistically significant at $p < 0.01$, $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively.

*, ** Differences between system 1 and system 2 are statistically significant at $p < 0.05$ and $p < 0.001$, respectively.

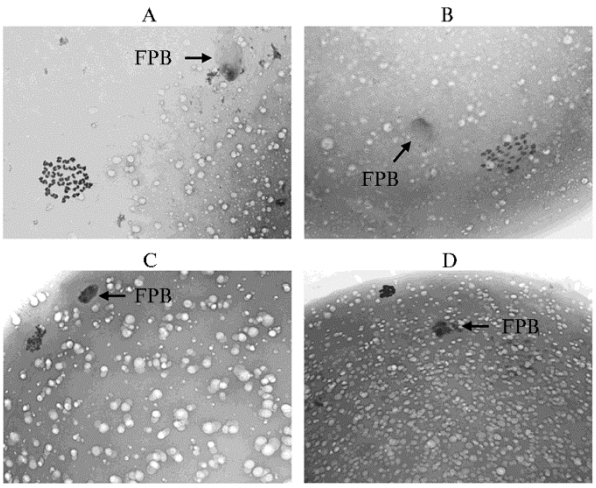

Fig. 2. Representative micro photos of chromosome morphology in cow oocytes at metaphase stage II (cytogenetic preparation): A — without signs of abnormality, B — decondensation, C — decondensation and partial agglomeration, D — dense lumpy structure. Arrows mark first polar bodies (FPB) (magnification ×1000, Axio Imager.M2, Carl Zeiss, Germany).

At absence of granulosa cells, effect of P4 on oocytes during the second phase of maturation resulted in further reduction of frequency of destructive changes of chromosomes during MII from 67.1±2.0 (control) to 51.2±2.9 % ($p < 0.01$), whereas placement of such cells in culture system eliminated positive effect of hormone ($p < 0.001$). Conversely, similar effect of PRL reducing the share of ageing oocytes with abnormal chromosome morphology at stage MII from 67.7±1.6 (control) to 46.5±5.0 % ($p < 0.001$) was found only in presence of granulosa cells. LH did not influence destructive chromosome changes in both systems.

Longstanding effect of P4 on apoptosis in ageing oocytes (Fig. 3, 4) was similar to that on abnormal chromosome transformations at MII. After maturing in the system free from granulosa cells and 24-hour prolonged culture, frequency of apoptotic degeneration was the least in oocytes subjected to P4 (17.6 against 23.5-25.2 % in other groups, $p < 0.05$). Granulosa cells did not suppress anti-apoptotic effect of P4; however in the presence of such cells only oocytes maturing in culture medium with P4 and LH differed significantly (18.3 % against 26.6 %, $p < 0.05$). Neither PRL, nor LH renders longstanding effect on apoptotic degeneration of ageing oocytes.

We did not find any differences in ageing medium concentration of P4 between all compared groups (Fig. 5). Thence, luteotropic factors affecting OCC during the second phase of in vitro maturation did not cause intensification of P4 secretion by cumulus cells surrounding ageing oocytes. Effect of P4 and PRL during maturation of oocytes on their resistance to age-related changes was not related to stimulation of endogenous P4 production in OCC during ageing.

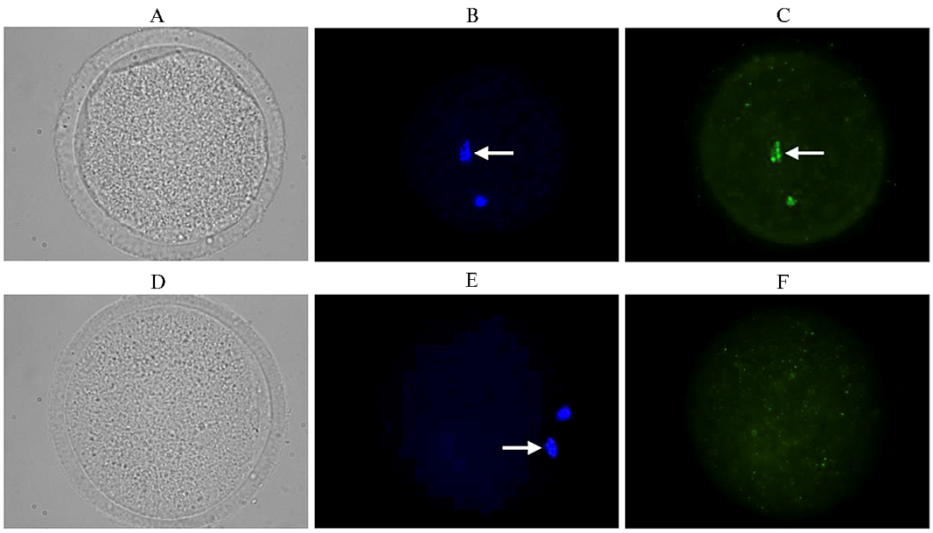

Fig. 3. Representative micro photos of cow oocytes with apoptosis (upper raw) **and without apoptosis** (lower raw)**:** A, D — morphology of ageing oocytes; B, E — DAPI staining of oocyte nuclear material (blue color), arrows mark metaphase II chromosomes; C, F — TUNEL staining of oocyte nuclear material (green color), arrows mark TUNEL-positive MII chromosomes (TUNEL method, magnification ×400, microscope Axio Imager.M2, Carl Zeiss, Germany).

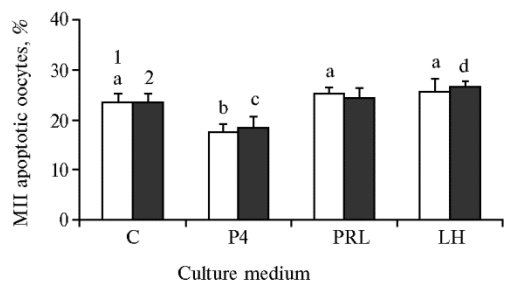

Fig. 4. Effect of luteotropic factors on apoptotic degeneration of cow oocytes surrounded by cumulus cells after 12-hour maturation at absence (1) **and in presence** (2) **of granulosa cells and further 24-hour ageing.** C — control, P4 — progesterone, PRL — prolactin, LH —luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 5$, independent tests).
[a, b]; [c, d] Differences between the compared groups are statistically significant at $p < 0.05$ and $p < 0.05$, respectively.

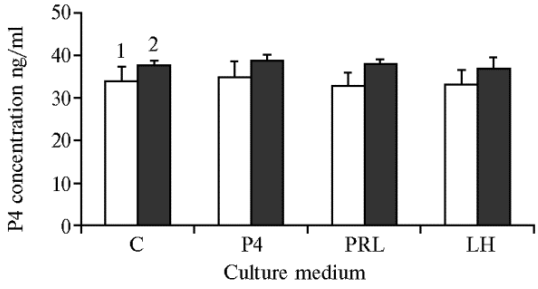

Fig. 5. Effect of luteotropic factors on progesterone (P4) **concentration in ageing medium during maturation of cow oocyte surrounded by cumulus cells at absence** (1) **and in the presence** (2) **of granulosa cells.** C — control, P4 — progesterone, PRL — prolactin, LH — luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 6$, independent tests).

In present paper we for the first time had studied longstanding effect of P4 and PRL on cow oocytes during the second phase of in vitro maturation. Previously, we had shown that adding of PRL into the commonly accepted one-phase IVM system (i.e. immediately prior to beginning of maturation) does not result in slowing down of destructive chromosome changes in oocytes

at MII stage during their further prolonged culture [22]. Besides, exogenous P4 in one-phase maturation system did not intensify competence of cattle oocytes to embryogenesis after in vitro fertilization [23]. Although LH is commonly considered as a stimulator of oocyte competence to further development and is widely used in routine IVM practice in cattle [1, 24, 25], in our two-phase system LH did not positively influenced the blastocyst yield [20]. In present paper, LH did not also increase the resistance of cow oocytes to age-related changes. I.e. the effects of P4, PRL, and LH on oocytes during the second phase of maturation (i.e. transfer from metaphase I to metaphase II) radically differ from those upon the influence on oocytes blocked at diplotene stage. Our findings agree with data on specific regulation of mice and pig oocyte maturation during MI to MII transformation of chromosomes [26, 27].

Therefore, effect of progesterone and prolactin on cow oocytes at the second phase of maturation in the two-phase in vitro system we proposed results in increase of their resistance to further age-related changes which reduce oocyte quality and, consequently, the yield of full-grade embryos after fertilization. Accordingly, these hormones could be used to optimize cattle oocyte maturation in two-phase in vitro system.

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