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NUCLEAR AND CYTOPLASMIC MATURATION OF BOVINE OOCYTES CULTURED WITH dbc AMP, FSH AND hCG

By

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LIEHMAN, P., T. GREVE and K. P. XU: *Nuclear and cytoplasmic maturation of bovine oocytes cultured with dbc AMP, FSH and hCG.* Acta vet. scand. 1986, 27, 566—574. — The present investigation was undertaken to study the effect of addition of dbc AMP on bovine oocyte maturation and fertilization in vitro. The bovine oocytes isolated from 2—8 mm follicles were cultured for 26 h in TCM-199. The maturation rate (71.4 %) did not significantly increase after supplementation of the culture medium with dbc AMP (86.3 %) or FSH + hCG (86.3 %). The in vitro fertilization rate of oocytes based on sperm penetration and presence of sperm tail in the ooplasm increased significantly in the dbc AMP (34.7 %) and the dbc AMP + FSH + hCG (33.9 %) treated groups when compared with untreated controls (17.9 %). However, dbc AMP treated oocytes were not able to secure the formation of male pronucleus 20 h after in vitro fertilization, while in oocytes matured in dbc AMP free medium both pronuclei were present in approximately 15 % of the penetrated oocytes. Also, the sperm head decondensation was blocked or slowed down by the dbc AMP treatment. It is concluded (1) that dbc AMP may improve the condition for the interaction of oocytes with spermatozoa, and (2) that the ooplasm of such dbc AMP treated oocytes apparently is not able to decandense the sperm head and transform it to the male pronucleus.

in vitro fertilization; IVF; cattle; gonadotrophins.

When released from follicles fully grown mammalian oocytes spontaneously resume meiosis and may mature under adequate culture conditions. The maturation of such oocytes has been investigated in different studies. Having elucidated basic events leading to reinitiation of meiosis *Eppig & Downs* (1984) suggested that cAMP plays an important role in the maturation process. However, the sensitivity to and effect of this cyclic nucleotide is

highly dependent on the mammalian species studied (mouse: *Stern & Wassarman* 1974, rat: *Magnusson & Hillensjö* 1977, *Rice & McGaughey* 1981, cattle, sheep, hamster: *Jagiello et al.* 1981, cattle: *Ball et al.* 1983).

Also the gonadotrophins FSH and hCG contribute to the process of maturation. They do not interfere with the germinal vesicle breakdown (GVBD) and chromosome condensation directly, but are believed to improve the quality of ooplasm substantially and are also responsible for the changes that take place in cumulus oophorus configuration (*Shalgi et al.* 1979, *Moor et al.* 1981).

The aim of the present study was to evaluate the influence of the cyclic adenosine monophosfate (cAMP) derivate dibutyryl cyclic adenosine monophosfate and the gonadotrophins hCG and FSH upon in vitro nuclear and ooplasmic maturation and in vitro fertilization (IVF) of bovine oocytes.

MATERIALS AND METHODS

Bovine oocytes from ovarian follicles 2—8 mm in diameter were aspirated at a local abattoir within 1 h after killing. The oocytes were transported to the laboratory in follicular fluid at temperature between 20—30°C. Only oocytes surrounded by compact cumulus were selected for the experiments.

Culture conditions

Sodium bicarbonate buffered TCM-199 medium (M-199) used for oocyte maturation, was supplemented with 0.73 mmol/l sodium pyruvate (Merck, FRG), 2.34 mmol/l calcium lactate (Lachema, Czechoslovakia), 0.32 mmol/l glutamine (Flow Laboratories, UK), 2.47 mmol/l glucose, 0.02 mmol/l estradiol-17β (E2) (Sigma), 40 i.u./ml penicillin (K salt), 40 i.u./ml streptomycin sulphate and 20 mg/ml freeze dried bovine serum growth protein (USOL, Czechoslovakia). The supplements were dissolved in redistilled water and for the E2 in absolute ethanol. The osmolarity of this medium was 289 mOsm and pH 7.3 after equilibration with 5 % CO_2 in air.

Where indicated, the medium was further supplement with either 1 mmol/l dbc AMP or 10 μg FSH and 5 i.u. hCG/ml. Ten-15 oocytes were then placed in 1 ml of this culture medium and incubated at 38°C under air with 5 % CO_2 and 100 % humidity

for 26—27 h. Following this culture period the oocytes were randomly assigned to two groups in order to study the maturation rate (Experiment I and experiment II).

Experiment I

The cumulus-oocyte complexes were treated with trypsin (Gibca, UK) and the cumulus cells removed be repeated suckling through a narrow pipette. The isolated oocytes were then mounted on slides, fixed in acetic acid: ethanol (1:3 v/v) for 24 h, stained with 1 % orcein and evaluated under interpherence phase contrast microscope. Abstriction of the first polar body (PB1) and a metaphase II configuration was regarded as an indication of completion of maturation (MII stages). Each treatment group was replicated at least 5 times.

Experiment II

The oocytes — cumulus — complexes were washed thouroghly in defined medium (DM) (*Brackett et al.* 1981) and placed into sperm suspension prepared as follows.

Fresh ejaculates from 4 bulls of proven fertility were mixed and the sperms washed twice in DM after 5—6 min centrifugation at 600 × g. After a third centrifugation the supernatant was removed and the pellet resuspended. Then 2 ml of DM were added carefully to the top of sperm suspension and incubated at 38°C. After 60 min the swimming up sperms were aspirated and after centrifugation treated with 40 i.u./ml heparin (Leo, Denmark) in DM without glucose for 15 min.

After removing of the heparin the suspension of final concentration 5×10^6 sperms/ml was prepared. Fifty µl microdrops of this suspension were covered with paraffin oil after adding 10—15 oocytes and incubated at 38°C under air with 5 % CO_2 and 100 % humidity.

Four replicates were carried out of each experimental group.

Twenty hours later the remaining cumulus cells and excess of spermatozoa were removed and the eggs mounted, fixed and stained according to the porcedure described above. The physiological stage of ooplasm was assessed according to its ability to be penetrated and secure decondensation of penetrated sperm head and formation of male pronucleus after in vitro fertilization. Only pronuclei (PN) with sperm tail in close proximity were considered as male (MP).

The data were evaluated by the χ^2 test at a P level of 0.05.

RESULTS

Experiment I

The results of the present study (Table 1) indicate that in vitro culture of bovine oocytes in medium containing gonadotrophins increased the rate of oocyte maturation (86.3 % and 86.3 % for dbc AMP and hCG/FSH, respectively) when compared with controls (71.4 %). This difference was not statistically significant.

Table 1. The maturation rate of bovine oocytes following 26 h culture medium containing dbc AMP or FSH + hCG.

Supplementation	No. of oocytes with	
	M II + PB extruded (%)	degenerated*
Controls	40/56 (71.4)	2/56
dbc AMP	44/51 (86.3)	1/51
FSH + hCG	44/51 (86.3)	2/51

* oocytes, in which the stage of maturation could not be recognized by interpherence phase contrast microscopy.

Experiment II

The penetration rate after in vitro fertilization was 17.9 % in the control group and supplementation of the culture medium with gonadotrophins did not increase this rate significantly (26 %). Both dbc AMP (34.7 %) and dbc AMP + FSH + hCG (33.9 %) treatments increased the rate of penetrated oocytes significantly (Table 2).

No male pronucleus was observed in the dbc AMP treated oocytes, while those matured without this substance transformed sperm head to male pronucleus in 14.3 % and 15 % respectively. The formation of female pronuclei was identical for all treatment groups.

Also, the sperm head decondensation was blocked or slowed down by the dbc AMP treatment (15.4 % and 5.3 %, respectively) when compared with the control group (35.7 %) and the groups cultured with gonadotrophins (45 %).

The appearance of cumulus oophorus after cultivation was also recorded. While cumulus cells in media containing no dbc AMP deposited large amounts of matrix to the intracellular space (gonadotrophins stimulated this proces) only tiny mucus forma-

Table 2. Sperm penetration rate, sperm head decondensation and formation of male pronuclei after in vitro fertilization of oocytes matured with dbc AMP and/or FSH + hCG.

Supplementation	No. of oocytes		No. of sperm heads			
	Total	Penetrated (%)	Non-decondensed (%)	Decondensed (%)	Transformed to MP (%)	Polyspermy
Nontreated controls	78	14 (17.9)[a]	7 (50.0)[a]	5 (35.7)	2 (14.3)	2
dbc AMP	75	26 (34.7)[b]	22 (84.6)[b]	4 (15.4)	0 (0)	4
FSH + hCG	77	20 (26.0)[ab]	8 (40.0)[a]	9 (45.0)	3 (15.0)	1
dbc AMP + FSH + hCG	56	19 (33.9)[b]	18 (94.7)[b]	1 (5.3)	0 (0)	6

[a,b] Values with different superscripts within the column differ significantly ($P < 0.05$) according to the χ^2-test.

tion was seen in the group of oocytes that were cultured with dbc AMP. Gonadotrophins in medium could not compensate this effect of dbc AMP.

DISCUSSION

Experiment I

While mouse and rat oocyte meiosis are reported to be almost totally blocked at the germinal vesicle (GV) stage by relatively low dbc AMP levels (*Stern & Wassarman* 1974, *Magnusson & Hillensjö* 1977), the pig oocytes seems to be less sensitive to this substance (*Rice & McGaughey* 1981). Also in cattle the inhibition of GVBD is either very low (*Jagiello et al.* 1981) or not observed at all (*Ball et al.* 1983) as in the present study. Our results support the suggestion of *Jagiello et al.* (1981) concerning the diversity of responses to dbc AMP in oocytes of different mammalian species.

Complete absence of the GVBD inhibition observed in this study could partially be explained by the long time which lapsed between oocytes aspiration and the subsequent culture. This time is believed to be critical for the GVBD inhibition (*Dekel et al.* 1984). However, in our pilot experiments where all oocytes were placed immediately after recovery in the medium containing dbc AMP they started to mature anyway. This is in good agreement with the findings of *Ball et al.* (1983). Contrary to their results, however, the present experiment revealed that dbc AMP treat-

ment could not prevent the completion of nuclear maturation i.e., a block at the MI stage did not occur. This difference may be caused by the above mentioned delay of oocyte culture (*Ball et al.* 1983).

Another possible explanation for the observed difference may be the source of oocytes, since changes connected with the acquisition of meiotic competence occur during the follicular growth from 1.6 to 3 mm size follicles (*Motlik et al.* 1984, *Crozet et al.* 1986). Oocytes from follicles about 1 mm in diameter may not resume of complete meiosis spontaneously and may also be more sensitive to the inhibiting effect of dbc AMP.

Experiment II

The present results indicate that dbc AMP added to culture medium can increase the penetration rate after fertilization in vitro. It is difficult to find an explanation for this improved interaction of the oocytes with spermatozoa. The possibility, that the rest of free dbc AMP in medium influenced the penetrating ability of sperms (*Fraser* 1981) can be excluded, since the oocytes were washed twice in fresh DM before transfer to the sperm suspension.

It has been generally accepted that, in contrast to the situation in vivo, many in vitro matured oocytes are not able to secure the proper structural changes of the penetrated sperm head. These changes in the ooplasma are a prerequisite for further development (*Usai & Yanagimachi* 1976, *Thadani* 1979, *Motlik & Fulka* 1981, *Fulka Jr. et al.* 1982). According to *Thibault et al.* (1975), sufficient activity of a "male pronuclear growth factor" (MPGF) is required for this sperm head decondensation and transformation to the male pronucleus. The lack of this activity in dbc AMP treated oocytes is probably caused by some deficiency of the MPGF synthesis. The fact, that dbc AMP affects the synthesis of several stage-specific (developmental) and also non-developmental polypeptides (*Richter & McGaughey* 1981) seems to support this idea.

Cumulus cells of immature follicles are connected with each other and with the oocyte by nutritive and regulative junctions. During both in vivo and in vitro maturation a mucous matrix is deposited in the intercellular spaces and the socalled cumulus expansion takes place.

Pronounced mucification of cumuli is generally considered

as a prerequisite for the sperm penetration (*Ball et al.* 1984), because non-expanded cumulus cells may function as a mechanical barrier for the penetrating sperm. However, our results in cattle confirmed observations made in mouse (*Schroeder & Eppig* 1984), that cumulus expansion in the sense of mucus deposition is not required.

In interpreting these results we must take into consideration, that intercellular uncoupling and mucification are two relatively independent processes (*Eppig & Ward-Bailey* 1982, *Salustri & Siracusa* 1983, *Motlik et al.* 1986), and that the cumulus, in which the uncoupling once took place, is easily penetrable by the sperm.

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SAMMENDRAG

Virkning af dbc AMP, FSH og hCG på den bovine oocytmodning in vitro.

Nærværende undersøgelse blev iværksat med henblik på at undersøge om dbc AMP påvirker in vitro modning og in vitro fertilisation af bovine oocyter opsamlet fra slagtehusmateriale (2—8 mm follikler). Isolerede oocyter omgivet af et tæt lag cumulusceller blev dyrket i 26 timer i TCM-199 tilsat enten dbc AMP eller FSH og hCG. In vitro modningsraterne i de 2 medier var henholdsvis 86,3 % og 86,3 %. In vitro penetrationsraterne baseret på tilstedeværelsen af sædcellens hale og hoved (dekondenseret) øgedes signifikant med tilsætning af dbc AMP (34,7 %) og dbc AMP + FSH + hCG (33,9 %) i forhold til kontrolgruppen (17,9 %). Imidlertid var ingen af de dbc AMP behandlede oocyter i stand til at udvikle normale hanlige forkærner, medens 15 % af de ikke behandlede oocyter var i stand til at understøtte dekondenseringsprocessen. Ud fra disse resultater kan det konkluderes (1) at dbc AMP behandling øger spermiepenetrationsraten, men (2) at dbc AMP behandling samtidig hindrer en normalt forløbende spermiekondenseringsproces.

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