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Maturation of pig oocytes *in vitro* in a medium with pyruvate

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Abstract

The aim of *in vitro* maturation oocyte systems is to produce oocytes of comparable quality to those derived *in vivo*. The present study was designed to examine the surface morphological changes of the cumulus-oocyte complex (COC) and nuclear maturation in a culture system containing pyruvate. Ovaries were obtained from a slaughterhouse and transported to the laboratory within 2 h at 35-39ºC, and rinsed three times in 0.9% NaCl. The COCs were harvested from the ovaries and *in vitro* maturation was evaluated in San Marcos (SM) medium, a chemically defined culture system containing 22.3 mM sodium pyruvate. Oocytes were cultured in SM, SM + porcine follicular fluid (pFF) and in SM + pFF + gonadotropins (eCG and hCG) for 20-22 h and then without hormonal supplements for an additional 20-22 h. After culture, the degree of cumulus expansion and frequency of nuclear maturation were determined. Oocytes matured in SM (40.9%) and SM + pFF (42.9%) showed moderate cumulus expansion, whereas oocytes matured in SM + pFF + gonadotropins (54.6%) showed high cumulus expansion. The maturation rate of cultured oocytes, measured in function of the presence of the polar corpuscle, did not differ significantly between SM (40.9 ± 3.6%) and SM + pFF (42.9 ± 3.7%). These results indicate that pig oocytes can be successfully matured in a chemically defined medium and suggest a possible bifunctional role of pyruvate as an energy substrate and as an antioxidant protecting oocytes against the stress of the *in vitro* environment.

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- Cumulus cells

Cumulus-oocyte complexes (COCs) are maintained by delicate cell-to-cell connections among cumulus cells and oocytes. Mammalian cumulus cells play a very important role during oocyte growth and maturation. They supply nutrients and/or messenger molecules for the development of the oocyte and mediate the effects of hormones on the COC (1). It is generally accepted that during the maturation period cumulus cells support the *in vitro* maturation (IVM) of oocytes to the metaphase-II stage and are involved in the cytoplasmic maturation needed for post-fertilization developmental competence such as male pronucleus formation in porcine oocytes (2). Studies of the importance of cumulus cells during oocyte IVM, mainly using serum containing IVM medium, have been reported. In cattle, when cumulus cells were removed before matura-

tion, there was a significant reduction in the rate of oocyte maturation, fertilization, and *in vitro* development (3).

The pattern of energy metabolism of the zygote is determined in the oocyte before fertilization and pyruvate is the main energy substrate which can be used directly by the oocyte and zygote. Cumulus cells can satisfy the energy requirements of oocyte and zygote by the metabolism of other substrates (4).

Early embryo development requires energy (ATP), which is produced through two possible mechanisms: glycolysis, using glucose as substrate, and oxidative phosphorylation, using pyruvate or oxaloacetate as substrate. Another key energy substrate is oxygen, essential for the conversion of ADP to ATP in oxidative phosphorylation through its role as an electron acceptor in the electron transport chain. However, the use of oxygen as an energy substrate also results in the production of reactive oxygen species, particularly superoxide anions and hydroxyl radicals. Reactive oxygen species are highly active electron acceptors, able to strip electrons from other molecules that, in turn, become free radicals (5).

The use of serum in culture systems generally does not permit an understanding of the functions of cumulus cells or of the chemical substance involved in the oocyte IVM. A chemically defined medium is useful for analyzing the physical action of substances such as inorganic compounds, energy substrates (pyruvate), hormones, cytokines, and vitamins on oocyte maturation and the development of preimplantation embryos, because it eliminates undefined factors present in serum or serum albumin (6).

Recent studies on cattle have demonstrated that the maturation and normal fertilization rates of cumulus-denuded oocytes (CDOs) cultured with sodium pyruvate and of COCs were higher than those of CDOs cultured without sodium pyruvate. The glutathione content of oocytes significantly decreased in CDOs after maturation culture (7).

The objective of the present study was to determine the effects of IVM of pig oocytes incubated in San Marcos (SM) medium in a serum-free culture system containing pyruvate.

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in sterile 0.9% NaCl at 35-39ºC within 2 h of slaughter. Oocytes were aspirated from follicles (3 to 6 mm in diameter) with an 18-gauge needle attached to a disposable syringe. Oocytes covered with multilayers of cumulus cells were selected. The base chemically defined medium for maturation culture of oocytes was SM containing 22.3 mM sodium pyruvate.

Oocytes were cultured at 37ºC in 100 µl of the following media: SM, SM + porcine follicular fluid (pFF) and in SM + pFF + equine chorionic gonadotropin (eCG) + human chorionic gonadotropin (hCG) (10 IU/ml each) for 20-22 h, and then without hormonal supplements for an additional 20-22 h, in six trials for each medium. Each drop of medium contained approximately 10 oocytes. Expansion of cumulus cells and nuclear maturation were evaluated at 44 h after maturation culture to assess the maturation rate.

Oocytes matured in SM and in SM + pFF media showed moderate cumulus expansion, whereas oocytes matured in SM + pFF + eCG and hCG showed high cumulus expansion (Figure 1). The maturation rate of cultured oocytes did not differ significantly ($P > 0.05$) between SM (40.9 ± 3.6%) and SM + pFF (42.9 ± 3.7%) media (Table 1).

The ovarian follicle is a morphological and functional unit in which the somatic and germ cell components are intimately associated and interdependent (8). Successful embryonic development not only depends on nuclear maturation but is also influenced by changes in the ooplasm and plasma membrane. All of these events must proceed in a

coordinated fashion (9). Therefore, a more detailed study of oocyte quality and of optimum conditions for *in vitro* maturation is extremely important.

Oocyte quality or developmental competence is acquired during the stages of oocyte maturation. Oocyte size and intercellular communication between oocyte and cumulus cells are important to acquire competition capacity (10). In the present study, follicles from pig ovaries measuring 3 to 6 mm and COCs with a minimum of three cell layers and homogeneous cytoplasm were selected. The cumulus-oocyte transzonal projections became disconnected between the metaphase I and metaphase II stages as a result of cumulus expansion; however, the cumulus-cumulus communications remained intact during these stages (11). The increased cumulus expansion observed for oocytes matured in SM + pFF and in SM + eCG and hGC could be attributed to the presence of pFF. Interestingly, in contrast to the observations in pig oocytes, no cumulus expansion was observed in mouse and hamster oocytes matured with gonadotropins and in the presence of bovine serum albumin and polyvinyl alcohol (12), respectively. On the other hand, under defined culture conditions (SM), cumulus expansion occurred in pig oocytes. These findings suggest that pig oocytes are capable of a certain degree of cumulus expansion in media with no serum supplementation. It is possible that some still unknown factors produced by the oocyte and/or cumulus cells stabilize the extracellular matrix, thus resulting in cumulus expansion.

Gamete metabolism is modified during the process of maturation, fertilization and development (13). Developing murine embryos prefer pyruvate as their main energy source until they reach the blastocyst stage (14). Isolated mouse cumulus cells formed pyruvate when incubated with glucose and lactate (15), and porcine oocyte-COCs are capable of producing pyruvate to meet metabolic needs (16). Pyruvate metabolism is increased during *in vitro* maturation, whereas glucose metabolism decreases during maturation of cattle oocytes (17). Even under basal conditions, aerobic metabolism entails the production of reactive oxygen species. Pyruvate plays a pivotal role in both primary energy metabolism and the redox potential. A possible function for pyruvate in protecting embryos against oxidative stress was suggested (18). Pyruvate may be secreted by some cells to function as an extracellular antioxidant (19). The present results show that *in vitro* oocyte maturation was similar in

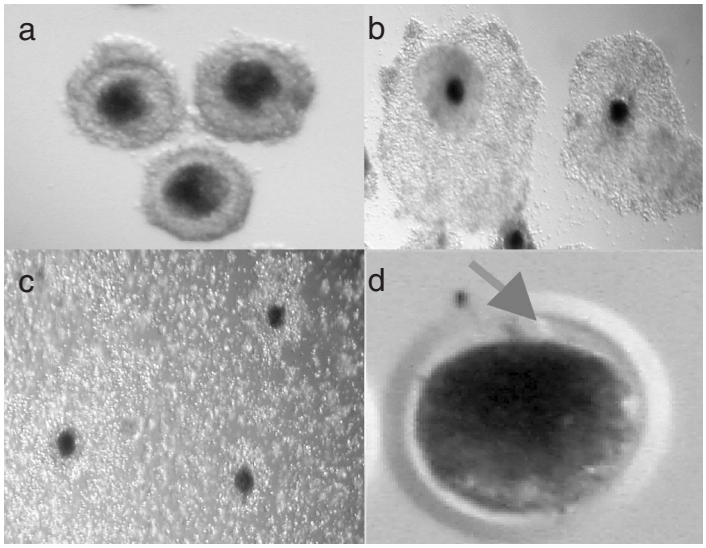

Figure 1. Pig cumulus cell expansion and nuclear maturation in San Marcos (SM) culture medium. *a*, Immature cumulus-oocyte complex in SM (30X); *b*, cumulus expansion at 24 h at 37ºC in SM (30X); *c*, cumulus expansion at 44 h at 37ºC in SM + porcine follicular fluid + gonadotropins (30X); *d*, mature oocyte with a polar corpuscle in SM (100X).

Table 1. Pig oocyte maturation *in vitro* in a culture system with pyruvate.

Media	No. of trials	No. of oocytes examined	Mature oocytes (%)
SM + pFF	6	182	42.9 ± 3.7[a]
SM + pFF + eCG + hCG	6	193	54.6 ± 3.6[b]
SM	6	185	40.9 ± 3.6[a]

Data are reported for matured oocytes as means ± SEM. SM = San Marcos medium; pFF = porcine follicular fluid; eCG = equine chorionic gonadotropin; hCG = human chorionic gonadotropin.
[a,b]Values with different superscripts differed significantly ($P < 0.05$, Z-test for comparison of two outcome proportions).

SM and in SM + pFF media, and suggest that pyruvate provides the necessary energy for oocyte primary metabolism and in addition regulates oxidative stress in the same way as follicular fluid, which contains antioxidants such as glutathione.

It is clear that pig oocytes have metabolic needs for maturation, undergoing cumulus expansion and extrusion of the polar corpuscle.

We conclude that cumulus cells probably supported oocyte maturation via metabolic cooperation, by passing pyruvate and other glycolytic products through gap junctions that synthesize glucose, enhancing oocyte quality as shown by the maturation rates of all COCs cultured with sodium pyruvate. Therefore, a possible bifunctional role of pyruvate is suggested, as an energy substrate and as an antioxidant protecting the oocyte against the stress of the *in vitro* environment.

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