INFLUENCE OF FOLLICULAR MATURATION AND CULTURE CONDITIONS ON THE MEIOSIS OF PIG OOCYTES *IN VITRO*

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The original finding of Pincus & Enzmann (1935) that isolated rabbit oocytes will mature spontaneously *in vitro* has been extended to nine other mammalian species (Donahue, 1972), including the pig (Edwards, 1965; Foote & Thibault, 1969; McGaughey & Polge, 1971). The main objectives of the present communication are (a) to describe a culture system for studying the maturation of pig oocytes which can also be used for other ovarian cell types, and (b) to compare the ability of oocytes isolated from follicles differing in their developmental stage to undergo maturation.

Oocytes were collected from pig ovaries obtained at a local slaughterhouse. Within 20 to 30 min after the pigs had been killed the ovaries were excised and placed in 0.9% NaCl plus 100 i.u. penicillin/ml and 100 µg streptomycin/ml at room temperature. Details of oocyte collection, culture, examination and classification have been described elsewhere (Tsafriri & Channing, 1975). Within 5 hr after the pigs had been killed, the oocytes were allocated to groups of ten to fifteen and placed in the compartments of an eight-chambered Lab-Tek culture slide (Miles Laboratories, Naperville, Illinois), each compartment containing 0.4 ml culture medium. The medium used was: TC 199 with Earle's salts and 25 mM-HEPES buffer (Grand Island Biological Co., GIBCO, Grand Island, N.Y.) supplemented with 15% pig serum (GIBCO), glutamine (1 mM, GIBCO) and gentamicin (50 µg/ml; Schering Diagnostics, Port Reading, N.J.). The porcine serum used contained low amounts of LH (<1 ng/ml in terms of NIH-ovine LH units), oestrogen (66 pg/ml) and progesterone (3 ng/ml), measured as detailed previously (Tsafriri & Channing, 1975). To this medium (TC 199 plus 15% pig serum and antibiotics), various amounts of sodium pyruvate (Eastman Organic Chemicals, Rochester, N.Y.), sodium lactate (2.5 mM; Sigma Chemical Co., St Louis, Mo.) and crystalline bovine zinc insulin (Lilly Laboratories, Indianapolis, Ind.) were added (Table 1). Unless mentioned otherwise, the oocytes were cultured for 43 to 48 hr at $37.5 \pm 0.5^\circ\text{C}$ in an incubator continuously flushed with air and 5% CO₂ saturated with water. At the end of the culture period, the oocytes were collected, mounted, fixed with alcohol-acetic acid (3:1), stained with aceto-orcein (1%; GIBCO) and examined microscopically. The stage of meiotic maturation was assessed according to stages described previously (Hunter & Polge, 1966). The oocytes were divided into three groups:

immature oocytes with an intact germinal vesicle, mature oocytes that had matured beyond first metaphase, and mature oocytes which had the first polar body and the second metaphase chromosomes. The percentage of oocytes at different meiotic stages in each chamber was calculated and the results were expressed as the mean $\pm$ S.E. of each experimental treatment. Each experimental treatment was repeated in at least two, and usually three or more, independent experiments. An example of fully matured oocytes is shown in Plate 1.

Table 1. Influence of pyruvate, insulin and lactate upon maturation of porcine oocytes grown in medium 199 plus 15% porcine serum

Treatment			No. of oocytes	Mean % of oocytes ($\pm$ S.E.)		
Pyruvate (mM)	Lactate (mM)	Insulin (mU/ml)		Dictyate	Mature	
					Total	With first polar body
0.03	—	—	40	19.7 $\pm$ 3.0	65.0 $\pm$ 8.5	50.5 $\pm$ 9.8
0.03	2.5	—	34	36.5 $\pm$ 12.8	63.4 $\pm$ 12.8	52.3 $\pm$ 7.3
0.03	2.5	1.25	41	22.0 $\pm$ 4.6	63.5 $\pm$ 3.4	43.7 $\pm$ 5.9
0.03	2.5	12.5	42	12.2 $\pm$ 3.7	78.6 $\pm$ 0.9	54.8 $\pm$ 3.3
0.03	2.5	125	70	14.7 $\pm$ 4.9	82.0 $\pm$ 4.6	58.5 $\pm$ 8.9
0.03	—	125	41	30.9 $\pm$ 4.9	58.7 $\pm$ 4.9	42.6 $\pm$ 7.6
0.03	25	—	36	56.7 $\pm$ 7.7	35.7 $\pm$ 10.4	30.7 $\pm$ 8.9
0.03	25	125	30	60.3 $\pm$ 8.4	33.3 $\pm$ 9.5	19.5 $\pm$ 8.6
—	2.5	125	55	38.1 $\pm$ 5.8	56.6 $\pm$ 5.9	29.8 $\pm$ 9.3
0.16	2.5	125	42	28.5 $\pm$ 11.5	69.4 $\pm$ 11.7	40.6 $\pm$ 6.7
0.33	2.5	125	45	34.5 $\pm$ 7.9	63.6 $\pm$ 8.1	43.3 $\pm$ 9.3

Table 2. Effect of oxygen concentration upon porcine oocyte maturation

O ₂ in air (%)	No. of oocytes	Mean % of oocytes ($\pm$ S.E.)		
		Dictyate	Total	With first polar body
20	102	15.9 $\pm$ 3.1	76.6 $\pm$ 3.9	52.7 $\pm$ 4.6
45	64	36.6 $\pm$ 17.6	56.6 $\pm$ 16.6	43.6 $\pm$ 12.8
95*	98	33.4 $\pm$ 8.6	53.2 $\pm$ 12.2	37.5 $\pm$ 8.0

* In 95% O₂, there were more degenerated oocytes (13%) than in 20% O₂ (7%) and 45% O₂ (8%).

In preliminary experiments using the above mentioned medium (without pyruvate, lactate and insulin), of the 237 oocytes isolated from medium-sized (3 to 5 mm in diameter) follicles, 65% matured beyond first metaphase and only 39% had the first polar body. The beneficial effects of pyruvate, lactate and insulin upon maturation of such oocytes are presented in Table 1. It is clear that addition of 0.03 mM-pyruvate, 2.5 mM-lactate and 12.5 to 125 mU insulin/ml produced optimal conditions for oocyte maturation and in the following experiments, 0.03 mM-pyruvate, 2.5 mM-lactate and 125 mU insulin/ml were added. Enriching the culture atmosphere with O₂ by flushing large mason jars containing the culture dishes with appropriate mixtures of O₂ and air at the

PLATE I

Photomicrograph of porcine oocytes matured *in vitro*: polar (a) and equatorial (b) view of the metaphase plate. The black mass is the polar body chromatin. Aceto-orcein stain, $\times 1000$.

(Facing p. 150)

beginning of the culture and after 24 hr caused some, but not complete, interference with oocyte maturation (Table 2). It is clear, however, that porcine oocytes were much less sensitive to noxious effects of O_2 than are hamster oocytes (Gwatkin & Haidri, 1974).

There was a graded increase in the competence of oocytes to resume meiosis *in vitro* according to the stage of follicular growth (Text-fig. 1). While $80.1 \pm 2.8\%$ (mean $\pm$ S.E.) of the oocytes isolated from large follicles (6 to 12 mm in diameter) gave off the first polar body, only 54.8 ± 3.3 and $15.6 \pm 5.1\%$ of the oocytes from medium-sized (3 to 5 mm) and small (1 to 2 mm) follicles, respectively, completed the first maturation division. Increasing the serum concentration to 50% was beneficial for maturation of oocytes derived from medium-sized follicles but

TEXT-FIG. 1. Influence of follicle size upon the ability of isolated porcine oocytes to mature *in vitro*. Oocytes were harvested from small (1 to 2 mm), medium (3 to 5 mm) and large (6 to 12 mm) follicles and were cultured for 43 to 48 hr. The vertical bars represent the S.E.M.

not for those from small ones. It is possible, however, that the altered responses in culture of the oocytes from the various follicular sizes represent different nutritional requirements, as has been shown for hamster oocytes (Haidri & Gwatkin, 1973). The percentage of maturing oocytes depends also upon the developmental stage of the animal: only 46% of the oocytes isolated from young rabbits matured while all the oocytes from mature oestrous animals matured (Thibault, 1972). In addition, in mouse ovarian explants treated with gonadotrophins, the medium-sized and small antral follicles contained oocytes whose maturation failed to go beyond the dictyate stage whereas large Graafian follicle oocytes usually completed the first maturation division (Neal & Baker, 1973). Collectively, these findings in pig, rabbit and mouse strongly suggest that not all the fully grown oocytes within the ovary are equally competent to mature. It seems that this competence is attained with follicular growth. The ability of

granulosa cells to luteinize in culture (Channing, 1970) as well as to bind chorionic gonadotrophin (Channing & Kammerman, 1973) also increases as the follicle matures. This may indicate that the ability of oocytes to mature and of granulosa cells to luteinize is controlled by similar mechanisms.

The system, employing porcine ovaries collected at the slaughterhouse, from animals at various stages of the oestrous cycle may be very useful for further studies on the physiology of oocyte maturation. Care must be taken to include only oocytes from medium or large follicles and to exclude oocytes found denuded of their cumulus. It is possible that oocytes without their cumulus have been obtained from atretic follicles. It should be emphasized that in the present study (as in most other studies on maturation of oocytes *in vitro*), only the morphological maturation of the oocyte nucleus was studied. The possibility exists that some proportion of the oocytes might have been degenerating. The best test for physiological maturation would be fertilization and subsequent normal development of the zygote. The possibility of culturing oocytes together with other ovarian constituents can be very useful for further studies on the oocyte-ovarian cell interrelationships. It has already been shown, utilizing this approach, that granulosa cells cultured with oocytes inhibited the spontaneous maturation of isolated oocytes (Tsafiriri & Channing, 1975).

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