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In vitro parthenogenesis of mouse oocytes under several experimental conditions

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Summary

Although the *in vitro* fertilisation index is a parameter commonly employed to investigate sperm functional activity, little attention has been given to the occurrence of parthenogenesis. The purpose of this study was to study at 6 h or 22 h incubation: (a) the cleavage-related events that occur in *in vitro* incubated mouse oocytes, in the absence (parthenogenesis) or presence of homologous spermatozoa; (b) the effect of mineral oil, commonly used in *in vitro* fertilisation assays; (c) the effect of piroxicam, a prostaglandin synthesis inhibitor, on the parthenogenetic rate; and (d) the influence on parthenogenesis of spontaneous loss of the cumulus oophorus coat during incubation. Under the experimental conditions employed, there was parthenogenetic activation and activation due to fertilisation. Both increased in a time-dependent manner. The mineral oil enhanced the parthenogenetic rate at 22 h incubation. However, it did not have any effect when the oocytes were inseminated. Since we can not discriminate how much of this activation was due to fertilisation and how much to parthenogenesis we must be very careful with this comparison. Piroxicam 10^{-8} M did not show any effect on the mouse oocyte parthenogenetic rate at neither 6 h or 22 h incubation. Our results suggest that oocyte susceptibility to spontaneous parthenogenetic activation may be modified by the presence of the cumulus and corona radiata cells. In conclusion, we consider that further rigorous studies on these influences are necessary in order to confer more reliability on the results.

Key words: Cumulus oophorus, *In vitro* fertilisation, Mineral oil, Mouse parthenogenesis, Piroxicam

Introduction

The spontaneous activation of mammalian oocytes (parthenogenesis) that occurs during *in vivo* or *in vitro* incubation is well known (Austin, 1956); it has been described in several species, such as golden hamster (Yanagimachi & Chang, 1961; Babcock & Pfeiffer, 1987), rabbit (Ozil, 1990), mouse (Stevens & Varnus, 1974; Stevens, 1975; Eppig, 1978; Anderson *et al*., 1984), etc. Psychological and/or chemical stimuli that can induce spontaneous oocyte activation in mammals include pricking, manipulation of oocytes *in vitro*, cooling, heating, enzymes such as trypsin or hyaluronidase, osmotic or ionic modifications in the incubation medium, anaesthetics or other drugs (Yanagimachi, 1988; Whittingham, 1980; Cuthberton, 1983; Rickords & White, 1992).

Parthenogenetically activated oocytes may often be difficult to distinguish from fertilised ones and can cleave, implant and occasionally develop as far as the forelimb-bud stage *in vivo* (Kaufman *et al*., 1977).

Although the *in vitro* fertilisation index is a parameter commonly employed to investigate sperm functional activity, little attention has been given to the occurrence of parthenogenesis under several experimental conditions. There exists evidence that the secretion of prostaglandins (PGs) by the oocyte–cumulus complexes plays a role in phenomena such as the regulation of embryonic development and the maintenance of a suitable hormonal environment for embryonic viability, among others (Gurevich & Shemesh, 1994). Moreover, the diminished synthesis of prostaglandin E by the oocyte–cumulus complexes has been associated with high parthenogenetic activation

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rates (Cebral *et al.*, 1998). On the other hand, mineral oil is frequently employed in *in vitro* fertilisation assays as an insulating coat. Hence we consider relevant the investigation of its possible effects in the experimental results after 6 h or 22 h incubation.

The purpose of the present study was to study (a) the cleavage-related events that occur in *in vitro* incubated mouse oocytes in the absence (parthenogenesis) or presence of homologous epididymal spermatozoa; (b) the effect of mineral oil; (c) the effect of piroxicam, a prostaglandin synthesis inhibitor, on the parthenogenetic rate; and (d) the influence upon the occurrence of parthenogenesis of spontaneous loss or the continuous presence of the cumulus oophorus coat during incubation.

Materials and methods

Animals were housed at 20 ± 2 °C under controlled illumination (12 h light : 12 h darkness). Water and pelleted food were supplied ad libitum.

Oocytes were harvested from albino Swiss mice. Superovulation was induced at sexually maturity (10–14 weeks) with an intraperitoneal injection of 5 IU PMS (Novormon, Syntex), followed (48 h later) by the injection of 10 IU hCG (Endocorion, Elea). The animals were killed by cervical dislocation 17–19 h after the hCG, and the cumulus–oocyte complexes were collected from the oviducts (by puncturing the swollen ampulla), pooled and then randomly allocated to the various treatment groups into centre-well dishes (60 × 15 mm; Falcon) with 1 ml of modified Tyrode's medium in the centre and 1.5 ml of Tyrode in the periphery.

The oocytes were incubated at 37 °C (5% CO_2 : 95% air) for 6 h or 22 h in the following conditions: (a) alone, to study spontaneous activation (parthenogenesis) or (b) in the presence of ~1×10^6/ml homologous pooled epididymal spermatozoa obtained from adult mice.

Evaluations were performed in an inverted microscope at ×400. In each group results are expressed as percentages of ova in the following stages: non-activated or activated; the latter were classified as pronuclear stage, 2 cells or more than 2 cells.

Tyrode's modified medium

The incubation was performed in a modified Tyrode's medium (Fraser, 1983) with the following composition (mM): $CaCl_2$ 1.8; $MgCl_2$ 0.929; KCl 2.68; $NaHCO_3$ 11.903; NaCl 136.86; NaH_2PO_4 0.287; glucose 5.56; penicillin 75 mg/l, streptomycin 75 mg/l; the medium was supplemented with 4 mg/ml BSA V (Sigma Chemical Co.). Osmolarity and pH were kept at 286 mosmol/l and 7.4 respectively. Medium was freshly prepared and sterilised by ultrafiltration in a 0.2 µm filter (Corning). The water employed was high-quality sterile, distilled, without pirogen, human injectable water (Fidex).

Treatment groups

Effects of mineral oil

Inseminated and non-inseminated oocytes were divided into two groups. In one of them, the centre of each dish was covered with 400 µl of equilibrated mineral oil (Sigma Chemical Co.) and in the other group oocytes were incubated in its absence (control).

Effect of in vitro *addition of piroxicam*

In non-inseminated oocytes, piroxicam (PIR) 10^8 M (4-hydroxy-methyl-3[pyrid-2-ylcarbomoyl]-2H-1,2-benzothiazine-1,1-dioxide) diluted in dimethylsulphoxide (DMSO) 2×10^{-8} M (Sigma Chemical Co.) was added to the medium at the beginning of the incubation.

Influence of the cumulus coat

When the non-inseminated oocytes were studied, the presence or absence (spontaneously lost) of the cumulus coat was taken into account.

At least five replicates of each treatment were made.

Statistical analysis

Results were analysed using the chi-squared test, the V-square and the Yates'-corrected chi-square for small-sized samples. When the p values in the three tests were < 0.05, the difference was considered significant.

Results

When oocytes from superovulated mice were incubated for 6 h or 22 h in the absence or presence of homologous epididymal spermatozoa, significant differences were found (Fig. 1*A* and Fig. 1*B*, respectively); it is evident that the percentage of activated gametes significantly increased over 22 h incubation in all experimental conditions studied here. Moreover, the percentages of activated oocytes at 22 h were higher in inseminated gametes, both in the presence or absence of mineral oil.

When oocytes were incubated without mineral oil for 22 h, parthenogenetic indexes were lower than those for oocytes incubated with mineral oil ($p < 0.01$). No significant differences in this parameter were found at 6 h. In the presence of spermatozoa the percentages of activated oocytes were similar in the presence or absence of mineral oil at 6 h and 22 h. The percentages of inseminated oocytes that reached the pronuclear

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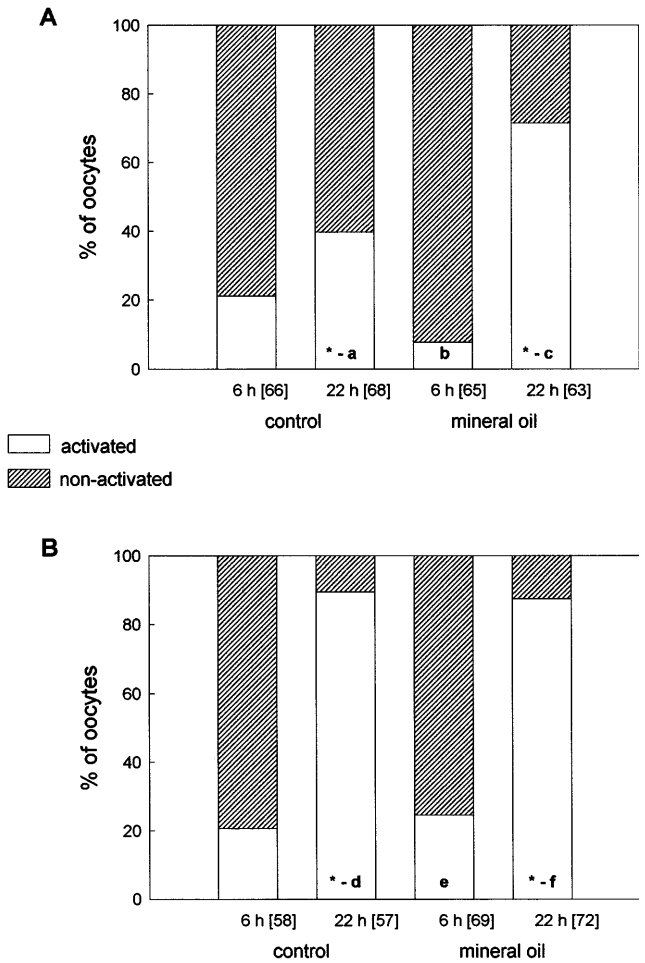


Figure 1 Activation of adult mouse superovulated oocytes in the absence (*A*, parthenogenetic index) or presence of ~1 × 10^6/ml pooled homologous epididymal spermatozoa (*B*). The evaluations were performed after 6 h or 22 h incubation in Tyrode's medium, covered or not covered (control) with 400 µl of equilibrated mineral oil. The figures in brackets are the number of oocytes. *Significantly different from the 6 h incubated group with the same treatment. a vs c or d, $p < 0.01$; b vs e, $p < 0.05$; c vs f, $p < 0.05$.

stage at 22 h were 62.8% ($n = 32$) and 38.1% ($n = 24$) ($p < 0.05$) in the absence or presence of mineral oil, respectively, while the percentage at the 2-cell stage (23.5% ($n = 12$) and 31.7% ($n = 20$) in the absence or presence of mineral oil, respectively) or with more than two blastomere (13.7% ($n = 7$) and 30.2% ($n = 19$) in the absence or presence of mineral oil, respectively) were similar in both conditions. The parthenogenetically activated oocytes achieved more than the 2-cell stage in all the experimental conditions, reaching 17.8% ($n = 8$) at 22 h in the presence of mineral oil.

Table 1 shows the results obtained when non-inseminated oocytes were incubated for 6 h or 22 h in the presence of PIR 10^{-8} M or PIR 10^{-8} M plus mineral oil. In this latter condition, the parthenogenetic index

Table 1 Percentage of parthenogenetically activated oocytes incubated for 6 h or 22 h in the presence of 10^{-8}M piroxicam (PIR) covered or not covered with equilibrated mineral oil

	% of parthenogenetic oocytes	
Treatment	6 h	22 h
PIR 10^{-8} M	8.8 [68]	49.2 [63]^{a*}
PIR + mineral oil	10.1 [79]	85.9 [77]^{b*}

The oocytes were obtained from superovulated mice and incubated in Tyrode's medium.
*Significantly different from the 6 h incubated group with the same treatment. a vs b, $p < 0.01$.

reached 85.9% at 22 h ($n = 77$), this value being significantly higher than that obtained with PIR 10^{-8} M without oil (49.2%, $n = 63$; $p < 0.05$) or in control conditions (39.7%, $n = 66$; $p < 0.01$) at the same time. The addition of only DMSO (PIR vehicle) did not significantly modify the parthenogenetic index at any time when compared with the PIR 10^{-8} M group or with the control group. Nevertheless, this value was lower than that obtained in presence of PIR 10^{-8} M plus mineral oil at 22 h ($p < 0.01$).

We also found that the parthenogenetic indexes in spontaneous cumulus-free oocytes were higher than those obtained from oocytes incubated in the presence of the cumulus oophorus at 6 h or at 22 h (Fig. 2).

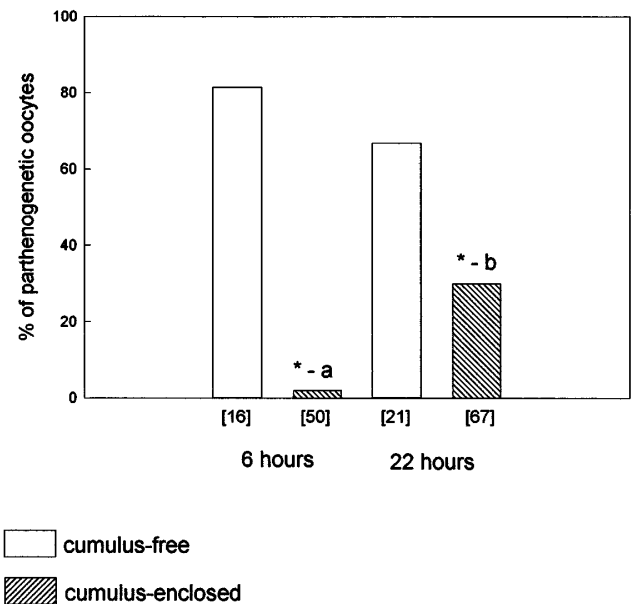


Figure 2 Parthenogenesis of superovulated mouse oocytes with (cumulus-enclosed) or without (cumulus-free, spontaneously lost) the cumulus oophorus coat. The evaluations were performed after 6 h or after 22 h incubation in Tyrode's medium. The figures in brackets are the number of oocytes. *Significantly different from the cumulus-free group after the same incubation time. a vs b, $p < 0.01$.

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Discussion

Since, in this study, the activation indexes of inseminated oocytes were higher than those of non-inseminated ones, it can be assumed that in the former, fertilisation did occur. In this respect, Behr *et al.* (1990) reported cleavage due to fertilisation *in vitro* as cleavage adjusted for parthenogenesis. However, in our experimental conditions, it is not possible to dismiss the possibility that the potentially parthenogenetically activated oocytes could not previously be fertilised. Thus, we claim that the report of results of *in vitro* fertilisation experiments requires further analysis.

The finding that in all experimental conditions the activation indexes increased during the incubation time seems to indicate that this phenomenon is a time-related event. Parthenogenesis may be in accordance with Presicce & Yang (1994), who demonstrated that ageing oocytes are parthenogenetically activated to a greater degree than younger ones, and that the nuclear events proceed faster in the former.

Mineral oil enhanced the parthenogenetic rate when evaluation was performed at 22 h incubation. However, it did not have any effect when the oocytes were inseminated. In this respect, Ozgur *et al.* (1995) found that mineral oil had some detrimental component which interferes with zona binding of human spermatozoa. Other authors, such as Strandell *et al.* (1998) and Miller *et al.* (1994), did not find any deleterious effects of the oil on the *in vitro* fertilisation assays, but they did not analyse the parthenogenetic rate. Since we can not discriminate how much of this activation is due to fertilisation and how much is due to parthenogenesis we must be very careful with this comparison. The potentially beneficial effects of mineral oil in this kind of technique must be revisited.

On the other hand, it must be considered that inseminated oocytes covered with mineral oil exhibited a proportion of pronuclear stage oocytes lower than the respective controls, which reached similar activation rates. This phenomenon would suggest some beneficial effect of oil in the embryo stage progression. It is noteworthy that we did not find reports from other workers regarding parthenogenetic embryos at more than the 2-cell stage.

Bouquet *et al.* (1995) found that the exposure of mouse oocytes to DMSO at room temperature induced an increase in the parthenogenetic activation rate, this difference not being significant with respect to results obtained in the absence of the cryoprotectant. Neither Shaw & Trounson (1989) nor Candy *et al.* (1994) found a deleterious effect when mouse oocytes were exposed to 1.5 M DMSO. Since, in our experimental conditions, DMSO was used as the PIR vehicle and the molarity employed was lower (2×10^{-8} M) than those added by other authors, the lack of any effect was predictable.

PG synthesis by the cumulus–oophorus complexes during oocyte maturation and *in vivo* or *in vitro* incubation is well known (Schuetz & Dubin, 1981). It has also been reported that non-steroidal anti-inflammatory drugs inhibit this synthesis (Schuetz & Dubin, 1981). However, the effects of these drugs on the parthenogenetic indexes of mouse oocytes have not been well studied. In our experimental conditions, PIR 10^{-8} M did not show any effect on the mouse oocyte parthenogenetic rate at either 6 h or 22 h incubation.

When the oocytes were covered with mineral oil during incubation with PIR 10^{-8} M, the parthenogenetic index increased at 22 h. From the comparison of this result with those obtained in presence of PIR 10^{-8} M or DMSO 2×10^{-8} M not covered with oil, or those only covered with oil, the parthenogenetic indexes found could be attributed to an effect of the mineral oil.

Our results suggest that oocyte susceptibility to spontaneous parthenogenetic activation may be modified by the presence of the cumulus and corona radiata, indicating that this structure may exert some 'protective' function, at least when spontaneous parthenogenesis is considered.

In this context, results from Marcus (1990) employing activator substances or Pinyopummin *et al.* (1994) incubating mouse oocytes with ethanol are not in agreement with ours. Differences in the experimental procedures, such as treatment with hyaluronidase to remove cumulus cells and/or chemical induction of parthenogenesis, could be a possible explanation for the above-mentioned divergence.

In conclusion, the experimental conditions employed here seem to be able to alter the results significantly. Other procedures, such as the time between hCG injection and ovum collection, have been reported as capable of modifying the parthenogenetic indexes (Behr *et al.*, 1990). In addition, the attempts to evaluate the results obtained in different experimental designs are often controversial. Hence, we consider that further rigorous studies on these influences are necessary in order to confer more reliability on the results.

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