Fertilization *in vitro* of cattle follicular oocytes with ejaculated spermatozoa capacitated in a chemically defined medium

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Summary. Cattle follicular oocytes were collected from the ovarian follicles and cultured for 28 h in m-KRB solution in a CO₂ incubator (5% CO₂ in air at 37°C, 95% humidity). After further culture for 15–18 h with spermatozoa, 78·6% of the oocytes had matured to the second metaphase. The highest fertilization rates (36–58%) were obtained when spermatozoa were collected 1 day before insemination, kept for 14–18 h at 20°C in a test tube (10×10^8 /ml), preincubated in m-KRB solution ($1\cdot1\text{--}2 \times 10^8$ /ml) in a CO₂ incubator for 0–12 h, and then inseminated at a concentration of $1\cdot5\text{--}2\cdot0 \times 10^6$ /ml. The optimum period of preincubation in the CO₂ incubator was 8 h: 6/15 denuded oocytes (40%) were fertilized with spermatozoa preincubated for 8 h. No polyspermic fertilization was observed in any of the 139 penetrated oocytes. When spermatozoa were preincubated in the uteri of oestrous cows and gilts for 4–4·5 h instead of the m-KRB solution, the fertilization rates were 80 and 86%, respectively. However, in this system, 13 and 24% of the penetrated oocytes were polyspermic. These results indicate that ejaculated bull spermatozoa can be capacitated in a chemically defined isotonic medium, and about half of the oocytes matured in culture are normally fertilized *in vitro*.

Introduction

We previously reported evidence of fertilization *in vitro* of cattle follicular oocytes that matured in culture with ejaculated spermatozoa capacitated in the isolated oviduct and uterus (Iritani & Niwa, 1977), but in those studies the fertilization rates, 18·5–20·7%, were comparatively low. The successful fertilization *in vitro* of 34·6% of preovulatory and 26·2% of ovulated cattle oocytes with spermatozoa treated with high ionic strength medium has been reported (Brackett, Oh, Evans & Donawick, 1980; Brackett *et al.*, 1982). In addition, it has been shown that 88·7% of the zona-free bovine oocytes after maturation in culture could be fertilized *in vitro* with epididymal spermatozoa (Fulka, Pavlok & Fulka, 1982). In the present experiment we attempted to find an effective procedure for capacitation of ejaculated spermatozoa in a chemically defined, isotonic medium followed by a repeatable and improved fertilization rate.

Materials and Methods

The medium used for manipulation of gametes was a modified Krebs–Ringer–bicarbonate solution (m-KRB containing 94·6 mM-NaCl, 4·78 mM-KCl, 1·71 mM-CaCl₂, 1·19 mM-KH₂PO₄, 1·19 mM-

MgSO₄, 25.07 mM-NaHCO₃, 21.58 mM-sodium lactate, 0.5 mM-sodium pyruvate, 5.56 mM-glucose, 4 mg crystalline bovine serum albumin (Sigma Chemical Co.)/ml, 50 µg streptomycin sulphate/ml and 75 µg potassium penicillin/ml) (Toyoda & Chang, 1974).

The ovaries were removed from cows at a local slaughterhouse and brought to the laboratory in saline (8.5 g NaCl/l) at 30°C within 1 h. Follicular oocytes were collected by puncturing follicles of diameter 2–5 mm with a needle. After washing twice with m-KRB solution, about 10 oocytes were introduced into 0.4 ml m-KRB solution covered with warm paraffin oil in a plastic culture dish (35 × 11 mm) and cultured for 28 h in a CO₂ incubator (5% CO₂ in air at 37°C, 95% humidity) until the second metaphase was achieved. In 2 trials the oocytes were freed from follicular cells by treatment with 370 NF units hyaluronidase (Sigma Chemical Co.)/ml for 5 min and by repeated passage through a fine pipette before insemination.

Semen was collected by artificial vagina from a bull of the Japanese Black breed. In the first series of experiments, the semen was collected on the day before insemination and half of it was washed once with m-KRB solution by centrifugation at 500 *g* for 10 min. Washed and unwashed semen samples (2–3 ml, 10 × 10⁸ spermatozoa/ml) were kept under a semi-aerobic condition in a stoppered 10 ml test tube at 20°C for 14–18 h until the next morning. Washed and unwashed spermatozoa were washed with m-KRB solution (first or second washing), and preincubated in 0.4 ml m-KRB solution (1–1.2 × 10⁸ spermatozoa/ml) in a CO₂ incubator for 0, 4, 8 or 12 h, i.e. kept at 20°C in the test tube for 12, 8, 4 or 0 h before insemination. Semen freshly collected on the day of insemination was washed once with m-KRB solution, resuspended to give a concentration of 10 × 10⁸/ml and also preincubated for 0, 4 or 8 h, i.e. kept at 20°C in the test tube for 8, 4 or 0 h. After preincubation, inseminations were made at a concentration of 1.5–2.0 × 10⁶ spermatozoa/ml in the fertilization medium by placing the culture dish on a warm plate at 37°C in air.

In the series of experiments in which isolated cow and gilt uteri were used as the environments for sperm capacitation, spermatozoa from each of the 3 pretreatment groups were preincubated in the isolated uteri from oestrous cows or gilts. The uteri were removed at a slaughterhouse after careful check of the integrity of the follicles and oestrous condition. Throughout the experiment the number of spermatozoa introduced into a cow or gilt uterine horn was 1.5–2.0 × 10⁸. The uterotubal junction and the cervical end of the uterus were ligated, and kept in saline for 4–4.5 h at 37°C. After incubation the spermatozoa were recovered by flushing the uteri with 5 ml m-KRB solution. Contaminating blood cells and cellular debris were removed by centrifugation at 150 *g* for 5 min. All sperm samples suspended in the supernatants were centrifuged at 500 *g* for 10 min, and resuspended spermatozoa were inseminated at a concentration of 1.5–2.0 × 10⁶/ml.

After culture of eggs for 28 h and additional culture with spermatozoa for 15–18 h, eggs were fixed for 2 days at room temperature in 25% acetic alcohol, stained with 1% aceto-orcein, and examined by phase-contrast microscopy. Oocytes that had matured and those undergoing fertilization according to the criteria shown in Plate 1 were identified.

Least-squares procedures as described by Harvey (1975) were used for statistical analysis of data.

Results

Throughout the present experiments 68–90% (78.6%, average) of the total number of oocytes examined had matured to the second metaphase (Tables 1 & 2). Table 1 shows the fertilization rates of oocytes with spermatozoa capacitated in a chemically defined medium under three different conditions. Since no sperm penetration was found in the oocytes that had not matured to second metaphase, the fertilization rates were expressed as the percentages of the number of oocytes maturing to second metaphase. Of the three different treatments for capacitation of spermatozoa significantly ($P < 0.05$) higher fertilization rates, 36–58%, were obtained when spermatozoa were collected 1 day before insemination, kept for 14–18 h at 20°C without washing and then

Table 1. Fertilization *in vitro* of cattle follicular oocytes with ejaculated spermatozoa capacitated in a chemically defined medium

Treatment of spermatozoa			No. of trials	No. of oocytes examined	No. of oocytes maturing to meta-phase II (%)†	No. of oocytes fertilized			
Day of semen collection	Sealed in 10 ml test tube	Preincubation at 37°C in CO ₂ incubator (h)				Total with evidence of fertilization (%)‡	Telo-phase II	Female pro-nucleus	Both pro-nuclei
One day before insemination	14–18 h at 20°C without washing	After 1st washing							
		0	4	38	30 (78)	12 (40)	4	2	6
		4	5	46	33 (72)	16 (48)	3	2	11
		8	8	81	62 (77)	36 (58)	5	6	25
		8*	2	19	15 (79)	6 (40)	1	1	4
		12	4	37	28 (76)	10 (36)	1	3	6
	14–18 h at 20°C after washing	After 2nd washing							
		0	4	35	28 (80)	9 (32)	2	0	7
		4	4	34	26 (76)	9 (35)	1	2	6
		8	4	36	26 (72)	11 (42)	1	3	7
		12	3	27	22 (82)	8 (36)	0	3	5
On the day of insemination	None	After 1st washing							
		0	3	29	26 (90)	6 (23)	2	0	4
		4	3	27	21 (78)	7 (33)	2	1	4
		8	3	25	17 (68)	7 (41)	0	2	5

* Denuded eggs were inseminated.

† Oocytes were fixed after culture for 28 h and additional culture with spermatozoa for 15–18 h.

‡ Percentages of the number of oocytes maturing to the second metaphase.

Table 2. Capacitation of bull ejaculated spermatozoa in the isolated uteri from oestrous cows and gilts, and fertilization *in vitro* of cattle follicular oocytes matured in culture

Treatment of spermatozoa			No. of trials	No. of oocytes examined	No. of oocytes maturing to meta-phase II (%)*	No. of oocytes fertilized			
Day of semen collection	Sealed in 10 ml test tube	Preincubation at 37°C for 4–4.5 h				Total with evidence of fertilization (%)†	Telo-phase II	Pro-nuclear (both)‡	Poly-spermic (%)§
One day before insemination	14–18 h at 20°C without washing	After 1st washing							
		Cow uteri	4	27	30 (81)	24 (80)	5	17 (15)	3 (13)
		Gilt uteri	6	55	44 (80)	38 (86)	6	29 (23)	9 (24)
	14–18 h at 20°C after washing	After 2nd washing							
		Cow uteri	6	56	45 (80)	29 (64)	8	21 (18)	2 (7)
		Gilt uteri	4	36	30 (83)	23 (77)	3	20 (14)	6 (26)
On the day of insemination	None	After 1st washing							
		Cow uteri	3	29	24 (83)	14 (58)	1	13 (9)	1 (7)
		Gilt uteri	3	27	23 (85)	16 (70)	2	14 (9)	5 (31)

* Oocytes were fixed after culture for 28 h and additional culture with spermatozoa for 15–18 h.

† Percentages of the number of oocytes maturing to the second metaphase.

‡ The number of oocytes with a female and at least one male pronucleus is shown in parentheses.

§ Percentages of the total no. of oocytes fertilized.

preincubated for 0–12 h in a CO₂ incubator after first washing; the optimum period (significant at $P < 0.05$) of preincubation was 8 h. In 2 trials with 19 denuded oocytes inseminated with spermatozoa preincubated for 8 h, a comparatively good fertilization rate, 40% (6/15), was obtained. When inseminations were made with spermatozoa preincubated in a CO₂ incubator after the second washing, the fertilization rates were 32–42%, and a significantly ($P < 0.05$) higher proportion, 42%, was again obtained with spermatozoa preincubated for 8 h. When spermatozoa were freshly collected, washed once and preincubated for 0–8 h in a CO₂ incubator, 23–41% of the oocytes were fertilized.

Of the total 137 oocytes fertilized, 90 (66%) were morphologically normal pronuclear eggs with both pronuclei, corresponding sperm midpiece and the second polar body, and 22 oocytes were at the second telophase. In 25 oocytes a delayed transformation of the male pronucleus was observed (Table 1; Plate 1).

As a control, cow and gilt uteri were used for capacitation of spermatozoa. The results are summarized in Table 2. Of the three different treatments, the significantly ($P < 0.05$) higher fertilization rates, 80 and 86%, were obtained when spermatozoa were collected on the day before insemination, kept for 14–18 h at 20°C without washing, washed once and preincubated in the uteri of cows or gilts. The fertilization rates were lower, 64 and 77%, when spermatozoa were washed before storage at 20°C in the test tube. In the experiments in which spermatozoa were freshly collected, washed once and preincubated in cow or gilt uteri, the fertilization rates were 58 and 70%, respectively.

The fertilization rates with insemination of spermatozoa preincubated in the isolated uteri were generally higher than those with spermatozoa pretreated in the m-KRB solution. When oocytes were inseminated with gilt uterine spermatozoa, the overall means of the fertilization rates, 79% (77/97) were significantly ($P < 0.05$) higher than those with cow uterine spermatozoa, 68% (67/99), suggesting that gilt uteri are more effective than cow uteri in capacitating spermatozoa. On the other hand, a high proportion of polyspermic fertilization, 26% (20/77) was seen after insemination of spermatozoa from gilt uteri, compared to 9% (6/67) with spermatozoa from cow uteri (Table 2; Pl. 1, Fig. 6).

PLATE 1

Fig. 1. An oocyte at the metaphase of the second meiotic division fixed 28 h after culture, showing first polar body (PB) and a metaphase plate of chromosomes (arrow). $\times 390$.

Fig. 2. The fertilizing spermatozoon in an oocyte fixed 15 h after additional culture with spermatozoa which were kept for 15 h at 20°C, washed and preincubated for 4 h in m-KRB solution, showing the beginning of nuclear swelling (ESH). The female condensed chromatin (FCC) and a second polar body (PB) are also present. $\times 390$.

Fig. 3. An egg at the pronuclear stage fixed 18 h after additional culture with spermatozoa which were kept for 18 h at 20°C, washed and preincubated for 8 h in m-KRB solution. The male pronucleus (M), the female pronucleus (F), the second polar body (PB) and the sperm midpiece (arrow) are present. $\times 390$.

Fig. 4. An egg at the pronuclear stage fixed 16 h after additional culture with spermatozoa which were freshly collected, washed and preincubated for 4 h in m-KRB solution. The same evidence of normal fertilization as in the egg shown in Fig. 3 can be seen. $\times 390$.

Fig. 5. An egg fixed 16 h after additional culture with spermatozoa which were kept for 14 h at 20°C, washed and preincubated in the isolated uterus from an oestrous cow for 4 h. The female pronucleus (F) and the second polar body (PB) are present, and an asynchronous transformation of the sperm head to the pronucleus was observed. $\times 390$.

Fig. 6. An egg showing polyspermic fertilization fixed 15 h after additional culture with spermatozoa which were freshly collected, washed and preincubated for 4 h in gilt uterus. The telophase of the second meiotic division (PB and FCC), enlarged sperm head (ESH) and male pronucleus (M) with corresponding sperm midpiece can be seen. $\times 390$.

PLATE I

(Facing p. 490)

Discussion

The overall mean maturation rate in culture of the oocytes to the second metaphase was 78.6% (530/674) (Tables 1 & 2). This value was much higher than the 60% obtained under almost identical conditions in our previous experiments in which the oocytes were cultured for 20–24 h before introduction of spermatozoa (Iritani & Niwa, 1977). One of the reasons for the higher maturation rate might be due to the increased cultivation period before insemination (28 h followed by 15–18 h with spermatozoa).

The highest fertilization rate, 58%, was obtained when spermatozoa were collected on the day before insemination, kept for 14–18 h at 20°C in a test tube, washed once and then preincubated for 8 h in a CO₂ incubator. This suggests that an early stage of capacitation, such as loosening of coating factors and the plasma membrane of the spermatozoa, might be initiated during the storage period at 20°C. As shown in Table 1, two washings of spermatozoa (the first at collection and the second just before incubation) were not advantageous for capacitation of spermatozoa compared with one washing of spermatozoa. Furthermore, it appears that preincubation of spermatozoa in a CO₂ incubator is not necessarily required for capacitation since 32% (27/84) of the oocytes were fertilized with spermatozoa without such preincubation (0 h). In our previous experiments (Iritani & Niwa, 1977), none of the oocytes were fertilized when inseminated with ejaculated spermatozoa that were washed once and preincubated at concentrations of $1-2 \times 10^7$ /ml in m-KRB solution in a CO₂ incubator. In the present experiment, however, concentrations of spermatozoa were 10×10^8 /ml at 20°C and $1-2 \times 10^8$ /ml at 37°C, suggesting that the sperm concentration during pre-treatment might be an important factor for capacitation. These results also indicate that ejaculated bull spermatozoa can be capacitated with an isotonic medium (290 mosmol/kg) instead of the high ionic strength medium (380 mosmol/kg) that was successfully used by Brackett *et al.* (1982).

As can be calculated from the data in Table 1, 66% of the fertilized oocytes had both male and female pronuclei and in 18% (25/137) of the penetrated oocytes male pronucleus formation had been arrested. This asynchronous pronuclear formation might be due to an incomplete cytoplasmic maturation in culture. Fulka *et al.* (1982) suggested that a cytoplasmic substance responsible for male pronucleus formation may be included in about 50% of randomly selected oocytes, and reported a higher incidence of the arrested male pronuclear formation. However, in their experiments zona-free oocytes were used after maturation in culture, whereas zona-intact oocytes were used in the present experiments.

As anticipated from the results of our previous paper (Iritani & Niwa, 1977), higher fertilization rates were obtained with insemination of spermatozoa preincubated in the isolated uteri of cows or gilts than those preincubated in the m-KRB solution. As shown in Table 2, gilt and cow uteri can be used, but the former offers a more favourable capacitation environment. However, considerably higher incidences of polyspermy (24–31%) were observed when spermatozoa were preincubated in gilt uteri. Although it might be advantageous to use uteri from oestrous cows to obtain good fertilization rates with a low proportion of polyspermy, it is very difficult to obtain appropriate uteri from cyclic cows which are not normally at the slaughterhouse when in oestrus, whereas gilt uteri are easily available.

The present results demonstrate that about half of the oocytes matured in culture can be normally fertilized and activated, at least to the pronuclear stage, with ejaculated spermatozoa pretreated in a chemically defined medium.

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