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PRELIMINARY STUDY OF BUFFALO SPERM PENETRATION INTO ZONA-FREE HAMSTER EGGS AFTER TREATMENT WITH CALCIUM IONOPHORE A23187

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In cattle, the study of in vitro fertilization (IVF) has greatly progressed, and it has become possible to obtain calves after the transfer of embryos which are matured, fertilized and cultured in vitro (Goto et al., 1988; Eyestone & First, 1989). However, the study of IVF in the buffalo is very limited, even concerning the induction of sperm fertilizing capacity (Singh et al., 1989).

Zona-free golden hamster eggs permit the entry of spermatozoa of many mammalian species provided the spermatozoa have completed capacitation and acrosome reactions, thus allowing them to be used in assessing the fertilizing capacity of spermatozoa of various species including the bull (Yanagimachi, 1984). However, there is no literature about the penetration of hamster eggs by buffalo spermatozoa. A previous study (Takahashi & Hanada, 1984) reported the penetration of zona-free hamster eggs by ejaculated bull spermatozoa after treatment with calcium ionophore A23187 (Ca-IA) in the presence of caffeine. Based on this study, successful IVF was accomplished not only in cattle (Hanada, 1986b) but also in goats and sheep (Hanada, 1986a) using spermatozoa treated with Ca-IA. In the present study, the effectiveness of Ca-IA treatment in inducing the fertilizing capacity of the river buffalo (*Bubalus Bubalis*) spermatozoa using zona-free hamster eggs, was examined.

Modified Krebs-Ringer bicarbonate solution (mKRB; Toyoda & Chang, 1974) was used for handling hamster oocytes. This medium was also used to make a solution of 0.1% hyaluronidase (lyophilised powder from bovine testes, Sigma Chem. Co., St.

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Louis, USA) and 0.05% trypsin (1 : 250, Difco, Detroit, USA). Ca-IA (Sigma Chem. Co., St. Louis, USA) was dissolved in a dimethyl sulfoxide-ethylalcohol mixture (3 : 1) at a concentration of 10 mM and stored in a freezer (−20°C). A chemically defined medium (DM; Brackett & Oliphant, 1975) supplemented with 15 mM theophylline without bovine serum albumin (DM+Theo) was used for washing sperm, and DM containing 20 mg/ml of crystalline bovine serum albumin (BSA; Fraction V, Sigma Chem. Co., St. Louis, USA) was used for the insemination droplet.

Fresh semen was collected from a fifteen-year-old river buffalo bull using an artificial vagina. One ml of the fresh ejaculated semen was diluted with 4 ml of DM+Theo and washed 3 times by centrifugation at 500 g for 10 min. After the third centrifugation and aspiration of the supernatant, the sperm pellet was resuspended in DM+Theo and the sperm concentration was adjusted to 1×10^7 cells/ml. The adjusted sperm suspension was exposed to 0.1−1.0 μM Ca-IA. After the exposure to Ca-IA for 0.5−2.0 min, 50 μl of sperm suspension was introduced into a droplet of 50 μl of DM supplemented with BSA under paraffin oil. The final concentration of spermatozoa in the insemination droplet was 5×10^6 cells/ml.

Mature female golden hamsters were induced to superovulate by intraperitoneal injection of 30 IU of pregnant mare's serum gonadotrophin followed 54 hr later by 30 IU of human chorionic gonadotrophin (hCG). Oocytes were recovered from the ampullar region of the oviducts 15−16 hr after the hCG injection, and were freed from cumulus cells by hyaluronidase. Cumulus-free oocytes were washed 3 times in fresh mKRB and treated with trypsin to remove the zonae pellucidae. Zona-free oocytes were washed 3 times in fresh mKRB and immediately put into a droplet containing Ca-IA-treated spermatozoa and incubated at 37°C in humidified air with 5% CO_2.

In experiment 1, sperm motility was examined after the introduction of sperm into a insemination droplet under an inverted-microscope at 1 hr intervals. Results are shown in Table 1. The motility of spermatozoa was impaired with the increase in the concentration and the length of the treatment period with Ca-IA. It was observed that sperm motility was lost within 1 hr when they were treated with 1.0 μM Ca-IA.

In experiment 2, the eggs were inseminated with spermatozoa that were treated with 0.1−1.0 μM Ca-IA for 1 min. The eggs were fixed with neutral formalin 3.5−4.0 hr after the insemination, and then stained with 0.025% aceto-lacmoide solution. Penetration was examined using a phase-contrast microscope, which detected the presence of a swollen sperm head or a male pronucleus with a tail within the egg cytoplasm as described previously (Takahashi & Hanada, 1984). Penetration results are shown in Table 2. The spermatozoa which were not treated with Ca-IA (control) attached heavily to the surfaces of zona-free hamster eggs as shown in Fig. 1a. However, they did not penetrate into the eggs. After the treatment with 0.1−0.25 μM of Ca-IA, spermatozoa also attached heavily to the eggs as in the

Table 1. Motility of buffalo spermatozoa after treatment with calcium ionophore A23187

Ionophore A23187		Motility score ; time after insemination		
Conc. (μM)	Time(min)	1	2	3(hr)
0	0	+++	+++	++
0.10	0.5	+++	++	+
	1.0	+++	++	+
	2.0	+++	+	±
0.25	0.5	++	+	±
	1.0	++	+	±
	2.0	++	±	±
0.50	0.5	+	±	−
	1.0	+	±	−
	2.0	−	−	−
1.00	0.5	−	−	−
	1.0	−	−	−
	2.0	−	−	−

Motility score : −, ±, +, ++ and +++ ; none, less than 25, 25 - 50, 50 - 75 and more than 75%, respectively.
All the ionophore-treated spermatozoa were introduced into insemination medium with 10 mg BSA/ml at the final concentration.

control. The removal of these attached spermatozoa from the eggs was quite difficult, and some of the inseminated eggs were broken during the removal of attached spermatozoa by pipetting. The examination of eggs was not possible due to the presence of attached spermatozoa after the treatment with 0.1 μM Ca-IA, and thus the penetration data could not be determined. Seventeen of 31 eggs (54.8%) had 1 to 6 enlarged sperm heads and/or male pronuclei (mean$\pm$SD$=1.7\pm1.4$) after insemination with spermatozoa treated with 0.25 and 0.5 μM Ca-IA (Fig. 1b). Two of 13 eggs were penetrated after insemination of sperm treated with 1.0 μM Ca-IA, probably due to the loss of sperm motility in a short period as shown in Experiment 1. To induce the fertilizing capacity of cattle, sheep and goat spermatozoa with Ca-IA, it had been recommended that spermatozoa should be exposed to 0.1–0.5 μM Ca-IA for 1.0 to 2.5 min in the presence of 2–10 mM caffeine (Hanada, 1986a, b). The present results are in close agreement with the above reports.

In the human, phosphodiesterase inhibitors such as caffeine and theophylline

Table 2. Effect of calcium ionophore A23187 concentration on the penetration of buffalo sperm into zona-free hamster eggs

Concentration of calcium ionophore (μM)	No. of eggs examined	No. of eggs penetrated
0 (control)	8	0
0.25	11	6
0.50	20	11
1.00	13	2

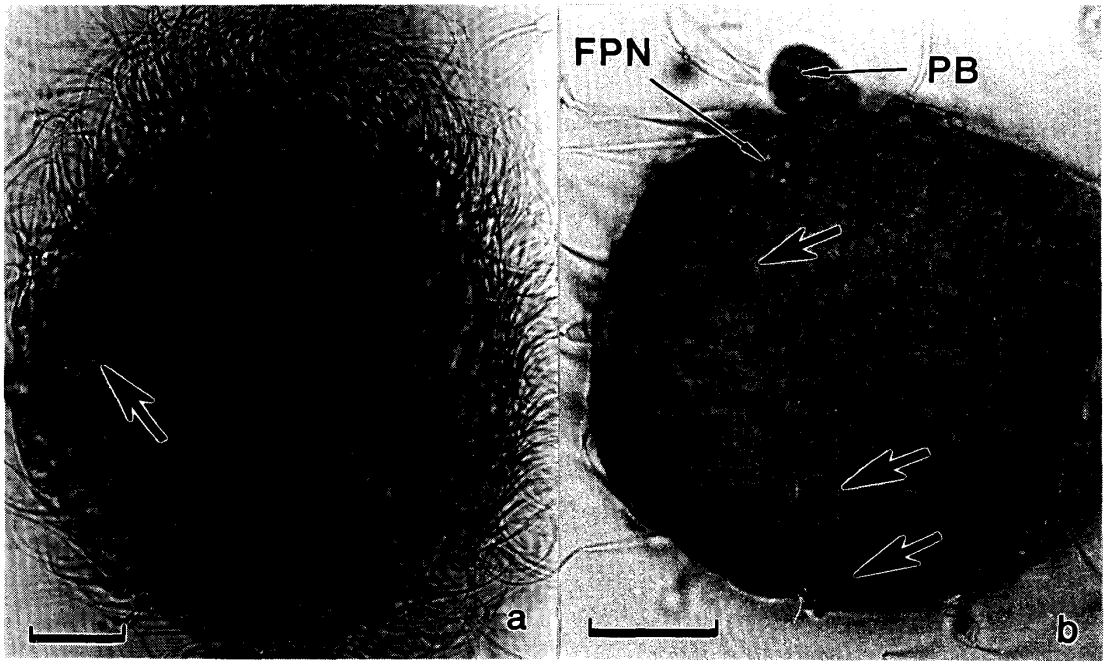

Fig. 1. Whole-mount preparations of zona-free hamster eggs after insemination with river buffalo sperm. (a) An egg showing a heavy attachment of sperm. The egg chromosomes (arrow) remained at metaphase II. (b) An egg showing the polar body (PB), a female pronucleus (FPN) and three enlarged sperm heads (arrows). Scale line=20 μm.

stimulate spermatozoa fertilizing capacity, and increase the penetration rates of zona-free hamster eggs by human spermatozoa (Perreault & Rogers, 1982; Chan et al., 1983). In a previous study (Takahashi & Hanada, 1984), bull spermatozoa were treated with Ca-IA in the presence of caffeine. However, theophylline was used in this study instead of caffeine, because the former showed a better penetration rate of zona-free hamster eggs after the insemination of bull spermatozoa than the latter (unpublished data).

In the present preliminary study, a limited number of eggs were examined and it was not possible to determine the actual penetration rate. Further experiments are needed to clarify the optimal Ca-IA treatment for buffalo spermatozoa. The present study, however, demonstrates that the fertilizing capacity of river buffalo spermatozoa can be induced using the Ca-IA treatment. It also suggests the possibility of direct chromosomal analysis using an interspecies IVF system between zona-free hamster ova and buffalo spermatozoa after treatment with Ca-IA as reported previously using bull spermatozoa (Tateno & Mikamo, 1987).

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