Zinc effects on mouse spermatozoa and in-vitro fertilization

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Summary. The fertilizing ability of mouse epididymal and capacitated spermatozoa was tested by mixing with normal or zona-free ova. In the presence of Zn^{2+} , the epididymal spermatozoa failed not only to penetrate the zona pellucida but also to fuse with zone-free ova, while no effect was observed on capacitated spermatozoa.

Zn^{2+} also inhibited fertilization with spermatozoa preincubated in Ca^{2+} -free medium. However, shortly after the addition of Ca^{2+} to the preincubated spermatozoa, Zn^{2+} lost its fertilization inhibitory activity.

Introduction

Prior to fertilization, spermatozoa undergo a series of physiological and morphological changes in the female reproductive tract (Austin, 1951; Chang, 1951). The zona pellucida is believed to help block polyspermy and protect the vitellus (Piko, 1969; Aonuma, Okabe, Kawai & Kawaguchi, 1978a). Studies to examine the interaction between spermatozoa and the zona pellucida (Hartmann & Gwatkin, 1971; Hartmann, Gwatkin & Hutchinson, 1972; Hartmann & Hutchinson, 1974a, b) have suggested that sperm capacitation may be a process of acquiring the capacity to penetrate the zona pellucida (Austin, 1965; Pavlok & McLaren, 1972).

We have shown that Zn^{2+} , which exists in high concentrations in the prostate, semen and spermatozoa, suppressed mouse sperm capacitation *in vitro* (Aonuma, Okabe & Kawaguchi, 1978b). The present study involved the suppression of capacitation by Zn^{2+} and investigation of the fertilizing ability of the spermatozoa when incubated with zona-free ova.

Calcium is known to be essential for the fusion of spermatozoa and ova (Iwamatsu & Chang, 1971; Johnson, 1975; Miyamoto & Ishibashi, 1975). Yanagimachi & Usui (1974) claimed that spermatozoa failed to undergo an acrosome reaction and the activation process in a calcium-free medium. Therefore, the fertilizing ability of spermatozoa preincubated in a calcium-free medium was also examined in the presence of Zn^{2+} .

Materials and General Methods

The spermatozoa were obtained from the cauda epididymidis of mature DDY mice (weighing 35–40 g) as previously reported (Aonuma *et al.*, 1978b). A modified Krebs–Ringer–bicarbonate (m-KRB) solution (Toyoda, Yokoyama & Hoshi, 1971) was changed by altering the pH from 7.4 to 8.0 (Aonuma, Okabe & Kawai, 1976) and the Ca^{2+} -free medium was prepared by deletion of CaCl_2 . The Zn^{2+} -containing medium was prepared by adding ZnCl_2 . For the 24-h incubation of the ova, the pH 7.4 medium was used. The ova were obtained from 4–5-week-old DDY mice injected intraperitoneally with 5 i.u. PMSG (Teikoku Zoki) 48 h before

administration of 5 i.u. hCG (Teikoku Zoki). The mice were killed at 14–16 h after hCG injection and the ova in cumulus cell clots were recovered by puncturing the ampullar portion of the oviduct with a needle. The collected ova were washed twice with 10 ml medium and used for the experiments. The zona-free ova were obtained by treating the ova with 0.1% bovine testicular hyaluronidase (Sigma) followed by 0.1% α -chymotrypsin (Sigma), each at 37°C, for 10 min in m-KRB. After two washes with fresh medium, the zona-free ova were collected and used for the experiments as indicated.

Throughout the experiments, 50 μ l aliquots of the sperm suspension ($0.8\text{--}1.2 \times 10^6$ spermatozoa/ml) were added to ova in 0.4 ml m-KRB. After incubation of normal ova, hyaluronidase (0.1% final concentration) was added to ova in m-KRB. The cumulus cells were removed rapidly by agitating the ova with a pipette. All ova were immediately examined for sperm penetration, formation of pronuclei and later stages of cleavage, as appropriate for the incubation time, with phase-contrast microscopy without staining.

Detailed Methods and Results

Effect of Zn^{2+} on the fertilization of mouse ova in vitro

In Exp. I, the fertilizing abilities of the epididymal spermatozoa and the capacitated spermatozoa were compared in the presence of 250 μM - Zn^{2+} . As shown in Table 1, few epididymal spermatozoa succeeded in penetrating the zona pellucida and/or forming pronuclei, but capacitated spermatozoa were not affected by the presence of Zn^{2+} . In Exp. II, epididymal or capacitated spermatozoa were introduced to zona-free ova and incubated for 5 h in the presence of 250 μM - Zn^{2+} . The capacitated spermatozoa penetrated the zona-free ova and formed pronuclei, but values for the epididymal spermatozoa were very low. In Exp. III, 1.5 or 5 h after sperm-egg mixing, ova were washed with m-KRB (pH 7.4) and transferred to 0.4 ml fresh m-KRB (pH 7.4). Zn^{2+} was then added and the incubation was continued until 24 h after insemination: there was no development to the 2-cell stage.

Table 1. Effects of Zn^{2+} on fertilization of mouse eggs *in vitro*

Exp.	Spermatozoa	Ova	Zn^{2+} addition	Fertilization (%)	
				Time after insemination	
				1.5 h ^d	5 h ^e
I	Capacitated	Normal	—	62 $\pm$ 8	55 $\pm$ 10
	Epididymal	Normal	+ ^a	0	7 $\pm$ 1*
	Capacitated	Normal	+ ^a	55 $\pm$ 9	55 $\pm$ 4
II	Capacitated	Zona-free	—	—	66 $\pm$ 7
	Epididymal	Zona-free	+ ^a	—	5 $\pm$ 3*
	Capacitated	Zona-free	+ ^a	—	50 $\pm$ 12
24 h ^f					
III	Epididymal	Normal	—	54 $\pm$ 2	
	Epididymal	Normal	+ ^b	2 $\pm$ 1*	
	Epididymal	Normal	+ ^c	0	

Zn^{2+} was added to the medium (a) at the time of insemination, (b) at 1.5 h after insemination and (c) at 5 h after insemination. Fertilized ova were examined for (d) zona penetration, (e) zona penetration and pronuclei formation, and (f) 2-cell ova. Each value shows mean $\pm$ s.e.m. of 3–5 independent tests; altogether 46–104 ova were examined in each group. *Significantly different from other values in that experiment ($P < 0.01$).

Effect of Zn²⁺ on the fertilizing ability of spermatozoa preincubated in Ca²⁺-free medium

In Exp. IV, spermatozoa were preincubated in Ca²⁺-free medium for 40 min. Ca²⁺ was then added and the spermatozoa were incubated at 37°C. Aliquots (50 µl) were taken from the sperm suspension at 10, 20, 30 and 40 min after the addition of Ca²⁺ and introduced to ova in Zn²⁺-containing medium. The ova (46–68/group) were examined for fertilization 90 min after the insemination. The preincubated spermatozoa were still Zn²⁺-sensitive at 10 min after the Ca²⁺ addition (no eggs fertilized). At 20 min, 26 ± 10% of eggs were fertilized, and at 30 and 40 min the values were 42 ± 12 and 46 ± 5% respectively. In Exp. V, spermatozoa were preincubated in Ca²⁺-free medium for 40 min and introduced to the ova in 4 test-tubes containing m-KRB (approximately 50 ova in each group). These 4 tubes were incubated for 10, 20, 30 and 40 min, respectively. Hyaluronidase (0.1% final concentration) was added to each tube at the end of the incubation. The fertilization percentages were 29 ± 9, 42 ± 7, 52 ± 13 and 52 ± 10 at 10, 20, 30 and 40 min respectively.

Discussion

Aonuma *et al.* (1978b) reported that Zn²⁺ inhibited penetration of the fresh epididymal spermatozoa into the ova, but did not inhibit penetration of the capacitated spermatozoa. Thus, the inhibitory activity of Zn²⁺ on the fertilization of mouse ova *in vitro* is not due to inhibition of sperm penetration, but to inhibition of sperm capacitation. However, since the observation was carried out 90 min after the insemination, the possibility that Zn²⁺ simply delayed capacitation could not be excluded. In the current studies, ova were inseminated by epididymal spermatozoa and incubated in the presence of Zn²⁺. After incubation for 5 h few ova were penetrated, suggesting that the effect of Zn²⁺ on sperm capacitation is based on the blocking rather than the delaying of the capacitation process. In spite of the presence of Zn²⁺, ova penetrated by capacitated spermatozoa developed to the pronuclear stage, indicating that Zn²⁺ has no effect on the fusion of capacitated spermatozoa with ova (Table 1, Exp. I). However, the zona pellucida might decrease the Zn²⁺ concentration in the perivitelline space. The experiment using zona-free ova inseminated by capacitated spermatozoa in the presence of Zn²⁺ (Table 1, Exp. II) confirmed that Zn²⁺ had no influence on the sperm–ova fusion. When zona-free ova were inseminated by epididymal spermatozoa under the same conditions, few pronuclei were observed during a 5-h incubation. This strongly suggests that the spermatozoa must be capacitated before they can fuse with the ova.

The fusion of spermatozoa to zona-free ova has been studied in mice (Pavlok & McLaren, 1972; Wolf, Inoue & Stark, 1976) and many other mammals (Yanagimachi, 1978), and the results indicate that plasma membrane in the post-equatorial segment is important for fusion. A change of membrane fluidity in the postnuclear cap region during capacitation has also been reported (Barros & Franklin, 1968; Barros, 1974; Barros & Berrios, 1977; Barros & Herrera, 1977; Yanagimachi, 1978). It is therefore likely that Zn²⁺ inhibits the membrane change in this region. However, it has also been suggested that the sperm trypsin-like enzyme participates in an acrosome reaction (Meizel & Lui, 1976; Lui & Meizel, 1979) and that the acrosomal enzymes may play a role in the modification of the postnuclear cap region (Barros, 1974). It remains to be determined whether or not the effect of Zn²⁺ on sperm–ova fusion is independent of an acrosome reaction. Since the zona pellucida was removed by enzymic treatment, an artificial change in the membrane might be inevitable. However, according to Hirao & Yanagimachi (1978), zona-free ova prepared in various ways (including enzymic treatment) showed no difference in fertilizing ability.

As shown in Exp. IV, spermatozoa preincubated in Ca²⁺-free medium remained sensitive to Zn²⁺, but lost this sensitivity after 20 min when incubated in normal medium. This suggests that the spermatozoa had not accomplished capacitation during the preincubation in Ca²⁺-free

medium and needed to spend about 30 min in the normal medium to become capacitated. However, when spermatozoa preincubated in the Ca^{2+} -free medium were introduced to the ova in the normal medium, sperm penetration occurred within 10 min after the insemination, indicating that the spermatozoa preincubated in Ca^{2+} -free medium achieved capacitation before the spermatozoa had lost their sensitivity to Zn^{2+} . It is not clear how many steps are involved in the capacitation process, or in how many steps Zn^{2+} has a regulatory activity. However, based on the results showing that the Zn^{2+} has no effect on capacitated spermatozoa, an interaction between the decapacitation factor(s) which is (are) released from the spermatozoa during capacitation (Aonuma *et al.*, 1973) and the Zn^{2+} is highly probable.

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