The effect of zinc ions on fertilization of mouse ova *in vitro*

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Summary. The fertilization rate of mouse gametes in a Krebs–Ringer bicarbonate medium was 60%. The rate fell to 0% when 250 μM - ZnCl_2 was added, but MgCl_2 , CaCl_2 , MnCl_2 , FeCl_2 and CuCl_2 (each at 250 μM) had no effect. The inhibitory effect of Zn^{2+} was not evident if the spermatozoa or eggs were preincubated with Zn^{2+} or if previously capacitated spermatozoa were used. The Zn^{2+} is thought to act by interference with capacitation.

Introduction

Bertrand & Vladesco (1921), aware of the relatively large amounts of zinc in the prostate gland, testes and semen of mammals, postulated that the element might play an important role in reproduction. Others have also noted that semen and spermatozoa are rich sources of zinc (Mann, 1945; Mawson & Fischer, 1953; Fujii, Utida & Mizuno, 1955). Zinc is known to exist in many metallo-enzymes (Li, 1966), some of which occur in the prostate gland or spermatozoa, e.g. carbonic anhydrase, non-specific acid and alkaline phosphatase and 5'-nucleotidase (Mann, 1964). However, all the zinc in semen cannot be explained from the amounts of these enzymes. The succinate-induced enhancement of the respiratory activity of human spermatozoa is known to be counteracted by Zn^{2+} (Eliasson, Johnsen & Lindholmer, 1971), and EDTA, histidine or cysteine treatment of human spermatozoa causes release of Zn^{2+} and an increase of oxygen uptake by and motility of the spermatozoa (Huacuja, Sosa, Delgado & Rosado, 1973). The spermatozoa of several species can be successfully capacitated *in vitro* (Toyoda, Yokoyama & Hoshi, 1971; Bavister, 1973; Toyoda & Chang, 1974; Brackett & Oliphant, 1975) and seminal plasma is known to contain decapacitating activity (Chang, 1957; Bedford & Chang, 1962; Weinmann & Williams, 1964; Dukelow, Chernoff & Williams, 1966; Pinsker & Williams, 1967), but the involvement of Zn^{2+} in capacitation and decapacitation phenomena has not been examined. The purpose of the present study was to investigate the effect of adding Zn^{2+} to the medium on the fertilization of mouse ova *in vitro*.

Materials and Methods

Preparation of sperm suspension and ova

The standard medium used throughout the experiments was a modified Krebs–Ringer bicarbonate (m-KRB) solution (Toyoda, Yokoyama & Hoshi, 1971). The amount of NaHCO_3 was increased from 2.01 to 5.00 g/l to bring the pH from 7.4 to 8.0, a value shown to be more suitable for in-vitro fertilization of mouse ova in our laboratory (Aonuma, Okabe & Kawai, 1976).

The ova were obtained from immature ddY female mice, 4–5 weeks old, which were injected intraperitoneally with 5 i.u. PMSG (Teikoku Zoki) 48 h before an i.p. injection of 5 i.u. hCG (Teikoku Zoki). The mice were killed 14–16 h after the hCG injection and the oviducts were removed and placed in 5 ml m-KRB in a watch-glass. The ova were released from the oviduct ampullae, washed twice with medium, and were introduced to 0.4 ml m-KRB (or m-KRB containing additional divalent cations) under paraffin oil in glass test-tubes (12 $\times$ 100 mm) and maintained at 37°C.

Epididymal spermatozoa were obtained from mice weighing 30–40 g. The connective tissues, blood vessels and fat pad over the cauda epididymidis were cut away and the spermatozoa were extruded from slits in the tubules into 2 ml m-KRB which had been previously equilibrated with 5% CO₂ in air. After 3 min, 1 ml sperm suspension was removed from the surface of the mixture: sperm concentration was $1.0\text{--}1.5 \times 10^6/\text{ml}$. For the experiments, 50 μl aliquots of the uncapacitated or capacitated sperm suspension (capacitated spermatozoa were obtained by incubating epididymal spermatozoa in m-KRB for 40 min at 37°C (Aonuma *et al.*, 1976)) were added to 0.4 ml m-KRB containing 15–20 ova.

Effect of divalent cations

Solutions of ZnCl₂, MgCl₂, CaCl₂, MnCl₂, FeCl₂, CuCl₂ and HgCl₂ were prepared in distilled water to a concentration of 5 mM. Calculated amounts of these stock solutions were added to the m-KRB to give the required concentrations of divalent cations for examination of their effects on fertilization of mouse ova *in vitro*.

In Exp. I, the ova were placed in 0.4 ml m-KRB containing 5–250 μM -ZnCl₂ or 250 μM -MgCl₂, CaCl₂, MnCl₂, FeCl₂, CuCl₂ or HgCl₂. In Exp. II the ova were placed in 0.4 ml m-KRB containing 250 μM -ZnCl₂ (Zn-KRB) and incubated with capacitated spermatozoa. In Exp. III, the ova were preincubated in 0.4 ml Zn-KRB for 30–60 min, washed twice in m-KRB and incubated in 0.4 ml m-KRB with epididymal spermatozoa. Because the epididymal spermatozoa needed to become capacitated before they could penetrate the eggs, the duration of their exposure to Zn²⁺ in Exps I and II was different. The effect of prolonged exposure of capacitated spermatozoa to Zn²⁺ was therefore examined. In Exp. IV, spermatozoa were incubated in Zn-KRB for 40 min before incubation with ova in m-KRB. In Exp. V, ZnCl₂ was added to the suspension of capacitated spermatozoa to give a concentration of 250 μM -ZnCl₂. The spermatozoa were incubated for a further 40 min before addition to ova in Zn-KRB.

In all the experiments the ova were incubated with spermatozoa for 90 min at 37°C under 5% CO₂ in air. They were treated with 0.01% hyaluronidase (Sigma) for 5 min and examined for the penetration of spermatozoa.

Results

The addition of Zn²⁺ to the medium inhibited sperm penetration through the zona pellucida slightly at 30 μM and completely at 250 μM (Table 1).

Table 1. The effect (mean $\pm$ s.e.m. of 3–6 independent tests) of various concentrations of Zn²⁺ on the fertilization of mouse ova

	Zn ²⁺ conc. (μM)						
	0	7.8	15.6	31.3	62.5	125	250 500
No. of replicates	6	3	3	3	6	6	6 3
Total no. of ova examined	104	59	66	66	108	113	95 53
Ova penetrated (%)	54 $\pm$ 6	56 $\pm$ 5	56 $\pm$ 2	35 $\pm$ 8	14 $\pm$ 3	7 $\pm$ 3	0 0

As shown in Table 2, Hg²⁺ also caused complete inhibition of fertilization *in vitro*. Moreover, after treatment with Hg²⁺, but not with Zn²⁺, the spermatozoa became immotile and the vitellus became darkened and opaque. The inhibitory effect by Cu²⁺ could not be regarded as significant.

Table 2. Effect (mean $\pm$ s.e.m. of values in 3 independent tests) of various divalent cations on the fertilization of mouse ova *in vitro*

Cation*	Total no. of ova examined	Ova penetrated (%)
—	52	60 $\pm$ 9
Zn ²⁺	45	0
Mg ²⁺	53	67 $\pm$ 4
Ca ²⁺	48	59 $\pm$ 9
Mn ²⁺	56	52 $\pm$ 10
Fe ²⁺	57	67 $\pm$ 8
Cu ²⁺	46	32 $\pm$ 13
Hg ²⁺	20	0

* Each at 250 μ M in m-KRB containing 1.19 mM-Mg²⁺ and 1.71 mM-Ca²⁺, except when these ions were increased as shown.

Table 3. Effect (mean $\pm$ s.e.m. of values in 3–5 independent tests) of Zn²⁺ on the fertilization of mouse ova *in vitro* under various conditions

Treatment*	Spermatozoa	Ova	Zn ²⁺ in final culture medium	No. of replicates	Total no. of ova examined	Ova penetrated (%)
None Zn ²⁺	Epididymal	Normal	—	4	88	68 $\pm$ 7
Exp. I	Epididymal	Normal	+	4	81	0
Exp. II	Capacitated	Normal	+	5	109	59 $\pm$ 8
Exp. III	Epididymal	Treated	—	4	77	54 $\pm$ 9
Exp. IV	Treated	Normal	—	3	52	52 $\pm$ 10
Exp. V	Treated epididymal capacitated	Normal	+	3	51	38 $\pm$ 11

* See 'Materials and Methods': 'treated' represents preincubation in 250 μ M-Zn²⁺.

Table 4. Relationship between mouse sperm capacitation and Zn²⁺

	Incubation time after insemination (min)	
	30	90
Epididymal spermatozoa	0	64 $\pm$ 13
Capacitated spermatozoa	58 $\pm$ 10	59 $\pm$ 8
Treated spermatozoa*	2 $\pm$ 2	52 $\pm$ 10

Values represent the % of ova penetrated (mean $\pm$ s.e.m. of values in 3 independent replicates).

* Preincubated with 250 μ M-Zn²⁺ for 40 min.

As shown in Table 3, the numbers of eggs fertilized were not influenced by any of the treatments of Exps II–V. Zn²⁺ no longer inhibited fertilization when capacitated spermatozoa were added to ova (Exp. II).

The time of zona penetration by the spermatozoa preincubated with Zn²⁺ for 40 min was compared with that of epididymal or capacitated spermatozoa (Table 4).

Discussion

Eliasson *et al.* (1971) reported that the increased respiratory activity of human spermatozoa which occurs in some conditions can be prevented by treatment with seminal plasma or by the addition of Zn^{2+} to the medium. The present study showed that the fertilizing ability of mouse capacitated spermatozoa cannot be depressed to the level similar to that of epididymal spermatozoa even after incubation for 40 min (Table 3). Therefore the inhibitory activity of Zn^{2+} on fertilization is not due to inhibition of sperm penetration, but to inhibition of sperm capacitation. Spermatozoa preincubated in the presence of Zn^{2+} were capacitated during subsequent incubation by decreasing the Zn^{2+} concentration (Exp. IV). The zona penetration by such spermatozoa was similar to that of epididymal spermatozoa in time (Table 4) and in the penetration ratio (Table 3). These results suggest that the fertilizing ability of epididymal spermatozoa was maintained but latent during incubation in the presence of $250 \mu\text{M-Zn}^{2+}$.

Zinc concentrations in seminal plasma have been reported as 2.06 mM in man (Mawson & Fischer, 1953), 349 μM in boar (Boursnell, Baronos, Briggs & Butler, 1972), 1.04 mM in dog (Saito, Zeitz, Bush, Lee & Whitmore, 1969) and 600 μM in rabbit (S. Aonuma, unpublished). Although the Zn^{2+} concentration in mouse seminal plasma is not known, the concentration of $250 \mu\text{M-Zn}^{2+}$ used in the present experiments may not differ greatly from that physiologically available in the seminal plasma of the mouse.

Although the decapacitating activity of Zn^{2+} was not observed at $250 \mu\text{M}$, the nature of the capacitation inhibitory activity of Zn^{2+} (which was not observed for Mg^{2+} , Ca^{2+} , Fe^{2+} and Cu^{2+}) described in this paper suggests that Zn^{2+} may play an important role, at least *in vitro*, in the regulation of fertilizing ability of spermatozoa, other than that of the decapacitation factor(s) found in seminal plasma (Chang, 1957), epididymal fluid (Weinman & Williams, 1964) or spermatozoa (Aonuma *et al.*, 1973).

It is widely accepted that capacitation may represent a change in the sperm membrane (Austin & Bavister, 1975). However, the functional groups of macromolecular components of biological membranes might react differently with metals when there is a change in membrane stability (Wills & Wilkinson, 1967; Chvapil, Ryan & Zukoski, 1972; Chvapil, 1973). Whether or not the capacitation inhibitory effect by Zn^{2+} described in the present report is related to the stability of the sperm membrane, it seems important that the participation of zinc is taken into account in studies of the functional properties of spermatozoa.

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