Importance of Ca^{2+} , K^{+} and Glucose in the Medium for Sperm Penetration through the Human Zona Pellucida

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HOSHI, K., TSUKIKAWA, S. and SATO, A. *Importance of Ca^{2+} , K^{+} and Glucose in the Medium for Sperm Penetration through the Human Zona Pellucida.* Tohoku J. Exp. Med., 1991, 165 (2), 99-104 — When salt-stored human oocytes were inseminated in the regular mBWW medium and examined 6 hr later, the zonae of almost all of the oocytes were penetrated by spermatozoa. In contrast, none of zonae were penetrated when the oocytes were inseminated in either Ca^{2+} -free, K^{+} -free or glucose-free medium. The failure of zona penetration by spermatozoa in Ca^{2+} -free and K^{+} -free media is likely to be due to the inhibition of the acrosome reaction. Spermatozoa were unable to exhibit hyperactivated motility in glucose-free medium, suggesting that the failure of zona penetration in this medium is due, at least in part, to the inhibition of hyperactivated motility of spermatozoa. ——— calcium ; potassium ; glucose ; sperm ; zona pellucida

Under normal conditions mammalian spermatozoa must pass through the zona pellucida before effecting fertilization. Proper interaction of sperm molecules with the complementary zona molecules, strong flagellar motion of the spermatozoa and ionic balance of the medium are all essential for successful penetration of spermatozoa through the zona pellucida (O'Rand et al. 1986; Yanagimachi 1988; Saling 1989; Wassarman 1990). We report here that human spermatozoa are unable to attach to and penetrate through the zona unless the medium contains millimolar concentrations of Ca^{2+} , K^{+} and glucose.

MATERIALS AND METHODS

Media

The standard medium used in this study was a modified Krebs-Ringer's solution, mBWW (Overstreet et al. 1980) containing 35 mg/ml human serum albumin (HSA; Sigma Chemical Co., St. Louis, MO, USA). This medium had an osmolality of 308 mOsmol and pH value of 7.5 ± 0.1 when equilibrated with 5% CO_2 in air. Ca^{2+} -free, Mg^{2+} -free, glucose-free, lactate-free and pyruvate-free media were prepared by replacing CaCl_2 , MgSO_4 , glucose, Na-lactate and Na-pyruvate, respectively, with appropriate amounts of NaCl to keep the osmolality of the media constant. K^{+} -free medium was prepared by replacing KCl and KH_2PO_4 with NaCl and NaH_2PO_4 , respectively. The pH of these media was adjusted, if necessary, to 7.5 by adding a small quantity of 1 M HCl or NaOH. All media were

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prepared immediately before use.

Preparation of salt-stored human oocytes

Oocytes were collected from isolated ovaries, allowed to mature in vitro and stored in a salt solution (0.5 M ammonium sulfate + 1 M magnesium sulfate + 0.1% dextran) as described by Yanagimachi et al. (1979). They were stored in the solution for up to 3 months at 4°C. When needed, the oocytes were thoroughly rinsed with a test medium (e.g., Ca^{2+} -free mBWW). Since the oocytes themselves were no longer alive after salt-storage, these oocytes were called zonae pellucidae (or zonae) throughout this study. Three to nine zonae were transferred into 0.3 ml of a test medium (e.g., Ca^{2+} -free mBWW), covered with mineral oil and kept at 37°C until they were inseminated.

Insemination and examination of zonae

An aliquot (0.5 ml) of semen from a man with normal semen parameters was placed in the bottom of a test-tube (15 × 100 mm) which contained 1.0 to 2.0 ml of a test medium (e.g., Ca^{2+} -free mBWW) and the spermatozoa were allowed to "swim up" into the medium (Overstreet et al. 1980). Over 80% of the spermatozoa thus obtained were actively motile. The spermatozoa were washed with the test medium twice by centrifugation (230 g, 5 min each), and suspended in a small volume (about 150 μl) of the test medium. Insemination of zonae was carried out by adding 10–30 μl of the sperm suspension to 0.3 ml of the test medium containing zona. The concentration of spermatozoa in the insemination medium was $1\text{--}2 \times 10^6$ cells per ml. After 6 hr of incubation at 37°C under 5% CO_2 in air, the zonae were transferred to a fresh medium, pipetted several times (to remove loosely bound spermatozoa from zona surface), and examined with a phase-contrast microscope as described by Yanagimachi et al. (1979) and Overstreet et al. (1980). A zona was recorded "penetrated" when at least one spermatozoon was found within the perivitelline space.

Sperm motility assessment

An aliquot (1.0 ml) of sperm suspension containing "swim up" spermatozoa were incubated under mineral oil at 37°C (gas phase, 5% CO_2 in air). At 1, 3 and 5 hr after the start of incubation, one drop (30 μl) of the sperm suspension was placed in a Makler Sperm Counting Chamber (SEFI Medical Instruments, Haife, Israel) and the percentage of progressively motile spermatozoa was determined by a Hamilton-Thorn Motility Analyzer Model 2030 (Hamilton-Thorn Research, Denver, MA, USA). The pattern of movement of individual spermatozoa (including hyperactivated motility) was analyzed with a high-speed videomicrograph (Nac Microscopic High Speed Video, Nac, Tokyo) (Hoshi et al. 1988).

RESULTS

When zonae were inseminated in the regular medium and pipetted at the end of 6 hr of incubation, more than 100 spermatozoa were found tightly bound to each zona and all of the zonae were penetrated by spermatozoa (Table 1). Deletion of Mg^{2+} from the medium had no effect on sperm binding and penetration of the zona. On the other hand, deletion of Ca^{2+} or K^+ from the medium impaired both sperm attachment and penetration of the zona. The absence of Ca^{2+} in the medium impaired the motility of spermatozoa, but K^+ -free condition, on the contrary, had little effect on sperm motility (Table 2).

Deletion of energy substrates, particularly that of glucose, impaired the ability of spermatozoa to penetrate the zona (Table 3). In glucose-free medium, virtually no spermatozoa exhibited hyperactivated motility at 5 hr after the start

TABLE 1. *Effects of Ca^{2+} , Mg^{2+} and K^{+} on sperm attachment to and penetration through the zona, examined 6 hr after insemination*

Test medium with (+) and without (-)			No. of zonae inseminated	No. of sperm bound to each zona	Zonae penetrated/Zonae inseminated
Ca^{2+} (1.71 mM)	Mg^{2+} (1.19 mM)	K^{+} (5.97 mM)			
+	+	+	3	>100	3/3
-	+	+	5	<10	0/5
+	-	+	3	>100	3/3
+	+	-	3	10-100	0/3

TABLE 2. *Effects of Ca^{2+} and K^{+} on the motility of spermatozoa*

Medium	% Motile sperm at various times after the start of incubation ^a			% Hyperactivated sperm in entire population at 5 hr after the start of incubation ^a
	1 hr	3 hr	5 hr	
Ca^{2+} -free	64.5±12.0	40.2±20.7	35.3±21.8	22.2±20.7
Regular	66.3±13.5	63.3± 6.0	61.2±11.5	40.3±15.6
<i>p</i> value	n.s. ^b	<0.05	<0.05	n.s.
Mg^{2+} -free	67.5± 9.3	64.5± 6.0	58.0±18.6	46.5±15.6
Regular	64.3± 9.4	66.3± 6.5	65.3±11.2	47.0±12.8
<i>p</i> value	n.s.	n.s.	n.s.	n.s.
K^{+} -free	63.0±10.8	63.8±10.5	56.5±21.8	44.0±22.7
Regular	67.5± 8.1	62.5±13.4	52.8±15.8	41.8±17.3
<i>p</i> value	n.s.	n.s.	n.s.	n.s.

^aResult are mean±s.d. of six experiments using spermatozoa from six different males.

^bn.s., not significant.

of incubation (Table 4).

DISCUSSION

In order to penetrate the zona pellucida, spermatozoa must be actively motile and undergo the acrosome reaction (Yanagimachi 1988). Any agents or conditions which inhibit sperm movement and the acrosome reaction would render spermatozoa incapable of penetrating the zona. Ca^{2+} in the medium is known to be essential for the acrosome reaction of spermatozoa (Yanagimachi 1982). Human spermatozoa are no exception (Thomas and Meizel 1988). According to Heffner et al. (1980), Ca^{2+} is important for the firm attachment (binding) of mouse spermatozoa to the zona. Failure of human spermatozoa to pass through the zona under Ca^{2+} -free conditions as reported in this paper (Table 1) is most likely due

TABLE 3. *Effects of energy substrates on sperm attachment to and penetration through the zona, examined 6 hr after insemination*

Test medium with (+) and without (—)			No. of zonae inseminated	No. of sperm bound to each zona	Zonae penetrated/ Zonae inseminated
Glucose (5.56 mM)	Lactate (21.58 mM)	Pyruvate (0.25 mM)			
+	+	+	9	>100	8/9
—	+	+	4	10–100	0/4
+	—	+	4	10–100	1/4
+	+	—	4	10–100	2/4
—	—	+	3	10–100	0/3
—	+	—	3	10–100	0/3
+	—	—	3	10–100	0/3
—	—	—	9	<10	0/9

TABLE 4. *Effects of glucose on the motility of spermatozoa*

Medium	% Motile sperm at various times after the start of incubation ^a			% Hyperactivated sperm in entire population at 5 hr after the start of incubation ^a
	1 hr	3 hr	5 hr	
Glucose-free	40.6±26.8	43.4±14.8	29.0±18.7	0.8±1.8
Regular	60.2±19.6	53.6±15.5	45.6±17.0	58.4±6.4
<i>p</i> value	n.s. ^b	n.s.	n.s.	<0.001
Lactate-free	51.8±10.0	50.8±6.1	41.3±9.4	55.3±18.4
Regular	61.5±12.5	54.8±6.2	45.0±11.5	54.3±13.7
<i>p</i> value	n.s.	n.s.	n.s.	n.s.
Pyruvate-free	79.0±14.6	71.0±16.5	63.9±17.2	42.0±11.9
Regular	81.2±11.2	72.9±16.1	66.7±17.2	48.0±9.3
<i>p</i> value	n.s.	n.s.	n.s.	n.s.
Lactate & Pyruvate-free	79.0±16.2	75.2±20.3	67.4±23.4	37.8±15.5
Regular	85.4±7.2	78.0±12.6	71.2±15.3	45.4±9.4
<i>p</i> value	n.s.	n.s.	n.s.	n.s.
Glucose, Lactate & Pyruvate-free	42.5±20.2	40.5±10.9	31.3±8.0	1.8±2.4
Regular	61.5±12.5	54.8±6.2	45.0±11.5	54.3±13.7
<i>p</i> value	n.s.	n.s.	n.s.	<0.001

^aResult are mean±S.D. of five experiments using spermatozoa from five different males.

^bn.s., not significant.

to the inhibition of the acrosome reaction.

K⁺ in the medium is not essential for capacitation and the acrosome reaction of guinea pig spermatozoa in vivo, but it is essential for zona penetration and sperm-egg fusion (Rogers et al. 1981; Yanagimachi and Bhattacharyya 1988). The reason for the failure of zona penetration by human spermatozoa in K⁺-free medium is not clear. Sperm attachment to the zona and the motility of spermatozoa were affected only slightly by K⁺-free condition. Like hamster and mouse spermatozoa (Mrsny and Meizel 1981; Fraser 1983), human spermatozoa may require extracellular K⁺ for the acrosome reaction.

Of these energy substrates tested, glucose had most pronounced effect on sperm penetration through the zona. In the mouse, glucose is required for initiation of the acrosome reaction and hyperactivation of spermatozoa, but not for capacitation (Fraser 1981; Fraser and Harod 1990). Like mouse spermatozoa, human spermatozoa may not be able to initiate hyperactivated motility in glucose-free medium (Table 4). We have previously reported that human spermatozoa incubated in glucose-free medium can penetrate into zona-free hamster eggs in the same medium (Hoshi et al. 1982), suggesting that human spermatozoa are able to undergo both capacitation and the acrosome reaction in glucose-free medium. Therefore, it must be the hyperactivated motility, not the acrosome reaction, which was inhibited under glucose-free conditions. The lack of strong motility of spermatozoa in glucose-free medium appears to be one of the major causes of the failure of zona penetration by spermatozoa in this medium.

References

- 1) Fraser, L.R. (1981) Dibutyryl cyclic AMP decreases capacitation time in vitro in the mouse spermatozoa. *J. Reprod. Fert.*, **62**, 63-72.
- 2) Fraser, L.R. (1983) Potassium ions modulate expression of mouse sperm fertilizing ability, acrosome reaction and hypereactivated motility in vitro. *J. Reprod. Fert.*, **69**, 539-553.
- 3) Fraser, L.R. & Harod, J.E. (1990) Expression of capacitation dependent changes in chlorotetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. *J. Reprod. Fert.*, **88**, 611-621.
- 4) Heffner, L.J., Saling, P. & Storey, B. (1980) Separation of calcium effects on motility and zona binding ability of mouse spermatozoa. *J. Exp. Zool.*, **212**, 53-59.
- 5) Hoshi, K., Saito, A., Suzuki, M., Hayashi, K. & Yanagimachi, R. (1982) Effects of substrates on penetration of human spermatozoa into the zona pellucida of human eggs and the zona-free hamster eggs. *Jpn. J. Fert. Ster.*, **27**, 439-444.
- 6) Hoshi, K., Yanagida, K., Aita, T., Yoshimatsu, N. & Sato, A. (1988) Changes in the motility pattern of human spermatozoa during in vitro incubation. *Tohoku J. Exp. Med.*, **154**, 47-56.
- 7) Mrsny, R.J. & Meizel, S. (1981) Potassium ion influx and Na⁺, K⁺-ATPase activity are required for the hamster sperm acrosome reaction. *J. Cell Biol.*, **91**, 77-82.
- 8) O'Rand, M.G., Welch, J.E. & Fisher, S.J. (1986) Sperm membrane and zona pellucida interactions during fertilization. In: *Molecular and Cellular Aspects of Reproduction*, edited by D.S. Dhindsa & O.P. Bahl, Plenum Press, New York, pp. 131-144.

- 9) Overstreet, J.W., Yanagimachi, R., Katz, D.F., Hayashi, K. & Hanson, F.W. (1980) Penetration of human spermatozoa into the zona-free hamster egg: A study of fertile donors and infertile patients. *Fertil. Steril.*, **33**, 534-542.
 - 10) Rogers, B.J., Ueno, M. & Yanagimachi, R. (1981) Fertilization by guinea pig spermatozoa require potassium ions. *Biol. Reprod.*, **5**, 639-648.
 - 11) Saling, P.M. (1989) Mammalian sperm interaction with extra cellular matrices of the egg. In: *Oxford Review of Reproductive Biology*, edited by S.R. Miligan, Vol. 11, Oxford Univ. Press, Oxford, pp. 339-348.
 - 12) Thomas, P. & Meizel, S. (1988) An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Res.*, **20**, 397-411.
 - 13) Wassarman, P.M. (1990) Profile of mammalian sperm receptor. *Development*, **108**, 1-17.
 - 14) Yanagimachi, R. (1982) Requirement of extracellular calcium ions for various stages of fertilization and fertilization related phenomena in the hamster. *Gamete Res.*, **5**, 323-344.
 - 15) Yanagimachi, R. (1988) Mammalian fertilization. In: *The Physiology of Reproduction*, edited by E. Knobil & J.D. Neill, Vol. 1, Raven Press, Ltd., New York, pp. 135-185.
 - 16) Yanagimachi, R. & Bhattacharyya, A. (1988) Acrosome-reacted guinea pig spermatozoa became fusion competent in the presence of extracellular potassium ions. *J. Exp. Zool.*, **248**, 354-360.
 - 17) Yanagimachi, R., Lopata, A., Odom, C.B., Bronson, R.A., Mahi, C.A. & Nicolson, G.L. (1979) Retention of biologic characteristics of zona pellucida in highly concentrated salt solution: The use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa. *Fertil. Steril.*, **31**, 562-574.
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