Effects of Caffeine on *In-Vitro* Fertilization of Pig Follicular Oocytes

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Abstract. Experiments were conducted to assess the effects of caffeine on the ability of frozen-thawed boar epididymal spermatozoa to penetrate oocytes that had been matured in culture. The spermatozoa were preincubated in modified TCM199 medium and subsequently incubated for 2 h in either a fertilization medium with 2 mM caffeine or in a fertilization medium without caffeine. Then a sample of the spermatozoa in the caffeine-containing medium was introduced into another caffeine-containing medium that contained matured oocytes, while another sample was introduced into a medium with matured oocytes without caffeine. This procedure was repeated for the spermatozoa that had been incubated in the caffeine-free medium. The two sets of manipulations resulted in higher rates of penetration of oocytes inseminated in the caffeine-containing medium than of oocytes in the caffeine-free medium. Furthermore, when oocytes with attaching spermatozoa were washed and transferred to caffeine-free medium after 1, 2 and 3 h of incubation in the presence of caffeine, penetration rates were 14%, 76% and 94%, respectively. Addition of caffeine to the fertilization medium caused as much as a 1.4-fold elevation in levels of cyclic AMP in sperm, the effect being maximal within about 0.5 h of incubation. These results indicate that during 2 to 3 h of incubation with oocytes in the caffeine-containing medium, the spermatozoa become highly fertile, and that a high concentration of cyclic AMP in sperm might not have any direct effects on the ability of sperm to penetrate oocytes.

Key words: Pig oocytes, *In-vitro* fertilization, Cyclic AMP, Spermatozoa.

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Successful *in vitro* fertilization of *in vivo* matured pig oocytes [1–3] has been reported. In such experiments, caffeine, a methylxanthine, was shown to be essential for the induction of the acrosome reaction, which results in the penetration of oocytes by sperm. Hyne and Garbers [4] reported that addition of 1-methyl-3-isobutylxanthine, together with the presence of Ca^{2+} ions in the culture medium induced the acrosome reaction in capacitated guinea-pig sper-

matozoa. They suggested that the increase in concentrations of cyclic AMP in sperm was related to the induction of the acrosome reaction. However, the mechanisms involved in these processes have not been clarified [4, 5].

In this study, the concentrations of cyclic AMP in sperm incubated in the presence and in the absence of caffeine were determined, and the effect of caffeine on *in vitro* fertilization of pig follicular oocytes was examined.

Materials and Methods

Culture of follicular oocytes

Oocyte-cumulus complexes from crossbred gilts of the Landrace, Large White and Duroc breeds were collected, washed and cultured as previously described [6].

Preparation of sperm

Epididymal spermatozoa were frozen, thawed and preincubated as previously described [3] with the single exception that the duration of incubation of the spermatozoa was 70 min and the concentration of spermatozoa during the incubation was $4\text{--}5 \times 10^8$ cells/ml.

Assessment of penetration of oocytes after preferential incubation of sperm

About 10 μl of the pre-incubated suspension of sperm was introduced into the fertilization medium (90 μl of Brackett and Oliphant solution with or without 2 mM caffeine) [7] and incubated (preferential incubation) for 2 h under an atmosphere of 5% CO_2 in air at 39 C before insemination. Oocytes were cultured for 46–48 h as described above, and then the cumulus cells were removed and the oocytes were transferred to the control fertilization medium (90 μl) or to the same medium supplemented with 2 mM caffeine. After the preferential incubation, a portion (about 10 μl) of each of the suspensions of sperm was introduced into the fertilization medium that contained oocytes so that the final concentration of spermatozoa at insemination was $4\text{--}5 \times 10^5$ cells/ml. The oocytes were incubated for 5 h with spermatozoa at 39 C and then transferred to modified BMOC-2 medium [8]. Spermatozoa were then removed from the surface of the oocytes by pipetting. The oocytes were transferred to 100 μl of the same medium and incubated for 5 h under the same conditions. After incubation, oocytes were fixed, stained and examined as previously described [9].

Assessment of penetration by sperm during insemination

After the preincubation, a portion (about 10 μl) of each suspension of sperm was diluted in fertilization medium (90 μl) that contained 2 mM caffeine and then 10 μl of each of these suspensions was introduced into the fertilization medium

that contained oocytes so that the final concentration of spermatozoa at insemination was $4\text{--}5 \times 10^6$ cells/ml. After incubation for 1, 2 and 3 h with spermatozoa at 39 C, oocytes were transferred to fresh fertilization medium without caffeine and treated as follows. Two-thirds of the inseminated oocytes were washed, with three changes of medium. A large-bore pipette was used in order to avoid removal of spermatozoa that were attached to the surface of the zona of oocytes. Then the oocytes were transferred to fertilization medium with or without caffeine. Spermatozoa were removed completely from the surface of the rest of inseminated oocytes by pipetting. After washing, these oocytes were transferred to the medium without caffeine. All the oocytes were subsequently incubated in fertilization medium such that the total duration of culture was 5 h. Then oocytes were transferred to modified BMOC-2 medium and treated as described above. After incubation, oocytes were fixed, stained and examined as previously described [9].

Determination of levels of cyclic AMP in sperm

A portion (about 20 μl) of the pre-incubated suspension of sperm was introduced into the fertilization medium (180 μl). After 0, 0.5, 1 and 2 h of incubation under the same conditions as described above, 50 μl of the suspension of sperm were pipetted into 1 ml of ice-cold 6% trichloroacetic acid (TCA), and the mixture was homogenized by sonication. The homogenate was centrifuged at $500 \times g$ for 15 min and the supernatant was collected. One milliliter of ice-cold 6% TCA was added to the pellet, and cyclic AMP was extracted again as described above. After removal of TCA from the supernatant by washing with saturated ethylether, the cyclic AMP was quantitated by radioimmunoassay using a radioimmunoassay kit (New England Nuclear, Dupon, Japan).

Statistics

Data were analyzed by the χ^2 -test.

Results

Almost all the oocytes had undergone germinal vesicle breakdown (91–100%). As shown in Table 1, when oocytes were inseminated with the sper-

matozoa in the presence of caffeine, the penetration rates were significantly higher (52% for the spermatozoa preincubated in the presence of caffeine and 62% for the spermatozoa preincubated in the absence of caffeine) than when oocytes were inseminated with spermatozoa in the absence of caffeine (2% and 7%, respectively).

As shown in Table 2, when inseminated oocytes were washed by a gentle pipetting and subsequently cultured in the presence of caffeine, the

penetration rates were high (85% to 98%) without regard to the duration of insemination (1 to 3 h). However, when the washed oocytes were cultured in the absence of caffeine, the rate of penetration of oocytes that were incubated with sperm for 1 h was significantly lower (14%) than in the case of 2- and 3-h incubations (76% and 94%, respectively). Moreover, when the inseminated oocytes were pipetted to remove the attached spermatozoa after 1 and 2 h of incubation with sperm, the rates of

Table 1. Effects of caffeine in the prefertilization^a medium on penetration by spermatozoa of oocytes

Prefertilization incubation of spermatozoa in presence (+) or absence (-) of caffeine (2 mM)	Fertilization in presence (+) or absence (-) of caffeine (2 mM)	No. of oocytes examined	No. of maturing oocytes ^b at examination (%)	No. of oocytes penetrated (%) ^c
+	+	69	65 (94)	34 (52) ^d
+	-	66	60 (91)	1 (2) ^e
-	+	47	47 (100)	29 (62) ^d
-	-	48	45 (94)	3 (7) ^e

^aAfter preincubation for 70 min in TCM199 (pH 7.8), spermatozoa were incubated in fertilization medium for 2 h before insemination.

^bOocytes that underwent germinal vesicle breakdown (GVBD).

^cPercentage of maturing oocyte.

^{d,e}Numbers with different superscripts are significantly different ($P < 0.05$).

Table 2. Effects of caffeine in the fertilization medium on penetration by spermatozoa of oocytes

Duration of incubation with sperm ^a	Pipetting ^b of inseminated oocytes	Culture of fertilized oocytes in presence (+) or absence (-) of caffeine (2 mM)	No. of oocytes examined	No. of maturing ^c oocytes at examination	No. of oocytes penetrated (%) ^d
1	-	+	34	34 (100)	29 (85) ^{fg}
	-	-	118	118 (100)	16 (14) ⁱ
	+	-	119	116 (98)	0 (0)
2	-	+	110	109 (99)	98 (90) ^f
	-	-	102	100 (98)	76 (76) ^g
	+	-	101	96 (95)	18 (19) ^j
3	-	+	131	129 (99)	128 (98) ^e
	-	-	103	101 (98)	95 (94) ^f
	+	-	107	107 (100)	66 (62) ^h

^aOocytes were inseminated with preincubated spermatozoa in fertilization medium that contained caffeine (2 mM) for the indicated time.

^bThe inseminated oocytes were freed of attached spermatozoa by vigorous pipetting (+) or washed by gentle pipetting (-).

^cOocytes that underwent germinal vesicle breakdown.

^dPercentage of maturing oocytes.

^{e,f,g,h,i,j}Numbers with different superscripts are significantly different ($P < 0.05$).

Fig. 1. Effects of caffeine on concentrations of cyclic AMP in sperm. Preincubated spermatozoa were incubated in fertilization medium with or without 2 mM caffeine for the indicated times. Results are means $\pm$ SE from three replicates.

penetration were significantly reduced (0% and 19%, respectively). However, after a 3-h incubation with sperm, the rate returned to a high value (62%).

As shown in Fig. 1, when preincubated spermatozoa were subsequently incubated in fertilization medium that contained caffeine, concentrations of cyclic AMP in sperm after 0.5, 1 and 2 h of culture were higher (267 ± 27 , 234 ± 14 and 107 ± 41 fmole/cell, respectively) than when they were incubated in the medium in the absence of caffeine (183 ± 13 , 171 ± 17 and 77 ± 28 fmole/cell, respectively; means $\pm$ SE: $n=3$).

Discussion

Methylxanthines are effective stimulators for the *in-vitro* fertilization in many mammalian species; e.g., pig [1–3], man [4], cattle [11], guinea pig [4] and mouse [12]. Fraser [12] demonstrated that caffeine significantly decreased the capacitation time for mouse spermatozoa and that it promoted rapid penetration of eggs. Our results indicate that caffeine also promotes penetration by sperm of pig oocytes matured *in vitro*.

To examine the effects of caffeine on the ability of spermatozoa to penetrate oocytes, preincubated spermatozoa were incubated for 2 h (prefertilization incubation) in fertilization medium in the

presence or in the absence of caffeine. Thereafter, the incubated spermatozoa were introduced into fertilization medium (with or without caffeine) that contained oocytes. If spermatozoa were fully capacitated during the prefertilization incubation in the presence of caffeine, they would be able to penetrate the oocytes in the caffeine-free medium. However, the spermatozoa that were incubated in the caffeine-containing medium penetrated only 2% of oocytes when the oocytes were inseminated in caffeine-free medium. Given that penetration rates were quite high (52% and 62%) in the case of oocytes inseminated in caffeine-containing medium, independently of the presence or absence of caffeine during the prefertilization incubation of sperm, it appears that a 2-h prefertilization incubation in caffeine-containing medium was not sufficient for the capacitation of spermatozoa. Alternatively, caffeine might promote the capacitation and/or the acrosome reaction of spermatozoa only in the presence of oocytes, that is to say, in the presence of zona pellucida. Critser *et al.* [13] reported that pretreatment of bovine spermatozoa with caffeine prior to insemination of oocytes did not increase the frequency of either the acrosome reaction or fertilization during incubation of sperm and oocytes, but they did not examine the effect of caffeine in the fertilization medium on penetration by sperm.

Following our observations of penetration by sperm of oocytes in fertilization medium that contained caffeine, we extended our studies to examine conditions that promoted penetration of oocytes by sperm. When oocytes were incubated with preincubated spermatozoa for 2 h in caffeine-containing medium and transferred to caffeine-free medium with spermatozoa attached to the zona, 76% of them were penetrated (Table 2). By contrast, when inseminated oocytes were freed of attached spermatozoa after a 2-h of incubation with sperm and then transferred to caffeine-free medium, the rate of penetration was significantly reduced (19%). It appeared that some spermatozoa had completed the process of capacitation and the acrosome reaction and had penetrated zona pellucida within the 2-h of incubation and that the spermatozoa that had attached to the zona during that time were able to penetrate oocytes in caffeine-free medium. However, if oocytes were incubated with spermatozoa for an additional hour in the presence of caffeine, the rate of

penetration rose considerably (62%). These results indicate that during 2 to 3 h of incubation with oocytes in the caffeine-containing medium the spermatozoa become highly fertile.

Although the mechanisms that control the production of cAMP in sperm are poorly understood [5], it was found that caffeine, a phosphodiesterase inhibitor, can elevate the concentration of cAMP in sperm (Fig 1). Treatments that should raise intracellular levels of cAMP promote fertilization *in vitro* in many mammalian species (reviewed by Fraser and Monks [5]). While the mechanism involved in this effect of cAMP on capacity of spermatozoa to fertilize oocytes is not clear, Fraser and Monks [5] suggested that high levels of cAMP may promote development of the hyperactivated motile sperm that are necessary for penetration of the zona pellucida. However, in the present experiments, no hyperactivated motile sperm were observed. In fact, the motility of the frozen and thawed epididymal spermatozoa was so low, as compared with that of freshly ejaculated spermatozoa, that only straight forward movements were observed. Given that the effect of caffeine on the concentration of cAMP was evident within only 0.5 h of incubation, while the effect of caffeine on the ability of sperm to penetrate oocytes was slow in developing and not evident until 2 to 3 h had elapsed, it is suggested that cyclic AMP might not influence the ability of sperm to fertilize oocytes directly. Thus, the effect of caffeine on sperm might be mediated by factors other than cyclic AMP or might be triggered by cyclic AMP.

It is of interest that the sperm acrosome reaction is associated with both the primary transport of Ca^{2+} ions and a Ca^{2+} -dependent increase in concentrations of cyclic AMP in guinea-pig sperm [4]. Moreover, significantly higher levels of cyclic

AMP were found in hamster spermatozoa incubated in the presence of calcium than in its absence [14]. In addition, Kopf *et al.* [15–17] reported that methylxanthines elevate concentrations of cyclic AMP in abalone sperm and induce the acrosome reaction. They suggested that the methylxanthine-induced acrosome reaction was mediated by a primary effect on the transport of Ca^{2+} ions and occurred through a verapamil-sensitive mechanism. They also reported that methylxanthines can affect the acrosome reaction via primary effects on the transport of Ca^{2+} ions and changes in Ca^{2+} conductance [17]. Furthermore, Okamura *et al.* [18] showed that when boar epididymal spermatozoa are incubated with or without caffeine in the presence of $^{45}\text{CaCl}_2$, incubation with caffeine results in a higher level of sperm-associated $^{45}\text{Ca}^{2+}$ ions as compared to that in spermatozoa incubated without caffeine. Therefore, it is suggested that caffeine might promote the association of external Ca^{2+} ions with spermatozoa and that the increased level of Ca^{2+} ions might induce the acrosome reaction when the spermatozoa are attached to the zona pellucida. Recently, it was shown that solubilized zona pellucida can bind to the acrosome of the boar spermatozoa when caffeine and Ca^{2+} ions are present in the incubation medium [19]. Furthermore, zona proteins have been reported to induce the acrosome reaction of ejaculated boar spermatozoa [20].

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