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RU486 Suppresses Progesterone-induced Acrosome Reaction in Boar Spermatozoa

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The effects of progesterone on the acrosome reaction, as well as the effects of RU486 on the progesterone-induced acrosome reaction in capacitated boar spermatozoa, were investigated. Progesterone, a major steroid that is secreted by the cumulus cells of oocyte, clearly induced the acrosome reaction in a dose-dependent manner in capacitated boar spermatozoa, even though it failed to show similar effects in non-capacitated spermatozoa. RU486, a potent antiprogestin, significantly reduced the effects of progesterone on the progesterone-induced acrosome reaction; however, when treated alone, it showed no inhibitory effects on the acrosome reaction. The inhibitory effects of RU486 were also shown to be dose-dependent. These results imply that in addition to the well-known inducer of the acrosome reaction, zona pellucida, progesterone can also induce the acrosome reaction through its specific receptors on spermatozoa after the spermatozoa undergo capacitation.

Keywords: Acrosome, Progesterone, RU486, Spermatozoa

Introduction

Successful fertilization of the oocyte by spermatozoa requires sequential intracellular events of spermatozoa during its journey in the female genital track following initial interaction with the oocyte (Yamagata *et al.*, 1999; Baldi *et al.*, 2002). Immediately after deposition in the female genital track, the male gamete does not have the ability to fertilize the egg, even though it has full motility. All mammalian spermatozoa undergo a process called capacitation and acrosome reaction, which give spermatozoa the ability to fertilize (Fraser, 1995). Capacitation is associated with changes in the sperm motility pattern, collectively referred to as sperm hyperactivation (Yanagimachi, 1994; Suarez and Dai, 1995). Capacitated spermatozoa can undergo an acrosome reaction, a fusion event between the overlying plasma membrane and the outer acrosomal membranes (Yanagimachi, 1994; Tulsiani *et al.*, 1998). Acrosome reaction is a modified form of exocytosis, which results in the release of acrosomal contents. Acrosome reaction is an important step in fertilization because only the acrosome-reacted sperm are capable of penetrating zona pellucida (ZP) and of fusing with the egg. An acrosome reaction can be induced by follicular fluid, cumulus cells (Tesarik *et al.*, 1993), or zona pellucida (Cross *et al.*, 1988). Progesterone (its receptor in spermatozoa has not yet been identified) is a major inducer of the acrosome reaction in follicular fluid (Meizel and Turner, 1991; Somanath *et al.*, 2000). The antiprogestin RU486 {RU38486, mifepristone, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-propynyl-estra-4,9-dien-3-one} binds with high affinity to the intracellular progesterone receptor in most vertebrate species (Baulieu, 1989). It has been reported that this potent antiprogestin has either a small or negligible inhibitory effect on the progesterone-mediated calcium influx into human sperm (Baldi *et al.*, 1991). In this study, we evaluated the effect of RU486 on the progesterone-induced acrosome reaction.

Materials and Methods

Materials Fresh boar testes were collected from the Deajean Meat Co. (Incheon, Korea). HAM's buffer was purchased from GIBCO Laboratories (Grand Island, USA). Progesterone, RU486, and chlorotetracyclin (CTC) were from Sigma Chemical Co. (St. Louis, USA). All of the other chemicals were obtained in molecular biology or extra-pure grade from Sigma Chemical Co. (St. Louis, USA) and Amersham Pharmacia Biotech (Buckinghamshire, England).

Methods

Preparation of spermatozoa Boar epididymides were dissected from freshly-excised tissue, and the spermatozoa were flushed with Ham's buffer (pH 7.4) that contained 50 mM benzamidine, which

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was added as an activation inhibitor of trypsin-like enzyme (Yu and Yi, 2001). The flushed spermatozoa were washed via centrifugation at $10,000 \times g$ through 11% ficoll that contained 50 mM benzamidine for 30 min (Yi, 1999). The spermatozoa pellets were then resuspended in Tyrodes solution for further study.

Induction of sperm capacitation Capacitation of boar spermatozoa was according to Fazeli *et al.* (1999). The spermatozoa pellets were washed twice in modified Tyrode's solution, mT-B25 medium (124.54 mM NaCl, 2.68 mM KCl, 0.49 mM $MgCl_26H_2O$, 25 mM $NaHCO_3$, 0.36 mM $NaH_2PO_42H_2O$, 5 μg/ml phenol red, 50 μg/ml kanamycin sulfate, 1.8 mM $CaCl_2$, 5.56 mM glucose, 4 mg/ml BSA, pH 7.4) at $300 \times g$ for 10 min, then diluted to a concentration of $2.5\sim5 \times 10^6$ cell/ml in the same medium (Sabeur *et al.*, 1996). Capacitation was induced by incubating the 200 μl aliquots of resuspended spermatozoa in 15 ml polypropylene conical centrifuge tubes (Falcon Blue Max, Lincoln Park, USA) at 37°C for 2 h in a humid atmosphere of 5% CO_2: 95% air (pH of medium, 7.4~7.6).

Effects of progesterone on the acrosome reaction of the capacitated and non-capacitated boar spermatozoa Experiments were designed to evaluate the acrosome reaction that was induced at different concentrations of progesterone according to Sabeur *et al.* (1996). The capacitated and non-capacitated boar spermatozoa were independently treated with different concentrations of progesterone (5 μM, 10 μM, and 20 μM) that were dissolved in 0.1% dimethysufoxide. Incubation was carried out at 37°C for 20 min in a humid atmosphere of 5% CO_2: 95% air.

Effects of antiprogestin RU486 on the acrosome reaction of the capacitated boar spermatozoa The capacitated boar spermatozoa were independently treated with different concentrations of RU486 (5 M, 10 M , 20 M) dissolved in 0.1% dimethysufoxide with or without 20 μM of progesterone. The incubation was carried out at 37°C for 20 min in a humid atmosphere of 5% CO_2: 95% air.

Assessment of acrosome reaction A chlorotetracycline (CTC) fluorescence assay was used to assess the functional status of the spermatozoa (Fraser and Herrod, 1990). A CTC solution was freshly prepared that contained 250 μM CTC in a buffer of 130 μM NaCl, 5 mM cysteine, and 20 mM Tris (final pH 7.4). The solution was wrapped in aluminum foil and kept at 4°C until use. To stain the cells, 50 μl of spermatozoa suspension was added to the 50 μl CTC solution and thoroughly mixed. The cells were then fixed by adding 10 μl of 12.5% (w/v) paraformaldehyde in a 0.5 M Tris buffer (pH 7.4) for 14 h. Thereafter, 10 μl of stained suspension was placed on a clean glass slide. A drop of mounting solution that contained 90% glycerol and 10% PBS was mixed carefully to retard the fading of the fluorescence. A coverslip was added, and the excess fluid was removed by compressing the coverslip to the slide. The slides were sealed with colorless nail polish, and the acrosome reaction was immediately assessed.

Statistical analysis Percentages of the acrosome reaction that were obtained in the different experiments were expressed as the mean ± standard error of the mean (SEM). In instances where one treatment and control were compared, the one-way ANOVA was applied.

Results

Effects of progesterone on the acrosome reaction in capacitated boar spermatozoa We tested to see if progesterone had any stimulatory effect on the acrosome reaction in capacitated-boar spermatozoa. Three different doses of progesterone (5, 10, and 20 μM) were added in the incubating medium that contained capacitated boar spermatozoa. The proportion of the acrosome-reacted spermatozoa in each experimental group was evaluated. The results showed that the proportion of the acrosome-reacted spermatozoa increased significantly in a dose-dependent manner with the increasing amounts of progesterone when compared to the non-treated control group. The proportion of the acrosome-reacted spermatozoa increased from 13.6% in the control group to 16.4%, 19.8%, and 27.4% in the progesterone-treated spermatozoa with 5, 10, and 20 μM of progesterone, respectively. These values represent 20.6%, 45.6%, and 101.5% increases when compared to the non-treated control group. The proportion of the acrosome-reacted spermatozoa that occurred spontaneously in the control group, 13.6%, was quite comparable with those that were reported by the other researchers (Fig. 1).

Effects of progesterone on the acrosome reaction in non-capacitated boar spermatozoa We next tested to see if progesterone would induce an acrosome reaction specifically with capacitated spermatozoa only, or nonspecifically with spermatozoa in general. The same progesterone doses were treated to the non-capacitated boar spermatozoa, and the proportion of the acrosome-reacted spermatozoa was

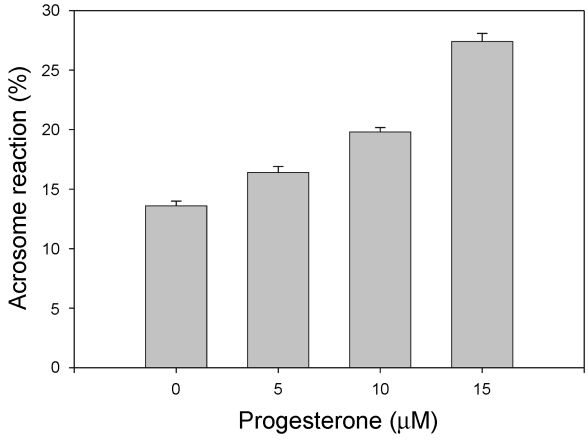

Fig. 1. Stimulatory effects of progesterone on the acrosome reaction in capacitated boar spermatozoa. Following capacitation, the boar spermatozoa were incubated with different concentrations of progesterone for 20 min. The spermatozoa, which underwent an acrosome reaction, were assessed by staining the spermatozoa with CTC. The results are presented as mean ± SEM ($n = 5$ with 200 spermatozoa scored for each treatment), and the statistical analysis showed that differences are significant at $P<0.05$. The error bar in each group denotes the variation in 5 different experiments.

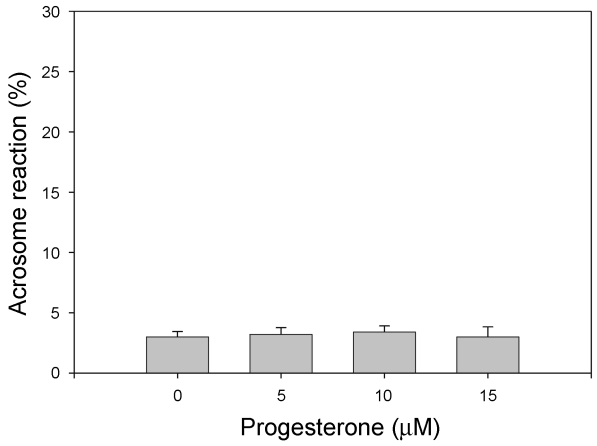

Fig. 2. Effects of progesterone on the acrosome reaction in non-capacitated boar spermatozoa. The boar spermatozoa were incubated with progesterone in the same way as described in Fig. 1 except that the capacitation was not induced for these spermatozoa. The assessment of proportion of boar spermatozoa that underwent the acrosome reaction was performed in the same way as described in Fig. 1. The results are presented as mean ± SEM (n = 5 with 200 spermatozoa scored for each treatment).

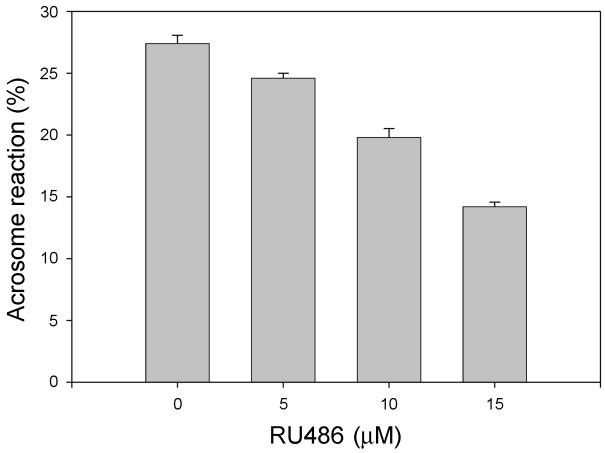

Fig. 3. Inhibitory effects of RU486 on the acrosome reaction in progesterone-treated boar spermatozoa. The boar spermatozoa were incubated with 20 μM of progesterone and different RU486 amounts. Following a 20-min incubation, the proportion of acrosome-reacted spermatozoa was evaluated in the same way as described in Fig. 1. The results are presented as mean ± SEM (n = 5 with 200 spermatozoa scored for each treatment). The statistical analysis showed significance at $P<0.05$.

evaluated. The results showed that the proportions of the acrosome-reacted spermatozoa were 3% in the non-treated control group, and 3.2%, 3.4%, and 3.0% in the 5, 10, and 20 μM of progesterone-treated spermatozoa, respectively. These results demonstrated the following: (1) With increasing amounts of progesterone, the dose-dependent increments of the acrosome-reacted boar spermatozoa proportion were abolished. (2) The proportion of the acrosome-reacted spermatozoa in the control and experimental groups were much lower when compared to the comparable groups in Figure 1. This shows that progesterone reacted and induced the acrosome reaction only in the capacitated-spermatozoa in boar (Fig. 2).

Inhibitory effects of RU486 on the progesterone-induced acrosome reaction in capacitated boar spermatozoa

Finally, we tested to see if the well-known antiprogestin, RU486, would reverse the stimulatory effects of progesterone on the acrosome reaction in capacitated boar spermatozoa. Different amounts of RU486 were mixed with 20 μM progesterone, and then treated to the capacitated boar spermatozoa. When the percentages of the acrosome-reacted spermatozoa were evaluated, there were clear and significant decreases in the proportion of the acrosome-reacted spermatozoa with increasing RU486 concentrations in a dose-dependent manner (Fig. 3). The proportion of the acrosome-reacted spermatozoa decreased from 27.4% of the control group (only treated with 20 μM progesterone) to 24.6%, 19.8%, and 14.2% in the 5, 10, and 20 μM of RU486 plus 20 μM progesterone-treated boar spermatozoa, respectively. These values represent 10.2%, 27.7%, and 48.0% decreases for the respective group when compared to the progesterone-only-treated control group. This clearly demonstrates that the potent antiprogestin, RU486, can reverse the stimulatory effects of progesterone on the acrosome reaction in capacitated boar spermatozoa. However, to demonstrate that RU486 can reverse the stimulatory effects of progesterone on the acrosome reaction, we needed to know whether or not RU486 would show inhibitory effects on the acrosome reaction when treated alone. When 5, 10, and 20 μM of RU486 were treated to the capacitated boar spermatozoa, 14.2%, 14.4%, and 14% of the acrosome-reacted spermatozoa were counted, respectively (Fig. 4). These values were quite close to the non-treated control value, 13.8%, demonstrating that RU486 does not exert any inhibitory effects on the acrosome reaction when treated alone to the capacitated boar spermatozoa.

Discussion

We report that the acrosome reaction is induced by progesterone in capacitated boar spermatozoa (Fig. 1) and that this stimulatory effect of progesterone on the acrosome reaction can be reversed by the antiprogestin, RU486 (Fig. 3). These results clearly demonstrate that progesterone induces the acrosome reaction, just like the generally known inducer, zona pellucida (Yanagimachi, 1994), in capacitated boar spermatozoa. Even though similar results of the progesterone-induced acrosome reaction were also reported from other species, such as humans (Osman *et al.*, 1989), mice (Melendrez *et al.*, 1994), stallions (Meyers *et al.*, 1995), golden hamsters (Meizel *et al.*, 1990), and goats (Somanath *et*

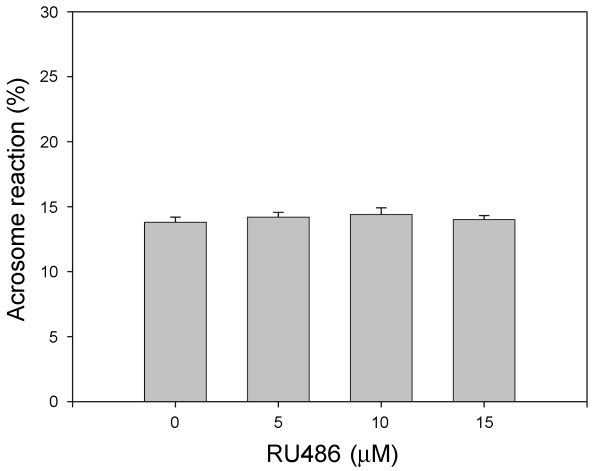

Fig. 4. Effects of RU486 on the acrosome reaction in the capacitated boar spermatozoa. The boar spermatozoa were incubated with 0, 5, 10, and 20 μM of RU486 in the absence of progesterone. Following a 20-min incubation, the proportion of the acrosome-reacted spermatozoa were evaluated in the same way as described in Fig. 1. The results are presented as mean ± SEM (n = 5 with 200 spermatozoa scored for each treatment). The statistical analysis showed significance at $P<0.05$.

al., 2000), the initiation of the acrosome reaction by progesterone has so far not been reported in boar. Although we could demonstrate that progesterone induces the acrosome reaction in capacitated boar spermatozoa, how progesterone specifically exerts its effects on the acrosome reaction could not be deduced from the progesterone treatment study alone. Therefore, we performed an inhibitor study using the potent antiprogestin, RU486. RU486 inhibits calcium uptake in sperm and counteracts the stimulation that is produced by progesterone (Yang *et al.*, 1994). The rationale for using the inhibitor of progesterone to determine whether or not the acrosome reaction is the result of a specific progesterone action was that antiprogestin reverses the progesterone effects by competing with the binding sites of progesterone, assuming that progesterone induces the acrosome reaction through its specific receptors in spermatozoa. The results of the inhibitor study clearly demonstrate that the antiprogestin RU486 reversed the progesterone effect (Fig. 3), while RU486 alone had no inhibitory effects on the acrosome reaction (Fig. 4). Therefore, these results strongly imply that the progesterone action is mediated by its specific receptors in spermatozoa. To our knowledge the inhibitory action of RU486 on the progesterone-induced acrosome reaction has not been previously reported. This led us to speculate that spermatozoa could possess the progesterone receptor in the non-genomic form, potentially in the membrane-bound form since the acrosome reaction occurs at the plasma membrane level that overlies the acrosome and outer acrosomal membrane. Evidence also suggests that the acrosome reaction that is induced by progesterone is mediated by a sperm-surface receptor through a nongenomic pathway (Revelli *et al.*, 1998); however, this receptor has not yet been identified. Currently, the search for the membrane-bound progesterone receptor in boar spermatozoa is underway in our laboratory.

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References

Baldi, E., Casano, R., Falsetti, C., Krausz, C., Maggi, M. and Forti, G. (1991) Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J. Androl.* **12**, 323-330.

Baldi, E., Luconi, M., Bonaccorsi, L. and Forti, G. (2002) Signal transduction pathways in human spermatozoa. *J. Reprod. Immunol.* **53**, 121-131. Review.

Baulieu, E. E. (1989) Contragestion and other clinical applications of RU486, an antiprogesterone at the receptor. *Science* **245**, 1351-1357.

Cross, N. L., Morales, P., Overstreet, J. M. and Hanson, F. W. (1988) Induction of acrosome reaction by the human zona pellucida. *Biol. Reprod.* **38**, 235-244

Fazeli, A., Duncan, A. E., Watson, P. F. and Holt, W. V. (1999) Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol. Reprod.* **60**, 879-886.

Fraser, L. R. (1995) Cellular biology of capacitation and acrosome reaction. *Hum. Reprod. Suppl.* **10**, 22-30.

Fraser, L. R. and Herrod, J. E. (1990) Expression of capacitation-dependent changes in chlorotetracycline fluorescence pattern in mouse spermatozoa requires a suitable glycosylable substrate. *J. Reprod. Fertil.* **88**, 611-621.

Meizel, S., Pillai, M. C., Diaz-Perez, E. and Thomas, P. (1990) Initiation of the human acrosome reaction by components of human follicular fluid and cumulus secretions including steroids. *Serono Symposia*. USA. 205-222.

Meizel, S. and Turner, K. O. (1991) Progesterone acts at the plasma membrane of human sperm. *Mol. Cell. Endocrinol.* **11**, R1-R5.

Melendrez, C. S., Meizel, S. and Berger T. (1994) Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction *in vitro*. *Mol. Reprod. Dev.* **39**, 433-438.

Meyers, S. A., Overstreet, J. W., Liu, I. K. M. and Drobnis, E. Z. (1995) Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. *J. Androl.* **16**, 47-54.

Osman, R. A., Andria, M. L., Jones, A. D. and Meizel, S, (1989) Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem. Biophys. Res. Commun.* **160**, 828-833.

Revelli, A., Massobori, M. and Tesarik, J. (1998) Nongenomic actions of steroid hormones in reproductive tissues. *Endocr. Rev.* **19**, 3-17.

Sabuer, K., Edwards, D. P. and Meizel, S. (1996) Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. *Biol. Reprod.* **54**, 993-1001.

Somanath, P. R., Suraj, K. and Gandhi, K. K. (2000) Caprine

sperm acrosome reaction: promotion by progesterone and homologous zona pellucida. *Small Rumin. Res*. **37**, 279-286.

Suarez, S. S. and Dai, X. (1995) Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. *Mol. Reprod. Dev.* **42**, 325-333.

Tesarik, J., Carreras, A., and Mendoza, C. (1993) Differential sensitivity of progesterone and zona pellucida-induced acrosome reactions to pertussis toxin. *Mol. Reprod. Dev.* **34**, 183-189.

Tulsiani, D.R., Abou-Haila, A., Loeser, C. R. and Pereira, B. M. (1998) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp. Cell Res*. **240**, 151-164.

Yamagata, K., Honda, A., Kashiwabara, S. I. and Baba, T. (1999) Difference of acrosomal serine protease system between mouse and other rodent sperm. *Dev. Genet*. **25**, 115-122.

Yanagimachi, R. (1994) Mammalian fertilization; in *The Physiology Of Reproduction*, Knobil, E and Neil, J. (eds), pp 135-186, Raven Press, New York.

Yang, J., Serres, C., Phlibert, D., Robel, P. and Baulieu, E. E. (1994) Progesterone and RU486: Opposing effects on human sperm. *Proc. Natl. Acad. Sci.* USA **91**, 529-533.

Yi, L. S. (1999) Amino acid composition analysis of the 32 kDa sperminogen. *J. Biochem. Mol. Biol.* **33**, 510-513.

Yu, H. and Yi, L. S. (2001) Purification and partial peptide sequence analysis of the boar 32 kDa sperminogen. *Mol. Cells* **31**, 107-111.