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Effect of norethisterone and its A-ring reduced metabolites on the acrosome reaction in porcine spermatozoa

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Summary. The synthetic progestin, norethisterone (NET), has been reported as a contragestational postcoital agent in humans, rodents and rabbits. The effect and molecular mechanisms of NET and its A-ring reduced metabolites, 5α-NET and 3β5α-NET, on the acrosome reaction (AR) are unknown. The aim of this study was to assess the effect of these compounds on an *in vitro* progesterone-induced AR in porcine spermatozoa. The spermatozoa were obtained from semen ejaculated by proven fertile adult pigs. Seminal plasma removed and incubated under capacitating conditions was performed in TALP-Hepes medium for 4 h. Progesterone (P_4) and three different progestins: norethisterone (NET), 5α-norethisterone (5α-NET) and 3β5α-NET were then added at equimolar doses, and the spermatozoa were incubated for 15 min. Double-staining with PSA-FITC and Hoechst-33258 assessed the AR and sperm viability. Both P_4 and NET induced the AR, while 5α-NET not only did not induce this process, but was able to block the effect of P_4 on the spermatozoa. 3β5α-NET was not able to inhibit P_4 action. These results suggest that NET and its A-ring reduced metabolites act in different ways on the progesterone-induced AR in porcine spermatozoa.

Introduction

Acrosome reaction (AR) is a crucial event for reproduction in many species including humans. Progesterone (P_4) is the main component of follicular fluid and induces the AR by means of a nongenomic membrane receptor, which triggers a Ca^{+2} influx in the spermatozoa (Foresta *et al.*, 1992; Baldi *et al.*, 1995; Sabeur *et al.*, 1996; Luconi *et al.*, 1998) and activates other intracellular pathways such as diacylglycerol generation (Murase & Roldan, 1996; Revelli *et al.*, 1998).

The P_4 receptor is mainly located on the head of human spermatozoa and has not been found in the median piece of the tail (Blackmore & Laltanzio, 1991). In capacitated pig spermatozoa, P_4 can trigger the AR *in vitro* at concentrations ranging from 10^{-9} to 10^{-6} molar. Melendrez *et al.* (1994) demonstrated that this steroid induces the AR in capacitated spermatozoa in the same manner as the solubilized zona pellucida. Contrary to the effects of P_4, the progestin RU486 is able to inhibit the AR in human spermatozoa, and also inhibits calcium influx and the lateral displacement of the sperm head (Uhler *et al.*, 1992; Yang *et al.*, 1994).

Other synthetic progestins such as NET and its A-ring reduced metabolites, 5α-NET and 3β5α-NET, show affinity for the nuclear progesterone and estrogen receptors, respectively (Chávez *et al.*, 1985). However, information regarding the influence of these progestins on sperm physiology is limited, and most of these studies assess the effects of these compounds on the sexual behaviour of males (Morali *et al.*, 1990), spermatogenesis, sperm count, motility and fertilization capability (Guerin & Rollet, 1988; Shetty *et al.*, 1997). Because these progestins exert effects on important reproduction events and may have a role in contraception in humans, we assessed the effect of NET and its A-ring reduced metabolites on the AR in pig spermatozoa.

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Materials and methods

Hoescht 33258, fluorescent isothiocyanate conjugated *Pisum sativum* agglutinin (FITC-PSA), polyvinylpyrrolidone (PVP, MW_{av} 40,000) and all capacitation medium components and chemicals were obtained from Sigma Chemical Company, St Louis, MO. 5α-norethisterone and 3β5α-NET were synthesized by norethisterone reduction (Schering Mexicana, SA, México) according to the method described by Bowers *et al.*, 1958) and was kindly provided by Dr Gustavo G. de la Mora from the School of Chemistry, UNAM. Progesterone was dissolved in PBS, while all the remainder compounds were dissolved in 52% ethanol. The final ethanol concentration in the incubation medium was not greater than 0.05%, and preliminary experiments proved that this concentration did not affect sperm viability, motility or the AR. Ionophore A23187 (10 μmol) dissolved in ethanol (0.1% final concentration) was used as the positive control.

Sperm samples were obtained from proven fertile adult pigs. Seminal plasma was removed and spermatozoa were capacitated according to the method described by Bonilla *et al.* (1994). Spermatozoa was centrifuged at 300 g for 5 min to eliminate the supernatant, and the sperm pellet was washed twice (300 g 5 min^{-1}) with fresh TALP-Hepes capacitation medium [100 mmol of sodium chloride, 0.29 mmol of dibasic sodium phosphate, 2.1 mmol of calcium chloride, 1.5 mmol of magnesium chloride, 25 mmol of sodium bicarbonate, 10 mmol of N-2-hydroxiethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 21.6 mmol of sodium lactate, 1 mmol sodium pyruvate, 3.1 mmol potassium chloride, 5 mg ml^{-1} bovine serum albumin, pH 7.4, osmolarity 290–300 mOsm kg^{-1}]. After each washing procedure the pH osmolarity was checked without any change. Afterwards, 50 μl of sperm suspension (15×10^6) was distributed in four well culture plates (Nunc, Denmark), adjusted to 1 ml by adding TALP-Hepes medium and incubated for 4 h at 39 °C in a 5% CO_2 atmosphere for capacitating conditions.

After the capacitating conditions, different steroids or steroid combinations were added and the spermatozoa were then incubated for 15 min at 39 °C. A dose–response curve proved that 10 μg ml^{-1} of progesterone (3.2×10^{-5} M) induced the greatest percentage of acrosome-reacted spermatozoa (10–13%) without causing toxicity and cell death (data not shown). Thus, we used this dose of progesterone for the assay, and progestins such as NET, 5α-NET and 3β5α-NET were added to the capacitation medium at equimolar doses. Double staining with Hoechst-PSA-FITC was performed to assess the AR as described by Berger (1990). Hoechst was added to each well to obtain a final 1 μg ml^{-1} concentration, and the suspensions were incubated for 8 min at 39 °C. In order to eliminate free Hoechst, 100 μl of sperm suspension was centrifuged at 600 g for 10 min in a 2% PVP-40 solution in PBS. The spermatozoa were fixed in absolute ethanol and placed on a slide, and 10 μl of lectin (200 μg ml^{-1} PBS) were added and incubated for 20 min at 39 °C in a 5% CO_2 humid atmosphere in the dark. A Zeiss epifluorescence microscope (Corl Zeiss, West Germany) was used to analyze sperm viability with UV (360 nm) and the acrosome conditions with 495-nm UV light (1000 ×). Two hundred spermatozoa were analyzed on each trial.

Data are expressed as mean and standard deviation. Differences in percentages of acrosome-reacted spermatozoa were tested by Student's *t*-test (Jandel Sigma Plot 3.0, Scientific Graphing Software) and *P*-values < 0.05 were considered significant.

Results

After capacitating conditions, progestin effect was evaluated only in those samples with 80% or more alive spermatozoa by Hoechst dye. The effect of equimolar doses of P_4, NET, 5α-NET, 3β5α-NET and different combinations of these compounds on the percentage of acrosome-reacted pig spermatozoa are shown in Figure 1. The effects of the progestins and their different combinations on the AR were compared using a group of spermatozoa treated with P_4 as the control. The mean percentage of spontaneously acrosome-reacted spermatozoa (2.6%) was subtracted from all the reported percentages of differently treated acrosome-reacted spermatozoa.

The addition of ethanol did not change the percentage of progesterone-induced acrosome-reacted spermatozoa (8.4 ± 2.0 vs. 11.9 ± 2.1, $t = 1.9$; $P = 0.091$). Norethisterone induced AR in 12.7 ± 4.2% of the spermatozoa, and this percentage was similar to that found in P_4-treated spermatozoa (11.9 ± 2.1) ($t = 0.26$; $P = 0.787$). However, no synergistic effect was observed when the spermatozoa were treated with P_4 or both P_4 and NET (11.9 ± 2.1 vs. 10.2 ± 2.1; $t = 0.970$; $P = 0.369$).

The addition of 5α-NET or 3β5α-NET alone did not induce the AR. However, when the spermatozoa were incubated in the presence of P_4 and equimolar doses of 5α-NET, the percentage of acrosome-reacted spermatozoa was 98.6% lower than that of the group treated only with P_4

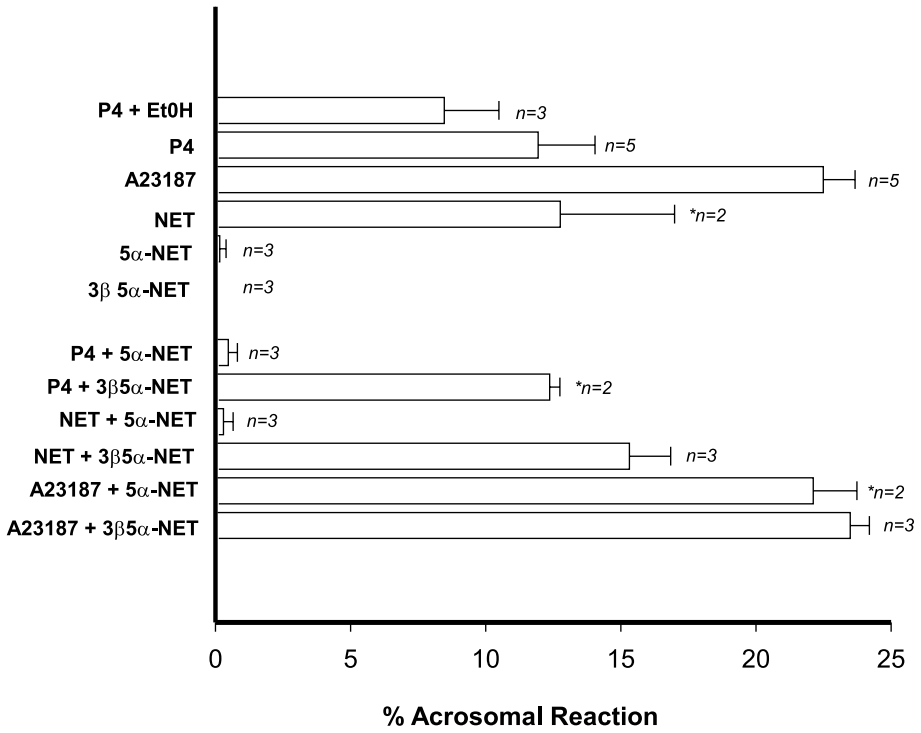

Figure 1. Effect of equimolar hormone concentrations on the sperm acrosomal reaction under capacitating conditions. Number of experiments by triplicate (*n*) or duplicate (**n*).

(0.1 ± 0.230 vs. 11.9 ± 2.1, $t = 8.03$; $P = 0.001$). Non-significant differences were found in the percentage of acrosome-reacted spermatozoa treated with P_4 alone or P_4 + 3β5α-NET (11.9 ± 2.1 versus 12.3 ± 0.3; $t = 0.23$; $P = 0.820$).

When the spermatozoa were incubated in the presence of NET and equimolar doses of 5α-NET, the percentage of acrosome-reacted spermatozoa decreased 98.4% as compared to the group treated with NET only (0.2 ± 0.153 versus 12.7 ± 4.23; $t = 3.97$; $P = 0.023$). The percentage of acrosome-reacted spermatozoa induced by the combination of NET plus 3β5α-NET was very similar to that induced by P_4, and the differences were not significant (15.3 ± 1.53 vs. 12.7 ± 4.23; $t = 0.743$, $P = 0.511$).

Finally, the A-ring reduced metabolites did not block the AR induction effect of the ionophore (iono) [22.4 ± 1.18 (iono) vs. 22.1 ± 1.62 (iono + 5α-NET) $t = 0.28$, $P = 0.789$; and 22.4 ± 1.18 (iono) vs. 23.4 ± 0.707 (iono + 3β5α-NET) $t = 1.14$; $P = 0.295$].

Discussion

Acrosomal reaction is an event that occurs after spermatozoa undergo a process of capacitation, following ejaculation. Normally it occurs in the female reproductive tract, however, it can also occur *in vitro* by adding progesterone into the experimental medium.

Even though the classic intracellular P_4 receptor does not exist in spermatozoa, progesterone appears to initiate the human AR via its interaction with one or two types of plasma membrane receptors (Blackmore *et al.*, 1990; Meizel & Turner, 1991; Tesarik *et al.*, 1992; Sabeur, 1996).

Some studies in the rat and rabbit have demonstrated the agonistic effect of norethisterone and the antagonistic effect of 5α-NET, both acting via a specific intracellular progesterone receptor (Pérez-Palacios *et al.*, 1992; Castro *et al.*, 1995; Pasapera *et al.*, 1995). However, to date there is no information on the effects of NET and its metabolites on sperm physiology nor is it known whether NET can be reduced to its metabolites in spermatozoa. We believed that 5α-NET could act as an antagonist of progesterone to induce AR and for that reason we considered to test this hypothesis.

Our results show that NET and progesterone function as AR inductors, while 5α-NET and 3β5α-NET alone were unable to increase the percentage of acrosome-reacted spermatozoa. This suggests that in porcine spermatozoa, NET acts as progestagen while 5α-NET and 3β5α- NET lack such activity. This is known to occur in other tissues (Pérez *et al.*, 1984; Garza *et al.*, 1986; Vilchis *et al.*, 1986; Larrea *et al.*, 1987). Interestingly, only 5α-NET, but not the 3β5α form, blocked the effect of

both P_4 and NET when administered at equimolar doses. This indicates that the antiprogesterone effect of 5α-NET cannot be explained by its conversion to 3β5α-NET. The mechanisms of action of these progestins in spermatozoa are unknown, but the rapid response to P_4 and synthetic progestins observed in only 15 min, suggest that these steroids might act through the activation of specific membrane receptors (Jacob *et al.*, 1998; Revelli *et al.*, 1994; Feng-Pang *et al.*, 1998), changes of intracellular calcium concentrations (Blackmore, 1990) or altering membrane fluidity (Shivaji & Jagannadham, 1992). These mechanisms have already been studied in humans, but remain to be tested in our model. Recently and in parallel with this study, we have carried out some experiments using mouse spermatozoa and we found the same results as we demonstrated in pig spermatozoa (unpublished data), but the hormone-binding receptor experiments demonstrated that neither NET nor 5α-NET were able to displace the binding to progesterone receptor. This means that probably the effects observed in both the mouse and the porcine spermatozoa might have occurred in different ways to that of the membrane progesterone receptor.

It has been recently demonstrated that P_4 increases intracellular calcium concentrations in human spermatozoa, inducing the AR, while 17β-estradiol induces a fast and sustained increase of intracellular calcium concentrations without inducing the AR when administered solely at nanomolar concentrations. Furthermore, 17β-estradiol shows a slight inhibitory effect of the progesterone-induced AR at nanomolar but not at micromolar concentrations (Luconi *et al.*, 1999). In accordance with the latter study, we found that micromolar concentrations of 3β5α-NET did not induce the AR when added alone. However, the addition of equimolar doses of P_4 (μM) and 3β5α-NET (μM) did not inhibit the acrosome reaction induced by progesterone. Whether this difference is because of differences of affinity for the 17β-estradiol and 3β5α-NET receptors remains to be verified. Furthermore, as 5α-NET and 3β5α-NET were unable to block the induction effect of the ionophore, the mechanism of action of these metabolites most probably affects a pathway different than that activated by the ionophore. As far as we know, this is the first report describing the effect of NET and its A-reduced ring metabolite 5α-NET on this crucial step in sperm physiology in pigs; no information in this regard is available on humans or any other species. Further studies are required to thoroughly understand the mechanism of action of these compounds in our model, and more importantly, in human spermatozoa.

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