Cell surface binding sites for progesterone on human spermatozoa

Madhavee Ambhaikar¹ and Chander Puri
Institute for Research in Reproduction (ICMR), Parel, Bombay 400012, India

This study demonstrates the presence of [3H]-progesterone binding protein on the cell surface of human spermatozoa. The binding protein is masked and is exposed after treatment of spermatozoa with surfactants such as digitonin. Specific binding of [3H]-progesterone is observed after the washed spermatozoa or the crude cell membrane fractions are treated with 0.1% digitonin at 0–4°C for 30 min R5020, 17α-hydroxyprogesterone and testosterone only partially compete with [3H]-progesterone for binding whereas the synthetic steroids STS557, RU486, ZK98.299 and ZK98.734 do not competitively inhibit the binding of [3H]-progesterone to spermatozoa. Progesterone binding sites are located in the acrosomal region of spermatozoa, as indicated by the use of fluorescein isothiocyanate-labelled progesterone–bovine serum albumin. The binding protein cross-reacts with the monoclonal antibodies to uterine progesterone receptor and has a molecular weight of ~85 kDa. This is the first study which demonstrates the presence of masked progesterone binding sites on the cell surface of human spermatozoa that are exposed after treatment with surfactants. These binding sites seem to have structural homology with the intracellular progesterone receptor but differ in terms of ligand specificity and location. The study further demonstrates that follicular fluid facilitates progesterone binding to spermatozoa.

Key words: follicular fluid/human spermatozoa/ligand specificity/progesterone binding

Introduction

Progesterone, which is primarily considered a female hormone, also has an important role in sperm function. Incubation of spermatozoa with progesterone results in an increase in intracellular calcium [Ca²⁺]i (Blackmore et al., 1990), induction of the acrosome reaction (Osman et al., 1989) and an increase in hyperactive motility (Uhler et al., 1992). Progesterone has also been shown to induce ovum penetration (Margolioth et al., 1988). When spermatozoa are capacitated in the presence of progesterone, the frequency of egg penetration is greater (Libersky and Boatman, 1995). It has also been shown that intracellular calcium increased and that the acrosome reaction in response to progesterone in human spermatozoa can be correlated with in-vitro fertilization (IVF) (Krausz et al., 1995). A clinical study has suggested the involvement of a defective progesterone receptor in some cases of unexplained male infertility (Tesarik and Mendoza, 1992). Since progesterone is present in high concentrations in follicular fluid and the cumulus matrix, it is possible that sperm metabolism is influenced by progesterone in the female genital tract.

The precise mechanism by which progesterone controls sperm function is not known. Tesarik and Mendoza (1993) have shown that the progesterone-induced Ca²⁺ influx and Ca²⁺-dependent acrosome reaction occur following aggregation of the progesterone receptors on the sperm membrane on ligand binding. The observation that the exposure of human spermatozoa to progesterone results in an increase in tyrosine phosphorylation of a phosphoprotein and in an increase in concentration of phosphotyrosine in the spermatozoa also suggests cell surface mediated action of progesterone (Tesarik et al., 1993). Progesterone has also been shown to induce a Ca²⁺-dependent cyclic AMP increase in human spermatozoa (Parinaud and Milhet, 1996). Since the progesterone-induced Ca²⁺ influx is a rapid process, with a maximum effect seen in <1 min (Blackmore et al., 1991), the mechanism of action of progesterone in the spermatozoa appears to be different from the classical genomic mechanism involving intracellular steroid hormone receptors and the processes of translation and transcription. It has been implicated that progesterone induces its effects of Ca²⁺ influx and acrosome reaction via a surface receptor (Blackmore et al., 1991; Meizel 1997). It has been shown that the progesterone-induced acrosome reaction involves the γ-amino butyric acid A (GABA A) receptor. The GABA A receptor/Ca²⁺ channel blockers picrotoxin or pregnenolone sulphate, and the GABA receptor antagonist, bicuculline, cause a reduction in progesterone-initiated human and pig sperm acrosome reaction (Wistrom and Meizel, 1993; Melendez and Meizel, 1995).

Cheng et al. (1981) have shown by autoradiographic techniques the presence of progesterone binding sites on spermatozoa, which are located on the midpiece of the tail as well as on the head and neck regions. However, these binding sites are not specific for progesterone and are displaced by 17β-oestradiol. On the other hand, using fluorescein isothiocyanate (FITC)-labelled progesterone–bovine serum albumin (BSA),...
progesterone binding sites have been shown to be present in the equatorial region of the spermatozoon (Blackmore and Lattanzio, 1991; Tesarik et al., 1992a). Using the conventional radioligand binding assays, the specific binding of progesterone to human spermatozoa was not observed. It is possible that the progesterone binding sites on spermatozoa are masked. These binding sites might be exposed during capacitation which involves rearrangement of lipids and proteins and unmasking of several antigens or in the presence of follicular fluid. A study was undertaken to investigate whether the progesterone binding sites on the spermatozoa are masked and if they could be exposed on treatment with surfactants. Previously, we have given a preliminary report of a similar study carried out in the rat (Ambhaikar and Puri, 1994). Studies were also conducted to localize and characterize the progesterone binding sites. The effect of follicular fluid on progesterone binding was also studied.

Materials and methods

Reagents

[3H]-progesterone (specific activity 91 Ci/mM) was obtained from Amersham International, (Amersham, UK). The other chemicals including detergents, urea, guanidine hydrochloride and FITC–progesterone–BSA were purchased from Sigma Chemical Company (St Louis, MO, USA). ZK98.734 [11β-(p-(dimethylamino)phenyl)-17β-hydroxy-17-{(Z)-3-hydroxypropenyl}-oestra-4,9-dien-3-one], ZK98.299 [11β–(p-(dimethylamino)phenyl)-17α-hydroxy-17-{(3-hydroxypropyl)-13α-oestra-4,9-dien-3-one} and R5020 (17,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione) were obtained from Schering AG (Berlin, Germany), RU486 [11β-(p-(dimethylamino)phenyl)-17β-hydroxy-17-(1-propynyl)-oestra-4,9-dien-3-one] was obtained from Roussel Uclaf (Paris, France). STS557 (17α-cyanomethyl-17β-hydroxy-oestra-4,9-dien-3-one) was a gift from the World Health Organization (WHO).

Preparation of spermatozoa for binding assays

Human semen samples from healthy donors were collected in sterile jars. The semen samples were allowed to liquefy at room temperature (~28°C) for 20 min. The sperm concentration and motility were determined in accordance with the WHO guidelines (WHO, 1987), and only samples that showed rapid progressive motility of >50% were used in binding studies. The semen samples were pooled and centrifuged at 4000 g for 5 min at 4°C. For binding assays, the sperm pellet was washed twice in TEMGD buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 10% glycerol, 25 mM sodium molybdate and 1 mM dithiothreitol) and resuspended in TEMGD buffer to achieve a final concentration of 40×10⁶ spermatozoa/ml.

Collection of human follicular fluid

Follicular fluid was obtained by aspiration from women undergoing laparoscopy for IVF and embryo transfer. The follicular fluid was pooled, centrifuged at 1000 g for 10 min at 0–4°C to remove cellular debris.

Preparation of crude membrane fraction

To determine that the binding of progesterone is to the intracellular sperm plasma membrane protein and is not merely the uptake and retention by the cytoplasm, the spermatozoa were subjected to 2000 kg pressure in hypotonic buffer using a French Press (Techno Sient Trading Corporation, New Delhi, India) to break the cell membranes and to release the intracellular proteins. The suspension was centrifuged at 800 g for 5 min to remove the unbroken spermatozoa. The membrane fraction was prepared following a modified method of Sidhu and Guraya (1993). The supernatant was centrifuged at 541 000 g for 1 h at 4°C to separate out the cell membrane fraction.

Treatment of spermatozoa with detergents

To study the effects of detergents on progesterone binding, the washed human spermatozoa were suspended in TEMGD buffer and treated with different concentrations of Triton X-100, digitonin, sodium dodecyl sulphate (SDS), sodium deoxycholate, Nonidet P-40 (octylphenoxy polyethoxyethanol), CHAPS (3-[3-cholamidopropyl]dimethylamino- l-1-propanesulphonate), urea or guanidine hydrochloride at 0–4°C for 30 min. The detergent-treated sperm suspension was centrifuged at 4000 g for 5 min at 4°C and the pellet was washed twice with TEMGD buffer. Both sperm suspension and supernatant were used for ligand binding assay. Since maximum specific binding of [3H]-progesterone was observed on treatment of spermatozoa with 0.1% digitonin, this concentration was used in all subsequent experiments.

Binding assays

The washed spermatozoa or the cell membranes were divided into two parts, one part was treated with 0.1% digitonin for 30 min at 0–4°C, and the other was treated similarly but without digitonin. Washed and digitonin treated spermatozoa, the cell membrane fraction and their fractions were incubated with 10 nM [3H]-progesterone with (non-specific binding, NSB) or without (total binding, TB) 1 μM progesterone for 24 h. Following incubation, the cell suspension was washed three times with TEMGD buffer by cycles of centrifugation and resuspension, resuspended in 0.5 ml buffer and counted for radioactivity using a liquid scintillation spectrometer (Kontron, Zurich, Switzerland).

The digitonin extract of spermatozoa was similarly incubated. The bound fraction was separated by incubating with 0.2 ml of 1% dextran-coated charcoal for 10 min at 4°C and centrifuging at 4000 g for 10 min at 0–4°C. The supernatant was decanted into scintillation vials and counted. To determine the optimum conditions for binding studies, the influence of sperm concentration, incubation time and temperature on binding of [3H]-progesterone to spermatozoa was studied. In case of experiments in which follicular fluid was used, the sperm suspension was incubated with [3H]-progesterone (10 nM) without (total binding) or with follicular fluid or with 100 fold molar excess of radioinert progesterone for 24 h at 0–4°C.

Ligand specificity

To determine the ligand specificity of binding, the spermatozoa were incubated with 10 nM [3H]-progesterone and increasing concentrations (0.039–5 μM) of radioinert progesterone, R5020, testosterone, RU486, ZK98.299, ZK98.734, 17α-hydroxyprogesterone or STS557.

Polyacrylamide gel electrophoresis and Western blot analysis

To identify and characterize the protein, the digitonin extract of spermatozoa, containing ~50 μg protein, was electrophoresed on 7.5% polyacrylamide gel (Laemmli, 1970) and the gel was processed as described by Estes et al. (1987). Briefly, the gel was transferred onto a nitrocellulose membrane. The blot was incubated overnight with monoclonal antibody to human uterine progesterone receptor at 4°C and developed using the Vectastain ABC–horseradish peroxidase kit (Vector laboratories, Burlingame, CA, USA). Normal mouse serum was used as the positive control. A similar gel was run and silver staining was carried out (Merril et al., 1979).
Localization of progesterone binding sites

FITC–progesterone–BSA conjugate was used to visualize progesterone binding sites on spermatozoa. The method described by Blackmore and Lattanzio (1991) was used with some modifications. The washed and digitonin treated spermatozoa were incubated with 1 μM FITC–progesterone–BSA in the absence or presence of 100-fold molar excess of progesterone at 25°C for 4 h. Following incubation, spermatozoa were pelleted by centrifugation and washed twice in TEMGD buffer (TEMGD buffer without glycerol) and examined at ×100 magnification for fluorescence pattern using a Zeiss Universal epifluorescence microscope. For each sample, 300 spermatozoa were assessed to determine the percentage of labelled spermatozoa.

Displacement of [3H]-progesterone by human follicular fluid

Human follicular fluid was diluted serially in TEMGD buffer. Spermatozoa were incubated with [3H]-progesterone in the absence (total binding) or presence of different dilutions of follicular fluid at 0–4°C for 24 h.

Flow cytometry

Flow cytometry was used to detect displacement of FITC–progesterone–BSA by human follicular fluid. Human spermatozoa (4×10⁶/assay) were incubated in the presence of FITC–progesterone–BSA (0.1 μM progesterone) with or without human follicular fluid (containing 1.84 μM progesterone) or progesterone (10 μM). The incubation was carried out at 4°C for 18 h. The cells were washed with phosphate-buffered saline (PBS, 0.01 M) and resuspended to achieve a final concentration of 1×10⁶ cells/ml. The cells were analysed using an EPICS Profile Flow Cytometer (Coulter Co, Miami, FL, USA). The sperm cell window was gated in a dot plot distribution of cells according to forward angle light scatter (Haas and Cunningham, 1984) and fluorescence intensity of 5000 cells was monitored in each sample. The data were analysed by an EPICS Flow Analyser. The data presented show the number of binding sites to increase as a function of x axis as channel number in logarithmic scale. The y axis represents the number of spermatozoa.

Effect of dialysis

To study whether the progesterone in the follicular fluid is bound to proteins or is free, 1 ml of follicular fluid was subjected to dialysis against 0.02 M PBS at 0–4°C for 48 h with several changes. The dialysed follicular fluid was lyophilized and the volume adjusted to original with TEMGD. Plain spermatozoa were incubated with [3H]-progesterone with or without dialysed follicular fluid.

Effect of temperature

To study the effect of heating of follicular fluid on the displacement of [3H]-progesterone, the follicular fluid was diluted so that it contained 30 mg/ml protein and heated at 30, 50 and 70°C. The samples were centrifuged at 3000 g for 10 min and the superantants were used for assay.

Estimation of protein

Protein was estimated by the method of Lowry et al. (1951) using BSA as the standard.

Estimation of progesterone

Progesterone in the follicular fluid was estimated by a specific radioimmunoassay (Sufi et al., 1988).

Statistical analysis

In case of binding assays, each assay was performed in triplicate and the results were expressed as the mean of three observations at each point. The assays were repeated at least three times using pools of samples to assess consistency of results. Student’s t-test was used where necessary. Differences were considered to be significant when P < 0.05.

Results

Binding of [3H]-progesterone to spermatozoa

Incubation of washed human spermatozoa with [3H]-progesterone at 4°C did not show any significant specific binding (Figure 1). Specific binding of [3H]-progesterone was also not observed on incubating the membrane preparations, and the sperm lysate obtained during preparation of the membranes. On the other hand, when the spermatozoa or the membrane preparations were treated with digitonin, specific binding of [3H]-progesterone to the spermatozoa as well as the membrane fraction was observed. Digitonin also solubilized at least a part of the progesterone binding protein, as specific binding of [3H]-progesterone was observed with the digitonin extract.

The specific binding of [3H]-progesterone increased with increasing number of spermatozoa and was stable up to 24 h at 25°C. The binding was stable at 0–4°C for up to 48 h of incubation. All subsequent assays were carried out at 0–4°C. Washed sperm samples were treated with different detergents for 30 min at 0–4°C, following which the binding of [3H]-progesterone was studied. The specific binding of [3H]-progesterone increased with increasing concentration of digitonin, and it was maximum on incubating the spermatozoa with 0.1% digitonin at 0–4°C for...
Figure 2. Effect of treatment with detergents on the binding of $[^{3}H]$-progesterone to human spermatozoa. Binding was measured as c.p.m. but was converted to nM for comparative purposes. Different concentrations of each detergent (0.001–0.5%) were evaluated and for comparison purposes the data with 0.1% concentration of each detergent are presented. Digitonin was most effective in facilitating binding of $[^{3}H]$-progesterone to spermatozoa and its effect was concentration dependent. Values are means ± SEM of triplicate determinations.

30 min (Figure 2). The specific binding was more when the spermatozoa were treated with digitonin as compared to Triton X-100. Treatment of spermatozoa with SDS, sodium deoxycholate, Nonidet P-40, CHAPS, urea and guanidine hydrochloride did not expose the binding sites. Urea and guanidine hydrochloride were used at 4 M concentration.

Ligand specificity

The binding of $[^{3}H]$-progesterone to spermatozoa could be displaced with increasing concentrations of radioinert progesterone (Figure 3). Progesterone agonists R5020, 17α-hydroxyprogesterone, and testosterone only partially displaced the binding of $[^{3}H]$-progesterone. On the other hand, RU486, ZK98.299, ZK98.734, or STS557 did not compete with $[^{3}H]$-progesterone for binding to spermatozoa.

Western blot analysis

The Western blot analysis of digitonin extract of human spermatozoa using monoclonal antibodies to human uterine progesterone receptor revealed a major protein band of 85 kDa (Figure 4).

FITC–progesterone–BSA binding

Specific binding of progesterone to human spermatozoa was also observed on incubation of digitonin treated spermatozoa with FITC–progesterone–BSA conjugate, and not with the spermatozoa which had not been treated with the detergent. The fluorescence could be localized in the acrosomal region and ~16 ± 3% spermatozoa showed specific binding.

Displacement of progesterone by human follicular fluid

Follicular fluid caused a dose dependent displacement of $[^{3}H]$-progesterone whereas radioinert progesterone did not cause any displacement (Figure 5). However, the displacement was not comparable to that of digitonin-treated spermatozoa (Figure 6).

In spermatozoa incubated with FITC–progesterone–BSA (0.1 µM progesterone), a fluorescence peak was observed (Figure 7). The fluorescence was quenched in the presence of 100-fold excess of progesterone (data not shown). FITC–BSA was used as a negative control. Dialysed follicular fluid also shows $[^{3}H]$-progesterone displacement and the amount of displacement was similar to that observed using neat follicular fluid (Figure 8). Heating the follicular fluid to 50°C inhibited displacement of $[^{3}H]$-progesterone in spermatozoa (Figure 9).
Figure 4. (A) Western blot of digitonin extract of human spermatozoa. Lanes 1, 2 and 3, digitonin extract of three semen samples. The binding protein corresponds to ~85 kDa. (B) Adjoining figure shows silver staining of gel with the same samples.

Figure 5. Dose-dependent displacement of [3H]-progesterone by cold progesterone and follicular fluid. Washed spermatozoa were incubated in the presence of serially diluted follicular fluid with progesterone concentrations in the range of 0–6.2 µM or with with approximately equimolar concentrations of progesterone. Values are means ± SEM of triplicate determinations.

Discussion
The results suggest the presence of a masked progesterone binding site on the surface of human spermatozoa and that progesterone binding is facilitated in the presence of digitonin and follicular fluid. The progesterone binding site is present in the cell membrane of human spermatozoa and has a molecular size of ~85 kDa. The binding protein differs in terms of location, ligand specificity and binding characteristics. As progesterone binding has been shown to promote Ca2+ influx and acrosome reaction in capacitated human spermatozoa, it is likely that the binding of progesterone to its corresponding binding site on spermatozoa triggers these biological responses.

[3H]-progesterone does not bind to washed spermatozoa, however, when the spermatozoa are treated with digitonin, specific

Figure 6. Comparison of [3H]-progesterone displacement in plain washed spermatozoa and digitonin treated spermatozoa by progesterone (P) and follicular fluid (FF). (i) Digitonin-treated spermatozoa were incubated with 10 nM [3H]-progesterone in the absence (TB, total binding) or presence of progesterone (1 µM)/ follicular fluid (equimolar progesterone). (ii) Washed spermatozoa were incubated similarly. Values are means ± SEM of triplicate determinations.

Figure 7. Flow cytometry. Spermatozoa were incubated in the presence of (i) fluorescein isothiocyanate (FITC)–progesterone (100 nM)–bovine serum albumin (BSA); (ii) FITC–progesterone–BSA + follicular fluid (progesterone = 1.84 µM); and (iii) FITC–BSA. Fluorescence intensity is displayed on the horizontal axis and the relative number of cells on the vertical axis.
that progesterone interacts with the membranes rather than intracellular receptor. The uterine progesterone receptor, in contrast, is intracellular. The difference in location suggests a difference in the mechanism of action. It is likely that Ca\textsuperscript{2+} influx, a rapid phenomenon, occurs via this membrane binding protein. This is supported by the work of Blackmore et al. (1991) which reports the location of progesterone receptor on the cell surface of human spermatozoa which was identified using progesterone immobilized on BSA. This complex increased intracellular Ca\textsuperscript{2+} in human spermatozoa suggest its action at the surface of spermatozoa.

Treatment with detergents increases the permeability and fluidity of the plasma membrane by dispersal of lipids and fragmentation of the membrane and thus exposes the binding sites. Digitonin and Triton X-100 have been successfully used to solubilize many membrane proteins without loss of binding activity (Tanford and Reynolds, 1976). Progesterone binding protein has also been demonstrated in the plasma membrane of Xenopus oocytes, which can be solubilized only on treatment of plasma membrane with a non-ionic detergent Brij 35 (polyethylene ether) (Sadler and Maller, 1984). Bramley and Menzies (1994) have demonstrated the presence of particulate progesterone binding sites in ovine corpus luteum membranes on treatment with digitonin. It is interesting that the binding of [\textsuperscript{3}H]-progesterone to spermatozoa was much less when Triton X-100 was used as the detergent, in comparison with the use of digitonin. The use of other detergents such as SDS, Nonidet P-40, CHAPS, sodium deoxycholate, and chaotrophic agents such as urea and guanidine hydrochloride neither facilitated binding to the spermatozoa nor did they solubilize the binding protein. The action of digitonin, thus, appears to be specific. Digitonin is known to have a number of effects on cells which include interaction with cholesterol, formation of cell surface membrane pores, unsealing of sealed vesicles and rapture of lysosomal and peroxisomal membranes (Bramley and Ryan, 1979; Bramley and Menzies, 1988). Digitonin stimulates progesterone binding, possibly by inducing conformational changes in the membrane to expose progesterone binding sites. During capacitation, a number of changes take place within the plasma membrane which include rearrangement of lipids and proteins and unmasking of several antigens. It could be possible that treatment with digitonin mimics at least some of the changes that take place in sperm plasma membrane during capacitation in the female genital tract. It is also likely that digitonin reacts with the sperm membrane cholesterol. Cholesterol sulphate has been shown to be a component of the human sperm membrane and represents ~20\% of the acrosomal surface area (Langlais et al., 1988). Cholesterol flux has been observed in spermatozoa incubated in the presence of human follicular fluid as well as in capacitating spermatozoa (Parks and Ehrenwald, 1989). It can be postulated that there are certain components in the follicular fluid that facilitate progesterone binding to spermatozoa and induce Ca\textsuperscript{2+} influx and the acrosome reaction.

The progesterone binding protein could be visualized predominantly in the acrosomal region of the spermatozoa with a FITC-labelled progesterone–BSA. Only 15–20\% of the spermatozoa showed specific localization of progesterone bind-

**Figure 8.** Effect of dialysis. Human spermatozoa were incubated in the presence of: (i) [\textsuperscript{3}H]-progesterone (total binding); (ii) [\textsuperscript{3}H]-progesterone and follicular fluid containing 6.2 µM progesterone and 6.2 mg protein per assay tube; and (iii) [\textsuperscript{3}H]-progesterone and dialysed follicular fluid 1.9 µM progesterone and 2 mg protein per assay tube. Values are means ± SEM of triplicate determinations.

**Figure 9.** Human follicular fluid was incubated at 30, 50 and 70°C for 30 min and centrifuged at 1000 g at 4°C for 10 min. The supernatants were incubated with spermatozoa in the presence of [\textsuperscript{3}H]-progesterone at 4°C for 24 h. Total binding was determined by incubating washed spermatozoa with [\textsuperscript{3}H]-progesterone (10 nM), represented by dark bar on left. Progesterone concentrations were 2.97, 3.05, 2.78 mg/ml respectively in supernatants of follicular fluid at 30, 50 and 70°C. Values are means ± SEM of triplicate determinations.
Spermatozoa and calcium response.

That there is no correlation between progesterone binding to intracellular calcium (Aitken 1992a). However, according to a recent study, 92–97% of spermatozoa exposed to progesterone showed increase in intracellular calcium (Aitken et al., 1996). Thus it appears that there is no correlation between progesterone binding to spermatozoa and calcium response.

The binding of [3H]-progesterone to digitonin treated spermatozoa was partially inhibited by R5020, 17α-hydroxy-progesterone and testosterone, and was not inhibited by RU486, ZK98.299 or ZK98.734. In the human uterine cytosol, the binding of [1H]-progesterone was displaced by R5020, RU486, ZK98.734 and ZK98.299 (unpublished results). The antiprogestins are known to act at the DNA level to inhibit progesterone binding and the relative binding affinity of RU486 is greater for the progesterone receptor than that of progesterone in the human endometrium. The binding of [3H]-progesterone to spermatozoa appears to be similar to that in the chick oviduct where RU486 does not compete with progesterone for binding to the progesterone receptor (Moudgil et al., 1986) and this is attributed to a difference of a single amino acid (Benhamou et al., 1992).

The ligand specificity results are thus suggestive of structural differences in the progesterone binding sites on human spermatozoa and in the human uterine cytosol. R5020 and some of the other potent progestins such as medroxyprogesterone acetate, norgestrel and noretisterone do not mimic the effects of progesterone to increase calcium influx in human spermatozoa, and progesterone receptor blockers RU486 and ZK98.299 do not inhibit the progesterone mediated increase in calcium influx in human spermatozoa, and progesterone receptor blocker blockers RU486 and ZK98.299 do not inhibit the progesterone mediated increase in calcium influx (Blackmore et al., 1991; Uhler et al., 1992). Contrary to these reports, Yang et al. (1994) reported that progesterone induced a rapid influx of calcium in capacitated human spermatozoa which could be counteracted by RU486. 10 µM RU486 was required to decrease the effect of 0.01 µM progesterone by ~69%. RU486 has also been shown to inhibit penetration of human spermatozoa into zona free hamster oocytes at concentrations of 50–100 µM (Yang et al., 1996). However, these concentrations of RU486 are very high and it is not known whether the same receptor is involved in the opposite effects of progesterone and RU486 on calcium intake in spermatozoa. Although there are differences in the reports, it may be concluded from our results and those of others that the antiprogestins do not interact or interact with a low affinity with the membrane binding site on spermatozoa.

In the Western blot analysis, a monoclonal antibody against human uterine progesterone receptor was used which recognized a single band of 85 kDa in the sperm extract. The same monoclonal antibody recognizes two bands in the uterine cytosol, 90 and 120 kDa. It is likely that the ligand binding domain of the two binding sites is common due to the common ligand, progesterone. This indicates that even though there are differences in the ligand specificity, there is some structural homology between the sperm and uterine progesterone receptors. Studies by other authors show the presence of different kinds of putative progesterone receptors/binding sites on spermatozoa. A 50–52 kDa protein was detected by an antibody raised against the C-terminal steroid binding domain of the human intracellular progesterone receptor, that blocked acrosome reaction (Sabour et al., 1996). A monoclonal antibody against 220 kDa surface antigen was shown to block progesterone initiated acrosome reaction and progesterone mediated Ca2+ influx (Brucker et al., 1992, 1994). A 94 kDa protein was reported to display progesterone mediated tyrosine phosphorylation (Tesarik et al., 1993). Interestingly, the tyrosine phosphorylation was independent of progesterone induced opening of a plasma membrane Ca2+ channel. Thus the effects of progesterone on plasma membrane Ca2+ channel and on tyrosine kinase activity are due to independent actions of the steroid ligand and may thus be mediated by different receptors (Mendoza et al., 1995; Tesarik et al., 1996). It is likely the binding site that we report here may be the one that is tyrosine phosphorylated. It needs to be determined which of these sites are involved in the progesterone induced effects on spermatozoa.

It was expected that capacitated and acrosome-reacted spermatozoa would show specific [3H]-progesterone binding. During capacitation and acrosome reaction, the sperm membrane undergoes changes to expose intrinsic membrane proteins. There was no specific binding in capacitated spermatozoa implying that such changes do not occur in the case of progesterone binding sites on spermatozoa. In acrosome-reacted spermatozoa, similar observations were made (unpublished results). This indicates that some components in the female tract, not present in the capacitation media, are required for the exposure of the progesterone binding sites or for progesterone interaction with the sperm membrane. Therefore, human follicular fluid which is the natural source of progesterone in the female tract, was used to study its effects on [3H]-progesterone displacement. When plain washed spermatozoa were exposed to follicular fluid, specific displacement of [3H]-progesterone was observed. However, radioinert progesterone did not cause displacement at the doses used. The study thus indicated the presence of a factor in the follicular fluid that is responsible for facilitating progesterone binding. Flow cytometric analysis also confirmed the above as follicular fluid caused quenching of the fluorescence in spermatozoa bound to FITC–progesterone–BSA. The quenching was not observed in presence of excess progesterone. The specific displacement of [3H]-progesterone caused by human follicular fluid in washed spermatozoa was not, however, comparable to that caused by radioinert progesterone observed in digitonin-treated spermatozoa. It was observed that free progesterone, even at concentrations as high as 10 µM, does not cause displacement of [3H]-progesterone in washed spermatozoa. In contrast, when
progesterone was present in follicular fluid at concentrations as low as 0.195 µM, there is a significant displacement. Thus some factors present in the follicular fluid facilitate the binding of progesterone to spermatozoa. Our results can be correlated with those of Miska et al. (1994) who observed that free progesterone at low concentrations did not show induction of acrosome of reaction in human spermatozoa. The results of Yang et al. (1994), Blackmore et al. (1991), Tesarik et al. (1992a) indicate that acrosome reaction is induced by free progesterone at concentrations as high as 2–3 µM (1–1.5 µg/ml).

The displacement of [3H]-progesterone caused by both dialysed and neat follicular fluid was equivalent even though the progesterone concentration in the dialysed follicular fluid was nearly half of that in the follicular fluid. It therefore appears that protein bound progesterone in the follicular fluid causes displacement of [3H]-progesterone. This was further supported by the observation that the displacement of [3H]-progesterone was inhibited when follicular fluid was heated to 50°C, possibly by denaturing the protein to which progesterone is conjugated. This observation correlates with that of Miska et al. (1994), in which the acrosome reaction inducing activity of human follicular fluid was lost upon treatment at 50°C or above. However, this is in contrast to the observation of other authors who heated human follicular fluid to 56°C to inactivate complement and still found acrosome reaction inducing activity (Suarez et al., 1986; Sitteri et al., 1988; Yudin et al., 1988; McClure et al., 1990; Seigel et al., 1990). Acrosome reaction inducing substances from the follicular fluid have been described in several reports. A protein of molecular mass 45 kDa that induces acrosome reaction has been reported (Miska et al., 1994). According to Saragueta et al. (1994), immunoglobulins from follicular fluid induce the acrosome reaction in the spermatozoa. It appears that progesterone in the follicular fluid is possibly associated with serum albumin, corticosteroids binding globulin, and possibly other proteins (Fleming and McGaughey, 1982). Fehl et al. (1995) have shown that progesterone in the follicular fluid is conjugated to corticosteroid binding globulin. A high molecular weight proteoglycan, proacrosin activator, present in high concentrations in peri-ovulatory human cumulus oophorus (Drahorad et al., 1988) has been shown to modulate the sperm response to progesterone (Mendoza et al., 1993). This activator-augmented progesterone induced acrosomal exocytosis. In the present study, it has been shown that follicular fluid influences the binding of free progesterone. It is likely that the proacrosin activator in the follicular fluid has some role to play in progesterone binding and this needs to be investigated.

Many factors in the follicular fluid have been implicated to have a major role in sperm–egg interaction. However, a study by Huyser et al. (1997) suggests that progesterone has a major role in these events. They showed that sperm–zona binding was enhanced by >100% when spermatozoa and zona pellucida were co-incubated in the presence of 10% follicular fluid. This opens up the possibility of use of follicular fluid to potentiate IVF. It is likely that components in the human follicular fluid and digitoxin act in a similar manner, since follicular fluid has been shown to cause cholesterol efflux in capacitating spermatozoa and induce membrane changes (Langlais et al., 1988). Progesterone after binding to its receptor probably acts via a mechanism of aggregation of the cell surface binding sites (Tesarik et al., 1992b; Tesarik and Mendoza, 1993). The protein component of the progesterone–protein conjugate present in the follicular fluid may have an active role in aggregation. However, the mechanism of how the binding of progesterone to spermatozoa initiates biologically relevant cellular responses, is not known. Further studies are required to elucidate the physiological significance of binding of progesterone to the sperm membranes.

**Acknowledgements**

The authors acknowledge the University Grant Commission, India, for the awards of Junior and Senior Research Fellowships to one of the authors (MA). The authors are grateful to Dr Meena Bhattacharya, King Edward Memorial Hospital, Bombay and to Dr Tarla Nandedkar for providing the clinical material. The antiprogestins were received from Schering AG, Berlin. The authors thank Dr P.G.Satyawaroop for the generous gift of antibody against human progesterone receptor.

**References**


