[Frontiers in Bioscience, 3, d1051-1059, November 1, 1998]

NONGENOMIC EFFECTS OF PROGESTERONE ON SPERMATOZOA: MECHANISMS OF SIGNAL TRANSDUCTION AND CLINICAL IMPLICATIONS

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Received 8/7/98 Accepted 9/14/98

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1. ABSTRACT

Progesterone (P) is one of the physiological stimuli of human sperm acrosome reaction. It is present in high levels at the site of fertilization (cumulus oophorus) and has been describe to affect several sperm functions including motility, capacitation and acrosome reaction. The effects of the steroid, which is present in high levels in the cumulus matrix that surrounds the oocyte, are mediated by an increase of intracellular calcium concentrations, efflux of chloride, stimulation of activity of phospholipases and phosphorylation of proteins. These effects are due to activation of a rapid/nongenomic pathway. Two different types of receptors for P, distinct from the genomic ones, have been recently identified on the surface of human spermatozoa. The affinities of P for these receptors are respectively in the nano- and in the micromolar range. Sperm responsiveness to progesterone is impaired in subfertile patients and is strictly correlated to the ability of fertilize the oocyte. In addition, the determination of sperm responsiveness is predictive of fertilizing ability with a positive predictive value of 90% and can be clinically useful for the preliminary assessment of the male partner to select the appropriate assisted reproductive technique.

2. INTRODUCTION

Sperm life after ejaculation is characterized by two essential events for the process of fertilization, i.e. capacitation and acrosome reaction. Capacitation occurs during sperm transit in the female genital tract and consists of several biochemical and functional modifications of sperm cells ultimately leading to increased hyperactivated motility (necessary to cross the cumulus matrix that surrounds the oocyte) as well as sensitivity to physiological agonists of acrosome reaction (AR) allowing fertilization of the oocyte. Molecular, biochemical and biophysical changes occurring during capacitation as well as the signal transduction pathways activated during this complex process have been reviewed elsewhere (1,2,3). AR occurs following contact of the spermatozoon with the zona pellucida of the oocyte and consists of fusion and fenestration of the outer acrosomal membrane with the plasma membrane and release of acrosomal enzymes which aid the spermatozoon to penetrate the various investments of the oocyte. Among the different agents that stimulates sperm AR, progesterone (P) is considered to be one the physiological together with the zona pellucida protein ZP3 (4,5). Indeed, high concentrations of the steroid (ranging from 1 to 10 mg/ml) are present in the cumulus matrix that surrounds the oocyte which must be necessarily crossed by the sperm to reach the zona pellucida. In addition, P and 17α-hydroxyP present in the follicular fluid are responsible of most of the biological effects of this fluid on spermatozoa (6,7,8). Whether the effects of P on spermatozoa are important for stimulation of capacitation, onset of hyperactivated motility, induction of AR or "priming" of the cells to the subsequent action of zona pellucida proteins is still matter of discussion. All these effects of P have been demonstrated, although some of them are still matter of discussion (reviewed in 4). The effects of P are mediated essentially by three signalling pathways (see section below for specific references): rapid increase of intracellular $[Ca^{2+}]_i$, efflux of Cl^{l-}, stimulation of phospholipases and phosphorylation of sperm proteins. It is now clear the effects of P on human spermatozoa are mediated by a pathway quite distinct from the classic genomic one. Indeed, most of its effects occurs within seconds following addition of the agonist and can be induced also by a non permeant P analogue (9,10) and thus cannot be attributed to a genomic effect. The field of rapid/nongenomic effects of steroids is evolved in recent years and it is now accepted that such effects cooperate with the genomic ones in the biological responses of several cell types to these hormones (11,12). The present review will focalize on the signal transduction pathways stimulated by progesterone in human spermatozoa (schematized in Figure. 1), the type of receptor involved

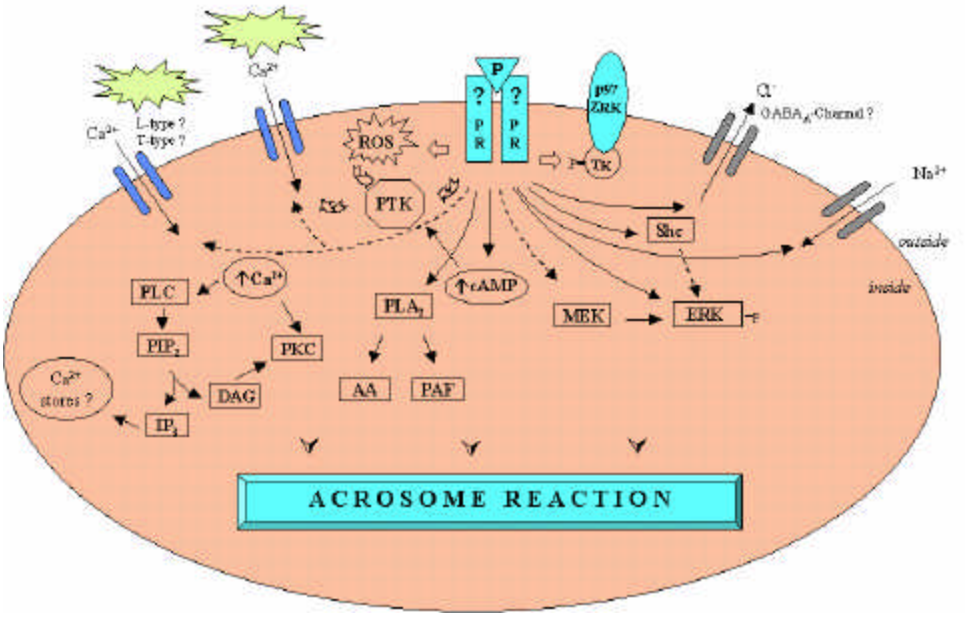

Figure. 1: Diagram illustrating the main signalling pathways stimulated by progesterone in human spermatozoa. Following interaction with specific membrane receptors present on human sperm surface, several signal transduction pathways are activated. The closed arrows indicate signalling pathways demonstrated to be activated by the steroid, while the dotted arrows indicate signalling pathways hypothesised to be involved in the response.

and on the possible physiological and clinical significance of these effects, updating previously published reviews on this topic (1,4,5).

3. PATHWAYS OF SIGNAL TRANSDUCTION

3.1. Effects on intracellular calcium and other ions

The effects of P on sperm $[Ca^{2+}]_i$ are characterized by an immediate increase of the ion (occurring within seconds after addition of the steroid) followed by a sustained increase (plateau phase) which may last for minutes (7,13,14). Both phases appear to be entirely due to influx of calcium from the extracellular space, since they are abolished if the ion in the external medium is eliminated or chelated by addition of EGTA (13,15). However, the possibility that release of calcium from putative intracellular stores evading conventional $[Ca^{2+}]_i$ monitors occurs in response to P cannot be ruled out. In particular, the finding that thapsigargin, although at high doses, elevates sperm $[Ca^{2+}]_i$ (16,17) and potentiates the effect of P (16), lead to the hypothesis that intracellular calcium stores may be present and active in sperm cells (16,18). On the other hand, P stimulates sperm phospholipase C activity (7,18-19) determining an increase of IP3 which is deputed to release calcium from intracellular stores. The recent finding of occurrence of IP3 receptors at the sperm acrosomal level (20) provide a possible target for the newly synthesized IP3. However, IP3 receptors do not appear to be active, since IP3 does not stimulate release of calcium from $45Ca^{2+}$ -loaded sperm plasma membranes vesicles or acrosome (19). Conversely, release of calcium from sperm plasma membranes vesicles is stimulated by synthetic PKC agonists, such as PMA, and from acrosomal membranes by cAMP (19). This result suggests that both the plasma and the acrosomal membranes contain calcium pumps which remove calcium from the cytosol (19), although their role during the process of acrosome reaction and calcium increase in response to P is still unclear.

The type of calcium channel mediating the influx of calcium stimulated by P is not yet known. Basically, three possibilities exist: a) voltage-dependent channels; b) second messenger-operated channels; c) progesterone receptor itself is a calcium channel.

a) Although pharmacological studies with L-type calcium channels blocking drugs suggest that voltage-dependent calcium channels (VOCs) are present in mammalian sperm and are involved in zona proteins effects (21-23), they do not seem to be involved in P-stimulated acrosome reaction. Indeed, pharmacological inhibitors of VOCs such as verapamil and nifedipine are ineffective in blocking the effects of the steroid (7,13,24). Goodwin *et al* (23) demonstrated that nifedipine was able to inhibit P-stimulated $[Ca^{2+}]_i$ increase and AR, if spermatozoa were prolonged exposed (at least 2 days) to the drug. However, this effect has been attributed to inhibition of surface expression of P receptors during capacitation rather than to blockade of P-activated calcium channels (23). Recent evidence indicate that the voltage-dependent calcium channels in mammalian spermatozoa are exclusively of the T-type (25-28). Indeed, only genes corresponding to T-type channels are present in spermatogenic cells in the mouse testis (25). In

addition, these channels are sensitive to amiloride and pimozide, while their sensitivity to L channels antagonists is from 10 to 100 times lower than that typically observed in somatic cells (26-28). At present, whether pharmacological inhibition of T-type calcium channels with pimozide or amiloride inhibits P-induced calcium increase or AR in human sperm is not known, although preliminary evidence from our lab seems to exclude the involvement of T-type channels (Bonaccorsi and Baldi, unpublished results).

b) Second messenger-operated calcium channels are activated following agonist-receptor interaction by one or more of the second messengers activated in the stimulated signalling cascade. This possibility is attractive, since calcium influx induced by zona proteins is sensitive to several inhibitors including tyrosine kinase and G proteins ones (29). However, in contrast to the effect of zona proteins, the effect of progesterone on $[Ca^{2+}]_i$ is pertussis toxin-insensitive (15,24,30) and is not inhibited by inhibitors of phospholipase C (15) or PKC (31,32). On the other hand, P-stimulated phospholipase C activation is secondary to calcium increase (7), ruling out the possibility that second messenger generated by this pathway may be involved in opening of calcium channels. The involvement of tyrosine kinase activation in the generation of calcium waves by P is a more attractive possibility, since the effect of the steroid on tyrosine phosphorylation is calcium-independent (see below). After 30 minutes incubation with genistein, Mendoza *et al* did not find modifications of calcium waves (33), while Bonaccorsi *et al* (17), with a short pre-incubation with the same inhibitor were able to demonstrate a suppression of the plateau phase. Later on, using a single cell analysis protocol to simultaneously monitor calcium influx and acrosomal exocytosis, Tesarik *et al* (34) demonstrated that tyrosine kinase inhibition strongly inhibit the secondary phase of calcium increase stimulated by the steroid which is primarily associated to induction of AR.

c) This possibility cannot be verified until membrane receptors for P will be cloned and its structure will be clarified. However, the insensitivity to pertussis toxin and to L-type calcium channels inhibitors make this hypothesis very attractive.

In addition to an increase of calcium, P stimulates chloride efflux (35,36) and sodium influx (24) causing a depolarization of sperm plasma membrane (24,37). Extracellular sodium appears to be important for development of the plateau phase of $[Ca^{2+}]_i$ influx and induction of AR by P (38). By contrast, Foresta *et al* (24) reported that P-initiated AR was unaffected in sodium-deficient medium. The effect on chloride has been attributed to stimulation of a chloride channel similar to the GABAa receptor (35-37, discussed below). Like the secondary phase of calcium influx stimulated by P, chloride efflux is also inhibited by tyrosine kinase inhibitors (36). The role of intracellular pH in the development of a normal response to P is also important. Although P itself does not modify internal pH (39), the pH of intracellular milieu must be kept alkaline in order to obtain full response to the steroid (40). Bicarbonate plays an essential role in regulating intracellular pH during capacitation, affecting the ability of spermatozoa to respond to P (40-42). Conversely, the role of cAMP in the regulation of sperm intracellular pH is discussed (40,42). On the other hand, internal sperm pH appears to be highly dependent by the external pH (39), suggesting lack of specific regulatory mechanisms.

3.2. Effects on phospholipases

As mentioned above, P stimulates phosphatidylinosotol hydrolysis (7, 18) with increased formation of diacylglycerol (DAG) and inositol phosphates. This effect is due to activation of calcium-dependent phospholipase C [other phospholipases do not appear to be involved (18)] and is inhibited by a tyrosine kinase inhibitor (43). Interestingly, previous exposure to P leads to increased DAG and inositol phosphates generation in response to zona protein 3 (18), indicating a "priming" effect of the steroid on the subsequent action of this protein on phospholipase C and subsequent AR. Phospholipase A2 activity is also stimulated by progesterone in capacitated spermatozoa (44,45). The newly generated arachidonic acid may be involved in stimulation of AR by the steroid, since arachidonate itself stimulates sperm AR (46). Besides generation of arachidonic acid, P increases also the formation of platelet-activating factor (44), an important mediator of cellular events, which is also involved (or may cooperate) in stimulation of AR by the steroid (47).

3.3. Effects on kinases

Tyrosine phosphorylation induced by P occurs in several protein bands, including one in the molecular weight range of 95-97 kDa (48-51). A protein with similar molecular weight is also phosphorylated in tyrosine during sperm capacitation (49,51,52) and following stimulation with zona proteins (53). This protein has been indicated as the possible sperm receptor for ZP3 (53). Among the other proteins whose phosphorylation is increased after stimulation with P, we have recently identified p42 extracellular-signal regulated kinase (ERK-2) (54). A 42 kDa sperm kinase with similar characteristics to ERK-2 has been also characterized by Berruti (55). We have shown that ERK-2 is activated and undergoes translocation from the neck to the equatorial segment of the sperm head following stimulation with P (54). ERK proteins are phosphorylated, activated and undergo translocation to the equatorial segment also during capacitation (56). Although they do not appear to be involved in P-stimulated AR (54), their role in the modulation of capacitation has been defined (56). In addition, translocation to the equatorial segment after AR and during capacitation (54,56) suggests a role of these proteins in the process of sperm-oocyte fusion. Phosphorylation of ERK-2 may occur through activation of ras/ERK cascade, since both ras (57) and MEK-1 (the upstream kinase responsible of ERK phosphorylation) (56) have been shown to be present in human sperm. Another sperm protein that undergoes tyrosine phosphorylation in response to P and during capacitation is p52Shc (58), a classical cytoplasmic substrate of activated tyrosine kinases in somatic cells. The

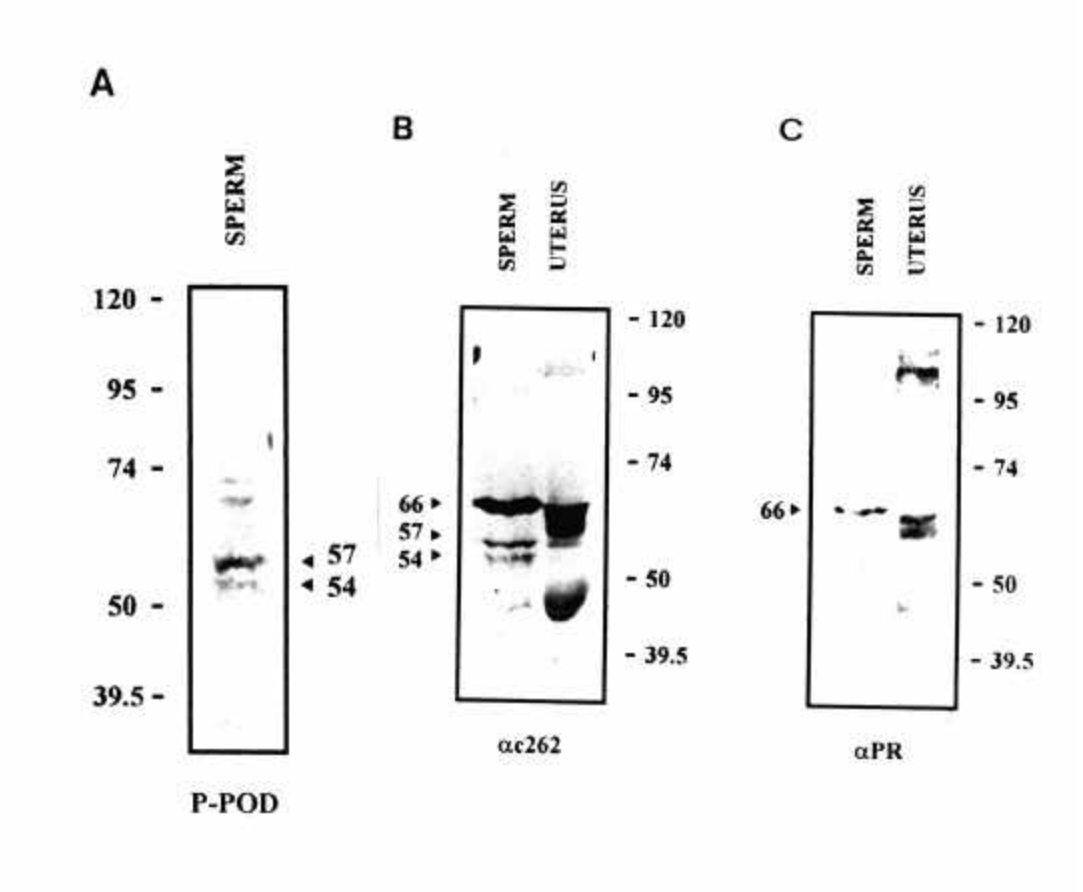

Figure. 2: A: ligand blot analysis of whole human sperm lysates using peroxidase-conjugated progesterone to reveal P-binding proteins. B and C: western blot analysis of sperm proteins with antibodies directed respectively against the ligand binding region (a -c262, B) and DNA binding region (a PR, C) of the progesterone genomic receptor. Note the identification of the same two proteins (indicated by the arrows) in panels A and B. From 68, with permission.

role of p52shc in P-mediated effects in human sperm has not yet been defined (58).

Tyrosine phosphorylation of proteins plays an essential role in stimulation of AR by P (48-50) and zona proteins (52,53), as well as in the process of sperm-zona penetration (59). The mechanism by which P stimulates phosphorylation of proteins in tyrosine residues is still unknown. We know that, at least for the 95-97 kDa band, it is calcium-independent (48, 60) and that it is inhibited by large spectrum tyrosine kinase inhibitors (49). On the other hand the p95/97 sperm receptor for ZP3 is a tyrosine kinase and resembles receptors for growth factors in somatic cells (53). However, a direct interaction of P with this protein is unlikely, since other proteins appear to be involved in the primary interaction of P with spermatozoa (see below). Recently, De Lamirande *et al* (51) have shown that reactive oxygen species (ROS) produced by spermatozoa are involved in P- and capacitation-stimulated increase of tyrosine phosphorylation. Similar results have been reported by others (61). However, the mechanism by which ROS activate this process (stimulation of kinases or inactivation of phosphatases) is not yet known and needs further investigation. Protein tyrosine phosphorylation in capacitating spermatozoa is regulated by cAMP/PKA pathway (61,62). Since P has been shown to stimulate weakly cAMP production (63), this pathway may represent an alternative route of tyrosine kinases activation by the steroid.

The presence of active protein kinase C in human spermatozoa has been well documented (for rev see 64). However, its activity in human sperm is very low (about 3% of that detected in rat brain). The possible involvement of PKC in P-stimulated AR is suggested by the rapid phosphoinositide hydrolysis stimulated by the steroid (se above) and by the direct effect of DAG and other PKC agonists on AR (64). Indeed, broad spectrum PKC inhibitors inhibit sperm AR induced by P (65,31,32). In agreement with the demonstration that its activity in spermatozoa is greatly stimulated by calcium (66), PKC activation appears to be downstream to the influx of calcium stimulated by the steroid (31,32) while conflictual results have been shown concerning its role in tyrosine kinase activation (32).

4. SPERM PROGESTERONE RECEPTOR

Although membrane perturbations induced by steroid have been reported (67), the majority of researches in the field of rapid/nongenomic actions of steroid agree about the existence of specific membrane receptors (11). In particular, in the case of P and sperm, down regulation of the $[Ca^{2+}]_i$ response observed after first addition of the steroid (15, 68) is consistent with a receptorial mechanism. In addition, induction of receptor aggregation by the agonist (69,70), a mechanism common to tyrosine-kinase-coupled receptors, the unusual steroid specificity of this receptor (71) and the lack of inhibition by classical P receptor antagonists (4, 15) are further evidence for a specific nongenomic receptor. In addition, the dissociation between the effects on calcium (dependent on tyrosine phosphorylation only in the plateau phase), on tyrosine phosphorylation (calcium-independent) and on efflux of chloride (dependent on tyrosine phosphorylation) led to the hypothesis of the presence of at least two membrane receptors for the steroid. The effect on chloride efflux, which occurs through a "unique" steroid receptor/chloride channel complex similar to the GABAA receptor (reviewed in 72), strongly indicates this protein as one of them. However, although the presence of sperm GABAA receptors has been reported (72,73), contradictory results have been obtained about the effects of GABA on human sperm acrosome reaction and on the involvement of GABA receptors on calcium influx induced by P [positive (37,74) and negative (15, 68, 75) results have been reported]. In an attempt to characterize the membrane receptors involved in the action of P, we have performed binding studies, ligand and western blot experiments on human spermatozoa (68). We demonstrated the presence of two surface receptors with different affinity for P (one in the nanomolar and the other in the micromolar range). By ligand and western blot experiments we identified two surface progesterone-binding proteins with molecular weights of 54 and 57 kDa, which appear to be involved in the biological actions of the steroid (68, Figure. 2A). In particular, the two proteins were detected using an antibody directed against the binding domain of the P genomic receptor (α-c262, Figure. 2B), while an antibody directed against the DNA binding domain did not detect any specific band (68, Figure. 2C), confirming the absence of nuclear P receptors (76). Consistent with the involvement of these two proteins in the biological effects of P, the α-c262 antibody inhibited both calcium influx (77) and AR (77,68) induced by the steroid. In agreement with these findings, mice lacking the gene for the P receptor (PRKO mice), recently generated in the Laboratory of Prof. O'Malley at Baylor College of Medicine (Houston, TX, USA) exhibited no difference in the steroid specificity of induction of AR (P was positive, testosterone, RU486, and E2 were negative), RU486 did not block progesterone effect and there were no differences in the percent of sperm that bound P-BSA (Lamb and O'Malley, personal communication).

5. CLINICAL STUDIES

The clinical significance of the biological effects of P on spermatozoa is under investigation. We and other Authors found that sperm responsiveness to P is reduced in oligozoospermic and infertile patients (78-80), suggesting a possible involvement of P in the process of fertilization. The "priming effect of P of ZP3-induced AR (18) as well its enhancing effect on oocyte penetration in infertile patients (81) are further evidence of such involvement. It is well known that medical/surgical treatment of male infertility is restricted to few clinical cases (such as hypogonadism, varicocele, epididymal obstructions), while in the cases of idiopathic infertility the only available treatment is represented by assisted reproduction techniques. At present, the appropriate technique [*in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI)] is chosen on the basis of the number of spermatozoa obtained after selection (swim up, mini-Percoll). However, in the 20-30% of cases, oocyte fertilization with IVF is not obtained even in normospermic subjects. In this light, the possibility of predicting sperm fertilizing ability or enhancing fertilization by treatment of spermatozoa *in vitro* with P (81) represent useful tools for the infertile couple. We investigated whether responsiveness to progesterone could represent a reliable test of sperm fertilizing ability by studying nearly 100 unselected couples undergoing *in vitro* fertilization. We found that both intracellular calcium increase and AR in response to P (ARPC) were significantly correlated to sperm fertilizing ability (82) and revealed to be predictive of fertlization outcome at determined cut-off values (83). In particular, we obtained positive predictive values > 90% for both intracellular calcium increase and AR inducibility respectively >1.2 fold and 7%. Both tests were highly sensitive, moderately specific and, at least for calcium determination, highly reproducible (83). Positive predictive values may rise >95% when the two tests are combined. Recently, others Authors confirmed these data (84-86). The analysis of sperm responsiveness to P is quite simple and highly specific, does not require expensive equipment and, for the evaluation of $[Ca^{2+}]_i$, is totally objective and thus independent from the operator.

In conclusion, the nongenomic effects exerted by P on human spermatozoa appears to be physiologically related to their fertilizing ability and therefore the evaluation of sperm responsiveness to the steroid may be of clinical usefulness for the preliminary assessment of the male partner to select the appropriate assisted reproductive technique.

6. ACKNOWLEDGMENTS

We thank Prof. Mario Serio (Endocrine Unit) for helpful advice and Dr. Antonio Cilotti for help in developing the HTLM file.

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Key words: Spermatoza, Testis, Signal Transduction, Tyrosione Kinase, Progestron, Hormone

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