Original Article

Progesterone Increases Capacitation and Exerts a Prosurvival Effect in Sperm via Akt Activation

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Abstract

Objective: Progesterone receptor is present in human sperm. Here, we determined if progesterone (P4) improves capacitation and stimulates sperm survival by suppressing apoptosis to evaluate its relevance to be used as a preservation agent to increase and prolong sperm viability and motility. **Materials and Methods:** Semen samples were obtained from healthy human donors. The sample was washed by Percoll gradient centrifugation and was divided into five groups of 500 µL, each containing approximately 10 million sperm cells. P4 was added to each group at the final concentrations of 0 (control), 250, 500, 750, and 1000 ng/mL, respectively, and was incubated at 37°C for 2 h. Sperm motility was assessed using a computer-assisted sperm analyzer. Tyrosine phosphorylation, Akt phosphorylation, and caspase-3 activation in sperm were analyzed by western immunoblotting. **Results:** P4 did not increase the sperm motility. Western blot analysis revealed that P4 at the concentration of 500 ng/mL increased tyrosine phosphorylation, indicating increased sperm capacitation. P4 at 750 mg/ml also increased Akt phosphorylation. Interestingly, the activation of Akt was associated with a decrease in caspase-3 activity, indicating the prosurvival effect of P4. **Conclusion:** This study results suggest that P4 does not influence sperm motility; however, it increases sperm tyrosine phosphorylation (at a specific concentration of 500 ng/ul), activates AKT (Protein kinase B), and suppresses caspase-3 (at a specific concentration of 750 ng/ul). The biochemical effects of P4 on sperm may warrant its use as a preservation agent to increase sperm viability.

Keywords: Akt, caspase-3, progesterone, sperm survival, tyrosine phosphorylation

Introduction

Infertility affects an estimated 15% of couples globally, and 50% of infertility cases are caused by male factors.[1] Assisted reproductive technology aims to overcome male infertility, but this method is often constrained by low sperm motility and survival.[2] Sperm cells are transcriptionally inactive, and the development of immature cells into mature cells capable of fertilizing the oocyte relies heavily on changes or modifications of pre-existing proteins.[3] Proteomic analysis of sperm cells is therefore essential for identifying proteins responsible for declining sperm quality and for identifying target proteins for regulating male fertility.[4] Proteomic analyses of human, mouse, and rat sperm have been conducted in the past 10 years.[5-7] These studies revealed the presence of some unexpected protein receptors on the surface of human sperm, including tyrosine kinase, glutamate-gated ion channel, and nongenomic progesterone receptors.[8] The presence of these receptors indicates a role for their ligands in the initiation of the biochemical pathways that regulate sperm functions. Previous studies have reported that P4 receptors were located on the sperm membrane, raising the question of whether P4 may affect the process of capacitation, indicated by global tyrosine phosphorylation in the sperm cells. Moreover, a recent study reported that progesterone has a survival effect on glioblastoma multiforme cells.[9] In this study, we aimed to determine if P4 affected sperm capacitation and stimulated a prosurvival effect by suppressing the apoptotic process in sperm cells.

Materials and Methods

Sperm preparation and P4 treatment

Normozoospermic samples were obtained from healthy volunteer donors by masturbation after at least 2 days of

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abstinence. The collection of human sperm samples was approved by the Biomedical Research Ethics Committee, Faculty of Medicine, Universitas Indonesia (No. 303/UN2. F1/ETIK/III/2018). Nine normozoospermic subjects were recruited, and semen samples were collected in sterile containers and were allowed to liquefy at room temperature for 15 min. Sperm was purified using discontinuous Percoll gradient centrifugation at 400 g for 30 min. The supernatant was discarded, and the pellet was resuspended in Biggers, Whitten, and Whittingham medium (BWW) and then was centrifuged again at 400 g for 15 min. The supernatant was discarded again and the sperm pellet was re-suspended in 1 mL of BWW and the concentration was measured. Sperm samples were divided into five groups of 10×10^6 cells in 500 µL of BWW medium: a control group without P4 and four experimental groups treated with 250, 500, 750, and 1000 ng/mL of P4 hormone (Sigma, MO, USA), respectively. The samples were incubated for 2 h at 37°C.

Sperm motility

Sperm motility was examined by using a computer assisted sperm analyzer (CASA) IVOS I machine (Hamilton Thorne, MA, USA). Ten microliters of sperm samples in medium BWW was added into examination chamber (Leja, The Netherlands) and inserted into the CASA machine. Sperm kinetic parameters examined in this study were average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL).

Protein isolation

Sperm cells were centrifuged at 5600 g for 5 min. The supernatant was removed and the pellet was dissolved in 50 µL sodium dodecyl sulfate (SDS) extraction buffer (2% SDS, 10% sucrose, 0.1875 M Tris pH 6.8, protease inhibitor). The sample was heated for 5 min at 100°C and then was centrifuged again for 10 min at 9000 g. The supernatant containing protein was transferred to a new tube, and the protein concentration was measured by the Bradford assay. The concentration of the sample was measured using NanoDrop 2000 spectrophotometry (Thermo Scientific, Waltham, MA, USA). Protein samples were stored at −20°C.

Western immunoblotting

The effects of P4 on tyrosine phosphorylation and activation of Akt and caspase-3 in sperm were measured by western immunoblot analysis. Sperm proteins (15 µg) were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was blocked by 3% bovine serum albumin for 1 h at room temperature and subsequently incubated with primary antibody overnight at 4°C. Tyrosine phosphorylation was detected using mouse anti-phosphotyrosine antibody (Sigma-Aldrich, St.Louis, MO, USA) at 1:500 dilutions. Apoptosis was detected using mouse anti-caspase-3 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 1:200 dilutions and Akt phosphorylation using anti-phospho-Akt (ser473) rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 dilutions. The following day, the membranes were washed three times with 1 ×Tris Buffered Saline with Tween 20 (TBST) for 5 min each and then were incubated with a secondary antibody which is rabbit anti-mouse IgG-conjugated horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Dallas, TX, USA) at 1:200 dilutions for 1 h at room temperature. Donkey anti-rabbit IgG HRP (1:200; Santa Cruz Biotechnology, Dallas, MA, USA) was used to detect the Akt phosphorylation. The membrane was then washed again with 1x TBST (3 min × 5 min), and complex antigen-antibody was visualized using western immunoblot detection system (GE Healthcare, Chicago, IL, USA). The signal was read and analyzed using ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, USA).

Statistical analysis

The data were analyzed by one-way ANOVA using SPSS 16 software (IBM Corporation, Armonk, NY, USA). *Post hoc* differences between groups were analyzed by the least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Sperm motility

The result showed that progesterone increased VAP, VSL, and VCL along with increasing P4 concentrations. The three velocity parameters reached the highest velocity at a P4 concentration of 750 ng/ml. However, at the concentration of 1000 ng/ml, all velocity parameters decreased. Although there were slightly increases in motility parameters (VAP, VSL, and VCL), especially at the concentration of 250–750 ng/ml, P4 did not seem to have a significant effect on sperm motility [Table 1].

Tyrosine phosphorylation during sperm capacitation

Sperm capacitation is characterized by an increase in intracellular cyclic adenosine mono phosphate (cAMP), leading to the activation of protein kinase A (PKA). This PKA will, in turn, cause global tyrosine phosphorylation on various sperm proteins that lead to hyperactivation. We assessed tyrosine phosphorylation on human sperm after being treated with progesterone to investigate whether progesterone affects

Table 1: Effects of progesterone on sperm motility

Progesterone concentration (ng/mL)	VAP (a) (µm/sec)	VSL (b) (µm/sec)	VCL (c) (µm/sec)	*P*
0	29.2±9.49	24.7±6.91	51.9±14.09	0.248 (a)
250	32.4±7.17	23.2±7.97	54.4±10.71	0.308 (b)
500	33.3±12.07	25.8±11.05	55.6±19.43	0.426 (c)
750	40.6±11.67	33.3±13.14	64.9±16.25	
1000	36.1±12.73	27.1±11.81	62.2±20.95	

Values given as mean±SE. Column (a) indicates the difference in VAP between the control and P4 treatment groups (250, 500, 750, and 1000 ng/ml); (b) indicates difference in VSL between the control and P4 treatment groups; (c) indicates the difference in VCL between the control and P4 treatment groups. SE: Standard error, VCL: Curvilinear velocity, VSL: Straight line velocity, VAP: Average path velocity

sperm capacitation. Western immunoblotting analyses showed an increase in tyrosine phosphorylation at the concentration of 500 ng/ml compare to the control. This indicates that P4 at 500 ng/ml stimulates sperm capacitation [Figure 1a, upper panel]. A 1.6-fold increase in tyrosine-phosphorylated protein bands (molecular weight range: 45–120 kDa) was observed in sperms treated with 500 ng/mL P4 compared with controls [Figure 1b, black bars].

Caspase-3 activity

Apoptosis is the default pathway for sperm after undergoing capacitation and fertilization. Proteins secreted from the epididymis as decapacitation factors, which prevent sperm from undergoing premature capacitation. We investigate whether progesterone can prevent sperm cells from entering apoptosis. Caspase-3 was activated by P4 at 500 ng/ml, whereas at the concentration of 750 ng/ml, a decrease in caspase-3 was observed [Figure 1a, middle panel]. A significant change in caspase-3 was observed at the concentration of P4 = 500 ng/ml ($P < 0.05$) [Figure 1b, gray bars].

Phosphorylation of Akt

AKT, also known as protein kinase B, is an important factor in cell survival and apoptosis inhibition. Western blot analyses using antibodies recognizing phosphorylated AKT showed an increase phosphorylation at the concentration of P4 = 750 ng/ml [Figure 1a, lower panel]. Concentration of progesterone 750 ng/ml seems to be the optimum amount of progesterone to activate the sperm prosurvival pathway via AKT activation. A significant increase in AKT phosphorylation was observed at P4 = 750 ng/ml [Figure 1c].

Discussion

In this study, progesterone slightly increases VAP, VSL, and VCL at the concentration of 750 ng/ml. However, in general, P4 did not significantly increase sperm motility. P4 is known to increase sperm motility even at low concentrations (~3 ng/mL).[10] Capacitation allows the sperm to undergo hyperactivation, interact with the zona pellucida, undergo the acrosome reaction, and initiate fusion with the oocyte.[11] The process of capacitation among other biochemical events is characterized by increased motility and phosphorylation of tyrosine residues.[12] Protein phosphorylation, controlled by the activity of protein kinases acting on serine, threonine, or tyrosine residues, is an important aspect of capacitation. Sumigama *et al*. demonstrated that P4 caused massive calcium influx in human sperm by activating the CatSper calcium channel, thus increasing sperm binding to the zona pellucida and triggering the acrosome reaction.[13] CatSper is located in the mid-piece of the sperm and plays an important role in hypermotility. Although there are many indicators of sperm capacitation, in our study, we used tyrosine phosphorylation as a capacitation indicator. The results showed that P4 (25–1000 ng/mL) had a dose-dependent effect on tyrosine phosphorylation. P4 is a steroid component of sperm and the female reproductive apparatus, which affects the penetration of sperm into the ovum. Semen contains P4 at a concentration of 0–2 ng/mL.[14]

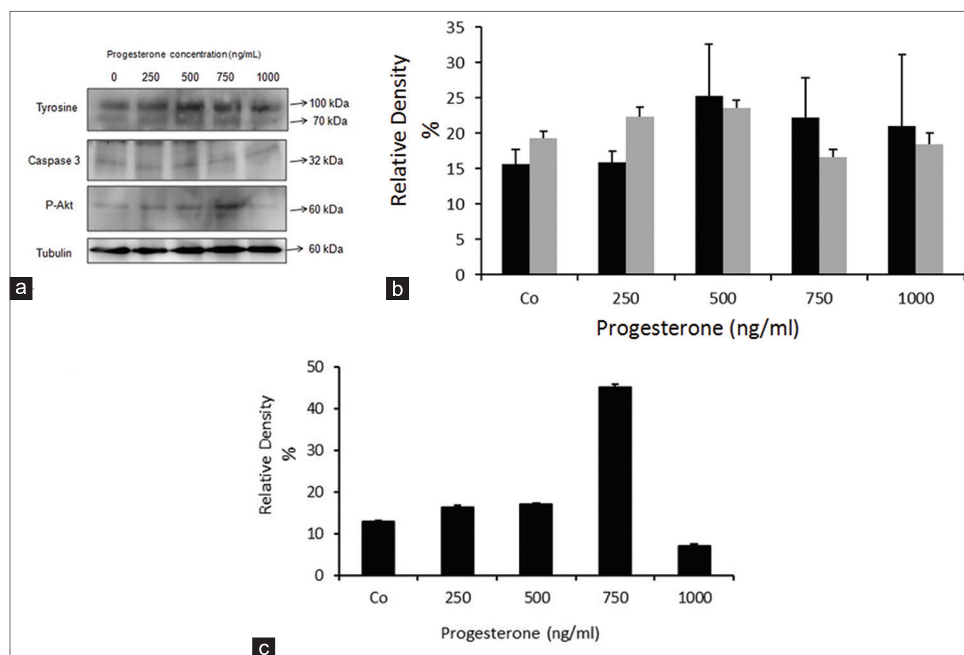

Figure 1: (a) Effects of P4 on tyrosine phosphorylation, caspase-3, and Akt phosphorylation were analyzed using western immunoblotting. Sperms were treated with progesterone (P4) at different concentrations indicated on top of the figure. P4 increased tyrosine phosphorylation and caspase-3 at P4 = 500 ng/mL. P4 stimulated Akt activation at P4 = 750 ng/ml at the same dose when caspase-3 activation decreased. (b) Band intensities for tyrosine phosphorylation (black bars) and caspase-3 activation (gray bars) were measured using ImageJ software. It showed an increase in tyrosine phosphorylation and caspase-3 at P = 500 ng/ml. (c) Band intensities of Akt phosphorylation in human spermatozoa after P4 treatment indicating a significant increase at a concentration of P = 750 ng/ml

Previous studies showed that P4 had antiapoptotic effects, depending on the cell type studied. A prosurvival effect of P4 associated with apoptotic suppression has been investigated in the heart, retina, granulosa cells, corpus luteum, mammary glands, and skin.[15-20] In this study, we investigated the prosurvival effect of P4 in sperm via apoptotic suppression. Pujianto *et al*. showed that increased sperm motility could also occur through the activation of Akt, which was one of the proteins involved in the suppression of apoptosis (prosurvival factor) in sperm after prolactin treatment.[21] In the current study, the treatment of sperm cells with P4 for 2 h showed an increase in caspase-3 at a dose of 500 ng/ml. These results were in accordance with the results reported by Lozano *et al*.,[22] using TUNEL and fluorometric assays. P4 is known to increase free Ca^{2+} concentrations in the cytosol, resulting in calcium overload in mitochondria where it can activate caspase-3 and phosphatidylserine externalization. P4 stimulates apoptosis by activating caspase-9, as the initiator of the intrinsic pathway of apoptosis in mitochondria.[23]

P4 in human sperm acts through the Akt signaling pathway, and the dose-dependent prosurvival effects of P4 in sperm involve maintaining survival and genomic integrity and suppressing caspase activation via mechanisms involving phosphoinositide 3-kinase (PI3K)/Akt phosphorylation. P4-induced Akt activation is a calcium-dependent process involving CatSper activation of the required PI3K-Akt pathway for motility and hyperactivation.[24] Gallardo Bolaños *et al*. revealed that Akt/protein kinase B promoted cell survival by preventing the apoptotic cascade,[25] while Li *et al*. showed that Akt inhibits the activation of caspase-9 and caspase-3.[26] Interestingly, our data showed that P4 stimulates sperm survival associated with increased Akt phosphorylation at a dose of 750 ng/ml when caspase-3 decreased significantly. The results suggest that P4 activates Akt pathway leading to the release of apoptosis inhibitory protein from binding with BAD, the pro-apoptotic Bcl2 family. Thus, apoptotic cascade is prevented from starting. In conclusion, progesterone (P4) administration *in vitro* into human sperm did not significantly increase VCL, VSL, and VAP. However, P4 increased sperm tyrosine phosphorylation, decreased caspase-3 activity, and increased Akt phosphorylation, supporting its role as a prosurvival factor. Thus, P4 increases sperm capacitation and stimulates a prosurvival effect on sperms.

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Conflicts of interest

There are no conflicts of interest.

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