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Desalted and lyophilized seminal plasma increases protein tyrosine-phosphorylation of frozen-thawed bull spermatozoa incubated with a cell-permeable cyclic AMP (cAMP) analog (cBiMPS)

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Abstract

The present study investigates the effect of desalted seminal plasma (SP) added to semen extender on hyperactivated motility and protein tyrosine-phosphorylation (PTP) of bull spermatozoa. The SP was harvested by centrifugation and desalted using Sephadex G-25 columns in order to be added to semen extender at 0 (control), 2.5, 12.5 and 25 mg/ml. Frozen-thawed spermatozoa were incubated with a cell-permeable cyclic AMP (cAMP) analog (cBiMPS) and examined subjectively for hyperactivated motility and for PTP by Western blotting. Although, the added SP sustains sperm motility at all incubation times especially in the presence of cBiMPS but without significant difference from the control samples. Moreover, total sperm motility of 12.5 and 25 mg/ml in the presence of cBiMPS at 60, 120 and 180 min were similar ($P \geq 0.05$). Surprisingly, cBiMPS-incubated spermatozoa in the presence of desalted SP were capable of exhibiting hyperactivated motility. Addition of SP increased and prolonged intracellular cAMP-induced PTP and in total 21 phosphorylated proteins with molecular weight ranging from 10 to >230 kDa were detected. The most prominent tyrosine-phosphorylated proteins (TPPs) were of 32, 38, 74 and 80 kDa which were more predominant in fertile bulls than subfertile bull. Furthermore, TPPs of 45 and 48 kDa were cBiMPS-dependent in fertile bulls whereas, in subfertile bull the latter was barely detectable and the former was cBiMPS-independent at only 0 min. This increase in PTP not only emphasizing the beneficial roles of desalted SP but excluding any detrimental effect of it on sperm cell functions during storage as well.

Key Words: Bicarbonate; cAMP; Desalted SP; Hyperactivation; Protein tyrosine-phosphorylation

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1. Introduction

Mammalian spermatozoa have their own particular idiosyncrasies as highly specialized cells. They are highly compartmentalized, transcriptionally inactive and unable to synthesize new proteins[38]. However, the reliance of mammalian spermatozoa on protein tyrosine-phosphorylation (PTP) as a means of altering their function is greater than in many other types of cell[38]. The sperm cell functions well known to associate with PTP are capacitation, hyperactivated motility, acrosome reaction and sperm-oocyte fusion[38].

Hyperactivated motility is a pattern of sperm flagellar movement characterized by an intensive and asymmetric whiplash beating of the middle and principal pieces of the sperm flagellum, which generates a strong driving force to penetrate extracellular matrix of oocytes[41]. Hyperactivated motility has been observed *in vitro* in various mammalian species especially if capacitation of spermatozoa was induced with Ca^{2+} containing capacitation media[41]. For instance, it was effectively induced *in vitro* in boar spermatozoa by incubation with a cell-permeable, phosphodiestrase-resistant cyclic AMP (cAMP) analog, "Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3´,5´-monophosphorothioate" (cBiMPS)[13]. During incubation, both the capacitation of sperm head and the tyrosine-phosphorylation of flagellar proteins were enhanced coincidently with the transition of motility[13]. These findings suggest that their simulation system can mimic intracellular changes leading to hyperactivation and PTP in *in vitro*-capacitated boar spermatozoa. Similar effects of cBiMPS on the transition of normal motility to hyperactivated motility were reported in frozen-thawed fertile and subfertile bull spermatozoa[28].

There are several cAMP signaling cascades that regulate hyperactivated motility of boar spermatozoa such as treatment with cBiMPS which activate protein kinase A (PKA) and PTP with a time lag of a few hours, via activation of protein tyrosine kinases in the connecting and principal pieces. Interestingly, tyrosine-phosphorylation is greatly enhanced in many flagellar proteins of boar spermatozoa coincident with hyperactivated motility[13].

Undoubtedly, freezing and thawing procedures are stressful to spermatozoa, diminish motility and cause membrane changes, including sperm capacitation and acrosome reaction[26]. There is substantial evidence that cryopreservation promotes capacitation-like changes in spermatozoa[30] leading to decreased fertilizing capacity of frozen semen in the female reproductive tract[39]. Sperm capacitation has been associated with an increase in the phosphorylation in tyrosine residues of several sperm proteins[38]. Phosphorylation of tyrosine residues on sperm proteins is one important intracellular mechanism regulating sperm function that is a meaningful index of capacitation[43]. Noteworthy, tyrosine-phosphorylation of proteins has also been observed in frozen-thawed bull[4] and boar[34] spermatozoa, suggesting that the cryopreservation procedures also induce PTP.

The post-thaw addition of seminal plasma (SP) to sperm increased motility and fertility in ram[24] and positively affect sperm quality in boar[10]. Moreover, although detrimental during cryopreservation, the post-thaw addition of SP to stallion sperm increased *in vivo* fertility[14]. SP contains several small molecules that bind to specific receptors on the sperm plasma membrane and act as first messengers causing biologically important changes in availability of the second messenger cAMP wherein; the most important one of these molecules is bicarbonate (HCO_3^-)[31]. Boatman and Robbins (1991)[7] found that HCO_3^- is essential for both capacitation and hyperactivation of hamster spermatozoa *in vitro*, but hyperactivation required higher concentrations of HCO_3^- than capacitation. It has long been believed that Ca^{2+} and cAMP are essential elements of the signaling cascades that regulate the expression of sperm fertilizing ability[17]. Because, the cAMP is produced from ATP mainly by soluble adenylyl cyclase (sAC) that stimulated directly via HCO_3^- and Ca^{2+} during capacitation process[17].

Impairment of acrosome reaction and/or hyperactivated motility might be cause fertilization failure leading to infertility or subfertility. Since, Ca^{2+} ionophore A23187-induced acrosome reaction and cBiMPS-induced hyperactivated motility may serve as useful tools to differentiate between fertile and subfertile bulls[28,29]. Moreover, premature capacitation (cryocapacitation), which is an important factor responsible for subfertility of frozen-thawed bull spermatozoa[18], could be reduced by adding desalted and lyophilized SP to semen extender[3]. Thus, it is necessary to investigate the effect of this SP powder on the hyperactivated motility and on the global PTP state of frozen-thawed fertile and subfertile bull spermatozoa in an attempt to exclude any detrimental effect and/or to detect any beneficial effect of this SP powder on sperm cell functions.

2. Materials and Methods

2.1. An animal use ethics statement: This study was approved by the Institutional Committee of Animal Experiments of Gifu University (Permission number: 12-10-10) and all experiments were carried out in accordance with the Gifu University Animal Experimentation Regulations.

2.2. Chemicals and reagents: Unless otherwise stated, all chemicals and reagents were procured from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Wako (Osaka, Japan).

2.3. Media: Tris based cryoprotective diluent consisting of 130 mM Tris, 29.8 mM sodium citrate, 41.3 mM lactose, 50.4 mM raffinose, 20% (v/v) egg yolk, 7% (v/v) glycerol, 0.6 mg/ml streptomycin and 600 IU/ml penicillin G potassium[21], was used for semen extension before freezing. The saline medium used for dilution of spermatozoa consisting of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes adjusted to pH 7.55 at 25°C with NaOH[32]. Saline medium containing 275 mM sucrose instead of NaCl was used for washing spermatozoa and designated as sucrose medium[32]. Both media also contained 0.1% (w/v) polyvinyl alcohol (PVA; molecular weight 30,000–70,000; Sigma, USA) and 0.1% (w/v) polyethylene glycol (PEG; Sigma, USA).

Brackett and Oliphant (BO)-Hepes medium[18], BO medium[8] modified by adding Hepes instead of sodium HCO_3^-, was used for incubation of spermatozoa in the experiments of hyperactivation. It consists of 112 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2$, 0.52 mM $MgCl_2$, 0.83 mM NaH_2PO_4, 37 mM Hepes, 13.9 mM glucose, 1.25 mM sodium pyruvate, 100 IU/ml potassium penicillin G and 0.1% (w/v) PVA, pH 7.55, at 20°C. For washing spermatozoa, BO-Hepes medium that did not contain $CaCl_2$ was used. BO-Hepes medium that contains 10% sucrose medium (BO-Hepes/sucrose) was used for adjustment of sperm concentration before incubation with cBiMPS. A stock solution of 10 mM cBiMPS (Biomol International, L.P., Plymouth Meeting, PA, USA) in 10% (v/v) dimethyl sulfoxide (DMSO) was prepared, and then added to the incubation medium to give a final concentration of 100 μM.

2.4. Collection and processing of SP: Semen was collected from four mature Japanese Black bulls (1.5 to 6.5 years-old) kept at Hida Beef Cattle Research Department, Gifu Prefectural Livestock Research Institute, Takayama, Japan. Two ejaculates from each bull were collected using an artificial vagina once for 4 occasions. From each ejaculate an aliquot (100 μl) of semen was used for standard semen analysis. The percentage of progressive motility was determined subjectively under a phase contrast microscope, and pH paper was used to determine pH of semen. Sperm concentration was counted by haemocytometer. Both % abnormal morphology and % intact-acrosome was determined with Glutaraldehyde fixation method according to Almadaly *et al.* (2012)[1]. All these ejaculates had $\geq$ 80% progressive motility, $> 10 \times 10^8$ sperm/ml, $<$ 10% abnormal morphology, $>$ 95% intact-acrosome and normal pH (6.4 to 6.8). These bulls had a field fertility

rate of more than 60% after AI with frozen-thawed semen. Immediately after collection, the collected semen was separated into spermatozoa and SP by centrifugation at 3,000×g for 15 min at 5°C; the supernatant (SP) per each ejaculate was collected, combined with that of the second ejaculate of the same bull and transferred to the laboratory at 5°C. The collected SP were centrifuged again at 12,000×g for 30 min at 4°C to eliminate the remaining spermatozoa and particulate debris. SP was desalted as described in our recent investigation[3], using gravity protocol according to the manufacturer's recommendations. To reduce individual variations, SP from four bulls was pooled, desalted and lyophilized. Briefly, the collected SP were pooled, mixed well and divided into known volumes in a 15 ml sterile cryogenic vial of known empty weight. Cryogenic vials were labeled and kept at −30°C overnight before inserted into the freeze-drying machine (FDU-1200; Tokyo Rikakikai Co., Ltd., Tokyo, Japan), previously stabilized at −50°C. After 24 h of freeze-drying, the vials containing the SP powder were tightly capped and weighed to determine the obtained amount of lyophilized SP powder. Lyophilization was done four times; desalted and lyophilized SP (designated as SP powder) was kept at −30°C until being added to the cryoprotective diluents before semen cryopreservation.

2.5. Supplementation of the cryoprotective diluent with SP powder and cryopreservation: One ejaculate was collected from four other mature Japanese Black bulls (2.5 to 5.5 years-old) using an artificial vagina. These bulls had more than 60% field fertility rate, a fact confirmed after AI. Similarly, one ejaculate was collected from one subfertile bull (3.5 years-old) of field fertility rate less than 30%. Each ejaculate was supplemented with four concentrations of SP powder before cryopreservation. Frozen straws prepared from these bulls were used to investigate the effect of SP powder on hyperactivation and PTP. One ejaculate from four fertile bulls and from only one subfertile bull, in total five ejaculates were diluted with Tris-based cryoprotective diluents[21], supplemented with SP powder, frozen and stored as in our recent report[3]. Briefly, ejaculated semen of each bull was initially diluted immediately after collection with glycerol-free Tris-based cryoprotective diluent at 37°C. Initial dilution was completed after cooling to 4°C for at least 2 h to produce a sperm concentration of $\approx 2 \times 10^8$/ml. The glycerolized portion of a Tris-based cryoprotective diluent containing four different concentrations of SP powder was used for the second dilution to produce a sperm concentration of $\approx 1 \times 10^8$/ml. Total protein content (g/dl) of the four different concentrations of each ejaculate was measured by refractometer and adjusted to 0 (as a control), 2.5, 12.5 and 25 mg/ml of diluted semen as described by Almadaly *et al.* (2015)[3]. Diluted semen of four different concentrations of SP powder for each bull was obtained. Dilution of semen with concomitant addition of SP powder was performed at 4°C. After equilibration at 4°C for at least 2 h, diluted semen was loaded into 0.5 ml straws. Cooled straws were frozen in liquid nitrogen vapor, 5 cm above liquid nitrogen, for 10 min and then the straws were plunged into liquid nitrogen for storage and transported to the laboratory for analysis. Supplementation of fresh semen with SP powder and subsequent cryopreservation was performed in Hida Beef Cattle Research Department, Gifu Prefectural Livestock Research Institute, Takayama, Japan.

2.6. Washing of spermatozoa: For each concentration of SP powder within the same bull, four straws of frozen semen were thawed at 39°C for 1 min in a water bath. Frozen-thawed semen was centrifuged at 830×g for 6 min then the supernatant was discarded and the resultant sperm pellet was resuspended in 500 µl saline medium containing 0.1% PVA and 0.1% PEG. After gentle mixing, the spermatozoa were washed through a sucrose medium by centrifugation at 400×g for 5 min followed by 1,000×g for 12 min[32]. The supernatant was discarded, and

the pelleted spermatozoa were resuspended in BO-Hepes medium without $CaCl_2$. After gentle mixing, the sperm suspension was centrifuged at 800×g for 8 min to obtain a sperm pellet and the sperm concentration in the resulting pellet was adjusted to 4×10^8 cells/ml using BO-Hepes/sucrose. All the washing procedures were performed at an ambient temperature of 20–25°C.

2.7. Incubation of spermatozoa with cBiMPS: The washed spermatozoa were pre-warmed for exactly 5 min at 38.5°C before resuspended in the incubation medium to adjust a final sperm concentration of 1×10^8/ml. Washed spermatozoa were incubated in a water bath (38.5°C) in air for 180 min. The incubation (BO-Hepes) medium contained 2.25 mM $CaCl_2$ and 0.1 mM cBiMPS during incubation[28]. To the control samples without cBiMPS, the same volume of 10% (v/v) DMSO was added to equalize the concentration of solvent. During incubation sperm suspensions were gently mixed well and then aliquots of them were recovered for the use in the following experiments:

2.8. Assessment of sperm motility: Sperm motility was subjectively assessed at specific intervals of 0, 60, 120 and 180 min of incubation. Briefly, aliquots (10 μl) of sperm suspension were taken and applied onto a glass slide prewarmed at 38.5°C. The sperm aliquots were coverslipped (18 mm × 18 mm), and sperm motility was examined at 200x magnification with a phase-contrast microscope (Olympus BX41, Tokyo, Japan), equipped with an automatic warming plate for total motility and swimming trajectories and at 400x for flagellar beating (symmetrical and asymmetrical) patterns. Total motility % includes all motile spermatozoa irrespective of their progressive motility. The swimming patterns of the motile spermatozoa were classified into 4 categories as following: straight forward swimming (% progressive motility), non-linear swimming (% non-linear motility), circular swimming with tails beating asymmetrically (% circles) and spermatozoa staying in a local area with vigorous whiplash flagellar beating (% whiplash). The latter 2 patterns were considered to be hyperactivated motility[23] and the percentage of each pattern to the total motile spermatozoa was calculated and expressed as % C and % W, respectively. Swimming spermatozoa with symmetrical flagellar beating were considered to have activated motility and were not considered to be hyperactivated.

2.9. SDS-PAGE and Western blotting: SDS-PAGE and Western blotting were performed as described previously[13] with slight modifications. Each aliquot (60 μl; sperm concentration: 1×10^8 cells/ml) of sperm suspensions was mixed with an equal volume of a double-strength sample buffer (pH 6.8) composed of 125 mM Tris-HCl, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.02% (w/v) bromophenol blue[19] and then incubated in a boiling water bath for exactly 5 min. The boiled samples were clarified by centrifugation at 10,000×g for 5 min at 4°C. After centrifugation, 20 μl of sperm extract obtained from 1×10^8 sperm cells was loaded on each lane of 10% polyacrylamide gel with laemmeli's buffer system[19]. Prestained SDS-PAGE broad range standards (10–230 kDa, BioLabs, New England) were used as molecular mass standards. Immediately after electrophoresis, the separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Immobilon-P®, Millipore, USA) in a semi-dry transfer cell for exactly 1 h at 2 mA/cm^2 in a transfer buffer composed of 48 mM Tris, 39 mM glycine and 20% (v/v) methanol[6] supplemented with 0.13 mM SDS.

The blotted membrane was blocked with 10% (v/v) fetal calf serum (FCS, Invitrogen Corp, Carlsbad, CA, USA) in PBS containing 0.1% (v/v) Tween-20 (PBS-Tween) for 60 min. Mouse anti-phosphotyrosine mAb (Clone 4G10, Upstate Cell Signaling Solutions, Charlottesville, VA, 1 : 10,000) was appropriately diluted with PBS-Tween containing 5% (v/v) FCS, and incubated with the membrane for 180 min. After washing three times for 10 min each in PBS-Tween, the

Table 1. Total motility of frozen-thawed fertile bull spermatozoa incubated at 38.5°C in the presence of either DMSO or cBiMPS

Conc. of SP (mg/ml)	No. of replicates	Total motility (%), incubation time (min)						
		0	60		120		180	
		DMSO	DMSO	cBiMPS	DMSO	cBiMPS	DMSO	cBiMPS
0	4	68.7 ± 3.1^{a}	65.0 ± 3.5^{ab}	72.5 ± 4.7^{A}	60.0 ± 2.0^{ab}	66.2 ± 5.9^{AB}	51.2 ± 4.2^{b}	$61.2 \pm 4.2^{B*}$
2.5	4	72.5 ± 2.5^{a}	70.0 ± 2.0^{a}	77.5 ± 1.4^{A}	65.0 ± 4.5^{a}	72.5 ± 1.4^{AB}	55.0 ± 3.5^{b}	66.2 ± 1.2^{B}
12.5	4	75.0 ± 2.0^{a}	70.0 ± 0^{a}	76.2 ± 2.3^{A}	67.5 ± 1.4^{ab}	71.2 ± 3.1^{A}	60.0 ± 3.5^{b}	63.7 ± 4.7^{AB}
25	4	67.5 ± 1.4^{a}	66.2 ± 1.2^{a}	73.7 ± 2.3^{A}	63.7 ± 2.3^{a}	$71.2 \pm 2.3^{A*}$	56.2 ± 2.3^{b}	$68.7 \pm 1.2^{AB*}$

Values were presented as mean ± SEM.
DMSO = Dimethyl sulfoxide; cBiMPS = Cell-Permeable cAMP Analog; SP = Seminal plasma.
Values bearing at least one common non-capitalized or capitalized superscript within the same column and within the same row were non-significantly different (Repeated measures ANOVA- Bonferroni's multiple comparison test, $P < 0.05$).
Within the same incubation time values bearing asterisk were significantly different (paired t-test, $P < 0.05$).

membrane was blocked in PBS-Tween containing 10% (v/v) FCS for 60 min and then treated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (1 : 5,000, Dako Cytomation Denmark A/S, Glostrup, Denmark) in the blocking buffer for 60 min. After washing three times, peroxidase activity was visualized using Western blotting Luminol Reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Western blots were scanned with an image analysis system with Image Master 1D Elite software Version 3.0 (GE Healthcare).

2.10. Statistical analyses: All the experiments were repeated at least five times (4 fertile + 1 subfertile) and normally distributed data were analyzed by ANOVA. Results are expressed as the means ± SEM. All the percentages were transformed into arcsin of square root [Y = sqrt(Y)] then subjected to repeated measures ANOVA. When F-test results were significant in ANOVA individual means were further tested by Bonferroni's multiple comparison test[27]. The proportion of total motility of cBiMPS-incubated and DMSO-incubated spermatozoa was subjected to Student's paired t-test. A probability of $P < 0.05$ was set as significance level. All analyses were carried out using a statistical software program (GraphPad Prism Version 5.0; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Sperm motility

Table 1 showed the total motility of fertile bull spermatozoa frozen stored with or without SP powder and incubated in the absence (DMSO) or presence of cBiMPS. However, the added SP powder sustained sperm motility in comparison to the control samples but without significant ($P \geq 0.05$) differences at all incubation times either with or without cBiMPS. Although, in the four concentrations of SP powder the total motile sperm % at 60, 120 and 180 min incubation with cBiMPS were apparently higher than those of the corresponding concentrations without cBiMPS but with no significant difference except at 120 and 180 min incubation for 25 mg/ml and at 180 min incubation for 0 mg/ml as presented in Table 1. Regarding the effect of incubation time on the total motility, our results revealed that the total motility of spermatozoa incubated without cBiMPS at 0 min was significantly ($P < 0.05$) higher than at 180 min but at 60 min the higher three (2.5, 12.5 and 25 mg/ml) concentrations of SP powder were greater than at 180 min. On the other hand, the total motility of spermatozoa incubated with cBiMPS either in the presence of 12.5 or 25 mg/ml SP powder at 60, 120 and 180 min incubation were similar ($P \geq 0.05$, Table 1).

3.2. Hyperactivated motility

Fertile bull spermatozoa frozen stored with

Table 2. Proportion of circles (% C) of frozen-thawed fertile and subfertile bull spermatozoa incubated with or without SP powder in the presence of cBiMPS

Conc. of SP (mg/ml)	Bull	No. of replicates	% C, incubation time (min)		
			60	120	180
0	Fertile	4	60.0 ± 4.5^{a}	49.0 ± 7.6^{a}	35.7 ± 5.3^{b}
	Subfertile	1	33.3 (15/45)	28.6 (10/35)	0 (0/20)
2.5	Fertile	4	67.9 ± 1.2^{a}	63.3 ± 2.8^{a}	45.3 ± 0.8^{b}
	Subfertile	1	28.6 (10/35)	16.7 (5/30)	0 (0/25)
12.5	Fertile	4	65.4 ± 3.3^{a}	62.7 ± 4.2^{a}	49.0 ± 3.0^{b}
	Subfertile	1	22.2 (10/45)	12.5 (5/40)	16.7 (5/30)
25	Fertile	4	67.7 ± 1.5^{a}	63.1 ± 1.4^{a}	43.7 ± 5.1^{b}
	Subfertile	1	12.5 (5/40)	12.5 (5/40)	20 (5/25)

cBiMPS = Cell-Permeable cAMP Analog; SP = Seminal plasma.
% C is the percentage of circles/total motile sperm.
In fertile bull values (means ± SEM) bearing at least one common superscript were non-significantly different from the other values within the same column and within the same row (Repeated measures ANOVA- Bonferroni's multiple comparison test, $P < 0.05$).
In subfertile bull values in parentheses indicate the percentage of circles/total motile sperm.

Table 3. Proportion of whiplash (% W) of frozen-thawed fertile and subfertile bull spermatozoa incubated with or without SP powder in the presence of cBiMPS

Conc. of SP (mg/ml)	Bull	No. of replicates	% W, incubation time (min)		
			60	120	180
0	Fertile	4	3.1 ± 1.7^{a}	7.7 ± 0.7^{ab}	16.5 ± 1.2^{c}
	Subfertile	1	11.1 (5/45)	14.3 (5/35)	25 (5/20)
2.5	Fertile	4	3.2 ± 1.8^{a}	10.0 ± 1.9^{b}	16.8 ± 3.4^{c}
	Subfertile	1	14.3 (5/35)	16.7 (5/30)	20 (5/25)
12.5	Fertile	4	1.5 ± 1.5^{a}	12.5 ± 2.1^{b}	23.9 ± 3.5^{c}
	Subfertile	1	22.2 (10/45)	25 (10/40)	16.7 (5/30)
25	Fertile	4	0^{a}	6.9 ± 2.9^{ab}	21.2 ± 2.0^{c}
	Subfertile	1	0 (0/40)	12.5 (5/40)	20 (5/25)

cBiMPS = Cell-Permeable cAMP Analog; SP = Seminal plasma.
% W is the percentage of whiplash/total motile sperm.
In fertile bull values (means ± SEM) bearing at least one common superscript were non-significantly different from the other values within the same column and within the same row (Repeated measures ANOVA- Bonferroni's multiple comparison test, $P < 0.05$).
In subfertile bull values in parentheses indicate the percentage of whiplash/total motile sperm.

or without SP powder exhibited cBiMPS-dependent hyperactivated motility in the form of circles and whiplash movements at 60, 120 and 180 min incubation. For the four concentrations of SP powder at 60 and 120 min % C was greater ($P < 0.05$) than at 180 min incubation (Table 2), whereas % W at 180 min was greater ($P < 0.05$) than at 60 and 120 min incubation (Table 3). However, in all incubation times % C of 2.5, 12.5 and 25 mg/ml SP powder spent a lot of time active and swim in circles which had a dose-dependent effect compared to 0 mg/ml: there were some tendency, but was no significant dose-dependent effect. Likewise, cBiMPS-dependent % W was nil at 60 min incubation in the presence of 25 mg/ml SP powder but without significant difference from the other concentrations of SP powder as shown in Table 3. Similarly, at 120 and 180 min incubation there was no significant difference in % W among the four concentrations of SP powder (Table 3). Furthermore, the range of % W induced in samples incubated without

cBiMPS was less than 5% and was not affected with the different doses of SP and incubation times.

Although, subfertile bull spermatozoa frozen stored with or without SP powder exhibited the two forms (circles and whiplash movements) of cBiMPS-dependent hyperactivated motility in the same pattern of fertile bulls but % C were lower than that of fertile bull spermatozoa within the same incubation time (Table 2). Regarding % W (Table 3) of subfertile bull at 60 and 120 min incubation it was higher than that of the fertile bulls in the four concentrations of SP powder except of 25 mg/ml at 60 min but at 180 min incubation % W was comparable between fertile and subfertile bull spermatozoa in all concentrations of SP powder. Notably, at 180 min incubation cBiMPS-dependent % C of both control and 2.5 mg/ml SP powder in subfertile bull was nil, whereas it was 16.7 and 20 for 12.5 and 25 mg/ml, respectively. Also, at 180 min incubation cBiMPS-dependent % W of the control was 25 but it was 20, 16.7, 20 for 2.5, 12.5 and 25 mg/ml, respectively (Table 3).

3.3. Protein tyrosine-phosphorylation (PTP)

Fig. 1A showed cBiMPS-dependent increases in tyrosine-phosphorylated proteins (TPPs) of frozen stored bull spermatozoa in absence (0 mg/ml) or presence of 25 mg/ml SP powder. The obtained results revealed that at 0 min the PTP state of frozen-thawed fertile bull spermatozoa in the absence of both SP powder and cBiMPS revealed a few TPPs of 32, 38, 74 and 80 kDa; these TPPs in addition to that of 14 and 33 kDa appear after 60 min incubation. Irrespective of presence or absence of cBiMPS in the control samples (0 mg/ml) TPPs of 32 and 33 kDa decrease in intensity after 120 min and completely disappear at 180 min incubation (Fig. 1A). On contrast, at 60 min incubation without SP powder in the presence of cBiMPS revealed several TPPs of 14, 30, 32, 33, 38, 45, 48, 62, 74, 80, 83, 94, 105, 123 and 230 kDa. These TPPs decrease gradually at 120 and 180 min incubation as depicted in Fig. 1A. It is worth noting that, incubation of frozen-thawed spermatozoa supplemented with 25 mg/ml SP powder revealed more TPPs which were strongly detected especially in the presence of cBiMPS at 60 and 120 min incubation compared to 0 mg/ml, particularly, those of 38, 45, 48, 62, 74, 80, 83, 94, 105, 123, 230 and $>$ 230 kDa. In total 21 TPPs of molecular weight range from 10 to $>$230 kDa were detected where those of 32, 38, 45, 74, 80, 105, 123 and 230 kDa were the major TPPs (Fig. 1A) and those of 10, 14, 22, 23, 24, 27, 30, 33, 48, 62, 83, 94 and $>$ 230 kDa were the minor TPPs. At 60 min incubation cBiMPS-dependent TPPs of 45, 62, 74, 80, 83, 94, 105, 123, 230 and $>$ 230 kDa were strongly detected in the presence of 25 mg/ml SP powder compared with the control samples. Also, at 120 min incubation the above-mentioned TPPs and those of 30, 33 and 48 kDa were strongly detected and at 180 min incubation TPPs of 32, 33 and 38 were strongly detected compared with the control samples as presented in Fig. 1A. The TPPs of frozen-thawed fertile bull spermatozoa supplemented with 2.5 and 12.5 mg/ml SP powder in the presence or absence of cBiMPS are presented in Fig. 1B. The PTP state of 2.5 and 12.5 mg/ml SP powder were similar to that of 25 mg/ml except the intensity of TPPs was SP powder dose-dependent. Thereby, much and prolonged TPPs were associated with the higher concentrations of the added SP powder.

Immunoblotting of subfertile bull spermatozoa at 0 min neither in the presence of cBiMPS nor SP powder revealed TPPs of 24, 27, 33, 38, 45, 48, 58, 62, 74, 80, 94, 105 and 230 kDa. Moreover, at 60 min incubation TPPs of 38, 58, 62 74, 80, 94 and 105 kDa decrease gradually toward 180 min incubation but TPPs of low molecular weight (22, 23, 24 and 27 kDa) were clearer after a prolonged time of incubation as shown in Fig. 1C. In general, incubation of subfertile bull spermatozoa with or without cBiMPS in the presence or absence of SP powder revealed lower TPPs except those of 24 and 27 kDa compared with fertile bulls. Additionally,

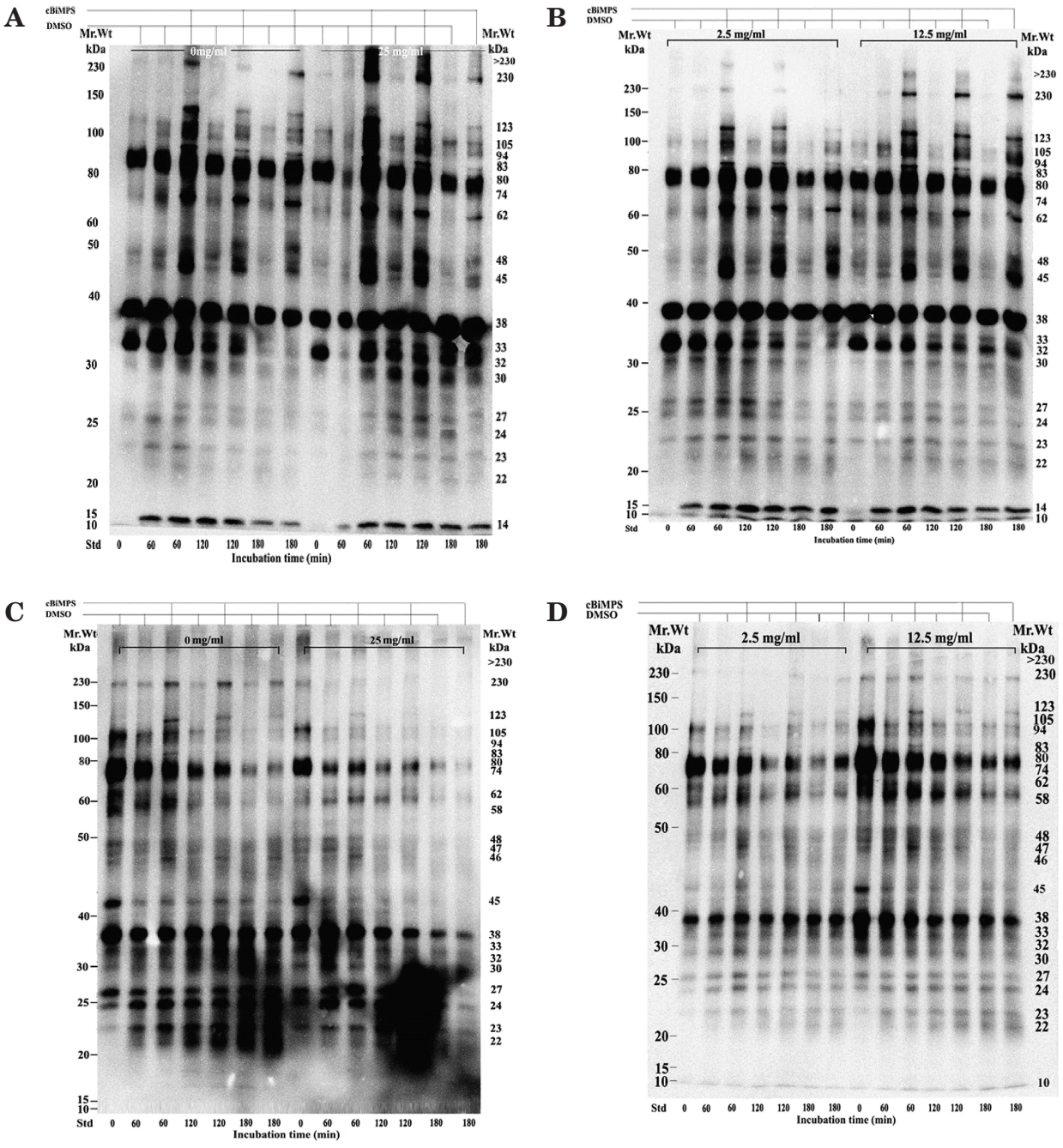

Fig. 1A: Western blots of TPPs extracted from frozen-stored fertile bull spermatozoa in the presence of 25 mg/ml and in the absence (0 mg/ml) of SP powder and incubated with $CaCl_2$ either with or without cBiMPS; **Fig. 1B:** Western blots of TPPs extracted from frozen-stored fertile bull spermatozoa in the presence of 2.5 and 12.5 mg/ml SP powder and incubated with $CaCl_2$ either with or without cBiMPS. **Fig. 1C:** Western blots of TPPs extracted from frozen-stored subfertile bull spermatozoa in the presence of 25 mg/ml and in absence (0 mg/ml) of SP powder and incubated with $CaCl_2$ either with or without cBiMPS. **Fig. 1D:** Western blots of TPPs extracted from frozen-stored subfertile bull spermatozoa in the presence of 2.5 and 12.5 mg/ml SP powder and incubated with $CaCl_2$ either with or without cBiMPS. In each experiment (Western blot; a representative of four replicates), aliquots of each sperm suspension were recovered at 0, 60, 120 and 180 min incubation and used for SDS-PAGE/Western blotting with mouse anti-phosphotyrosine mAb (Clone 4G10, 1 : 10,000) followed by horseradish peroxidase (HRD)-conjugated goat anti-mouse immunoglobulin polyclonal antibody (1 : 5,000). TPPs = Tyrosine-phosphorylated proteins; Std = Molecular weight standards; Mr. Wt = Molecular weight.

PTP state of subfertile bull shown in Fig. 1C and 1D revealed that the major TPPs were of 38, 74, 80 kDa, whereas the minor bands were of 10, 22, 23, 24, 27, 30, 32, 33, 45, 46, 47, 48, 58, 62, 83, 94, 105, 123, 230 and >230 kDa. Collectively, the TPPs in fertile bulls were SP powder dose-dependent especially in the presence of cBiMPS but in subfertile bull it was higher at 0 min incubation in the absence of cBiMPS (Fig. 1C, D). Moreover, in subfertile bull the TPP of 45 kDa was strongly detected at 0 min incubation either with or without SP powder in the absence of cBiMPS, whereas in fertile bulls it was strongly detected at 60, 120 and 180 min incubation either with or without SP powder in the presence of cBiMPS. Furthermore, the effect of SP powder in prolonging TPPs was less pronounced in subfertile bull spermatozoa compared with fertile bull spermatozoa. Interestingly, TPPs of 24, 27, 32, 33, 38, 45, 48, 74, 80, 83, 94, 105, 123 and 230 kDa were different between fertile and subfertile bulls.

4. Discussion

This study is apparently the first report to describe the impacts of desalted and lyophilized SP on the hyperactivated motility and PTP of mammalian spermatozoa. In the current study electrolytes specifically, K^+ and HCO_3^- were removed from the SP to be desalted SP in order to be added to semen extender before freezing. Our intention was to remove low molecular weight molecules specifically, HCO_3^- that disrupt the sperm plasma membrane and induce cryocapacitation which consider one of the main causes of reduced fertility of frozen semen in cattle industry[18].

The existence of both beneficial and detrimental factors for sperm in SP has been known for many years. However, the fact that bovine SP (BSP) proteins acts like a double edged-sword that is both beneficial and detrimental to sperm depending on the context (capacitation or preservation) is surprising. The first functional role identified for BSP proteins is that they promote the capacitation of bull sperm, which involves a complex series of events[35]. Two additional functions were proposed for BSP proteins. The first one is *in vitro* activity of protein kinase C (PKC) and of tyrosine protein kinase (TPK) are inhibited by BSP protein-A1/-A2 (BSP-A1/-A2) and proposed that the inhibition of PKC may serve to prevent the premature acrosome reaction of sperm in the female reproductive tract[42]. The second function is that BSP proteins mediate the binding of sperm to the oviductal epithelium and proposed that they are involved in prolonging sperm survival during storage and in the maintaining sperm motility in the oviduct[11]. Additionally, BSP proteins could repair the damage caused by cold shock and restoring the plasma membrane integrity[5]. Concentration of BSP proteins in the added SP powder may be correlated with sperm resistance to freeze-thaw damage. Thus, the functional plasma membrane integrity of frozen-stored spermatozoa with SP powder assumed to be higher than that of the control samples because BSP proteins and other organic components, such as lipids particularly cholesterol in the added SP powder[3], may have a protective effect against freeze-thaw damage. The obtained data suggest that, BSP proteins play multiple roles in sperm cell functions and have a beneficial effect on sperm cell fertility.

Both cAMP and Ca^{2+} regulating hyperactivation through different pathways and they also modulate the actions of each other but it appears that Ca^{2+} is the most important factor regulating hyperactivation, and although cAMP is required, it is not sufficient for expression of hyperactivation[17]. Moreover, internal Ca^{2+} stores could provide sufficient Ca^{2+} for the induction of hyperactivated motility and Ca^{2+} influx is required to maintain intracellular Ca^{2+} levels sufficient to sustain hyperactivated motility[22]. Although, the process of hyperactivation involve increase in intracellular cAMP, which at least is required to support

motility, but neither % C nor % W showed any significant dependence on SP powder. Thus, while fructose content of the added SP powder is required for motility in general; it is not involved in switching on hyperactivated motility in the axoneme.

In mature spermatozoa, sAC is localized in the middle piece of the sperm flagellum[15] and involved in ATP synthesis. Moreover, it also plays a crucial role in the regulation of capacitation-associated PTP, motility activation and hyperactivation[15]. Because sAC is also stimulated via the direct binding with not only HCO_3^- but also Ca^{2+} though, hyperactivated motility has been induced in intact spermatozoa of several mammalian species by capacitation media containing Ca^{2+} in the mM range[41]. From these results, it is postulated that hyperactivation of frozen-stored spermatozoa with desalted SP in absence of HCO_3^- may initially be triggered by the influx of extracellular Ca^{2+} and subsequently be modulated via the action of cBiMPS. This might be the most reasonable explanation for the non-significant effect of the added SP powder on % C and % W of frozen-thawed bull spermatozoa. As a consequence of changes in membrane structure, spermatozoa tend to aggregate head-to-head as previously reported in boars[12]. The motility of aggregated spermatozoa, however, cannot be analyzed by computer-aided sperm motion analyzer and therefore capacitation with mM Ca^{2+} hampers the analysis of hyperactivated motility, at least in boar spermatozoa. Similarly in the current study hyperactivated motility was induced by capacitating medium containing 2.25 mM Ca^{2+} which leads to agglutination of spermatozoa. Accordingly, % C and % W revealed non-significant difference among the four concentrations of SP powder whereas after immunoblotting clear differences were detected among the four concentrations of SP powder (Fig. 1 A, B).

Indeed, glucose is required for sperm cell hyperactivation[40], zona pellucida penetration[37], and promotes PTP[36]. Thereby, in absence of glucose, both sperm-oocyte fusion and PTP in the mid-piece are inhibited, indicating that PTP in the whole flagellum is glucose-dependent and mandatory for sperm-oocyte fusion[36]. Regarding our results, it revealed that the reason for the prolonged and increased PTP of frozen-thawed bull spermatozoa supplemented with SP powder might be high amount of fructose in the added SP powder which utilized by sperm to produce high amount of cAMP from ATP in the presence of sAC enzyme in agreement with the findings of Urner *et al.* (2001)[36]. Addition of membrane permeable cAMP analogs in the capacitating medium of mouse spermatozoa restored HCO_3^- induced capacitation response[9] which was consistent with our findings because incubation of frozen-thawed bull spermatozoa supplemented with desalted SP (HCO_3^- free) in the presence of cBiMPS induced hyperactivated motility and PTP.

Taken these findings together, it is obvious that by adding more SP powder containing more fructose increases the PTP of frozen-thawed spermatozoa incubated in the presence cBiMPS and Ca^{2+}. This might be another acceptable explanation for our findings because the higher concentrations (12.5 and 25 mg/ml) of the added SP powder maintained motility up to 180 min better than 2.5 mg/ml of SP powder and the control samples in the presence of cBiMPS (Table 1). Similarly, the mean percentage of hyperactive bull spermatozoa tends to be slightly more constant in the presence than in the absence of SP powder in agreement with the results of Schmidt and Kamp (2004)[33]. However, herein, head-to-head agglutination was observed in the presence not in the absence of SP inconsistent with Schmidt and Kamp (2004)[33]. In the presence of sufficient ATP during incubation in a capacitation medium at 38.5°C, Ca^{2+} switches on hyperactivation by enabling curvature of the principal bend[16] which might be the reason for the non-significant effect of the added SP powder containing much fructose and no HCO_3^- on the hyperactivated motility of frozen-thawed bull spermatozoa.

A number of physiological factors, such as Ca^{2+}, cAMP, HCO_3^- and metabolic substrates, are essential for the initiation and maintenance of sperm hyperactivation *in vitro*. The crucial site for the action of Ca^{2+} is the axoneme of sperm cell[20]. It should be emphasized that Ca^{2+} is responsible for increasing flagellar asymmetry, which is a hallmark of hyperactivated motility, whereas cAMP is a major factor in initiation and maintenance of flagellar beats. Moreover, HCO_3^- induced tyrosine phosphorylation is relatively slow in the natural situation because it is dependent on membrane changes and removal of cholesterol which are indirectly guided by HCO_3^-. Recently, we found that the added SP powder has high cholesterol content[3] which interfere with membrane depolarization and Ca^{2+} influx inside the sperm cell required for PTP and this might be another plausible explanation for the prolonged PTP of frozen-stored bull spermatozoa with SP powder compared with those frozen-stored without SP powder. Another possibility is the albumin content of the added SP powder[3] might be responsible for this increased PTP of frozen-thawed spermatozoa because albumin is sterol acceptor which serves to remove cholesterol from sperm plasma membrane[25] to allow greater Ca^{2+} influx and stimulation of sAC/cAMP/PKA signaling cascade of PTP.

With respect to the difference in hyperactivated motility and PTP states between fertile and subfertile bull spermatozoa. This difference may be attributable to the different plasma membrane integrity because it was 52.1% for fertile bulls versus $\leq$ 17% for subfertile bull used in the current study in propidium iodide stained semen smear[2]. Our results support the finding of Murase *et al.* (2010)[28] who reported that the hyperactivated motility especially % C of subfertile bull was lower than that of fertile bull. Suggesting that, cBiMPS-induced hyperactivated motility might be useful for identifying subfertile bulls in AI center. Additionally, the beneficial effect of the added SP powder on hyperactivated motility and associated PTP was obvious in fertile bulls than subfertile bull. Meanwhile, there was a close correlation between the ability of sperm to display hyperactivated motility and their ability to fertilize oocyte *in vitro*[7] and also our findings revealed non-significant effect of the added SP powder on hyperactivated motility. Thus, the added SP powder at least has no detrimental effect on the fertilizing ability of sperm cells.

In conclusion, addition of desalted and lyophilized SP to the cryoprotective diluents not only protects frozen-thawed spermatozoa from cryocapacitation but also increase its PTP state. Consequently, not only has no detrimental effect on sperm cell functions, but also enhancing the fertilizing ability of frozen semen. These findings highlight the novelty of addition of desalted and lyophilized SP to semen extender to minimize cryocapaciatation. We hope that this study will create renewed interest in the significance of desalted and lyophilized SP to alleviate reduced fertility of frozen semen.

Conflict of Interest

We certify that there is no conflict of interest with any people or organizations that could prejudice or bias the content of this research paper.

Acknowledgments

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