Global Veterinaria 13 (5): 753-766, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.13.05.8684

Plasma Membrane Integrity and Morphology of Frozen-Thawed Bull Spermatozoa Supplemented with Desalted and Lyophilized Seminal Plasma

[1,2]Essam Almadaly, [3]Foad Farrag, [4]Mostafa Shukry and [1]Tetsuma Murase

[1]Laboratory of Theriogenology, Division of Veterinary Medicine,
Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan
[2]Department of Theriogenology, Faculty of Veterinary Medicine,
Kafrelsheikh University, Kafrelsheikh 33516, Egypt
[3]Department of Anatomy, Faculty of Veterinary Medicine,
Kafrelsheikh University, Kafrelsheikh 33516, Egypt
[4]Department of Physiology, Faculty of Veterinary Medicine,
Kafrelsheikh University, Kafrelsheikh 33516, Egypt

Abstract: Cryopreservation induces extensive changes in the sperm cell and the success of artificial insemination (AI) with frozen semen implies reduction of the deleterious effects on sperm plasma membranes induced by this technique. This study compare three methods for evaluating plasma membrane integrity of frozen-thawed Japanese Black bull spermatozoa supplemented with desalted and lyophilized seminal plasma (SP): eosin-nigrosin (EN); Propidium iodide (PI); and hypoosmotic swelling test (HOST). SP was desalted using Sephadex G-25 desalting column and lyophilized before added to semen extender at final concentrations 0, 2.5, 12.5 and 25 mg/ml. Plasma and acrosomal membranes integrity as well as abnormal sperm morphology were assessed. The results showed that the proportions of membrane-intact sperm with EN, PI and HOST were not significantly different and all methods failed to detect any significant effect of the added SP. Centrifugation, washing and HOST solution adversely affect acrosomal membrane. The proportions of abnormal spermatozoa identified by Glutaraldehyde fixation was equivalent to that of EN. Overall, EN, PI and HOST have no significant difference in identifying the proportions of membrane-intact bull spermatozoa supplemented with desalted and lyophilized SP. Subsequently, the added SP has no beneficial effect on either physical or functional integrity of the sperm membranes.

Key words: Japanese Black Bull · Cryopreservation · Fluorescent Stain · Acrosome · Giemsa Stain

INTRODUCTION

Artificial insemination (AI) is one of the most successful reproductive technologies developed for rapid dispersal of superior germplasm to improve productivity of domesticated animals. For successful AI, an accurate, repeatable and cost-effective means of determining sperm quality is required. Hence, assessment of characteristics of plasmalemma is considered to be useful for predicting the fertilizing ability of a sperm [1,2]. Among the cellular components including the head, intermediate piece, flagellum and DNA content, the integrity of both plasma and acrosomal membranes are good parameters of the sperm quality and fertility. Numerous methods have been designed to assess the status of sperm plasma membranes. These methods range from the simplest hypoosmotic swelling test (HOST) [1] and the exclusion of impermeable dyes such as eosin by intact cells to more sophisticated techniques based of fluorescent stains [3,4]. Eosin-Nigrosin (EN) is an exclusion stain that is simple and economical. Eosin binds to and stains the DNA of sperm that have a disrupted post-acrosomal plasma

Corresponding Author: Essam Almadaly, Department of Theriogenology, Faculty of Veterinary Medicine,
Kafrelsheikh University, Kafrelsheikh 33516, Egypt.
Tel/Fax: +02-(0)47-323-1311.

membrane [5], whereas nigrosin produces a dark background. Sperm of intact post-acrosomal plasma membrane [5] exclude the eosin stain and appear white, whereas sperm with loss of post-acrosomal membrane integrity allow permeation of eosin and appear pink [6]. Staining with eosin and nigrosin has long been used to assess mammalian sperm viability but has a disadvantage that the dye eosin is easily incorporated into live spermatozoa which results in underestimation of sperm viability particularly when used with frozen-thawed semen containing glycerol [7].

Fluorescent stains have been used to label sperm and determine sperm membrane integrity [4,8]. Propidium iodide (PI) is a DNA-specific fluorescent stain which penetrates non-intact plasma membrane, probably entering the nuclear compartment through pores in the nuclear membrane [4]. Fluorescent microscopy has been used to count immobilized sperm, determining the percentage of sperm stained by various fluorescent stains including that assessing plasma membrane integrity [2,9].

The HOST developed by Jeyendran and coworkers, was designed to evaluate the function of the sperm membrane [1]. Its principle is based on the observation of the morphological alterations in spermatozoa exposed to hypoosmotic conditions [10]. Drevius and Erikson [10] demonstrated an increase in cell volume of spermatozoa placed in hypoosmotic solutions. This volume increase was associated with the spherical expansion of the cell membrane covering the tail, thus forcing the flagellum to coil inside the membrane. Coiling of the tail begins at the distal end and proceeds toward the mid-piece and the head as the osmotic pressure of the suspending medium is lowered [1]. It has been shown that a good correlation exists between the percentage of coiled spermatozoa in a seminal specimen and the outcome of the zona-free hamster oocyte assay as well as *in vitro* fertilization outcome in humans [1]. The HOST may also prove useful tool in assessing cryopreservation-induced membrane damage of frozen-thawed semen [11].

Seminal plasma (SP) affects the physiology of spermatozoa, although its effects on different species are very variable, depending also on the condition of the sample [12]. In boar, incubation of fresh or cryopreserved sperm in media supplemented with 10% SP seems to prevent and possibly reverse, capacitation-related changes [13]. Post-thawing addition of SP improved membrane and acrosomal integrity and enhanced the *in vivo* fertilization [14] and use of 10% SP after thawing rendered good fertility results when combined with a modified freezing/thawing protocol [15]. SP supplementation prior to freezing was of most benefit [16] where, SP act as a protective medium during *in vitro* processing of ram spermatozoa, but this does not appear to be the case for bull spermatozoa. Although, we recently [17] reported that premature capacitation during freeze-thaw processes could be reduced by adding desalted and lyophilized SP but no information are available on the effect of desalted and lyophilized SP on physical and/or functional membrane integrity of bull spermatozoa. Therefore, this study aimed to:

- Investigate the possibility that EN and/or PI and/or HOST could detect any significant effect of desalted and lyophilized SP on the physical and/or functional integrity of frozen-thawed spermatozoa.
- Investigate the effect of freezing-thawing, washing and HOST solution on the acrosomal membrane.
- The possibility that Glutaraldehyde fixation and/or EN staining methods could detect any beneficial effect of desalted and lyophilized SP on the sperm cell morphology was examined.

MATERIALS AND METHODS

Semen: Semen used for recovery of SP was collected with an artificial vagina from four mature Japanese Black bulls kept at Hida Beef Cattle Research Department, Gifu Prefectural Livestock Research Institute, Japan. Two ejaculates from each bull were collected once for 4 occasions. These ejaculates had >75% progressively motile, >830 million sperm/ml, <11% morphologically abnormal sperm, >97% intact-acrosome and normal pH (6.4). For experiments where semen was frozen being supplemented with SP, one ejaculate was collected from another four Japanese Black bulls. 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 desalted and lyophilized SP on the sperm membrane integrity. All bulls were of proven fertility and had conception rates >50% after AI with frozen-thawed semen. Frozen semen from each bull was evaluated in four replicates by the same technician for all examined parameters in the present study.

Chemicals and Reagents: All chemicals and reagents were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA), Wako Pure Chemicals Industries (Osaka, Japan) unless otherwise stated.

Media: Tris-based extender used for semen dilution was prepared according to Masuda [18] and the media used for sperm washing were as described previously [19]. The saline medium consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes adjusted to pH 7.55 at 25°C [20]. Sucrose medium containing 275 mM sucrose in place of NaCl was used for washing spermatozoa [20]. Saline and sucrose media also contained 0.1% (w/v) polyvinyl alcohol (PVA) and 0.1% (w/v) polyethylene glycol (PEG). Freshly prepared 2% Glutaraldehyde in 0.165 M sodium cacodylate buffer, adjusted to pH 7.3 at 25°C was used for fixation of spermatozoa. A stock solution of PI (P4170; Sigma-Aldrich, Germany) 0.5 mg/ml H_2O was prepared and stored frozen at -30°C in the dark. Bovine serum albumin (BSA; Fraction V) was dissolved in H_2O at 100 mg/ml and kept frozen at -30°C. Eosin yellowish (CI 45380) and Giemsa were from Merck, Germany whereas, nigrosin was from Sigma-Aldrich, Germany.

Recovery and Processing of SP: Recovery of SP with subsequent desalting and lyophilization were performed as described in our recent report [17]. Briefly, SP was recovered by centrifugation at 3000 x g for 15 min at 5°C; the supernatant (SP) was re-centrifuged at 12000 x g for 30 min at 4°C. SP was desalted by using Sephadex G-25 desalting columns and the collected SP were pooled, mixed and divided into known volumes in cryogenic vials. Cryogenic vials were kept at -30°C overnight before lyophilization and then inserted into the freeze-drying machine previously stabilized at -50°C. After 24 hrs of freeze-drying, the vials were tightly capped and weighed to determine the obtained amount of lyophilized SP powder. Desalted and lyophilized SP (designated as SP powder) was kept at -30°C until being incorporated into the cryoprotective diluents during semen processing.

Processing of Frozen Semen Supplemented with SP Powder: One ejaculate from four bulls, in total four ejaculates were diluted with Tris-based diluents [18], supplemented with SP powder and frozen stored according to protocol of Almadaly *et al*. [17]. Each ejaculate was diluted with glycerol-free Tris-based diluent at 37°C. Initial dilution was completed after equilibration for at least 2 h at 4°C to produce a sperm concentration of 2 x 10^8/ml. The glycerolized portion of a Tris-based diluent containing four different concentrations of SP powder was used for the second dilution to produce a sperm concentration of 1 x 10^8/ml. Total protein concentration (g/dl) of each ejaculate was measured by refractometer and according to the concentration; the weight of SP powder to be added was adjusted to 0 (as a control), 2.5, 12.5 and 25 mg/ml of diluted semen [17]. Finally, frozen straws of four different concentrations of SP powder for each bull was obtained which plunged into liquid nitrogen for storage and transported to the laboratory for analysis.

Evaluation of Plasma Membrane Integrity: Two straws from each concentration of frozen semen supplemented with SP powder as well as from the control group were thawed at 39°C for 1 min, combined and gently mixed. Plasma membrane integrity was evaluated by EN, PI and HOST as following:

EN Staining: The EN stain was prepared by mixing 1% eosin and 5% nigrosin in 3% sodium citrate (dihydrate) according to Swanson and Bearden [6]. An EN stained semen smear was prepared immediately after thawing and the proportion of membrane-intact sperm was determined by examining 100 spermatozoa in each of two different smears (200 spermatozoa in total) under magnification 1000x. Smears were prepared by mixing 10 µl of semen and 10 µl of stain on a warm slide and spreading the mixture with the edge of a warm slide, held at a 30-40° angle. The slide was placed back on a warming plate for several min and then it was stored at room temperature until sperm were evaluated. Sperm that did not uptake the stain (white) were counted as membrane-intact (Fig. 1EN - panel- a), whereas sperm with any detectable eosin showing either partial (Fig. 1EN - panels- b and c), or complete (Fig. 1EN - panel- d), pink staining were counted as membrane-damaged. The percentage of membrane-intact sperm was determined for each sample as the percentage of unstained sperm in the two hundred sperm counted.

PI Staining: Membrane-intact sperm % was examined using PI stain according to Harrison and Vickers [3] with some modifications. Briefly, 10 µl of PI stock (0.5 mg/ml in H_2O) was added to saline medium (920 µl). Frozen-thawed semen (80 µl) was added and incubated in saline medium for 5 min at room temperature and spermatozoa were immobilized with formaldehyde [final concentration: 0.002% (w/v)]. After incubation, 10 µl of BSA (100 mg/ml) was added to the sperm suspension. The stained suspension (4 µl) was applied onto a glass slide and covered with a coverslip (18 mm x 18 mm). Spermatozoa were examined under a fluorescence microscope equipped with a green excitation filter (WIG; excitation filter wavelength, 530-550 nm; barrier filter wavelength, 575nm;

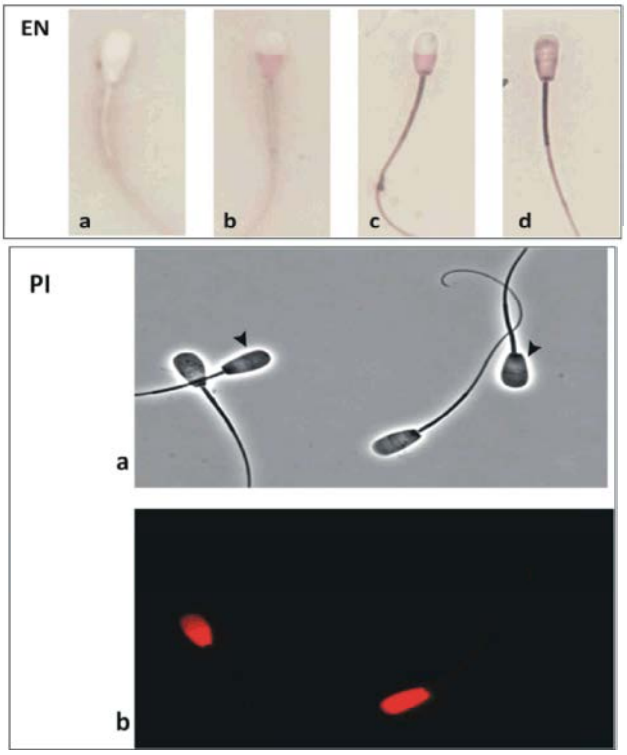

Fig. 1: Patterns of bull spermatozoa supplemented with SP powder and stained with Eosin-nigrosin (EN) and Propidium iodide (PI) stains

EN a) Represents the membrane-intact sperm b, c & d) Represents the three patterns of membrane damaged sperm.

PI Spermatozoa were incubated with PI (final concentration 5 µg/ml) for 5 min and examined a) under phase-contrast optics (arrow head are membrane-intact spermatozoa), b) Using the filter for PI.

Note that with PI two spermatozoa are red-stained

Olympus, Tokyo, Japan). Using combination of phase-contrast optics and epifluorescence spermatozoa were examined at 400x magnification and a minimum of 100 spermatozoa were counted in each of two different slide preparations (200 spermatozoa in total). Spermatozoa that fluoresced red (PI-positive; Fig. 1PI - panel- b) were considered being membrane-damaged and unstained (PI-negative; Fig. 1PI - panel- a, arrow head) spermatozoa being membrane-intact; the percentages of PI-negative spermatozoa were calculated as membrane-intact sperm %.

HOST Technique: To assess the functional status of sperm plasma membrane, the spermatozoa were subjected to HOST [1]. The HOST was performed by incubating 100 µl of frozen-thawed semen in 1 ml of a pre-warmed 150 mOsm hypoosmotic solution (13.51 g fructose plus 7.35 g sodium citrate. $2H_2O$ per 1000 ml H_2O; [1]) at 37°C for 60 min. After incubation the functional integrity of plasma membrane and % intact-acrosome was examined by the following methods:

Giemsa Staining: An aliquot (5 µl) of well-mixed HOST exposed sample was smeared on a warm glass slide and dry on a warming plate of 38.5°C. Semen smear was fixed with methanol for a minimum of 10 min and rinsed briefly with tap water. The fixed smear was stained with 7.5% Giemsa/5mM phosphate buffer saline (PBS) of pH 7 [21]. Giemsa stain solution consisted of 3 ml Giemsa stock solution [22], 2 ml PBS and 35 ml H_2O. After staining for at least 3 hrs, slides were rinsed, dried and mounted with rapid mounting medium for microscopy (Entellan, Merck-Germany). A minimum of 200 spermatozoa were examined under a phase-contrast microscope at 1000x magnification and spermatozoa were sorted according to their HOST response (HOST-positive: cells with an intact plasma membrane indicated by coiled tails; HOST-negative: cells with a damaged plasma membrane indicated by straight tails) and their acrosomal status (acrosome positive: cells having the acrosomal cap; acrosome negative: absent or damaged acrosomal cap). Using this technique, the sperm cells were sorted into four subpopulations as shown in

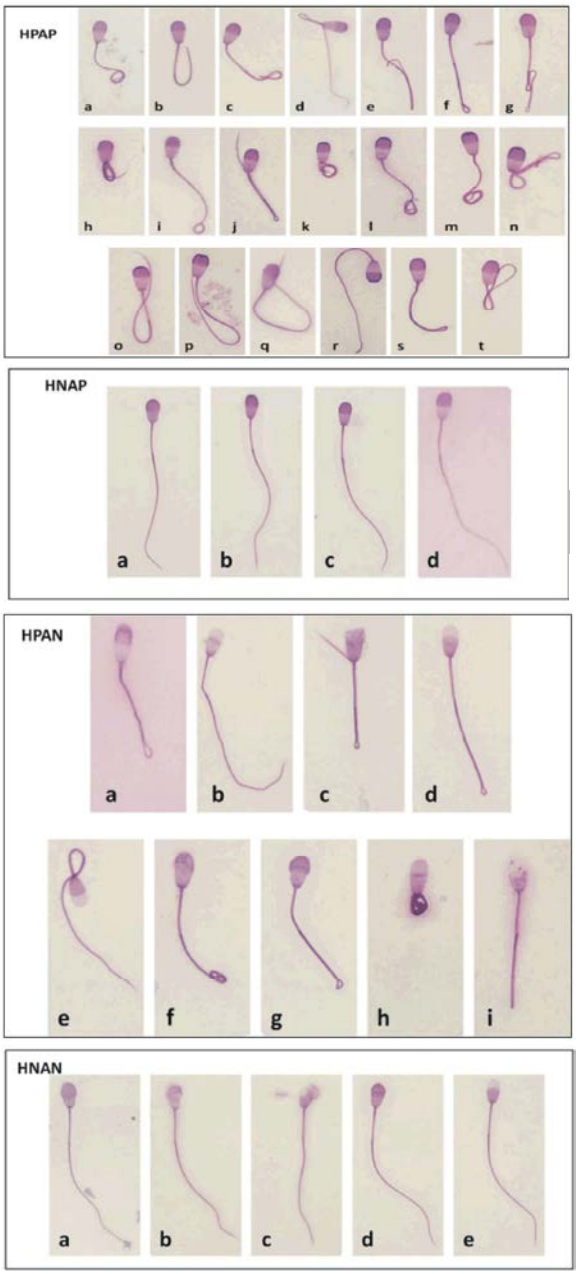

Fig. 2: Categories of HOST-Giemsa stained bull spermatozoa supplemented with SP powder. Bull spermatozoa subjected to HOST and stained with Giemsa stain (7.5% Giemsa/5mM PBS of pH 7). Spermatozoa were examined under a phase-contrast microscope (1000x) and classified according to both the response to HOST and the acrosomal status into four categories:

- HPAP (Hypoosmotic swelling test positive and acrosome positive), this category has different staining patterns (panels a - t).
- HNAP (Hypoosmotic swelling test negative and acrosome positive), this category has four staining patterns (panels a - d).
- HPAN (Hypoosmotic swelling test positive and acrosome negative), this category has different staining patterns (panels a - i).
- HNAN (Hypoosmotic swelling test negative and acrosome negative), this category has five staining patterns (panels a - e).

Figure 2: (1) HPAP (HOST-positive and acrosome-positive), (2) HNAP (HOST-negative and acrosome positive), (3) HPAN (HOST-positive and acrosome-negative) and (4) HNAN (HOST-negative and acrosome-negative). Both HPAP and HPAN subpopulations were added together to calculate % HOST-positive, whereas, HPAP and HNAP subpopulations were added together to calculate % intact-acrosome. The proportion of coiled tail spermatozoa prior to HOST was determined and subtracted from the proportion of live spermatozoa that underwent a hypoosmotic swelling response to obtain the actual proportion of HOST-positive sperm cells (True HOST positive; [23]).

Glutaraldehyde Fixation Method: Both the response of frozen-thawed spermatozoa supplemented with SP powder to HOST and % intact-acrosome were determined by Glutaraldehyde fixation method [24]. Briefly, 50 µl of HOST exposed semen was fixed with 1% Glutaraldehyde/ 0.165 M sodium cacodylate buffer (pH 7.3 at 25°C) at room temperature for a minimum of 30 min. Wet-mounts (2 µl each) of Glutaraldehyde fixed sperm cells were overlaid with coverslip and examined under phase-contrast microscope (Olympus BX41) at 1000x magnification. Two hundred spermatozoa on each slide were scored for the presence of acrosomal dense apical ridge and tail swelling and two wet-mounts were counted for a total of 400 spermatozoa per sample. Spermatozoa showing a dense, thick apical ridge on the head were considered acrosome-intact [25] whereas, spermatozoa showing ruffled or vesiculated acrosomal membrane were considered acrosome-damaged. Likewise, the sperm cells were sorted into four subpopulations according to their HOST response and acrosomal status based on the microscopic observation that were HPAP, HNAP, HPAN and HNAN as previously described.

Evaluation of Acrosomal Membrane Integrity: The acrosomal membrane integrity was evaluated by Glutaraldehyde fixation method according to Almadaly *et al.* [24]. Briefly, Fresh (10 µl) and frozen-thawed (50 µl) semen was diluted with 50 µl of 0.16 M NaCl. Diluted spermatozoa were fixed with 1% Glutaraldehyde/ 0.165 M sodium cacodylate buffer at room temperature for a minimum of 30 min. Glutaraldehyde fixed sperm cells were examined for the presence of the acrosomal ridge as mentioned above. Two wet-mounts were counted for a total of 400 spermatozoa per sample and spermatozoa categorized into acrosome-intact and acrosome-damaged.

The remaining volume (~700 µl) of frozen-thawed semen from each concentration of SP powder was centrifuged at 830 x g for 6 min. The supernatant was discarded and the concentrated semen was diluted with saline medium and overlaid onto a layer of sucrose medium. The sample was washed by centrifugation at 400 x g for 5 min followed by 1000 x g for 12 min; the supernatant was removed by aspiration. Aliquot (50 µl) of washed sperm cells were fixed by mixing with equal volume of 2% Glutaraldehyde/0.165 M cacodylate buffer. Finally, spermatozoa were examined under phase-contrast microscope (1000x), for acrosomal membrane integrity using Glutaraldehyde fixation method as mentioned before.

Morphological Characteristics

EN Staining: Morphological assessment was performed by examining EN stained semen smear [26], using bright-field microscopy at 1000x magnification and 200 sperm cells were examined according to the criteria described by Barth and Oko [27]. Spermatozoa were also assessed for sperm defects other than those associated with acrosomal cap origin such as head, mid-piece and tail defects.

Glutaraldehyde Fixation Method: The morphology of Frozen-thawed spermatozoa supplemented with SP powder was evaluated by Glutaraldehyde fixation method [24] using the same technique of acrosomal evaluation as described above. Glutaraldehyde fixed spermatozoa were examined for sperm morphology (% abnormal morphology) and abnormal sperm cells were categorized into head (Fig. 3I), mid-piece (Fig. 3II) and tail (Fig. 3III) defects.

Statistical Analyses: Results are presented as mean ± SEM). The obtained data were subjected to paired *t*-test or repeated measures ANOVA. When difference was significant by ANOVA, individual means were further tested by Tukey's multiple comparison test [28]. A $p<0.05$ was considered statistically significant. All analyses were carried out using a statistical software program (GraphPad Prism Version 5.0; GraphPad Software, San Diego, CA, USA).

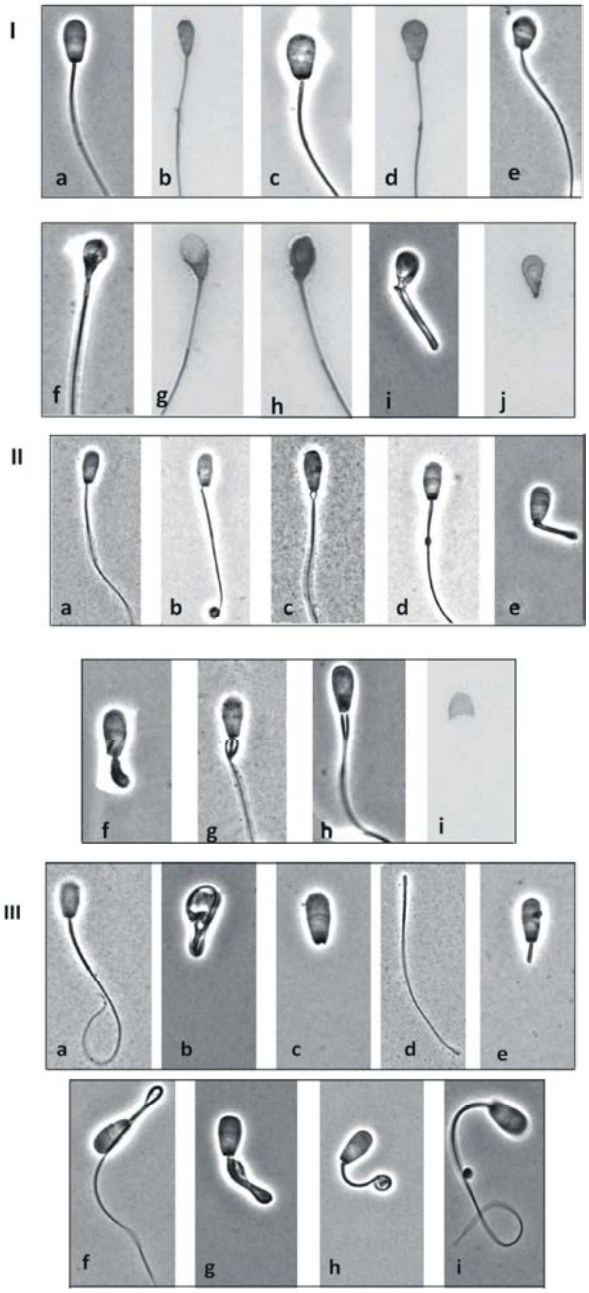

Fig. 3: Categories of head (I), mid-piece (II) and tail (III) sperm cell defects of bull spermatozoa supplemented with SP powder Bull spermatozoa were fixed with 1% Glutaraldehyde and examined under phase-contrast microscope (1000x).

- Sperm head defects where the observed abnormalities were microcephalic head (panel-b), macrocephalic head (panel-c), pyriform head (panel-d), abnormal shaped head (panels- e - i) and malformed sperm cell (panel-j), but normal head was presented in panel a.
- Sperm mid-piece defects which include abaxial implantation (panel-a), fractured neck (panel-b), proximal (panel-c) and distal (panel-d) cytoplasmic droplet, short (panel-e), kinked (panel-f), swollen (panel-g), double (panel-h) mid-piece and detached acrosome (panel-i).
- Sperm tail defects were bent tail (panel-a), Dag defect (panel-b), detached head (panel -c), detached tail (panel-d), short tail (panel-e), looped tail (panels-f - i)

RESULTS

Plasma Membrane Integrity: Repeated measures ANOVA followed by Tukey's multiple comparison test among the different methods used to determine membrane-intact sperm % were shown in Table 1. Over all samples, EN yielded higher membrane-intact sperm % than PI and PI yielded higher % than the two methods (Glutaraldehyde fixation and Giemsa staining) of True HOST-positive. A significant difference ($p<0.05$) was not detected among all the different methods used in the present study. All methods used for assessment of membrane-intact sperm % failed to detect any significant effect of the added SP powder in terms of plasma membrane integrity. Though, the proportion of HOST positive and true HOST positive sperm cells were higher with Glutaraldehyde fixation method than that of Giemsa staining but there was no significant difference between the two methods as shown in Table 1.

Acrosomal Membrane Integrity: The aim of this experiment was to assess the effect of freezing-thawing, washing and HOST solution on % intact-acrosome of bull spermatozoa. The proportions of sperm with intact-acrosome of fresh, frozen-thawed, washed and HOST-treated semen were presented in Table 2. The results revealed that the freezing-thawing process has no significant effect on % intact-acrosome of all concentrations of SP powder. On contrary, washing procedures significantly damaged the acrosomal membrane in comparison with fresh semen but not with frozen-thawed semen as shown in Table 2. Additionally, incubation of sperm cells in HOST solution significantly damaged the acrosomal membrane in comparison with both fresh and frozen-thawed semen. The added SP powder has no beneficial effect on % intact-acrosome of fresh, frozen-thawed, washed and HOST treated bull spermatozoa (Table 2).

Morphological Characteristics: The morphological evaluation of bull spermatozoa supplemented with desalted and lyophilized SP was shown in Table 3. The added SP powder has non-significant effect on the head, mid-piece and tail defects as well as on the total % abnormal morphology. Also, Glutaraldehyde fixation has no more advantage than EN staining in evaluation of both differential and total abnormal morphology of frozen-thawed bull spermatozoa. The observed forms of head defects were presented in Figure. 3I which include microcephalic (panel-b), macrocephalic (panel-c), pyriform (panel-d), abnormal shaped head (panels e - i) and malformed sperm cell (panel-j). The mid-piece defects were shown in Figure. 3II which include abaxial implantation (panel-a), fractured neck (panel-b), proximal cytoplasmic droplet (panel-c), distal cytoplasmic droplet (panel-d), short (panel-e), kinked (panel-f), swollen (panel-g), double (panel-h) mid-piece and detached acrosome (panel-i). Moreover, the forms of tail defects were bent tail (panel-a), Dag defect (panel-b), detached head (panel-c), detached tail (panel-d), short tail (panel-e), looped tail (panels-f - i) as shown in Figure. 3III.

DISCUSSION

Although the sperm plasma membrane covers the entire cell, it consists of three distinct membrane compartments, one which covers the outer acrosomal membrane, one which covers the post acrosomal portion of sperm head and one which covers the middle and principal pieces [5]. Most viability tests assess whether the plasma membrane is intact or not. However, because the plasma membrane is composed of these different compartments, different viability assays assess the integrity of different plasma membrane compartments. Classical stains, such as EN as well as more recent fluorescent stains, such as PI bind to and stain the DNA of the sperm that possess a post-acrosomal plasma membrane that is not intact. However, these probes will not assess the integrity of the plasma membrane covering the acrosome or principal piece. The integrity of the plasma membrane covering the principal piece can be assessed using sperm motility or the HOST [1,29].

In the present study using EN stain, partially stained sperm were observed (Fig. 1 EN, panel b and c) and those were considered to be dead. Because of the clear correspondence of the unstained portion of sperm head with the acrosomal region in these cases. The tight juxtaposition of the plasma membrane with the nuclear membrane just after the posterior portion of the acrosome in the equatorial region may prevent the diffusion of the eosin dye to the anterior portion of the head cytoplasm [2].

There was high correlation and good agreement between the results obtained with vital stains, with excellent agreement observed between EN and trypan-blue (TB) and between carboxyfluorescein diacetate (CFDA)/PI and SYBR/PI, indicating that these stains are measures of the same sperm attribute (plasma membrane integrity; [2]). Many reports revealed that the proportion of membrane-intact sperm was overestimated by EN and

Table 1: Membrane-intact sperm % (mean ± SEM) of frozen-thawed bull spermatozoa supplemented with SP powder*

		Membrane-intact sperm %					
				HOST			
				HOST-positive		True HOST-positive	
Conc. of SP (mg/ml)	No. of replicates	EN	PI	1%Glutaraldehyde fixation	Giemsa staining	1%Glutaraldehyde fixation	Giemsa staining
0	4[n]	57.5±6.2	56.5±3.9	56.0±2.6	54.8±2.2	50.5±2.9	49.3±2.1
2.5	4[n]	60.0±6.4	55.2±5.3	58.7±5.8	54.6±3.7	53.8±5.9	49.7±3.7
12.5	4[n]	53.8±4.7	52.3±5.2	54.7±6.9	53.2±4.7	50.0±6.9	48.5±4.5
25	4[n]	53.2±5.5	52.1±5.2	55.2±6.6	55.2±5.8	50.2±6.7	50.2±5.8

*For each concentration, membrane-intact sperm % was examined in the same sample combined from 2 straws of semen.

[n]Frozen semen prepared from two straws for each concentration was used in four bulls in total 8 straws for each concentration.

Repeated measures ANOVA and Tukey's multiple comparison test, $p<0.05$.

Key: SP-Seminal plasma, EN-Eosin-nigrosin, PI-Propidium iodide, HOST-Hypoosmotic Swelling Test.

Table 2: Percentages of intact-acrosome (mean ± SEM) of fresh, frozen-thawed, washed and HOST treated bull spermatozoa examined by Glutaraldehyde fixation method*

		% intact-acrosome			
Conc. of SP (mg/ml)	No. of replicates	Fresh	Frozen-thawed	Washed	HOST treated
0	4[n]	98.4±0.2[a]	90.9±0.7[ab]	83.0±1.5[bc]	72.1±6.1[c]
2.5	4[n]	98.4±0.2[a]	91.6±0.6[ab]	82.0±1.1[bc]	73.0±5.2[c]
12.5	4[n]	98.4±0.2[a]	91.5±0.7[ab]	80.8±1.1[bc]	74.2±5.0[c]
25	4[n]	98.4±0.2[a]	91.6±0.9[ab]	78.8±1.1[bc]	74.2±5.9[c]

*For each concentration, % intact-acrosome was examined in the fresh semen and in the same sample combined from 2 straws of frozen semen.

[n]Frozen semen prepared from two straws for each concentration was used in four bulls in total 8 straws for each concentration.

Values with different superscripts was significantly different from the other values in the same column and row (Repeated measures ANOVA – Tukey's multiple comparison test, $p<0.05$.

Key: SP-Seminal plasma

Table 3: % abnormal morphology (mean ± SEM) of frozen-thawed bull spermatozoa supplemented with SP powder*

			% abnormal morphology	
Conc. of SP (mg/ml)	No. of replicates	Site of abnormality	1% Glutaraldehyde fixation	EN staining
0	4[n]	Head	1.3±0.5	1.7±0.8
		Mid-piece	2.5±0.2	1.5±0.5
		Tail	6.5±0.7	8.3±0.8
		Total	10.3±0.4	11.6±0.5
2.5	4[n]	Head	1.5±0.6	1.2±0.6
		Mid-piece	2.2±0.6	1.3±0.4
		Tail	6.5±0.6	8.2±1.1
		Total	9.6±1.8	10.8±0.8
12.5	4[n]	Head	2.0±0.4	2.2±0.4
		Mid-piece	2.2±0.2	3.2±1.0
		Tail	5.8± 0.6	5.7±0.7
		Total	10.1±0.6	11.2±0.6
25	4[n]	Head	1.7±0.6	2.7±0.4
		Mid-piece	2.5±0.7	2.7±0.8
		Tail	6.6±0.9	6.7±0.6
		Total	10.8±1.2	12.2±0.8

*For each concentration, % abnormal morphology was examined in the same sample combined from 2 straws of semen.

[n]Frozen semen prepared from two straws for each concentration was used in four bulls in total 8 straws for each concentration.

Key: SP-Seminal plasma

TB stains when compared to PI-combined fluorescent stains [2,9,30]. On contrary, two reports revealed that no significant difference was observed in the proportion of membrane-damaged sperm in samples stained with EN or PI [31,32] in agreement with our finding. The possible explanations for these contradictory results might be the different experimental conditions used such as the experimental animal, type of semen (fresh or frozen), type and composition of semen extender, the freezing protocol, staining technique and the concentration of stain. Also the different time of exposure to stain might be implicated, which was only a few seconds for EN and 10-30 min for PI. In the present study the sperm was exposed for few seconds and 5 min with EN and PI respectively. Although, two reports investigated the frozen-thawed bull semen [2,9] but one report used a different composition of EN stain which might affect pH of stain and followed by Giemsa staining [9]. Since, Eosin dye has acidic pH, then the more concentration of EN stain the lower pH (more acidic) of the stain therefore, the more membrane-intact sperm because the ability of membrane-damaged sperm to exclude an acidic stain (Eosin) was better than its ability to exclude a basic dye (PI) according to Foster *et al*. [33]. In the other report the semen was frozen using Triladyl extender followed by three times dilution with the same extender before examination [2] which might affect the plasma membrane integrity according to Amirat *et al.* [34] where they found that more than 80% of bull spermatozoa were injured after incubation for 4 hrs in Triladyl extender. Additionally, dual fluorescence was used (CFDA/PI and SYBR-14/PI), but in our study EN and PI were used lonely.

Though, evaluation of membrane-intact sperm with EN has been widely used as a field procedure in clinical practice, due to its speed and simplicity. However, this technique has many limitations, including a relatively low number of sperm typically counted and subjectivity in assessment [35]. Staining time, concentration of sperm and stain used and diluent characteristics which may interfere with available eosin or with stability of sperm membranes, are considered additional factors that may alter the proportion of membrane-intact sperm detected using this stain [36].

When frozen-thawed semen sample stained with EN or exposed to HOST solution the proportion of membrane-intact sperm was overestimated with EN than HOST. Based on this observation, some suggestions have been made that HOST and EN stain could be used to selectively evaluate the sperm membrane over the tail and head, respectively. However, since the plasma membrane is continuous over the sperm tail and head, it seems more likely that the plasma membrane of some HOST-positive sperm were damaged during incubation with the hypoosmotic solution and these sperm become HOST-negative during examination [2].

High correlations were observed between HOST and vital stains in bulls [37]. In addition, previous reports found that the proportions of viable sperm identified by HOST and vital stains were similar [38]. The obtained data revealed that, the proportions of membrane-intact sperm identified by either HOST or true HOST were similar to those identified by either EN or PI among all concentrations of SP powder. This finding indicated that both the physical and the functional integrity of sperm plasma membrane were similar at least in the frozen-thawed bull spermatozoa used in this study. Based on this finding we infer that proper semen collection, processing and storage might be maintaining both the physical and functional plasma membrane integrity of frozen-thawed bull spermatozoa.

Post-thaw HOST was the only method that had a significant relationship with *in vitro* fertilization rate [2]. When evaluated separately, HOST predicted a similar proportion of the variation in fertilization rate *in vitro* than sperm morphology, motility and acrosome integrity and significantly increased the proportion of variation accounted for when these methods were evaluated all together [2]. The proportions of normal, motile, acrosome-intact and HOST-positive bull spermatozoa had similar correlations with non-return rate after AI [39]. Moreover, when the number of viable sperm inseminated was calculated as a function of the HOST, or as a combined function of morphology, motility and the HOST, bulls with higher fertility had greater numbers of viable sperm than bulls with lower fertility. However, viable spermatozoa, calculated as a simple function of morphology, motility or intact-acrosome, were not different between high and low fertility bulls. Post-collection handling of SP as well as the point at which SP was introduced and the protein concentration of SP applied influences the final outcome of SP on sperm cell quality and function [40]. There was no significant effect of the added SP powder on the plasma membrane integrity which might be due to the added SP powder has no electrolytes especially Ca^{2+} and bicarbonate which are responsible for disruption of sperm plasma membrane. Also, egg yolk binds to SP proteins and avoids their detrimental effects on plasma membrane. Moreover, there was no significant effect of the added SP powder on the response of bull spermatozoa to HOST. In light of these

findings; the added SP powder at least had no harmful effect on the fertilizing potential of bull spermatozoa. Worth noting, this report emphasizing our recent finding [17] which revealed that desalted SP could be harnessed to protect frozen-thawed bull spermatozoa from cryocapacitation without harmful effect on either physical or functional plasma membrane integrity.

The integrity of the plasma membrane covering the acrosome is generally assessed in conjunction with the integrity of the outer acrosomal membrane. The acrosomal ridge, present on the sperm of several species, can be used to assess the integrity of this plasma membrane [25]. Although, several non-fluorescent and fluorescent staining combinations [41] have been developed to permit assessment of acrosomal membrane integrity of fresh and fixed sperm samples but Glutaraldehyde fixation method was used to examine sperm cell for acrosomal status in all experiments of the current study. Freezing-thawing non-significantly damaged the acrosomal membrane. Skillful semen collection, dilution, freezing-thawing and storage might be responsible for this non-significant effect. Nevertheless, as the proportion of intact-acrosome did not differ between fresh and frozen-thawed semen, it is more likely that freezing-thawing induced alterations involves changes in the mechanism responsible for acrosome reaction rather than acrosomal membrane integrity.

There are a number of sperm selection techniques for use with bovine sperm. These techniques are used for removing SP, dead and abnormal sperm cells, cryoprotective agents and other factors [42]. The techniques include Percoll density gradient centrifugation [42,43], swim-up migration, washing by centrifugation and glass wool filtration [42]. Acrosome integrity, as well as enzyme maintenance, is crucial to successful fertilization [44]. Similarly, our data revealed that, washing frozen-thawed sperm cell with saline and sucrose media damaged acrosomal membrane but significant effect was not detected in comparison with unwashed frozen-thawed semen.

Previous study [21], reported that sperm acrosomes developed resistance to hypoosmotic stress during incubation, the proportion of normal acrosomes remaining more or less stable from the beginning to the end of HOST. Acrosome was labile to excessive processing steps for evaluation of acrosomal status. Therefore, excessive centrifugation/washing steps and HOST solution result in loss, selection, or damage the acrosomal membrane of bull spermatozoa in agreement with Almadaly *et al.* [24].

Sperm morphology evaluation is an essential component of semen analysis and provides invaluable information for assessing the breeding soundness of a bull and the potential fertility of individual semen samples. Differences in sperm morphology results among different evaluation methods were attributed to introduction of artifacts, poor resolution/definition of sperm structures and unfamiliarity of the evaluator with the distinctive appearance of sperm processed by a particular method. Studies comparing methods of sperm morphology evaluation have either used only the proportion of normal sperm, which by itself is not a reliable endpoint from which to draw conclusions, or have made little effort to explain the possible causes of differences in the proportions of different sperm defects [45]. Although, EN stain recommended by the Society For Theriogenology for evaluation of bull sperm morphology [46] and is widely used, mainly because of its ease of use. The sperm with bent/coiled tails were more susceptible to head-tail detachment during smearing onto slides. On the other side, the use of wet-mount preparations seemed to reduce the introduction of some artifacts (detached sperm heads), but increased others (bent/coiled mid-pieces) in agreement with Brito *et al.* [47]. In the current study, sperm head, mid-piece and tail defects were enumerated in an attempt to paint a comprehensive picture of differences among evaluation methods. Brito *et al.* [47], reported that the use of wet-mount preparations and phase-contrast facilitated the observation of acrosome defects, nuclear vacuoles and cytoplasmic droplets, as demonstrated by the increased proportions of these defects when compared to stained smears; these results were consistent with our finding where the cytoplasmic droplets were apparently higher with Glutaraldehyde fixation (wet-mount) than EN staining but significant difference was not detected. On contrary EN staining showed higher proportions of detached head and detached tail defects than Glutaraldehyde fixation without significant effect on total tail defects.

Notably, in the present study although one bull had high % (>30%) of abnormal morphology (Data not shown) mainly coiled and bent tail but has high conception rate after AI using frozen-thawed semen. This might be due to these abnormalities appear to be compensable, meaning that fertility can be improved if more sperm were inseminated, while other are uncompensable [48], those are associated with sperm that are incompetent for fertilization [49]. The obtained data revealed, there were no significant differences among all concentrations of SP powder for all sperm cell defects using two evaluation

methods. This might be explained by that SP has crucial roles on sperm cell function not quality. Adjusting the volume (2 µl) of sample and the size of coverslip and letting the sample settle and dry for 5 min on warming plate before examination minimize the proportions of improperly oriented sperm. In addition the use of warm slides and stain combined with quick drying of the smear to minimize the time of contact of sperm with stain prevented any increase in bent and coiled sperm tails in the present study.

In conclusion, the added SP powder neither had harmful effect on the physical and/or the functional plasma membrane integrity nor on the fertilizing potential of frozen-thawed spermatozoa in terms of HOST results. Acrosome was labile to excessive centrifugation, washing and HOST solution. Sperm morphology did affected by neither the added SP powder nor the evaluation method. This manuscript could prove useful in studies involving addition of desalted SP to semen diluents before cryopreservation.

ACKNOWLEDGMENTS

This study was supported in part by the Grants-in-Aid from Japan Society for the Promotion of Science to T. M. (21580389). The authors are indebted to all staff members of Hida Beef Cattle Research Department, Gifu Prefectural Livestock Research Institute, Takayama, Japan, for donation of bull semen.

REFERENCES

1. Jeyendran, R.S., H.H. Van der-Ven, M. Perez-Pelaez, B.G. Crabo and L.J.D. Zaneveld, 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reproduction and Fertility, 70: 219-228.
2. Brito, L.F.C., A.D. Barth, S. Bilodeau-Goeseels, P.L. Panich and J.P. Kastelic, 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with *in vitro* fertilization rate. Theriogenology, 60: 1539-1551.
3. Harrison, R.A.P. and S.E. Vickers, 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J. Reproduction and Fertility, 88: 343-352.
4. Garner, D.L. and L.A. Johnson, 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction, 53: 276-284.
5. Mocé, E. and J.K. Graham, 2008. *In vitro* evaluation of sperm quality. Animal Reproduction Sci., 105: 104-118.
6. Swanson, E.W. and H.J. Bearden, 1951. An eosin-nigrosin stain for differentiating live and dead bovine spermatozoa. J. Animal Sci., 10: 981-987.
7. Mixner, J.P. and J. Saroff, 1954. Interference by glycerol with differential staining of bull spermatozoa. J. Dairy Sci., 37: 652.
8. Christensen, P., J.P. Stenvang and W.L. Godfrey, 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. J. Andrology, 25: 255-264.
9. Pintado, B., J. Fuente and E.R.S. Roldan, 2000. Permeability of boar and bull spermatozoa to the nuclei acid stains propidium iodide or Hoechst 33258, or to eosin: accuracy in the assessment of cell viability. J. Reproduction and Fertility, 118: 145-152.
10. Drevius, L.O. and H. Erikson, 1966. Osmotic swelling of mammalian spermatozoa. Experimental Cell Research, 42: 136-156.
11. Zavos, P.M., 1990. Hypoosmotic swelling test (HOS)/functional integrity of sperm membrane. J. and Association of Reproductive Technology and Andrology, 2: 215-216.
12. Rovegno, M., W.B. Feitosa, A.M. Rocha, C.M. Mendes, J.A. Visintin, M.E.O and D'Avila Assumpção, 2013. Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank, 14: 333-339.
13. Vadnais, M.L. and K.P. Roberts, 2007. Effects of seminal plasma on cooling induced capacitative changes in boar sperm. J. Andrology, 28: 416-422.
14. Okazaki, T., S. Abe, S. Yoshida and M. Shimada, 2009. Seminal plasma damages sperm during cryopreservation, but its presence during thawing improves semen quality and conception rates in boars with poor post-thaw semen quality. Theriogenology, 71: 491-498.
15. Okazaki, T. and M. Shimada, 2012. New strategies of boar sperm cryopreservation: development of novel freezing and thawing methods with a focus on the roles of seminal plasma. J. Animal Sci., 83: 623-629.

16. Leahy, T., J.I. Marti, G. Evans and W.M.C. Maxwell, 2009. Seminal plasma proteins protect flow-sorted ram spermatozoa from freeze-thaw damage. J. Reproduction Fertility and Development, 21: 571-578.
17. Almadaly, E., Y. Hoshino, T. Ueta, K. Mukoujima, M. Shukry, F. Farrag, I. El-Kon, K. Kita and T. Murase, 2014. Desalted and lyophilized bovine seminal plasma delays induction of the acrosome reaction in frozen-thawed bovine spermatozoa in response to calcium ionophore. Theriogenology (In press).
18. Masuda, H., 2006. Preservation of semen in liquid and frozen state. In Artificial Insemination Association of Japan, Textbook for the Course of Artificial Insemination in Domestic Animals, Fuji Planning Press, pp: 343-352 (In Japanese).
19. Murase, T., K. Mukohjima, S. Sakaguchi, T. Ohtani, T. Tsubota and I. Kita, 2001. Characterization of frozen-thawed Japanese black bull spermatozoa by standard semen analysis, mucus penetration test and the ability to undergo the acrosome reaction in response to calcium and the calcium ionophore A23187. J. Reproduction and Development, 47: 237-43.
20. Roldan, E.R.S. and T. Murase, 1994. Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. Effect is not mediated by protein kinase C. J. Biological Chemistry, 269: 23583-23589.
21. Tamuli, M.K. and P.F. Watson, 1994. Use of a simple staining technique to distinguish acrosomal changes in the live sperm sub-population. Animal Reproduction Sci., 35: 247-254.
22. Watson, P.F., 1975. Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. Veterinary Record, 97: 12-15.
23. Thundathil, J., A.T. Palasz, A.D. Barth and R.J. Mapletoft, 2002. Plasma membrane and acrosomal integrity in bovine spermatozoa with the knobbed acrosome defect. Theriogenology, 58: 87-102.
24. Almadaly, E., I. El-Kon, B. Heleil, E. Fattouh, K. Mukoujima, T. Ueda, Y. Hoshino, M. Takasu and T. Murase, 2012. Methodological factors affecting the results of staining frozen–thawed fertile and subfertile Japanese Black bull spermatozoa for acrosomal status. Animal Reproduction Sci., 136: 23-32.
25. Shams-Borhan, G. and R.A.P. Harrison, 1981. Production, characterization and use of ionophore-induced, calcium- dependent acrosome reaction in ram spermatozoa. Gamete Res., 4: 407-432.
26. Dott, H.M. and G. Foster, 1972. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential live/dead stain. J. Reproduction and Fertility, 29: 443-445.
27. Barth, A.D. and R. Oko, 1989. Abnormal morphology of bovine spermatozoa. Ames: Iowa State University Press.
28. Motulsky, H., 1995. Intuitive Biostatistics. New York: Oxford University Press.
29. Colenbrander, B., B.M. Gadella and T.A.E. Stout, 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reproduction in Domestic Animals, 38: 305-311.
30. Gadea, J. and C. Matas, 2000. Sperm factors related to *in vitro* penetration of porcine oocytes. Theriogenology, 54: 1343-1357.
31. Graham, J.K., E. Kunze and R.H. Hammerstedt, 1990. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biology of Reproduction, 43: 55-64.
32. Vazquez, J.M., E.A. Martinez, P. Martinez, C. Garcia-Artiga and J. Roca, 1997. Hypoosmotic swelling of boar spermatozoa compared to other methods for analyzing the sperm membrane. Theriogenology, 47: 913-922.
33. Foster, M.L., C.C. Love, D.D. Varner, S.P. Brinsko, K. Hinrichs, S. Teague, K. LaCaze and T.L. Blanchard, 2011. Comparison of methods for assessing integrity of equine sperm membranes. Theriogenology, 76: 334-341.
34. Amirat, L., M. Anton, D. Tainturier, C. Chatagnon, I. Battut and L.J. Courtens, 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction, 129: 535-543.
35. Johansson, C.S., F.C. Matsson, H. Lehn-Jensen, J.M. Nielson and M.M. Petersen, 2008. Equine spermatozoa viability comparing the NucleoCounter SP-100 and the eosin-nigrosin stain. Animal Reproduction Sci., 23: 24-25.

36. Björndahl, L., I. Söderlund, S. Johansson, M. Mohammadieh, M. Reza Pourian and U. Kvist, 2004. Why the SHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. Andrology Lab Corner. J. Andrology, 25: 671-678.
37. Correa, J.R. and P.M. Zavos, 1994. The hypoosmotic-swelling test: its employment as an assay to evaluate the functional integrity of frozen-thawed bovine sperm membrane. Theriogenology, 42: 351-360.
38. Lin, M., M. Morshedi, C. Srisombut, A. Nassar and S. Oehninger, 1998. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, water test and eosin-Y staining. Fertility Sterility, 70: 1148-1155.
39. Correa, J.R., M.M. Pace and P.M. Zavos, 1997. Relationships among frozen-thawed sperm characteristics assessed via routine semen analysis, sperm functional tests and fertility of bulls in artificial insemination program. Theriogenology, 48: 721-731.
40. Leahy, T., J.I. Marti, G. Evans and W.M.C. Maxwell, 2010. Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Animal Reproduction Sci., 119: 147-153.
41. Silva, P.F.N. and B.M. Gadella, 2006. Detection of damage in mammalian sperm cells. Theriogenology, 65: 958-978.
42. Morrell, J.M. and H. Rodriguez-Martinez, 2009. Biomimetic techniques for improving sperm quality in animal breeding: a review. Open Andrology J., 1: 1-9.
43. Mehmood, A., M. Anwar and S.M. Saqlan-Naqvi, 2009. Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or Percoll gradient method from frozen-thawed buffalo semen. Animal Reproduction Sci., 111: 141-148.
44. Öura, C. and K. Toshimori, 1990. Ultrastructural studies on the fertilization of mammalian gametes. International Review of Cytology, 122: 105-151.
45. Freneau, G.E., P.J. Chenoweth, R. Ellis and G. Rupp, 2010. Sperm morphology of beef bulls evaluated by two different methods. Animal Reproduction Sci., 118: 176-81.
46. Chenoweth, P., J. Spitzer and F. Hopkins, 1992. A new bull breeding soundness evaluation form. Society for Theriogenology, Montgomery, pp: 63-70.
47. Brito, L.F.C., L.M. Greene, A. Kelleman, M. Knobbe and R. Turner, 2011. Effect of method and clinician on stallion sperm morphology evaluation. Theriogenology, 76: 745-750.
48. Walters, A.H., W.E. Eyestone, R.G. Saacke, R.E. Pearson and F.C. Gwaszdauskas, 2005. Bovine embryo development after IVF with spermatozoa having abnormal morphology. Theriogenology, 63: 1925-1937.
49. Saacke, R.G., S. Nadir and R.L. Nebel, 1994. Relationship of semen quality to sperm transport, fertilization and embryo quality in ruminants. Theriogenology, 41: 45-5.