The Thai Journal of Veterinary Medicine

Volume 49
Issue 3 *September, 2019*

Article 7

9-1-2019

Comparison of different methods for sperm vitality assessment in frozen-thawed Holstein bull semen

Kakanang Buranaamnuay

Original Article

Comparison of different methods for sperm vitality assessment in frozen-thawed Holstein bull semen

Kakanang Buranaamnuay

Abstract

The present study evaluated the accuracy of four methods, i.e. eosin-nigrosin, trypan blue, hypo-osmotic swelling test (HOST) and Hoechst 33342 (H342)/propidium iodide (PI) for assessing bovine sperm vitality. Frozen-thawed semen (n = 30) of Holstein bulls was layered on 40%/80% percoll solutions to isolate viable spermatozoa. An aliquot of viable spermatozoa was kept at 37°C (live sample); the rest was submitted to cold shock (dead sample). The two aliquots were mixed in three proportions, corresponding to 0%, 50% and 100% of viable cells; the sperm vitality was analyzed. The percentages of viable spermatozoa evaluated with the four methods significantly correlated with the expected vitality (r_s = 0.92 – 0.94, P<0.001). Comparing methods of evaluation, the sperm vitality assessed by eosin-nigrosin and trypan blue was comparable (P>0.05) when live and dead samples were used. For the live sample, eosin-nigrosin (82.37 ± 1.48%) and trypan blue (81.00 ± 1.67%) yielded significantly greater results than HOST (50.14 ± 1.68%) and H342/PI (56.69 ± 1.76%) (P<0.001). The percentage of viable spermatozoa in the dead sample (13.45 ± 0.86%) was highest when HOST was exploited (P<0.001); still, the sperm vitality acquired by this technique become the lowest when the live sample was evaluated (P<0.001 to P=0.03). In conclusion, while HOST and fluorescence stains H342/PI with the protocol used in this study are not trustworthy methods, eosin-nigrosin and trypan blue are accurate techniques for sperm vitality assessment in frozen-thawed Holstein bull semen.

Keywords: cattle, spermatozoa, membrane integrity, staining techniques, freezing

Molecular Agricultural Biosciences Cluster, Institute of Molecular Biosciences (MB), Mahidol University, Nakhon Pathom 73170, Thailand

****Correspondence**: ningkakanang@yahoo.com* (K. Buranaamnuay)

Introduction

Vitality of spermatozoa is requisite for successful fertilization. Therefore, irrespective of animal species, sperm vitality is always included in semen analysis (Vasan, 2011; Morado *et al*., 2015; Sutovsky, 2015). Currently, there are several methods for assessing the vitality of spermatozoa. Dye exclusion assays, traditional vitality tests, such as eosin, eosin-nigrosin and trypan blue are of the most common methods (Brito *et al*., 2003), and eosin-nigrosin is recommended as a vitality test by the World Health Organization (WHO) for use in clinical Andrology laboratories (WHO, 2010). The dye exclusion tests are grounded in the permeability of the plasma membrane. The plasma membrane of viable spermatozoa is a barrier to dye penetration; therefore, after incubation with the dyes, viable spermatozoa remain colorless. On the other hand, damaged or dead spermatozoa lack membrane structural integrity and, as a result, allow the entry of the dyes (Eliasson and Treichl, 1971). It has been reported that trypan blue contained in the triple staining technique and eosin-nigrosin have been effective for vitality evaluation in bovine fresh spermatozoa but in frozen-thawed semen the eosin-nigrosin technique overestimated the number of viable cells that were able to fertilize oocytes (Felipe-Perez *et al*., 2008).

In addition to the permeability of the plasma membrane, sperm vitality can also be evaluated by determining the ability of the sperm membrane to maintain equilibrium between the intracellular-fluid and extracellular-fluid compartments using the hypo-osmotic swelling test (HOST) (WHO, 2010). During the HOST procedure, spermatozoa are exposed to hypotonic solutions; influx of the solutions results in the expansion of the cytoplasmic space especially in the tail. Coiling of sperm tails can be easily observed with a phase contrast microscope (Jeyendran *et al*., 1992; Hossain *et al*., 1998). A higher percentage of swollen spermatozoa indicate the presence of viable spermatozoa having a functional plasma membrane (Ramu and Jeyendran, 2013). HOST is considered to be a useful method for clinical and practical applications apart from its diagnostic value in selecting viable but immotile spermatozoa for intracytoplasmic sperm injection (ICSI) although, in humans, lower fertilization rates have been obtained from this sperm population as compared with cases where motile and viable spermatozoa were used (Tsai *et al*., 1997). In boars, it has been shown that HOST test correlated significantly with *in vivo* fertility and indicated possible sperm damage due to cold shock (Perez-Llano *et al*., 2001). Contrarily, HOST test is not sufficiently sensitive to discriminate between bovine semen samples of intermediate fertility (Rota *et al*., 2000).

The assessment of sperm vitality has been enhanced by the availability of new fluorescent stains targeting sperm DNA. The combination of these nucleic acid stains, one stain to identify viable spermatozoa e.g. Hoechst 33342 (H342) and SYBR-14 and another to stain only dead spermatozoa e.g. Hoechst 33258 (H258), propidium iodide (PI) and ethidium bromide, have been effective methods for determining sperm vitality in boars (Garner *et al*., 1996), monkeys (Cai *et al*., 2005) and other mammals (Garner and Johnson, 1995). Using H342/PI for rhesus monkey ejaculates, the correlation between percentages of expected vitality and viable spermatozoa stained only with H342 was found to be very strong both when evaluated by fluorescence microscopy and flow cytometry (Cai *et al*., 2005). Besides vitality evaluation, H342 has been utilized in computer assisted sperm analysis (CASA) (Farrell *et al*., 1996; Oliveira *et al*., 2013) and flow cytometric sorting of spermatozoa (Garner, 2009). For CASA, H342 helps distinguish spermatozoa from other particulate material such as somatic cells in semen and egg yolks or whole milk in freezing media while in the process of flow sorting, H342 is used to measure DNA mass in X- and Y-chromosome-bearing mammalian spermatozoa. According to previous results, it has been proved that exposure of spermatozoa to H342 did not affect sperm DNA, the fertilizing capacity of the viable spermatozoa or the developmental competence of the resultant embryos (Morrell and Dresser, 1989; Seidel and Garner, 2002; Cran, 2007).

To date, there are no comparative studies on the effectiveness of using eosin-nigrosin, trypan blue, H342/PI as well as HOST for assessing sperm vitality. The present study was therefore designed to compare the accuracy of these methods for evaluating the sperm vitality of frozen-thawed Holstein bull semen. The results obtained will be useful for selecting the appropriate effective method for sperm vitality assessment.

Materials and Methods

Chemicals: All reagents, unless otherwise other specified, were from Sigma-Aldrich (St. Louis, MO, USA).

Ethics Statement: No live animals were included in any of the experiments described in this article. Frozen bovine semen used for sperm vitality evaluations was supplied from a commercial dairy farm named Farm Chokchai®, which is situated in Nakhon Ratchasima province in the northeastern region of Thailand.

Specimens: Frozen semen in mini-(0.25 mL) plastic straws (Minitub GmbH, Tiefenbach, Germany) was produced from 30 ejaculates of Holstein bulls, whose their fertility had been proved, for daily use in artificial insemination on the farm. The protocol for freezing semen according to a disclosure by a member of staff is briefly described here. At room temperature (27 ± 2°C), raw semen with the progressive motility of 65% or more was extended in Tris-citric acid-egg yolk-fructose diluent (Buranaamnuay *et al*., 2015) to attain $120x10^6$ spermatozoa/mL in final sperm concentration. The diluted semen was placed at 4°C for 4 h, loaded into the straws and frozen by a rapid freezing method in a styrofoam box.

Sperm preparation: In order to assess the vitality of the spermatozoa, 2 – 3 frozen semen straws from the same ejaculates and the same bulls were thawed (37°C for 30 s) and three defined percentages of the sperm vitality (i.e. 0%, 50% and 100%) were made by the following

procedure. Immediately after thawing, the total motility of spermatozoa was evaluated visually under a light microscope (400x; Helmut Hund GmbH, Wetzlar-Nauborn, Germany) to check whether the post-thaw spermatozoa were viable. The thawed semen that contained viable spermatozoa was then placed on the topmost layer of the 40% and 80% Percoll gradient (semen: 40% Percoll: 80% Percoll = 1:3:3, v/v) and centrifuged at 400 x g for 15 mins. Percoll solutions were prepared beforehand by the dilution of Percoll® (GE Healthcare, Little Chalfont, UK) in sperm-TALP (10X and 1X) (Buranaamnuay, 2013). The sperm pellet was washed in 2 mL sperm-TALP by single centrifugation (200 x g, 5 min); and then the washed pellet was resuspended in sperm-TALP to acquire approximately 80 x 10^6 spermatozoa/mL. This sperm portion was assumed to be 100% viable sample, since the total sperm motility checked at this stage was not less than 75%.

To provide quantifiable proportions of viable and dead spermatozoa, the 100% viable sample was divided into two parts. The first part was placed in a water bath at 37°C to maintain sperm vitality. The other part was submitted to cold shock to induce cellular damage by two cycles of suddenly placing it in a -80°C freezer (model 720, capacity 0.566 m^3; Thermo Fisher Scientific Inc., Waltham, MA) for 7 min and thawing at 37°C. At this stage, motile spermatozoa were not found according to the sperm motility evaluation. This sperm portion was therefore deemed to be a 0% viable sample. Then, a sample with 50% viable spermatozoa was made by mixing aliquots of 100% and 0% viable samples at a ratio of 1:1. In each semen sample, three defined percentages of sperm vitality (i.e. 0%, 50% and 100%) were prepared in total and used for sperm vitality evaluations.

Evaluations of sperm vitality: In this study, the vitality of spermatozoa was appraised by four techniques, as described below.

One-step eosin-nigrosin staining: The eosin-nigrosin test is a dye exclusion method recommended by WHO to be used as a sperm vitality test. For each sample, a 20 µl aliquot was placed on a pre-warmed glass slide; the same amount of eosin-nigrosin dyes [0.6% (w/v) eosin Y and 5% (w/v) nigrosin dissolved in distilled water] was dropped on. After mixing for 30 secs, 10 µL of the mixture was taken to spread on another clean glass slide and dried on a 37°C warm plate. The dried smear is then observed for the vitality of spermatozoa using bright-field microscopy (1000x). The viable spermatozoa with intact plasma membrane are not stained by the eosin dye. On the other hand, the dye penetrates the membrane-damaged spermatozoa; dark pink or red sperm heads are seen. In each sample, the percentage of sperm vitality was calculated based on 200 total spermatozoa counted.

Trypan blue staining: The vitality of spermatozoa was also evaluated by the vital stain Trypan blue, which penetrates into the post acrosomal region of dead cells (Felipe-Pérez *et al.*, 2008). The sperm sample was diluted with 0.4% trypan blue solution in 1:1.2 (v/v) ratio and then incubated at 37°C for 15 min. To stop the movement of spermatozoa during evaluation, 10 µL of formol saline [0.9% (w/v) NaCl and 0.1% (v/v) 40% formaldehyde in distilled water] (Dott and Foster, 1975) was added to the sample. Microscopic slides were prepared by placing a drop of the stained sample on the slide and covering with a cover slip. Slides were examined under the 1000 x magnification, with immersion oil, of a bright-field microscope. A total of 200 sperm heads was counted, and the percentage of viable (unstained) spermatozoa was calculated and noted.

Hypo-osmotic swelling test (HOST): The HOST is an alternative to the dye exclusion test for the assessment of sperm vitality (WHO, 2010). It estimates physiological functionality not structural integrity as evaluated by the dye exclusion method, of the sperm plasma membrane (Lin *et al.*, 1998). A swelling solution (75 mOsm) had been prepared by combining Na-citrate dihydrate with D-fructose in distilled water. A total of 50 µL sample was mixed with 300 µL of the swelling solution. After incubation for 20 min at 37 °C, a small drop of the mixture was placed on a microscope slide overlaid with a coverslip and 200 spermatozoa were analyzed by phase-contrast microscopy (400x; Nikon, Melville, NY, USA). Only swollen cells of different types were considered positive and reported as a percentage of all spermatozoa analyzed. For an accurate assessment, sperm samples without the swelling solution were also examined for spontaneous swelling or spermatozoa with coil tails. The number of swollen cells in the unincubated samples was deducted from the HOST score in order to obtain the percentage of viable spermatozoa.

H342/PI staining: The H342/PI staining procedure used in the present study was modified from Garner *et al.* (1996). A 22 µM working solution of H342 was prepared from a stock solution (22 mM) by dilution in distilled water. The working solution of PI was 1 mM in phosphate buffered saline (PBS). An aliquot (75 µL) of sample was stained with 4 µL of H342 and 4 µL of PI working solutions. The sample was incubated in darkness for 30 min at 37°C. Immediately prior to assessment, a drop of formol saline was added to prevent sperm movement. Fluorescent staining was monitored using a fluorescence microscope (400x; Axioskop, Carl Zeiss Jena GmbH, Jena, Germany). The viable, dead and moribund spermatozoa emit bright blue, pinkish red and both blue and red, respectively when the H342/PI-stained samples are excited with the ultraviolet (UV) light. The percentage of sperm vitality was determined from a total of 200 spermatozoa counted.

Statistical analysis: All statistical analyses were performed using the SPSS software (version 17.0 for Windows, SPSS Inc., Chicago, IL, USA). An assessment of the normality of data was undertaken by the Kolmogorov-Smirnov test. Correlation coefficients between expected (i.e. 0%, 50% and 100% vitality) and measured (i.e. % vitality obtained through four tests) values were made using linear regression analysis. Correlations among pairs of measured variables were calculated using a Spearman correlation. One-way

ANOVA and Tukey's HSD post-hoc test were used to determine the difference in the percentages of sperm vitality among tests. Data are represented as means ± SEM; the level of significance was set at P<0.05.

Results

In the present study, the mean percentages of viable spermatozoa assessed by eosin-nigrosin and trypan blue, for ratios of 0:100, 50:50 and 100:0 of the live and dead mixtures were 6.50%, 46.63% and 82.37% and 6.73%, 50.77% and 81.00%, respectively. The correlations between the vitality evaluated by these two techniques with the expected percentages were r_s = 0.94 (*P*<0.001) (Table 1 and Fig 1 A and B). Similarly, very high positive correlations (r_s = 0.92 – 0.94, *P*<0.001) were found between the expected values and the viable population detected by HOST and H 342/PI (Table 1 and Fig 1 C and D).

Comparing the methods of vitality evaluation, the percentages of viable spermatozoa assessed by eosin-nigrosin and trypan blue were commensurate (*P*>0.05) when the ratios of viable spermatozoa in the sample were 0% and 100% (Table 2). However, when the proportion of viable spermatozoa was 50%, trypan blue gave significantly, but marginally, higher in the results than eosin-nigrosin staining (P=0.04). The results in the 50% and 100% viable samples evaluated with eosin-nigrosin and trypan blue were significantly greater compared with the vitality assessed by HOST and H342/PI (P<0.001). The percentage of viable spermatozoa in dead samples (13.45 ± 0.86%) was the highest when the HOST method was used (*P*<0.001 for all). On the other hand, the sperm vitality acquired by this technique (50.14 ± 1.68%) became the lowest when the samples containing 100% of living cells were evaluated (*P*<0.001 to *P*=0.03). Regardless of the mixture ratio, the percentages of viable spermatozoa were positively and significantly associated among the four evaluation methods; the correlation coefficients were between 0.84 and 0.95 (*P*<0.001, Table 1).

Table 1 Coefficients of correlation (r_s) and significance level (P value) between the percentages of sperm vitality, evaluated by four different methods and expected values

	Eosin-nigrosin	Trypan blue	HOST[1]	H342/PI[2]
Expected values	0.94 (P<0.001)	0.94 (P<0.001)	0.92 (P<0.001)	0.94 (P<0.001)
Eosin-nigrosin		0.95 (P<0.001)	0.86 (P<0.001)	0.92 (P<0.001)
Trypan blue			0.84 (P<0.001)	0.93 (P<0.001)
HOST				0.86 (P<0.001)

[1]The sperm vitality detected by the hypo-osmotic swelling test
[2]The sperm vitality evaluated by staining with Hoechst 33342 and propidium iodide (PI)

Table 2 The mean percentages (± SEM) of viable frozen-thawed bovine spermatozoa assessed by four different methods, for ratios of 100:0, 50:50 and 0:100 of the living and killed mixtures

Expected percentage of sperm vitality	Method of sperm vitality evaluation			
	Eosin-nigrosin	Trypan blue	HOST[1]	H342/PI[2]
0%	6.50 ± 1.19 [b]	6.73 ± 1.19 [b]	13.45 ± 0.86 [a]	0.73 ± 0.31 [c]
50%	46.63 ± 0.96 [b]	50.77 ± 1.00 [a]	32.07 ± 1.21 [c]	31.47 ± 1.16 [c]
100%	82.37 ± 1.48 [a]	81.00 ± 1.67 [a]	50.14 ± 1.68 [c]	56.69 ± 1.76 [b]

Values with different letters (a, b, c) indicate significant difference within row
[1]The sperm vitality detected by the hypo-osmotic swelling test
[2]The sperm vitality evaluated by staining with Hoechst 33342 and propidium iodide (PI)

Discussion

In the present study, the accuracy of techniques for sperm vitality evaluation was analyzed in frozen-thawed bovine semen. This has yet to be carried out in dairy cattle, to the author knowledge. The results showed that all four techniques studied (three assessed with vital stains and one evaluated by measurement of osmoregulatory capacity) correlated significantly with the expected vitality percentages. This indicates that both measurements of structural integrity using eosin-nigrosin, trypan blue and H342/PI and functional integrity using HOST of sperm plasma membrane are usable for sperm vitality assessment in Holstein bulls. In accordance with the present results, previous studies have demonstrated the usefulness of such tests in the evaluation of sperm vitality in several mammalian species including humans (Eliasson and Treichl, 1971; Bjorndahl *et al.*, 2003), monkeys (Cai *et al.*, 2005), horses (Kutvolgyi *et al.*, 2006), pigs (Zhou *et al.*, 2004) and mice (Al-Saleh and Al-Otaiby, 2014).

Besides high correlations with the expected vitality, the percentages of viable spermatozoa obtained with the four methods correlated significantly with one another especially between the results obtained with vital stains, for which correlation coefficients of more than 0.90 were shown. The present finding is in agreement with a previous report where vital stains eosin-nigrosin, trypan blue and PI in combination with carboxyfluoresceindiacetate (CFDA) or SYBR-14 were tested in beef bull semen and high correlations between the results were investigated (Brito *et al.*, 2003). This could suggest that these vital stains provide measures of the same sperm trait, i.e. plasma membrane integrity. The mechanism of action of these

dyes depends on the properties of the cell membrane which separates the inside of the cell from the external environment. Eosin and trypan blue, that in an aqueous solution at physiological pH are anionic, do not penetrate the intact plasma membrane of viable cells due to the negative charge of the cell membrane; in contrast, these dyes penetrate dead or damaged cells, staining the cytoplasm (Kwolek-Mirek and Zadrag-Tecza, 2014). To interpret the results, for eosin staining, viable cells are colorless or faint pink and dead cells show red or dark pink. In cases of combining with eosin, nigrosin helps provide a dark background for increased contrast and yields reliable evaluations using ordinary light microscope optics (WHO, 2010). For trypan blue staining, colorless cells are scored as viable cells while, in spermatozoa, cells with blue color at the post acrosomal region are classified as dead cells (Felipe-Pérez *et al.*, 2008). Comparing single- (trypan blue) and dual- (eosin-nigrosin) staining methods, differentiation of viable from dead cells using bright field microscopy is easier for dual- than single-staining methods. This could be a possible reason explaining why in the 50% viable samples of the present study the sperm vitality evaluated with eosin-nigrosin was different from that assessed with trypan blue; nonetheless, such difference was marginally significant (P=0.04). Disregarding a trivial difficulty during assessment, both eosin and trypan blue staining techniques require neither of expensive material nor highly sophisticated equipment (Bjorndahl *et al.*, 2003; Felipe-Pérez *et al.*, 2008); it is therefore highly possible for a small laboratory and even in the field to adopt these methods in semen analysis.

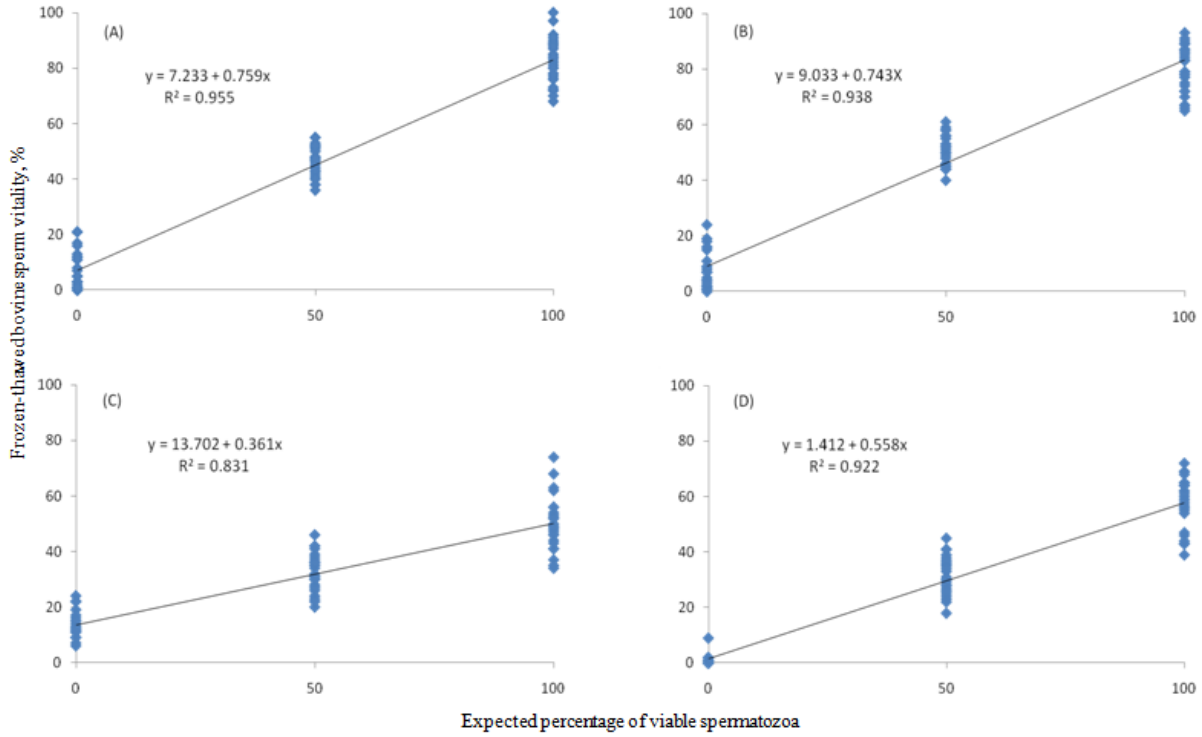

Figure 1 Regression plots for correlations between the percentages of viable frozen-thawed bovine spermatozoa evaluated by eosin-nigrosin staining (A), trypan blue staining (B), hypo-osmotic swelling test (C) and Hoechst 33342 and propidium iodide staining (D) and the expected percentages.

Apart from eosin-nigrosin and trypan blue, the vitality of frozen-thawed bovine spermatozoa was also evaluated with nucleic acid specific-stains H342/PI. The proportions of membrane-intact spermatozoa identified by H342/PI were significantly inferior to those detected with eosin-nigrosin or trypan blue stains. The difference in the proportions of viable cells acquired by these staining methods is potentially due to the fact that these dyes bind to different cell components, i.e. eosin and trypan blue stain the cytoplasm while H342/PI stain the nucleus. Furthermore, for H342 staining, cell types and staining conditions such as concentrations of the dye, acidity and alkalinity of media as well as incubation temperature and time are important factors in dye uptake by the cells (Lalande *et al.*, 1981; Weisenfeld, 2007) and, hence, the percentage of cell vitality. For bovine spermatozoa, it has been speculated that, other than staining conditions, spermatozoa from different bulls and even some spermatozoa within an ejaculate may differ in dye permeability; this results in variations in staining efficiency among cells (Garner, 2009). Nonetheless, it is worth noting that results of the present study were in accord with those observed in beef bull semen, for which CFDA/PI or SYBR-14/PI fluorescent stains instead of H342/PI were used and yielded significantly lower sperm vitality compared with eosin-nigrosin and trypan blue (Brito *et al.*, 2003).

As stated above, the HOST and vital stains measure different aspects of the sperm plasma membrane, i.e. the HOST evaluates functional activity while vital stains assess structural integrity (Lin *et al.*, 1998). This fact may be used to explain differences in the sperm vitality percentages between the HOST and vital stains

in the present study. The HOST yielded significantly greater results in the dead (0% viable) sample but provided significantly lower results in the 100% viable sample. The present finding, in the dead sample, agreed with Munuce *et al.* (2000), who reported higher vitality values in the HOST than in the eosin-nigrosin when freeze-killed human spermatozoa were evaluated. The authors suggested that when spermatozoa are killed by freezing, structural integrity of the sperm plasma membrane is lost before the capacity to regulate osmotic equilibrium (Munuce *et al.*, 2000). Similarly, in humans, it has been revealed that poor quality spermatozoa with borderline membrane integrity stained red with eosin can exhibit swelling in HOST (Chan *et al.*, 1991). In addition, the results of the present study found that the 100% viable sample was in concurrence with previous findings which indicated that the response of spermatozoa to hypo-osmotic stress does not depend only on the morphological integrity of the sperm plasma membrane (Brito *et al.*, 2003) and that during the HOST procedure hypo-osmotic shock on its own induces membrane damage and therefore decreases the percentages of viable spermatozoa (Ramirez *et al.*, 1992).

Although the proportions of viable spermatozoa acquired by means of H342/PI staining and HOST were more likely to deviate from the expected values, in comparison to eosin-nigrosin and trypan blue, vitality assessment by these two techniques offers some advantages over staining with eosin-nigrosin or trypan blue. The most prominent one is that viable spermatozoa detected with these techniques can be further used for therapeutic procedures such as ICSI (Tsai *et al.*, 1997), and the resultant embryos and offspring are normal (Cran *et al.*, 1995; Garner *et al.*, 1996; McNutt and Johnson, 1996). Moreover, using HOST as a vitality assessment method, proportions of HOST positive spermatozoa were significant predictors of sperm fertility in beef bulls (Brito *et al.*, 2003) and boars (Perez-Llano *et al.*, 2001). Therefore, to confirm the usefulness of sperm vitality assessment techniques for Holstein bulls, the relationships between the percentage of sperm vitality evaluated with these four methods and fertility *in vitro* and *in vivo* should also be determined.

In conclusion, while the fluorescence stains H342/PI with the protocol used in this study are not trustworthy stains to assay sperm vitality, eosin-nigrosin and trypan blue staining techniques can be taken as effective diagnostic tests for the discrimination of viable from nonviable spermatozoa in Holstein bulls. Nevertheless, *in vitro* and/or *in vivo* fertility of frozen-thawed spermatozoa should also be investigated to confirm the benefit of these vitality assessment techniques for this animal.

Conflicts of interest: There are no conflicts of interest regarding the publication of this article.

Acknowledgements

Frozen Holstein bull semen was kindly supplied by Farm Chokchai®. The author is grateful to Assist. Prof. Kulnasan Saikhun and Ms. Kornkanok Promthep for providing the laboratory facilities.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

References

Al-Saleh AA and Al-Otaiby AM 2014. Evaluation effect of dacarbazine on mice testis tissues and sperm parameters. Int Res J Pharm App Sci. 4: 9–13.

Bjorndahl L, Soderlund I and Kvist U 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod. 18: 813–816.

Brito FC, Barth AD, Bilodeau-Goeseels S, Panich PL and Kastelic P 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 60: 1539–1551.

Buranaamnuay K 2013. Sperm-TALP: an alternative extender for retrieving and diluting epididymal sperm in the domestic cat. Reprod Dom Anim. 48: 912–917.

Buranaamnuay K, Sangsuwan P, Changsangfa C, Faisaikarm T and Kaeoket K 2015. Influence of discontinuous PureSperm® and OptiPrep™ gradient centrifugations on bovine sperm quality and the sex ratio of in vitro produced embryos. Chiang Mai J Sci. 42: 637–649.

Cai K, Yang J, Guan M, Ji W, Li Y and Rens W 2005. Single UV excitation of Hoechst 33342 and propidium iodide for viability assessment of rhesus monkey spermatozoa using flow cytometry. Arch Androl. 51: 371–383.

Chan PJ, Tredway DR, Corselli J, Pang SC and Su BC 1991. Combined supravital staining and hypoosmotic swelling test. Hum Reprod. 6: 1115–1118.

Cran DG 2007. XY sperm separation and use in artificial insemination and other ARTs. Soc. Reprod. Fertil. (Suppl) 65: 470–491.

Cran DG, Johnson LA and Polge C 1995. Sex preselection in cattle: a field trial. Vet Rec. 136: 495–496.

Dott HM and Foster GC 1975. Preservation of differential staining of spermatozoa by formol citrate. J Reprod Fertil. 45: 57–60.

Eliasson R and Treichl L 1971. Supravital staining of human spermatozoa. Fertil Steril. 22: 134–137.

Farrell PB, Foote RH and Zinaman MJ 1996. Motility and other characteristics of human sperm can be measured by computer-assisted sperm analysis of samples stained with Hoechst 33342. Fertil Steril. 66: 446–453.

Felipe-Perez YE, Juarez-Mosqueda ML, Hernandez-Gonzalez EO and Valencia JJ 2008. Viability of fresh and frozen bull sperm compared by two staining techniques. Acta Veterinaria Brasilica 2: 123–130.

Garner DL 2009. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 71: 11–21.

Garner DL, Dobrinsky JR, Welch GR and Johnson LA 1996. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology 45: 1103–1113.

Garner DL and Johnson LA 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod. 53: 276–284.

Hossain AM, Rizk B, Barik S, Huff C and Thorneycroft IH 1998. Time course of hypo-osmotic swellings of human spermatozoa: Evidence of ordered transition between swelling subtypes. Hum Reprod. 13: 1578–1583.

Jeyendran RS, Van der Ven HH and Zaneveld LJ 1992. The hypoosmotic swelling test: an update. Arch Androl. 29: 105–116.

Kutvolgyi G, Stefler J and Kovacs A 2006. Viability and acrosome staining of stallion spermatozoa by Chicago sky blue and Giemsa. Biotech Histochem. 81: 109–117.

Kwolek-Mirek M and Zadrag-Tecza R 2014. Comparison of methods used for assessing the viability and vitality of yeast cells. FEMS Yeast Res. 14: 1068–1079.

Lalande ME, Ling V and Miller RG 1981. Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells. Proc Natl Acad Sci. 78: 363.

Lin MH, Morshedi M, Srisombut C, Nassar A and Oehninger S 1998. Plasma membrane integrity of cryopreserved human sperm: an investigation of the results of the hypoosmotic swelling test, the water test, and eosin-Y staining. Fertil Steril. 70: 1148–1155.

McNutt TL and Johnson LA 1996. Flow cytometric sorting of sperm: influence on fertilization and embryo/fetal development in the rabbit. Mol Reprod Dev. 43: 261–267.

Morado S, Pereyra V, Breininger E, Sara R and Cetica P 2015. Study of sperm evaluation parameters to estimate cryopreserved bovine semen fertility. Austin J Vet Sci & Anim Husb. 2: 1005.

Morrell JM and Dresser DW 1989. Offspring from inseminations with mammalian sperm stained with Hoechst 33342 either with or without flow cytometry. Mutat Res. 224: 177–183.

Munuce MJ, Caille AM, Berta CL, Perfumo P and Morisoli L 2000. Does the hypoosmotic swelling test predict human sperm viability? Arch Androl. 44: 207–212.

Oliveira LZ, de Arruda RP, de Andrade AF, Celeghini EC, Reeb PD, Martins JP, dos Santos RM, Beletti ME, Peres RF, Monteiro FM and Hossepian de Lima VF 2013. Assessment of *in vitro* sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Anim Reprod Sci. 137: 145–155.

Perez-Llano B, Lorenzo JL, Yenes P, Trejo A and Garcia-Casado P 2001. A short hypoosmotic swelling test for the prediction of boar sperm fertility. Theriogenology 56: 387–398.

Ramirez JP, Carreras A and Mendoza C 1992. Sperm plasma membrane integrity in fertile and infertile men. Andrologia 24: 141–144.

Ramu S and Jeyendran RS 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. In: Spermatogenesis. Methods in molecular biology (Methods and Protocols), vol 927. D Carrell and K Aston (eds) Totowa, NJ: Humana Press.

Rota A, Penzo N, Vincenti L and Mantovani R 2000. Hypoosmotic swelling (HOS) as a screening assay for testing in vitro fertility of bovine spermatozoa. Theriogenology 53: 1415–1420.

Seidel Jr GE and Garner DL 2002. Current status of sexing mammalian sperm. Reproduction 24: 733–743.

Sutovsky P 2015. New approaches to boar semen evaluation, processing and improvement. Reprod Dom Anim. 50 (Suppl 2): 11–19.

Tsai YL, Liu J, Garcia JE, Katz E, Compton G and Baramki TA 1997. Establishment of an optimal hypo-osmotic swelling test by examining single spermatozoa in four different hypo-osmotic solutions. Hum Reprod 12: 1111–1113.

Vasan SS 2011. Semen analysis and sperm function tests: How much to test? Indian J Urol. 27: 41–48.

Weisenfeld RB 2007. Mass transport kinetics of the DNA-binding dye Hoechst 33342 into bovine spermatozoa. Bioorg Med Chem. 15: 6361–6366.

World Health Organization 2010. WHO Laboratory manual for the examination and processing of human semen. WHO Press, Geneva, Switzerland.

Zhou JB, Yue KZ, Luo MJ, Chang ZL, Liang H, Wang ZY and Tan JH 2004. Effect of extenders and temperatures on sperm viability and fertilizing capacity of Harbin White boar semen during long-term liquid storage. Asian-Aust J Anim Sci. 17: 1501–1508.