



Hypo-Osmotic Swelling Test Modification to Enhance Cell Membrane Integrity Evaluation in Cryopreserved Bull Semen

Baiee, F. H.^{1,3}, Wahid, H.^{1*}, Rosnina, Y.¹, Ariff, O. M.², Yimer, N.¹, Salman, H.¹, Tarig, A. A.¹ and Khumran, A. M.¹

¹Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

²Department of Veterinary Pre-Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

³Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Kufa, Najaf, Iraq

ABSTRACT

The objective of this study was to enhance the hypo-osmotic swelling test evaluation when it reads under light microscope using 5% formaldehyde-fixed sperm solution buffer (FSSB). Twenty four ejaculates were collected from six crossbred bulls using electro-ejaculator (EE). Tris-egg yolk extender was used to cryopreserve the semen. Concentration, volume, motility, morphology and viability rates of fresh semen were evaluated and samples were cryopreserved in liquid nitrogen. After two weeks of liquid nitrogen treatment (freezing), the motility, morphology and viability rate of the semen were evaluated. In order to carry out a hypo-osmotic swelling test, post-thaw semen was divided into four aliquots based on period of incubation (30 or 60 minutes) adding FSSB to half the samples. The components of FSSB were 5% formaldehyde and 1% eosin-nigrosin stain in PBS. Results showed that F₆₀ (61.48 ± 0.89%) resulted in higher percentage (P<0.05) of total membrane intact compared with F₃₀ (54.40 ± 1.34%) and N₃₀ (53.96 ± 1.17%), but did not differ (P>0.05)

with N₆₀ (60.90 ± 0.70%). In conclusion, adding 10 µl of FSSB after 60 minute of incubation with hypo-osmotic swelling solution (HOSS) will enhance evaluation of the hypo-osmotic swelling test (HOST) under light microscope.

ARTICLE INFO

Article history:

Received: 05 April 2016

Accepted: 21 February 2017

E-mail addresses:

falalhali@uokufa.edu.iq (Baiee, F. H.),
wahidh@upm.edu.my; wahidharon@gmail.com (Wahid, H.),
Rosninanuris@upm.edu.my (Rosnina, Y.),
mo_ariff@upm.edu.my (Ariff, O. M.),
nurhusien@upm.edu.my (Yimer, N.),
salman_hammadi@yahoo.com (Salman, H.),
tarignazar@hotmail.com (Tarig, A. A.),
khumranmada@yahoo.com (Khumran, A. M.)

* Corresponding author

Keywords: Hypo-osmotic swelling test, bull, sperm, cryopreservation

INTRODUCTION

Super-vital staining and hypo-osmotic swelling tests (HOST) are used to validate the membrane integrity of spermatozoa; HOST however, is the most preferred technique because it is easy, simple, frugal, and quick (Tartagni et al., 2002).

Sperm cell swelling was first discussed by Drevius and Eriksson (1966), and its deformities were illustrated by developing a HOST for human (Jeyendran et al., 1984) and bull sperm (Revell & Mrode, 1994) based on sperm cell permeability to allow it to swell under hypo-osmotic situation. Therefore, HOST can determine the viability of sperm by increasing cell volume to obtain the balance between inside and outside solutions.

Swelling of the spermatozoon usually begins at the tail as the membrane at this area was loosely held, and then moves toward mid piece and head, while the dead, non-bioactive sperm has a normal shape because of their leaky membrane (Jeyendran et al., 1984). Hence, coiled tail, shortening of the tail and swelling head are shown in the test. This study was conducted to determine the effect of using formaldehyde and eosin-nigrosin stain on bull sperm. .

MATERIALS AND METHODS

Animal Selection

Six fertile bulls (four Brangus–Simmental crossbred and two Sahiwal-Friesian crossbred) were selected for this study. They were between three and five years old, and

weigh between 570 kg and 650 kg. They were kept in a separate pen at Universiti Putra Malaysia farm. All bulls were fed with *Brachiaria decumbens* and additional 3 kg/BW palm kernel cakes concentrate. The water and mineral block were provided *ad libitum*.

Semen Collection and Processing

Semen from all bulls were collected using an electro-ejaculator (EE) (Ideal® Instruments Neogen Corporation, Lansing, Michigan, USA) as described by Sarsaifi et al. (2013). Freshly collected semen were evaluated and accepted at a concentration $\geq 600 \times 10^6/\text{ml}$, motility $\geq 75\%$, morphology and viability $> 85\%$. After evaluation, semen was diluted to final concentration of 20×10^6 sperm per 0.25ml straw using the formula below:

- Number of Straws = $\frac{\text{Concentration of semen} \times \text{Volume} \times \text{Motility}\% *}{N^{**}}$
- The total volume = number of straws x volume of one straw
- Volume of extender = total volume – volume of semen

* Percentage of motility to cancel the percentage of immotile sperm

** N = number of sperm per straw (in this study was $20 \times 10^6/\text{sperm/ml}$)

Tris-egg yolk extender was prepared according to Amirat-Briand et al. (2010).

It was divided into two fractions, the first fraction contained 0% glycerol (Tris: 2.24 g, citric acid: 1.48 g, fructose: 1 g, and 20% egg yolk in 100 ml extra pure of distilled water) while the second fraction had the same ingredients except for 12.8% glycerol. The first fraction of extender (without glycerol) was added to the semen and maintained at between 2°C and 5°C for three hours. The second fraction of extender (with 12.8% glycerol) was added at chill temperature and then immediately concussed and loaded in 0.25 ml French straws (Berndtson & Foote, 1969) and frozen in liquid nitrogen using the following steps: a) all straws were placed approximately 6.5 cm over the liquid nitrogen for five minutes, b) the distance was lowered to approximately 3.0 cm over liquid nitrogen for five minutes, and c) the distance lowered again to about 1.0 cm above the liquid nitrogen for three minutes. All straws were plunged into liquid nitrogen immediately after that and stored in a cryogenic tank. Straws were thawed after 14 days for further evaluation.

Hypo-Osmotic Swelling Solutions Preparation

All chemicals used in this study were purchased from Sigma Co. (Sigma Chemical Co., St. Louis, MO, USA). Hypo-osmotic swelling solution (HOSS) was prepared as described by (Jeyendran et al., 1984; Revell and Mrode, 1994). It contains 0.9 g fructose and 0.49 g sodium citrate dissolved up to 100 ml in distilled water. For preparation of 5% formaldehyde-fixed sperm solution

buffer (FSSB), 5.0 ml of formaldehyde was added and mixed with 95.0 ml of phosphate buffered saline (stock solution). Then, 1.0ml from the stock solution was removed and added 1.0ml of eosin-nigrosin stain to achieve a final concentration of 5% formaldehyde and 1% eosin-negrosin stain.

Semen Evaluation

Sperm motility and concentration were evaluated using computer assisted sperm analyser (CASA; Hamilton Thorne Bioscience). Frozen straw is first thawed in a water bath at 37°C for 30 sec. About 10 µl of semen was placed on a dry warm dual sided sperm analysis chamber and covered with a cover glass. After one to two minutes the slide was placed in CASA, and at least three microscopic fields were read from each chamber.

The viability (Memon et al., 2012) and morphology (Chemineau et al., 1991) of sperm were evaluated using eosin-nigrosin stain. For this, 5 µl of semen was mixed with 15 µl of eosin-nigrosin for one minute at room temperature. Then, the smear was allowed to dry on hotplate stage (Copens Scientific (M) Sdn. Bhd.) maintained at 37°C and subsequently examined under a light microscope (Nikon Japan, ECLIPSE E200) at 40X magnification. Stained sperm head was classified as dead sperm while white sperm head was indicated as live. Sperm abnormalities were also determined where at least 200 sperms were evaluated at 100X.

Hypo-Osmotic Swelling Test

This experiment was divided into four different groups. For each group, 10 µl of post-thawed semen was incubated with 80 µl of HOSS in water path at body temperature. The 1st group (F₃₀) and 2nd group (N₃₀) were incubated for 30 minutes, while 3rd group (F₆₀) and 4th group (N₆₀) were incubated for 60 minutes. At the end of incubation period, 10 µl of FSSB was added to F₃₀ and another 10 µl of FSSB was added to F₆₀ to stop reaction, fix the shape of sperm and to give the medium some colours (F refers to that group that had FSSB at the end of incubation period, while N means none. On the other hand, the subscript ₃₀ refers to the incubation period of 30 minutes while the subscript ₆₀ refers to the incubation period of 60 minutes). For groups N₃₀ and N₆₀, immediately at the end of incubation period, 10 µl of the mixture was placed on a warm microscopic slide and covered with cover slip and evaluated under microscope (400X). Groups F₃₀ and F₆₀ underwent similar process but they were evaluated

after 5 minutes of adding FSSB. Sperm with intact membranes swelled and their tails curled while sperms with damages in their membranes remain unchanged. At least 200 sperm with intact and non-intact membranes were recorded.

Statistical Analysis

Data was analysed as mean ± standard error of the mean (SEM). Descriptive statistic and one way ANOVA were used to analysis HOST, fresh and cryopreserved semen while t-test pairs analysis was used to examine semen parameters to compare motility, morphology and viability before freezing and after thawing using SPSS version 22 for windows (SPSS Inc.; Chicago, IL, the USA).

RESULTS

Fresh and cryopreserved semen analysis

Table 1 shows that the concentration of fresh sperm was greater in bull B231 1270.25 X 10⁶ ± 66.91 sperm/ml, and it

Table 1
Sperm parameters of fresh and cryopreserved bull semen (Mean±SEM)

Bull ID	N	Concentration X 10 ⁶ Spermatozoa/	Fresh semen			Post-thawed semen			
			Volume/ ml	Motility%	Morphology %	Viability%	Motility%	Morphology %	Viability%
T104	4	995.00 ± 81.80 ^{ab}	8.88 ± 2.42	86.25 ± 2.39 ^{ab}	97.00 ± 0.41 ^a	94.00 ± 0.58 ^a	41.00 ± 2.61	94.75 ± 0.32	65.13 ± 2.29
B9001	4	775.00 ± 47.87 ^b	9.25 ± 1.60	77.00 ± 1.00 ^b	93.00 ± 1.39 ^{ab}	89.25 ± 1.20 ^{ab}	33.25 ± 1.80	89.63 ± 0.92	55.75 ± 3.71
B229	4	1175.00 ± 125.00 ^{ab}	5.87 ± 0.72	84.25 ± 2.95 ^{ab}	93.75 ± 1.36 ^{ab}	91.12 ± 1.33 ^{ab}	53.50 ± 6.30	92.87 ± 0.52	71.75 ± 2.25
B231	4	1270.25 ± 66.91 ^a	8.50 ± 0.29	86.25 ± 2.39 ^{ab}	86.87 ± 0.77 ^b	87.37 ± 0.97 ^b	47.75 ± 5.57	86.75 ± 0.48	70.75 ± 1.88
B206	4	1176.25 ± 33.25 ^{ab}	3.78 ± 0.13	86.75 ± 1.18 ^a	94.75 ± 0.25 ^{ab}	87.50 ± 0.65 ^{ab}	51.75 ± 1.80	90.88 ± 0.37	69.25 ± 1.11
T902	4	845.00 ± 90.97 ^{ab}	5.00 ± 0.71	77.25 ± 0.95 ^b	96.13 ± 0.37 ^{ab}	87.00 ± 0.41 ^{ab}	47.00 ± 4.10	92.12 ± 0.43	69.25 ± 1.31
Total ¹	24	1039.42 ± 47.81	6.88 ± 0.63	82.96 ± 1.13 [*]	93.60 ± 0.76 [*]	89.37 ± 0.62 [*]	45.71 ± 2.06	91.17 ± 0.56	66.98 ± 1.39

¹Parameter of fresh semen with asterisk (*) compared with the same parameter of post-thawed semen.

^{a, b} and ^{ab} – different superscripts in the values of each bull in the column 'concentration', 'motility', 'morphology' and 'viability' differ significantly at P<0.05

was significantly high ($P < 0.05$) compared with bull 9001 $775 \times 10^6 \pm 47.87$ sperm/ml. There were no significant differences ($P > 0.05$) in the volume of fresh semen. The motility rate of fresh semen was greater in bull B206 and it was highly significant ($P < 0.05$) compared with bull 9001 and T902. Sperms with good integrity and viability were recorded in bull T104 $97 \pm 0.41\%$ and $94 \pm 0.58\%$ respectively. They were significantly higher ($P < 0.05$) compared with bull 231.

Results show that the concentration, volume, motility, morphology and viability of fresh crossbred bulls semen were $1039.42 \times 10^6 \pm 47.81$ (sperm/ml), 6.88 ± 0.63 ml, $82.96 \pm 1.13\%$, $93.60 \pm 0.76\%$ and $89.37 \pm 0.62\%$ respectively. Results showed that motility, morphology and viability of frozen semen were reduced where ($P < 0.01$) $45.71 \pm 2.06\%$, $91.17 \pm 0.56\%$ and $66.98 \pm 1.39\%$ respectively compared with fresh semen.

Cell membrane integrity

Membrane integrity of post-thawed semen was read via two incubation periods, 30 and 60 minutes with HOSS and with or without adding FSSB. According to Table 2, the ratio of membrane integrity in F_{60} $61.48 \pm 0.89\%$ was high ($P < 0.01$) compared with F_{30} $54.40 \pm 1.34\%$ and N_{30} $53.96 \pm 1.17\%$. However, there were no significant differences between F_{60} and N_{60} that underwent the same incubation period. Moreover, adding FSSB gave some darkness to the solution and enabled the coiled and swollen sperm to be easily located (Figure 1).

DISCUSSION

In the present study, some semen parameters were different based on the age of bulls (Al-Kanaan et al., 2015). Significant differences were recorded in semen volume, motility,

Table 2
Cell membrane integrity using hypo-osmotic swelling test of cryopreserved bull semen

Group	N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum
				Lower Bound	Upper Bound	
F_{30}			1.34	51.63	57.16	43.50
N_{30}	24	53.96 ^a	1.17	51.53	56.38	45.00
F_{60}	24	61.48 ^b	0.89	59.65	63.31	52.00
N_{60}	24	60.90 ^b	0.70	59.45	62.34	52.00

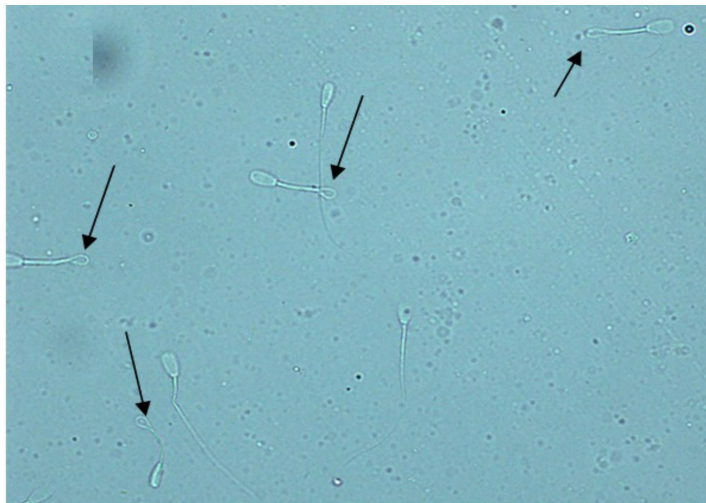
^a and ^b – different superscripts within column differ significantly at $P < 0.01$

F_{30} : 10 μ l of thawed semen incubated with 80 μ l of HOSS for 30 min at 37°C, and then FSSB was added at the end of incubation period.

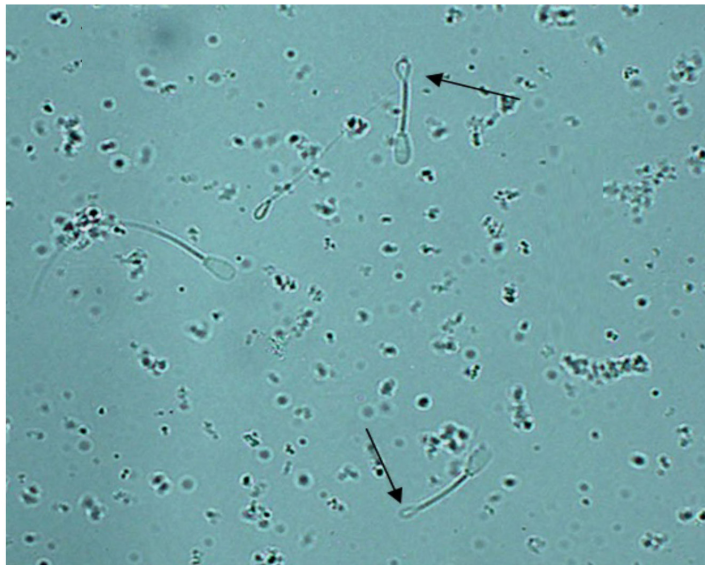
N_{30} : 10 μ l of thawed semen incubated with 80 μ l of HOSS for 30 min at 37°C, without adding FSSB.

F_{60} : 10 μ l of thawed semen incubated with 80 μ l of HOSS for 60 min at 37°C, and then FSSB was added at the end of incubation period.

N_{60} : 10 μ l of thawed semen incubated with 80 μ l of HOSS for 60 min at 37°C, without adding FSSB.



(a)



(b)

Figure 1. Frozen bull sperm under hypo-osmotic condition; coiled sperm tail indicating intact membranes of sperm. a) without adding FSSB; b) after adding FSSB

concentration, and output between older and younger bulls. These findings are consistent with previous works (Brito et al., 2002) on genetic impact (Carabano et al., 2007), and season (Al-Kanaan et al., 2015). However, this study was conducted in a tropical region which is limited by two seasons .

Semen response to the freezing varies among males of the same species and in different species, as shown in Table 1 when motility rate was compared between bull 9001 ($33.25 \pm 1.80\%$), and bull B231 ($53.50 \pm 6.30\%$). This difference may be due to age (Foote, 2002; Snoj et al., 2013), or genetic

material variations (Gürler et al., 2015a; Snoj et al., 2013). However, differences were not due to nutrition variation, since all bulls were reared under similar conditions.

The protocol of freezing processes, three steps of freezing in liquid nitrogen, using tris-egg yolk extender obtained a motility rate $45.71 \pm 2.06\%$, with 66.98 ± 1.39 of viability rate; this is very near to the universal acceptance of semen motility and viability after cryopreservation (Hu et al., 2011; Lessard et al., 2000; Watson, 2000).

The quality of freezing straw is limited by many factors, such as genetic material of the bull, motility of sperm before and after freezing, number of sperms that can pass the freezing stress with normal cell membrane, acrosome, and DNA material (Foote & Kaproth, 1997). In addition, the collection of semen and its procedure, age of sperm before using, shipping, and the skill of insemination are important to ensure the fertility rate of cryopreserved semen (Rycroft & Bean, 1992). All these factors are important to reduce the motility, viability, and morphology rate significantly of post thawing semen compared with fresh semen (Yimer et al., 2014, 2015; Zhang et al., 2015). Our findings are consistent with those of earlier studies.

One of the most important parameters to detect male fertility is morphology of the sperm (Lasiene et al., 2013), and abnormalities of sperm indicate a genetic disorder in spermatogenesis (Veeramachaneni & Sawyer, 1996). Therefore, it might decrease the fertility of semen (Nagy et al., 2013; Saacke, 2008;

Serafini et al., 2015). In the present study, sperm morphology of frozen-thawed semen was significantly reduced compared with fresh sperm as previously highlighted (Al-Makhzoomi et al., 2008).

Sperm have three layers and are rich with polyunsaturated fatty acids, plasma, mitochondrial, and acrosome membranes. They are highly susceptible to oxidative stress during cryopreservation processes (Moussa et al., 2002). Furthermore, when sperms are frozen, they are exposed to increasingly hypertonic condition as a result of the freezing water conditions and intracellular solutes are at maximum concentration. These may rupture the cell membrane and affect the motility of the sperm. Cell membrane rupture is clearly associated with sperm viability loss (De Leeuw et al., 1993). El-Nour et al. (2001) used immotile sperm for ICSI that were divided into two groups: one group was tested using HOST (HOST-group) and the other was not tested (no-HOST). It was found that the pregnancy rate of HOST-group (40%) was higher than the no-HOST group (14%). Hence, if the sperm pass HOST, it tends to increase chances of pregnancy (El-Nour et al., 2001; Stanger, 2010). In addition, HOST is easy, inexpensive and simple (Hossain et al., 1998; Tartagni et al., 2002). All these explain the value of HOST.

This study was designed to focus on the reading method of the HOST to make it easier and more precise for searchers via adding FSSB immediately at the end of incubation period of sperm in HOSS.

Results showed that addition of FSSB after 60 minutes of incubation with HOSS showed positive impact to enhance the test of membrane integrity. The reason for adding formaldehyde at 5% is to stop the reaction of sperm with HOSS solution and keep the final shape of sperm at the end of the incubation period. In addition, under microscope, some sperm were seen moving that leads to misreading of the exact number of sperm that are active biologically. Thus, formaldehyde at 5% will prevent the sperm from moving and conserve their shape if the osmolarity is changed. Moreover, adding 1% eosin-nigrosin stain provides some darkness to the solution that will enable easy spotting of coiled and swollen sperm.

HOST is good to test male fertility (El-Nour et al., 2001; Liu et al., 1997; Perez-Llano et al., 2001; Stanger et al., 2010). The present study and previous ones (Revell and Mrode, 1994; Zubair et al., 2013) depended on HOST (Jeyendran et al., 1984) to prepare the HOSS using sodium citrate in tri form and fructose at osmolarity 150 mOsm/kg, and tested on bull semen. In our study, adding FSSB to the post thawed semen that was incubated for 60 minutes seems to increase the total percentage of membrane intact and this was consistent with a study conducted on Nili-River bulls (Zubair et al., 2013). the authors had tested different osmolarities of HOSS (70, 90, 100, 120, 140, 150, 190, 230, 280, and 300 mOsm/kg) at similar incubation period (60 minutes) and found that 150 mOsm/kg showed high percentage of membrane integrity.

However, our observations are in contrast with those of Revell and Mrode (1994), The authors had attempted to look for the best osmolarity in best incubation period of membrane osmolarity resistant correlated with pregnancy rate (49 days non-return rate after AI), and found that the best osmolarity for fresh bull semen was 150 mOsm/kg and 125 and 100 mOsm/kg for frozen semen in 20 minutes of incubation for both fresh and frozen semen. The difference between their findings and the present study may due to number of bulls that were tested, genotype and diet of animals and number of samples from each bull. A handfull of studies found that a diet rich in poly unsaturated fatty acid can affect the structure of sperm membrane (Gürler et al., 2015b; Khoshvaght et al., 2016) that can lead to a change in the permeability of cell membranes.

CONCLUSION

This study showed that supplementation of HOST with FSSB at the end of incubation period has a beneficial effect by enhancing the membrane integrity test reading under light microscope 40 X. In terms of incubation period, 60 minutes resulted in better membrane integrity.

ACKNOWLEDGEMENT

The authors thank the management and staff of Universiti Putra Malaysia farm (TPU) and Theriogenology and Cytogenetics laboratory of the Faculty of Veterinary Medicine, UPM for their cooperation during this study.

REFERENCES

- Al-Kanaan, A., König, S., & Brügemann, K. (2015). Effects of heat stress on semen characteristics of Holstein bulls estimated on a continuous phenotypic and genetic scale. *Livestock Science*, *177*, 15-24.
- Al-Makhzoomi, A., Lundeheim, N., Håård, M., & Rodriguez-Martinez, H. (2008). Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology*, *70*(4), 682-691.
- Amirat-Briand, L., Bencharif, D., Vera-Munoz, O., Pineau, S., Thorin, C., Destrumelle, S., & Shmitt, E. (2010). In vivo fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: Preliminary results of artificial inseminations. *Animal Reproduction Science*, *122*(3), 282-287.
- Berndtson, W., & Foote, R. (1969). The survival of frozen bovine spermatozoa following minimum exposure to glycerol. *Cryobiology*, *5*(6), 398-402.
- Brito, L., Silva, A., Rodrigues, L., Vieira, F., Deragon, L., & Kastelic, J. (2002). Effect of age and genetic group on characteristics of the scrotum, testes and testicular vascular cones, and on sperm production and semen quality in AI bulls in Brazil. *Theriogenology*, *58*(6), 1175-1186.
- Carabano, M., Diaz, C., Ugarte, C., & Serrano, M. (2007). Exploring the use of random regression models with Legendre polynomials to analyze measures of volume of ejaculate in Holstein bulls. *Journal of Dairy Science*, *90*(2), 1044-1057.
- Chemineau, P., Cagnie, Y., Guerin, V., Orgeur, P., & Vallet, J. (1991). Training manual on artificial insemination in sheep and goats. *Animal Reproduction and Health*, *83*, 115-130.
- De Leeuw, F., De Leeuw, A., Den Daas, J., Colenbrander, B., & Verkleij, A. (1993). Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology*, *30*(1), 32-44.
- Drevius, L. O., & Eriksson, H. (1966). Osmotic swelling of mammalian spermatozoa. *Experimental Cell Research*, *42*(1), 136-156.
- El-Nour, A. M., Al-Mayman, H. A., Jaroudi, K. A., & Coskun, S. (2001). Effects of the hypo-osmotic swelling test on the outcome of intracytoplasmic sperm injection for patients with only nonmotile spermatozoa available for injection: a prospective randomized trial. *Fertility and Sterility*, *75*(3), 480-484.
- Foote, R. (2002). The history of artificial insemination: Selected notes and notables. *Journal of Animal Science*, *80*(2), 1-10.
- Foote, R., & Kaproth, M. (1997). Sperm numbers inseminated in dairy cattle and nonreturn rates revisited. *Journal of Dairy Science*, *80*(11), 3072-3076.
- Gürler, H., Calisici, O., & Bollwein, H. (2015a). Inter-and intra-individual variability of total antioxidant capacity of bovine seminal plasma and relationships with sperm quality before and after cryopreservation. *Animal Reproduction Science*, *155*, 99-105.
- Gürler, H., Calisici, O., Calisici, D., & Bollwein, H. (2015b). Effects of feeding omega-3-fatty acids on fatty acid composition and quality of bovine sperm and on antioxidative capacity of bovine seminal plasma. *Animal Reproduction Science*, *160*, 97-104.
- Hossain, A., Rizk, B., Barik, S., Huff, C., & Thorneycroft, I. (1998). Time course of hypo-osmotic swellings of human spermatozoa: evidence of ordered transition between swelling subtypes. *Human Reproduction*, *13*(6), 1578-1583.

- Hu, J. H., Zhao, X. L., Tian, W. Q., Zan, L. S., & Li, Q. W. (2011). Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. *Animal*, 5(01), 107-112.
- Jeyendran, R., Van der Ven, H., Perez-Pelaez, M., Crabo, B., & Zaneveld, L. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility*, 70(1), 219-228.
- Khoshvaght, A., Towhidi, A., Zare-shahneh, A., Noruozi, M., Zhandi, M., Davachi, N. D., & Karimi, R. (2016). Dietary n-3 PUFAs improve fresh and post-thaw semen quality in Holstein bulls via alteration of sperm fatty acid composition. *Theriogenology*, 85(5), 807-812.
- Lasiene, K., Gedrimas, V., Vitkus, A., Glinskyte, S., Lasys, V., Valanciute, A., & Sienkiewicz, W. (2013). Evaluation of morphological criteria of sperm quality before in vitro fertilization and intracytoplasmic sperm injection. *Polish Journal of Veterinary Sciences*, 16(4), 773-785.
- Lessard, C., Parent, S., Leclerc, P., Baileys, J. L., & Sullivan, R. (2000). Cryopreservation alters the levels of the bull sperm surface protein P25b. *Journal of Andrology*, 21(5), 700-707.
- Liu, J., Tsai, Y. L., Katz, E., Compton, G., Garcia, J. E., & Baramki, T. A. (1997). High fertilization rate obtained after intracytoplasmic sperm injection with 100% nonmotile spermatozoa selected by using a simple modified hypo-osmotic swelling test. *Fertility and Sterility*, 68(2), 373-375.
- Memon, A. A., Wahid, H., Rosnina, Y., Goh, Y. M., Ebrahimi, M., & Nadia, F. (2012). Effect of antioxidants on post thaw microscopic, oxidative stress parameter and fertility of Boer goat spermatozoa in Tris egg yolk glycerol extender. *Animal Reproduction Science*, 136(1), 55-60.
- Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., & Anton, M. (2002). Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 57(6), 1695-1706.
- Nagy, S., Johannisson, A., Wahlsten, T., Ijäs, R., Andersson, M., & Rodriguez-Martinez, H. (2013). Sperm chromatin structure and sperm morphology: Their association with fertility in AI-dairy Ayrshire sires. *Theriogenology*, 79(8), 1153-1161.
- Perez-Llano, B., Lorenzo, J., Yenes, P., Trejo, A., & Garcia-Casado, P. (2001). A short hypoosmotic swelling test for the prediction of boar sperm fertility. *Theriogenology*, 56(3), 387-398.
- Revell, S., & Mrode, R. (1994). An osmotic resistance test for bovine semen. *Animal Reproduction Science*, 36(1), 77-86.
- Rycroft, H., & Bean, B. (1992). Factors influencing non-return data. In *Proceedings of the 14th Technical Conference on Artificial Insemination and Reproduction, National Association of Animal Breeders* (pp. 43-46).
- Saacke, R. (2008). Sperm morphology: Its relevance to compensable and uncompensable traits in semen. *Theriogenology*, 70(3), 473-478.
- Sarsaifi, K., Rosnina, Y., Ariff, M., Wahid, H., Hani, H., Yimer, N., & Abas, M. (2013). Effect of Semen Collection Methods on the Quality of Pre-and Post-thawed Bali Cattle (*Bos javanicus*) Spermatozoa. *Reproduction in Domestic Animals*, 48(6), 1006-1012.
- Serafini, R., Romano, J. E., Varner, D. D., Di Palo, R., & Love, C. C. (2015). Sperm DNA assays and their relationship to sperm motility and morphology in bulls (*Bos Taurus*). *Animal Reproduction Science*, 159, 77-86.

- Snoj, T., Kobal, S., & Majdic, G. (2013). Effects of season, age, and breed on semen characteristics in different *Bos taurus* breeds in a 31-year retrospective study. *Theriogenology*, *79*(5), 847-852.
- Stanger, J. D., Vo, L., Yovich, J. L., & Almahbobi, G. (2010). Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. *Reproductive Biomedicine Online*, *21*(4), 474-484.
- Tartagni, M., Schonauer, M., Cicinelli, E., Selman, H., Ziegler, D., Petruzzelli, F., & D'addario, V. (2002). Usefulness of the Hypo-Osmotic Swelling Test in Predicting Pregnancy Rate and Outcome in Couples Undergoing Intrauterine Insemination. *Journal of Andrology*, *23*(4), 498-502.
- Veeramachani, D., & Sawyer, H. R. (1996). Use of semen as biopsy material for assessment of health status of the stallion reproductive tract. The Veterinary Clinics of North America. *Equine Practice*, *12*(1), 101-110.
- Watson, P. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, *60*, 481-492.
- Yimer, N., Muhammad, N., Sarsaifi, K., Rosnina, Y., Wahid, H., Khumran, A., & Kaka, A. (2015). Effect of honey supplementation into Tris Extender on Cryopreservation of Bull Spermatozoa. *Malaysian Journal of Animal Science*, *18*(2), 47-54.
- Yimer, N., Noraisyah, A., Rosnina, Y., Wahid, H., Sarsaifi, K., & Hafizal, A. (2014). Comparison of cryopreservative effect of different levels of omega-3 egg yolk in citrate extender on the quality of goat spermatozoa. *Pakistan Veterinary Journal*, *34*(3), 347-350.
- Zhang, X. G., Hu, S., Han, C., Zhu, Q. C., Yan, G. J., & Hu, J. H. (2015). Association of heat shock protein 90 with motility of post-thawed sperm in bulls. *Cryobiology*, *70*(2), 164-169.
- Zubair, M., Ahmad, I., Ahmad, M., & Iqbal, Z. (2013). Development of the best hypo-osmotic swelling solution for evaluation of functional membrane integrity of spermatozoa of Nili-Ravi buffalo bull. *Asian Journal of Agricultural Biology*, *1*(2), 63-66.

