CODEN (USA): IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF

PHARMACEUTICAL SCIENCES

Available online at: http://www.iajps.com Research Article

EFFECT OF DIFFERENT DILUTION MATERIALS USAGE ON INDONESIAN PERANAKAN ONGOLE BULL SPERM QUALITY DURING COOLING PROCESS

Nolasco Da Costa, Trinil Susilawati*, Nurul Isnaini* and Moh. Nur Ihsan*

Doctoral student at Animal Production Study Program, Faculty of Animal Husbandry, Brawijaya University

*Department of Animal Production, Faculty of Animal Husbandry, Brawijaya University, Jalan Veteran, Malang, East Java, 65145 Indonesia.

Abstract:

The research aimed to examine the effect of dilution based on tris and egg yolk with CEP-2 on motility, viability and sperm membrane integrity of Peranakan Ongole bull during cooling process. The randomized block design with 10 replications from 10 ejaculations of Peranakan Ongole bull was applied in this study. Treatments applied were T0 = Tris aminomethane + 20% egg yolk, T1 = CEP-2 + 10% egg yolk, T2 = CEP-2 (Non BSA) + 10% egg yolk, T3 = CEP-2 (Non BSA) + 15% soya bean extract and T4 = CEP-2 + 15% soya bean extract. Observations on individual sperm motility, viability and sperm membrane integrity were directly carried out after dilution each day up to the tenth day and after dilution and kept cooled. The study showed that dilution based on tris aminomethane + egg yolk could maintain the semen quality included sperm motility, viability and cattle sperm membrane integrity during cooling process. Spermatozoa of Peranakan Ongole bull diluted with tris aminomethane + 20% egg yolk at the seventh day of storage had a progressive motility (45.75%), viability (49.25%) and membrane integrity (42.70%).

Keywords: *Tris aminomethane, egg yolk, motility, membrane integrity, viability.*

Corresponding author:

Nolasco Da Costa,

Doctoral student at Animal Production Study Program,
Faculty of Animal Husbandry,
Brawijaya University,
Jalan Veteran, Malang, East Java,
65145 Indonesia.
E-Mail:dacostanolasco@yahoo.co.id

QR code

Please cite this article in press as Nolasco Da Costa et al, ***Effect of Different Dilution Materials Usage on Indonesian Peranakan Ongole Bull Sperm Quality during Cooling Process****, Indo Am. J. Pharm. Sci, 2016; 3(4).*

INTRODUCTION:

The Government of Indonesia through the Directorate General of Livestock Services has been promoted a livestock development program targeting self-sufficiency for meat. In order to achieve the target, the government intends to gradually reduce both the number of imported beef cattle and imported frozen meat. In addition, the government has also been offered projects for scientists, researchers and academicians to improve the productivity of the native cattle breeds in Indonesia [1]. Peranakan Ongole cattle have been acknowledged as one of indigenous cattle breeds that still exist in East Java province. Artificial insemination (AI) is the good available tools for reproduction improvement because this technology could maximize utilized the best reproduction capacity of bull [2]. The fertility power of spermatozoa could be maintained using freezing or cooling system in which semen were diluted using dilution material which could provide the physical and chemical needs. However, during the storage process, the semen could suffer from spermatozoa membrane damage resulted by the cold shock effect and could end up with spermatozoa mortality [3]. Therefore in an attempt to minimize the cell damage, substances which could protect the spermatozoa during cooling need to be added [4] . Dilution materials for diluting fresh semen should contain nutrition source, buffer, anti cold shock material, antibiotic and cryoprotectant which could protect spermatozoa during cooling process. The most utilized nutrition source is fructose because it could be easily metabolized by spermatozoa [5]. Buffer has the function to adjust pH and to neutralize lactic acid which is produced from leftover spermatozoa metabolism. Tris aminomethane buffer is most commonly used since it has a good buffer action with low toxicity in high concentration [6]. While Ducha et al (2013) [7] showed that semen diluted with CEP-2 + 10% egg yolk could stored for 8 days. The common anti cold shock substance added were either egg yolk or soya bean [4] which could protect spermatozoa from room temperature changes into cold temperature. Based on description above there is a need to conduct a research to find out the effect of diluted material based on tris aminomethane and CEP-2 with different cryoprotectant added on sperm quality of Peranakan Ongole bull during cooling process.

MATERIALS AND METHODS:

This research was conducted from October to December 2014 in Beef Cattle Research Station in Grati, Pasuruan East Java province and the material used in this study were 2 PO bulls aged 5 – 6 years with body weight of 520 – 545 kg and placed in individual cage. These bulls were fed with fresh grass and concentrate and drink water were given ad libitum.

Research Method

This study was conducted as laboratorium experimental with five treatments namely: T0 = Tris aminomethane + 20% egg yolk; T1 = CEP-2 + 10% egg yolk; T2 = CEP-2 (Non BSA) + 10% egg yolk; T3 = CEP-2 (Non BSA) + 15% soya bean extract; T4 = CEP-2 + 15% soya bean extract and each treatment was replicated 10 times. Parameter measured were motility percentage, viability and spermatozoa percentage with good integrity. The spermatozoa quality observations were carried out from the first to the tenth day.

CEP-2 Dilution Preparation

Dilution materials were prepared by several steps as follow: (1) weight 15 mmol/l NaCl, 42.9 mmol/l citric acid, 4 mmol/l $MgCl_2(H_2O)$. 11.9 mmol/l $NaHCO_3$, 8 mmol/l NaH_2PO_4, 20 mmol/l KH_2PO_4, 55mmol/l fructosa, 1 g/l sorbitol, 133.7 mmol/l BSA, 2 g/l tris, 0.05 NaH_2PO_4, 20 mmol/l KH_2PO_4, 55 ml mmol/l fructosa, 1 g/l sorbitol, 133.7 mmol/l BSA, 2 g/l tris, 0.05 g/l gentamicin Verberckmous et al (2004). Furthermore, CEP – 2 dilution were added with egg yolk or soya bean extract according to each treatment [8] .

Semen Collection and Semen Quality Assessments

Bull was cleaned thoroughly at all belly parts and preputium before semen collection was conducted and collection was carried out using artificial vagina. Sperm motility evaluation was subjectively conducted by dropping semen in a covered glass object, and observed under a light microscope at 400 x magnification. Motile spermatozoa were indicated by a forward movement compared to the one that stand still without movement and the results were presented as percentage [9,10,11].

$$\%\text{Motility}=\frac{\text{amount progresive spermatozoa}}{\sum \text{spermatozoa observed}}\times 100\%$$

Sperm viability percentage test was carried out individually by dropping one drop of fresh semen on an object glass and then added with one drop of eosin – negrosin. Moreover, this mixture was subsequently examine using a light microscope at 400 x magnification. Dead spermatozoa were indicated by red color (absorbed color) and live sperm had no color (transparant). Thus, the percentage of sperm viability was done by finding the proportion of viable sperms and observed sperms [9,12].

$$\%\text{Viability/alive} = \frac{\text{amount of viable spermatozoa}}{\sum \text{spermatozoa observed}} \times 100\%$$

Sperm concentration was evaluated using Haemocytometer according to Ax et al (2008) [9]. Semen was absorbed using erythrocyte pipette to level 0.5 and add 3% of NaCl to level 101. The pipette was then shaked to homogenize semen and NaCl. One to two drops of this mixture were discarded and then dropped into a covered glass object. The number of spermatozoa was manually calculated using a light microscope at 400 magnifications.

Hypoosmotic Swelling Test (HOST) was used to measure sperm membrane integrity adopted from Correa et al (1997) [13] and Susilawati (2011) [12] as follow: 1 ml of hypoosmotic125 mOsmol were added with 0.1 ml spermatozoa sample and then incubated at 37°C for 30 minutes. This mixture was examined under a light microscope at 400 magnification. Sperm with good membrane integrity had a circled tail, while damage sperm had a straight tail. The results were presented as percentage.

Semen Dilution

Fresh semen was diluted with the treated dilution which was already prepared previously. Dilution was gradually conducted by mixing semen and dilution materials and then shaked slowly to homogenize it. The sperm concentration in each treatment was 100 millions/ml. The tubes containing semen were then put in a water bath before placing it into a refrigerator, and then put it into a water jacket until the temperature dropped gradually from 30°C to 5°C in 1 hour [9].

Data Analysis

This study applied a randomized block design (RBD) which consisted of 5 treatments and each treatment was replicated 10 times. If there were significant effect (P<0.01) among the variables the analysis were then continued by Duncan Multiple Range test [14].

RESULTS AND DISCUSSION:

Semen Quality before Treatment

The fresh sperm quality of Peranakan Ongole bull include sperm volume, sperm motility percentage, sperm viability percentage, sperm membrane integrity and sperm concentration is presented in Table 1.

Table 1. Average volume, motility, viability, sperm membrane integrity and concentration semen evaluation just after collected using artificial vagina on the Indonesian Peranakan Ongole bull

Parameter	Means ± SD
Volume (ml)	7.10±1.68
Motility (%)	65.50±1.58
Viability (%)	78.18±12.25
Sperms with good membrane integrity (%)	75.52±12.66
Sperm concentration (10^6/ml)	524.10±428.67

The preliminary evaluation showed that fresh semen used in the experiment had good quality. Table 1 shows that semen volume was 7 ml and this could be classified as normal category based on Maud et al (2012) [15] who stated that a normal semen volume for local cattle ranged between 3 and 7 ml/ejaculate. The sperm volume differences were affected by several factors such as cattle age, feed and species, the frequency sperm collection [9] and testis size [16]. Another findings showed that the motile sperm was 65.50 ± 1.58% and this was normal based on Garner and Hafez (2008) [17] and Michael et al (2010) [18] who mentioned that the minimum standard of motile sperm was 60 – 65%. With regards to sperm viability, the study observed that the number of viable sperms was 78.18 ± 12.25%. This result is not in agreement with Cooper et al (2006) [19] and Gunalp et al (2001) [20] who found that average sperm viability was 89%. The quality of sperm membrane relates to sperm motility and viability. The study found that sperms with good membrane integrity was 75.52±12.66%. Another result found that fresh semen concentration was 524.10±428.67 x10^6, and it was considered as normal category. In daily basis, bulls can produce approximately 3.2-6.7 (10^9) spermatozoa [12]. By considering these results, the experiment could be continued to further process.

Individual Mortality, Viability and Sperm Membrane Integrity Percentage During Cooling Storage

The study found that the percentage of motile sperm of Peranakan Ongole bull in different dilution had a highly significant difference (P<0.01) as shown in Table 2.

Table 2. Average individual sperm motility of the Indonesian Peranakan Ongole bull during cooling process

Storage time (days)	Treatments				
	T0	T1	T2	T3	T4
1	66.25[c]	66.75[cd]	66.75[cd]	63.25[a]	64.00[ab]
2	65.25[bc]	64.00[bc]	63.50[ab]	60.00[a]	61.50[ab]
3	64.25[cd]	62.75[cd]	62.00[c]	53.80[a]	58.75[b]
4	61.50[d]	57.50[cd]	56.75[c]	46.00[a]	50.50[b]
5	57.25[d]	5100[cd]	48.50[bc]	39.50[a]	43.25[ab]
6	53.75[d]	47.00[cd]	43.50[c]	32.25[a]	35.50[ab]
7	49.25	40.25	35.25	21.50	30.00
8	45.7	34.25	31.00	16.00	21.2[b]
9	38.50[e]	22.00[cd]	19.25[bc]	6.75[a]	9.00[ab]
10	30.00[d]	12.25[bc]	9.50[bc]	3.00[a]	5.00[ab]

Information: Notations which are different in same column were highly significant different at P<0.01.

Table 2 discovers that addition of tris amino methane with 20% of egg yolk provided the best individual sperm motility up to seventh day, and this was still beyond the standard (45.73%) for artificial insemination purposes. The statistical analysis showed a highly significant different (P<0.01) at the first up to ninth day of cooling storage. This indicated that citric acid and fructose in tris aminomethane + 20% of egg yolk had a function to maintain sperm motility percentage. Whereas, the results showed no significant difference (P >0.05) during the ninth and tenth day of cooling storage. Barrios et al (2005) [21] argues that Low Density Lipoprotein (LDL) fraction especially phospholipids which already existed in egg yolk were effective component in preventing motile sperms from cold shock. The statistical analysis indicated that CEP – 2 + 15% of soya bean extract treatment had a lower result compared to other treatments. This is because the longer the duration of the cooling storage the lower the motile sperms [22].

As discovered by Schulze et al (2013) [23], type of tris-egg yolk dilution materials at the optimum declining temperature rate had the highest sperm motility percentage compared to other type of dilution materials. This was due to the formation of intracellular ice crystal on tris – egg yolk dilution material during cooling storage created smoother texture and prevented cell. Whereas, the formation of intracellular ice crystal on soya bean and egg yolk dilution materials had a rough texture and could damage sperm cell [25] . Table 3 presents the number of viable sperms of the Indonesian Peranakan Ongole bull during cooling process.

Table 3. Average sperm viability percentage of the Indonesian Peranakan Ongole bull during cooling process

Storage time (days)	Treatment				
	T0	T1	T2	T3	T4
1	90.05[d]	88.75[bc]	88.10[bc]	85.35[a]	87.30[b]
2	87.95[d]	86.55[cd]	85.35[bc]	82.80[a]	84.35[ab]
3	86.30[d]	84.85[cd]	83.90[cd]	79.85[a]	83.15[bc]
4	85.20[d]	83.35[bc]	82.20[bc]	77.55[a]	81.45[b]
5	83.00[d]	80.85[bc]	79.40[bc]	74.70[a]	78.95[b]
6	80.60[d]	78.65[bc]	77.35[bc]	72.05[a]	75.95[b]
7	78.05[d]	75.60[cd]	73.95[bc]	68.00[a]	72.10[b]
8	75.05[d]	70.45[cd]	67.65[bc]	59.75[a]	64.75[ab]
9	70.75[e]	65.05[cd]	62.65[c]	51.60[a]	55.55[ab]
10	62.60[e]	53.70[cd]	51.05[bc]	43.20[a]	46.75[ab]

Information: Notation which are different in same column showed a highly significant different (P<0.01).

Viable sperm are one of most important indicator to determine spermatozoa quality during dilution. The study found that the tenth day of cooling storage resulted the highest viable sperms in tris amino methane + 20% of egg yolk (49.25%) followed by CEP –2 + 10% of egg yolk (40.00%), CEP – 2 (Non BSA) + 10% of egg yolk (37.25%) and CEP – 2 + 15% of soya bean extract (32.95%) respectively. Whereas, the lowest viable sperm (29.05%) was found in CEP – 2 (Non BSA) + 15% of soya bean extract during cooling process. The decreasing number of viable sperms was due to the inconsistent temperature changes during storage time as a result of activities when opening and closing the refrigerator through the observation. Lopez et al (2012) [26] stated that viable sperm changes could occur during storage and decreased gradually. Sperm metabolism then produced lactic acid that became one of inhibitor factors which could decrease sperm viability.

The best sperm viability percentage was found on tris amino methane + 20% of egg yolk treatment which could maintain sperm plasma membrane compared to other treatments. This result indicated that sperm viability percentage during cooling storage was highly significant different (P<0.01). This study reaffirmed previous study conducted by Sildivane et al (2013) [27] in which the addition of 20% of egg yolk into tris amino methane dilution material could maintain bull sperm viability during cooling process with highly significant different results (P<0.01).

The best spermatozoa membrane integrity percentage

Hypoosmotic Swelling Test (HOST) was used to find out the best sperm membrane integrity percentage as shown in Table 4.

Table 4. Average sperm membrane integrity percentage of the Indonesian Peranakan Ongole bull during cooling process

Storage time (days)	Treatment				
	T0	T1	T2	T3	T4
1	87.55^{d}	86.45^{bc}	85.40^{bc}	82.90^{a}	84.70^{b}
2	85.35^{cd}	83.70^{bc}	83.20^{bc}	80.90^{a}	82.35^{ab}
3	84.15^{bc}	82.15^{bc}	81.35^{ab}	77.65^{a}	79.30^{ab}
4	81.85^{cd}	79.40^{bc}	78.95^{bc}	75.25^{a}	77.05^{ab}
5	79.90^{cd}	77.55^{bc}	76.60^{bc}	73.30^{a}	75.45^{ab}
6	78.15^{cd}	75.35^{bc}	73.90^{bc}	70.25^{a}	72.50^{ab}
7	75.70^{d}	71.70^{bc}	70.30^{bc}	67.10^{a}	69.50^{ab}
8	72.40^{cd}	67.35^{bc}	66.40^{bc}	58.05^{a}	61.75^{ab}
9	67.90^{d}	62.70^{cd}	60.10^{bc}	50.40^{a}	54.35^{ab}
10	64.45^{d}	54.50^{bc}	52.35^{bc}	44.50^{a}	47.80^{ab}

Information: Notation which are different in same column showed a highly significant different (P<0.01).

Table 4 presents the highest percentage of sperm membrane integrity cool stored at the first up to tenth day was observed at treatment with tris aminomethane + 20% egg yolk around 42.70%, followed by treatment with CEP-2 + 10% egg yolk around 34.95%, CEP – 2 +10% (Non BSA) egg yolk around 33.10%, CEP-2 + 15% soya bean extract around 31.45% respectively. Whereas the lowest number of sperm membrane integrity (29.45%) was found on CEP-2 + 15% (Non BSA) of soya bean extract. Enciso et al (2011)[28] found that incapable nutrition needed by spermatozoa in the dilution material could not stimulate the sperm membrane integrity, and hence decreased the sperm quality. Figure 1 shows sperm in various conditions. Firstly, the figure shows that motile sperms have a good membrane as shown in Figure 1A. Secondly, Figure 1B shows abnormal sperms with straight tail.

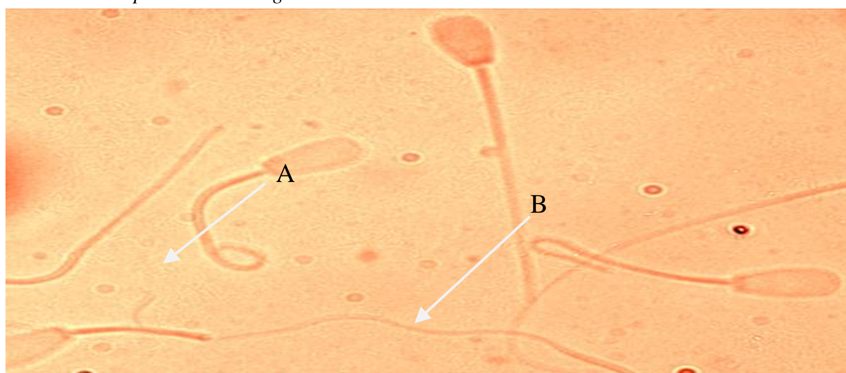

Fig1: Observation result of ongole filial bull sperm integrity plasma membrane percentage with curled tail i.e. good membrane integrity (A) and spermatozoa with straight tail indicated as bad membrane integrity (B) using light microscope at 1000 X magnification.

These figures indicated that a proper dilution material selection strongly very affected sperm membrane integrity. The addition of tris amino methane + egg yolk therefore showed the better capability to maintain sperm membrane integrity and permeability, and thus, could reduce membrane cell damage caused by osmotic pressure [29]. Forouzanfar et al (2010) [30] reported that lipoprotein and lecithin in egg yolk played important role in maintaining and protecting sperm membrane integrity. Furthermore, egg yolk could act as cryoprotectant and protect sperm acrosome from damage [31]. Also, glucose in egg yolk provide viscosity which could maintain spermatozoa life [32]. Moreover, cryoprotectants e.g. low density lipoprotein and hyaluronidase actively act in cryopreservation process [33].
Phospholipids and lecithin in egg yolk have functions to protect sperm membrane, maintain membrane integrity and protect the cell from its environment [34], hence extracellular matrix deformation and ion regulation system changes in sperm membrane were minimized. Therefore, calcium ion influx could be neutralized by a good and functional membrane. This was supported by previous research conducted by Sharafi et al (2009) [35] in which lecithin could maintain and protect sperm membrane integrity. Besides, lecithin also consisted of fatty acid glycerol, phosphate acid and choline which had a function to develop membrane metabolism structure [31]. The increasing sperm membrane integrity function will then increase substrate absorption on spermatozoa which could promote its motility [36].

CONCLUSION:

Tris amino methane + 20% of egg yolk was better than CEP-2 basic dilution materials to maintain sperm motility, viability and membrane integrity of Indonesian Peranakan Ongole bull during 7 days of cooling process.

REFERENCES:

1.Nugroho E, Azizah S, Susilawati T and Novianti I Socio-economic potential of Indonesian native cattle in supporting meat self-sufficiency in Indonesia. Livestock Research for Rural Development,2013, 25 (11):2013.
2.Thurston L M, P F Watson, A J Mileham and W V Holt Morphologically distinct sperm subpopulations defined by fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J. Androl; 2001;22:382–394.
3. Perez-Pe R, P Grasa, M Fernandez-Juan, M L Peleato, J A Cebrian-Perez and T Muino-Blanco Seminal plasma proteins reduce protein tyrosine phosphorylation in the plasma membrane of cold-shocked ram spermatozoa. Mol. Reprod Dev;2002; 61:226–33.
4.Aboagla E M E and T Terada , Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology;2004;* 62:1160-1172.
5.Ponglowhapan S, B Essén-Gustavsson and C Linde Forsberg, Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology,2004; 62:1498–517.
6.Kaeoket K, P Chanapai, P Junchiyaphoom and P Chanapiwat, The effect of using long term and short term extenders during cooling process on the quality of frozen boar semen. Thai J. Vet. Med,2011;41(3):283-288.
7.Ducha N, T Susilawati, Aulanni‘am, S Wahjuningsih and M Pangestu, Ultrastructure and fertilizin ability of limosin bull sperm after storage in extender CEP-2 with or without egg yolk. Pakistan journal of biological sciences,2012; 15 (20) : 979-985.
8.Aires V A, Hinsch K D, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S and Hinsch E, *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology,2003;* 60, 269-279.
9.Ax R L, M R Dally, B A Didion, R W Lenz, C C Love, D D Varne., B Hafez and M E Bellin, Semen evaluation in farm animal reproduction 7th edition ed. by E.S.E Hafez and B Hafez. Blackwell Publisher;2008; 365-375.
10.Kirkman-Brown J and L Björndahl, Evaluation of a disposable plastic Neubauer counting chamber for semen analysis. *J Fert Ster*,2009; 91:627-631.
11. Martínez-Rodríguez C M, Alvarez L Ordás, C A Chamorro, F Martinez-Pastor, L Anel and P de Paz, Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test. Theriogenology,2012; 77(8):1575-1586.
12.Susilawati T, Rahayu S, Udrayana S, Sudarwati H and Nugroho E, Effect of different centrifugation duration on *Simmental* bull sperm quality and membrane status after sexing, cooling and freezing processes. American-Eurasian Journal of Sustainable Agriculture,2011; 8 (7), Special, Pages: 28-34.
13.Correa J R, M M Pace and P M Zavos, Relationships among frozen–thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program *Theriogenology*,1997; 48:721-731.
14.Steel R G D dan J H Torrie 1991, Statictic principle and procedure. PT Gramedia Pustaka utama. Jakarta.
15.Maud B, C K Cornwallis, I A Malecki, P K Rybnik-Trzaskowska and S W P Cloete, The effect of temperature and pH on the motility and viability

of ostrich sperm. Animal Reproduction Science,2012; 133(1–2):123-128, ISSN 0378-4320.
16.Ayelet A, H Ron, P Gedalia, E Sandra, Kleiman L Ofer, Y Haim and Y Leah 2014 Thawed human sperm quality is influenced by the volume of the cryopreserved specimen. Fertility and Sterility; 640-646.
17.Garner D L and Hafez E S E 2008 Spermatozoa and seminal plasma. *Reproduction in Farm Animal*. 7th eds. Edited by Hafez ESE, Hafez, B. Baltimore. Lippincott and Williams. 7: 96-109.
18.Michael A J, C Alexopoulos, E A Pontiki, D J Hadjipavlou-Litina, P Saratsis and H N Ververidis 2010 Effect of N-acetyl-L-cysteine supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Reprod Domest Anim; 45:201–7.
19.Cooper T G and C H Yeung 2006 Computer-aided evaluation of assessment of'grade a' spermatozoa by experienced technicians. Fertil Steril; 85:220–224.
20.Gunalp S, C Onculoglu, T Gurgan. T F Kruger and C J Lombard 2001 A study of semen parameters with emphasis on sperm morphology in a fertile population: an attempt to develop clinical thresholds. Hum Reprod; 16:110–114.
21.Barrios B, M Fernández-Juan, T Muino-Blanco and J Cebrián-Pérez, Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. J Androl,2005; 26:539.
22.Abdul Malik, L Muhammad and I Z Muhammad, Effects of long term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed Frisian Holstein bull spermatozoa, Asian Pacific Journal of Reprod,2015;4:22-25.
23.Schulze M, H Henning, K Rüdiger, U Wallner and D Waberski 2013 Temperature management during semen processing: impact on boar sperm quality under laboratory and field conditions. Theriogenology; 80:990–8.
24.Vasudevan K and J M Sztein 2011 Treatment of sperm with extracellular adenosine 5'-triphosphate improves the in vitro fertility rate of inbred and genetically modified mice with low fertility. Theriogenology; 76:729–36.
25.Zhang S S, Hu Q W Li, Z L Jiang and Z Xiao-Ying, The croyoprotective effect of soybean lecithin on boar spermatozoa quality. *Afr. J. Biotechnol*,2009; 8:6476-6480.
26.Lopez Rodriguez A, T Rijsselaere, P Vyt, A Van Soom and D Maes 2012 Effect of dilution temperature on boar semen quality. Reprod. Domest. Anim. 47: 63–66.
27.Sildivane V S, T S Adriana, M B André, C A Felipe, F N José, A P Christina and M P G Maria 2013 Addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. Animal Reproduction Science; 137:37-44.
28.Enciso M Cisale, H Johnston, S D Sarasa, J Fernández, J L Gosálvez J 2011 Major morphological sperm abnormalities in the bull are related to sperm DNA damage, Theriogenology 23-32.
29.Aksoy A, DbC Dowell and C Snyder 2010 A multicase comparative assessment of the ensemble Kalman filter for assimilation of radar observations. Part II: Short-range ensemble forecasts. *Mon. Wea. Rev*; 138:1273-1292.
30.Forouzanfar M, M Sharafi, S M Hosseini, S Ostadhosseini, M Hajian, L Hosseini, P Abedi, Nili, N H R Rahmani and M H Nasr-Esfahani 2010 In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. *Theriogenology;* 73: 480–487.
31.Hammadeh M E, Zeginiadov, T P Rosenbaum, T Georg, W Schmidt and E Strehler 2001 Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. Arch Androl; 46:99-104.
32.Said T M, A Agarwal, R K Sharma, A J Thomas and S C Jr Sikka 2005 Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. Fertil Steril; 83:95-103.
33.Bucak MbN, A Atessahin, O Vansli, A Yuce, N Tekin and A Akcay 2007 The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen. Microscopic and oxidative stress paramaters after freeze-thawing process, Theriogenology; 67:1060-1067.
34.Moussa M, V Martinet, A Trimeche, D Tainturier and M Anton 2002 Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*; 57:1695-1706.
35.Sharafi M, S Eghbalsaied, N Nili and M H Nasr-Esfahani 2009 Ram semen in vitro fertility after cryopreservation using soybean lecithin and egg yolk based extenders. Reprod. Domest. Anim; 44: 90-95.
36.Amirat L, D Tainturier, L Jeanneau, C Thorin, O Gerard, J Courtens and M Anton 2004 Bull semen *in vitro* fertility after cryopreservation using egg yolk LDL: A comparison with Optidyl a commercial egg yolk extender. *Theriogenology;* 61: 895-907.