Impact of replacing egg yolk with lecithin on quality of pre-freeze and post-thaw buffalo spermatozoa

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1. Introduction

Artificial insemination is one of the assisted fertility tools which

is used to improve the genetic potential of livestock breeds and
to exploit the spermatozoa from superior ones. Semen from farm

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ABSTRACT

Objective: To estimate the result of egg yolk replacement with alternative cryopreservatives
such as plant-derived lecithin from soybean on sperm quality parameters pre and post
freezing in buffalo bulls. Methods: The control cryopreservation extender was tris-citric
acid-fructose-egg yolk-glycerol (TCFYG) diluent. Semen samples were extended gradually
1:10 with TCFYG control extender and tris-citric acid-fructose-glycerol (TCFG) extender
plus variable concentrations of soybean lecithin (0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) to
ensure 60 million active spermatozoa/mL of the extended semen. The diluted semen samples
were refrigerated slowly (roughly for 2 h) up to 5 °C and equilibrated for 2 h. Semen was
filled into 0.25 mL polyvinyl French straws (IMV, France). After equilibration period, the
straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen
for 10 min and were then dipped stored in liquid nitrogen at -196 °C. Results: The respective
overall percentages of forward motile spermatozoa, live spermatozoa, morphologically
normal spermatozoa, acrosome integrity and hypo-osmotic swelling reactivity observed
primarily in fresh semen, after equilibration (pre-freeze stage) and post freezing (post-thaw
stage) in TCFYG (control) extended semen declined progressively and statically (P<0.01)
during these periods of study. Pre-freezing stage: replacement of egg yolk into TCFG with
soybean lecithin at concentrations of 1.0% and 1.5% significantly (P<0.01) ameliorated
the maintenance of (motility, viability, acrosome and membrane integrity %), meanwhile it
had significantly (P<0.01) reduced the abnormality % of spermatozoa to the lowest value
compared to control TCFYG and to some other concentrations in use. Post-thaw stage: the
replacement of egg yolk with 1.0% soybean lecithin (SL) showed significantly (P<0.01)
higher percentage of sperm progressive motility compared to 1.5% SL and TCFYG control.
These values were significantly (P<0.01) higher than 0.5%, 2.0%, 2.5% and 3.0% SL. The
post thawing live sperm percentage mean values were significantly (P<0.01) higher in 1.0% SL and 1.5% SL compared to control. These values were significantly (P<0.01) higher than in 0.5%, 2.0%, 2.5% and 3.0% SL. The mean values of post-thaw morphological normal sperm percentage did not differ between 1.0% SL and control groups but significantly (P<0.01) higher than 0.5%, 1.5%, 2.0%, 2.5% and 3.0% SL. The respective percentage mean values of post-thaw sperm with head, mid-piece and tail abnormalities were significantly (P<0.01) lower in 1.0% SL than all other SL concentrations. Concerning the post-thaw percentages of acrosome and sperm membrane integrity, the respective mean values were significantly (P<0.01) higher in 1.0% SL and 1.5% SL as compared to control. Mean values of both parameters in the 0.5% SL were intermediate between 1.0% and 1.5% SL versus control groups. The previously mentioned mean values in acrosome/membrane integrity were significantly (P<0.01) higher than 2.0% SL, 2.5% SL and 3.0% SL. Conclusions: Lecithin-based diluent can be a potent proper alternative extender for preservation of spermatozoa during pre- and post-freezing process. SL 1.5% extenders have supplied an optimal environment and condition for ameliorating the quality of pre-freezing and post-thaw buffalo spermatozoa by means of improved motility, viability, functional acrosome, sperm membrane integrity and morphologically normal spermatozoa.

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animals used for these purposes could be preserved either for short-
term at 4 °C in the liquid form or for long-term in cryopreserved
state with liquid nitrogen[1]. However, declines of about 50% in
spermatozoal motility, livability and sperm membrane status are
major problems occurring during the freezing process, caused by
severe deterioration in sperm membrane during the freezing process.
Recent studies have been performed to constitute semen diluents
for protecting bull spermatozoa in post-freezing and post-thawing
process[2-7].

Commonly, buffalo semen is preserved in milk[8-11], tris-egg
yolk[12-14] and egg yolk-citrate[3,15] diluents. These diluents contain
additives of animal source (egg yolk and/or milk) which may pose
an extreme hazard of microbial contaminants[16,17]. This sanitary
risk may reduce fertility of frozen semen directly through producing
hazardous metabolites and toxins which deteriorates the semen
characteristics, or indirectly through local infection leading to
abortion[6,18]. Also, egg yolk in semen diluent can lower the activity
and motility of ram spermatozoa[19]. Furthermore, high density
lipoproteins in egg yolk lowers the quality of semen by causing
cholesterol efflux out of the spermatozoal membrane, which leads
to change in flexibility and increases the liability to cold shock[20].
The main functional portion of egg yolk is low density lipoproteins
fraction like lecithin, which protects the membrane integrity all
through preservation[21,22]. A high quality alternative instead of the
ingredients of animal origin in extenders for freezing of semen is
soybean lecithin (SL), a phospholipid that is the principal component
of soybean[23]. SL may decrease the hygienic risks and improve
freezability and fertilizing capacity of bovine spermatozoa[6,24].

Therefore, the current investigation was designed to estimate the
result of egg yolk replacement with alternative cryopreservatives
such as plant-derived lecithin from soybean on sperm quality
parameters pre and post freezing in buffalo bulls.

2. Materials and methods

2.1. Semen collection and initial evaluation

Four buffalo bulls (aged 3.5-5 years) kept at the Abassia Buffalo
Semen Freezing Center, Central Organization for Veterinary
Services, Ministry of Agriculture, Egypt, were chosen to be the
source of semen. The buffalo bulls were kept under uniform standard
nutrition and managerial practices. They were in healthy conditions
(600-800 kg body weight), free from general and genital diseases.
The bulls were under constant weekly intervals semen collection
program with an artificial vagina. Semen collections were carried out
early in the morning. Two successive ejaculated semen samples were
collected using an artificial vagina at each collection process with
10-15 min interval. The ejaculates were pooled to enlarge the semen
volume for different aliquots and to avoid the evaluated samples
variability. The semen ejaculates (10 ejaculates per bull, total 40
ejaculates in each experiment) after collection were immediately
transferred into a water bath at 35 °C for 10 min and evaluated for
visual motility using a high power ordinary microscope (at 400 ×)
with closed circuit television, sperm concentration using Neubauer
haemocytometer and abnormality % using eosin-nigrosin stain.
The semen samples with 70% motility, total sperm defects lower
than 20% and with concentration of 600 ×10⁶ spermatozoa/mL of
the ejaculates were selected for processing. Additionally, the hypo-
osmotic swelling test was performed to assess the sperm membrane
functional integrity of % as recorded by Jeyendran et al[25]. The sperm
with swollen twisting tail was considered intact. Sperm
acrosomal integrity % was undertaken as mentioned by Watson[26].
Normal acrosome was identified by normal apical ridge.

2.2. Experimental design

This experiment was designed to investigate the effect of variable
levels of SL, as an alternative for egg yolk in buffalo semen diluent
on post-cooling and post-thawing functional sperm quality. The
control cryopreservation extender was tris-citric acid-fructose-egg
yolk-glycerol (TCFYG) diluent prepared by dissolving 3.028 g
tris, 1.678 g citric acid and 2.000 g fructose in 100 mL bi-distilled
water, then by adding 20% egg yolk and 7% glycerol combined with
antibiotics according to Ijaz et al[27]. Semen samples were extended
gradually 1:10 with TCFYG control extender and tris-citric acid-
fructose-glycerol (TCFG) extender plus variable concentrations of
SL (0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) to ensure 60 million
active spermatozoa/mL of the extended semen. The diluted semen
samples were refrigerated slowly (roughly for 2 h) up to 5 °C and
equilibrated for 2 h. Semen was filled into 0.25 mL polyvinyl French
straws (IMV, France). After equilibration period, the straws were
placed horizontally on a rack and frozen in a vapor 4 cm above liquid
nitrogen for 10 min and were then dipped in liquid nitrogen at -196 °C.

2.3. Statistical analysis

The data are tabulated as mean±standard deviation (mean±SD).
All data were submitted to one way analysis of variance by using
computerized statistical analysis.

The analytical design was factorial design (general linear
model procedure) to clarify the result of cryoprotectant (type and
concentration) in extenders including egg yolk (control) and SL
on sperm motility, viability, abnormalities, sperm membrane and
acrosome integrities pre and post freezing. Treated means were
compared by the least significant difference test at 5% and 1% levels
of probability. Each treatment was repeated five times, for each
replicate; four straws were thawed and pooled for assessing of sperm
characteristics. All statistical methods were performed as recorded
by Snedecor and Cochran[28].

3. Results

3.1. Effects of freezing stage on semen quality traits

As shown in Table 1, the respective overall percentages of forward
motile spermatozoa, live spermatozoa, morphologically normal
spermatozoa, acrosome integrity and hypo-osmotic swelling
reactivity observed primarily in fresh semen (initial stage), after
3.2. Effect of egg yolk– and lecithin–based extender on semen quality traits

3.2.1. Pre–freezing stage

Concerning the results in Table 1, replacement of egg yolk into TCFG with SL at concentrations of 1.0% and 1.5% significantly (P<0.01) ameliorated the maintenance of motility%, viability %, acrosome % and membrane integrity %, while it had significantly (P<0.01) reduced the abnormality % of spermatozoa to the lowest value compared to control TCFYG and to some other concentrations in use.

The highest mean values of motility percentage were observed in 1.0% SL and 1.5% SL as compared to control. These values were significantly (P<0.01) higher than 0.5%, 2.0%, 2.5% and 3.0% SL pre-freezing.

Similarly, the highest mean values of live sperm percentage were detected in 1.0% and 1.5% SL as compared to control. These values were significantly (P<0.01) higher than 0.5%, 2.0%, 2.5% and 3.0% SL pre-freezing.

Table 2

Segment wise sperm abnormalities in buffalo bull semen at different stages of cryopreservation in TCFYG extender and egg yolk free extender (TCFG) containing different concentration of SL (n=4) (Mean±SD).

| Freezing stage | Extender | Progressive motility % | Live sperm % | Morphological normal sperm % | Intact acrosome % | Hypo-osmotic swelling reactive sperm % |
|----------------|----------|------------------------|--------------|------------------------------|------------------|--------------------------------------|
| Initial        | --       | 85.54 ± 9.45           | 89.18 ± 9.06 | 91.26 ± 8.89                 | 93.38 ± 9.49     | 78.52 ± 8.00                        |
| Pre-freeze     | TCFYG    | 72.62 ± 7.26           | 81.12 ± 7.69 | 83.25 ± 8.68*                | 85.39 ± 9.22*    | 69.26 ± 6.83*                       |
|                | TCFG + 0.5% SL | 71.50 ± 6.29         | 75.11 ± 7.22 | 87.11 ± 8.92*                | 81.15 ± 8.32*    | 67.43 ± 6.24*                       |
|                | TCFG + 1.0% SL | 80.72 ± 7.91          | 86.42 ± 8.28 | 88.19 ± 8.87*                | 90.66 ± 9.52*    | 74.11 ± 7.37*                       |
|                | TCFG + 1.5% SL | 79.12 ± 8.21          | 85.15 ± 8.33 | 89.36 ± 9.09*                | 88.75 ± 8.83*    | 74.36 ± 7.06*                       |
| Post-thaw      | TCFYG    | 46.36 ± 4.82           | 48.48 ± 4.75 | 78.34 ± 7.26*                | 71.39 ± 7.42*    | 56.33 ± 5.82*                       |
|                | TCFG + 0.5% SL | 15.05 ± 3.29         | 42.16 ± 4.38 | 68.52 ± 7.08*                | 76.13 ± 7.36*    | 59.24 ± 6.12*                       |
|                | TCFG + 1.0% SL | 58.24 ± 6.00          | 58.18 ± 5.25 | 78.73 ± 7.42*                | 86.27 ± 8.11*    | 68.92 ± 7.32*                       |
|                | TCFG + 1.5% SL | 48.12 ± 5.30          | 55.62 ± 5.28 | 73.23 ± 7.56*                | 85.49 ± 8.53*    | 67.83 ± 6.75*                       |
|                | TCFG + 2.0% SL | 33.28 ± 3.41          | 42.23 ± 4.04 | 71.26 ± 7.13*                | 65.18 ± 6.74*    | 52.25 ± 5.14*                       |
|                | TCFG + 2.5% SL | 25.71 ± 2.54          | 43.38 ± 4.24 | 68.11 ± 6.89*                | 66.54 ± 6.82*    | 50.34 ± 4.82*                       |
|                | TCFG + 3.0% SL | 17.82 ± 2.01          | 42.54 ± 4.29 | 69.36 ± 7.06*                | 65.43 ± 6.50*    | 51.62 ± 4.93*                       |

Means bearing different superscripts between different extenders pre-freeze (a, b, c, d, e) and post- thawing (f, g, h, i, j) stages differ at 5% and 1% levels of probability. TCFYG: Tris-citrate-fructose-egg yolk-glycerol; TCFG: Tris-citric acid-fructose-glycerol; SL: soybean lecithin.

Table 3

| Freezing stage | Extender | Head | Mid-piece | Tail | Overall |
|----------------|----------|------|-----------|------|---------|
| Initial        | --       | 3.33 ± 0.03| 1.38 ± 0.01| 5.03 ± 0.04| 8.74 ± 0.07|
| Pre-freeze     | TCFYG    | 5.56 ± 0.05*| 2.46 ± 0.03*| 9.73 ± 0.09*| 16.75 ± 1.57*|
|                | TCFG + 0.5% SL | 4.59 ± 0.05*| 1.52 ± 0.02*| 6.68 ± 0.68*| 12.89 ± 1.28*|
|                | TCFG + 1.0% SL | 4.99 ± 0.05*| 1.50 ± 0.02*| 5.75 ± 0.06*| 11.81 ± 1.20*|
|                | TCFG + 1.5% SL | 4.15 ± 0.04*| 1.79 ± 0.01*| 4.60 ± 0.05*| 10.64 ± 1.07*|
|                | TCFG + 2.0% SL | 5.02 ± 0.05*| 2.28 ± 0.02*| 11.56 ± 1.10*| 18.86 ± 1.85*|
|                | TCFG + 2.5% SL | 5.52 ± 0.06*| 3.53 ± 0.03*| 12.62 ± 1.25*| 21.67 ± 2.20*|
| Post-thaw      | TCFYG    | 6.21 ± 0.06*| 3.20 ± 0.03*| 12.25 ± 1.36*| 21.66 ± 2.09*|
|                | TCFG + 0.5% SL | 6.76 ± 0.06*| 3.64 ± 0.04*| 21.08 ± 1.83*| 31.48 ± 3.55*|
|                | TCFG + 1.0% SL | 5.86 ± 0.07*| 3.00 ± 0.03*| 12.40 ± 1.39*| 21.27 ± 2.43*|
|                | TCFG + 1.5% SL | 7.25 ± 0.07*| 3.21 ± 0.03*| 16.31 ± 1.45*| 26.77 ± 2.61*|
|                | TCFG + 2.0% SL | 6.50 ± 0.06*| 4.00 ± 0.04*| 18.24 ± 1.40*| 28.74 ± 2.35*|
|                | TCFG + 2.5% SL | 6.00 ± 0.05*| 4.05 ± 0.04*| 21.68 ± 1.99*| 31.89 ± 3.03*|
|                | TCFG + 3.0% SL | 6.54 ± 0.06*| 4.58 ± 0.05*| 19.50 ± 2.02*| 30.64 ± 3.11*|

Means bearing different superscripts between different extenders pre-freeze (a, b, c, d, e) and post- thawing (f, g, h, i, j) stages differ at 5% and 1% levels of probability. TCFYG: Tris-citrate-fructose-egg yolk-glycerol; TCFG: Tris-citric acid-fructose-glycerol; SL: soybean lecithin.
3.2.2. Post–thaw stage

Table 1 revealed that the replacement of egg yolk with 1.0% SL showed significantly ($P<0.01$) higher percentage of sperm progressive motility compared to 1.5% SL and TCFYG control. These values were significantly ($P<0.01$) higher than 0.5%, 2.0%, 2.5% and 3.0% SL. The post thawing live sperm percentage mean values were significantly ($P<0.01$) higher in 1.0% SL and 1.5% SL compared to control. These values were significantly ($P<0.01$) higher than in 0.5%, 2.0%, 2.5% and 3.0% SL.

The mean values of post-thaw morphological normal sperm percentage did not differ between 1.0% SL and control groups but significantly ($P<0.01$) higher than 0.5%, 1.5%, 2.0%, 2.5% and 3.0% SL. The respective percentage mean values of post-thaw sperm with head, mid-piece and tail abnormalities (Table 2) were significantly ($P<0.01$) lower in 1.0% SL than all other SL concentrations.

Concerning the post-thaw percentages of acrosome and sperm membrane integrity, the respective mean values were significantly ($P<0.01$) higher in 1.0% SL and 1.5% SL as compared to control. Mean values of both parameters in the 0.5% SL were intermediate between 1.0% and 1.5% SL versus control groups. The previously mentioned mean values in acrosome/membrane integrity were significantly ($P<0.01$) higher than 2.0% SL, 2.5% SL and 3.0% SL.

4. Discussion

The present overall mean percentages of semen quality characteristics recorded primarily in fresh buffalo semen had declined progressively and statistically significantly ($P<0.01$) after extension with control TCFYG extender during pre-freezing and post-thaw stages. This finding is in accordance with Fukui et al[29], Khalifa and Abdel-Hafez[30,31], Chaudhari et al[7] and El-Sisy et al[32], who reported deterioration in sperm characteristics during refrigeration and freezing. This marked reduction was related to changes in the pH of extension and osmolarity in addition to bacterial and fungal contamination present in egg yolk-based extender[33]. The microbial contamination results in endotoxins that decrease the liveability of sperm[34].

The replacement of egg yolk into TCFG with SL alleviated to a great extent the maintenance of semen characteristics (motility, livability, acrosome and membrane integrity %), it had significantly ($P<0.01$) reduced the spermatozoa with total, head, mid-piece and tail abnormalities during pre-freezing and post-thaw stages of cryopreservation. Increasing vitality of spermatozoa obtained with SL-based extender is caused by phosphotidyl choline from SL that restores the membrane phospholipids to preserve the membrane integrity and maintains sperm motility at low temperature. Furthermore, SL plays a vital physiological function in reducing the cooling point and lowering the replacement of plasmalogenos to reduce the possible mechanical injuries of the sperm membrane[4].

Results of the present investigation showed that among the wide range (0.5% to 3.0% SL) tested, concentrations of SL for cooling and freezing of buffalo semen were 1.0% and 1.5%. In particular 1.0% SL, these concentrations showed best semen parameters after both stages of cryopreservation. The reported optimal concentrations of SL in extender used for semen freezing in the literatures were ranged from 0.8% in dogs[35], 1.0% in rams[31,36-38], humans[39] and cats[40] and 1.5% in bovine bulls[41-46] and goats[47-52].

The current results regarding greater effectiveness of 1.0% and 1.5% lecithin-based extender than egg yolk-based extender in preserving forward motility, live, morphologically normal and acrosomal status and membrane integrity of buffalo bull spermatozoa are in accordance with those reported by Amirate et al[20], Bard[53], El-Sherbieny[6] and El-Sisy et al[32] in buffaloes but are in confliction with those recorded in buffalo bulls[3,7,54,55] and bovine bulls[56,57]. These authors observed no differences in the effect of SL extender and egg yolk-based extender concerning percentage values of the aforementioned semen traits of buffalo. Apparent differences between these findings are attributed to the variations in cooling and freezing rates and type of commercially available SL-based extenders, but is also likely attributed to breed and species variations with subsequent changes in sperm membrane and seminal plasma composition[58,59].

According to our findings, optimal SL concentration (1.0% and 1.5% SL) is the best for protection of spermatozoa during temperature variation. Concentration of SL below or above the optimal may have deleterious effect and this may be the effect with 0.5%, 2.5% and 3.0% SL extenders. The reduction in most of semen characteristics in extenders containing 0.5% SL may be related to insufficient support to offer great cryoprotection of sperm membrane integrity[6]. SL at concentrations higher than 1.5% were toxic for sperm motility and viability and this was likely that higher concentration of SL amplified thickness of extenders with much debris observed in the extender with 2% and more lecithin. Also, spermatozoa are able to move more easily in semen diluents containing optimum levels of SL than in other extenders which would lead to better sperm motility.

The sperm membrane/acrosome integrity of spermatozoa is important to keep sperm functionality during storage in the female genital tract[60]. The deterioration of sperm membrane fluidity due to disarrangement of lipids within the membrane during cooling and freezing may induce further cellular and subsequent sperm damage[61]. In the current study, 1.0% and 1.5% SL resulted in higher membrane and acrosome integrity compared to other SL-based and control egg yolk-based extenders. Amirate et al[20] reported higher sperm percentage with normal acrosome frozen in SL-based diluent as compared to an egg yolk-based diluent and suggested that presence of higher calcium ions of egg yolk might be concerned with the acrosomal damage. It was declared that the acrosome status and normal membrane of spermatozoa has direct impact of 1.0% and 1.5% SL on progressive motility of sperm may be partly related to plasma membrane/acrosome integrities. Although
the perfect mechanism by which lecithin induces its effect on plasma membrane of spermatozoa during pre- and post-freezing process is not clear, it has been explained that lecithin in soybean protects sperm membrane phospholipids by occupying sites on the sperm membrane and ameliorates its tolerance to the freezing process.[21,60].

It has been identified that sperm motility is done via propelling forces of its tail in conjugation with lateral displacement of its head based on the energy supplied by mitochondria in the mid-piece[64,65]. The findings of this study show a considerable correlation between the morphological structures of the three segments of live spermatozoa with motility percentage. The present findings documented a strong positive correlation between morphologically normal spermatozoa with its three segments and progressive motility in 1.0% SL-based extender pre and post freezing. Evaluation of frozen-thawed spermatozoa with Rhodamine 123 fluorescent dye showed that the percentage of active mitochondria was superior in 1.0% SL with respect to 2.0% SL diluent[66].

Our findings postulated that the percentages of live spermatozoa pre and post freezing were considerably higher in 1.0% and 1.5% SL extenders compared to control egg yolk-based extender. Emamverdi et al[60] confirmed that 1.0% and 1.5% SL extenders contain low percentages of early apoptotic spermatozoa compared to egg yolk-based extender. Del Valle et al[67] reported that lecithin is able to perfectly preserve sperm against cooling and cryoinjury, as its addition resulted in increased percentage of viable and non-apoptotic spermatozoa. In the existing study, we have not evaluated the lipid peroxidation in spermatozoa, but it seems that SL-based extenders can decrease damage in spermatozoa via decreasing fatty acid peroxidation in plasma membrane[68,69] which may cause apoptosis in these cells.

In conclusion, our findings documented that lecithin-based diluent can be a potent proper alternative extender for preservation of spermatozoa during pre- and post-freezing process. Between lecithin-based extenders tested in this study and, to a lesser extent, 1.5% SL extenders have supplied an optimal environment and condition for ameliorating the quality of pre-freezing and post-thaw buffalo spermatozoa by improving motility, viability, functional acrosome, sperm membrane integrity and morphologically normal spermatozoa.

**Conflict of interest statement**

All authors declare that there is no conflict of interest.

**References**

[1] Maxwell WMC, Watson PF. Recent progress in the preservation of ram semen. *Anim Reprod Sci* 1996; 42(1): 55-65.

[2] Andrabi SMH. Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod Domest Anim* 2009; 44(3): 552-569.

[3] Akhter S, Ansari MS, Rakha BA, Ullah N, Andrabi SMH, Khalid M. *In vitro* evaluation of liquid-stored buffalo semen at 5 °C diluted in soya lecithin based extender (Bioxcell™), tris-citric egg yolk, skim milk and egg yolk-citrate extenders. *Reprod Domest Anim* 2011; 46(1): 45-49.

[4] Singh AK, Singh VK, Narwade BM, Mohanty TK, Atreja SK. Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5 °C) in egg yolk-and soya milk-based extenders. *Reprod Domest Anim* 2012; 47(4): 596-600.

[5] Ahmad I, Jatoi SU, Zubair M, Younis M. Comparative efficacy of different cryoprotectants for deep freezing of buffalo bull semen. *Adv Anim Vet Sci* 2017; 2(3): 150-154.

[6] El-Sherbieny MA. Impact of replacing egg yolk with lecithin on sperm characteristics, bacterial count and fertilizing ability of cryopreserved buffalo semen. *J Anim Poult Prod Mansoura Univ* 2014; 5(6): 353-364.

[7] Chaudhuri DV, Dhami AJ, Hadiya KK, Patel JA. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (-196 °C) of buffalo semen. *Vet World* 2015; 8(2): 239.

[8] Kumar S, Sahni KL, Bistha GS. Cytomorphological characteristics of motile and static semen of buffalo bulls. *Buffalo J* 1993; 2: 117-127.

[9] El-Azab AI, Khadr NA, Zahran K. Effect of non-protein nitrogen in the ration on ram semen quality. *Small Rumin Res* 1998; 27(1): 73-77.

[10] Pramanik PS, Raina VS. Refrigerator (4-7 °C) preservation of buffalo semen in various extenders. *Indian J Dairy Sci* 1998; 51: 375-379.

[11] Akhter S, Ansari MS, Andrabi SMH, Ullah N, Quayyum M. Effect of antibiotics in extender on bacterial and spermazoal quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Reprod Domest Anim* 2008; 43(1): 272-278.

[12] Rasul Z, Anzar M, Jalali S, Ahmad N. Effect of buffering system on post-thaw motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa. *Anim Reprod Sci* 2000; 59(1): 31-41.

[13] Rasul Z, Ahmad N, Anzar M. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. *J Androl* 2001; 22(2): 278-283.

[14] Andrabi SMH, Ansari MS, Ullah N, Anwar M, Mehmood A, Akhter S. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. *Anim Reprod Sci* 2008; 104(2): 427-433.

[15] Sansone G, Nasti MJF, Fabbrocini A. Storage of buffalo (*Bubalus bubalis*) semen. *Anim Reprod Sci* 2008; 62(1): 55-76.

[16] Bousseau S, Brillard JP, Marquant L, Guienne B, Guerin B, Camus A, et al. Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology* 1998; 50(5): 699-706.

[17] Thibier M, Guerin B. Hygienic aspects of storage and use of semen for artificial insemination. *Anim Reprod Sci* 2000; 62(1): 233-251.

[18] Althouse GC. Sanitary procedures for the production of extended semen. *Reprod Domest Anim* 2008; 43(1): 374-378.

[19] Watson PF, Martin IC. Artificial insemination of sheep: The effect of semen diluents containing egg yolk on the fertility of ram semen. *Theriogenology* 1976; 6(5): 559-564.
[51] Yotov S. Effect of TFC-based extenders with soybean lecithin and/or low concentration of glycerol on the quality of goat chilled-stored semen. *Int Curr Microbiol App Sci* 2015; 4(3): 752-761.

[52] Masoudi R, Sharafi M, Shahneh AZ, Towhid M, Kohram H, Esmaeili V, et al. Fertility and flow cytometry study of frozen-thawed sperm in cryopreservation medium supplemented with soybean lecithin. *Cryobiology* 2016; 73(1): 69-72.

[53] Bard MR. Cryopreservation of buffalo spermatozoa in soy lecithin based extender. *Assia Vet Med J* 2008; (54): 272-284.

[54] Akhter S, Ansari MS, Rakha BA, Andrahi SMH, Iqbal S, Ullah N. Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell® extender. *Cryobiology* 2016; 73(1): 69-72.

[55] Akhter S, Ansari MS, Rakha BA, Aziz T, Andrahi SMH, Ullah N, et al. Effect of milk based extenders on motility and acrosomal integrity of buffalo bull (*Bubalus bubalis*) spermatozoa at 5°C. *Pak J Zool* 2015; 47(6): 1645-1648.

[56] Celeghini ECC, Arruda RP, Andrade AFC, Nascimento J, Raphael CF, Rodrigues PHM. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. *Anim Reprod Sci* 2008; 104(2): 119-131.

[57] Leite TG, Filhoa VR, Arrudab RP, Andradeb AF, Emericka LL, Zaffalonb FG, et al. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. *Anim Reprod Sci* 2010; 120(1): 31-38.

[58] Manjunath P, Nauc V, Bergeron A, Ménard M. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen’s egg yolk. *Biol Reprod* 2002; 67(4): 1250-1258.

[59] Bencharif D, Amirat L, Anton M, Schmitt E, Desherces S, Delhomme G, et al. The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen. *Theriogenology* 2008; 70(9): 1478-1488.

[60] Emamverdi M, Zhandi M, Zare Shahneh A, Sharafi M, Akbari-Sharif A. Optimization of ram semen cryopreservation using a chemically defined soybean lecithin-based extender. *Reprod Domest Anim* 2013; 48(6): 899-904.

[61] Holt WV, North RD. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. *Biol Reprod* 1994; 51(3): 414-424.

[62] Gil J, Rodriguez-Irazoqui M, Lundheim N, Soderquist L, Rodriguez-Martinez H. Fertility of ram semen in Bioexcell and used for cervical artificial insemination. *Theriogenology* 2003; 59(5): 1157-1170.

[63] Salmani N, Nabi MM, Vasgehi-Dodaran H, Rahman MB, Mohammadi-Sangehshimeh A, Shakeri M, et al. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. *Small Rum Res* 2013; 112(1): 123-127.

[64] Eddy EM, O’Brien D. The spermatozoon. In: Knobil E, Neill JD, editors. *The physiology of reproduction*. 2nd ed. New York, NY, USA: Raven Press; 1994, p. 29-77.

[65] Moustacas VS, Zaffalon FG, Lagaeres MA, Loaiza-Eccheverri AM, Varago FC, Neves MM, et al. Natural, but not lyophilized, low density lipoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen. *Theriogenology* 2011; 75(2): 300-307.

[66] Martinez-Pastor F, Fernandez-Santos MR, Del Olmo E, Dominguez-Rebolledo AE, Esteso MC, Montero V, et al. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. *Reprod Fertil Dev* 2008; 20(5): 547-556.

[67] Del Valle I, Gomez-Duran A, Holt WV, Muino-Blanco T, Cebrian JA. Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *J Androl* 2012; 33(4): 717-725.

[68] Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J Androl* 1992; 13(3): 232-241.

[69] Hammerstedt RH. Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: A review of the effect on design of storage preservation systems. *Reprod Fertil Dev* 1993; 5(6): 675-690.