Research Article

In-vitro and *In-vivo* Fertilizing Potential of Kamohri Buck Semen Frozen in Tris-Based Egg Yolk Extender Supplemented with Selenium

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Abstract | To assess the influence of selenium on semen cytological parameters, 48 ejaculations were collected from 4 Kamohri bucks using an artificial vagina twice a week. Following the semen collection, ejaculates were assessed through macroscopic and microscopic evaluation. Semen ejaculates having ≥ 80% motility, morphology, and live-dead ratio were pooled and diluted in Tris-based Egg Yolk (TEY) extender supplemented with various concentrations of selenium, i.e., group A control (0mM), group B (2mM), group C (4mM) and group D (6mM). Results showed significantly (P<0.05) higher motility (78.1±0.22), morphology (86.5±1.08), membrane integrity (85.6±0.30) and live dead ratio (85.3±0.37) for chilled semen in group B than the other groups. Significantly (P<0.05) improved post-thawed motility (68.1±0.42), morphology (73.5±0.3), membrane integrity (70.8±0.36) and live dead ratio (77.1±0.41) were observed in group B as compared to other groups. For *in vivo* fertility assessment, twenty goats were selected and divided into two experimental groups, viz., group A and B (n=10/group). The goats of both groups were synchronized using the Ovsynch protocol. Group A goats were inseminated with semen extended in a TEY extender without selenium supplementation (control) and group B goats were inseminated with 02 mM selenium supplementation. After 45 days of insemination, pregnancy was confirmed utilizing a transabdominal probe and real-time B mode ultrasonography. The results showed that group B had a significantly (P<0.05) higher conception rate (50%) than group A (30%). In conclusion, supplementation of TEY extender with 2 mM selenium improved *in-vitro* (chilled and frozen-thaw sperm traits) and *in-vivo* conception rate of Kamohri buck semen.

Keywords | Kamohri Buck semen, Tris-based Egg Yolk extender, Selenium, Sperm motility, Fertility rate

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INTRODUCTION

Pakistan is primarily an agricultural land; most of the rural and peri-urban population is engaged in livestock farming and agriculture for their livelihood. Pakistan possesses a variety of goat breeds, including 34 native breeds (Salami et al., 2011). Sindh province is the hub of renowned goat breeds, including the Kamohri, Bari, Bugi toori, Pateri, and Tapri (Khan et al., 2008). Kamohri is one of the reputed goat breeds regarding milk yield and is also idiomatically called the poor man's cow for his milk production. Kamohri has a distinguishing body structure

with long ears, a neck, and a broad body. Its body is dark brown with small coffee-colored or dark patches over its whole body. Popularly, this breed is found in Tando Adam, Hyderabad, Saeedabad, Hala, Bhit Shah, Matiyari, Tando Allah Yar and Dadu districts of Sindh province. (Kunbhar et al., 2016).

Artificial insemination (AI) is a biotechnological technique for breed betterment programs and is broadly considered a breeding technique in farm animals (Sansone et al., 2000). A. I in goats reported a pregnancy rate of 7 to 79% with post-thawed buck semen (Bispo et al., 2012). Semen handling and cryopreservation cause some damage to the DNA of spermatozoa, motility, acrosomal cap, membrane integrity, leakage of intracellular enzymes of sperm, and low fertility (Guthrie & Welch, 2006). The extension of caprine semen is a complicated process that involves balancing many hurdles to achieve optimum results in terms of quality and fertilizing potential. Buck spermatozoa need special attention to achieve a high post-thawing live-dead ratio and fertility. In general, around 50-60% of the sperm population survived under cryopreservation with standard protocols.

Low fertility of cryopreserved semen has been a challenge and remains a problem for the goat breeders and scientists. Buck spermatozoa contain a high proportion of polyunsaturated fatty acids, which is why it is particularly susceptible to peroxidative damage (Asadpour et al., 2012). This results from increased free radical production of Reactive Oxygen Species (ROS), i.e., superoxide, hydroxyl radical and peroxide. This risk increases significantly after cryopreservation, which leads to an imbalance between ROS production and the scavenging system and can cause morphological and functional damage to spermatozoa. To reduce these destructive effects of ROS, seminal plasma has an antioxidant system that protects sperm from oxidative damage to some extent (Alvarez & Storey, 1989). To combat the high level of ROS and encourage motility and survival of sperm, various antioxidants have proven favorable effects to ameliorate buck fertility (Azawi & Hussein, 2013).

Selenium (Se) is an antioxidant and essential mineral for development and growth in humans and animals. Deficiency of Selenium is related to reproductive disorders and slow sperm quality of various species, i.e., rats, mice, chickens, pigs, sheep, and cattle (Zubair et al., 2015). High selenium concentrations in testicles and seminal material were essential to fertility. Inadequacy of selenium is evident with low sperm counts and increased abnormalities in sperm development (Tang et al., 1991). The favorable effect of adding selenium to semen extenders of farm animals has been reported by many researchers (Khalil et al., 2019). Nateq et al. (2020) investigated the addition of 1µg of nano-selenium (Se) in a semen extender to enhance the post-thaw quality of ram semen. Similarly, Fouad and Ashour (2021) stated that adding 0.5mg/100 ml selenium in the extender positively affected the post-thaw semen characteristics of the Friesian bull.

The *in-vivo* effect of selenium supplemented with 1.0 µg/ml in cryopreservation of semen was studied by Khalil et al. (2019) reported a 90% pregnancy rate in Friesian cattle. In another study, Jamali et al. (2019) found a better conception rate with the selenium-treated group, 60%, than the control group, 30% in Kundhi buffalos. To the best of our knowledge, the effect of selenium on the semen characteristics of Kamohri buck is not evaluated yet. In the present study, selenium was used as an antioxidant in the TEY extender with a hypothesis of improving results of *in-vitro* and *in-vivo* post-thawed semen of Kamohri buck.

MATERIAL AND METHODS

Animals and semen collection

All experimental protocols were approved by the Departmental Board of Studies of the Department of Animal Reproduction. Four healthy bucks of the Kamohri breed at the age of 6-8 months were maintained and trained for semen collection at the facility of Livestock farm of the Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam. Bucks were kept in a separate cage with sufficient ventilation. Seasonal green grass, 500g wheat grain, and 250g wheat bran were offered to each buck daily. Water was available *ad libitum* throughout the trial. A total of 48 (n=12/buck) ejaculates of semen were collected by the Artificial Vagina method. Right after collection, the semen ejaculates were moved to the laboratory and kept in a water bath maintained at 37°C. Initially, semen was assessed for color, volume, pH, wave motion, motility, morphology, live-dead ratio, concentration, and membrane integrity.

Assessment of macro and microscopic semen parameters

The color of semen was recorded by visual examination. The volume of semen observed directly from a graduated collection tube (Memon et al., 2011). The pH of semen was recorded using a digital pH meter (RoHS company). Wave motion was assessed on a warm, sterile, dry slide by placing a drop of semen (undiluted) under a phase contrast microscope (Nikon, Germany) at (10x) magnification. Wave pattern semen was noted and classified as described by Rehman et al. (2014).

Sperm motility percentage was assessed by diluting 1 ml of semen with 100 ml of normal saline. Then, 10µl of dilut-

ed semen was placed on a pre-warmed slide and covered with a cover slip. The prepared slide was observed under a phase contrast microscope at (20x) magnification. At least 200 spermatozoa were randomly recorded, those moving in the forward direction from four microscopic fields, and the mean of four fields examination was concluded as the final motility percentage.

The hemocytometer method was used for the determination of sperm concentration. The normal morphology and live-dead ratio were estimated using the eosin-nigrosine staining technique (Evans & Maxwell, 1987), as Memon et al. (2011) described. The sperm membrane integrity was determined by the Hypo Osmotic Swelling test (HOST) as described by Memon et al. (2011).

The ejaculate volume ≥ 1-2ml, concentration ≥ $2.5x10^9$ sperm/ml with motility ≥ 70% normal morphology and live dead ratio ≥ 80% were pooled and further processed for extension and freezing. Before the processing of the semen, the seminal plasma of the semen was washed using a ringer solution as described by Anand et al. (2017).

Semen processing

Semen samples qualifying the above criteria were pooled and diluted in TEY extender according to groups given in Table 1. Dilution of semen was processed at 5 °C. Then, the diluted semen was in a cold cabinet for 4 hours. The rate of dilution was adjusted to 200 million sperm/ml. After cooling, the four mini-straws of 0.25ml of different colors, i.e., white (control), red (2mM), blue (4mM), and yellow (6mM) corresponding to each group, were filled with the help of a filling machine (Repulsion Motor, MULTIFLEX Betriebsart: DB Schutzart: IP 21). The straws' open end was manually sealed with polyvinyl chloride powder (PVC). The vapor freezing of chilled straws was carried out for 7 minutes, 4 cm above the liquid nitrogen vapors, and then plugged into a liquid nitrogen container for 24 hours. The semen quality parameters, i.e., motility, morphology, membrane integrity, and live-dead ratio, were observed at pre-freezing and post-thawing using the same methodology applied to fresh semen.

Fertility rate

For *in vivo* fertility assessment, twenty goats were selected and divided into two experimental groups, viz., group A and B (n=10/group). The goats of both groups were synchronized using the Ovsynch protocol. Day 0 = Bosol (buseriline acetate 0.0042mg), 1ml dose (Selmore, Pvt Ltd) a synthetic GnRH analog. Day 7 = Serilin (Lecirelin), a synthetic prostaglandin (Selmore, Pvt Ltd), 1ml dose. On day 9, the same hormone of day 0 was repeated. Artificial insemination was carried out after 16 hours of the last treatment of the synchronization protocol. Group A goats were inseminated with semen extended in a TEY extender without selenium supplementation (control) and group B goats were inseminated with 02 mM selenium supplementation. After 45 days of insemination, pregnancy was confirmed utilizing a transabdominal probe and real-time B mode ultrasonography (HS2000, Honda Electronics Co. Ltd. Toyohashi, Japan) (Khand et al., 2021).

Table 1: The composition of the extender

Composition of extender	Treatment Groups			
	A	B	C	D
Tris (g)	3.81	3.81	3.81	3.81
Citric acid (g)	1.97	1.97	1.97	1.97
Fructose (g)	1.25	1.25	1.25	1.25
Egg yolk (ml)	20	20	20	20
Glycerol(ml)	07	07	07	07
Penicillin (I.U/ml)	1000	1000	1000	1000
Streptomycin (µg/ml)	1000	1000	1000	1000
Water(ml)	100	100	100	100
Selenium (mM)	0mM	02mM	04mM	06mM

Statistical analysis

Results recorded on semen quality parameters were analyzed, with one-way analysis of variance (ANOVA) Statistics 8.1 version (2006) and LSD was used to determine the difference between means of different groups.

RESULTS

Data for the effect of different concentrations of selenium in TEY extender on chilled semen characteristics are depicted in Fig 1. Significant differences ($P<0.05$) were observed in motility, morphology, live-dead ratio and membrane integrity in all groups. All the treated groups showed higher values of semen compared to the control group. The result reveals that the mean semen characteristics percentage was significantly ($P<0.05$) higher in group B (78.1±0.42) supplemented with Selenium 02mM compared to the rest of the groups.

Effects of selenium in TEY extender on post-thaw semen quality are depicted in Fig. 2. Significantly higher results were obtained by adding selenium. Moreover, the mean (± SE) motility, morphology, live-dead ratio and membrane integrity percentage are significantly ($P<0.05$) higher in group B (02 mM selenium) followed by group C (04 mM), D (06 mM) and A (0 mM).

In vivo fertility rate of post-thawed Kamohri buck semen extended in TEY extender supplemented with selenium (02mM) and control is shown in Fig. 3. Significantly ($P<0.05$) higher (50 %) *in vivo* fertility was recorded in

group B (supplemented with selenium (2mM) than group A (30%) (Without selenium supplementation).

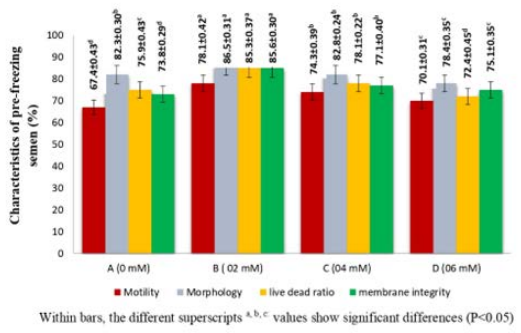

Figure 1: Assessment of pre-freezing semen characteristics of Kamohri buck (Mean % ± SEM) supplemented with various concentrations of Selenium in tris-based egg yolk extender

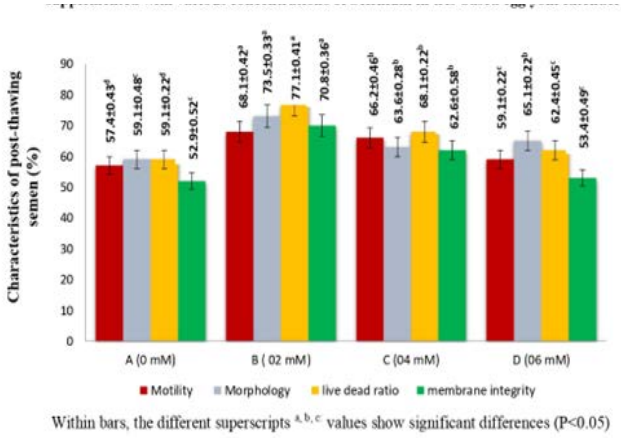

Figure 2: Assessment of Post thawing semen characteristics of Kamohri buck (Mean % ± SEM) supplemented with various concentrations of Selenium in tris-based egg yolk extender

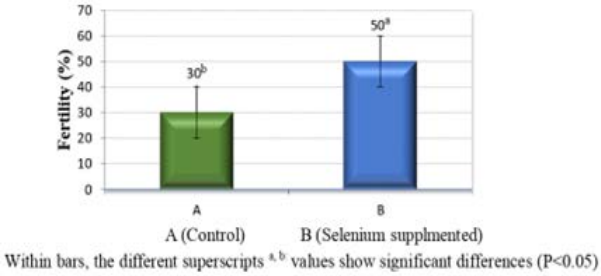

Figure 3: *In vivo,* fertility rate with post-thawed Kamohri buck semen extended in tris-based egg yolk extender supplemented with selenium (2mM) and control (0mM)

DISCUSSION

Cryopreservation of semen causes chemical and physical stress to spermatozoan membranes, disrupting sperm physiological activity. The mammalian sperm membrane comprises phospholipids and fatty acids in the unsaturated form, which are always vulnerable to peroxidation of lipids. Thus, the increased rate of lipid peroxidation causes membrane damage (Aitken et al., 1993; Ball et al., 2001). In addition to this, there is some evidence that the cooling and freezing process diminishes the fraction of intact sperms, lessens viable sperm by 50%, and increases the number of ROS production within the semen, thus leading to reduced semen quality (Chatterjee et al., 2001). Many studies have focused on modifying the extender as it can improve the quality of stored semen. Therefore, in the current study, the exogenous antioxidant (i.e., selenium) was added during the extension of Kamohri buck semen with the hypothesis that it may improve semen quality.

The assessment of post-thawed quality parameters is critical in determining the frozen-thaw quality and fertilization of sperm after freezing and thawing (Lone et al., 2018). The present study findings showed that the inclusion of selenium (2, 4 and 6mM) in the TEY extender improved the quality of pre-freezing and post-thawing semen compared to the control group (Without selenium supplementation). In the present study, the inclusion of 02mM selenium in a TEY-based extender showed significantly (P<0.05) higher motility of pre-freezing (78.1±0.42) and post-thawing (68.1±0.42) sperm compared to other groups. A similar result of chilled (78.7 ± 1.49) semen was reposted by Maidin et al. (2014) in Jermasia bucks. They extended the semen in an extender supplemented with sodium selenite 2 mg/ml. Similarly, Memon et al. (2012) observed 68.60 ± 0.56 frozen-thawed motility of sperm in TEY based extender supplemented with ascorbic acid. Enhancement of sperm motility might be attributed to the fact that selenium enhanced the rate of adenosine triphosphate (ATP) utilization and alterations in sperm metabolism pathway by using higher oxygen (Pratt et al., 1980; Marin-Guzman et al., 2000). However, the lower results of chilled (74.75± 1.9**)** and frozen-thawed **(**57.00±0.5) sperm motility were observed by Jamali et al. (2019) in Kundhi buffalo bulls even though used same quantity of selenium (02mM). Similarly, Channo et al. (2023) (58.31±0.86) and Memon et al. (2022) (57.00±0.5) observed post-thawed sperm motility in Tharparkar bulls. They diluted the semen with BioXcell™ supplemented vitamin E (α-Tochopherol) 0.02 mM/ml and soya been milk-based extender, respectively. The variation in sperm motility percentage may be due to species, extender and antioxidant differences.

In our study the addition of 2mM of selenium in the TEY extender significantly (P<0.05) improved pre-freezing (86.5±0.31) post-thawing (73.5±0.33) sperm morphology. The current findings agreed with Jamali et al. (2019). They observed chilled (86.6± 1.2) and post-thawed (73.50±1.5) morphologically normal spermatozoa in Kundhi buffa-

lo bulls supplemented with 2mM selenium. Similarly, Memon et al. (2011) observed pre-freeze morphological normal sperm (85.80 ± 0.47) in Boer goat semen extended with TEY extender supplemented with (2 mM) of butylated hydroxytoluene (BHT). Likewise, Channo et al. (2023) observed post-thaw (76.22±1.04) viable sperm in Tharparkar bull extended the semen with BioXcell™ supplemented vitamin E (α-Tochopherol) 0.02 mM/ml. From these findings, it is said that selenium and other antioxidants (i.e., vitamin E and BHT) play the same role in protecting the sperm's shape during cooling, freezing, and thawing by inhibiting ROS production. Maybe it modulates the antioxidant defense mechanism and thus can improve the normal morphology of the sperm.

However, a significantly (P<0.05) higher morphology percentage (82.20 ± 0.66) of post-thawed sperm was observed by Memon et al. (2011) in Boer goat semen. They extended with TEY extender supplemented with (01, 0.5, 02, and 03 mM) butylated hydroxytoluene (BHT**).** This difference may be due to breed (Boer goat), environment, and semen processing techniques.

The live dead ratio of pre-freezing (85.3±0.37) and pot-thawing (77.1±0.41) of Kamohri buck sperm significantly (P<0.05) improved by supplementation of 2mM selenium in the TEY extender. The current results were close to earlier reported by Jamali et al. (2019). They found 87.62±07 chilled and 72.00±0.8 post-thaw viable percentage of spermatozoa in Kundhi buffalo bulls supplemented with 2mM selenium. It is thought that adding 02mM selenium may improve the live-dead ratio percentage of spermatozoa. The significantly higher results (98.76± 1.89) of post-thawed sperm live-dead ratio were observed by Fouad and Ashour (2021) in Friesian-bull by adding 0.5mg of selenium in the treatment group. They examined post-thawed semen traits using the CASA (computer-assisted semen analyzer), which may be the reason for obtaining the higher results.

While the current result is higher than Dorostkar et al. (2012), they observed after equilibration time (79.6 ± 1.8) and frozen-sperm (67.4 ± 1.9) live dead ratio percentage of water buffalo bull by adding (02µg mL^{-1}) of selenium during extension of semen. Similarly, in an earlier study, equilibrated (68.67±2.9) and post-thawed (58.67±2.33) sperm live-dead ratios were observed by Fouad and Ashour, (2021) in Friesian-bull by adding 0.5mg of selenium during semen dilution. The lower results may be due to the low quantity of selenium and species difference. From these findings, it was noted that adding 02mM of selenium during semen processing is the optimum dose for the improvement of the live-dead ratio percentage of sperm.

In our study, supplementing the 2mM of selenium in the treatment extender improved the membrane integrity percentage of pre-freezing (85.6±0.30) and post-thawing sperm (70.8±0.36). The current result aligns with Jamali et al. (2019). They added (2mM/ml) selenium in the treatment group and observed post-chilled (87.87± 0.6) and post-thawed (70.00±0.1) sperm membrane integrity. The same post-thaw results (70.00±0.1) were also observed by Memon et al. (2022) in Tharparkar bull. They used 9ml of soya bean milk in the treatment group.

However, the current findings are higher than those of Memon et al. (2011). They observed before-freezing (72.20 ± 0.47) and post-thaw (59.40 ± 0.52) results in Boer goat by treating the semen with BHT (2mM). Similarly, Khalil et al. (2019) used 1µg/ml of selenium nano-particles in Friesian-bull, Fouad and Ashour (2021) used 0.5mg of selenium in Friesian-bull), Dorostkar et al. (2012) used 02µg mL^{-1} sodium selenite in buffalo bulls, and Channo et al. (2023) used BioXcellTM+ vitamin E (α-tocopherol) 0.02 mM/ml in Tharparkar bulls, find the lower results than our study. This variation in results may be due to using different semen extenders, antioxidants and species. Moreover, the lower selenium concentration was used in previous studies compared to ours.

Improvement in membrane integrity percentage is due to Selenium, which is vital in the antioxidant system of cells and protects them by establishing catalytic sites for antioxidant enzymes such as GPx (Alvarez & Storey, 1989). Furthermore, the cold shock to sperm starts a chain reaction of free radical production, which leads to structural component damage in the plasma membrane (Alkhedaide et al., 2016; Fonseca et al., 2005). Selenium's ability to preserve sperm membranes has been demonstrated in various animals (Angrimani et al., 2017; Dorostkar et al., 2012; Sanchez et al., 2008).

The fertility rate is significantly (P<0.05) higher in a group containing 2mM selenium than the non-supplemented group. The current report agrees with Jamali et al. (2019). They reported a 60% *in vivo* conception rate in buffaloes with a selenium-treated group compared to the non-supplemented group. However, Khalil et al. (2019) reported a 90% conception rate using 1.0 µg/ml selenium supplementation than 59% control non-supplemented semen. The difference may be due to species, season (Tuli & Holtz, 1995), breed (Qureshi et al., 2013), age of the donor male animal (Toe et al., 1994), and management practices (Hanmante et al., 2009).

CONCLUSION

In conclusion, the findings of this study underscore the

significance of the supplementation of TEY extender with 2 mM selenium, which positively affects the *in-vitro* semen quality and *in-vivo* conception rate of Kamohri buck semen.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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NOVELTY STATEMENT

To the best of our knowledge, the effect of selenium on the semen characteristics of Kamohri buck has not been evaluated yet. The purpose of this study is to enhance the quality traits of Kamohri buck semen by adding selenium with a commonly used TEY semen extender.

AUTHOR CONTRIBUTIONS

Concept – A.A.M., N.A.; Design – A.K.; Supervision – A.A.M., A.K., A.H.M., Resources – M.I.P.; Materials – N.A.S., M.A.V.; Data Collection and/or Processing – N.A., F.U.R.; Analysis and/or Interpretation – A.H.M., N.A.; Literature Search – M.I.P., A.H.M.; Writing Manuscript – M.I.P., N.A.; Critical Review – A.A.M., A.K, K.A.M., N.A.S.; Other – N/A.

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