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Vitamin E supplement in Tris-pumpkin seed extenders on sperm viability and fertilizing parameters of West African dwarf goat bucks chilled at 4^{o}C.

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***Target Audience:** Animal scientists, ruminant farmers, animal breeders*

Abstract

This study investigated the protective effects of vitamin E in Tris-pumpkin seed extenders on sperm viability and fertilizing parameters of West African dwarf goat bucks chilled at 4^{o}C. Pooled semen from four matured WAD bucks was diluted in Tris-pumpkin extenders containing vitamin E at 0mM, 2mM, 4mM, 6mM and 8mM/100ml respectively. Microscopic assessments of diluted semen samples were carried out on sperm progressive motility, acrosome and membrane integrities, livability, sperm abnormality, arginase and acrosin activities, leukocytes, in vitro sperm capacitation and acrosome reaction after in vitro storage at 4^{o}C for 96 hours. The concentration of malondialdehyde (MDA) in the stored semen was measured in thiobarbituric acid reactive substances. The results showed Tris-pumpkin based extender supplemented with 4mM, 6mM and 8mM inclusion levels of vitamin E had higher ($p<0.05$) percentage sperm progressive motility, membrane and acrosome integrities and livability compared to other inclusion level and the control group. Furthermore, Spermatozoa with Tris based extender supplemented with 2mM and 8mM inclusion levels of vitamin E had lower ($p<0.05$) percentage abnormality. The results showed lower ($p<0.05$) concentrations of MDA and leukocyte at 8mM inclusion levels of vitamin E and higher value of arginase activity at 4mM and 8mM inclusion levels. However, Tris- pumpkin extenders supplemented with 6mM and 8mM inclusion of vitamin E had highest ($p<0.05$) percentage of spermatozoa that underwent in vitro acrosome reaction and sperm capacitation compared to other levels and the control. The findings indicate protective potential of vitamin E on sperm viability of chilled semen stored at 4^{o}C.

***Key words:** Bucks, vitamin E, spermatozoa viability, Pumpkin seed based extender*

Description of Problem

Chilled-stored semen in artificial insemination has become a technique in sheep and goat breeding (7). Preservation of sperm for longer periods by cooling or freezing requires dilution with a protective solution to preserve fertilizing capacity of spermatozoa during *in vitro* storage at low temperatures. Irrespective of extenders nature however, viability of spermatozoa deteriorates at low temperatures during storage (36). Sperms are subject to oxidative stress resulting from lipid peroxidation, which can lead to membrane damage, reduced sperm viability and lower fertility (18). Although semen contains antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (30), the endogenous antioxidative capacity of semen is not sufficient during storage (37). Moreover, during the preservation process the levels of antioxidant decreased by

dilution of semen with extender and excessive generation of reactive oxygen species (ROS) molecules (4; 29), Extenders with various additives help to maintain fertilizing ability of semen for a long time. Efforts to improve the preservation of ruminant semen are focused on the modification of extenders (35), as well as on the addition of various components to maintain motility, fertilizing capacity and preserve sperm membrane integrity (44; 49). Egg yolk is generally accepted to be an effective agent in semen extenders for protection of spermatozoa against cold shock and the lipid-phase transition effect (1). However, the use of chilled-stored semen diluted in egg yolk-based semen extenders is limited by its relatively short-time fertilization capacity (6) and individual differences in egg yolk due to different period of egg´s storage. Though a lot of successful semen dilutions have been done globally with egg yolk but with shortcomings and therefore, removal of chicken egg yolk from a semen extender would improve consistency in the components of semen extenders and eliminate hygiene risk. Therefore, it is reasonable to develop synthetic semen extenders free of egg yolk. A soybean lecithin based extender (AndroMed, Minitube, Tiefenbach, Germany) has been developed and utilized for bovine (3), mountain gazelle (47) and sheep (21) semen with satisfactory fertility results. The potential cause of the decline in motility and fertility during hypothermic storage of liquid semen is an oxidative damage of spermatozoa (8). Therefore, to maintain sperm for longer period during storage, it is necessary to dilute semen in a protective solution (7). A wide variety of antioxidants, such as glutathione (GSH), oxidized glutathione (GSSG), cysteine, taurine, hypotaurine, bovine serum albumin, trehalose or hyaluronan have been tested to minimize the damage caused by cooling and freezing-thawing on bull (50;55), water bufallo (23), stallion (8), goat (48) and ram (43;54) semen. Glutathione can influence cell metabolism through detoxication (17) and by preventing the formation of free radicals (56) in spermatozoa. The addition of glutathione to ram sperm diluents improved sperm motility, viability and plasma membrane characteristics, protected the spermatozoa from free radical damage (53) and improved sperm survival following six (6) hours cooling storage at 5 °C (11). *In vitro* studies suggested that the addition of some antioxidants to extended semen could improve the motility and survival of spermatozoa (40; 10).The use of vitamin E as an antioxidant is generally accepted and proven to be effective in many biological systems (46). Several reports have indicated beneficial effects of vitamin E arising from its potent antioxidant on preservation of functional parameters of mammalian spermatozoa (12; 45). Therefore, the study aims to determine the effect of vitamin E supplement in Tris-pumpkin seed extenders on sperm viability and fertilizing parameters of West African dwarf goat bucks chilled at 4^{o}C.

Materials and Methods

The experiment was carried out at the Goat Unit of Directorate of University Farm, Federal University of Agriculture, Abeokuta, located on latitude 7^{0} 10' N and longitude 3^{0} 2' E, and altitude 76m above sea level with a mean annual rainfall of 1,037mm and average temperature of 34.7^{0}C. Six intact West African Dwarf goat bucks and one matured teaser doe were used for the experiment. The bucks ranged between 4 and 5 years with average weight of 18kg. The animals were kept under intensive management and maintained under a

uniform and constant nutritional regimen with concentrate feed supplemented with guinea grass (*Panicum maximum*).

Semen collection, dilution and storage

Semen samples were collected from six WAD goat bucks with the aid of artificial vagina. Only ejaculates showing >80% motility were pooled. Pooled semen sample (each pool originating from six males) was diluted with Tris-pumpkin seed milk based extender. Prior to dilution, the extender was supplemented each with vitamin E at 2mM, 4mM, 6mM and 8mM /100ml of the diluents respectively. Following dilution, the semen was dispensed into 5ml tubes sealed and gradually chilled at 4^{o}C for 96 hours. The pH of the pumpkin seed milk extenders (Control: 7.05 and vitamin E extender 6.89) was determined using digital pH meter.

Table 1: Chemical composition of Tris pumpkin seed milk based extender

Composition	Quantity (g/100mL)
Trishydroxyaminomethane	2.42
Citric acid	1.36
Glucose	1.0
Penicillin	0.028
pumpkin seed milk	20
Distilled water	76

Source: (4)

Semen Evaluation

Sperm Motility

Sperm motility was determined as described by (9).Briefly, semen was thawed in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37^{0}C and accessed for sperm motility using Celestron PentaView microscope (LCD-44348 by RoHS, China) at 400 X magnifications. A semen mount was made using 5µl semen and the semen was placed directly on a microscope slide and covered with cover slip. For each sample, five microscopic fields were examined to observe progressive sperm motility and the mean of the five successive evaluations was recorded as the final motility score.

Acrosome Integrity

The percentage of spermatozoa with intact acrosomes was determined according to (2). Briefly,50 µl of each semen sample was added to a 500µl formalin citrate solution (96 ml 2.9% sodium citrate, with 4 ml 37% formaldehyde) and mixed carefully. A small drop of the mixture was placed on a microscope slide and a total of 200 spermatozoa were counted in at least three different microscopic fields for each sample, using Celestron PentaView LCD microscope (400 X magnifications).Intactness of acrosome characterized by normal apical ridge of spermatozoa was assessed using Celestron PentaView LCD microscope.

Sperm Membrane Integrity

Hypo-osmotic swelling test (HOST) assay as described earlier (26) was used to determine sperm membrane integrity and this was done by incubating 10 µl semen in 100 µl Hypo-osmotic solution (fructose and sodium citrate) at 37^{0}C for 30minutes, 0.1 ml of the mixture was spread over a warmed slide, covered with a cover slip and observed under Celestron PentaView LCD digital microscope (400 X magnifications). Two hundred spermatozoa (200) were counted for their swollen characterized by coiled tail, indicating intact plasma membrane.

Sperm Abnormality

Sperm abnormality was evaluated as described by (9) with the use of eosin-nigrosin smears. A thin smear of mixture of semen and eosin-nigrosin solution was drawn across the slide and dried. Abnormality of sperm cells located in the head, midpiece and tail were observed under Celestron PentaView LCD microscope (400 x magnifications).

Live sperm

Live sperm was evaluated as described by (9) with the use of eosin-nigrosin stain. A thin smear of mixture of semen and eosin-nigrosin solution were drawn across the slide and dried. Semen samples were examined under a digital microscope (Celestron Penta®) LCD view at 400xmagnification for live spermatozoa. Spermatozoa that appear white were recorded as live spermatozoa and those that pick up the stain were recorded as dead spermatozoa.

MDA concentrations

MDA concentration as index of lipid peroxidation in the stored semen was measured in thiobarbituric acid reactive substances (TBARS) according to (57). For this assay, 0.1ml of sperm suspension was incubated with 0.1 ml of 150 mM Tris-HCl (pH7.1) for 20 minutes at 37°C. Subsequently, 1ml of 10% trichloroacetic acid (TCA) and 2 ml of 0.375% thiobarbituric acid was added followed by incubation in boiling water for 30 min. Thereafter, it was centrifuged for 15 minutes at 3000 rpm inside the blank tube and the absorbance was read with UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 532 nm. The concentration of MDA was calculated as follows: The concentration of malondialdehyde MDA (nmol/ml) = AT – AB/1.56×10^5; Where: AT = the absorbance of the sample serum, AB = the absorbance of the blank, 1.56×10^5 molar absorptivity of MDA

Arginase activity

Arginase activity was carried out according to the procedure of (32). Briefly, 0.1g bovine serum albumin (BSA) as standard in 10ml of water was used. The tubes containing 1ml alkaline copper reagent (a mixture of copper sulfate reagent, sodium dodecyl sulfate solution, and sodium hydroxide solution (1:2:1) and 0.1ml supernatant sample were mixed and incubated for 10min at room temperature. After this, 4ml folin Ciocalteu"s phenol reagent was added to the tubes, mixed and incubated for 5min at 55°C. The absorbance of the samples was recorded at 650nm in spectrophotometer (UV spectrophotometer, SW7504 model by Surgifriend Medicals, England).

Leukocytes

Peroxidase test as recommended by WHO was used as follows: A stock solution was prepared by mixing 50ml distilled water with 50 ml 96% ethanol plus 125 mg benzidine. The working solution was obtained by adding 5µl 30% H_2O_2 to 4 ml of stock solution. Twenty (20)µl of working solution was mixed with 20µl of cryopreserved semen in a small test tube. After incubation for 5min at room temperature, 20µl of working solution was mixed with 20µl of phosphate-buffered saline. Then, 10µl was placed in a haemocytometer, and peroxidase-positive cells (dark brown round cells) were counted.

***In vitro* acrosome reaction**

Following cryopreservation, spermatozoa were thawed by plunging straws into a water bath (37°C) for 1min and the proportion of acrosome reaction was

determined as described by (51) with modification as follows: Samples of cryopreserved spermatozoa were washed with non culture medium (Phosphate-Buffered Saline [PBS]), and the pellets were re-suspended in culture medium (Calcium chloride di hydrate 265mg/L, Magnesium chloride anhydrous 46 mg/L, Potassium chloride 200mg/L, Sodium chloride 8000 mg/L, Sodium dihydrogen phosphate anhydrous 50 mg/L, D-Glucose 1000 mg/L). Immediately after the inclusion of 0.9% wt/vol PBS (15µg/ml), the acrosome reaction was induced by incubating spermatozoa for 20min with progesterone (2.5mg/ml) at 38.5°C (5% CO_2 in air; 100% humidity). To determine the proportion of spontaneous acrosome reaction, progesterone was omitted but an equal volume of PBS was added. Spermatozoa were observed in an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics, and 100 cells were counted per slide. Spermatozoa with intense fluorescence over the acrosome were classified as acrosome intact and those with no fluorescence or a dull fluorescence along the equatorial segment as acrosome reacted.

***In vitro* capacitation**

In vitro capacitation of the spermatozoa was evaluated using the CTC fluorescence assay as described by (13). In brief, CTC (750 µM*)* was prepared in 20mM Tris buffer containing 130mM NaCl and 5 mM DL-vitamin E (final pH 7.8). Sperm suspension (5µl) was mixed with 5µl of CTC solution on a warmed slide (37° C). After 30sec, 5µl of 0.2% glutaraldehyde in 0.5 M Tris pH 7.4 was added. Finally, 5µlof 90% glycerol and 10% PBS (pH was adjusted to 8.6) were added to retard fluorescence fading. After adding a cover slip, slide was examined with an upright Carl Zeiss Fluorescent Microscope (Primo Star, Germany) equipped with phase contrast and epifluorescence optics, and 100 cells were counted per slide. The proportion of cryopreserved spermatozoa that exhibited pattern B according to the CTC assay was determined. Spermatozoa characterized by bright anterior head and faint fluorescence in the post-acrosomal region were classified as capacitated spermatozoa while non capacitated sperm had bright uniform fluorescence over the head.

Statistical Analysis

Data obtained were subjected to analysis of variance (ANOVA) using SAS 1999. While Tukey HSD was used to separate significantly different means. The model that was used to analyze the data is stated below:

$Y_{ij} = \mu + A_i + L_j + \sum_{ij}$

Where,

Y_{ij}= Dependent variables

μ = Population mean

A_i = Effect due to i^{th} vitamin E,

L_j = Effect due to j^{th} level of inclusion, j = 0, 2, 4, 6, 8

$\sum_{ij}$ =Experimental Error

Results

Results in Table 2 showed higher ($p<0.05$) sperm motility, acrosome integrity, membrane integrity and live sperm in Tris pumpkin extenders supplemented with vitamin E compared to the control. Spermatozoa chilled with 4mM, 6mM and 8mM inclusion of vitamin E had the highest ($p<0.05$) percentage sperm motility, acrosome integrity, live sperm and membrane integrity which also includes 2mM compared to other inclusion levels of vitamin E and the control. The results showed lower ($p<0.05$) percentage abnormalities at all levels of vitamin E supplementation compared to the control. However, lowest ($p<0.05$) percentage

abnormalities were observed at 2mM and 8mM inclusion levels of vitamin E compared to the control.

Seminal oxidative stress parameters of semen cryopreserved with varying concentrations of vitamin E as antioxidant is presented in Table 3. The results showed that semen cryopreserved with tris based extender supplemented with different vitamin E levels had lower p>0.05 MDA concentrations compared with the control group. However, semen cryopreserved with vitamin E at 8mM inclusion level had lowest p>0.05 MDA concentrations compared to 2mM, 4mM, 6mM and the control group while lower p>0.05 leukocytes was obtained in 4mM, 6mM and 8mM compared to other inclusion levels and the control . However, higher arginase activity p>0.05 was obtained at 4mM and 8mM inclusion levels of vitamin E as compared the control group.

The results in figure I and II showed effect of Tris pumpkin seed milk extenders supplemented with vitamin E on *in vitro* acrosome reaction (%) and *in vitro* sperm capacitation of WAD buck spermatozoa chilled at 4^{o}C. Higher (p<0.05) percentage of spermatozoa cryopreserved that underwent acrosome reaction and sperm capacitation were obtained in Tris pumpkin milk extenders supplemented with 6mM and 8mM vitamin E. More spermatozoa (p<0.05) cryopreserved with Tris based extenders supplemented with vitamin E induced acrosome reaction and capacitation compared to control group.

Table2: Effect of Tris pumpkin seed milk extenders supplemented with vitamin E on sperm viability of WAD buck spermatozoa chilled at 4^{o}C

	Extenders				
Parameters (%)	**0mM**	**2mM**	**4mM**	**6mM**	**8mM**
Sperm Motility	33.20 ± 4.57^{c}	53.40 ± 3.40^{b}	63.40 ± 9.72^{a}	67.60 ± 0.60^{a}	70.20 ± 8.14^{a}
Acrosome Integrity	45.50 ± 1.89^{c}	59.00 ± 5.44^{b}	72.00 ± 4.08^{a}	67.00 ± 1.91^{a}	76.00 ± 2.16^{a}
Membrane Integrity	51.50 ± 1.26^{b}	72.50 ± 4.19^{a}	75.00 ± 3.69^{a}	76.00 ± 2.16^{a}	78.00 ± 1.41^{a}
Live Sperm	55.00 ± 2.89^{c}	65.00 ± 2.89^{b}	72.50 ± 2.50^{a}	80.00 ± 0.00^{a}	77.50 ± 2.50^{a}
Abnormality	4.00 ± 0.00^{a}	2.75 ± 0.48^{c}	3.00 ± 0.41^{b}	3.50 ± 0.65^{b}	1.25 ± 0.25^{c}

$^{a, b, c}$ Values within rows with different superscripts differ significantly (P < 0.05), SE: standard error.

Table 3: Effect of Tris pumpkin seed milk extenders supplemented with vitamin E on seminal oxidative stress parameters of WAD buck spermatozoa chilled at 4^{o}C

	Extenders				
Parameters	**0mM**	**2mM**	**4mM**	**6mM**	**8mM**
MDA Concentration (nmol/ml)	0.39 ± 0.04^{a}	0.14 ± 0.02^{c}	0.22 ± 0.00^{b}	0.15 ±0.04^{c}	0.07 ± 0.01^{d}
Arginase Activity (units/mg protein)	0.58 ± 0.00^{d}	0.67 ± 0.00^{c}	3.21 ± 0.00^{a}	1.03 ± 0.00^{b}	3.21 ± 0.00^{a}
Leukocytes (x10ml)	6.46 ± 0.11^{a}	3.96 ± 0.05^{b}	3.51 ± 0.06^{b}	3.21 ± 0.08^{b}	2.69 ± 0.07^{c}

$^{a, b, c,d}$ Values within rows with different superscripts differ significantly (P < 0.05), SE: standard error.

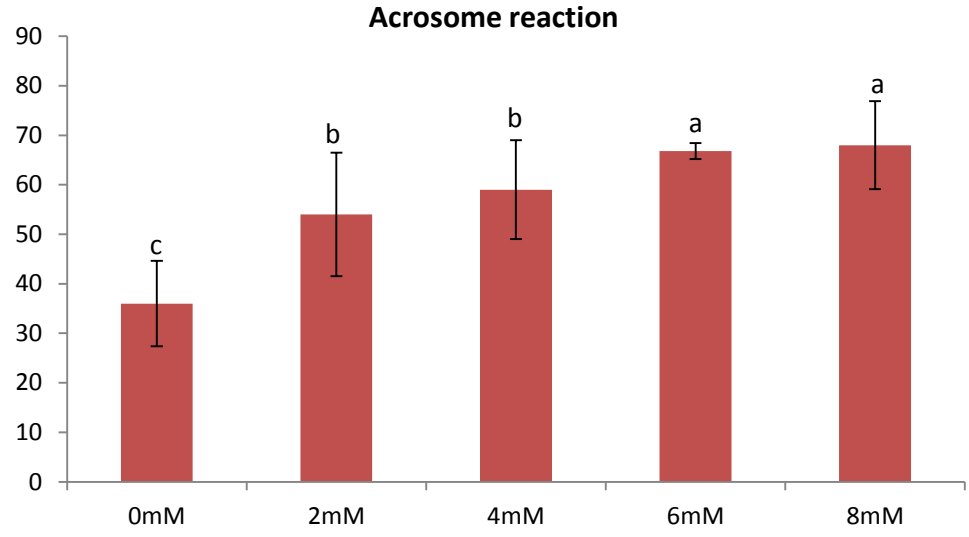

Figure I: Effect of Tris pumpkin seed milk extenders supplemented with vitamin E on *in vitro* acrosome reaction (%) of WAD buck spermatozoa chilled at 4^{o}C

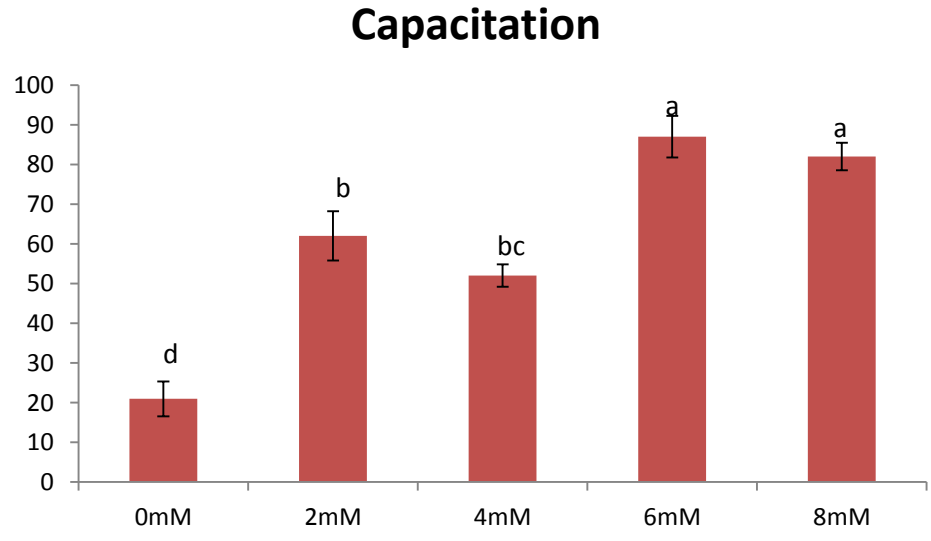

Figure II: Effect of Tris pumpkin seed milk extenders supplemented with vitamin E on *in vitro* Capacitation (%) of WAD buck spermatozoa chilled at 4^{o}C

Discussion

Sperm are exposed to a range of temperatures during storage process, this promote structural (16) or functional modifications (25). The modifications are evident in mitochondrial and membrane malfunction. This reduces the quantity of viable and progressive motile sperm with uncapacitated and intact acrosomal cap prior to artificial insemination, by affecting the permeability of the sperm surface to water and ions (24; 42). Capacitation and acrosomal cap destruction are cellular exocytic like processes which affect sperm fertilizing ability and are a critical requirement for successful pregnancy after insemination (31). The antioxidant vitamin E protected the integrity of chilled goat sperm which is shown in its ability to effectively preserve the sperm at different supplemented levels. This was consistent with previous research that reported that supplementing vitamin E as an antioxidant enhanced post-thawed sperm qualities by lowering faulty sperm or excessive ROS produced by leukocytes or sperm (14). It is generally understood that motility is an important factor in determining the quality of fresh and post-thawed sperm. Progressive motility was found to be significant when vitamin E was used as an antioxidant source when supplemented with extender, which could be due to the presence of a-tocopheryl acetate bioavailability as an energy source (33) is required to maintain sperm metabolism and to survive stress induced inhibition caused by freezing. Energy is required to keep sperm metabolism going and to withstand the stress induced dormancy caused by storage (22; 57).

Vitamin E contains a high concentration of phospholipids (5), which makes it an excellent additive to semen extender. Phospholipids stabilize the cell membrane, and protect sperm from cold shock (38; 20). The considerable effect of phospholipid content on sperm viability, acrosome, and membrane integrities when extender is supplemented with vitamin E could be attributable to its phospholipid content (5). The current findings are consistent with previous findings that sperm motility and viability were higher in goats and roosters (27; 39). When vitamin E is supplemented in an extender, the acrosome and membrane integrities are better preserved and this increases the extender's ability to maintain sperm functional integrity.

Lower oxidative parameter values suggested that vitamin E added with an extender could minimize oxidative stress in sperm during storage. Mammalian sperm contain a high concentration of polyunsaturated fatty acids in their plasma membrane, rendering them susceptible to lipid peroxidation (41; 34). Vitamin E as an antioxidant maintained sperm viability by scavenging lipid peroxidation after freezing (52). Leukocytes or sperm function tests, such as acrosome response and capacitation, are more accurate indicators of fertility than standard sperm characteristics (28). *In vitro* acrosome response and sperm capacitation were improved with vitamin E supplemented with tris- pumpkin extender at 6mM and 8mM, indicating that these procedures may improve the fertilizing potential of chilled sperm. Significant changes in sperm quality parameters on the other hand, have already been documented (58).

Conclusion and Application

1. The findings of the present study indicated that chilled WAD goat buck semen supplemented with 6 mM and 8 Mm resulted in improved viability and fertilizing ability parameters.
2. Chilled WAD goat buck semen supplemented with 6 mM and 8 Mm

resulted in reduced oxidative stress parameters.

3. Preservation of semen samples obtained from WAD goat bucks with tris-pumpkin seed milk based extender supplemented with vitamin E as antioxidant is satisfactory for AI programme.

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References

1. Aboagla, E.M.E.; Terada, T. (2004). Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology*, 62, 1160-1172.
2. Ahmad, Z. A., Zar, M., Shahab, N., Ahmad, S. and Andrabi, M. H. (2003). Sephadex and sephadex ionexchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. *Theriogenology*, 59, 1189-1202.
3. Aires, V.A., Hinsch, K.D., Schloesser, F.M., Bogner, K., Schloesser, S.M., Hinsch, E. (2003). In vitro and in vivo comparision of egg yolk based and soyabean extenders for cryopreservation of bovine semen. *Theriogenology*, 60, 269-279.
4. Andrabi, S. M. H. (2009). Factors affecting the quality of cryopreserved buffalo (Bubalus bubalis) bull spermatozoa. *Reproduction in domestic animals*, 44(3), 552-569.
5. Atkinson J, Epand, R.F. and Epand, R.M. (2008). Tocopherols and tocotrienols in membranes: a critical review. *Free Radical Biology Medicine*, 44, 739-764.
6. Aurich, J.E., Schonherr, U., Hoppe, H., Aurich, C. (1997). Effects of antioxidants on motility and membrane integrity of chilled stored stallion semen. *Theriogenology*, 48:185-192.
7. Ax , R.L., Dally, M.R., Diddion, B. A., Lenz, R.W., Love, C.C., Varner, D.D., Hafez, B. and Bellin, M.E. (2001). *Artificial insemination. In Reproduction in farm animals*. London Pp 376-389.
8. Ball, B., Medina, V., Gravance, C., Baumber, J. (2001). Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5ºC. *Theriogenology*, 56, 577-589.
9. Bearden, H. J. and Fuquay, J. W. (1997). Semen evaluation. In: Applied animal reproduction. 4th ed. *New Jersey: Prentice* Hall; pp. 158-169.
10. Bilodeau, J. F., Blanchette, S., Cormier, N., and Sirard, M. A. (2002). Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*, 57(3), 1105-1122.
11. Bucak MN, Tekin N, (2007). Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Ruminant Research,* **7**, 103-108.
12. Cerolini, S., Maldjian, A., Surai, P., and Noble, R. (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Animal Reproduction Science*, 58(1-2), 99-111.
13. Collin, S.; Sirard, M.A.; Dufour, M. and Bailey, J.L. (2000). Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. *Journal of Andrology*,

21, 938-943.

14. Daramola, J. O. and Adekunle, E. O. (2017). Effects of Washing Protocols on Cryosurvival of Spermatozoa from West African Dwarf Goat Bucks. *CryoLetters*, 38(3), pp.210-215.
15. Daramola, J. O., Adekunle, E. O., Oke, O. E., Ogundele, O., Saanu, E. O. A., and Odeyemi, A. J. (2016). Effect of vitamin E on sperm and oxidative stress parameters of West African Dwarf goat bucks. *Tropical and Subtropical Agro ecosystems*, 19(2).44-49
16. De Leeuw, F.E., Chen, H.C., Colenbrander, B. and Verkleij, A.J., (1990). Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology*, 27(2), 171-183.
17. De matos, D. and Furnus, C. (2000). The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development: Effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology*, 53(3), 761-771.
18. Donoghue, A. M., and Donoghue, D. J. (1997). Effects of water-and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poultry Science*, 76(10), 1440-1445.
19. Duncan, B. D. (1955). Multiple Range Test and Multiple F-test, Biometrics, 11: 1-42.
20. Ezeh O., Gordon M. H., Niranjan K. (2014). Tiger nut oil (Cyperus esculentus L.): A review of its composition and physico-chemical properties. *European Journal of Lipid Science Technology*, 16, 783–794.
21. Fukui, Y., Kohno, H., Togari, T. and Hiwasa, M. (2007). Fertility of ewes inseminated intrauterinally with frozen semen using extender containing bovine serum albumin. *Journal of Reproduction and Development*, 53, 959-962.
22. Gadea J. (2003). Review: Semen extenders used in the artificial insemination of swine. *Spanish Journal of Agricultural Research*, 1, 17–27.
23. Ghosh, I. and Datta, K. (2003). Sperm surface hyaluronan binding protein (HABPI) interacts with zona pellucida of water buffalo (Bubalus bubalis) through its clustered mannose residues. *Molecular Reproduction and Development*, 64, 235-244.
24. Hagiwara, M., Choi, J.H., Devireddy, R.V., Roberts, K.P., Wolkers, W.F., Makhlouf, A. and Bischof, J.C., (2009). Cellular biophysics during freezing of rat and mouse sperm predicts post-thaw motility. *Biology of reproduction*, 81(4), 700-706.
25. Hammersted t, R.H., Graham, J.K. and Nolan, J.P., (1990). Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of andrology*, *11*(1), 73-88.
26. Jeyengran, R. S. Van der Ven, H. H. Peter-Perez, M., Crabo, B. G. and Zaneveld, L. J. (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 Fertilility*, 70 (10), 219-28.
27. Kargar, R., Mohsen, F., Gholamreza, G. and Mohammad, H.N.E. (2017). Dietary flax seed oil and/or Vitamin E improve sperm parameters of cloned goats following freezing-thawing. *Cryobiology*, 74,110–114.
28. Katsuki T., Hara T., Ueda K., Tanaka J., Ohama K. 2005. Prediction of outcomes of Assisted Reproduction Treatment using the calcium ionophore-induced acrosome reaction. *Human Reproduction*, 20, 469–475.
29. Kumar, M., Verma, V., Nagpal, R., Kumar, A., Gautam, S. K., Behare, P.

V. and Aggarwal, P. K. (2011). Effect of probiotic fermented milk and chlorophyllin on gene expressions and genotoxicity during AFB1-induced hepatocellular carcinoma. *Gene,* 490(1-2), 54-59.

30. Lewis, S.E.M. Sterling, E.S.L. and Young, L.S. 1997. Comparison of individual antioxidant of semen andseminal plasma in fertile and infertile men. *Fertility and Sterility*, 67,142-147.
31. Lindsay, G, Evans G, Maxwell WMC. (2005). Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology,* 63,445–457
32. Lowry, O. H., Roseenbroug, N. J., Farr, A. and Randall, L. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.
33. Mah, E., Sapper T.N. and Chitchumroonchokchai C. (2015). alphaTocopherol bioavailability is lower in adults with metabolic syndrome regardless of dairy fat co-ingestion: a randomized, double-blind, crossover trial. *American Journal of Clinical Nutrition*, 102, 1070 -1080
34. Mandal R., Badyakar D., Chakrabarty J. (2014). Role of membrane lipid fatty acids in sperm cryopreservation. Advances in *Andrology*, *Article* ID 190542, 9 p, from http://dx.doi.org/10.1155/2014/190542
35. Marti, J. I. Marti, E. Cebrian-perez, J. E. and Muino-blanco, T. (2003). Survival rate of antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. *Theriogenology,* 60, 1013-1020.
36. Maxwell, W. M. C., and Watson, P. F. (1996). Recent progress in the preservation of ram semen. *Animal Reproduction Science,* 42(1-4), 55-65.
37. Maxwell, W. M., and Salamon, S. (1993). Liquid storage of ram semen: a review. *Reproduction, Fertility and Development,* 5(6), 613-638.
38. Medeiros C. M. O., Forell F., Oliveira A. T. D., and Rodrigues, J. L. (2002). Current status of sperm cryopreservation: Why isn't it better? *Theriogenology*, 57: 327–344.
39. Moghbeli M, Kohram H, Zare-Shahaneh A, Zhandi M, Sharideh H. (2016). Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? *Cryobiology*, 72: 264–268
40. Molinia, F. C., Evans, G., Casares, P. Q., and Maxwell, W. M. C. (1994). Effect of monosaccharides and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Animal Reproduction Science*, 36(1-2), 113-122.
41. Niki E., Yoshida Y., Saito Y., Noguchi N. (2005). Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochemical and Biophysical Research Communications*, 338, 668–676
42. Oldenhof, H., Friedel, K., Sieme, H., Glasmacher, B. and Wolkers, W.F. (2010). Membrane permeability parameters for freezing of stallion sperm as determined by Fourier transform infrared spectroscopy. *Cryobiology*, 61(1), 115-122.
43. Paulenzen, H., Söderquist , L., Pérez-pé, R. and Berg, K.A. (2002). Effect of different extenders and storage temperatures on sperm viabiliáty of liquid ram semen. *Theriogenology*, 57, 823-836.
44. Riha, L., Apolen, D. Pivko, J. Grafenau, P. and Kubovicova, E. (2006). Influence of implememtors on

sheep fertility out of season. *Slovak Journal of Animal Science*, 4, 180-182

45. Sahin, S., and Sumnu, S. G. (2006). Physical properties of foods. *Springer Science and Business Media*, 44, 739-764
46. Salamon, S., and Maxwell, W.M.C. (2000). Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science*, 38,1–36.
47. Saragusty, J., Gacitua, H., King, R., Araw, A., (2006). Post-mortem semen cryopreservation and characterization in two different endargered Gazelle species (Gazelle gazelle and Gazelle dorcis) and one subspecies (Gazelle gazelle acuiae). *Theriogenology*, 60,775-784.
48. Salvador, I., Yantz, M., Viudes-de-castro, P., Gomez, E. A. and Silvestre, M. A. (2006). Effect of solid storage on caprine semen conservation at 5C. *Theriogenology*, 64, 252-260.
49. Sarlos, P., Molnar, A., Kokai, A., Gabor, G. and Ratky, J. (2002). Evaluation of the effect of antioxidants inthe conservation of ram semen. *Actabulum Veterinary Hungry*, 50(2), 235-245.
50. Stradaioli, G., Noro, T., Sylla, L., and Monaci, M. (2007). Decrease in glutathione (GSH) content in bovine sperm afer cryopreservation: Comparison between two extenders. *Theriogenology*, 15, 1249-1255.
51. Tardif, S., Sirard, M. A., Sullivan, R. and Bailey, J. (1999). Identification of capacitation-associated phosphoproteins in porcine sperm electroportated with ATP-γ-^{32} P. *Molecular Reproduction and Development*, 54, 292-302.
52. Traber, M.G. and Atkinson, J. (2007). Vitamin E, antioxidant and nothing more. *Free Radical Biology Medicine*, 43, 415.
53. Triwulanningsih, E.P., Situmorang, P. Sugiarti, T., Sianturi, R.G. and Kusumaningrum, D. A. (2003). The effect of glutathione addition in sperm diluent on the quality of bovine chilled semen,, 8(2), 91-97.
54. Uysual, O., Bucak, M. N., Yavas, I. Varish, O. and Gürcan, I. S. (2005). Evaluation of ram sperm frozen with various taurine concentration. *Indian Veterinary Journal*, 82, 1059-1061.
55. Uysal, O., Bucak, M. N., Yavas, I. and Varish, O. (2007). Effect of various antioxidants on the quality of frozen thawed bull semen. *Journal Animal Veterinary Advances*, 6(12), 1362-1366.
56. Wijaya, A. (1996). Radikal bebas and parameter status antioksidan. Forum Diagnostikum No.1. Lab. Klinik Prodia.
57. Yagi, K., 1998. Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. *Free Radical and Antioxidant Protocols*, 108, 101-106.
58. Yancey PH, (2005). Organic osmolytes as mompatible, metabolic and counter-acting cryoprotectant in high osmolarity and other stresses. *Journal of Experimental Biology*, 15, 2819-2830.
59. Zhu Z, Fan X, Lv Y, Zhang N, Fan C, Zhang P. (2015). Vitamin E Analogue Improves Rabbit Sperm Quality during the Process of Cryopreservation through Its Antioxidative Action. *PLoS ONE*, 10(12)