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Antioxidant effect of lycopene in chilled buck semen

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Extension and chilling are important methods of semen preservation practiced widely to improve livestock productivity. Lycopene is a powerful *in vitro* antioxidant, among naturally occurring carotenoids. This study aimed to evaluate the effects of lycopene supplementation in egg yolk extender on semen quality of chilled buck semen. This research involved the use of 5 sexually matured bucks. Egg yolk citrate extender was prepared. Freshly collected semen samples were macro and microscopically examined, good quality samples were pooled and divided into five aliquot, each sample was extended by dispensing 0.1 mL semen into 0.5 mL of egg yolk, the extender was supplemented with (0.0µM (control), 0.5µM, 1.0µM, 1.5µM and 2.0µM) lycopene. The samples were chilled in a refrigerator at 4-5°C. The samples were evaluated for mass motility, livability and sperm abnormalities at 24h, 48h and 72h. The fresh and chilled semen were subjected to antioxidant enzymes studies (Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase) and malandoaldehide (MDA) to determine the level of lipid peroxidation. Mean gross motility decreased across all groups from 0 hours to 72 hours. At 72 hours of chilling, 1.5 µM group had the highest mean gross motility of 45.00 ± 2.6%. At 48 hours of chilling, 1.5 had significantly higher values (70.00 ± 3.4%) compared to all groups. Glutathione peroxidase (GPx) was significantly lower in control group (216.75 ± 2.3) when compared to all other groups. Superoxide dismutase (SOD) and Catalase (CAT) were found to be significantly lower in control and 0.5 µM Lycopene, no significant difference was observed in the remaining groups. The MDA was found to be significantly lower in 1.5 and 2.0 µM lycopene (0.08 ± 0.39 and 0.09 ± 0.33 respectively). Therefore, lycopene can be used as an antioxidant supplement to reduce oxidative stress (OS) and its complications in extended chilled semen.

Keywords: Antioxidant, Lycopene, Buck, Semen, Nigeria.

INTRODUCTION

Extension and chilling are important methods of semen preservation practiced widely to improve livestock productivity (Hussain et al., 2018). Applying this technique using chilled and cryo-preserved semen has become a procedure in sheep breeding (Fernández Abella et al., 2014). The choice of semen extender is an important aspect of semen processing. The effect of extender on semen is to protect and maintain the sperm cells during processing and storage. Thus, an efficient diluent should serve as energy source, maintain osmolality level, pH and concentration of the spermatozoa, in order to protect the sperm cells from cold shock (Hussain et al., 2018; Rodriguez et al., 2017).

Animal - based extenders like skimmed milk and egg yolk- citrate has been used for freezing ram semen (Hameed et al., 2024). However, during storage, there were irreversible damages to some semen qualities over time due to lipid perioxidation or excessive production of reactive oxygen species ROS (Silvestre et al., 2021). There is an increase global concern about microbiological contamination of sperm cells, excessive ROS production and lipid peroxidation, following the usage of egg yolk as constituent of semen extender (Rao et al., 2014). (Solihati et al., 2018) reported that some constituent of egg yolk inhibits sperm cell metabolism and respiration which lead to decrease in sperm motility.

Lycopene on the other hand is a powerful in vitro antioxidant, among naturally occurring carotenoids,

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Lycopene has shown the strongest ability to scavenge free radicals (Bin-Jumah et al., 2022) and chemically quench singlet oxygen (Przybylska & Tokarczyk, 2022) being 10-fold more effective at quenching singlet oxygen than α-tocopherol (Wan et al., 2022). Supplementation of a reasonable level of antioxidants in the media might be effective to obtain optimum ROS neutralization and maintenance of equilibrium during in vitro oocyte maturation and embryo development.

Animal-based extenders such as Egg yolk has been reported to cause irreversible loss of semen quality due to excessive production of ROS that leads to lipid peroxidation (Benko et al., 2022). There is paucity of information on goat semen extension and preservation using extenders supplemented with antioxidant and their effects on semen characteristics. The study will proffer solution to the problem of irreversible loss of semen quality due to semen contamination, excessive production of ROS in animal-based extenders. This study will also provide information on the effect of lycopene in semen extender on some semen quality parameters. The aim of this study was to evaluate the effects of lycopene supplementation in egg yolk extender on some quality parameters of chilled buck semen.

MATERIALS AND METHODS

Study Area

The study was conducted at the Andrology laboratory, department of Theriogenology and Animal Production, Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria. The state is located between latitude 4^0 and 6^0 40'N and longitudes 11^0 30' and 15^0 50'E of the extreme north-western part of Nigeria (SOGD, 2007). The state falls within the Sudan Savannah ecological zone, it is characterized by two extreme temperatures, experienced in the months of March/April (45^0day time temperature) and lowest temperature during harmattan between November and February (about 28.3^0C day time temperature). The annual average temperature is 28.3^0C, while the climate of the state is basically of two seasons; the dry and wet seasons (SMANR, 2007). The rainy season is from June to October, the mean annual rainfall is 750 mm and potential evaporation rate has been reported to be 102cm. Major livestock raised in the state include camel, cattle, sheep, goat and poultry (Mamman *et al.,* 2000) .

Study Animals

This research involved the use of 5 (n = 5) sexually matured bucks aged between (1 – 2 years) weighing 25 – 30 kg. The animals were purchased from livestock Market and housed at the animal house of the Veterinary Teaching Hospital Usmanu Danfodiyo University, Sokoto. The bucks were allowed to acclimatize for two weeks before the commencement of the study, during which blood and fecal samples were collected and sent to the appropriate laboratories for screening of haemo and gastrointestinal parasites. The animals were fed with a mixture of wheat bran and bean offal together with groundnut hay, and clean water was provided *ad libitum.*

Preparation of Extender

Exactly, 2.9% w/v of sodium citrate, $NaC_6 H_5O_7 . {}_2H_2O$ was prepared and 2.9 gms of the salt was dissolved in 100mls of double distilled H_2O, it was then mix thoroughly and store at 5^oC. The egg was washed in warm water with mild detergent and rinsed in 70% ethyl alcohol. The egg was broken midway and transfer the yolk back and forth from each other between the two shells until all of the white is separated from the yolk. The yolk was placed on a wide white filter paper and roll yolk to remove traces of albumin from the yolk. The yolk membrane was punctured with a sterile glass rod to permit the yolk to run through the separator unto the graduated cylinder containing the extender. The cylinder containing both egg yolk and extender was shaken several times for thorough mixing (Jibril *et al*., 2011)

Preparation of Lycopene

For preparing the stock solution of lycopene, 1 mg of lycopene (molar mass: 536.873; Cat No. L9879, Sigma) was dissolved in 5 mL of DMSO (Cat No. D2650, Sigma), thereafter, 1 mL of distilled water was added. Aliquots of 20 µL of lycopene stock was made and stored at -20^0C. For preparing the lycopene working solution on the day of experiment, 10 µL of lycopene stock solution was added into 1990 µL of distilled water to obtain 0.2µM/1µL (Sidi *et al* 2022). After that, the lycopene working solution (0.0µM lycopene (control), 0.5µM, 1.0µM, 1.5µM and 2.0µM) was added into egg yolk extender.

Semen Collection

A total of fifteen (15) Semen samples were collected from five (5) experimental bucks once weekly for three (3) weeks, using a portable electro-ejaculator (EE) for bucks at the rate of 12 volts DC at 5 to 10 seconds interval. The bucks were properly restrained. The hair around the preputial area was clipped, cleaned with chlorhexidine using guaze. The probe of the electro-ejaculator was lubricated with KY-Jelly, .it was then inserted gradually into the rectum and controlled by one person; another person was holding the tube around the preputial area to collect the semen. The electro-ejaculator was switched on to cause an erection and subsequent ejaculation. The semen was collected in a calibrated tube and transported to the andrology laboratory in water bath set at a temperature of 37°C. The ejaculate was collected in a pre-warmed calibrated test tube. The samples were stored in a water-bath and maintained at 37°C (Jibril *et al*., 2011).

Experimental design

Freshly collected semen samples were macro and microscopically examined, good quality samples were pooled and divided into five (5) aliquot, each aliquot sample was extended by dispensing 0.1 mL semen into 0.5 mL of egg yolk, the extender was supplemented with (0.0μM (control), 0.5μM, 1.0μM, 1.5μM and 2.0μM) Lycopene. The samples were chilled in a refrigerator at 4-5°C. The samples were evaluated for mass motility, livability and sperm abnormalities at 24h, 48h and 72h. The fresh and chilled semen were subjected to antioxidant enzymes studies (Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase) and malandoaldehide (MDA) to determine the level of lipid peroxidation.

Materials for Antioxidative Assay

a.MDA Assay kit (colorimetric/ fluorometric) ab118970
b.GPx activity (U/mg) protein was evaluated using Randox RANSEL commercial kit (Randox Laboratories)
c.SOD activity (U/mg) protein was assessed using Randox RANSOD commercial kit (Randox Laboratories)

Semen Evaluation

The gross semen characteristics that were examined include volume, colour and motility as described by (Maina VA, 2006).

Volume and colour

The semen was collected and read from the graduated collecting tubes immediately. Semen colour was observed immediately after collection and recorded as Watery (1), Milky (2), or Yellowish/ Creamy (3).

Gross motility

Gross motility was assessed by observing the sperm cells' movement pattern. A drop of undiluted semen was placed on a warmed glass slide, covered with cover slip, and examined under a microscope at 10x magnification to determine motility, which was then quantified as a percentage.

Sperm concentration

This was determined using the haemocytometer. Semen was drawn into the pipette for dilution with red blood cells until it reached the 0.1 mark, and then the volume was adjusted to the 101 mark using a 10% formal saline solution and thoroughly mixed. A drop of this mixture was placed under a cover-slip on the haemocytometer after discarding a few drops, and the cells were allowed to settle before counting under x 40 magnification. Sperm cells were counted diagonally from the top left to the bottom right in 5 small squares of the improved Neubauer haemocytometer. The sperm concentration of each individual buck was then calculated following the method outlined by (Verstegen J, 2002).

Live spermatozoa

The viability of sperm was assessed using Eosin-Negrosin stain which was prepared as 1% solution in 0.9% NaCL. Esteso *et al.* (2006) outlined the method used. A small amount of the semen was spread thinly on glass slides and then stained with Eosin-Nigrosin. Dead sperm cells were distinguished by their absorption of the stain, while live cells remained unstained. A minimum of 200 sperm cells, taken from about 4 different microscopic views, were counted sequentially using a light microscope at 40x magnification.

Sperm abnormalities

The determination of abnormalities followed the protocol outlined by Esteso *et al*. (2006). Semen samples were thinly smeared onto clean, grease-free glass slides and then fixed using buffered formal saline. Each slide was meticulously examined under a light microscope at 100x magnification with oil immersion, ensuring a systematic inspection.

A total of 400 well-distributed sperm cells were scrutinized in each preparation. Subsequently, the percentage of abnormalities in the head, mid-piece, and tail of the spermatozoa were calculated with precision.

Data Analysis

Obtained data was analyzed using SPSS and presented in Mean ± Standard deviation. Analysis of Variances (ANOVA) and repeated measure anova was used to compare between the differences in mean group and variables with p-values less than 0.05 was considered statistically significant, result were presented as tables.

RESULTS

Pre-extension Evaluation

The volume of semen recorded in this study ranged from 0.30 to 1.1 mL. The gross sperm motility observed ranged from 55 to 95 %. The sperm concentration observed in this study ranged from 213 x 10^6/mL, while the least was 65.0 x10^6/mL. The values for colour ranged from 2.0 to 3.0. (Table 1)

Table 1: Pre-extension Evaluation of the Semen

Buck No.	Semen Vol.	Semen Colour	Sperm motility (%)	Sperm Conc. (X 10^6/mL)	Livability (%)
1	0.7	2	75	65	75
2	0.5	3	65	213	85
3	1.1	3	90	193	65
4	0.8	2	95	187	60
5	0.6	2	85	78	70

Table 2: Effect of Lycopene on semen motility (%) of Red Sokoto Bucks in egg-yolk citrate extender for 3 days

	Groups				
Hours	Control	0.5 μM LYC	1μM LYC	1.5μM LYC	2μM LYC
0	84.00 ± 6.7	84.00 ± 6.7	84.00 ± 6.7	84.00 ± 6.7	84.00 ± 6.7
24	54.00 ± 6.7^a	55.00 ± 3.5^a	57.00 ± 2.7^a	65.00 ± 3.8^b	63.00 ± 6.2^{bc}
48	45.00 ± 2.6^a	50.00 ±11.0^a	55.00 ± 3.6^b	63.00 ± 6.4^b	57.00 ± 4.7^b
72	25.00 ± 3.4^a	30.00 ± 2.7^a	35.00 ± 3.7^b	45.00 ± 2.6^c	35.00 ± 21.7^b

^{abc} Different superscript between rows indicate differences (P < 0.05). Data expressed as Means ± SEM

Table 3: Effect of Lycopene on semen live cells (%) of Red Sokoto bucks in egg-yolk citrate extender for 3 days

	Groups				
Hours	Control	0.5 μM LYC	1μM LYC	1.5μM LYC	2μM LYC
0	90.00 ± 6.3	90.00 ± 6.3	90.00 ± 6.3	90.00 ± 6.3	90.00 ± 6.3
24	74.00 ± 3.7^a	70.00 ± 3.5^a	73.00 ± 2.6^a	75.00 ± 6.4^a	73.00 ± 5.2^a
48	65.00 ± 6.6^a	67.00 ± 71.0^a	65.00 ± 3.6^a	70.00 ± 3.4^b	64.00 ± 6.7^a
72	45.00 ± 5.4^a	47.00 ± 2.9^a	50.00 ± 4.7^a	55.00 ± 6.5^b	53.00 ± 21.7^b

^{abc} Different superscript between rows indicate differences (P < 0.05). Data expressed as Means ± SEM

Post-extension Evaluation

Mean gross motility decreased across all groups from 0 hours to 72 hours (Table 2). At 72 hours of chilling, 1.5 μM group had the highest mean gross motility of 45.00 ± 2.6%. This value was significantly (P < 0.05) higher than values obtained from all other groups. The control and 0.5 μM groups had gross motility of 25 ± 3.5% and 30.00 ± 2.7% respectively (Table 2). This value was significantly (P < 0.05) lower than values obtained from other groups. At 48 hours of storage, control and 0.5 μM had significantly lower values compared to all other groups. Live sperm ratio decreased as chilling time also increased across all groups (Table 3). At 72 hours post storage, 1.5 and 2.0 μM Lycopene group had the highest live ratio (55 ± 6.5 and 53 ± 2.7% respectively), which was significantly (P < 0.05) higher than values obtained for all other groups. At 48 hours of chilling, 1.5 had

Table 4: Effect of Lycopene on semen morphology (%) of Red Sokoto bucks in egg-yolk citrate extender for 3 days

	Groups				
Hours	Control	0.5 µM LYC	1µM LYC	1.5µM LYC	2µM LYC
0	8.700 ± 5.3	8.700 ± 5.3	8.700 ± 5.3	8.700 ± 5.3	8.700 ± 5.3
24	18.23 ± 5.3^a	20.46 ± 3.6^a	15.51 ± 2.9^a	12.60 ± 6.4^b	14.73 ± 9.2^a
48	25.42 ± 3.2^a	27.34 ± 4.1.0^a	23.54 ± 2.8^a	18.40 ± 67.4^b	24.50 ± 6.3^a
72	33.36 ± 2.8^a	30.00 ± 3.20^a	27.67 ± 2.3^a	25.63 ± 7.3^a	31.27 ± 6.7^a

^{abc} Different superscript between rows indicate differences (P < 0.05). Data expressed as Means ± SEM

Table 5: Concentration of GPx, SOD, CAT and MDA in extended semen (72hs) supplemented with graded level of Lycopene

[redacted]

^{abc} Different superscript between rows indicate differences (P < 0.05). Data expressed as Means ± SEM

significantly higher values (70.00 ± 3.4%) compared to all groups. At 24 hours of storage, no significant difference was observed.

There were no significant differences in semen abnormalities across all groups except in 1.5 µM where significantly lower values were observed at 24 and 48 hours post storage (Table 4). The concentration of GPx was significantly lower in control group (216.75 ± 2.3) when compared to all other groups. SOD and CAT were found to be significantly lower in control and 0.5 µM Lycopene, no significant difference was observed in the remaining groups. The MDA was found to be significantly lower in 1.5 and 2.0 µM lycopene (0.08 ± 0.39 and 0.09 ± 0.33 respectively) Table 5.

DISCUSSION

Spermiogram of bucks was established to aid in the selection of bucks with good semen quality. The semen volume recorded in this study is similar to that reported in goats (0.78 ± 0.27 mL) by Marjuki, (2011). Semen colours observed were normal and varied among the bucks corroborating a previous study (Catunda *et al*., 2011).

The average semen motility recorded in this study is similar to that reported in red Sokoto bucks by (Belllo et al., 2022), with a range of 71-91%. Semen motility is influenced by nutrition as reported by Bello et al. (2020a), who obtained the highest semen motility of rams fed crude protein levels of 15% as compared to those fed 10 and 20%. The semen concentration in this study is different to what was reported in Nubian goats by Elsheikh & Elhammali (2015). This difference might be due to breed difference and climatic conditions of study locations. The values for semen concentration observed in this study were also different from 437.5 ± 65.24 x 106/mL reported by Itodo et al. (2020) in red Sokoto goats. This difference could be due to the period of semen collection, as season has been reported to affected semen quality (Elsheikh & Elhammali 2015).

The live sperm in this study is consistent to what was reported by Bello et al., (2022) in red Sokoto goats with values of 80.00 ± 3.50 – 95.00 ± 6.92%.

Beneficial effects of lycopene supplementation on semen preservation has been confirmed and parameters of spermatozoa and enzymatic activities also supported the protective action of lycopene as it reduces the concentration of MDA and improved the functions of GPX, SOD and CAT.

This result is in agreement with that of Bello et al., (2022), who reported that the optimal concentration of BHT required for semen preservation vary according to the animal species and range between 0.05-2.0 mM/mL BHT.

Differences in sperm motility between control group and treated groups in this study contradicted the work of Khumran et al. (2017) in bulls, where no significant difference was observed between the treated groups and controls for general motility. This might be due to species variations differences in the semen make-up of bucks as compared to bulls.

The decreasing sperm motility observed was attributed to depletion of nutrients Gibb & Aitken (2016), which inhibit fructolysis and respiration of sperm cells (Gündoğan et al., 2003)

In this study, beneficial effects were observed by addition of 1.5 μM and 2 μM lycopene in semen of red Sokoto bucks stored in citrate egg-yolk extender. This is in accordance with reports by Singh et l. (2017) in bulls, where beneficial effects were observed by addition of 1.0mM BHT which improved post thaw semen quality as evident by higher progressive motility. Bucks have the bulbo-urethral secretions which are detrimental to sperm survival. Supplementation with lycopene might have mitigated the detrimental effects of the secretions, hence the reason for the increased motility observed in supplemented groups compared to the control.

A growing body of investigations done on OS proposes that OS is one of the important factors leading to abnormal sperm parameters and subsequent infertility in men (Agarwal et al., 2012).

Appropriate amounts of ROS play an important role in normal sperm function, including hyperactivation, capacitation, acrosomal reaction, and finally oocyte fusion. An impaired balance between antioxidant capacity and ROS production results in the accumulation of oxidative products (Drevet et al., 2022)

Enzymatic antioxidants catalyze ROS to neutral products, and non-enzymatic antioxidants directly neutralize free radicals (Agarwal & Majzoub 2017). Three predominant antioxidant enzymatic components in semen include SOD, GPX, and CAT (Akbari et al., 2022)

Common ROS are superoxide anion (O-,), hydroxyl radical (OH), and the strong oxidizer hydrogen peroxide (H2O2) (Tripathy et al., 2020). In the presence of SOD, O-, is converted to H2O2. It is a powerful membrane-permeable oxidant that induces oxidative damage to lipids, proteins, and DNA in the cell and has to be rapidly eliminated from the cell (Ros & Madkour, 2019). Spermatozoa are highly susceptible to the cytotoxic effects of H2O2, and the elimination of H2O2 is either affected by CAT or GPX (Nenkova et al., 2017). GPX, with significant concentrations in both the mitochondrial matrix and nucleus, maintains mitochondrial function and protects DNA by reducing H2O2. CAT also counteracts OS by converting H2O2 to O2 and H2O (Mar et al., 2022).

Our results showed that with decreasing antioxidant capacity and increasing ROS, SOD activity increased in the treatments groups to balance between ROS and H2O2 levels. In addition, the level of MDA was highest, and sperm motility was lowest in the control group, which confirms the destructive effect of excessive ROS in increasing membrane LPO and consequent DNA damage.

Interestingly, this result revealed that supplementation of lycopene in the treatment groups, especially at the dose of 1.5 and 2 μM, reduced the ROS levels by increasing CAT enzyme activity. Thus, reduced DNA damage and LPO and finally an increased in sperm motility and livability observed in the treatment groups compared to control group.

Several studies have been performed on the mechanism of action of lycopene to reduce the risk of chronic diseases caused by OS such as cancer (Puah et al., 2021), osteoporosis (Rao et al., 2014), hyperten-sion (Agarwal & Majzoub, 2017), neuro-degeneration (Saini et al., 2020), and cardiovascular disease (Petyaev et al., 2018). These mechanisms include oxidative and non-oxidative mechanisms (Durairajanayagam et al., 2014). Lycopene has 11 conjugated double bonds (Joshi et al., 2020); hence, it contains many electrons that can be donated to free radicals such as O-, H2O2, NO, OH•, and thus neutralize them (Kwatra, 2020). As an antioxidant, lycopene reduces the burden of ROS and thus OS, thereby preventing oxidative damage to lipids, proteins, and DNA (Kwatra, 2020). Furthermore, lycopene is lipophilic and tends to accumulate in cell membranes and lipoproteins, thus, directly neutralizing ROS by acting as a singlet oxygen quencher, hence causing the overall amount of ROS to decrease (Riaz et al., 2021). Additionally, lycopene may have other beneficial effects via non-oxidative mechanisms in the testis, such as gap junction communication, modulation of gene expression, regulation of the cell cycle, and immune-enhancement (Oluwaseun et al., 2021).

In 2020, a study has been conducted on the effect of lycopene at a dose of 1 mg/kg intra-peritoneally on varicocele in rats (Antonuccio et al., 2020). They reported that treatment with lycopene significantly increased weight of testes and decreased MDA in rats with varicocele.

Various studies investigated the effect of lycopene on fertility in men and animals and showed promising results. Our results clearly showed that lipid peroxidation, the level of catalase, and SOD improved in groups treated with lycopene especially at the dose of 1.5 and 2 μM. Tripathy et al. (2020) investigated the direct role of lycopene on cyproterone acetate (CPA)-induced infertility in rat. They showed antioxidant enzyme activities such as catalase, peroxidase, SOD, and GST were recovered after direct exposure of lycopene to the CPA-treated (CPA + lycopene-treated) infertile animals may be due to the potent antioxidant activity of lycopene by rapid destruction of free radical (Tripathy et al., 2020).

This study illustrated that addition of lycopene in egg yolk extender decreased the ROS level and lipid peroxidation and protected sperm from DNA damage. A study about antioxidant effects of lycopene on bovine sperm and oxidative profile following cryopreservation showed that lycopene exhibited significant reactive oxygen species-trapping and antioxidant properties which may prevent oxidative damage to frozen-thawed sperm, and, thus, decreased lipid peroxidation and oxidative DNA damage (Babaei et al., 2021).

Similar to our results, Chib et al. (2020) illustrated that oral lycopene therapy seems to have a role in the management of idiopathic male infertility and maximum improvement seems to occur in the sperm concentration and motility. Lycopene was also reported to have improved embryo development and quality when supplemented in oocytes maturation media (Sidi et al., 2022).

Other studies also explained that lycopene reduces sperm DNA fragmentation, LPO of the plasma membrane, and improves concentration, motility, viability, and morphology in sperm of infertile man (Mohanty et al., 2020) and animals (Bucak et al. 2015; Mangiagalli et al., 2012).

CONCLUSION

According to previous studies and the present study on the effects of lycopene on infertility, lycopene can be used as an antioxidant supplement to reduce OS and its complications in extended chilled semen.

Lycopene has the potentials to improve semen motility, livability, and reduced oxidative stress.

RECOMMENDATION

Based on the findings of this research, we recommend that lycopene should be supplemented in egg yolk citrate extender for better extension and chilling.

Lycopene potentials should be investigated in other semen extenders.

Lycopene should also be supplemented in freezing and vitrification medium for semen storage in order to discover its full potentials in semen extension and cryo-preservation.

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