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Quality of Chilled Ram Semen in Tris Egg Yolk Extender Added with Different Concentrations of Glutamine

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Abstract

Glutamine is a non-essential amino acid that can reduce free radicals and act as an antioxidant. This study aims to determine the effect of adding glutamine in egg yolk tris diluent at concentrations of 0 mM (K), 2,5 mM (G2,5), 5 mM (G5), and 7,5 mM (G7,5) on the quality of liquid semen and kept in a refrigerator at 4 °C. Microscopic evaluation was performed immediately after dilution (H0) and every 24 hours until day 5 (H5). The results showed that the G 7,5 mM group could maintain better motility and viability ($P < 0,05$) than the K, G 2,5, and G 5 groups. The addition of glutamine did not have a significant effect ($P > 0.05$) in maintaining the MPU of spermatozoa. Glutamine with a concentration of 7.5 mM is the best concentration to maintain the quality of sheep's liquid semen.

Keywords: antioxidant, egg yolk, glutamine, ram, semen quality

Introduction

Artificial insemination (AI) technology in sheep to increase the population has been applied in Indonesia using liquid semen or frozen semen. The success rate of artificial insemination in sheep is still very low due to the complex anatomical structure of the cervix so that deposition only reaches the cervical mouth (Kumar & Naqvi, 2014). Another factor that also affects the success of AI is the quality of the spermatozoa used. During the processing and preservation of spermatozoa, there is often a decrease in the quality or motility of spermatozoa, mainly due to changes in the environment from anaerobic to aerobic.

Spermatozoa are exposed to an anaerobic environment in a natural mating, thereby reducing the damage that may occur due to reactive oxygen species (ROS) because of the oxidation process. In addition, the oviduct fluid contains high amounts of taurine (Miller & Shultz, 1987), which regulation in overcoming the accumulation of ROS when spermatozoa

are exposed to an aerobic environment (Alvarez & Storey, 1983; Holmes et al., 1992). During the preservation process, spermatozoa will be exposed to aerobic conditions and direct light. This condition can cause excessive ROS formation because spermatozoa cell membranes contain high concentrations of unsaturated fatty acids, so they are susceptible to lipid peroxidation by O_2 to H_2O_2 (Alvarez & Storey, 1989; Storey, 1997; Dawra et al., 2015). The small cytoplasmic volume caused the endogenous antioxidant content to below so it was not sufficient to protect spermatozoa from ROS that might be formed. Excessive reactive oxygen will cause damage to the genomic integrity of spermatozoa and cause a decrease in sperm motility (Storey, 1997; Aitken et al., 1998; Bilodeau et al., 2001; Kasimanickam et al., 2006).

Free radicals are defined as free molecules that contain unpaired electrons in atomic orbitals. Free radicals are unstable and highly reactive (Cheeseman & Slater, 1993). Cells use oxygen to produce energy, free radicals are created because of the production of ATP (adenosine triphosphate) by the mitochondria. The product is generally Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) generated from cellular redox processes. ROS and RNS at high concentrations will produce oxidative stress (Droge, 2002). Oxidative stress arises because of an imbalance between free radical production and antioxidant defense which is associated with various types of molecular damage (McCord, 2000). Oxidative stress is one of the important factors that contribute to the quality of semen (Bucak et al., 2010). Cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress (Agarwal et al., 2008). Semen contains various unsaturated fatty acids which are easily oxidized to produce reactive oxygen species (ROS) (Sinha et al., 1996). Mammalian spermatozoa showed to generate ROS when incubated under aerobic conditions. Highly the ROS caused oxidation and caused the structural and functional changes that result in cell damage (Lamirande & Gagnon, 1995; Guerin et al., 2001; Agarwal et al., 2005). Lipid peroxidation occurs in fatty acids located on cell membranes and then proceeds with radical chain reactions (Lovell et al., 1995). The continuous formation of free radicals causes the endogenous antioxidant defense system to be unable to detoxify the changes caused by free radicals (Sikka, 2004). Conditions like this require a material that can eliminate and neutralize free radicals such as exogenous antioxidants that come from outside the body (Halliwell & Guttridge, 2006). Antioxidants are stable molecules that donate electrons to free radicals and neutralize them, thereby reducing their capacity to suffer damage. Antioxidants delay or inhibit cell damage (Halliwell, 1995). Antioxidants have low molecular weights that can interact with free radicals safely and stop chain reactions before important molecules are damaged (Shi et al., 1999). One of the compounds that have an important role in cellular metabolic processes (such as oxidative energy, and lipogenic precursors) is glutamine (Curi et al., 2005).

Glutamine is a non-essential amino acid that acts as an energy source to accelerate cell regeneration (Erbil et al., 2005). Another function of glutamine can reduce damage caused by oxidative stress because glutamine is also an antioxidant and anti-inflammatory (Tihan et al., 2011). Glutamine has metabolic effects including protein synthesis, regulation of acid-base balance, anabolic, gluconeogenesis, nucleic acid synthesis, glutathione (GSH) synthesis, and regulation of immune cells (Erbil et al., 2005). The amino acid glutamine also plays a role in regulation during cellular metabolic processes, maintains the structure of cell membranes, and participates in the oxidation-reduction activity of protein structures, and gene expression, (Curi et al., 2007). Cruzat & Terapegui (2009) reported that glutamine is involved in the maintenance and synthesis of glutathione (GSH) which functions to maintain a balance between antioxidant and oxidant capacity, maintain cellular integrity, and prevent tissue damage. Administration of glutamine at the extracellular level can increase the motility of spermatozoa in horses (Khlifaoui et al., 2005), humans (Renald et al., 1996), frozen semen of sheep (Bucak et al., 2009), cattle (Dawra et al., 2015), roaster (Khiabani

et al. 2017). The addition of glutamine in sheep liquid semen has not been reported, so this study was conducted to determine the quality of liquid semen from sheep with the addition of glutamine at various concentrations.

Meterials and Methods

Animals and Semen Collection

Semen sample from two mature sheep, taken from the Reproductive Rehabilitation Unit (URR) SKHB IPB. The sheep used as experimental animals were mature sheep with a bodyweight of about 30 kg. The feed is given in the form of grass and concentrated twice a day. The semen collection was performed using an artificial vagina. Immediately after collection, the ejaculates are assessed in the laboratory and then prepared to preserve.

Preparation of the Extenders and Sperm Dilution

The research used a tris egg yolk extender, which consisted of Tris hydroxymethyl aminomethane 0,3028 g, d-fructose 0,125 g, and citric acid monohydrate 0,17 g in 10 mL milli-Q water. The antibiotics penicillin (100,000 IU) and streptomycin (100 mg) were added to the mixture. The egg yolk was added to the tris buffer as much as 20%. The semen samples were diluent without glutamine as a control (K), added 2,5 mM glutamine (G2,5), 5 mM glutamine (G5), and 7,5 mM glutamine (G7,5). The liquid semen is then stored in a refrigerator at a temperature of 3-5 °C using the water jacket method. Microscopic evaluation was carried out as in fresh semen immediately after dilution (H0) and every 24 hours until day 5 (H5). Repetition in the study was carried out five times.

Assessment Sperm Quality

1. Sperm Motility

Sperm motility (%) was assessed subjectively using a microscope with an objective lens magnification of 40x were observed in 10 fields of view. The percentage of sperm motility is a percentage of sperm that are progressive. The number of immobilized spermatozoa was calculated first, then after all the spermatozoa did not move, the total sperm was calculated.

2. Sperm Viability

Semen sample mixed with eosin 2%, then smeared on object-glass. The percentage of live spermatozoa sperm viability (%) was assessed using a microscope set at magnification 400x. The viable spermatozoa with intact plasma membranes are not stained by the eosin dye. On the other hand, the eosin can penetrate the membrane-damaged spermatozoa showed dark pink or red sperm heads. This value is calculated from the change in the color of the spermatozoa (Fig. 1C) head at least 200 spermatozoa stained with eosin-nigrosine stain (Felipe et al., 2008).

3. Sperm Membrane Integrity

Sperm membrane integrity (%) was determined by calculation of the percentage of spermatozoa having intact plasma membrane of Sperm or plasma membrane integrity (%) was assessed using a hypo-osmotic swelling test (HOST) by Jeyendran *et al.* (1984). Hypo-osmotic solution composition comprising: 1,35 g of fructose and 0,73 g of sodium citrate were dissolved with water up to a volume of 100 ml. A total of 200 µL of the hypo-osmotic solution was added to 20 µL semen, mixed until homogeneous, then incubated at 37°C for 45 minutes. Semen samples were smeared on a glass object and evaluated with 400X magnification. One hundred spermatozoa were assessed and the percentage of spermatozoa with curled tail (swelling) were calculated (Fig. 1B).

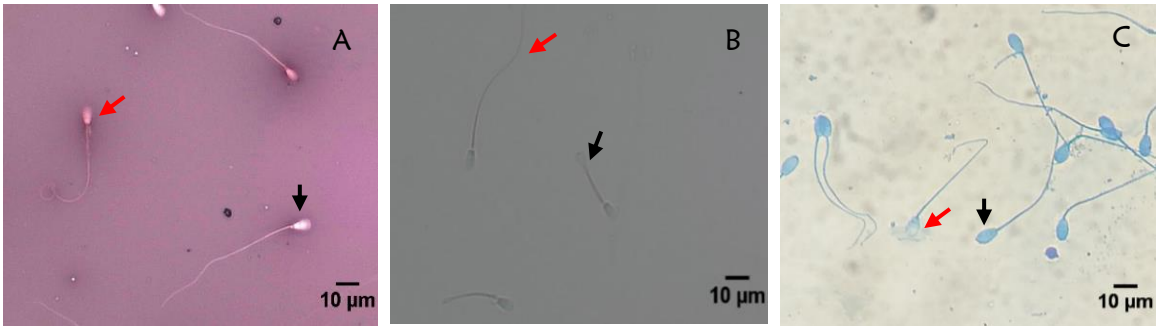

Fig. 1 Microscopic evaluation of sperm. A) Viability, black arrow: viable spermatozoa, red arrow: dead spermatozoa B) Membrane integrity, black arrow: intact plasma membrane, red arrow: incomplete plasma membrane C) Acrosome integrity, black arrow: acrosome cap intact, red arrow: acrosome is not intact.

4. Acrosome integrity

One mL of Tyrode's albumin lactate pyruvate (TALP) medium was put into a centrifuge tube and mixed with 10 L of liquid sheep semen. The mixture was centrifuged at 1400 rpm for 8 minutes. After completion of centrifugation, the supernatant was discarded, and 4% paraformaldehyde was added. The mixture was allowed to stand for 5 minutes, then the supernatant was discarded. Ammonium acetate pH 9 with the addition of 3 M NaOH was prepared, then check the pH using pH indicator paper. Added one mL of ammonium acetate pH 9 and centrifuged again, then the supernatant was discarded. The precipitate from the centrifuge was taken and dripped onto an object glass and mixed with one drop of Coomassie blue dye. Observations were made under a light microscope with an objective lens magnification of 400x viewed in 10 fields of view or a sperm cell count of at least 200 (Galantino-Homer et al. 2004 modified from Larson and Miller 1999). The damaged acrosome is indicated by an incomplete and colorless acrosome head (Fig. 1C).

Statistical analysis

The data are presented in the form of an average with a standard deviation (Mean±SD). The data were analyzed using Analysis of Variance (ANOVA) by SPSS 17 software program. Data were analyzed using a completely randomized design and if there was a significant difference and continued with Duncan's test (95%).

Results and Discussion

The results showed that the quality of spermatozoa generally decreased with increasing storage time. A decrease in the quality of spermatozoa during storage can occur due to metabolic processes that cause the energy of spermatozoa to be decreased and the semen diluents can induce damage to the plasma membrane (Nuranti, 2005). Spermatozoa motility was decreased during preservation time. Alvarez et al. (2012) reported that sheep spermatozoa were more appropriate to use tris fructose diluent with 20% egg yolk concentration. This is in accordance with the results of the study which showed that up to day 4 the percentage of spermatozoa motility at 4 °C storage temperature was still in the range of 50%-70% for all treatments.

The sperm motility decrease occurred starting on day 1, in the K group and the G2,5 group, there was no significant difference, but significantly lower from the G5 and G7,5 groups ($P<0,05$). On day 3, the G2,5 group showed significantly different results from the K group ($P<0,05$) and continued to decrease until day 5 of preservation. On day 5, the G7,5 group could maintain motility above 60%, while the G2,5 and G5 groups had 40% motility.

Spermatozoa motility is important to determine the ability of spermatozoa to fertilize (Bearden et al., 2004). The lecithin contained in egg yolk acts as a protective agent that can protect spermatozoa from cold shock at cold temperatures to maintain sperm motility (Souhoka et al., 2009).

Table 1. Progressive motility (%) of sheep sperm at extender with various concentrations of glutamine

Groups	Time					
	HO(%)	H1(%)	H2(%)	H3(%)	H4(%)	H5(%)
K	83±2.7A	76±2.2a,B	69±2.2a,C	62±2.7a,D	50±3.5a,E	36±4.2a,F
G2,5	83±2.7A	78±2.7a,B	72±2.7a,C	67±2.7b,D	58±4.5b,E	40±5.0ab,F
G5	83±2.7A	82±2.7b,A	76±2.2b,B	72±2.7c,B	67±4.5c,C	46±5.5b,D
G7,5	83±2.7A	82±2.7b,A	77±2.7b,B	74±2.2c,BC	70±3.5c,C	61±4.2c,D

^{A,B,C,D,E,F} Different letters in the same row and ^{a,b,c} different letters in the same column show a significant difference (P<0,05). Liquid semen without glutamine (K), added glutamine 2,5 mM (G2,5), added glutamine 5 mM (G5), added glutamine 7,5 mM (G7,5). Time preservation of liquid semen on day 0 (H0), day 1 (H1), day 2 (H2), day 3 (H3), day 4 (H4), day 5 (H5)

Spermatozoa viability of sheep at egg yolk tris extender with various concentrations of glutamine for five days of preservation at 4 °C in Table 2. The viability of spermatozoa decreased significantly on day 2 to day 5 in groups K, G2,5, and G5 (P<0,05). The sperm viability in the G7,5 group showed decreased significantly (P<0,05) on day 3 and remained until day 4 (P>0,05). The G7,5 group showed significantly different (P<0,05) from the G2,5 group on day 3 to day 5, but had the same with the G5 group (P>0,05). Five days after preservation, the spermatozoa viability of group G5 was not statistically different from that of groups K and G2,5 (P>0,05). The data obtained in this study indicated that the addition of glutamine was able to maintain viability (above 70-80%) until the last day of preservation. The glutamine concentration of 7,5 mM was better than that of another group.

Table 2. Sperm viability (%) of sheep at extender with various concentrations of glutamine

Groups	Time					
	H0(%)	H1(%)	H2(%)	H3(%)	H4(%)	H5(%)
K	95.8±1.4a,A	93.4±3.1a,A	89.5±3.5a,B	84.8±1.2a,C	79.2±3.7a,D	73.4±2.3a,E
G2,5	96.9±1.3ab,A	95.8±2.1ab,A	93.3±2.1b,B	88.8±1.4b,C	84.4±1.8b,D	78.6±2.3b,E
G5.0	98.1±0.7bc,A	96.8±1.5b,AB	94.2±2.3b,BC	91.5±1.2c,C	88.0±2.6c,D	82.9±4.0bc,E
G7,5	98.6±0.4c,A	97.1±1.5b,A	95.4±1.8b,AB	92.9±1.6c,BC	90.0±1.9c,C	84.8±4.5c,D

^{A,B,C,D,E,F} Different letters in the same row and ^{a,b,c} different letters in the same column show a significant difference (P<0,05). Liquid semen without glutamine (K), added glutamine 2,5 mM (G2,5), added glutamine 5 mM (G5), added glutamine 7,5 mM (G7,5). Time preservation of liquid semen on day 0 (H0), day 1 (H1), day 2 (H2), day 3 (H3), day 4 (H4), day 5 (H5)

The viability of spermatozoa can be maintained by egg yolk which contains phospholipids and lipoproteins (Kulaksiz et al., 2010). The addition of tris (hydroxymethyl aminomethane) was also able to maintain the viability of spermatozoa. Because tris is a buffer capable of supporting the stability of the pH (Hafez & Hafez, 2000). The viability of spermatozoa in

this study is higher than motility. This is in accordance with the statement of Campbell et al., (2003) that viable spermatozoa are not necessarily able to move forward progressively.

The percentage of sperm membrane integrity until day 5 of preservation was still above 90% (Table 3). These data indicated that preservation of spermatozoa at 4 °C until day 4 did not cause membrane damage. A slight significant decrease (P<0,05) showed on day 5. The addition of glutamine in this study did not have a significant effect (P>0,05) in maintaining the sperm membrane integrity of spermatozoa.

Table 3. Sperm membrane integrity (%) of sheep at extender with various concentrations of glutamine

Groups	Time					
	HO(%)	H1(%)	H2(%)	H3(%)	H4(%)	H5(%)
K	98.4±0.6A	98.2±0.7A	97.7±1.2AB	97,5±1.1AB	97.2±1.1AB	96.6±1.4B
G2,5	98.8±0.6A	98.5±0.6AB	98.3±0.7AB	98.1±0.7AB	97.7±1.1AB	97.4±1.2B
G5	98.9±0.6A	98.8±0.6AB	98.5±0.7AB	98.3±0.8AB	97.9±0.9AB	97.8±1.0B
G7,5	99.1±0.4A	98.9±0.5AB	98.6±0.7AB	98.5±0.7AB	98.2±0.8AB	98.0±0.9B

A,B Different letters in the same row show a significant difference (P<0,05). Liquid semen without glutamine (K), added glutamine 2,5 mM (G2,5), added glutamine 5 mM (G5), added glutamine 7,5 mM (G7,5). Time preservation of liquid semen on day 0 (H0), day 1 (H1), day 2 (H2), day 3 (H3), day 4 (H4), day 5 (H5)

The percentage of acrosome integrity of the spermatozoa showed > 90% until day 5 of preservation in all groups. The acrosome integrity of the spermatozoa showed K group decreased significantly (P<0,05) at day 5 (Table 4). These results indicated that the addition of glutamine was significantly able to maintain the integrity of the spermatozoa acrosome integrity. The research data obtained showed that the addition of glutamine had no significant effect (P>0,05) on day 4 and day 5.

Table 4. The acrosome integrity (%) of sheep at extender with various concentrations of glutamine

Group	Time					
	HO(%)	H1(%)	H2(%)	H3(%)	H4(%)	H5(%)
K	98.7±0.5a,A	98.4±0.6a,A	98.1±0.9a,AB	97.8±0.8a,AB	97.4±1.1AB	96.9±1.5B
G2,5	99.2±0.3b,A	99.0±0.3b,A	98.7±0.2ab,AB	98.3±0.6ab,ABC	97.8±1.2BC	97.6±1.3C
G5	99.4±0.3b,A	99.2±0.3b,AB	98.9±0.3b,ABC	98.6±0.6ab,BCD	98.4±0.7CD	98.1±0.8D
G7,5	99.4±0.2b,A	99.3±0.3b,A	99.1±0.3b,AB	98.8±0.5b,ABC	98.6±0.6BC	98.3±0.8C

A,B, C,D Different letters in the same row and a,b show a significant difference (P<0,05). Liquid semen without glutamine (K), added glutamine 2,5 mM (G2,5), added glutamine 5 mM (G5), added glutamine 7,5 mM (G7,5). Time preservation of liquid semen on day 0 (H0), day 1 (H1), day 2 (H2), day 3 (H3), day 4 (H4), day 5 (H5)

The plasma membrane regulates all the electrolytes needed so that it is related to the metabolism of spermatozoa (Herdis et al., 2003). Plasma membrane integrity is also related to successful fertilization because the plasma membrane functions in capacitation and penetrate the oocyte membrane (Ansari et al., 2010). Spermatozoa plasma membrane damage is usually accompanied by damage to the integrity acrosome. The damaged of acrosome integrity was characterized by the apical region of the spermatozoa head not being stained (Larson & Miller, 1999). The damage of acrosome caused fertilization process does not occur. According to Setiadi et al., (2006) stated that the factor that affects the integrity

of the plasma membrane of spermatozoa during storage at a temperature of 5 °C is the composition of the extender. Herdis and Darmawan (2012) stated that the membrane integrity was able to maintain the motility and viability of spermatozoa.

The decreased motility can be caused by extracellular oxidative stress and the formation of endogenous free radicals/ROS (Kasimanickam et al., 2006). Axonemal damages and depletion of the ATP by generated ROS are the major causes of motility reduction in spermatozoa preservation. Glutamine has the potential to elevate the catalase activity in frozen–thawed ram spermatozoa (Bucak et al., 2009), and lead to the elimination of the ROS in the frozen–thawed spermatozoa. Free radicals can damage the unsaturated fatty acids contained in the plasma membrane of spermatozoa so disrupt the integrity of the spermatozoa cell membrane (Storey, 1997). The integrity of the spermatozoa plasma membrane will affect the motility, viability, and integrity acrosome of the spermatozoa (Kasimanickam et al., 2006). The preservation process will cause the spermatozoa to be exposed to aerobic conditions. These conditions can accelerate the formation of ROS (Alvarez and Storey, 1989; Storey, 1997).

The results of this study showed that the glutamine concentration of 7.5 mM was able to maintain the quality of liquid semen spermatozoa compared to all groups. Glutamine is a non-essential amino acid capable of binding ROS that can reduce oxidative damage (Erbil et al., 2005; Tihan et al., 2011). Glutamine was regulated in the synthesis of glutathione (GSH) which functions to maintain a balance between antioxidant and oxidant capacity, maintain cellular integrity, and prevent tissue damage (Cruzat & Terapegui, 2009). The non-essential amino acid is charged molecules, it is possible that they interact electrostatically with the phosphate groups of the sperm plasma membrane phospholipids, thereby forming a layer on the sperm surface. This can protect spermatozoa from cold shock (Kundu et al., 2001).

Glutamine can act as an antioxidant so that it can maintain the integrity of the plasma membrane of spermatozoa (Tihan et al., 2011). A study showed that 50 mM glutamine was able to increase the motility of post-thaw horse spermatozoa (Khlifaoui et al., 2005). Tuncera et al., (2011) reported that the addition of 3 mM glutamine in the extender can increase motility, plasma membrane and acrosomes integrity in frozen bovine semen. The study showed that glutamine was also able to act synergistically with raffinose and sucrose in maintaining semen quality. Mercadoa et al., (2009) in their research showed that glutamine will improve the survival of spermatozoa in cryopreservation of porcine. Glutamine is an amino acid that can improve sperm survival which is associated with metabolic, cryoprotectant, oxidative, or osmoregulation properties (Martins-Bessa et al., 2007). Glutamine acts at the extracellular level, being able to increase post-thaw human spermatozoa motility and fertilization (Renard et al., 1996). The results of other studies also showed that 8 mM glutamine in egg yolk tris extender could maintain motility, viability, and acrosome integrity of post-thaw bovine spermatozoa (Dawra et al., 2015).

Although the mechanism of action of glutamine as a cryoprotectant is still unclear, many authors have reported that glutamine has an anti-oxidative capacity which may be related to its cryoprotective properties. However, it is believed that Glutamine is an antioxidant that functions to maintain the integrity of the membrane and acrosome after cryopreservation and maintain spermatozoa motility and viability (Dawra *et al.* 2015). Glutamine is an antioxidant that is naturally produced by the testes and acts as a cryoprotectant in

maintaining the integrity of the plasma membrane of post-thaw spermatozoa. The addition of glutamine will increase the antioxidant activity of spermatozoa which maintains viability and motility of spermatozoa (Koohestanidehagh *et al.* 2020).

Conclusion

The addition of glutamine in sheep semen extender which was preserved for 5 days at 4 °C was able to maintain the quality of spermatozoa. Glutamine concentration of 7,5 mM showed the best results compared to group control and added glutamine concentrations of 2,5 mM and 5 mM.

Conflict of Interest

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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