Jurnal Veteriner
pISSN: 1411-8327; eISSN: 2477-5665
Terakreditasi Nasional, Dirjen Penguatan Riset dan Pengembangan,
Kemenristek Dikti RI S.K. No. 36a/E/KPT/2016

September 2018 Vol. 19 No. 3: 390-396
DOI: 10.19087/jveteriner.2018.19.390
online pada http://ojs.unud.ac.id/index.php/jvet

Lactose-Astaxanthin Increased the Frozen Semen Quality of Gembrong Goat in Conservation Efforts

(LAKTOSA-ASTAXANTHIN MENINGKATKAN KUALITAS SEMEN KAMBING GEMBRON DALAM UPAYA KONSERVASI)

[1]Wayan Bebas, [2]Wayan Gorda, [1]I Gusti Ngurah Bagus Trilaksana, [1]Desak Nyoman Dewi Indira Laksmi, [1]Tjok Gede Oka Pemayun

[1]Department of Veterinary Reproduction,
[2]Department of Clinic, Faculty of Veterinary Medicine,
Udayana University
Jln Sudirman, Denpasar, Bali, Indonesia 80234
E-mail: w_bebas@unud.ac.id

ABSTRACT

This research was conducted to produce a formula of diluent for the manufacture of frozen semen of Gembrong goat. Yolk phosphate was used as a basic diluent with the addition of anti-cold shock, antioxidants and combination of anti-cold shock-antioxidant. The research design used a completely randomized design with three kinds of treatments; T1: the addition of lactose 0.6% (anti-cold shock), T2: the addition of astaxanthin 0.004% (antioxidant), and T3: a combination of 0.6% lactose-Astaxanthin 0.004% (combination of anti-cold shock and antioxidants). The addition of DMSO 6% was used in each treatment as intracellular cryo_protectants. The freezing process was done with the gradual freezing with conventional techniques. Examination of the quality of semen by thawing prior included progressive motility, viability, and abnormalities. The results showed that anticoldshock-antioxidant combination (0.6% lactose -Astaxanthin 0.004%) produced the best semen quality with progressive motility, viability, and less abnormalities as follow 44.00 $\pm$ 3.46%, 59.00 $\pm$ 1.85%, and 14.00 $\pm$ 0.76% respectively. It was concluded that the addition of a combination of 0.6% lactose-Astaxanthin 0.004% on the phosphate yolk diluent with 6% DMSO as intracellular produced best quality of frozen goat Gembrong semen that suitable for use in artificial insemination and in vitro fertilization

Keywords**:** lactose; astaxanthin; frozen semen quality; Gembrong goats

ABSTRAK

Penelitian ini dilakukan untuk menghasilkan formula pengencer pembuatan semen beku kambing Gembrong. Penelitian ini menggunakan pengencer dasar fosfat kuning telur dengan penambahan *anticold shock*, antioksidan dan kombinasi *anticold shock*-antioksidan. Rancangan penelitian yang digunakan adalah rancangan acak lengkap dengan tiga macam perlakuan masing- masing T1 : penambahan laktosa 0,6% (*anticold shock)*, T2 : penambahan astaxanthin 0,004% (antioksidan), dan T3 : kombinasi laktosa 0,6%-Astaxanthin 0,004% (kombinasi *anticoldshock*-antioksidan). Dilakukan penambahan DMSO 6% pada masing-masing perlakuan sebagai krioprotektan intraseluler. Proses pembekuan dilakukan dengan teknik pembekuan bertahap secara konvensional. Pemeriksaan kualitas semen dengan melakukan *thawing* terlebih dahulu meliputi motilitas progresif, daya hidup dan abnormalitas, Hasil penelitian menunjukkan, kombinasi *anticold shock*-antioksidan (laktosa 0,6%-Astaxanthin 0,004%) menghasilkan kualitas semen paling baik dengan motilitas progresif, daya hidup, dan abnormalitas masing-masing 44,00 $\pm$ 3,46%, 59,00 $\pm$ 1,85%, dan 14,00$\pm$ 0,76%. Dapat disimpulkan bahwa penambahan kombinasi laktosa 0,6%-Astaxanthin 0,004% pada pengencer fosfat kuning telur dengan DMSO 6% menghasilkan kualitas semen beku kambing Gembrong dengan kualitas baik dan layak digunakan untuk inseminasi buatan dan pembuahan secara *in vitro*.

Kata-kata kunci: laktosa; astaxanthin; kualitas semen beku; kambing gembrong

INTRODUCTION

According to the research conducted by Agricultural Technology Assessment Center of Bali by DNA test, Gembrong goats are from Karangasem and this strain is only one in the world. Gembrong goat is one of germplasm which in critical status to extinction. Therefore it need to be saved because loosing of livestock family is a big loss for the community, nation, and state, especially for our children.

Zein *et al.* (2012) conducted a study of Gembrong goats DNA by using microsatellite markers, the result showed a very low heterozygosis of Gembrong goats that can be used to describe the genetic diversity of livestock populations (Moioli *et al.,* 2004). Heterozygosity value can deduce the coefficient of inbreeding in a population. Inbreeding can result in decreased physical and physiological performance of cattle, causes a decrease in resistance of cattle to the environmental changes, and decreased reproductive activity to infertility.

The efforts that can be taken to protect the germ plasma is by conservation to promote the increment of population through the production of frozen semen technology with the application of Artificial Insemination (AI) which has been believed to be able to rescue germ plasma. Frozen semen can also be used as a genetic bank, besides that it can avoid mating between individuals who have a close kinship level.

In the process of semen freezing there is an extremely decreased temperature until -190^{o}C that can give negative effect to the spermatozoa. Low temperatures (below the freezing point) will occur physical and chemical changes in the cells, will form ice crystals and increase the concentration of intracellular electrolyte that causes cold shock. Cold shock will result in damage to the cell membrane of both the plasma membrane and membrane acrosome which greatly affect the motility, cell abnormalities and the cell eventually becomes dead. During the process of sperm freezing, it also occur peroxidation process that can result in free radicals. Free radicals are highly reactive and can cause lipid peroxidation of plasma membrane (Douard *et al*., 2004). Lipid oxidations in plasma membrane of the spermatozoa are toxic to the cells which also can cause damage to the DNA (Edyson, 2002; Sanocka *et al*., 2004).

To overcome this effect, it is necessary to add some substance into the diluent as cryo protectant when working in extracellular and intracellular (Budiono, 2006) and antioxidant. Lactose 0.6% as extracellular cryo protectant in phosphate egg yolk diluent can maintain spermatozoa's plasma membrane permeability of green jungle fowl stored at 5°C (Bebas and Laksmi, 2012).

Astaxanthin, a carotenoid group of antioxidant which is currently the most powerful antioxidant (capable was able to tackle free radicals by means of "scavengers" (Hussein *et al*., 2006). The addition of astaxanthin with concentration of 0.004% in the yolk phosphate diluents was able to maintain the spermatozoa's plasma membrane of green jungle fowl stored at 3-5^{o}C for 48 hours (Bebas and Laksmi, 2012).

This study was designed to determine the effect of lactose 0.6%, astaxanthin 0.004% and combination of lactose 0.6% and astaxanthin 0.004% during the semen freezing of Gembrong goats to increase the quality of spermatozoa post thawing.

RESEARCH METHODS

This study used a two years old male Gembrong goat. Semen collection was conducted using an artificial vagina. Semen put in the tube and placed in a water bath at 37°C to be evaluated both macroscopically (volume, pH, consistency/ viscosity and color) and microscopically (mass movement, individuals movement, abnormalities and the concentration of spermatozoa). The diluent base used in this study was a yolk phosphate with the addition of anti-cold shock, antioxidants and the combination of anti-cold shock-antioxidant. The study used a completely randomized design with three kinds of treatment; T1: the addition of 0.6% lactose as anti-cold shock, T2: the addition of 0.004% astaxanthin as an antioxidant and T3: a combination of 0.6% lactose and, 004% astaxanthin as a combination of anti-cold shock and antioxidants. The addition of 6% DMSO in every treatment was as intracellular cryo protectants. The freezing process included the dilution of semen with each treatment, filling and sealing in the 0.25 ml straw, equilibration at 4°C for 4 hours, aeration above liquid nitrogen vapor with a distance of 10 cm for 10 minutes, freezing with liquid nitrogen and stored in containers. The thawing of frozen semen prior to the tests, the evaluation semen qualities included progressive motility, viability and cell abnormalities.

Progressive Motility Examination

Drop 0.05 mL cement in above of warm (37°C) objects glass then covered, observed under a microscope with a magnification of 400 times. Count the spermatozoa that have progressive movement with percent, observations conducted on five fields of vision (Breininger *et al.,* 2004).

Viability Measurement

Viability examination of spermatozoa was done by eosin-negrosin staining methods. Eosin-negrosin prepared by mixing 6.7 g/L Eosin Y and 9 g/L Nigrosin in 9 g/L sodium chloride. Mix and homogenize 50 µl semen with 50 µl eosin-nigrosin, after 30 second, smears are made, air dried, and examined in an ordinary light microscope. Dead sperm will appear red, while the life sperm will not stained/transparent. Count in percent of the life spermatozoa.

The Abnormalities of Spermatozoa

The examination of spermatozoa abnormalities was done by the similar techniques of semen viability examination used eosin-negrosin techniques, observed 200 cells under a microscope with a magnification of 400 times, abnormalities were seen from the shape of the head, body and tail and counted in percent. Data were analyzed by using analysis of variance, if there is a significant result then continued by Duncan test.

RESULTS AND DISCUSSION

Results

The quality of fresh Gembrong goat semen is presented in Table 1.

The quality of frozen Gembrong goat semen is presented in Tab. 2.

The Quality of Fresh Semen

The fresh semen produced by male Gembrong goat had good qualities, this can be seen from macroscopic and microscopic sperm examination result (Tab. 1). The criteria of goat's semen that can be process to be frozen semen should have the following requirements i.e. motility >50%, viability ≥ 80%, abnormalities <15%, and concentration 2.10^9/mL (Tambing *et al.,* 2000).

Semen volume that collected in this study was 0.6 mL, the similar result to the research conducted by Pamungkas *et al.* (2014) (0.5 ±0.1 mL) and Rizal (2008) in crossbreed of Etawah goat with 0.68±0.18 mL. Our finding was lower than the research result by Husein *et al.* (2007); Kostaman and Sutama (2006) in Boer goats with following volume 0.83 ± 0.29 mL and 0.8 ± 0.2 mL. Normally, the goat semen volume ranged between 0.1-1.5 mL. The volume of semen is influenced by the breed, age, weight, season, food and frequency of cement collection.

Semen color of fresh Gembrong goat was a bit cream with a thick consistency and the concentration $4{,}452.10^6$ cells/mL. This study supported the result reported by Rizal *et al.* (2008) in crossbreed of Etawah goat which with color was look like a milky white with a thick consistency and the concentration ranged between $4{,}148 \pm 198.60.10^6$/mL. The color of goat semen ranging from milky white to cream, but on Boer goats were white to cream (Kostaman and Sutama, 2006).

The pH value of Gembrong goat was 6.6, these result was still within the pH range of Pamungkas *et al*. (2014) results that reported pH values of 6.4; Husein *et al.* (2007) reported 6.67 ± 0.76 in Nubian goat, 6.50 ± 0.50 in Nubian-Etawah crossbreed and 6.83 ± 0.29 in Boer goat. The normal pH of fresh goat semen ranged from 5.9 to 7.3.

Abnormalities of the spermatozoa found in fresh Gembrong goat semen was 4%, the result was still in the range of abnormalities reported by Pamungkas *et al.* (2014) (5.74 ± 1.59%), but these results were much smaller when compared with abnormalities in Kacang goat (8.6 ± 2.4%) (Bintara, 2011). According to Siregar (2005) if the abnormalities are more than 20%, it will decrease the fertility.

The Quality of Post Thawing Semen

Semen freezing process can lead a cold shock which can cause damage to the structure and function of the cell due to the extremely temperature drop, which affects the structural damage of the cell membrane (Ejarah, 2007). Cold shock resulted the phospholipid changes that make up the plasma membrane during the transition phase from the liquid phase to the gel phase (Ghetler *et al.,* 2005), and this occurs at temperatures below 20°C. The change of fatty acid and protein chains in plasma membrane caused leakage of the membrane and decreased its selectivity (Dziekoñska and Strze¿ek, 2011). Coldshock is the most important process in decreasing membrane permeability that can caused death of the cell (Dziekoñska and Strze¿ek, 2011). Cold shock caused the loosing

Table 1. Macroscopic and microscopic examination of fresh Gembrong goat semen

Examination	Parameter	Result
	Volume (mL)	0.6
	Color	Cream
Macroscopic	Ph	6.6
	Smell/odor	Distinctive
	Consistency	Thick
Microscopic	Mass motility	+++
	Individual motility (%)	89
	Concentration (10^6/mL)	4452
	Abnormalities (%)	4
	Viability (%)	97

Legends: +++: very good mass motility, ++: good mass motility, +: not good mass motility,-: bad mass motility

Table 2. The qualities of post thawing frozen Gembrong goat semen

Semen quality	Treatments		
	Lactose 0.6%	Astaxanthin 0.004%	Lactose 0.6%-Astaxanthin 0.004%
Motility	40.75 ± 1.49^a	41.25 ± 1.67^a	44.00 ± 3.46^b
Viability	52.88 ± 2.03^a	54.25 ± 1.91^a	59.00 ± 1.85^b
Abnormalities	16.00 ± 0.76^a	15.63 ± 0.74^a	14.00 ± 0.76^b

of glukosa-6-fosfatase dehydrogenase enzyme from cytoplasm, which generally leads to decrease intra cellular ATP concentrations. If the center of spermatozoa's plasma membrane is damaged, there will be leakage of enzymes that play a role in the process of metabolism such as aspartate aminotransferase (AspAT) and *ATP-ase-linkked sodium-potasium pump* (Na+/K+-ATPase) enzyme (Colenbrander *et al.,* 1992; Arifiantini and Purwantara, 2010), causing a dislocation of proteins plasma membrane such as the glucose transporter group) (Kokk *et al.,* 2005).

Lactose is a disaccharide class of carbohydrate consisting of two monosaccharide units, the glucose unit and the galactose unit that binded to the lipid (glycolipid) and/or to the protein (glycoprotein) called cell shell or glycocaly (Palaez and Long, 2005). Normally, lactose also able to substitute the water in polar hydrated group (Viswanath and Shannon, 2000). This means lactose helps to stabilize cell plasma membranes during transition through a critical temperature zone, as well as changing the mechanical properties of the diluent through increase viscosity. Lactose can also directly interact with the central polar of phospholipid during freezing process and decreased the van der Walls bond (Aisen *et al.,* 2002).

Lactose is a disaccharide class of carbohydrate that can be metabolized by spermatozoa through glycolysis or Krebs cycle to produce energy (ATP). Spermatozoa have the necessary equipment for the glycolytic metabolism, cycle of citric acid and oxidative phosphorylation in mitochondria (Dziekonska *et al.,* 2009).

Blesbois *et al.* (2005) indicated lipid peroxidation occurred during ejaculation. During the process of semen collection and storage, spermatozoa are exposed to oxygen from the atmosphere, which can cause lipid peroxidation in the plasma membrane and produce ROS (Reactive Oxygen Species) (Douard *et al.,* 2004). High ROS production also occurs in young spermatozoa, sperm that have abnormalities in the head and tail, indicated that ROS production is correlated to the spermatozoa abnormalities index (Azis *et al.,* 2004).

The impact of ROS on spermatozoa is causing damage to the cell membrane thus increasing membrane permeability to the enzymes and other substrates, finally decreased activity of cell metabolism (Storey, 1997). The

impact of spermatozoa cell membrane damage due to ROS affected the activity of aspartate aminotransferase enzyme (AspAT) or alanine aminotransferase (AlnAT) (Colebrander *et al.,* 1992). High levels of AspAT and AlnAT enzymes which is found in seminal plasma have a correlation with cell plasma membrane damage (Pesch *et al.,* 2006).

Astaxanthin is an antioxidant that is classified into a class of carotenoids called xantopi (Liu and Osawa, 2007). Astaxanthin has a unique molecular structure with oxygen as a hydroxyl groups (OH) and carbonyl groups or a combination of both. The presence of hydroxyl and carbonyl functional groups in ketocarotenoids, make astaxanthin a powerful antioxidant (Hussein *et al.,* 2006). Astaxanthin has the power of counteracting free radicals 65 times stronger than Vitamin C, 14 times stronger than Vitamin E and 54 times stronger than â-carotene.

Astaxanthin with its unique structure with two ring terminals work at the surface of the membrane while the ketocarotenoids chain work on the inside of the membrane. Thus astaxanthin can be effective in tackling ROS on the surface of membrane while its ketocarotenoid chains inhibit oxidative chain reactions inside the membrane. Based on ketone groups, hydroxyl groups, double bonds and open rings of astaxanthin are capable of being a powerful antioxidant that very well protects all cellular components from degenerative damage and ROS attacks.

Progressive motility examination of postthawing spermatozoa with addition of 0.6% lactose, 0.04% astaxanthin and a combination of lactose 0.6% -astaxanthin 0.004% were as follow: 40.75 ± 1.49%, 41.25 ± 1.67% and 44.00 ± 3.46%, respectively. The addition of combination of lactose 0.6% and astaxanthin 0.004% provide and significantly better results (P<0.05) compare to other addition. Our results were similar to those reported by Rabadan *et al.* (2012) in Blanca-Celtiberica goat with motility of 43.4% and similar to the report by Ahmad *et al.* (2014) with 42.3±7.5%. This result was lower than the research result conducted by Pamungkas *et al.* (2014) and Tambing *et al.* (2003) respectively on Gembrong and Seanen goats which were 49% and 48.33%.

The result of sperm viability with addition of 0.6% lactose, 0.04% astaxanthin and a combination of lactose 0.6% and astaxanthin 0.004% were as follow: 52.88 ± 2.03%, 54.25 ± 1.91% and 59.00 ± 1.85%, respectively. The addition of combination of lactose 0.6% and staxanthin 0.004% provided better significant result (P<0.05) compared to both other treatments. Our finding supported he result of Ahmad *et al.* (2014) in Beetal goat which got 81.00±5.7% of normal sperm.

From the result of motility, viability and abnormalities examination of post thawing Gembrong goat sperm, it showed a good quality and it could be able to be used for artificial insemination and in vitro fertilization. One criterion to determine the quality of sperm can be seen from the progressive motility of post thawing sperm which was 44.00±3.46%. Frozen semen that able to be for artificial insemination should have minimum 40% of progressive sperm motility.

CONCLUSION

The addition of combination of lactose 0.6% and astaxanthin 0.004% resulted the best quality of Gembrong goat frozen semen which feasible to be used for artificial insemination and in vitro fertilization.

SUGGESTION

Artificial insemination is needed to determine the fertility of frozen semen

ACKNOWLEDGEMENT

The research was funded by the Decentralized Research Grant of Year 2016, Contract No.: 486.3/UN14.2/PNL.01.03.00/2016. The authors thank to Research and Community Service Department of Udayana University who facilitated the implementation of this grant project.

REFERENCES

Ahmad M, Nasrulla R, Riaz H, Sattar A. Ahmad N. 2014, 'Changes in motility, morphology, plasma membrane and acrosome integrity during stages of cryopreservation of buck sperm'. *Journal of the South African Veterinary Association* 85(1): 1-4

Aisen EG, Medina VH, Venturino. 2002. Cryopreservation and post-thawed fertility of ram frozen semen in different trehalose concentrations. *Theriogenology* 57: 1801-1808.

Arifiantini RI, Purwantara B, Putra WW. 1999. Pengujian keutuhan membran plasma spermatozoa semen cair domba menggunakan larutan hipoosmatik. *Proseding Seminar Hasil Hasil Penelitian Bidang Ilmu Hayat*. 16 September 1999. Bogor.

Aziz N, Saleh R, Sharma R. 2004. Association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* 81: 349-354.

Bebas W, Laksmi DNDI. 2012. Laktosa-astaxanthin mencegah cold Shock dan radikal bebas selama penyimpanan semen ayam hutan hijau meningkatkan fertilitas dan daya tetas telur ayam kampung. *Laporan Hibah Unud 2012. Universitas Udayana*.

Bintara S. 2011. Rasio spermatozoa X:Y dan kualitas sperma pada kambing kacang dan peranakan ettawa. *Sains Peternakan* 9(2): 65-71

Blesbois E, Graseau I, Seigneurin F. 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. *Reprod* 129: 371-378.

Breininger E, Beorlegui NB, O'Flaherty CM, Beconi MT. 2005. Alpha-Tocopherol Improves Biochemical and Dynamic Parameters in Cryopreserved Boar Semen. *Theriogenology*. 63: 2126-2135.

Budiono A. 2006. Kriopreservasi dan viabilitas in-vitro.Modul "pemanfaatan bioteknologi embrio pada hewan laborataorium sebagai model dalam penelitian biologi perkembangan dan produksi bahan biologis" Pelatihan dosen universitas/perguruan tinggi.Bogor 28 Agustus- 4 Desember 2006.

Colenbrander B, Fazeli AR, Vanbuiten A, Parleviet J, Gadella M. 1992. Assessment of sperm cell-membrane integrity in the horse. *Acta Vet Scand Suppl* 88: 49-58

Douard W, Hermier D, Magistrini M, Labbe C, Blesbois F. 2004. Impact of change in composition of storage medium on lipid content and quality of turkeys spermatozoa. *Theriogenology* 61: 1-13.

Dziekoñska A, Fraser L, Strze¿ek J. 2009. Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. *J Anim Feed Sci*. 18: 638-649.

Dziekoñska A, Strze¿ek J. 2011. Boar variability effects sperm metabolism activity in liquid stored semen at 5°C. *Polish Journal of Veterinary Sciences* 14(1): 21-27.

Edyson 2002. Pengaruh pemberian kombinasi vit C dan E terhadap aktivitas superoxide dismutase (SOD) dan kadar malondialdehyde (MDA) pada eritrosit *Rattus norvegicus* Galur Winstar yang diinduksi L-Tiroksin. *Tesis*. Surabaya. Universitas Airlangga.

Ejarah AH. 2007.Effect of cryopreservation and constituents of semen extender on mitochondrial fuction of bull spermatozoa. *Disertation*. Louisiana. Lousiana State University.

Ghetler Y, Yavin S, Ruth SR, Amir AA. 2005.The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Human Reproduction* 20(12): 3385–3389.

Hussein G, Goto H, Oda S, Sankawa U. 2006. Antihypertensive potential and mechanism of action of astaxanthin III.Antioxidant and histopathological effects in spontaneosly hypertensive rats. *Biol Pharm Bull* 29: 684-688.

Husein N, Suteky T, Kususiyah. 2007. Uji kualitas semen kambing Nubian dan peranakannya (kambing Nubian x PE) serta kambing Boer berdasarkan lama penyimpanan. *Jurnal Sain Peternakan Indonesia* 2: 57-65.

Kokk K, Verajankorva E, Laato M, Wu XK, Tapfer H and Pollanen P. 2005. Expression of insulin receptor substrates 1-3, glucose transporters glut-1-4, signal regulatory protein 1 alpha, phosphatidylinositol 3-kinase and protein kinase B at the protein level in the human testis. *Anatomical Sci Intl* 80: 91-96

Kostaman T, Sutama IK. 2006. Studi motilitas dan daya hidup spermatozoa kambing Boer pada pengencer tris-sitrat-fruktosa. *J Sain Vet* 24(1): 58-64.

Liu X, Osawa T. 2007. Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity in vitro compared to the all-trans isomer. *Biochem Biophys Res Commun* 357(1): 187-193.

Moioli B, Napolitano F, Catillo G. 2004. Genetic diversity between Piedmontese, Maremmana, and Podolica cattle breeds. *Journal of Heredity* 95: 250-256.

Naijian H. Kohram RH, Shahneh AZ,and Sharafi M. 2013. Effects of various concentrations of BSA on microscopic and oxidative parameters of Mahabadi goat semen following the freeze–thaw process. *Small Rum Res* 113: 371-375.

Paleaz J, Long JA. 2005. Lectin Characterization of Membrane Susface Carbohydrates in *Poultry Spermatozoa. J Androl* 36 (Suppl.): 58.

Pamungkas FA, Batubara A, Sutoro. 2014. The quality of spermatozoa of Gembrong goats during cryopreservation process. *Media Peternakan* 37(2): 95-100

Pesch S, Bergmann M, Bostedt H. 2006. Determination of some enzymes and macro and microelements in stallion seminal plasma and their correlations to semen quality. *Theriogenelogy* 66: 307-317

Rabadan PJ, Ramon M, Alvarez OG, Morales AM, Olmo ED, Guzman MDP, Bisbal AM. Santos MRF, Garde JJ, Soler AJ. 2012. Effect of semen collectionmethod (artificial vagina vs. electroejaculation), extenderand centrifugation on post-thaw sperm quality of Blanca Celtibérica buck ejaculates. *Anim Reprod Sci* 132: 88-95.

Rizal M, Herdis, Surachman M, Nalley WMM. 2008. Pengaruh plasma semen domba Priangan terhadap daya hidup spermatozoa kambing Peranakan Etawah yang disimpanpada suhu 3–5 °C. *Jurnal Ilmu Ternak dan Veteriner* 13: 23-29.

Sanocka D, Kurpisz M. 2004. Reactive oxygen species and sperm cells. *Reproductive Biology and Endocri-nology* 2 (12): 1-7

Storey BT. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod* 3: 203-213.

Tambing SN, Toelihere MR, Yusuf TL, Sutama IK. 2000. Pengaruh gliserol dalam pengencer tris terhadap kualitas semen beku kambing peranakan Etawah. *Jurnal Ilmu Ternak dan Veteriner* 5: 84-99.

Viswanath R, Shanon P. 2000. Storage of bovine semen in liquid frozen. *Anim Reprod Sci* 62: 23-53.

Zein MS, Sulandri AS, Muladno, Subandriyo, Riwantoro. 2012. Diversitas genetik dan hubungan kekerabatan kambing lokal Indonesia menggunakan marker DNA mikrosatelit. *Jurnal Ilmu Ternak dan Veteriner* 17: 25-35.