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Effect of Cryopreservation Duration Time on Post-Thawing Sperms' Characteristics of Goat Semen

(*PENGARUH LAMA WAKTU KRIOPRESERVASI TERHADAP KARAKTERISTIK SPERMATOZOA KAMBING SETELAH SEMEN DI-THAWING*)

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ABSTRACT

Cryopreservation is a technique utilized to store spermatozoa with the aim of preserving the quality of frozen semen. The quality of spermatozoa after thawing is a critical factor influencing the success of Artificial Insemination (AI). This decline in quality can be attributed to factors such as exposure to extreme temperatures, formation of ice crystals, reduced membrane permeability and the generation of Reactive Oxygen Species (ROS). Given this decline in quality, it becomes imperative to assess spermatozoa quality at the cellular level. This study was aimed to investigate the impact of storage time on frozen goat semen on spermatozoa membrane integrity, levels of Malondialdehyde (MDA), and sperm DNA fragmentation. The samples utilized included frozen goat semen stored for less than two years and six years, subjected to a thawing process at 37°C for 20 seconds. Data pertaining to the percentage of spermatozoa with intact membrane integrity, sperm DNA fragmentation, and MDA levels (nmol/L) were analyzed using a paired t-test with a 95% confidence level (α = 0.05). The findings of this study revealed that the duration of cryopreservation may not significantly impact post-thawing DNA fragmentation and MDA levels ($p>0.05$). However, a moderate decrease in sperm viability and intact plasma membrane (IPM) was observed with increasing cryopreservation duration ($p<0.05$). Furthermore, the IPM parameter emerged as a potentially suitable indicator for assessing the quality of post-thawing frozen semen, alongside sperm viability and motility.

Keywords: Cryopreservation; duration; goat semen; spermatozoa membrane; malondialdehyde

ABSTRAK

Cryopreservation adalah teknik yang digunakan untuk menyimpan spermatozoa dengan tujuan mengawetkan kualitas semen beku. Kualitas spermatozoa setelah thawing adalah faktor kritis yang memengaruhi keberhasilan inseminasi buatan. Penurunan kualitas dapat disebabkan sejumlah faktor, antara lain, terpapar ke suhu yang ekdtrim, terbentuknya Kristal es, menurunnya permeabilitas membrane dan terbentuknya *Reactive Oxygen Species* (ROS). Mengingat penurunan kualitas ini, penilaian kualitas spermatozoa pada tingkat sel menjadi penting untuk dilakukan. Penelitian ini bertujuan meneliti pengaruh waktu pe-nyimpanan semen beku kambing dalam hal keutuhan membrane spermatozoa, kadarmalondialdehyde (MDA) dan fragmentasi DNA spermatozoa. Sampel yang digunakan meliputi semen beku kambing dengan lama simpan di bawah dua tahun dan enam tahun. Sampel tersebut kemudian diberi perlakuan proses *thawing* pada suhu 37°C selama 20 detik. Data yang diambil berupa persentase spermatozoa dengan membran utuh, fragmentasi DNA spermatozoa dan kadar MDA (nmol/L). Data tersebut dianalisis menggunakan uji-t berpasangan dengan tingkat kepercayaan 95% ($\alpha = 0,05$). Temuan studi ini mengungkapkan bahwa lama simpan dengan cara cryopreservasi tidak memengaruhi secara nyata fragmentation DNA spermatozoa dan kadar MDA ($p>0.05$) setelah thawing. Namun, ditemukan penurunan dengan tingkatan sedang pada viabilitas spermatozo dan membran plasma utuh pada semen dengan bertambahnya lama waktu penyimpanan menggunakan cara cryopreservasi ($p<0.05$). Selain itu, parameter membrane plasma utuh dapat dijadikan sebagai indikator yang berpotensi cocok untuk menilai kualitas semen beku pasca thawing, selain viabilitas dan motilitas spermatozoa.

Kata-kata kunci: Cryopreservasi; lama simpan; semen kambing; membrane spermatozoa; malondialdehyde

INTRODUCTION

Artificial Insemination (AI) is an application of reproductive technology which aims to improve the quality of animals and increase the number of populations of certain farm animals. To achieve this goal, it is necessary to have good-quality semen so that the success rate of AI becomes satisfied (Ardhani *et al*., 2020). The application of AI technology is still not as expected. The low pregnancy rate of goats indicates this after AI. Reports studies of AI in goats show that the pregnancy rate obtained varies from 40-46% (Achlis *et al*., 2013). According to Pamungkas (2014), the success rate of AI is around 33-73%, even only reaching 36-46% in the field.

Sperm quality affects success in the artificial insemination (AI) process. The availability of quality semen is crucial for applying reproductive biotechnology and cell biology in livestock. One of the causes of the low pregnancy rate of goats in the AI procedure is the poor quality of the semen used. Poor semen quality in goats can be caused by damage to the sperm plasma membrane. Plasma membrane damage is caused by lipid peroxidation caused by free radicals produced by metabolic processes (Dwinofanto *et al*., 2018).

Cryopreservation in semen is a complex process that requires a balance of various factors to achieve maximum results. Sperm (especially goat semen) requires special attention to maximize post-thawing viability and fertility of sperm. In general, 50-60% of the total spermatozoa population is still alive if cryopreservation is carried out according to the protocol. If the total number of viable spermatozoa is less than the specified number, spermatozoa fertility will be reduced (Gangwar *et al*., 2016). The spermatozoa membrane is a pivotalstructure

that affects spermatozoa morphology and function under normal and pathological conditions. The fatty acid profile determines the performance of not only spermatozoa

motility but also the acrosome reaction and spermatozoa-oocyte fusion. The proportion of saturated fatty acids was quite high in goat spermatozoa, approximately 67% of saturated fatty acid chains in goat spermatozoa are also associated with the phospholipid fraction.

As mentioned above, the difficulty in the semen storage stage is mainly due to the damage to the plasma membrane of spermatozoa. The longer the time in semen storage, the higher the abnormality in semen caused by cold shock and osmotic pressure imbalance due to metabolic processes that continue in the semen storage process. The longer the storage period, the more cellular changes happen, especially in the sper-matozoa membrane, which will experience alterations that can potentially reduce the quality of post-thawing spermatozoa. Extended semen storage time will produce side effects in spermatozoa metabolism (Kusumawati *et al*., 2019).

Unfortunately, the freeze–thaw process of cryopreservation is known to inevitably trigger excessive production of free radicals called reactive oxygen species (ROS). The spermatozoa plasma membrane contains highly polyunsaturated fatty acids, which are susceptible to lipid peroxidation and prone to oxidation by ROS or other free radicals, with the main side-product being malondialdehyde (MDA). Malondialdehyde compounds are weak free radical comp-ounds, but their accumulation is toxic to cells and negatively impacts spermatozoa fertility (Nahar *et al*., 2013). Excess MDA has the potential to disrupt the integrity of the spermatozoa membrane and induceDNA fragmentation, changes in mitochondrial structure, and the death of spermatozoa (Longobardi *et al*., 2020). The over-production of free radicals also alters contamination in spermatozoa which affects the integrity of the membrane, thus affecting the quality of frozen semen (Salim *et al*., 2012). Therefore, examining DNA fragmentation can confirm the diagnosis of male fertility, and the level of MDA levels affects damage to spermatozoa during the cryopreservation process. Based on this background, this study was conducted to determine the effect of the storage time of semen on DNA fragmentation and intact plasma membranes of goat spermatozoa with different storage periods.

RESEARCH METHODS

The research was carried out at the Animal Disease and Diagnostic Laboratory, Universitas Brawijaya Malang for one month, starting from March to April 2021. This research was a comparative study in which the frozen goat semen was divided into two groups according to storage / cryopreservation duration time (less than two year and six years). These two groups of frozen goat semen were thawed and spermatozoa characteristics such as via-bility, integrated plasma membrane (IPM), DNA fragmentation, and malon-dialdehyde (MDA) concentration were analyzed. The viability of spermatozoa was analyzed using the staining method of 2% Eosin-nigrosin. Spermatozoa that absorbed the stain were determined dead sper-matozoa, while spermatozoa that did not absorb the stain were determined live sperms. The inte-grated plasma membrane (IPM) of spermatozoa was analyzed using Hypo-osmotic swelling test (HOS-test) procedures in which spermatozoa with swollen tails were determined to have intact membranes, while spermatozoa with unswollen tails were determined to have damaged mem-branes. The DNA integrity of spermatozoa was determined using Toluidine Blue Stain method. Spermatozoa with good DNA integrity showed staining results with bright blue or transparent chromatin/DNA. Conversely, spermatozoa with poor DNA integrity showed blue or dark blue chromatin/DNA in color. The malon-dialdehyde (MDA) level of post-thawed frozen goat semen was examined using the Enzyme-Linked Immunosorbent Assay/ELISA technique (Bioenzy® Goat MDA Kit). The MDA

concentration of post-thawing spermatozoa was expressed in nmol/mL.

Data Analysis

Data analysis was carried out by testing IPM data, DNA fragmentation and MDA level with a normality test; customarily distributed data ($p>0.05$) could be analyzed using an Independent T-Test to determine differences between groups.

RESULTS AND DISCUSSIONS

Spermatozoa Viability

Spermatozoa viability was observed using 2% Eosin-nigrosin staining by dropping semen onto an object glass and then dripping stain at a ratio of 1:1. After that, it was homogenized and smear preparations were made, then observed using a light microscope with a magnification of 1000x. The results of the viability examination in groups T1 and T2 were presented in Figure 1.

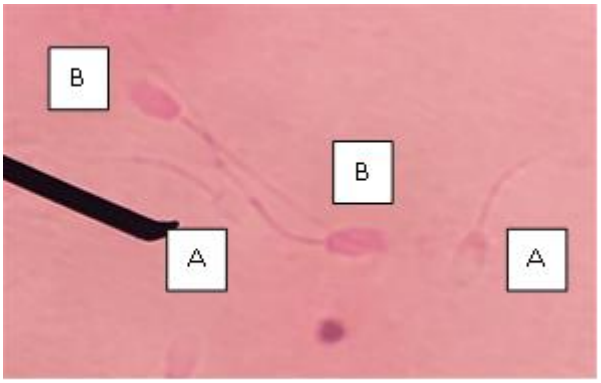

Figure 1. Viable Sperm: colorless (A) and dead spermatozoa: pink colored (B)

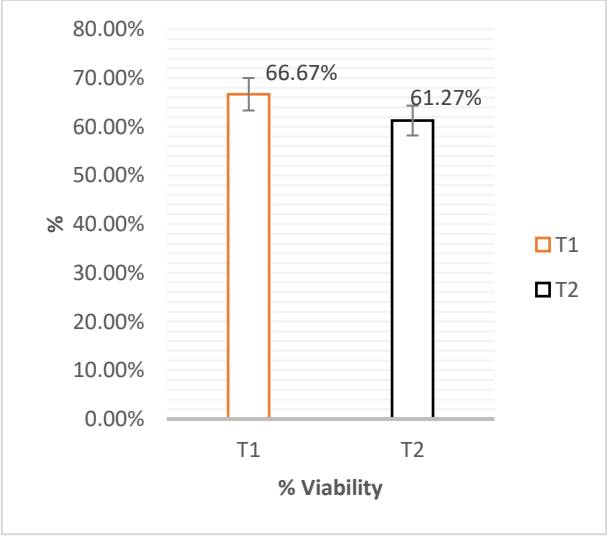

Figure 2. Statistical result of post-thawed viability (%)
*T-independent Sig (2-tailed) : 0.019 ($p < 0.05$)

The viability of thawed spermatozoa refers to their ability to maintain themselves after the freezing process. This is a common indicator used to assess the quality of spermatozoa. According to existing standards, for artificial insemination using frozen goat spermatozoa, a minimum via-bility of at least 50% is necessary. The T1 group showed a viability of 66.67%, while the T2 group had a viability of 61.27%.

The Examination of Post-thawed IPM of Goat Spermatozoa

Figure 5. The Hypoosmotic Swelling Test (HOS Test) result. The yellow arrows show circular spermatozoa tails (indicating spermatozoa with a suitable plasma membrane), and the red arrows show straight spermatozoa tails (indicating a damaged plasma membrane) at 1000X magnification

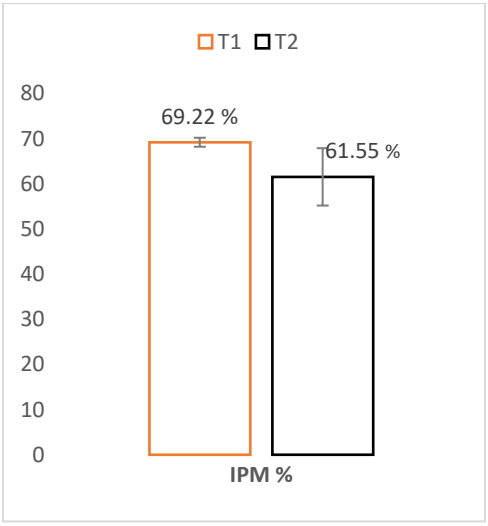

Figure 6. Statistical result of post-thawed IPM (%)
*T-independent Sig (2-tailed) : 0.030 (p<0.05)

In this study, the calculation results show that the average IPM of T1 and T2 were 69.22% and 61.55%, respectively. These results are considered good, as the minimum IPM value was 58% (Hess *et al*., 2005). According to an unpaired T-test, which yielded a P-value of 0.030 (less than 0.05), it can be concluded that there is a significant difference in post-thawed IPM between groups T1 and T2.

The Examination of Post-thawed DNA Fragmentation of Goat Spermatozoa

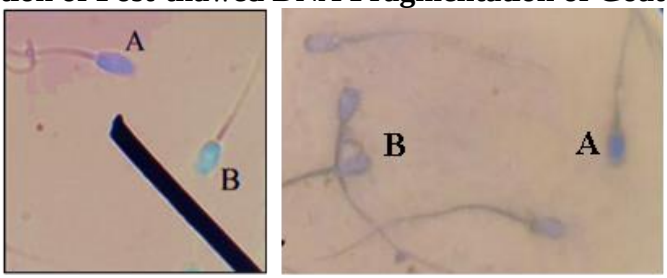

Figure 3. Sperm with a low density (A) and spermatozoa with good density (B)

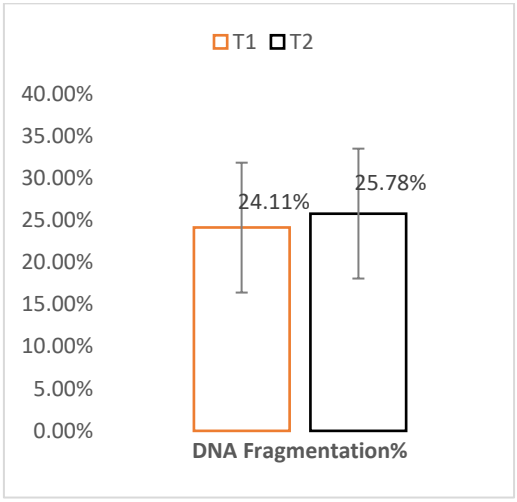

Figure 4. Statistical result of post-thawed DNA fragmentation (%)
*T-independent Sig (2-tailed) : 0.692 (p > 0.05)

The results of observations (Figure 3) of spermatozoa chromatin density using Image J software were analyzed using SPSS Statistic 22 software, which showed that the data were normally distributed (p > 0.05) and homogeneous (p > 0.05) in the nor-mality test and homogeneity test. However, in the results of data analysis using the independent T-test, no significant difference was found between the T1 group and the T2 group. According to the results (Figure 4) the DNA fragmentation of spermatozoa between two treatment groups with semen storage of one years (T1) showed a result of 24.11%, while in the treatment group with more than five years (T2) was 25.78%. This possibly happen because the storage at a very low tem-peratures in a long time can change the condition of both the structure and function of spermatozoa. However, based on the statistical analysis shows no significant difference between the two treatments on DNA fragmentation (p>0.05). This statistical analysis in line with previous research (Petrushko *et al.*, 2017) that cryopreservation does not affect the DNA fragmentation of normal spermatozoa.

The Examination of Post-thawed MDA Concentration

The analysis of MDA concentration in post-thawing goat semen were carried out using the ELISA technique (Bioenzy® Goat MDA Kit). The ELISA results (Figure 7) wells were read using the ELISA Reader with a wave length of 450 nm. Microsoft Excel processed the standard absorbance value for the concentration obtained. The value equation obtained is y=23.01 x -3.98 with a coefficient of determination R^2= 0.917. The expression of MDA levels in the T1 has a value of 8.25 ± 2.41 nmol/L, while the second treatment group (P2) has a value of 8.36 ± 2.79 nmol/L. The T2 treatment group had higher post-thawing MDA levels than in the T1 treatment group. Even though according to statistical ana-lysis, there was no significant difference between the two groups of frozen semen storage time (p>0.05).

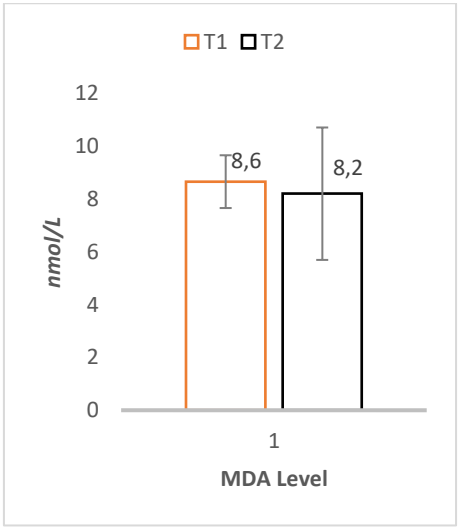

Figure 7. Statistical result of post-thawed MDA level (nmol/L)

Cryopreservation with suitable media will keep spermatozoa fertile and maintain their quality. However, cryopreservation techniques for semen cause a significant reduction in motility, viability, chromatin stability and membrane integrity due to decreased morphological function (Sharma *et al.*, 2015).

After the cryopreservation process, an evaluation of post-thawing semen was conducted to determine the quality of post-thawing semen. The semen evaluation process is vital to determine the success of AI. Various tests, including viability, intact plasma membrane and intact acrosome cap, can be used to asses post-thawing semen quality. Viability, intact plasma membrane (IPM), and intact acrosome cap are the benchmarks of frozen semen quality. If the viability of spermatozoa decreases, it will also reduce the success level of AI because only live spermatozoa can fertilize an oocyte (Susilawati, 2011). Spermatozoa viability according to SNI ideally should be at least 50% (Kusumawati *et al.*, 2019) and not less than 40%. The results of this study indicate that both groups (T1 and T2) meet the minimum criteria for post-thawing sperm viability, with rates of 66.67% and 61.27% respectively. Alongside viability testing, maintaining plasma membrane integrity is crucial for ensuring sperm survival and successful fertilization (Rizal *et al.*, 2015). The sperm plasma membrane, comprised of phospholipids, glycolipids, cholesterol and proteins (Isnaini, 2011), plays a vital role in regulating intracellular metabolic activity, ionic composition and pH while maintaining permeability. In this study, the average intact plasma membrane (IPM) percentages for T1 and T2 were 69.22% and 61.55%, respectively, both falling within the ideal minimum IPM value of 58%.

It was also stated by Mustofa (2021) that spermatozoa with intact plasma membranes were characterized by a circular tail, whereas a straight tail marked sper-matozoa with a damaged plasma membrane. Adding HOS solution to the semen made the spermatozoa inner cell pressure become hyper osmotic compared to the outside of the cell. In order to achieve an isoosmotic state, the cell will absorb water associated with K^+ and Cl^- and be driven by Na^+ or mediated by Na^+/H^+. The entry of water into the plasma membrane will expand the entire area of the plasma membrane. The tail will coil when the plasma membrane is inflated hypoosmotic.The tail coiling occurs because the tail is more flexible than the other parts, so it is possible to coil as a sign that the plasma membrane of spermatozoa is still intact (Larsen and Hoffmann, 2020).

Plasma membrane damage can be caused by significant temperature changes that will damage the lipoproteins in the spermatozoa membrane, the presence of free radicals in the metabolic process, or a decrease in spermatozoa quality due to changes in osmotic pressure in the sper-matozoa (Cahya *et al.*, 2017). Dramatic shifts of temperatures during the freezing process also stimulate some alterations in the polarity of the atoms/molecules that make up the membrane due to changes in the polarity of these atoms, causing membrane instability and reducing the physiological function of the membrane. This is mainly caused by a high level of lipid content (phospholipids, glycolipids and cholesterol) in goat semen that easily binds to free radicals. Examination of DNA fragmentation after thawing of spermatozoa can also be done to identify spermatozoa abnormalities due to cryopreservation in males. Fragmentation examination of spermatozoa DNA uses toluidine blue staining (Kim *et al.*, 2013), spermatozoa was stained bright blue or transparent chromatin. Conversely, spermatozoa with poor chromatin are blue or dark blue in color.

Toluidine blue is a dye that has metachromatic acidophilic properties and is selective for tissues with acidic properties, so that toluidine blue dye can bind to sulfate, carboxylate and phosphate (Nasr-Esfahani *et al*., 2021).The working principle of this dye is that the dye will be absorbed by the phosphate group of the DNA strands of spermatozoa that have less chromatin density. Spermatozoa with low chromatin density will absorb Toluidine blue dye so that the spermatozoa will be colored dark blue. Conversely, spermatozoa with optimal chromatin density will not be stained so the image will show a bright or unstained color.

Prolonged storage in liquid nitrogen media can induce spermatozoa damage, primarily through oxidative stress, characterized by the generation of reactive oxygen species and subsequent harm to sperm cells. Additionally, the freezing process itself contributes to the production of reactive oxygen species and sperm cell damage. Sudden drop of temperature can lead to cold shock in spermatozoa. Another factor contributing to increased DNA fragmentation in spermatozoa is the temperature change during the transfer of semen from a large container to a small one. Despite being often overlooked, this temperature shift can cause damage and reduce spermatozoa quality (Pardede *et al*., 2020). Consistent with these findings, the report of Priyanto *et al*. (2019) demonstrated that the semen freezing process decreased motility, viability, intact plasma membrane (IPM), and intact acrosome, but did not significantly affect DNA integrity. These findings align with our study, which also observed a low percentage of DNA damage following the freezing process, with no significant difference observed based on storage duration.

The fragmentation testing of DNA spermatozoa was also conducted to assess the fertility level of bulls. Various factors can contribute to DNA damage, including apoptosis during spermatogenesis, alterations in chromatin bonds, oxidation reactions and exposure to toxic environments (Sakkas and Alvarez, 2010). Sperm DNA damage can result from the production of MDA compounds, which are by-products of lipid peroxidation during oxidative stress (Jeremias *et al*., 2021).

Previous research conducted by Febrianti *et al.*, (2014) showed an increase in MDA levels in frozen semen stored for about two until three months. Unfor-tunately, the elevating level of MDA had negative correlation with the number of viable spermatozoa. Another study con-ducted by Dwinofanto *et al.* (2019) on Bali cattle semen that was frozen and thawed had MDA levels of 6.8 ± 0.3 µM at 4 hours of storage and 6.4 ± 0.4 µM.

Even though the results of the study statistically confirmed that storage time has no significant effect on post-thawing MDA levels, it does not mean that accumulation of MDA does not harm the quality of

spermatozoa in the future. Malondialdehide is a by-product of lipid peroxidase and suitable as a marker of oxidative stress. Lipid peroxidation in the plasma membrane of spermatozoa can cause structural alterations in spermatozoa. Changes occur due to the decreased of motility and damage to cell components, one of which is the nucleus. Thus, high concentrations of MDA in seminal plasma can reduce the quality and function of spermatozoa.

CONCLUSION

The length storage of cryopreservation method does not affect the MDA levels and DNA fragmentation of sperm after thawing. However, the duration of storage at freezing temperatures is closely linked to decreased post-thaw motility and viability of sperm. This parameter relating to membrane integrity could be considered when assessing the quality of frozen semen after thawing, alongside sperm viability and motility.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Achlis R, Anwar H, Hidanah S, Srianto P. 2013. Frozen Semen Quality of Etawa Crossbreeds in Various Diluents. *Veterinary Medicine* 6(1): 69-74.

Ardhani F, Mufidah H, Samsuriati R. 2020. Long Effects of Storage of Frozen Bali Cow on Artificial Insemination Posts on Plasma Membranes, Whole Acrosome Captures, and Spermatozoal DNA. *Jurnal Ilmu Peternakan Terapan* 3(2): 58-66 DOI:10.25047/jipt.v3i2.1950

Febrianti KI, Rahayu S, Agung-Pramana WM, Soewondo A. 2014. MDA Spermatozoa Levels After Freezing Process. *Biotropics J Trop Bio* 2: 142–147

Dwinofanto H, Mustofa I, Susilowati S, Hernawati T. 2018. The effect of duration of preservation on the quality, MDA level, and DNA damage of post-thawed Bali cattle bull sperm. *Iraqi Journal of Veterinary Sciences* 32(2): 249-252. 10.33899/ijvs.2019.153857

Gangwar C, Kharche SD, Kumar S, Jindal SK. 2016. Cryopreservation of goat semen: status and prospects. *Indian J Small Ruminants* 22(1): 1-10. https://doi.org/10.5958/0973-9718.2016.00005.2

Hess KC, Jones BH, Marquez B, Chen Y, Ord T, Kamenetsky M, Miyamoto C, Zippin J, Kopf GS, Suárez SS, Levin LR, Williams CJ, Buck J, Moss SB. 2005. The "Soluble" Adenylyl Cyclase in Sperm Mediates Multiple Signaling Events Required for Fertilization. *Dev Cell* 9(2): 249-59. https://doi.org/10.1016/j.devcel.2005.06.007

Isnaini N. 2011. Boer Goat Spermatozoa Viability Post Cooling and Freezing Using Tris Base Diluent with Different Trehalose Levels. *J Ternak Trop* 12: 27–37.

Jeremias JT, Belardin LB, Okada FK, Antoniassi MP, Fraietta R, Bertolla RP, Intasqui P. 2021. Oxidative origin of sperm DNA fragmentation in the adult varicocele. *Int Braz J Urol* 47: 275–283. https://doi.org/10.1590/s1677-5538.ibju.2019.0827

Kim HS, Kang MJ, Kim SA, Oh SK, Kim H, Ku SY, Kim SH, Moon SY, Choi YM. 2013. The utility of sperm DNA damage assay using toluidine blue and aniline blue staining in routine semen analysis. *Clin Exp Reprod Med* 40: 23. https://doi.org/10.5653/cerm.2013.40.1.23

Kusumawati ED, Rahadi S, Nurwathon S, Yulianti DL. 2019. Quality of Post Thawing Spermatozoa of Etawa Peranakan (PE) Goats at 37°C with

Different Times. *Jurnal Ilmu dan Teknologi Peternakan Tropis* 6; 246. https://doi.org/10.33772/jitro.v6i2.7152

Larsen EH, Hoffmann EK. 2020. Volume Regulation in Epithelia, in: Hamilton KL, Devor DC (Eds.). *Basic Epithelial Ion Transport Principles and Function, Physiology in Health and Disease*. Springer International Publishing, Cham, Hlm. 395–460. https://doi.org/10.1007/978-3-030-52780-8

Longobardi V, Zullo G, Cotticelli A, Salzano A, Albero G, Navas L, Rufrano D, Claps S, Neglia G. 2020. Crocin Improves the Quality of Cryopreserved Goat Semen in Different Breeds. *Animals* 10(6): 1101. https://doi.org/10.3390/ani10061101

Lubis TM, Dasrul, Thasmi CN, Akbar T. 2013. The Effectiveness of Adding Vitamin C in Egg Yolk Skim Milk Diluent on Spermatozoa Quality of Boer Goats after Cold Storage. *J Sains Pertanian* 3: 347–361.

Mustofa I, Susilowati S, Wurlina W, Hernawati T, Oktanella Y. 2021. Green tea extract increases the quality and reduced DNA mutation of post-thawed kacang buck sperm. *Heliyon* 7: e06372. https://doi.org/10.1016/j.heliyon.2021.e06372

Nahar Z, Ali M, Biswas S, Kamrun N, Bashar T, Arslan M. 2013. Study of Seminal MDA Level as an Oxidative Stress Marker in Infertile Male. *J Sci Foundation* 9(1&2): 85–93. https://doi.org/10.3329/jsf.v9i1-2.14651.

Nasr-Esfahani M, Tavalaee M., 2021. Sperm Chromatin Structure: Toluidine Blue Staining, in: Majzoub A, Agarwal A, Henkel R. (Eds.). *Manual of Sperm Function Testing in Human Assisted Reproduction*. Cambridge. Cambridge University Press. Hlm. 156–162. https://doi.org/10.1017/9781108878715.021

Pamungkas FA, Batubara A, Anwar A. 2014. Spermatozoa cryopreservation of Boer goats: Comparison of two diluents on post-thawing quality and fertilizing ability. *Jurnal Ilmu Ternak Veteriner* 19. https://doi.org/10.14334/jitv.v19i2.1041

Petrushko MP, Pavlovich EV, Pinyaev VI, Volkova NA, Podyfaliy VV. 2017. Apoptosis and the processes of DNA fragmentation in native and cryopreserved human sperm cells with normo- and pathosperma. *Cytol Genet* 51: 278–281. https://doi.org/10.3103/S0095452717040065

Poulos A, Brown-Woodman PDC, White IG, Cox RI. 1975. Changes in phospholipids of ram spermatozoa during migration through the epididymis and possible origin of prostaglandin F2α in testicular and epididymal fluid. *Biochim Biophys Acta* 388: 12–18.

Priyanto, L., Budiyanto, A., Kusumawati, A., Kurniasih, -, Arifiantini, I., 2019. Comparison of Post Thawing Spermatozoa DNA Damage Examination between Sperm-Bos-Halomax® dan Toluidine Blue. *Jurnal Peternakan Sriwijaya* 7(1): 30-39. https://doi.org/10.33230/JPS.7.1.2018.7081

Rana APS, Majumder GC, Misra S, Ghosh A. 1991. Lipid changes of goat sperm plasma membrane during epididymal maturation. *Biochim Biophys Acta* 1061: 185–96.

Rizal M, Herdis N., Riyadhi M. 2015. Cryopreservation Of Garut Ram Semen with Tris Extender Ethylene Diamine Tetraacetic Acid. *Jurnal Veteriner* 16(2): 249-255.

Roostika I. 2016. Development of Applications of Cryopreservation Techniques for Conservation and Supporting Plant Breeding Programs. *J. AgroBiogen* 9(1): 39-48.

Sakkas D, Alvarez JG. 2010. Sperm DNA Fragmentation: Mechanisms of Origin, Impact on Reproductive Outcome, and Analysis. *Fertil Steril* 93: 1027–1036. https://doi.org/10.1016/j.fertnstert.2009.10.046

Salim MA, Susilawati T, Wahyuningsih S. 2012. The Effect of the Thawing Method on the Quality of Frozen Semen for Bali Cattle, Madura Cattle and PO Cattle. *J Agripet* 12: 14–19. https://doi.org/10.17969/agripet.v12i2.197

Sharma R, Kattoor AJ, Ghulmiyyah J, Agarwal A. 2015. Effect of Sperm Storage and Selection Techniques on Sperm Parameters. *Syst Biol Reprod Med* 61(1): 1–12

Sridharan, G., Shankar, A., 2012. Toluidine blue: A Review of Its Chemistry and Clinical Utility. *J Oral Maxillofac Pathol* 16: 251. https://doi.org/10.4103/0973-029X.99081