

The Effect of Glutathione on The Quality of Aceh Local Catfish (*Clarias gariepinus*) Spermatozoa After Cryopreservation

Raudhah Mahfudhah¹, Kartini Eriani^{1,2*}, Zainal Abidin Muchlisin³

¹Master Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Indonesia

²Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Indonesia

³Department of Aquaculture, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Indonesia

*Email: kartini_eriani@unsyiah.ac.id

Submitted: 5 December 2019. Revised: 11 February 2020. Accepted: 20 March 2020

Abstract. The cryopreservation process might reduce the quality of spermatozoa due to an increase in the production of reactive oxygen species (ROS) compounds during cooling, freezing, and thawing. The quality of spermatozoa can be maintained by adding glutathione as an exogenous antioxidant into cryo-diluent. This study aimed to examine the effect of the addition of different doses of glutathione in cryopreservation of Aceh Local catfish (*Clarias gariepinus*) spermatozoa after freezing. The method used was a completely randomized design (CRD) with four treatments and four replications. Fresh semen was diluted in Ringer, 15% DMSO, and 20% Fetal Bovine Serum (FBS) and then added with glutathione antioxidants of 0.0 mgL⁻¹, 0.5 mgL⁻¹, 1.0 mgL⁻¹, and 2.0 mgL⁻¹. The parameters observed in this study were motility, integrity of the plasma membrane, fertility, and DNA integrity. The results showed that the concentration of glutathione had no effect on motility, integrity of the plasma membrane, or fertility, but had an effect on DNA integrity. The highest percentage of motility and plasma membrane integrity respectively was 40.50% (P3) and 70.87% (P2). Furthermore, the assessment of DNA integrity showed that there was no DNA fragmentation both treatments and fresh spermatozoa. This research is the first study regarding glutathione supplementation in cryo-diluent of Aceh Local catfish spermatozoa. Finally, the results obtained can provide information about the exact concentration of glutathione in the extender on the quality of spermatozoa of Aceh Local catfish (*C. gariepinus*) after the cryopreservation process. These results can also increase the success of fertility be used by the seed hall unit and the aquaculture industry to increase the productivity and supply high quality seeds.

Key words: Antioxidants; Glutathione; Cryopreservation; Spermatozoa

How to Cite: Mahfudhah, R., Eriani, K. & Muchlisin, Z. A. (2020). The Effect of Glutathione on The Quality of Aceh Local Catfish (*Clarias gariepinus*) Spermatozoa After Cryopreservation. *Biosaintifika: Journal of Biology & Biology Education*, 12 (1), 125-131

DOI: <http://dx.doi.org/10.15294/biosaintifika.v12i1.22353>

INTRODUCTION

Aceh Local catfish (*Clarias gariepinus*) is a commercial freshwater fish and one of the most popular aquaculture species in the world (Asraf et al., 2013). The fish have high economic value for consumption purposes. Aceh Local catfish are resistant to disease, have high growth rates as well as the ability to tolerate various environmental conditions. In addition, catfish contain high nutritional value including 15-18% protein, 5-10% fat, 1.2% vitamins, and 1.2% minerals (Wachirachaikarn et al., 2009). Aceh Local catfish are labeled in the red list category of endangered species in the LC (least concern) category according to the International Union for Conservation of Nature (Konings et al., 2018). The existence of this fish needs to be preserved through breeding with the aim of cultivation for both consumption and conservation. However, the obstacle regarding the mentioned program is that the gonad maturity of Aceh Local catfish only occurs in the rainy season and affecting the reduction of the gametes supply (Mahyudin, 2013). Also, in artificial spawning, male catfish must be sacrificed which results in a reduced

number of male broodstock at the hatchery (Adipu et al., 2014).

One effort offered to overcome this problem is by applying the spermatozoa cryopreservation method (Sulmartiwi et al., 2011; Muchlisin et al., 2015; Muthmainnah et al., 2018). The cryopreservation method allows unlimited genetic material to be stored to ensure the availability of germplasm. Cryopreservation can eliminate the time and place limits in the supply of spermatozoa for artificial insemination. The use of cryopreservation method is more efficient, economical, and effective (Cabrita et al., 2010). However, cryopreservation has the disadvantage of causing damage to the spermatozoa plasma membrane due to cooling, freezing, and thawing which can reduce their quality and viability (Dhanju et al., 2001; Zilli et al., 2005).

This negative effect is due to an increase in the production of reactive oxygen species (Bucak et al., 2010). Reactive oxygen species (ROS) are compounds produced naturally in cellular metabolism that oxidize reactive oxygen derivatives (Valko et al., 2007). This compound is easy to bind with polyunsaturated fatty acids which constitute the majority of the structural composition of the spermatozoa mem-

brane. Excess ROS will weaken the enzymatic defense of antioxidants and causes oxidative stress on cells as well as damage to DNA, protein, and fat which results in decreased quality of spermatozoa (Eriani et al., 2018). Antioxidants are nucleophilic compounds that have the ability to reduce and suppress free radical reactions, slowing down and preventing lipid oxidation. The quality of spermatozoa can be maintained by the addition of antioxidants (Shui et al., 2004). Glutathione is one of the exogenous antioxidant compounds that reduces the occurrence of lipid peroxidation reactions in the plasma membrane of spermatozoa cells (Raijmakers et al., 2003). Lahnsteiner et al. (2011) reported that the addition of 1 mmol/l glutathione to the cryopreservation of Brook trout (*Salvelinus fontinalis*) sperm resulted in a fertility rate of 76.3%. Muthmainnah et al. (2018), conducted a cryopreservation study on Seurukan fish (*Osteochillus vittatus*) with the addition of 0.5 mg/ml glutathione resulting in a fertility rate of 51.33%. However, the application of glutathione in the cryopreservation of Aceh Local catfish (*C. gariiepinus*) spermatozoa has not been studied. Therefore, further research was needed regarding the administration of glutathione in cryopreservation of Aceh Local catfish sperm.

The results of this study were expected to provide information about the optimum concentration of glutathione to maintain the quality of spermatozoa after cryopreservation. The results could also be utilized by the seed hall unit and aquaculture industry to increase the productivity and supply of high quality seeds. The seeds produced from the cryopreservation process will be released into their natural habitat to support conservation programs.

METHODS

Broodstock Preparation and Sperm Extraction

Samples of Aceh Local catfish (*C. gariiepinus*) broodstock were obtained from the market, as many as 20 Aceh Local catfish were recorded with a weight of 1000 g. Ovaprim at concentration of 0.3 ml/kg body weight was injected intramuscularly to male broodstock to stimulate spermiation. The spermiation process took about 10 hours, beginning after the hormone injection. Following that, the male broodstock was dissected to obtain the gonad. During the dissection, the sperm was protected from water and any ash to avoid sperm death. Next, the quality of the sperm was evaluated.

Spermatozoa Dilution

The cryopreservation process of Aceh Local catfish spermatozoa began with the preparation of diluent (extender) in the form of Ringer's solution. The

Ringer's solution was made based on Muchlisin et al., (2004), which details that 50 ml Ringer's solution requires 0.375g NaCl, 0.01 g KCL, 0.01g CaCl₂, and 0.01g NaHCO₃. Next, each 6.5 ml of the Ringer's solution were distributed to four different tubes (vol. 10 ml). Two (2) ml of fetal bovine serum (FBS) and 1.5 ml of DMSO were added to each tube resulting in a concentration of 20% FBS and 15% DMSO respectively. Then, 0.5 ml of sperm was added to the tube resulting in final semen dilution rate of 1:20. The dilution was then distributed into four cryotubes (as repeats), as each cryotube is filled with 2 ml of diluted sperm. Finally, 0.005 mg of glutathione was added to the cryotube to get a concentration of 0.5 mgL⁻¹, 0.01 mg glutathione for 1.0 mgL⁻¹ and 0.02 mg glutathione for 2.0 mg L⁻¹.

Freezing and Thawing

The samples were stored in a refrigerator for equilibration at temperature of 4-5°C for 5 minutes. The samples were then evaporated at a distance of 6 cm above the surface of the liquid nitrogen (-79°C) for 5 minutes. The samples then were immediately put into a container of liquid nitrogen with a temperature of -196°C to be stored for two weeks. After those two weeks, Cryotubes were taken from the container and placed in a water bath at a temperature of 37°C for 5 minutes (Muchlisin et al., 2015).

Motility and Integrity of Plasma Membrane

The percentage of sperm motility was observed by dropping a few drops of sperm on a microscope slide, then covered with a cover glass. Parameters were observed based on five different fields of view under a binocular electric microscope with a magnification of 400x. The integrity of the plasma membrane was examined by using the Hypo-osmotic Swelling Test (HOS-Test) method. Healthy spermatozoa had a circular or swollen tail, while damaged spermatozoa showed a straight tail when exposed to a hypo-osmotic solution. They were then incubated at 37°C for 15 minutes. Spermataozoa was observed based on Eriani, et al (2017) using a binocular electric microscope with a magnification of 100x.

Fertilization

Ovaprim at a concentration of 0.3 ml/kg body weight were injected intramuscularly into Female broodstocks. The process of ovulation took over 10 hours after injection. Next, the broodstocks were stripped and the eggs were placed in a container. Four (4) ml of the eggs were placed into petri dishes and mixed with 1ml of sperm solute (ratio of sperm to egg 1: 4). Two drops of water were then added to activate the sperm, stirred using a feather, and left for two minutes. Fertility rate was calculated two hours after

mixing the sperm with the eggs (Dardiani & Sary, 2010).

Analysis of DNA Damage

The quality of fresh and frozen spermatozoa DNA was analyzed using a Genetic DNA Purification Kit (Promega, 2017). The process then was continued with electrophoresis using 1.5% agarose gel in TBE buffer solution in an electrophoresis chamber at 135V voltage. The electrophoresis process was stopped after 30 minutes. The method was carried out by placing an agarose gel on transilluminator platform and the band was compared to the markers and the amplicon band. The results were then documented. The DNA that experienced damage due to freezing was characterized by the formation of fragments when visualized using the transilluminator platform after electrophoresis (Yusoff et al., 2018).

Data analysis

Data were analyzed using ANOVA and SPSS Version 17, and continued with the Duncan Test. DNA fragmentation data were analyzed descriptively and presented in the form of images.

RESULT AND DISCUSSION

Quality of Fresh Semen

Fresh semen of Aceh Local catfish used in this study had met the standards to be continued to freez-

ing process. Semen was collected from healthy male catfish characterized by a body without any defects (Figure 1). The average concentration of Aceh Local catfish spermatozoa in this study was $4.2 \times 10^9 \text{ ml}^{-1}$, milky white in color, and with a thick consistency. This results are not much different from the results reported by Rustidja (2000) and Faqih (2011). Mass movements show a slightly slow movement with an average sperm motility of 62.25% (Table 1).

Table 1. Quality assessment of Aceh Local Catfish (*C. gariepinus*) fresh semen

Parameter	Result
Sperm cell concentration (10^9 /ml)	4.2 ± 1.9
pH	8
Color	Milky white
Consistency	Thick
Mass movement	++
Motility (%)	62.25 ± 0.95
Viability (%)	89.66 ± 3.54

+++ : mass movement of spermatozoa moving forward, slightly slow and resembling thick clouds

Quality of Spermatozoa after Cryopreservation

Motility is one of important parameters to determine the success of fertilization (Kang et al., 2004). The percentages of sperm motility of Aceh Local catfish are shown in Table 2.

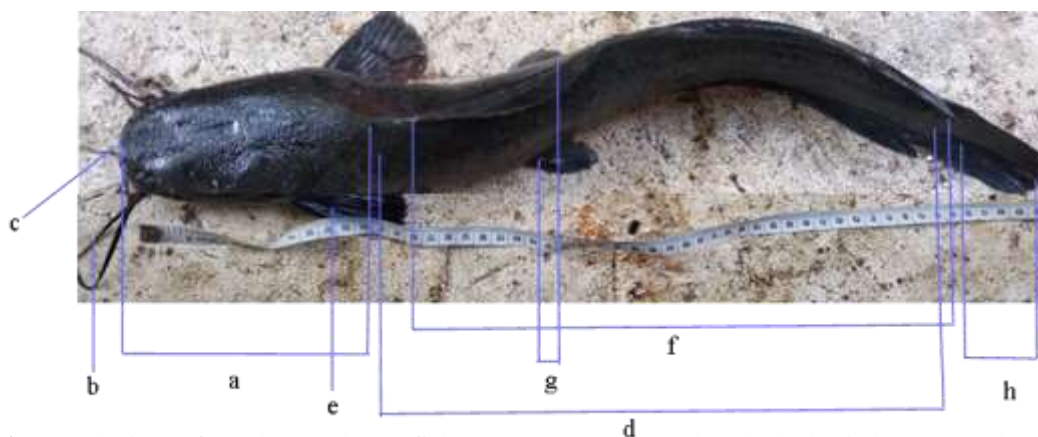


Figure 1. Morphology of Aceh Local Catfish (*C. gariepinus*); (a) head, (b) barbel, (c) mouth, (d) abdomen, (e) pectoral, (f) dorsal, (g) anal, (h) caudal

Table 2. Percentage of motility, plasma membrane integrity (PMI), and fertility of Aceh Local catfish (*Clarias gariepinus*) after freezing

Glutathione Concentration (mg L^{-1})	Motility (%)	PMI (%)	Fertility (%)
0 (Control)	$33.25^a \pm 4.43$	69.62 ± 7.00^a	70.50 ± 4.79^b
0.5	$34.50^a \pm 4.43$	57.00 ± 15.1^a	68.75 ± 9.17^a
1.0	$39.25^a \pm 5.16$	70.87 ± 8.86^a	70.50 ± 7.14^a
2.0	$40.50^a \pm 5.74$	62.25 ± 7.59^a	72.50 ± 11.09^a

Note: Means in the same column with different superscripts are significantly different ($P < 0.05$)

The results of motility in this study show there was no significant difference among all treatments. The motility of Aceh Local catfish spermatozoa in this study were 40.50% (P3), 39.25% (P2), 35.50% (P1), and 33.80% (P0). These values indicate that the higher concentration of glutathione, the higher rate of motility. This is in line with Rajmakers et al., (2003) who stated that high concentrations of glutathione can maintain spermatozoa motility.

The percentage of motility in P3 (40.50%) is higher than in the control (33.25%) due to the presence of glutathione. Glutathione is considered as an exogenous antioxidant that can react against ROS by acting as a cofactor for glutathione peroxidase which serves as a catalyst to reduce the toxic effects of hydrogen peroxide (Bilodeau et al., 2000). Glutathione acts as a free radical scavenger, stabilizing the membrane structure by eliminating or suppressing the formation of peroxide in lipid peroxide reactions (Price et al., 1990; Maia et al., 2009; Suryohudoyo 2000).

However, the low concentration of glutathione is unable to protect spermatozoa during the cryopreservation process. As shown in Table 1 and 2, the decrease in motility of Aceh Local catfish spermatozoa from fresh semen (62.25%) to P1 (34.50%) is presumably caused by the low concentration of glutathione that cannot maintain the motility of spermatozoa. This is in accordance with Holt (2000) who stated that glutathione at low concentration cannot work effectively in protecting spermatozoa from damage that caused by ROS. Excessive and unbalanced ROS production weakens the antioxidant enzymatic defense system, causing oxidative stress. Oxidative stress on spermatozoa destabilize the plasma membrane causing a reduction in sperm motility (Rizal, 2010).

The process of spermatozoa collection is also considered to be the cause of low rates of motility as reported by Urbanyi et al. (1999) in cryopreservation of Aceh Local catfish using the combination of 12% DMSO and fructose resulted in sperm motility at 25% after thawing. Lutfi et al. (2011) also reported that the addition of Ringer's with 15% DMSO resulting in Aceh Local catfish motility at 33.6%. The low motility rate in this study is probably due to the dissection process during sperm collection. Akçay et al. (2004) reported that the cryopreservation of carp semen using Ringer's and 2% of DMSO with the application of stripping method in gaining sperm, resulted in a motility rate of 55%. In comparison, the decrease in motility of Aceh Local catfish spermatozoa is considered due to the unfinished process of development, likely in the stages of spermatogonia or spermatocytes.

The concentration of glutathione used for Aceh Local catfish in this study was lower compared to the study carried out by Muthmainnah et al. (2018) in Kawan fish (*Osteochilus vittatus*) with an optimum

concentration of glutathione was 40 mgL⁻¹ which resulted in a rate of motility of 48.88%. The results of this study are in line with Lahnsteiner et al. (2011) who reported that the addition of 3 mmol/l glutathione in the cryopreservation of Trout (*Salvelinus fontinalis*) spermatozoa resulted in a motility rate of 28.7%. Therefore, it is assumed that by considering the high economic value of glutathione, the optimum concentration of glutathione for the motility of Aceh Local catfish spermatozoa is 1.0 mgL⁻¹.

Plasma membrane integrity is one of determinants of the quality of spermatozoa. The highest percentage of plasma membrane integrity in this study were P2 (70.87%), P0 (69.62%), P3 (62.25%), and P1 (57.00%). The statistical test showed that treatments P0, P1, P2 and P3 were not significantly different. These results indicate that glutathione is able to protect MPU from damage after freezing. In the other hand, FBS (which was also added into dilution) contain various elements such as hormones, vitamins, proteins, and other growth factors. The addition of FBS is an important factor in protecting the integrity of the plasma membrane during freezing (Garzón et al., 2008). In this study, FBS is used as a substitute for egg yolk (extracellular cryoprotectant) because FBS has a positive effect on protein, serum, and lipids, which play an important role in the clotting process as a defense mechanism in the instability of plasma membranes. In addition, it turns out that FBS also acts as a cryoprotectant compound (Koh et al., 2010).

Integrity of the plasma membrane is an important factor for the survival of spermatozoa. Damaged plasma membrane will affect the metabolic processes of spermatozoa which are related to the viability of the spermatozoa produced (Rizal 2005). Metabolism can work well as long as the plasma membrane still has its integrity. It can regulate traffic in and out of all the electrolytes needed in the metabolic process (Herdis et al., 2003). In addition, the integrity of plasma membrane is important to allow penetration of egg cell during the fertilization process (Ansari et al., 2010).

Fertilization is the ability of fish spermatozoa to fertilize an egg. The statistical tests show that there was no significant effect on fertilization rate among all treatments. The fertilization rate in this study is higher than Muthmainnah (2018) who reported that the fertilization rate of seurukan fish (*O. vittatus*) by the supplementation of 40 mgL⁻¹ DMSO is 45.67%. The higher percentage of fertilization in this study is due to the fact that serum (FBS) can increase the chance of interaction between the pellucid zone and spermatozoa and thereby increase the fertilization. In this case, the presence of serum stimulates the occurrence of capacitations in spermatozoa and acrosome

reactions. These results are in line with those reported by Alcay et al. (2019) who used Bovine Serum Albumin (BSA) as a substitute for egg yolk in the extender. BSA has a multifunctional effect on spermatozoa because of its macromolecular structure and antioxidants content.

The hormone content in the serum has a positive effect on the mechanism of fertilization. Some researchers report that there are certain components that are contained in serum such as protein, hemoglobin, glucose, insulin, cortisol, hormones, parathyroid, and prostaglandin E. These components make a serum that protects against the threats that come from the environment (Garzón et al., 2008; Yusoff et al., 2018). The results of this study also showed that the relationship between motility and fertilization was not directly proportional. This is presumably because the fertilization rate is not only influenced by the motility of spermatozoa but also influenced by the quality of the egg. This is consistent with the findings of Ardias (2008) who stated that the success of fertilization is strongly influenced by the quality of spermatozoa and the condition of the egg. Another cause of low fertilization rate is likely because spermatozoa unable to penetrate the eggs. Egg hole will be closed approximately one minute after exposed to water.

Assessment of DNA Integrity (Post-Thawing)

This study used a DNA laddering analysis method through gel electrophoresis by separating DNA fragments based on molecular weight. DNA analysis needs to be administered to examine DNA damage after cryopreservation. The results of the analysis of DNA integrity in frozen spermatozoa cells diluted in extenders plus various concentrations are presented in Figure 2.

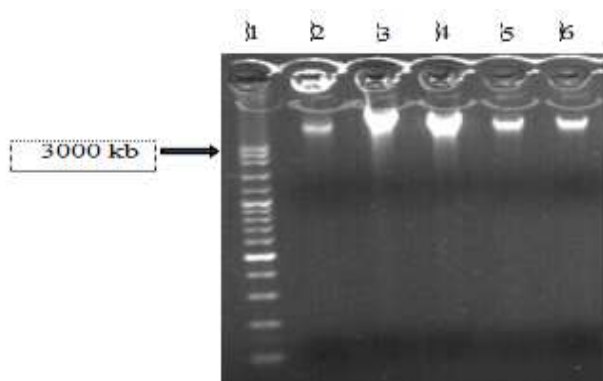


Figure 2. Electrogram of spermatozoa DNA of Aceh Local catfish Line 1=marker DNA, Line 2=DNA of fresh sperm, Line 3=DNA P0, Line 4=DNA P1, Line 5=DNA P2, Line 6=DNA P3

Figure 2 shows that higher glutathione concentrations can maintain DNA integrity after cryopreservation as described by Raijmakers et al., (2003) and

Bucak et al., (2010). Glutathione is thought to play a role as chelating agents as reported by Bucak et al., (2010) who states that antioxidants such as glutathione are capable of binding free radicals produced in the freezing process. Glutathione also takes part in the process of amino acid transport as well as DNA and protein synthesis. In line with the results of this study, Putranti et al., (2010) reported that the protein in diluents also help in maintaining the stability and permeability of spermatozoa plasma membranes by protecting the membrane, so that, only a few phospholipids of the plasma membrane undergo peroxidation. In this case, the addition of glutathione maintains the structure of DNA, making it remain stable, but the mechanism still unknown certainly. However, Yusoff et al., (2018) states that DNA laddering cannot be used as a benchmark in determining motility and fertilization.

This research is the first study to investigate the quality and DNA of spermatozoa after cryopreservation using Ringer's extender and cryoprotectant (DMSO and FBS) added with glutathione as an antioxidant.

The results obtained can provide information about the exact concentration of glutathione in the extender on the quality of spermatozoa of Aceh Local catfish (*C. gariepinus*) after the cryopreservation process. These results can also increase the success of fertility be used by the seed hall unit and the aquaculture industry to increase the productivity and supply high quality seeds.

CONCLUSION

The addition of 2.0 mg L⁻¹ of glutathione can maintain the quality of motility (40.50^b ± 5.74) and DNA integrity of Aceh Local catfish spermatozoa.

ACKNOWLEDGEMENT

This research was supported by the Kemenristekdikti through Master Thesis Research Grant (Contract Number 099/SP2H/LT/DPRM/2019). Therefore, the authors thank Kemenristekdikti for the financial support. Also thanks to BPBAP Ujong Batee where this research was conducted.

REFERENCES

- Adipu, Y., Sinjal, H. J., & Watung, J., (2011). Ratio Pengenceran Sperma Terhadap Motilitas Spermatozoa, Fertilitas Dan Daya Tetas Ikan Lele (*Clarias* sp.). *Jurnal Perikanan dan Kelautan Tropis*, 7(1), 48-55.
- Akçay, E., Bozkurt, Y., Seçer, S., & Tekin, N. 2004. Cryopreservation of Mirror Carp Semen. *Turkish*

- Journal of Veterinary and Animal Sciences*, 28(5), 837-843.
- Alcay, S., Toker, M. B., Gökçe, E., Önder, N. T., Üstüner, B., & Zekariya, N. U. R. (2019). Long Term Incubation Resilience of Post-Thaw Ram Semen Diluted with Lecithin-Based Extender Supplemented with Bovine Serum Albumin. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, 25(3), 291-297.
- Ansari, M. S., Rakha, B. A., Ullah, N., Andrabi, S. M. H., Iqbal, S., Khalid, M. & Akhter, S. (2010). Effect of exogenous glutathione in extender on the freezability of nili-ravi buffalo (*Bubalus bubalis*) Bull Spermatozoa. *Animal Science Papers and Reports*, 3 (28), 235-244.
- Ardias, N. (2008). Peranan NaCl terhadap derajat pembuahan, penetasan telur dan kelangsungan hidup larva Ikan Koi *Cyprinus carpio*. [Skripsi]. Fakultas Perikanan dan Ilmu Kelautan, Institut Pertanian Bogor, Bogor, 48 hlm.
- Asraf, A., Muchlisin, Z. A., & Siti-Azizah, M. N. (2013). Removal of eggs adhesiveness of african catfish (*Clarias gariepinus*) at different concentrations of urea solution. *Aceh International Journal of Science and Technology*, 2(3), 94-97.
- Bilodeau, J. F., Chatterjee, S., Sirard, M. A., & Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development: Incorporating Gamete Research*, 55(3), 282-288.
- Bucak, M. N., Tuncer, P. B., Sariözkan, S., Başpınar, N., Taşpınar, M., Çoyan, K., Bilgili, A., Akalın, P. P., Büyükleblebici, S., Aydos, S. & Ilgaz, S. (2010). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect dna integrity against cryodamage. *Cryobiology*, 61(3), 248-253.
- Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirao, J., Pérez-Cerezales, S., & Herráez, M. P. (2010). Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology*, 26(5), 623-635.
- Dardiani & I. R. Sary (2010). Mata Diklat 4 Manajemen Penetasan Telur dan Pemeliharaan Larva. Pusat Pengembangan dan Pemberdayaan Pendidik dan Tenaga Kependidikan. Pertanian Direktorat Jenderal Peningkatan Mutu Pendidik dan Tenaga Kependidikan. Departemen Pendidikan Nasional.
- Dhanju, C. K., Cheema, R. S. & Kaur, S. P. (2001). Effect of freezing on proteins and protein profiles of sperm membrane extracts and seminal plasma of buffalo bulls. *Asian-Australasian Journal of Animal Sciences*, 14(12), 1678-1682.
- Eriani, K., Azhar, A., Ihdina, M., Rosadi, B., Rizal, M., & Boediono, A. (2018). Quality enhancement of aceh swamp buffalo (*Bubalus bubalis*) frozen semen by supplementing β -carotene. *Tropical Animal Science Journal*, 41(1),1-7.
- Eriani, K., Sari, N., Rosnizar, Dasrul, Suhartono, S., Rizal, M. (2017). Cryopreservation of Aceh swamp buffalo (*Bubalus bubalis*) semen with combination of glycerol and lactose. *Biosaintifika*, 9(3), 409-416.
- Faqih, A. R. (2011). Penurunan motilitas dan daya fertilitas sperma ikan lele dumbo (*Clarias Sp*) pasca perlakuan stress kejutan listrik. *The Journal of Experimental Life Science*, 1(2), 72-82.
- Herdis, Toelihere M. R, Supriatna I, Purwaritara, B, dan Adikara R. T. S. (2003). Integritas dan daya hidup spermatozoa pada pembekuan semen domba garut (*Ovis arios*) dengan pengenceran dasar tris dan susu skim kuning telur. *Jurnal Sains dan Teknologi Indonesia*, 2 (3), 62-68.
- Holt, W. V. (2000). Basic Aspects Of Frozen Storage Of Semen. *Animal Reproduction Science*, 62(1-3), 3-22.
- Garzón, D. L., Peñaranda, D. S., Pérez, L., Marco-Jiménez, F., Espert, X., Müller, T., & Asturiano, J. F. (2008). Effects of pH, sodium bicarbonate, cryoprotectants and fetal bovine serum on the cryopreservation of european eel sperm. *Reproduction in Domestic Animals*, 43(1), 99-105.
- Kang, K. H., Kho, K. H., Chen, Z. T., Kim, J. M., Kim, Y. H., & Zhang, Z. F. (2004). Cryopreservation of Filefish (*Thamnaconus septentrionalis* Gunther, 1877) Sperm. *Aquaculture Research*, 35(15), 1429-1433.
- Koh, I. C. C., Hamada, D., Tsuji, Y. A., Okuda, D., Nomura, K., Tanaka, H. & Ohta, H. 2017. Sperm cryopreservation of japanese eel, *Anguilla japonica*. *Aquaculture*. 473 : 487-492.
- Konings, A., Freyhof, J. & FishBase team RMCA, Geelhand, D. (2018). *Clarias gariepinus*. The IUCN Red List of Threatened Species 2018: e.T166023A115860648.
- Lahnsteiner, F., & Mansour, N. (2010). A comparative study on antioxidant systems in semen of species of the *Percidae*, *Salmonidae*, *Cyprinidae*, and *Lotidae* for improving semen storage techniques. *Aquaculture*, 307,130-140.
- Lahnsteiner, F., Mansour, N., & Kunz, F. A. (2011). The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). *Theriogenology*, 76 (5), 882-890.
- Lutfi., Arifiantini, R. I., & Purwantara, B. (2011). Pembekuan Semen Lele Dumbo (*Clarias gariepinus* Burchell 1822) Sebagai Model Kriopreservasi Semen Ikan. *Jurnal Ikhtologi Indonesia*, 11 (2), 169-175.

- Mahyudin, K. (2013). *Panduan Lengkap Agribisnis Lele*. Penebar Swadaya; Jakarta.
- Muchlisin, Z.A., Hashim, R. & Chong, A.S.C. (2004). preliminary study on the cryopreservation of tropical bagrid catfish (*Mystus nemurus*) spermatozoa; the effect of extender and cryoprotectant on the motility after short-term storage. *Theriogenology*, 62 (1-2) : 25-34.
- Muchlisin, Z. A., Nadiyah, W. N., & Siti-Azizah. M. N. (2015). Exploration of natural cryoprotectants for cryopreservation of african catfish, *Clarias gariepinus*, Burchell 1822 (*Pisces: Clariidae*) spermatozoa. *Czech Journal of Animal Science*, 60 (1), 10-15.
- Muthmainnah, C. R., Eriani, K., Hasri, I., Irham, M., Batubara, A. S., & Muchlisin, Z. A. (2018). Effect of glutathione on sperm quality after short-term cryopreservation in surukan fish *Osteochilus vittatus* (Cyprinidae). *Theriogenology*, 122, 30-34.
- Promega. (2017). *Wizard® Genomic DNA Purification Kit*. USA.
- Putranti, O., Kustono & Ismaya. (2010). Pengaruh Penambahan Crude Tannin pada Sperma Cair Kambing Peranakan Ettawa yang Disimpan Selama 14 Hari Terhadap Viabilitas Spermatozoa. *Buletin Peternakan*, 34 (1), 1-7.
- Raijmakers, M. T., Roelofs, H. M., Steegers, E. A., Mulder, T. P., Knapen, M. F., Wong, W. Y., & Peters, W. H. (2003). Glutathione and glutathione s-transferase A1-1 and P1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa. *Fertility and Sterility*, 79(1), 169-172.
- Rizal, M. (2005). Daya hidup spermatozoa epididimis domba garut yang dikriopreservasi menggunakan modifikasi pengencer tris. *Hayati Journal Of Biosciences*, 12(2), 61-66.
- Rizal, M., & Herdis. (2010). Peran Antioksidan dalam Meningkatkan Kualitas Semen Beku. *Wartazoa*, 20 (3), 139-145.
- Rustidja. 2000. Prospek Pembekuan Sperma. Fakultas Perikanan Universitas Brawijaya. Malang.
- Shui, G., Wong, S.P. & Leong, L.P., (2004) . Characterization of Antioxidants and Change of Antioxidant Levels During Storage of *Manilkara Zapota* L. *Journal of Agricultural and Food Chemistry*, 52(26) : 7834-7841.
- Sulmartiwi, L., Ainurrohmah, E., & Mubarak, A. S (2011). Pengaruh konsentrasi air kelapa muda dan madu dalam NaCl fisiologis terhadap motilitas dan lama hidup spermatozoa ikan patin (*Pangasius pangasius*). *Jurnal Ilmiah Perikanan Dan Kelautan*, 3(1), 67-72.
- Urbanyi, B., Horvath, A., & Dinnyés, A. (1999). Cryopreservation of African catfish (*Clarias gariepinus*) sperm using different cryoprotectants. *Theriogenology*, 51(1), 296.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J. (2007). free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, 39(1), 44-84.
- Wachirachaikarn A., Rungsin, W., Srisapoom, P. and Na-Nakorn, U. (2009). Crossing of African catfish (*Clarias gariepinus*) strains based on strain selection using genetic diversity data. *Aquaculture*, 290: 53-60.
- Yusoff, M., Hassan, B. N., Ikhwanuddin, M., Sheriff, S. M., Hashim, F., Mustafa, S. & Koh, I. C. C., (2018). Successful sperm cryopreservation of the brown-marbled grouper, *Epinephelus fuscoguttatus* using propylene glycol as cryoprotectant. *Cryobiology*, 81, 168-173.
- Zilli, L., Schiavone, R., Zonno, V., Rossano, R., Storelli, C. % Vilella, S. (2005). Effect of Cryopreservation on Sea Bass Sperm Proteins. *Biology of Reproduction*, 72(5), 1262-1267.