

The Effect of Sanrego Wood (*Lunasia amara* Blanco) Extract Addition to the Andromed® Diluent on Sperm Quality of Belgian Blue Crossbreeds Bull

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ABSTRACT

Sperm quality is vital for livestock reproduction. Without good sperm quality, reproduction will not occur properly, which will cause a hamper in the increase of livestock population. Sanrego (*Lunasia amara* Blanco) is a plant that has an aphrodisiac effect that can increase libido, so this plant is very interesting to study whether it can maintain sperm quality during sperm processing and storage. Belgian Blue Crossbreeds is crossed between Belgian Blue cattle and Brahman cattle which needs to be improve local cattle in Indonesia. The purpose of this study was to determine the effect of adding Sanrego wood extract (*Lunasia amara* Blanco) to the Andromed® diluent on the sperm quality of Belgian Blue Crossbreeds bull. The treatment of this research consisted of Andromed® diluent (P0) as control, Andromed® diluent and 2% Sanrego wood extract (P1), Andromed® diluent and 3% Sanrego wood extract (P2), and Andromed® diluent and 4% Sanrego wood extract. (P3). The measured variables were motility and viability of spermatozoa. Observations were made in the first 3 hours and then every 24 hours until the fifth day. Data obtained were analyzed using analysis of variance based on Completely Randomized Design (CRD). The results showed that the addition of Sanrego wood extract could increase ($P < 0.05$) motility and viability of spermatozoa stored at 5 °C. The motility of spermatozoa on the fifth day at P0, P1, P2, and P3 were 35.4±1.5%, 42.3±0.4%, 44.4±2.1% and 43.2±1.8%, respectively. The viability of spermatozoa on the fifth day at P0, P1, P2, and P3 were 55.0±1.9%, 64.0±1.2%, 70.2±2.3% and 68.2±1.6%, respectively. The conclusion of this study was that the addition of Sanrego wood extract to the Andromed® diluent could improve the sperm quality of Belgian Blue Crossbreeds bull when compared to controls such that it might be utilized as a diluent alternative to improve liquid sperm quality.

Keywords: Sanrego wood extract; Andromed®; Sperm quality; Belgian Blue Crossbreeds.

1. INTRODUCTION

The difficulty of increasing population and productivity was one of the issues confronting the Indonesian beef cattle industry. To increase productivity, a cross-breeding program between local and European cattle breeds is an option that can be implemented in Indonesia. One of the cross-breeding breed was the crossbreed of Belgian Blue cattle with the Brahman cattle and produced another Indonesian BB cattle breeds. The “double muscling” characteristic of Belgian Blue cattle is a distinct phenotypic[1]. The double muscling phenomenon in Belgian Blue cattle can increase carcass percentage by deleting 11 bases in the third exon of the myostatin gene, which caused the phenomenon and made the myostatin gene a major gene candidate for animal growth. Because of this

phenomenon, the Belgian Blue is highly preferred in the crossing program and successfully raised meat production in a number of countries with artificial insemination (AI) methods[2] which collected BB cattle semen and put to the Brahman cows. Following sperm ejaculation, spermatozoa undergo irreversible aging, which eventually leads to death. It is thus the goal of animal breeders to extend the life of sperm cells by using cryopreservation agent in semen diluent [3]. Several herbal extracts and plant-derived pure molecules have been shown to have an effect on the reproductive process. Furthermore, recent research has shown that administration of plant extracts improves sperm parameters, androgen status, fertility index, and has a positive effect on sperm quality in males [4], [5].

Sanrego (*Lunasia amara* Blanco) has been used in folk medicine to increase and/or treat male fertility [6]. Sanrego is a herb which contains steroid that has aphrodisiac features and can increase sexual libido [7]. In vivo, Sanrego extract increased male fertility and libido significantly by increasing testosterone levels and antioxidant enzyme activity [8]. However, there is no scientific evidence supporting the Sanrego's significant effect as diluent on liquid sperm quality. The aim of this study was to identify the effect supplementing extract of *Lunasia amara* Blanco in sperm diluent had on sperm quality, specifically sperm motility and viability as well as knowing the proper amount of sanrego concentration to use as a diluent.

2. MATERIAL AND METHODS

2.1. Animal and chemicals

Two reproductive tracts of mature Belgian Blue Crossbreeds bull were maintained under standard field, laboratory conditions and performed following the University guidelines. The chemicals used were diluent liquid andromed (Minitube, Germany), eosin (Sigma-Aldrich, USA), liquid nitrogen, and aquabidest.

2.2. Semen collection and initial evaluation

Semen was collected using an artificial vagina initially evaluated for sperm quality. Semen have normal color, 0.8–0.2 mL volume, 88.2 ± 0.8% motility and 90.8 ± 1.1% viability and 3180 ± 204.9 10⁶/mL concentration, <10% abnormality. Based on these findings, the quality of fresh bull sperm can be assessed in subsequent processing. Sanrego extract made by mixed Sanrego plant which diluted in 96% ethanol used maceration method for 24 h with three times stirring for three days

2.3. Post chilled semen evaluation

The semen ejaculates were pooled to avoid individual effects. Initially, the pooled semen was diluted with andromed 1:4. The diluted samples were split into four aliquots according to Sanrego extract concentration (P0 0% control; P1:2%; P2: 3%; and P3:4%). The samples were kept in a cold cabinet for 120 h at 5 °C and monitored for sperm motility and viabilities in D-1, D-4 and D-5. The sperm motility was observed by putting and homogenizing 10 µL of diluent mixed with NaCl (1:4) and then placed on the microscope (Olympus CH 20). The view was taken from ten fields at a magnification of 100x400, scores were given in the range 0-100% with a 5% scale. Eosin staining procedure was used for sperm viability. A total of 200 spermatozoa were counted per sample using light microscope (Olympus CH 20) to differentiate the reacted and non-reacted spermatozoa. The death sperm

with damaged acrosome emitted the strong red color whereas non-reacted with were live sperm emitted with light pink or no-color.

2.4. Statistical analysis

Result were expressed as mean ± standard deviation (SD). The statistical significant between the mean values was determined by one-way analysis of variance (ANOVA) followed by Duncan post hoc test with $p < 0.05$ as the statistically significant criterion. A total of four independent experiments were performed.

3. RESULT AND DISCUSSION

The advancement of animal reproduction has been aided by the use of cryopreservation and artificial insemination technology. However, a significant proportion of spermatozoa undergoes alterations and loses fertility during cryopreservation, making frozen-thawed sperm unsuitable for routine use. Cryopreservation has been shown to reduce sperm longevity and fertility. Cryosurvival of spermatozoa from different bulls, and even from the same bull, varies in artificial insemination (AI) centers [3]. The goal of cryopreservation is to preserve as many post-thawed viable normal spermatozoa as possible while maintaining the original pre-freezing sperm quality parameters such as structural integrity, viability, motility, DNA integrity, and biological function related to fertilization competence. However, the cryopreservation process reduces sperm function and fertility. Spermatozoa are confined to cold shock during cryopreservation, leading to lower post-thaw sperm survival and, for those that survive but are cryocapacitated, a shorter lifespan in the female reproductive tract. The negative effects of cryopreservation on sperm include, in addition to cold shock, other damages such as osmotic stress and changes in membrane fluidity and permeability, which leads to lower motility and viability numbers. [6]

Sperm motility refers to the ability of sperm to move properly toward an oocyte. It is well understood that sperm motility is an important factor in determining the quality of sperm. One of the most common causes of subfertility or infertility is a lack of sperm motility [9]. Sperm fertilization capacity is affected not only by motility but also by other factors such as viability and sperm quality. DNA fragmentation is becoming increasingly recognized as an important factor in this [10], [11]. Sperm motility was measured in both the treated and control groups. During the counting of all motile and immotile spermatozoa, the sperm motility was evaluated. The effect of *Lunasia amara* Blanco on the total motility and viability of sperm in all the experimental and controlling group were reported in Table 1. In comparison to the control, incubating

Belgian Blue Crossbreeds bull semen Sanrego extract (treatment group) for all holding times (D-5) had significant effect ($P < 0.05$) on total sperm motility and viability. However, no significant differences were found in each treatment concentration group. Washed spermatozoa are used in artificial insemination procedures. Poor sperm motility is a common issue in many cases of infertility. In these procedures, advanced techniques for optimizing sperm function are clearly beneficial [12]. Many herbal medicines have been shown to improve sperm motility. According to the studies on the effect of Sanrego extract on Belgian Blue Crossbreeds bull sperm motility and viability in vitro, this extract could significantly increase sperm motility and viability in cattle, which is similar to previous research in mice [6], [13]. The mechanism by which this action occurs is still unknown and requires further investigation. This biological activity could be related to one or more of the extract's compounds/phytochemicals.

The previous study found that Sanrego extract has antioxidant activity with IC_{50} was $69.46 \mu\text{g/ml}$ (0.069 mg/ml) and it contained steroid, phenolic, saponin, alkaloid and coumarine compounds [14] so that it can be used as an antioxidant to improve liquid sperm quality. Lutfi [6] stated that supplementation *Lunasia amara* Blanco in Vivo could increase the fertility potential of male rats directly or indirectly, primarily by acting directly on the pituitary gland and influencing its secretion or the pituitary gland– spermatogenic axis. Because our experiments were conducted in vitro, the stimulatory effects on motility could not have been obtained through androgenic mechanisms but more likely via increasing antioxidant mechanism to against oxidative free radicals or via increasing intracellular calcium cyclic nucleotides. A significant stimulatory effect on bull sperm motility could be associated to trace elements, particularly Ca^{2+} , found in Sanrego extract. Ca^{2+} could inhibit the enzyme phosphate diesterase, restricting cyclic adenosine monophosphate degradation and increasing sperm motility [11]. Furthermore, free radicals play a crucial role in male infertility since antioxidants could preserve sperm from their harmful effects.

Antioxidants have been shown to have the greatest affect on sperm motility [15]. Sanrego extract contains polyphenols, a class of compounds that includes phenolic acids and flavonols [14]. These elements have a strong relationship with antioxidant activity [16]. This extract has antioxidant activity, which could be another effective mechanism for improving bull sperm motility. Oxidative stress negatively affects sperm function and reduces male fertility by decreasing sperm viability. High NO concentrations could have a cytotoxic effect on sperm viability, which is most possibly mediated by oxidative stress and lipid peroxidation of sperm membranes [17]. ROS levels that are too high directly damage oocytes and sperm DNA and cause sperm

apoptosis. ROS disrupts the integrity of sperm DNA and contributes to lipid peroxidation in the male reproductive system [16]. Excessive ROS production in the reproductive tract can damage both the fluidity of the sperm plasma membrane and the integrity of the DNA in the sperm nucleus, leading to spermatogenesis dysfunction and toxic effects on sperms, resulting in lipid peroxidation of the sperm membrane [17]. Antioxidants like in Sanrego could directly scavenge ROS, inactivate them, and repair the damage. They also demonstrated a wide range of biological activities as a result of their ability to mimic endogenous estrogen actions, inhibit hormone actions, and modulate hormone production. This antioxidants also reduced lipid peroxidation levels when compared to control samples. Because of the ROS-induced damage in ATP utilization, the levels of lipid peroxidation increase during incubation, altering sperm motility [9]. The use of these natural antioxidants like in Sanrego would be dependent on the technique used in the fertilization process, with them being useful in procedures such as intrauterine insemination that are compatible with a limited lifespan of the sperm. As a result of the not significant differences found in each treatment group, future research with a higher concentration range is required to evaluate a better dose of effectiveness in administering Sanrego extract.

4. CONCLUSION

The conclusion of this study was that the addition 2-4% of Sanrego wood extract to the Andromed® diluent could improve the sperm quality of Belgian Blue Crossbreeds bull when compared to controls such that it might be utilized as a diluent alternative to improve liquid sperm quality.

AUTHORS' CONTRIBUTIONS

All authors contributed to the design, execution, analysis, and write up of this article.

ACKNOWLEDGMENTS

The author would thanks the Faculty of Deputy for Strengthening Research and Development, Ministry of Research and Technology/National Research and Innovation Agency, PT Widodo Makmur Perkasa, PT Pasir Tengah, and Bengkel Sapi Kalijeruk Yogyakarta for partially sponsoring the research.

REFERENCES

- [1] P. P. Agung, S. Said and A. Sudiro. 2016 Myostatin gene analysis in the first generation of the belgian blue cattle in Indonesia. *J. Indones. Trop. Anim. Agric.* 41 (1):13–20, 2016. DOI: <https://doi.org/10.14710/jitaa.41.1.13-20>

- [2] G. Domingo, J. Cantalapidiera, I. B. Pernodo, R. P. Carriera, L. Monserrat and L. Sanchez. 2014. Performance of crossbreed fattened calves in commercial farms in Spain,” *J. Anim. Plant Sci.* 24(3):722–729 ISSN: 1018-7081.
- [3] M. P. B. Nagata, J. Egashira, N. Katafuchi, K. Endo, K. Ogata, K. Yamanaka, T. Yamanouchi, H. Matsuda, Y. Hashiyada and K. Yamashita. 2019. Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. *J. Anim. Sci. Biotechnol.* 2019. 10 (1): 1–14. DOI: 10.1186/s40104-019-0395-9.
- [4] M. Askaripour, A. Hasanpour, F. Hosseini, M. Moshrefi, G. Mostaghi, M. hasannejad, S. Rajabi, S. Noueddin and N. Mahani. 2018. The effect of aqueous extract of *Rosa damascena* on formaldehyde-induced toxicity in mice testes. *Pharm. Biol.* 56 (1):12–17. DOI: 10.1080/13880209.1413663
- [5] F. Rahmouni, S. Daoud and T. Rebai. 2019. *Teucrium polium* attenuates carbon tetrachloride-induced toxicity in the male reproductive system of rats. *Andrologia.* 51 (2):1–8. DOI: 10.1111/and.13182.
- [6] M. J. Luthfi. 2015. Effect of *Lunasia amara* Blanco on Sperm Number, Sperm Motility, and Testicular Histology of Male Rats. *Biol. Med. Nat. Prod. Chem.* 4 (2):31. DOI: 0.14421/biomedich.2015.42.31-33
- [7] N. Rahmawati, A. Prichatin and K. Dewi. 2012. Aphrodisiac Effect of *Lunasia amara* Blanco , *Centella asiatica* and *Curcuma domestica* Combination Infusion on Male Rat Libido. *Int. Conf. Res. Appl. Tradit. Complementary Altern. Med. Heal. Care.* 1 (1):22–23.
- [8] R. Nor-Raidah and M. N. Mahanem. 2015. Enhancement of fertility and libido in male sprague dawley rats following the administration of aqueous extract of *Lunasia amara*. *Malaysian Appl. Biol.* 44 (1): 125–131. DOI: <http://journalarticle.ukm.my/8719/>
- [9] M. Remya, R. C. Sharma, H. Shoaib, R. J. U. A. Asad and S. Swati. 1999. Effect of cyclosporine on human sperm motility in vitro. *Arch. Androl.* 43 (3): 215–220. DOI: 10.1080/014850199262526
- [10] G. López, R. Lafuente, M. A. Checa, R. Carreras and M. Brassesco. 2013. Diagnostic value of sperm DNA fragmentation and sperm high-magnification for predicting outcome of assisted reproduction treatment. *Asian J. Androl.* 15 (6): 790–794. DOI: 10.1038/aja.2013.81.
- [11] S. Khaleghi, M. Bakhtiari, A. Asadmobini and F. Esmaeili. 2017. *Tribulus terrestris* Extract Improves Human Sperm Parameters In Vitro. *J. Evidence-Based Complement. Altern. Med.* 22(3):407–412. DOI: 10.1177/2156587216668110
- [12] J. Liu, P. Liang, C. Yin, T. Wang, H. Li, Z. 2004. Effects of several Chinese herbal aqueous extracts on human sperm motility in vitro. *Andrologia.* 36 (2):78–83. DOI: 0.1111/j.1439-0272.2004.00607.x
- [13] M. J. far Luthfi, A. Kamalrudin and M. M. Noor. 2017. Effects of *Lunasia amara* blanco (Sanrego) on male fertility: A preliminary study on sperm proteomic analysis. *J. Appl. Pharm. Sci.* 7 (8):085–091. DOI: 10.7324/JAPS.2017.70812
- [14] H. Hasnaeni and A. Aminah. 2019. Uji Aktivitas Antioksidan dan Profil Fitokimia Ekstrak Kayu Beta-beta (*Lunasia amara* Blanco.). *J. Farm. Galen. (Galenika J. Pharmacy).* 5 (1):101–107. DOI: 10.22487/j24428744.2019.v5.i1.12404
- [15] M. N. Banday, F. A. Lone, F. Rasool, M. Rashid and A. Shikari. 2016. Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation. *Cryobiology.* 74 :25–30. DOI: 10.1016/j.cryobiol.2016.12.008
- [16] J. S. Len, W. S. D. Koh and S. X. Tan. 2019. The roles of reactive oxygen species and antioxidants in cryopreservation. *Biosci. Rep.* 39 (8). DOI: 10.1042/BSR20191601
- [17] E. Tvrda, Z. Knazicka and N. Lukac. 2012. Selected heavy metals versus antioxidant parameters in bull seminal plasma - A comparative study. *J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng.* 47 (9): 1261–1266. DOI: 10.1080/10934529.2012.672117

Table 1. Effect of Sanrego extract (*Lunasia amara* Blanco) addition in semen extender on Belgian Blue cattle sperm motility and viability stored at 5°C for 5 days

Tomato extract	Motility (%)			Viability (%)		
	D-1	D-4	D- 5	D-1	D-4	D- 5
Control	80.0±1.6 ^a	37.0±1.6	35.4±1.5 ^{Ab}	84.4±1.1 ^a	57.4±1.8	55.0±1.9 ^{Ab}
2%	80.6±1.1 ^a	44.8±1.8	42.3±0.4 ^{Bb}	84.8±1.5 ^a	68.3±1.9	64.0±1.2 ^{Bb}
3%	81.0±1.9 ^a	47.2±0.8	44.4±2.1 ^{Bb}	86.2± 1.5 ^a	75.7±1.6	70.2±2.3 ^{Bb}
4%	81.6±1.3 ^a	46.2±1.3	43.2±1.8 ^{Bb}	87.2±1.1 ^a	74.6±1.9	68.2±1.6 ^{Bb}

^{ABCD}Means in a row not sharing superscript show significant differences ($p < 0.05$) for of Sanrego extract