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Effect of Vitamin E Supplementation in Diluent on Fertility Parameters of Different Quality Fresh Bull Sperm

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ABSTRACT

This study aimed to determine the effect of vitamin E supplementation on tris-egg yolk diluent using different quality fresh semen. The research material was ejaculated from six Simmental bulls and then classified into standard quality (SQ) and low quality (LQ) according to progressive motility. The research method was experimental with a randomized factorial design (2X5), with the group by fresh semen with 3 replications. The first factor was the quality of fresh semen, while the second factor was the level of vitamin E addition (B0: 0 mg/ml; B1: 0.5 mg/ml; B2: 1 mg/ml; B3: 1.5 mg/ml; and B4: 2 mg/ml). After thawing, the observation parameters were motility, viability, abnormality, plasma membrane integrity, and MDA levels. The result shows that the quality of fresh semen has a very significant effect ($p<0.01$) on the motility of frozen semen (66.28±8.66%) and a significant impact ($p<0.05$) on the plasma membrane integrity (91.31±2.87%). The use of vitamin E at 1 mg/ml showed the best results on motility (66.11±7.64%), viability (70.19±12.13), abnormality (12.00±3.13), and MDA (2.11±0.77). This study concludes that adding vitamin E to the diluent can increase motility, viability, and plasma membrane integrity while reducing abnormality and MDA levels. Low-quality fresh semen can be processed into frozen semen after adding vitamin E. The best level of vitamin E addition is 1 mg/ml.

Keywords: Spermatozoa, Fertility, Frozen Semen, Vitamin E,

INTRODUCTION

The frozen semen quality indicates the success of artificial insemination (Salan et al., 2021). The semen freezing process causes around 50% of spermatozoa to become immotile, which ultimately reduces fertilization ability (Hossain et al., 2023). The addition of diluent to fresh semen is an effort to maintain the viability of spermatozoa when inseminated (Arifiantini et al., 2012). During the cooling, freezing, and thawing process, the spermatozoa membrane is exposed to physical and chemical stresses that cause a decrease in viability, spermatozoa fertilization ability, lipid composition, and changes in the composition of the plasma membrane due to oxidative stress by ROS (Reactive Oxygen Species) (Alyethodi et al., 2021). The ROS are by-products of various enzymatic reactions from cells, including the cytoplasm, cell membrane, endoplasmic reticulum, mitochondria, and peroxisomes (Forrester et al., 2018). It produces free radicals with unpaired electrons, making them highly reactive. Free radicals react quickly with surrounding molecules to steal electrons (Kujoana et al., 2024). The free radical reaction causes the oxidation of polyunsaturated fatty acids (PUFA), which is referred to as lipid peroxidation (Nam, 2011). The oxidation can be prevented by adding antioxidants to the balance of the free radicals (Nurkhasanah et al., 2023), while the imbalance between ROS and available antioxidants results in oxidative stress (Slimen et al., 2014). This will reduce motility and damage spermatozoa cell macromolecules and the plasma membrane integrity of the spermatozoa (Raeeszadeh et al., 2022). Furthermore, controlling ROS levels by antioxidants is a mechanism to maintain cell homeostasis conditions, thereby reducing the production of free radicals that cause oxidative stress (Ayudiah et al., 2023).

One of the antioxidants that can be added to prevent oxidative stress is Vitamin E, a synthetic and non-enzymatic antioxidant. (Luo et al., 2023). Vitamin E is a fat-soluble antioxidant that donates its hydrogen groups so that free radicals become non-reactive (Akhter et al., 2023). It can also inhibit free radical reactions and lipid peroxidation that will cause reduced motility, reduced fructolysis, and cell membrane respiration. (Haris et al., 2020), increase motility, viability, and plasma membrane integrity in frozen semen (Priyanto et al., 2023), and reduce plasma membrane susceptibility to lipid peroxidation lipid (Feradis, 2009).

This study aims to determine the effect of vitamin E addition at different levels on fertility parameters of frozen semen processed using different fresh semen quality. Then, this study aims to determine whether, by adding vitamin E, low-quality fresh semen can be used in the manufacture of frozen semen. Finally, the most appropriate dose of vitamin E added to diluents will be known to produce the best post-thawing frozen semen.

MATERIAL AND METHOD

The research was conducted from September to December 2024 at the Tuah Sakato Regional Technical Implementation Unit Laboratory and Resource Development Center and the Livestock Biotechnology Laboratory of Andalas University. The research material used fresh semen from six Simmental bulls, which were collected using an artificial vagina. Each ejaculation is then analyzed and grouped based on progressive motility.

Research Design

The research design used was a Factorial Randomized Group Design with two factors and five levels (2x5) with groups in the form of fresh semen of the same quality of ejaculation. Repetition was carried out three times based on the quality of ejaculation. Factor A in this study was fresh semen quality by progressive motility, which is SQ for standard quality (motility of fresh semen >70%) and LQ for Low Quality (progressive motility of fresh semen <70%). Factor B is the level of vitamin E addition in diluent, namely B0: 0mg/ml (control); B1: 0.5 mg/ml; B2: 1 mg/ml; B3: 1.5 mg/ml; and B4: 2 mg/ml. Data analysis was performed using analysis of variance (ANOVA) with further tests using the Duncan Multiple Range Test (DMRT) to determine if there was a significant effect using the SPSS (Statistical Package for Social Sciences) Version 26 application.

Semen Collection and Dilution

Semen is collected from bulls that have been programmed for artificial insemination using an artificial vagina. The collection is carried out in a special collection location using another bull as a teaser in the morning with a frequency of collection twice a week. Based on the results of progressive motility evaluation, fresh semen was classified based on motility, standard quality, and low quality. This classification is based on the Indonesian national standard (National Standardization Agency of Indonesia, 2021). Each fresh semen was then diluted with egg-yolk tris diluent, which is a 100 ml extender containing 74% tris buffer (TRIS-hydroxymethyl-aminomethane, monohydrated citric acid, and fructose), 20% egg yolk, and 6% glycerol. The diluent is then added with vitamin E liquid (α-tocopherol water-miscible, Emprove®) with different levels of 0mg/ml, 0.5mg/ml, 1mg/ml, 1.5 mg/ml, and 2 mg/ml. Following the dilution of the semen, the freezing and storage procedures were executed by established standards. Furthermore, semen was analyzed after 48 hours submerged in liquid nitrogen. The research parameters were progressive motility, viability, abnormality, plasma membrane integrity, and malondialdehyde (MDA) level after thawing.

Semen Analysis

Progressive motility was evaluated by thawing frozen semen in a 37°C-38°C water bath for 30 seconds which was assessed in percent. (Nagata et al., 2019). It was observed by dripping 7µl of sample semen on a glass object then covered with a cover glass and analyzed using CASA (Computer Assisted Semen Analyzer Vers 3.7.5 SpermVision Software) (Hendri et al., 2024). Viability and abnormality parameters were calculated conventionally using the eosin-nigrosin staining method. (Tanga et al., 2021). Observations were made under a microscope at 400 times magnification in ten fields of view or at least 200 cells. (Saili et al., 2023). Viability count by identifying the colorless sperm as live sperm with the pink sperm as dead (Akhter et al., 2023). Abnormality values were calculated using the same sample. (Wijayanti et al., 2023) by observing sperm head shape, midribs, bent tails, and coiled tails (Hanson et al., 2023).

Plasma membrane integrity was calculated using the HOS (hypo-osmotic swelling) test. A total of 50 µl of the sample was incubated with 500 µl of HOS solution for 30 min at 37°C (Ahmed et al., 2024). 10 µl of the sample was dripped on the glass object and, covered with a cover glass, and counted using a microscope at 400 times magnification (Wang et al., 2023). Two hundred spermatozoa cells were counted by comparing cells with bulging or coiled tails, which means they have an intact plasma membrane. (Khalil et al., 2023).

Lipid peroxidation in frozen semen was quantified by calculating the level of malondialdehyde (MDA) (Su et al., 2025). According to Kumaresan et al. 2006, MDA

production in frozen semen was tested by thawing frozen semen back into microtubes. The sample was centrifuged at 3000 rpm for 15 minutes. After discarding the seminal plasma, 2 milliliters of PBS were added. The addition of TBA regent was then used to calculate lipid peroxidation. After 15 minutes of heating in a boiling water bath, the sample was centrifuged for another 15 minutes. The supernatant was separated and counted with a 535 nm spectrophotometer. MDA concentration was determined with a specific absorbance coefficient of $1.56x10^5/mol/cm^3$.

RESULT AND DISCUSSION

Motility After Thawed

Motility value is the main parameter in assessing male fertility (Pardede et al., 2020). Post-thawing frozen semen motility can be seen in Table 1. The study results showed that adding vitamin E can increase the motility of frozen semen ($P < 0.01$), with an SQ value of 66.28 ± 8.66% and LQ of 53.59 ± 9.45%. The addition of vitamin E level shows an increase in the value of frozen semen motility with the highest average at the level of addition of 1 mg/ml, which is 66.11 ± 7.64% ($p < 0.05$).

Table 1. Post-thawing Frozen Semen Motility (%)

Factor A	Factor B (Vitamin E Level)					Average
	B 0 (0 mg/ml)	B 1 (0.5 mg/ml)	B 2 (1 mg/ml)	B 3 (1.5 mg/ml)	B 4 (2 mg/ml)	
Standard Quality	59.88±12.59	69.91±2.10	72.06±3.86	67.56±10.20	62.03±9.07	66.28±8.66^{A}
Low Quality	43.33±8.40	55.27±10.15	60.15±4.97	63.91±6.34	48.34±6.18	53.59±9.45^{B}
Average	51.6±13.18^{a}	62.59±10.36^{ab}	66.11±7.64^{c}	64.24±8.28^{c}	55.18±10.22^{ab}	

Note: Superscripts with uppercase letters indicate a very significant effect ($p<0.01$), Superscripts with lowercase letters indicate a significant effect ($p<0.05$), and superscripts with the same letter indicate no significant difference.

Frozen semen standards are declared feasible in Indonesia if it has a post-thawing motility value of at least 40% (National Standardization Agency of Indonesia, 2021). The results showed that the motility of post-thawing frozen semen at the control level was 59.88±12.59% at SQ and 43.33±8.40% at LQ. This result shows that the motility value of frozen semen is more than the standard set, which means that frozen semen is suitable for insemination. Adding vitamin E as much as 0.5 mg/ml resulted in a higher frozen semen motility value than the control, which was 69.91±2.10% in SQ and 55.27±10.15% in LQ. This means that the addition of vitamin E can increase the value of post-thawing motility. According to the research of Nagata et al. (2019), the supplementation of vitamin E will positively affect progressive and non-progressive motility. The increase in frozen semen motility after the addition of vitamin E is due to the protective effect that can reduce oxidative damage during the freezing and thawing process (Houshaimy et al., 2018). Vitamin E is the most powerful antioxidant in inhibiting ROS production through the prevention of peroxyl and alkoxyl radicals during the lipid peroxidation process (Zhao et al., 2015). Furthermore, Mousavi et al. (2019) explained that the protective effect provided by Vitamin E is through the mechanism of scavenging free radicals produced by ROS. Then, damage to the genetic material of spermatozoa cells due to oxidation that occurs during freezing and thawing will also decrease (Espinosa-García et al., 2023).

Subsequently, the addition of Vitamin E at higher levels decreased the motility, although it was still better than the control. The highest SQ motility value was at the level of 1 mg/ml (72.06 ± 3.86%), the addition of higher levels of vitamin E decreased the motility value of frozen semen, 67.56 ± 10.20% (level 1.5 mg/ml) and 62.03 ± 9.07 (level 2 mg/ml). In LQ, the highest frozen semen motility was found at the level of 1.5 mg/ml which was 63.91 ± 6.34%. Adding a higher concentration of vitamin E, 2 mg/ml, decreased the motility value of frozen semen to 48.34 ± 6.18%. Adding vitamin E at higher levels decreases frozen semen motility, causing pro-oxidants that eventually form free radicals. This leads to increased damage to PUFAs exposed to free radicals, thus increasing lipid peroxidation. (Daramola et al., 2016).

Viability Spermatozoa

The effect of supplementation vitamin E in the diluent on the viability of frozen semen after thawing can be seen in Table 2.

Table 2. Viability After Thawing (%)

Factor A	Factor B (Vitamin E Level)					Average
	B 0 (0 mg/ml)	B 1 (0.5 mg/ml)	B 2 (1 mg/ml)	B 3 (1.5 mg/ml)	B 4 (2 mg/ml)	
Standard Quality	65.24±7.83	74.02±5.60	78.38±7.62	72.91±5.96	67.83±12.41	71.67±8.47 ^{ns}
Low Quality	58.22±7.92	60.14±9.00	61.99±8.48	59.40±5.78	63.04±12.33	60.56±9.44 ^{ns}
Average	61.73±8.77^{ns}	67.08±10.91 ^{ns}	70.19±12.13 ^{ns}	66.16±9.44 ^{ns}	65.43±12.63 ^{ns}	

Note: Superscript ns means no significant

In SQ, the highest viability was at the 1 mg/ml addition level, which was 78.38±7.62%, followed by the 0.5 mg/ml (74.02±5.60%) and 1.5 mg/ml (72.91±5.96%) levels. Slightly different results were found in LQ, that the highest viability was at the level (2 mg/ml) followed by 1 mg/ml (61.99 ± 8.48%), and 0.5 mg/ml (60.14 ± 9.00%). Based on the analysis of variance, the highest average viability value was found in the addition of vitamin E at the level of 1 mg/ml (70.19 ± 12.13%). The study shows that supplementation of vitamin E can increase the recovery value and viability compared to the control. Oxidative damage to spermatozoa during freezing and thawing can be protected by vitamin E by breaking the lipid peroxidation chain and the scavenging effect of vitamin E on free radicals. (Motemani et al., 2017), inhibiting lipid peroxidation and DNA damage (Yuan et al., 2023), while showing a protective effect on cryopreservation (Khan et al., 2021) and accelerates the viability of frozen semen after thawing (Abdel-khalek et al., 2022).

Spermatozoa Abnormalities

Table 3 shows the result of supplementation with vitamin E for abnormal spermatozoa. The quality factor of fresh semen does not significantly affect the abnormality of frozen semen. The abnormality of frozen semen in SQ is lower than that of LQ ($p>0.05$). The abnormality of SQ supplemented with 1 mg/ml vitamin E had the lowest value, ranging from 10.51 to 3.50 percent. Increasing the vitamin E level by 1.5 mg/ml and 2 mg/ml increased the abnormality of frozen semen. In the LQ, 2 mg/ml of vitamin E supplementation had the lowest abnormality value.

Table 3. Effect Supplementation of Vitamin E to Frozen Semen Abnormalities (%)

Factor A	Factor B (Vitamin E Level)					Average
	B 0 (0 mg/ml)	B 1 (0.5 mg/ml)	B 2 (1 mg/ml)	B 3 (1.5 mg/ml)	B 4 (2 mg/ml)	
Standard Quality	15.11±2.64	15.21±4.42	10.51±3.50	11.43±3.69	14.98±2.86	13.45±3.63
Low Quality	20.29±0.67	17.25±0.81	13.49±2.37	15.33±1.92	11.57±2.73	15.59±3.51
Average	17.70±3.32^{Bc}	16.23±3.06^{ABbc}	12.00±3.13^{Aa}	13.38±3.39^{ABab}	13.27±3.12^{ABab}	

Note: Superscripts with uppercase letters indicate a very significant effect ($p<0.01$), Superscripts with lowercase letters indicate a significant effect ($p<0.05$), and superscripts with the same letter indicate no significant difference.

The results showed that the quality factor of fresh semen did not significantly affect the abnormality of frozen semen. Abnormality of frozen semen in SQ was lower than in LQ ($p>0.05$). In the SQ, the abnormality level of 1,5 mg/ml supplementation had the lowest value (11.43±3.69%). The abnormality of frozen semen increased when the level of vitamin E was increased by 1.5 mg/ml and 2 mg/ml. In the LQ factor, the supplementation of vitamin E at the level of 2 mg/ml had the lowest abnormality. Analysis of variance showed that the addition of vitamin E at 1 mg/ml had a very significant effect ($p<0.01$) on the abnormality of frozen semen (12.00 ± 3.13%), which means that the addition of vitamin E at 1 mg/ml can reduce the abnormality of frozen semen compared to control (17.70 ± 3.32%). The decrease in abnormality due to the addition of vitamin E to frozen semen is explained by Ghirardosi et al. (2018) Which states that freezing and thawing will cause spermatozoa abnormality, especially in the middle part of the spermatozoa body in its distal part. This is due to the accumulation of ROS formed in freezing semen and thawing. (Motemani et al., 2017). The accumulation of ROS eventually causes head anomalies and neck integrity and affects the structure and function of the tail. (Dementieva et al., 2024).

The addition of vitamin E in a diluent that exceeded the dose of 1 mg/ml increased the abnormality of frozen semen. Adding vitamin E as much as 1.5 mg/ml and 2 mg/ml resulted in abnormality values of 13.38 ± 3.39% and 13.27 ±

3.12%, respectively. Savitri et al. (2014)This is due to the reduced antioxidant effect of adding high concentrations to cause prooxidants.

The results of the study on the effect of vitamin E addition on the plasma membrane of frozen bovine semen can be seen in Table 4.

Plasma Membrane Integrity

Table 4. Effect of vitamin E addition on Plasma Membrane Integrity of frozen semen after thawing (%)

Factor A	Factor B (Vitamin E Level)					Average
	B 0 (0 mg/ml)	B 1 (0.5 mg/ml)	B 2 (1 mg/ml)	B 3 (1.5 mg/ml)	B 4 (2 mg/ml)	
Standard Quality	89.28±1.86	90.63±3.36	91.74±1.46	93.18±3.97	91.99±3.36	91.31±2.87[a]
Low Quality	76.08±11.28	84.43±4.75	86.64±1.59	89.89±4.03	86.45±8.47	84.70±7.58[b]
Average	82.68±10.23	87.40±4.91	88.19±3.11	91.54±4.00	89.22±6.51	

Note: Superscripts with lowercase letters indicate a significant effect ($p<0.05$), and Superscripts with the same letter indicate no significant difference.

The results showed that there was a significant effect ($p<0.05$) between the use of SQ and LQ after being treated with vitamin E on the integrity of the plasma membrane. SQ has an average value of plasma membrane integrity of 91.31 ± 2.87%, while LQ has a plasma membrane integrity of 84.70 ± 7.58%. This means that the use of fresh semen of standard quality and lower quality has an influence on the plasma membrane's integrity of frozen semen after thawing. Standard-quality frozen semen has better plasma membrane integrity than lower quality. According to Giaretta et al. (2025), this difference is due to the advanced oxidative protein products in the plasma membrane that affect the quality of fresh semen.

In the current study, the supplementation of vitamin E does not significantly affect the integrity of the plasma membrane ($p>0.05$). The level of vitamin E addition as much as 1.5 mg/ml is known to produce the highest value of plasma membrane integrity, 93.18 ± 3.97% of SQ and 89.89 ± 4.03% to LQ. These results indicate that the addition of vitamin E produces better values than control. Although not significantly affected, the addition of vitamin E was able to increase the value of plasma membrane integrity of frozen semen after thawing. This result supports Ullah et al.*'s* (2019) research, which found that lipid peroxidation can be decreased by adding 1.5 mg/ml. Lipid peroxidation is triggered by oxidative stress and the freezing process, both of which produce ROS. Aldehydes produced by this lipid peroxidation attach to proteins on lecithin, lysine, and cysteine residues to create free radicals. Furthermore, decreased spermatozoa also produce ROS, which damages the plasma membrane of surviving spermatozoa cells and causes hyperviscosity (Upadhyay et al., 2022).

Due to their high PUFA content, plasma membranes are more vulnerable to lipid peroxidation, which damages them. (Lone et al., 2018). According to Akhter et al. (2023), vitamin E in frozen semen can neutralize free radical activity while shielding sperm from ROS activity. Agarwal et al. (2012), state that adding vitamin E as an antioxidant lessens damage to plasma membranes because it prevents frozen semen from oxygen species interactions that occur during freezing. By scavenging free radicals, vitamin E lessens this type of oxidative damage. As a result, spermatozoa function and fertility decline (Motemani et al., 2017).

MDA Level of Frozen Semen

The effect of Vitamin E addition on MDA levels in semen of different quality can be seen in Table 5. The table shows that adding vitamin E to SQ and LQ has no significant effect on MDA levels in frozen semen ($P>0.05$). The incorporation of vitamin E into SQ yielded reduced MDA levels compared to LQ, with measurements of 2.30 ± 0.82 and 2.42 ± 0.59, respectively. In SQ, the minimum MDA level was observed with the addition of 2 mg/ml of vitamin E, measuring 1.69 ± 0.50 in comparison to the control group. LQ exhibited slightly varied results, with the lowest MDA levels at a 1 mg/ml vitamin E concentration, 2.11 ± 0.77. The optimal concentration of vitamin E for minimizing MDA levels is 1 mg/ml, resulting in MDA values of 2.11 ± 0.77.

Table 5. Effect of vitamin E addition on post-thawing frozen semen MDA levels

Factor A	Factor B (Vitamin E Level)					Average
	B 0 (0 mg/ml)	B 1 (0.5 mg/ml)	B 2 (1 mg/ml)	B 3 (1.5 mg/ml)	B 4 (2 mg/ml)	
Standard Quality	3.01±0.86	1.99±0.40	2.07±1.15	2.73±0.64	1.69±0.50	2.30±0.82
Low Quality	2.67±0.78	2.27±0.81	2.16±0.37	2.40±0.84	2.60±0.22	2.42±0.59
Average	2.84±0.76	2.13±0.59	2.11±0.77	2.57±0.69	2.14±0.60	

MDA is the outcome of lipid peroxidation resulting from the interaction of reactive oxygen species (ROS) with polyunsaturated fatty acids (PUFA). The findings of this study align with the research by Sharafi et al., (2022), which indicates that the incorporation of antioxidants can diminish MDA levels in post-thaw frozen semen. Vitamin E, as an antioxidant, can mitigate oxidative damage caused by the production of MDA. (Maleki et al., 2023). A study by Ramazani et al., (2023) Similarly, it indicated that the incorporation of antioxidants did not significantly alter MDA levels in frozen semen. However, antioxidants effectively reduced MDA levels by mitigating oxidative stress, thereby enhancing spermatozoa quality during storage.

CONCLUSION

The addition of vitamin E to frozen semen was able to improve the fertility parameters of spermatozoa in frozen semen. The addition of vitamin E to standard-quality semen and low-quality semen increased progressive motility, viability, and higher plasma membrane integrity while reducing abnormalities and MDA levels of frozen semen compared to without the addition of vitamin E. The use of standard quality semen with supplementation of vitamin E in diluent was able to improve the quality of frozen semen. Using standard quality semen added with vitamin E in diluent improved the quality of frozen semen. Low-quality semen can be frozen when supplemented with vitamin E. The best level of vitamin E addition is at a concentration of 1 mg/ml.

REFERENCES

Abdel-Khaled, E. A., Dowidar, Y., El-Nagar, H., Wafa, W., El-Ratel, I. T., Mousbah, A., & Authors, C. (2022). A Review on Various Antioxidants Utilized in Bovine Semen Extenders. Journal of Applied Veterinary Sciences, 7(2), 13–24. https://doi.org/10.21608/javs.20

Agarwal, S., Jammu, T., Pandey, A. K., Verma, P. K., & Jammu, T. (2012). Effect of anti-oxidant supplementation on post-thaw sperm characteristics , membrane integrity , migration capability and lipid peroxidation in bull semen Effect of anti-oxidant supplementation on post-thaw sperm characteristics , membrane integrity , mig. Indian Journal of Animal Sciences, 82 (5)(May), 457–460.

Ahmed, H., Ijaz, M. U., Riaz, M., Ullah, F., Samir, H., Shuaib, M., & Swelum, A. A. (2024). Taxifolin: A flavonoid in the freezing medium augments post-thaw semen quality and in vivo fertility potential of buffalo bull spermatozoa. Cryobiology, 117 (December), 104980. https://doi.org/10.1016/j.cryobiol.2024.104980

Akhter, S., Zubair, M., Mahmood, M., Murtaza, S., Andrabi, H., Hameed, N., Ahmad, E., Saleemi, M. K., Andrabi, S. M. H., Hameed, N., Ahmad, E., & Saleemi, M. K. (2023). Effects of vitamins C and E in tris citric acid glucose extender on chilled semen quality of Kail ram during different storage times. Scientific Reports, 13(1), 1–8. https://doi.org/10.1038/s41598-023-43831-2

Alyethodi, R. R., Singh, A., Karthik, S., Tyagi, S., Perumal, P., Singh, U., Sharma, A., & Kundu, A. (2021). Cryobiology Role of seminal MDA , ROS , and antioxidants in cryopreservation and their kinetics under the influence of ejaculatory abstinence in bovine semen. Cryobiology, 98(October 2020), 187–193. https://doi.org/10.1016/j.cryobiol.2020.11.002

Arifiantini, I., Yusuf, T. L., & Graha, N. (2012). Longivitas dan Recovery Rate Pasca Thawing Semen Beku Sapi Fresian Holstein Menggunakan Bahan Pengencer yang Berbeda. Buletin Peternakan, 29(2),

53. https://doi.org/10.21059/buletinpeternak.v29i2.1163

Ayudiah, R. U., Sitompul, D. T., & Felly, T. (2023). Artikel Review : Stres Oksidatif dan Penyakitnya. Research Gate, January. https://www.researchgate.net/publication/366903090_Artikel_Review_Stres_Oksidatif_dan_Penyakitnya#fullTextFileContent

Badan Standardisasi Nasional. (2021). SNI 4869-1:2021 Semen Beku-bagian 1: Sapi.

Daramola, J. O., Adekunle, E. O., Oke, O. E., Ogundele, O., Saanu, E. O. A., & Odeyemi, A. J. (2016). Effect of vitamin E on sperm and oxidative stress parameters of West African Dwarf goat bucks. Tropical and Subtropical Agroecosystems, 19(2), 151–158. https://doi.org/10.56369/tsaes.2159

Dementieva, N. V, Dysin, A. P., Shcherbakov, Y. S., Nikitkina, E. V, Musidray, A. A., Petrova, A. V, Mitrofanova, O. V, Plemyashov, K. V, Azovtseva, A. I., Griffin, D. K., & Romanov, M. N. (2024). Risk of Sperm Disorders and Impaired Fertility in Frozen – Thawed Bull Semen : A Genome-Wide Association Study. animals, 14 (2)(251), 1–19. https://doi.org/https://doi.org/10.3390/ani14020251

Espinosa-García, L. N., Véliz-Deras, F. G., Delgado-González, R. A., Gaytán-Alemán, L. R., Morales-Cruz, J. L., Carrillo-Moreno, D. I., Moran-Martínez, J., & Arvind kumar. (2023). Effect of Adding α-tocopherol on Fertility Parameters in Bovine Semen Cryopreservation. Agricultural Science Digest, 43(1), 113–117. https://doi.org/https://arccjournals.com/doi 10.18805/ag.DF-461

Feradis. (2009). Peran Antioksidan dalam Pembekuan Semen. Jurnal Peternakan, 6(2), 63–70. https://doi.org/http://dx.doi.org/10.24014/jupet.v6i2.379

Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q., & Griendling, K. K. (2018). Reactive oxygen species in metabolic and inflammatory signaling. Circulation Research, 122(6), 877–902. https://doi.org/10.1161/CIRCRESAHA.117.311401

Ghirardosi, M. S., Fischman, M. L., Jorge, A. E., & Cisale, D. C. H. (2018). Relationship between morphological abnormalities in commercial bull frozen semen doses and conception rate. Andrologia, 50(3)(June 2017), 1–5. https://doi.org/10.1111/and.12884

Giaretta, E., Damato, A., Zennaro, L., Bonfatti, V., Mislei, B., Vigolo, V., Falomo, M. E., Bertuzzo, F., Gabai, G., & Bucci, D. (2025). Metabolome and oxidative stress markers in the seminal plasma of Holstein bulls and their relationship with the characteristics of fresh and frozen/thawed sperm. Theriogenology, 235(January), 262–274. https://doi.org/10.1016/j.theriogenology.2025.01.015

Hanson, R., Reddick, S., Thuerauf, S., Webb, K., Kasimanickam, V., & Kasimanickam, R. (2023). Comparison of bull sperm morphology evaluation methods under field conditions. Clinical Theriogenology, 15, 1–9. https://doi.org/10.58292/ct.v15.9425

Haris, F. Z., Ondho, Y. S., & Samsudewa, D. (2020). Effect of vitamin e addition to frozen Simmental bull semen extender on post-thawing quality. E3S Web of Conferences, 142. https://doi.org/10.1051/e3sconf/202014202002

Hendri, Jaswandi, Indriastuti, R., & Ananda. (2024). Sperm Kinematics of Pesisir Bull Thawed at Different Temperatures and Times. Buletin Peternakan, 48 (4)(November), 233–241. https://doi.org/Doi: 10.21059/buletinpeternakv%vi%i.96459 Sperm

Hossain, M. K., Howlader, M. M. R., & Alam, M. G. S. (2023). Effects of semen quality on pregnancy rate in artificially inseminated dairy cows. Bangladesh Veterinarian, 39(1–2), 1–15. https://doi.org/10.3329/bvet.v39i1-2.69061

Houshaimy, K., Ţogoe, D., Constantin, T., Micșa, C., & Șonea, A. (2018). Preliminary Study Regarding the Additional Effect of Adding

Antioxidants on Bull Frozen Semen. “Agriculture for Life, Life for Agriculture” Conference Proceedings, 1(1), 440–444. https://doi.org/10.2478/alife-2018-0067

Khalil, W. A., Hassan, M. A. E., El-Harairy, M. A., & Abdelnour, S. A. (2023). Supplementation of Thymoquinone Nanoparticles to Semen Extender Boosts Cryotolerance and Fertilizing Ability of Buffalo Bull Spermatozoa. Animals, 13(18), 2973. https://doi.org/10.3390/ani13182973

Khan, I. M., Cao, Z., Khan, A., & Rahman, S. U. (2021). Addition of L-Cysteine and Vitamin E to Semen Diluent Enhances Freeze-thawed Spermatozoa Characteristics in Crossbred Cattle Bulls under Subtropical Environment. Pakistan J. Zool, August, 1–11. https://doi.org/DOI: https://dx.doi.org/10.17582/journal.pjz/20191006091046

Kujoana, T. C., Sehlabela, L. D., Mabelebele, M., & Sebola, N. A. (2024). The potential significance of antioxidants in livestock reproduction: Sperm viability and cryopreservation. Animal Reproduction Science, 267(May), 107512. https://doi.org/10.1016/j.anireprosci.2024.107512

Kumaresan, A., Ansari, M. R., Garg, A., & Kataria, M. (2006). Effect of oviductal proteins on sperm functions and lipid peroxidation levels during cryopreservation in buffaloes. Animal Reproduction Science, 93(3–4), 246–257. https://doi.org/10.1016/j.anireprosci.2005.06.030

Lone, S. A., Prasad, J. K., Ghosh, S. K., Das, G. K., Balamurugan, B., & Verma, M. R. (2018). Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. Andrologia, 50(4), 1–8. https://doi.org/10.1111/and.12970

Luo, X., Wu, D., Liang, M., Huang, S., Shi, D., & Li, X. (2023). The effects of melatonin, glutathione and vitamin E on semen cryopreservation of Mediterranean buffalo. Theriogenology, 197, 94–100. https://doi.org/10.1016/j.theriogenology.2022.11.035

Maleki, K., Ayen, E., Khaki, A., & Soleimanzadeh, A. (2023). Comparison of sperm characteristics and antioxidant and oxidant levels in bull semen frozen with four widely used extenders. Veterinary Research Forum, 14(7), 373–379. https://doi.org/10.30466/vrf.2023.562594.3631

Motemani, M., Chamani, M., Sharafi, M., & Masoudi, R. (2017). Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen. Spanish Journal of Agricultural Research, 15(1), e0401. https://doi.org/10.5424/sjar/2017151-9761

Mousavi, S. M., Towhidi, A., Zhandi, M., Amoabediny, G., Mohammadi-Sangcheshmeh, A., Sharafi, M., & Hussaini, S. M. H. (2019). Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. Animal Reproduction Science, 209. https://doi.org/10.1016/j.anireprosci.2019.106171

Nagata, M. P. B., Egashira, J., Katafuchi, N., Endo, K., Ogata, K., Yamanaka, K., Yamanouchi, T., Matsuda, H., Hashiyada, Y., & Yamashita, K. (2019). Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. Journal of Animal Science and Biotechnology, 10(1), 1–14. https://doi.org/10.1186/s40104-019-0395-9

Nam, T. G. (2011). Lipid peroxidation and its toxicological implications. Toxicological Research, 27(1), 1–6. https://doi.org/10.5487/TR.2011.27.1.001

Nurkhasanah, M. A., Si, A., Mochammad, S., Bachri, S., Si, M., Si, D. S., & Yuliani, M. P. (2023). Antioksidan dan Stres Oksidatif (G. A. Sabilla (ed.); Cetakan Pe). UAD PRESS.

Pardede, B. P., Supriatna, I., Yudi, Y., & Agil, M. (2020). Decreased bull fertility: Age-related changes in sperm motility and DNA fragmentation. E3S Web of Conferences, 151, 01010.

https://doi.org/10.1051/e3sconf/202015101010

Priyanto, L., Herdis, Santoso, Sitaresmi, P. I., Priyatno, T. P., Anwar, R. I., Mahari, D. A., Lupitasari, F. B. I., Surachman, M., Darmawan, I. W. A., Abrar, A., & Setiawan, A. (2023). The Effects of Various High Dosage of αTocopherol and Ascorbic Acid in Tris Egg Yolk Extender on Post-Thawed Sperm Quality in Tropical Brahman Bulls. Pakitan Journal of Zoology, 1–9. https://doi.org/https://dx.doi.org/10.17582/journal.pjz/20230131020144

Raeeszadeh, M., Shokrollahi, B., khademi, N., & Akbari, A. (2022). Superior effect of broccoli methanolic extract on control of oxidative damage of sperm cryopreservation and reproductive performance in rats: A comparison with vitamin C and E antioxidant. Theriogenology, 181, 50–58. https://doi.org/10.1016/J.THERIOGENOLOGY.2022.01.010

Ramazani, N., Mahd Gharebagh, F., Soleimanzadeh, A., Arslan, H. O., Keles, E., Gradinarska-Yanakieva, D. G., Arslan-Acaröz, D., Zhandi, M., Baran, A., Ayen, E., & Dinç, D. A. (2023). Reducing oxidative stress by κ-carrageenan and C60HyFn: The post-thaw quality and antioxidant status of Azari water buffalo bull semen. Cryobiology, 111(May), 104–112. https://doi.org/10.1016/j.cryobiol.2023.04.003

Saili, T., Nafiu, L. O., Pagala, M. A., Bain, A., Aku, A. S., Rahadi, S., Rusdin, M., & Lopulalan, F. (2023). Sperm quality of Bali bull following sexing and freezing using different cryoprotectants. IOP Conference Series: Earth and Environmental Science, 1241(1). https://doi.org/10.1088/1755-1315/1241/1/012137

Salan, B., Dethan, A. A., & Purwantiningsih, T. I. (2021). Analisis Faktor Keberhasilan Inseminasi Buatan Pada Ternak Sapi Bali di Kecamatan Atambua Selatan Kabupaten Belu. Jurnal of Animal Science, 6(4), 72–75. https://doi.org/10.32938/ja.v6i4.1496

Savitri, F. K., Suharyati, S., & Siswanto. (2014). Kualitas semen beku sapi Bali dengan penambahan berbagai dosis vitamin C pada bahan pengencer skim kuning telur. Jurnal Ilmiah Peternakan Terpadu. Jurnal Ilmiah Terpadu Peternakan, 2(3), 30–36. https://doi.org/10.23960/jipt.v2i3.p%25p

Sharafi, M., Borghei-Rad, S. M., Hezavehei, M., Shahverdi, A., & Benson, J. D. (2022). Cryopreservation of Semen in Domestic Animals: A Review of Current Challenges, Applications, and Prospective Strategies. Animals, 12(23), 1–24. https://doi.org/10.3390/ani12233271

Slimen, I. B., Najar, T., Ghram, A., Dabbebi, H., Mrad, B., & Abdrabbah, M. (2014). Reactive oxygen species , heat stress and oxidative- induced mitochondrial damage . A review. 30(7), 513–523. https://doi.org/10.3109/02656736.2014.971446

Su, G., Liu, Z., Xue, H., Zhao, X., Yang, L., Wu, D., Hai, C., Liu, X., Song, L., Bai, C., Li, G., & Li, G. (2025). Spirulina polysaccharides improve postthaw sperm quality in bulls by inhibiting the activation of pathways related to protein kinase A. International Journal of Biological Macromolecules, 296(January 2025), 139796. https://doi.org/10.1016/j.ijbiomac.2025.139796

Tanga, B. M., Qamar, A. Y., Raza, S., & Bang, S. (2021). Semen evaluation : methodological advancements in sperm quality-specific fertility assessment — A review. 34(8), 1253–1270. https://doi.org/10.5713/ab.21.0072

Ullah, Z., Khan, H., Hussain, S. M., Tunio, M. T., Dilshad, S. M. R., Gohar, A., Zahid, H., & Ali, A. (2019). Enhancement of extender excellence of frozen bull semen using α-tocopherol as an antioxidant. Acta Scientiae Veterinariae, 47(1), 1–6. https://doi.org/10.22456/1679-9216.90447

Upadhyay, V. R., Ramesh, V., Dewry, R. K., Yadav, D. K., & Ponraj, P. (2022). Bimodal interplay of reactive oxygen and nitrogen species in physiology and pathophysiology of bovine sperm function. Theriogenology, 187, 82–94. https://doi.org/10.1016/j.theriogenology.2022.04.024

Wang, M., Wu, S., Yang, B., Ye, M., Tan, J., Zan, L., & Yang, W. (2023). Grape Seed Proanthocyanidins Improve the Quality of Fresh and Cryopreserved Semen in Bulls. Animals, 13(17), 2781. https://doi.org/10.3390/ani13172781

Wijayanti, A., Suprayogi, T. W., Prastiya, R. A., Hernawati, T., Sardjito, T., Saputro, A. L., Amaliya, A., & Sulistyowati, D. (2023). Effect of Addition of Green Tea Extract (Camellia sinensis) in Egg Yolk Tris Diluter on Spermatozoa Quality in Bali Cattle (Bos sondaicus) After Freezing. Jurnal Medik Veteriner, 6(1), 66–74. https://doi.org/10.20473/jmv.vol6.iss1.2023.66-74

Yuan, C., Zhang, K., Wang, Z., Ma, X., Liu, H., Zhao, J., Lu, W., & Wang, J. (2023). Dietary flaxseed oil and vitamin E improve semen quality via propionic acid metabolism. Frontiers in Endocrinology, 14. https://doi.org/10.3389/fendo.2023.1139725

Zhao, X. L., Li, Y. K., Cao, S. J., Hu, J. H., Wang, W. H., Hao, R. J., Gui, L. S., & Zan, L. S. (2015). Protective effects of ascorbic acid and vitamin E on antioxidant enzyme activity of freeze-thawed semen of qinchuan bulls. Genetics and Molecular Research, 14(1), 2572–2581. https://doi.org/10.4238/2015.March.30.16