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REVIEW ARTICLE

Antioxidant Glutathione Supplementation in Semen Extenders and its Effect on the Quality of Cryopreserved Bull Sperm: A Meta-Analysis

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ABSTRACT

Glutathione, an organic antioxidant, is believed to suppress the damaging effects caused by reactive oxygen species on post-thaw quality of bull semen. This meta-analysis aimed to evaluate the role and optimal dosage of glutathione in reducing the adverse effects of cryopreservation on post-thaw semen quality of bulls. The analysis used data from 24 articles following various selection processes. The parameters observed were total and progressive sperm motility, acrosomal integrity, sperm viability, morphological abnormalities, plasma membrane integrity, DNA damage, curvilinear velocity, straight linear velocity and fertility. Data were analyzed using the mixed model methodology, with glutathione dosage as the fixed effect and different studies as the random effect. A significant effect of glutathione was found on post thaw total sperm motility ($P<0.05$), progressive motility ($P<0.05$) and plasma membrane integrity ($P<0.05$). The supplementation of glutathione to semen extenders significantly suppressed the increase in post-thaw sperm abnormalities ($P<0.05$). Glutathione supplementation in semen extenders had varying effects among different bull breeds ($P<0.05$). Supplementing glutathione is recommended to improve sperm quality in freezing bull semen. Glutathione supplementation can maintain post-thaw sperm quality with an optimal dosage of 4.49 mM.

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INTRODUCTION

An effective and efficient artificial insemination (AI) program depends on ability of sperm to fertilize the ovum. In bovines, artificial insemination is mostly performed using frozen thawed semen under field conditions. The efficacy of the field AI program is contingent upon the quality of the frozen semen. The quality of semen after cryopreservation is influenced by various factors, including the type of diluent, glycerol concentration, semen packaging method, cooling rate, and semen handling during the cryopreservation and thawing process (Lyashenko, 2015). Semen cryopreservation suppresses sperm cell metabolism through rapid temperature reduction (Sharafi *et al.,* 2022). Freezing and subsequent thawing of semen generate reactive oxygen species (ROS), impairing sperm motility, membrane integrity and fertilization capability (Büyükleblebici *et al.,* 2014). Sperm mitochondria may undergo alterations in fluidity during cryopreservation, resulting in the release of substantial

quantities of ROS, such as hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anion (O_2–), and hydroxyl radical (OH) (Johnson *et al.,* 1999; Said *et al.,* 2010). ROS-induced ATP consumption or damage to the flagellar contractile apparatus affects sperm motility (Guthrie and Welch, 2012). Sperm capacitation and acrosome reactions require ROS but can be harmful at high concentrations (Ugur *et al.,* 2019).

The free radical proliferation in post-thaw bull semen can be reduced through the addition of antioxidants to the extender (Hayanti *et al.,* 2022; Sapanidou *et al.,* 2023). Antioxidants are typically categorized into enzymatic and non-enzymatic types (Flieger *et al.,* 2021). Enzymatic antioxidants facilitate the reduction of hydrogen peroxide and organic peroxides, including phospholipid peroxides, within the antioxidant system of semen (Irato and Santovito, 2021). In contrast, non-enzymatic antioxidants impede superoxide production, thereby safeguarding against sperm dysfunction (Kowalczyk, 2022). Glutathione is an organic compound that serves as an enzymatic antioxidant in various cell types, including spermatozoa. It is found in the cytosol of both animal and plant cells (Meister, 1988; Wu *et al.,* 2004; Hasanuzzaman *et al.,* 2017). In body cells, glutathione is present in two forms, namely reduced glutathione (GSH) and oxidized glutathione (GSSG) (Enns and Cowan, 2017). The GSH functions as an antioxidant by scavenging electrophilic species and oxidants, both directly and via enzymatic catalysis (Gaucher *et al.,* 2018). It protects cells against free radicals, reactive oxygen species, lipid hydroperoxides, xenobiotic toxins and heavy metals (Narayanankutty *et al.,* 2019). It is also a cofactor for antioxidant and detoxification enzymes such as glutathione peroxidase, glutathione S-transferase and glyoxalase. It enables cells to adapt and adjust according to changes in the internal and external environment (Panday *et al.,* 2020).

Previous studies have suggested that supplementation of GSH to semen extenders improves the ability of post-thawing sperm homeostasis (Karaji *et al.,* 2014; Nadri *et al.,* 2020). However, several other studies have indicated that the dosage of GSH administered in frozen bovine semen extenders varied widely. Meta-analysis can be used as a logical technique to analyze data from several studies using comparable variables to obtain a final dosage recommendation that refers to the results of various previous studies. Therefore, the present meta-analysis aimed to determine the effect of Reduced Glutathione (GSH) supplementation to semen extenders on the quality parameters of cattle bull sperm after freezing and thawing.

MATERIALS AND METHODS

Search through search engine: The articles were obtained through extensive online search on library databases, such as PubMed, Google Scholar, Semantic Scholar, Science Direct, and Scopus. The process involved the use of specific keywords such as "bovine", "bull", "semen", "spermatozoa", "Glutathione", "reduced Glutathione", "GSH", "amino acids", "antioxidants", "supplements", "cryopreserved", "frozen" and "post-thaw". The compilation comprised a comprehensive collection of scholarly publications, including primary research articles, literature reviews and conference proceedings, totaling 508 items published between 1987 and 2023 (Fig. 1).

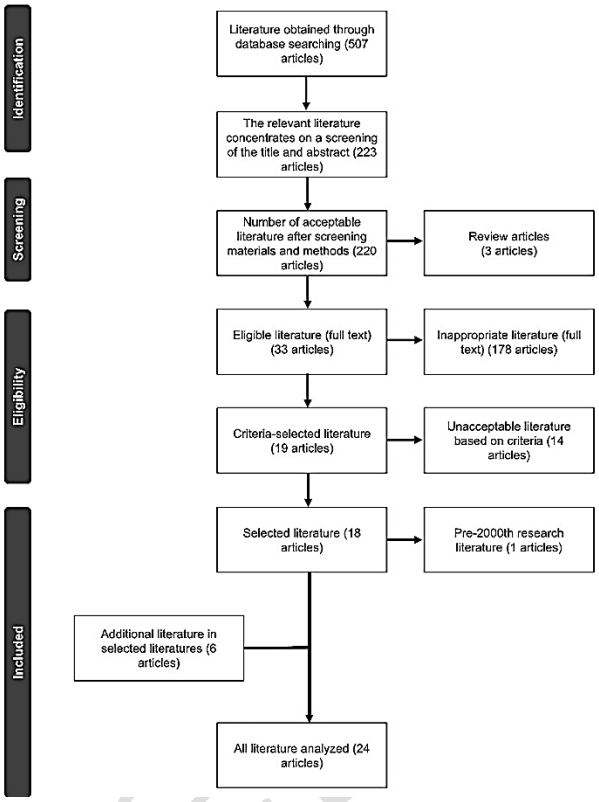

Fig. 1: The literature selection process for meta-data analysis of the effects of glutathione supplementation on cryopreserved bull sperm.

Selection of articles: The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol, which considers previously available reviews, was followed for selection of articles. The methods were used to ensure that the articles selected were relevant and of high quality for inclusion in the meta-analysis and the possibility of bias in the selection process was minimized (Sholikin *et al.,* 2023). For this purpose, the following six points were considered: i), the title and abstract of the article was related to key-words; ii), articles were selected based on experimental research; iii), articles had sufficient information about materials and methods and were well designed experimentally; iv), articles had information on glutathione type, levels and units; v), articles described the effect of GSH supplementation only on post-thawing sperm; vi), bull semen was used as raw material in the article. A representative selection process and the amount of literature collected are presented in Fig. 1. Articles that did not meet the selection process requirements were discarded.

Meta-data collection and verification: A total of 24 articles obtained through the selection process are presented in Table 1. All of this literature was used for the meta-analysis. Data collected from 24 articles consisted of databases and parameters. The database contained primary information about the dosage of glutathione supplementation to semen extenders. The final unit dosage of glutathione was converted into mM, with the lowest value being 0 mM and the highest value being 10 mM. Apart from the dosage, the glutathione type and breed of bulls were also included in the database. Meanwhile, the parameters observed were total sperm motility (%), progressive motility (%), acrosomal integrity (%), sperm

Table 1: List of literature used in meta-analysis studies of the effect of glutathione supplementation on cryopreserved sperm.

No	Article	Dosage (mM)	Country	Breed	Sperm parameters examined
1	Li *et al.* (2023)	0, 4	China	Holstein	Total motility, acrosome integrity, viability, abnormality, plasma membrane integrity, fertility
2	Ogata *et al.* (2022)	0, 1, 5, 10	Japan	Holstein Japanese Black	Total motility, acrosomal integrity, viability, DNA damage, curvilinear velocity, fertility
3	Almeida *et al.,* (2021)	0, 2	Brazil	Nellore	Total motility, acrosomal integrity, abnormality, plasma membrane integrity, fertility
4	Salman *et al.,* (2021)	0, 5	Austria	Holstein	Total motility, progressive motility, viability, DNA damage, curvilinear velocity
5	Nadri *et al.,* (2020)	0, 1, 2.5, 5	Iran	Holstein	Total motility, progressive motility, acrosomal integrity, viability, abnormality, plasma membrane integrity, curvilinear velocity, straight linear velocity
6	Pinto *et., al* (2020)	0, 2.5	Brazil	Nellore	Total motility, acrosomal integrity, abnormality
7	Yadav *et al.,* (2019a)	0, 0.5, 1	India	Hariana	Plasma membrane integrity
8	Yadav *et al.,* (2019b)	0, 0.5, 1	India	Hariana	Viability
9	Yadav *et al.,* (2019c)	0, 0.5, 2	India	Hariana	Progressive motility, sperm viability
10	Murtaza *et al.,* (2019)	0, 0.5, 1, 2	Pakistan	Sahiwal	Total motility, acrosomal integrity, viability, plasma membrane integrity DNA damage
11	Shah *et al.,* (2017)	0, 0.5, 1	India	Hariana	Total motility, abnormalities, plasma membrane integrity
12	Uysal *et al.,* (2007)	0, 5	Turkey	Holstein	Total motility, acrosomal integrity, viability, plasma membrane integrity, fertility
13	Eidan, (2016)	0, 2	Iraq	Holstein	Total motility, progressive motility, curvilinear velocity, straight linear velocity
14	Sattar *et al.,* (2016)	0, 1	Pakistan	Sahiwal	Total motility, acrosomal integrity, viability, plasma membrane integrity
15	Ansari *et al.,* (2014)	0, 0.5, 1	Pakistan	Sahiwal	Total motility, progressive motility, viability, plasma membrane integrity, DNA damage, curvilinear velocity, straight linear velocity
16	Daghigh-Kia *et al.,* (2014)	0, 5	Iran	Holstein	Total motility, progressive motility, viability, plasma membrane integrity, DNA damage, curvilinear velocity, straight linear velocity
17	Karaji *et al.,* (2014)	5, 7.5	Iran	Holstein, Simental	Total motility, acrosomal integrity, fertility
18	Badr *et al.,* (2012)	0, 0.5, 1, 2, 3, 5	Egypt	Nellore	Total motility, progressive motility, curvilinear velocity
19	Perumal *et al.,* (2011)	0, 5	India	Jersey crossbred	Total motility, viability
20	El-Harairy *et al.,* (2011)	0, 0.2, 0.4, 0.8	Egypt	Friesian	Total motility, viability, abnormality, plasma membrane integrity, DNA damage, curvilinear velocity, straight linear velocity, fertility
21	Tuncer *et al.,* (2010)	0, 0.5, 2	Turkey	Holstein	Total motility, acrosome integrity, acrosome abnormality, abnormality, plasma membrane integrity, fertility
22	Sariözkan *et al.,* (2009)	0, 2	Turkey	Holstein	Total motility, viability, abnormality, plasma membrane integrity, DNA damage, curvilinear velocity, straight linear velocity, fertility
23	Perumal, (2008)	0, 5	India	Jersey crossbred	Total motility, progressive motility, acrosomal integrity, viability, curvilinear velocity, straight linear velocity, fertility
24	Gadea *et al.,* (2008)	0, 1, 5	Spain	Spanish breed 'Asturiana de Valles'	Total motility, progressive motility, acrosomal integrity, viability, curvilinear velocity, straight linear velocity, fertility

liveability (%), morphological abnormalities (%), plasma membrane integrity (%), DNA damage (%), curvilinear velocity (VCL) (μM/s), straight linear velocity (VSL) (μM/s), and fertility (%).

Construction of mathematical model: The current study utilized quantitative, qualitative and interaction meta-regression techniques, also known as a linear mixed model (LMM), as meta-analysis methods (St-Pierre, 2001; Sauvant *et al.*, 2008). The mathematical model is presented below:

$$Y_{ij} = \mu + s_i + \tau_j + s\tau_{ij} + \beta_0 + \beta_1 X_{ij} + (Breed * GSH) + b_i X_{ij} + e_{ij} \quad (1)$$

Note: the dependent variable is represented as Y_{ij}, the average value is represented as μ, the i-th random factor is represented as s_i, and the j-th fixed factor is represented as τ_j. The random interaction factor between the difference in the study and the fixed factor is represented as $s\tau_{ij}$. The point at which the average value of all studies intersects with the X-axis is represented as β_0, while the coefficient is represented as β_1. The term "Breed*GSH" represents the cumulative impact of all studies. The variable "e_{ij}" denotes the residual error value, whereas "$b_i X_{ij}$" represents the random effect of the heterogeneity in studies on the regression coefficient Y in the X variable of the i-th study. The meta-regression test employed in the advanced stage was based on least-squares means, as described by Searle *et al.* (1980).

Statistical model test: The statistical analysis in this meta-analysis was conducted using Core Team (2022) version 4.2.0. The residual mean square error (RMSE) was utilized for statistical testing. The Nakagawa determination value or RGLMM(c)2, as described by Nakagawa *et al.* (2017) and Nakagawa and Schielzeth (2013), was also employed. The Core Team (2022) was the source of the R software. The equations for RMSE and Nakagawa's R-squared (R^2) were as follows:

$$RMSE = \sqrt{\frac{\sum(O-P)^2}{NDP}} \quad (2)$$

$$R_{GLMM}(c)^2 = \frac{(\sigma^2{}_f + \sum(\sigma^2{}_l))}{(\sigma^2{}_f + \sum(\sigma^2{}_l) + \sigma^2{}_e + \sigma^2{}_d)} \quad (3)$$

Here, O represents the observed value, P represents the predicted value and NDP represents the number of data points. Additionally, the formula included the following variables: 〖σ^2〗_f, which represented the variant of a fixed factor; ∑(〖σ^2〗_l), which represented the total of all variants of the component; 〖σ^2〗_e, which

represented the variant owing to the predictor dispersion; and ⟦σ^2⟧_d, which represented the particular distribution of the variant. The significance of the model was subsequently evaluated using a variance analysis test, which was considered significant if the p-value was less than 0.05 and tended to be non-significant if the p-value was more than 0.05.

Determination of the optimal dosage: The research determined the optimal point of the response surface methodology (RSM) to ascertain the ideal dosage of Glutathione (Kamarudin *et al.*, 2017). This approach involved one factor, namely the Glutathione dosage (mM). Four responses were selected for assessment: response 1 for total sperm motility (%), response 2 for progressive motility (%), response 3 for abnormalities (%) and response 4 for plasma membrane integrity (%). These responses pertained to the performance parameters observed throughout the entire process of supplementing frozen bovine semen with Glutathione. It was crucial to acknowledge that not all responses were appropriate for analysis using RSM; only explicitly expressed responses were taken into consideration. The optimization process identified the most favorable value for each factor and response. The RSM and optimization procedures were conducted using Design Expert version 13.

RESULTS

The effect of GSH supplementation on frozen semen quality parameters after thawing is presented in Table 2. The quadratic effect of GSH supplementation was found on sperm total motility (P-quadratic (Pq) <0.001), abnormalities (Pq=0.039) and plasma membrane integrity (Pq=0.005) (Table 2). GSH also had a significant linear effect on sperm total motility (P-linear (Pl)=0.006), progressive motility (Pl=0.024), abnormalities (Pl=0.009) and plasma membrane integrity (Pl<0.001). However, it had a non-significant linear effect on acrosome integrity, sperm viability, DNA damage, curvilinear velocity, straight linear velocity and fertility (Table 2).

Glutathione had a significant effect (P<0.05) in preventing a decrease in total sperm motility after thawing (Table 2, Fig. 2a). The optimal response of GSH supplementation at a dosage of 4.49 mM on total spermatozoa motility was 57.2% (Fig. 4). Moreover, supplementation of GSH had a significant effect (P<0.05) on progressive motilit (Table 2, Fig. 2b). The optimal response of GSH at a dosage of 4.49 mM to progressive motility after thawing was 39.2% (Fig. 4). Similarly, GSH supplementation had a significant effect (P<0.05) in preventing an increase in sperm abnormalities in frozen semen (Table 2, Fig. 2e). Optimal supplementation of GSH

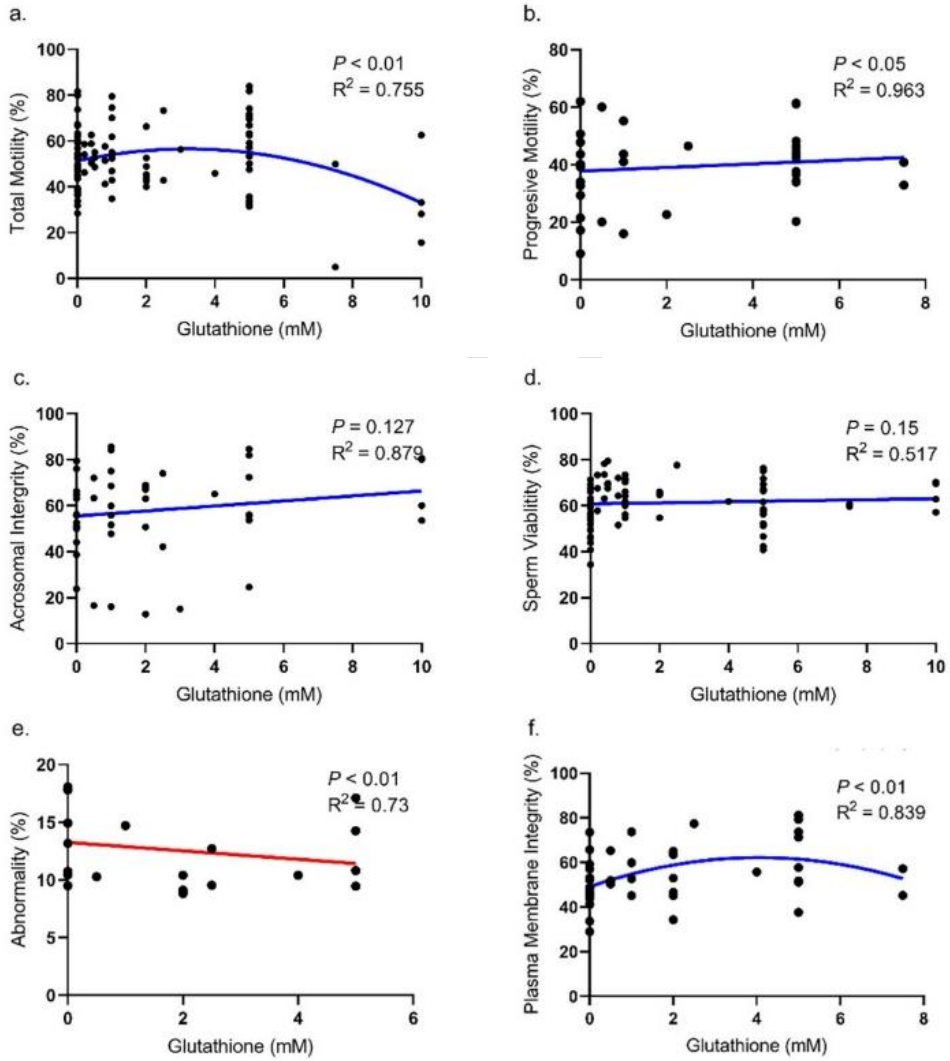

Fig. 2: Response of total and progressive motility, acrosomal integrity, sperm viability, abnormality and plasma membrane integrity due to glutathione supplementation in cryopreserved bovine sperm. The blue line represents the effect of increasing and then decreasing, and the red line corresponds to reducing impact.

at a dosage of 4.49 mM can suppress the percentage of abnormalities by up to 11.7% (Fig. 4). Likewise, GSH had a significant effect (P<0.001) on sperm plasma membrane integrity (Table 2, Fig. 2f). The optimal dosage of GSH at 4.49 mM can increase the percentage of plasma membrane integrity up to 62% (Fig. 4). However, glutathione showed a non-significant effect (P>0.05) on acrosomal integrity (Fig. 2c), sperm viability (Fig. 2d), DNA damage (Fig. 3a), VCL (Fig. 3b), VSL (Fig. 3c) and fertility (Fig. 3d). The desirability value obtained at the optimal dosage of 4.49 mM based on the significantly affected parameters was 0.6 (Fig. 4).

The alterations in the values of all parameters prior to and after glutathione administration in post-thaw sperm are presented in Table 2. The quadratic model indicated that both total motility and plasma membrane integrity exhibited a decline in value following the administration of glutathione. Concurrently, in other quadratic parameters, specifically abnormality, a positive change in value was observed following the administration of glutathione. Following the thawing of sperm, parameters such as total motility, progressive motility, acrosome integrity, sperm viability, plasma membrane integrity, DNA damage, VSL, and infertility, analyzed through a linear model, exhibited an increase in value with a positive slope after the administration of glutathione. Concurrently, sperm exhibiting abnormal parameters and VCL demonstrated a decline in value following the administration of glutathione.

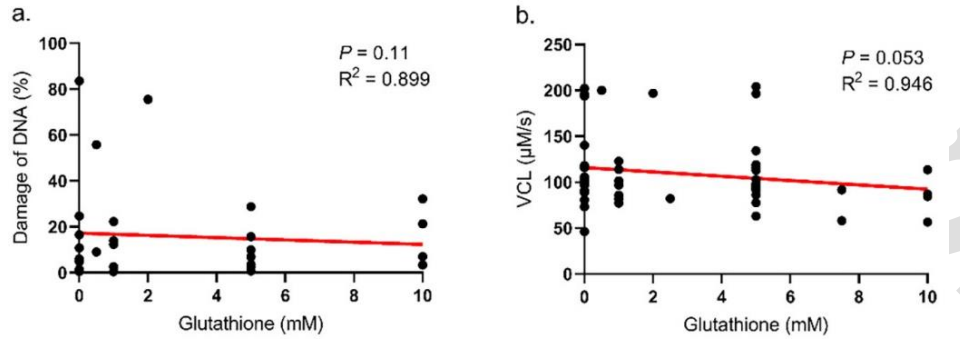

Fig. 3: Value of DNA damage, VCL, VSL, and fertility of frozen semen supplemented with glutathione (mM). The blue line represents the effect of increasing and then decreasing, and the red line corresponds to reducing impact.

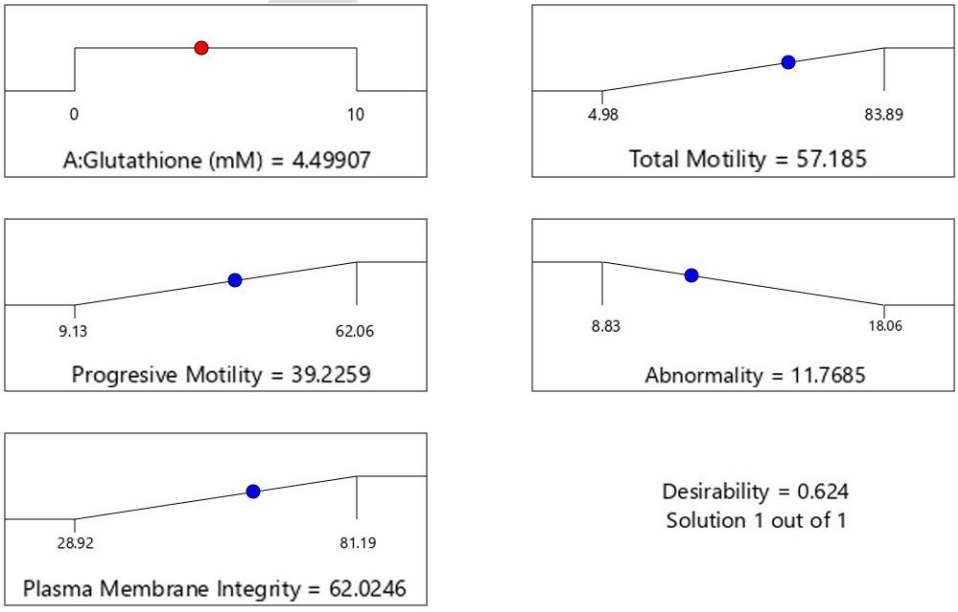

Fig. 4: Glutathione (mM) optimization is based on total and progressive motility parameters, sperm abnormalities, and plasma membrane integrity. The optimal dosage of glutathione in the cement extender is 4.49 mM. Glutathione optimization responses were total motility (57.2%), progressive motility (39.2%), abnormalities (11.7%), and plasma membrane integrity (62%).

Table 2: Effect of glutathione supplementation on various quality parameters of cryopreserved sperm.

No.	Parameter	Intercept		Slope		p-value	RMSE	R^2	B vs G
		Value	SE	Value	SE				
1.	Total motility (%)	52	2.45	2.39	0.833	0.006	6.17	0.755	0.56
				-0.416	0.102	<0.001			
2.	Progressive motility (%)	38	3.74	0.498	0.205	0.024	2.14	0.963	0.135
3.	Acrosomal Integrity (%)	57.8	3.94	0.494	0.316	0.127	4.64	0.879	0.654
4.	Sperm viability (%)	59.4	1.7	0.458	0.314	0.15	5.87	0.517	0.33
5.	Abnormality (%)	14.1	0.946	-2.43	0.769	0.009	1.23	0.743	0.127
				0.376	0.162	0.039			
6.	Plasma membrane integrity (%)	49.5	2.62	5.1	1.12	<0.001	3.79	0.839	0.674
				-0.589	0.192	0.005			
7.	DNA damage (%)	13.5	6.93	0.689	0.411	0.11	5.74	0.899	0.221
8.	Curvi linear velocity (µM/s)	114	9.14	-0.892	0.444	0.053	7.53	0.946	0.032
9.	Straight linear velocity (µM/s)	57.1	8	0.075	0.273	0.789	2.16	0.986	0.025
10.	Fertility (%)	48.8	7.04	0.515	0.511	0.325	5.74	0.901	0.587

B = bull breed; G = glutathione dosage in mM; R^2 = R squared Nakagawa for validate linear mixed model, RMSE = root mean square error, and SE = standard error.

Table 3: Effect of glutathione supplementation on various quality parameters of cryopreserved sperm across multiple cattle bull breeds

No.	Parameter	B1	B2	B3	B4	B5	B6	B7	B8	B9	SEM	p-value
1.	Total motility (%)	60ab		50.1a	39a	69.4b	40.2ab	55.6ab	52.2ab	44.7ab	3.64	0.013
2.	Progressive motility (%)	41.6	54.4	34.		52		12.6	32.1		6.26	0.071
3.	Acrosomal Integrity (%)	55.8ab		65.1b	63.3b		43.9ab	67.6b		18.1a	7.68	0.014
4.	Sperm viability (%)	59.5	62.4	57.5	66.2	66.4		66.2	59		1.45	0.47
5.	Abnormality (%)			13.1		13.7	9.72				1.24	0.259
6.	Plasma membrane integrity (%)		59.4	54.7		68.8	31.6	47.7	45.9		5.21	0.097
7.	DNA Damage (%)		13.1	19.1	12.8	13.1					1.52	0.986
8.	Curvi linear velocity (µM/s)	116		135	86.6	107		61.7	74.9		11.1	0.316
9.	Straight linear velocity (µM/s)	86.7		52		59.5		49.3			8.53	0.692
10.	Fertility (%)	55.7		60.6	11.5	57.5	35.7			39.4	7.62	0.48

Note: B1 = Asturiana de Valles, B2 = Hariana, B3 = Holstein, B4 = Japanese Black, B5 = Jersey Crossbred, B6 = Nellore, B7 = Sahiwal, B8 = Simental, B9 = Friesian, SEM = sum error of mean. Different superscripts in breeds indicate differences between breeds only in that parameter. Different superscripts indicate significant differences between bull breeds for each parameter (P<0.05).

The model's efficacy, indicated by an R^2 value nearing 1.0, is also illustrated in Table 2. Among all parameters, those approximating 1.0 were VSL, VCL and progressive motility. In contrast, total motility, acrosomal integrity, sperm viability, abnormalities, plasma membrane integrity, DNA damage, and fertility exhibited inferior model goodness relative to VSL, VCL, and progressive motility. Table 2 also illustrates the interaction between bulls and glutathione. Bull and glutathione exhibited significant interaction on VCL (P=0.032) and VSL (P=0.025). Meanwhile, there was no interaction between bull and glutathione concerning total motility, progressive motility, acrosomal integrity, sperm viability, plasma membrane integrity, sperm abnormalities, DNA damage and fertility.

The results of the analysis showed that different bull breeds had significant differences in two parameters of the quality of post-thawing bull sperm given GSH (Table 3).

The analysis of GSH supplementation to bulls of several breeds showed significant differences (P<0.05) in total sperm motility and acrosomal integrity. The total sperm motility of Holstein and Japanese Black bull sperm given GSH was significantly lower (P<0.05) compared to the total sperm motility of Jersey Crossbred bulls, but was non-significantly different from Asturiana de Valles, Nellore, Sahiwal and Friesian bulls. Following GSH supplementation, the acrosomal integrity of Friesian bull sperm was significantly lower (P<0.05) compared to the acrosomal integrity of Holstein, Japanese Black and Sahiwal bull sperm but was non-significantly different from the Asturiana de Valles and Nellore bulls.

DISCUSSION

Many studies have recorded variable effects of GSH supplementation as an antioxidant in cryopreserved semen extenders on sperm quality, especially in bovine species. Some of these studies strongly recommend adding the antioxidant GSH because it increases sperm fertilization ability, with increasing the percentage of motile sperm (Gilmore *et al.*, 2021). Several previous studies on GSH have also suggested the best dosage of GSH that should be supplemented with the semen extender to optimize the improvement in sperm quality parameters. The addition of antioxidants to semen extenders is anticipated to enhance sperm viability during cryopreservation while preserving fertilization capacity of each sperm (İnanç *et al.*, 2018). Unfortunately, the recommended dosages vary greatly, even though studies have been conducted on bovine species (Ogata *et al.*, 2022). The relationship between the supplementation of GSH in the semen extender and various sperm quality parameters was investigated in the present study (Table 1). This meta-study was prepared based on a compilation of literature studies over a decade that have researched and found multiple effects of GSH supplementation with various supplementation dosages and on several bull breeds.

The primary function of GSH in the reproductive system is related to its interaction with other systems as a preventive mechanism against reactive oxygen species (ROS) (Adeoye *et al.*, 2018). Previous studies have clearly stated that GSH deficiency can cause instability of the sperm midpiece, which can cause motility disorders (Adeoye *et al.*, 2018; Abdullah *et al.*, 2021). GSH protects the plasma membrane from lipid peroxidation, scavenging superoxide and preventing oxygen formation (Ayala *et al.*, 2014; Abdullah *et al.*, 2021). Furthermore, it is known that mitochondria are organelles located in the midpiece of the sperm (Pardede *et al.*, 2023).

Mitochondria help produce energy in sperm, which is needed for sperm to be motile and progressive (Pardede *et*

al., 2023). Pardede *et al.* (2024) reported decreased spermatozoa motility related to decreased mitochondrial activity. Mitochondrial damage disrupts adenosine triphosphate (ATP) production (Pardede *et al.,* 2023; Pardede *et al.,* 2024). Mitochondria are the primary oxidative energy source for cells that produce ATP through the electron transport chain (Pardede *et al.,* 2024). Castellini *et al.,* (2021) added that energy in the form of ATP is the result of metabolism in the mitochondrial membrane, which plays a role in the work of microtubules so that spermatozoa can move freely.

Furthermore, motile and progressive sperm movement is essential for the success of the fertilization process (Pardede *et al.,* 2024). So, it is unsurprising that motility is one of the main factors or sperm quality parameters most widely used as a standard for determining the feasibility of freezing semen and the distribution of frozen semen (Pardede *et al.,* 2020a; Pardede *et al.,* 2024). It is often used as a determining parameter for the fertility of bulls (Pardede *et al.,* 2020a).

The findings in the current study support the above hypothesis. The supplementation of GSH in the extender to frozen semen significantly improves several sperm quality parameters (Table 2). Based on these parameters, the optimum dosage that can be supplemented to the extender as part of the cryopreservation process was 4.49 mM (Fig. 4), with a desirability value of 0.6. The desirability value of 0.6 indicates that the dosage of 4.49 mM obtained through the meta-analysis is optimal but has the potential to be increased. With a dosage of 4.49 mM, it is believed that the supplementation of antioxidant will be more optimal and can provide maximum impact and improve various aspects of sperm quality parameters. Furthermore, based on analysis, the value of 4.49 mM is believed to apply to specific and all bull breeds. Nineteen of the twenty-four articles used in this meta-analysis study were close to the optimum dosage, while the rest varied with dosages only reaching 4.0 mM. Despite this dosage, several previous studies showed a significant impact, but only in certain bull breeds, such as Holstein and Nellore bulls (Table 3).

There was a significant increase in the percentage of total and progressive sperm motility and plasma membrane integrity and decrease in sperm abnormalities following supplementation of semen extender with GSH (Table 2). GSH supplementation may increase the possibility of successful fertilization and improve fertility rates. However, the results show that the GSH supplementation did not significantly improve the fertility rates in this study. However, besides the quality of the sperm (Butler *et al.,* 2019), many other factors can also influence fertility rate in bulls. These factors include the cow, AI technicians in the field, extender and such others (Butler *et al.,* 2019). However, when talking about the fertility of bulls, sperm quality is an essential factor (Kusumawati *et al.,* 2023). According to Pardede *et al.* (2020a) and Pardede *et al.* (2024), the fertility of bulls is greatly influenced by the quality of semen, where good quality semen must contain spermatozoa that are motile (above 80%), live (above 80%), have intact plasma membranes (above 80%), intact acrosomal membranes (above 90%), and have low abnormal morphology (below 45%), which is good for fertilizing the oocyte. However, recently, it has been suggested that there are intrinsic spermatozoa factors, including molecules such as RNA and protein, which play an essential role and are considered more accurate in determining the fertility of bulls (Pardede *et al.,* 2020b; Indriastuti *et al.,* 2022; Rosyada *et al.,* 2023).

Glutathion was also found to improve plasma membrane integrity with a optimum effect value of 62% (Fig. 3). Plasma membrane parameters are related to the function of spermatozoa movement (Shan *et al.,* 2021). The stability of the plasma membrane affects ATP production, whereas damage to the membrane causes ATP production to stop so that spermatozoa are unable to move (Gallo *et al.,* 2021). It was further explained that the integrity of the spermatozoa plasma membrane is essential for optimizing spermatozoa function (Shan *et al.,* 2021) because only spermatozoa with intact plasma membranes can survive, go through a series of complex changes in the female reproductive tract and can fertilize oocytes (García-Vázquez *et al.,* 2016). According to Boe-Hansen *et al.* (2018), midpiece damage contributes the most to bovine sperm abnormalities, namely 9.97%. As it was explained that GSH deficiency is related to the midpiece, this may be related to the abnormalities found in this study (Adeoye *et al.,* 2018). However, the various studies compiled did not explain what morphological abnormalities were intended in the study. However, several other studies have demonstrated a connection between abnormalities in the plasma membrane and spermatozoa motility (Pardede *et al.,* 2020a; Palacin *et al.,* 2020). The various sperm quality parameters play their respective roles in the normal function of sperm, which is closely related to fertility (Pardede *et al.,* 2020a; Pardede *et al.,* 2022). In a study involving infertile men with unilateral varicocele or genital tract inflammation, glutathione caused a significant improvement in sperm quality (Adeoye *et al.,* 2018). The glutathione/reductase system protects against lipid peroxidation in the spermatozoa plasma membrane (Atig *et al.,* 2012). It scavenges lipid peroxides, thereby stopping the progressive chain reaction of lipid peroxidation (Ayala *et al.,* 2014; Adeoye *et al.,* 2018). GSH also scavenges hydrogen peroxide (H_2O_2), which is responsible for lipid peroxidation (Chaudhary *et al.,* 2023). Glutathione reductase reduces glutathione disulfide to reduced glutathione, thereby recycling it (Vašková *et al.,* 2023).

Specifically, this research found that bull breeds had different and varied response after GSH supplementation to the frozen semen extender, especially on total motility and acrosome integrity. It cannot be denied that bull breeds have different sperm quality characteristics, and this has been widely reported before (Barth and Waldner 2002; Novianti *et al.,* 2020; Pardede *et al.,* 2021; Pardede *et al.,* 2022). Moreover, the qualities of semen are also influenced by various factors, most of which are influenced by genetics and the environment (Barth and Waldner, 2002; Donkin and Barrès, 2018). Different bull-rearing environments can significantly affect the quality of the semen, not to mention genetic factors that have been carried since the bull was still in embryo form (Donkin and Barrès, 2018). However, of course, these findings are quite relevant in determining the particular dosage of GSH to be given, which at least needs to consider the breed factor or at least can be material for further studies to group them first based on species, such as *Bos taurus*, *Bos indicus*, and *Bos sondaicus*. However, as discussed previously, the meta-analysis results show that the

dosage of 4.49 mM is optimal and can be applied to all bull breeds without considering breed factors. However, further studies with these findings would be beneficial and enrich the results of this meta-analysis study.

Conclusions: Glutathione supplementation in the semen extender for the cryopreservation of bull semen may maintain some sperm quality parameters post-thawing. The optimal dosage of Glutathione recommended as an antioxidant in semen extenders during cryopreservation is 4.49 mM, with the expected positive impacts including increasing total and progressive motility, plasma membrane integrity, and minimizing sperm abnormalities.

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