Protective effects of trehalose on ruminant sperm characteristics: A meta-analysis

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Abstract. Sperm cryopreservation adds to the extension of reproductive techniques. The composition of the semen diluent's extender, such as trehalose, is one of the most fundamental factors affecting cryopreservation. The objective of the research is to find out the cryoprotectant impacts of various concentrations of trehalose because of the post-thaw quality of ruminant spermatozoa. The data was based on scientific articles that were distributed via Web engines. The following stage was the assessment of reasonableness concerning the exploration subject and study goals, after which 22 papers were chosen for the data set. To retrieve statistically investigated data from the literature, a mixed model was used. A fixed effect was used to analyze the varying degrees of fixation of trehalose in the semen diluent on ruminant sperm quality. The findings indicated that increasing the trehalose content in the semen diluent increased the motility percentage and the hypoosmotic swelling test while decreasing the abnormalities. The recommended optimal level of trehalose addition to maximize sperm motility is around 98.2 mM, or to optimize the hypoosmotic swelling test value, it is about 74 mM. Trehalose improves the quality of ruminant spermatozoa after being frozen and thawed, so it is recommended that trehalose be used to protect spermatozoa during the cryopreservation process.

1 Introduction

The cryopreservation of sperm is crucial for producing and conserving high-quality hereditary material and transferring genetic material in the future. The cryopreservation process has an undesirable effect on spermatological characteristics. Ice crystals occur in the extracellular environment during the freezing of sperm testing. Ice crystals produce strain on the cell and organelles' membranes, disrupting and impairing membrane permeability [1-2]. Membrane lipid peroxidation is essential for sperm survival and motility, and its development is inhibited by reactive oxygen species [3]. Oxidative pressure causes cellular damage when (reactive oxygen species) ROS production surpasses the organism’s antioxidant agent limit. In any event, cold shock and oxidative pressure during cryopreservation result in cryodamage to the spermatozoa's structure [4]. Different cryoprotective chemicals are added to diluents

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before freezing to mitigate the negative effects of oxidative stress and cold shock on semen [5].

Cryobiology has experimented with several cryoprotective chemicals due to their success in cryopreserving gamete cells from different species. Following the freeze-thaw process, glycerol [6] and ethylene glycol [7] have comparable effects, making them the most supportive cryoprotectants. Additionally, sugars contribute to sperm cryopreservation through their osmotic effects and their interaction with the phospholipid bilayers during freezing, which maintains their equilibrium [8]. Sugar, for instance, contains trehalose, a disaccharide called α-D-glucopyranosylα-D-glucopyranoside, which binds to extracellular ice crystals and exhibits its cryoprotective effect by interacting with membrane phospholipids and increasing the membrane's resilience against cryoinjury [9-10]. This disorder results in extracellular hypertonicity, which causes water to exit the cell and delays the formation of intracellular ice crystals upon freezing [2,11]. As a non-saturating cryoprotectant, trehalose causes spermatozoa to dry out because of the water's osmotically regulated progression. This slight drying out causes spermatozoa to have less intracellular water, which in turn reduces the production of intracellular ice crystals [2]. This state is advantageous to sperm since intracellular ice crystal development arrangements trigger cell death, which lowers the cryopreserved semen's fertility [12].

Experiments, including the impact of trehalose concentrations of cryoprotectants that can further develop post-thaw sperm boundaries in ruminants, have produced varied experimental results. The variable aftereffects of these investigations are likely to result in a variety of extender creations or species with varying levels of resistance to trehalose, semen collection, and experimental methodology [8,13]. Consequently, it is difficult to draw definite conclusions about the protective effects of trehalose on ruminant sperm parameters such as motility, progressive motility, hypoosmotic swelling test, and abnormalities due to the diversity in experimental results. Hence, a meta-analysis methodology is an alternative option [14]. A meta-analysis study employs data from carefully and statistically conducted prior studies to draw correct findings [15]. Thus, the goal of this study was to do a meta-analysis of the existing literature on how trehalose consumption affects the protective qualities of bovine sperm characteristics.

2 Materials and Methods

2.1 Database development

The meta-analysis employs the methodology outlined in Selcuk's Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [16]. The review data set started with dispersed scientific publications that were searched for using the terms "trehalose" and "semen diluent" on websites like Scopus and Google Scholar. Although we initially found 125 articles, only 46 of them were deemed appropriate based on the abstract. From these, 22 reports were retrieved and included in the database after the full text was reviewed (Table 1). The database contained the following parameters: 1) motility; 2) progressive motility; 3) path velocity; 4) acrosome integrity; 5) hypoosmotic swelling test; 6) viability; and 7) abnormality. The level of trehalose in semen diluent ranged from 0 (control) to 435 mM, with the basic extender in the form of tris (Table 1). To assess the impact of trehalose supplementation at varying dosages during cryopreservation on post-thawing quality, semen is often collected using an artificial vagina attached to the dummy and then sent to the laboratory. Importantly, data tabulation made processing easier by analyzing all the parameters in one unit.

2.2 Data analysis

Table 1. A list of the chosen papers that served as the data sources for this meta-analysis

No.	Animal	Semen collecting	Trehalose inclusion in diluent (mM)	Basic textender	Reference
1.	Goat	artificial vagina	0 – 100	tris	[17]
2.	Ram	artificial vagina	0 – 435	tris	[18]
3.	Ram	artificial vagina	0 – 100	tris	[19]
4.	Goat	artificial vagina	0 – 100	tris	[20]
5.	ram	artificial vagina	0 – 150	tris	[21]
6.	bull	artificial vagina	0 – 200	tris	[22]
7.	goat	artificial vagina	33.1 – 264	tris	[23]
8.	ram	artificial vagina	0 – 100	tris	[24]
9.	bull	artificial vagina	0 – 100	tris	[12]
10.	ram	electro ejaculator	0 – 100	tris	[25]
11.	buffalo and bull	artificial vagina	0 – 100	tris	[26]
12.	ram	artificial vagina	0 – 100	soybean	[10]
13.	goat	artificial vagina	0 – 150	tris	[27]
14.	buffalo	artificial vagina	0 – 100	tris	[28]
15.	bull	artificial vagina	0 – 200	tris	[29]
16.	bull	testicular tissue	0 – 25	DMEM	[30]
17.	buffalo	artificial vagina	0 – 60	tris	[31]
18.	buffalo	artificial vagina	0 – 45	tris	[32]
19.	bull	artificial vagina	0 – 25	tris	[33]
20.	ram	electro ejaculator	0 – 50	tris	[34]
21.	goat	artificial vagina	0 – 150	tris	[8]
22.	ram	artificial vagina	0 – 100	tris	[2]

DMEM = Dulbecco's modified Eagle's medium.

Based on the meta-analysis approach, a mixed model was used to examine the contents of the database [35-37]. The meta-analysis included two types of statistical models because the indicator variable was either discrete or persistent (see Equations a and b). Trehalose's addition to the sperm diluent was referred to as a "fixed effect," but the other impacts-which were implied by the RANDOM statement-were referred to as "random effects" because they weren't entirely predetermined. The first-order linear mixed model (LMM) is shown in Equation 1, and the second-order LMM model is shown in Equation 2:

$$Y_{ij} = \mu + s_i + \tau_j + s\tau_{ij} + \beta_0 + \beta_1 T_{ij} + b_i T_{ij} + e_{ij} \quad \text{(a)}$$

$$Y_{ij} = \mu + s_i + \tau_j + s\tau_{ij} + \beta_0 + \beta_1 T_{ij} + \beta_2 T^2{}_{ij} + b_i T_{ij} + e_{ij} \quad \text{(b)}$$

Where the Y_{ij} is a dependent variable, μ is the average value of the overall trehalose level, s_i is the i-th random factor from various studies, τ_j is the j-th fixed factor of the predictor, $s\tau_{ij}$ is a factor of random interaction between the difference of the research and the fixed factor of the trehalose level, β_0 is the value of the average intersection of all studies with the X axis, β_1 is the linear regression coefficient, β_2 is the quadratic regression coefficient, T_{ij} is addition level of trehalose, $b_i T_{ij}$ is the random effect of the difference in studies from the regression coefficient Y in the X of the i-th study, and e_{ij} is the unexplained error.

Furthermore, an interaction analysis was carried out between the addition of trehalose to the type of livestock and the type of treatment, as well as the combination of interactions described in the following equation,

$$Interaction = T + A + Exp + T * A + T * Exp + A * Exp + T * A * Exp \quad (c)$$

Where T is the level of trehalose addition (in mM), A is the type of animals (buffalo, bull, goat, and ram), and Exp is the type of treatment, i.e., control and trehalose addition.

In situations where the individual quadratic regression model was not significant at $p < 0.05$, a linear regression model was used. The significance test was performed at p-value <0.05 using the model statistics of p-value and root means square error. Furthermore, a p-value somewhere in the range of 0.05 and 0.10 indicated an impact tended to be significant. To validate the LMM equation, root means square error (RMSE) and R squared Nakagawa (R^2) were used [38]. One-way ANOVA and the least-squares mean were used to compare livestock, breed, semen collection, and basic extender. Every quantifiable analysis was carried out using R software, version 4.2.0 [39]. The findings of the descriptive statistics were presented as mean ± standard deviation. The mean ± standard error was used to present the meta-analysis findings.

3 Results and Discussion

A statistical description of the data used are shown in Table 2. Based on all the results, the mean dosage for supplementing semen diluent with trehalose was 58.5 ± 69.9 mM. Trehalose inclusion was present at a minimum of 0 mM (control). Trehalose was employed as a protective semen diluent in all the control treatments. The maximum use of trehalose in semen diluent in this study was 435 mM. The average motility was 47.4 ± 15.5 %, the average progressive motility was 27.6 ± 17.2 %, and the average path velocity was 84.7 ± 27.7 µm/s. The average acrosome integrity was 43.1 ± 24.9 %. The average of the hypoosmotic swelling test (HOST), viability, and abnormality were 40.3 ± 14.3, 61.9 ± 17.5, and 9.48 ± 3.3 %, respectively.

Table 2. An overview of the descriptive statistics for the variables and parameters that were observed during this study

Parameter	Mean	SD	Minimum value	Maximum value
Level of trehalose, mM	58.5	69.90		435
Motility, %	47.4	15.5	1.45	78
Progressive motility, %	27.6	17.2	7.4	64.2
Path velocity, µm/s	84.7	27.7	57.4	170
Acrosome integrity, %	43.1	24.9	1.3	84.6
HOST, %	40.3	14.3	0.67	67.4
Viability, %	61.9	17.5	31.7	115
Abnormality, %	9.5	3.3	5.4	16.4

HOST = hypoosmotic swelling test

The results of a meta-analysis concentrate on uncovering a further developing impact of trehalose supplemented with a basic extender on semen quality, giving the best protection regarding post-thaw motility parameters, progressive motility, and membrane integrity while diminishing the abnormal sperm morphology. The cryoprotective limit of trehalose differed, relying upon the concentration of supplementation in the extenders [23]. In this review, the

control treatment of trehalose in semen diluent was 0 mM, with the highest use of trehalose concentration in semen diluent was 435 mM.

Table 3. Regression parameters for effect of trehalose in semen diluent on ruminant sperm characteristic.

Parameter	Model	N	Intercept	SE Intercept	Slope	SE Slope	*p*-value	RMSE	R^2
Motility, %	Q	74	44.3	3.37	0.112 -0.000608	0.0437 0.000222	0.0137 0.0082	7.18	0.72
Progressive motility, %	L	43	26.4	4.65	0.0304	0.0117	0.014	4.3	0.9
Path velocity, µm/s	L	32	86.6	11.7	-0.0316	0.0312	0.322	5.2	0.97
Acrosome integrity, %	L	42	42.5	6.98	0.0134	0.0153	0.39	4.69	0.94
HOST, %	Q	54	37.2	3.69	0.157 -0.00106	0.0552 0.000332	0.007 0.00277	6.63	0.72
Viability, %	L	39	60.6	5.25	0.0079	0.0245	0.751	8.64	0.72
Abnormality, %	Q	18	11.5	1.18	-0.0491 0.00013	0.0173 0.0000435	0.0125 0.00938	2.21	0.46

HOST = hypoosmotic swelling test; L = linear; N = total of analyzed data; p-value = significant value if the *p*-value is less than 0.05 and tends to be significant if it is less than 0.1; Q = quadratic; RMSE = root mean square error.

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The effect of using trehalose content in semen diluents on the observed parameters of semen characteristics are shown in table 3. The level of trehalose concentration significantly affected the main parameters of ruminant semen characteristics, namely motility, progressive motility, hypoosmotic swelling test, and abnormalities. The level of trehalose concentration expanded the level of motility, progressive motility ($p < 0.05$), the hypoosmotic swelling test ($p < 0.01$), and diminished the percentage of abnormality ($p < 0.05$). This significant impact demonstrates that the expansion of trehalose concentration in the semen diluent can function as a protective material in the cryopreservation process of spermatozoa. The increase in trehalose concentration in the semen diluent did not significantly affect the path velocity, acrosome integrity, or viability. Based on the parameters of motility, hypoosmotic swelling test, and abnormality, the following equations were obtained,

$$Motility\ (\%) = 44.3 - 0.0006X^2 + 0.112X \quad (d)$$

$$Hypoosmotic\ swelling\ test\ (\%) = 37.2 - 0.0011X^2 + 0.157X \quad (e)$$

$$Abnormality\ (\%) = 11.5 + 0.0001X^2 - 0.049X \quad (f)$$

These equations are used to determine the optimal level of trehalose addition.

Motility is generally considered to be a facet of viability and structural integrity and may be the primary factor associated with sperm's capacity to fertilize. The meta-analysis showed that adding trehalose to the semen extender further developed post-thaw motility and progressive motility parameters. The osmotic effect and particular interactions with membrane phospholipids are therefore thought to be connected to the cryoprotective effect of trehalose [22]. According to Aboagla [17] and Reddy [40], these sugars work with the phospholipids in the plasma membrane to reduce cryodamage of spermatozoa. They also improve the fluidity of the membrane, which further prevents spermatozoa from being damaged by freeze-thaw cycles. On the other hand, according to a different theory, trehalose probably shields biomolecular structures by capturing basic hydration water particles [11] and substituting water in hydrogen bonds [41]. Aboagla [17] announced that freezing goat sperm at trehalose brought about significantly more prominent post-thaw total motility and progressive motility than without even a trace of trehalose. A trehalose concentration positively affected the total and progressive sperm motility in ram semen [25].

During cryopreservation, sperm plasma membranes are susceptible to reactive oxygen species (ROS) assault; damage to these membranes results in decreased fertility [42]. It has been determined that the addition of trehalose to sperm extender significantly impacted the integrity of the protective membrane [33,43-44] and gave lower abnormal spermatozoa ratio compared to the control in bull [29], ram [19], goat [23] sperm cryopreservation studies. The specific defensive component of trehalose is obscure, yet it is proposed that its activity would be through balancing out the membrane bilayers and adjusting membrane fluidity [45]. Trehalose also reduces the production of ice crystals by balancing cell dehydration and influencing the crystallization example of the solute divers in the unfrozen regions of water [9].

Therefore, improved sperm capabilities, such as motility characteristics, would result from this enhancement in membrane integrity and functioning. It is also suggested that the osmotic effects and direct interactions with membrane phospholipids are linked to the cryoprotective effects of trehalose included in extenders [31]. Trehalose can protect cells from several environmental stressors, such as oxidation, dehydration, heat, and cold [46]. The formation of a non-hygroscopic glass state gave it excellent settling qualities and shielded lipid and protein membranes from deterioration during the freeze-drying procedure [29]. When trehalose was used, membrane integrity was better preserved. Membrane breakdown may result from sperm freezing due to changes in membrane phases occurring in different regions of the very specific sperm plasma membrane [47]. These situations may allow the sperm membrane to sustain damage during freezing since trehalose settles natural membranes, perhaps due to its interaction with the polar heads of the membrane phospholipids [48]. Trehalose may lead to more advanced post-thaw sperm motility by influencing the usable function of acrosomes and mitochondria, which are responsible for generating energy from intracellular stocks of ATP [40].

Table 4. The p-value of the interaction between the level of addition of trehalose, animals, and experimental treatments and their combinations.

Parameter	T*A	T*Exp	A*Exp
Motility, %	0.065	0.366	0.253
Progressive motility, %	0.945		0.687
Path velocity, μm/s	0.513		0.226
Acrosome integrity, %	0.197		0.425
HOST, %	0.946	0.415	0.0994
Viability, %	0.442		0.531

Abnormality, %	0.0009	0.0093	0.0085

A = animals; Exp = experimental treatments; HOST = hypoosmotic swelling test; T = addition level of trehalose; p-value = significant value if the *p*-value is less than 0.05.

The result of the interaction test between the level of trehalose, the type of animals, the type of experimental treatment, and their combinations are shown in Table 4. The interaction between the level of trehalose in the type of animals, the level of trehalose in the kind of experimental treatment, and the type of animals in the type of experimental treatment influenced the significant value of abnormality ($p < 0.05$). Meanwhile, other parameters are not influenced by these interaction factors.

The addition of trehalose on semen characteristics was not affected by animal and breed. This is possible because, even though each breed and species of animal has a varied body size, including testes volume, testes size mainly refers to the quantity of sperm produced per ejaculate [49]. Sonseeda [50], the only statistically significant effect of breed on semen quality is semen volume. Furthermore, Adamu [51] claims that close genetic affinities may be the cause of the breed's lack of influence on semen quality. In contrast, Greiser [52] demonstrates that breed effects account for a sizable and considerable part of diversity in fresh and frozen-thawed semen features.

The effects of trehalose addition on semen properties influenced by animal breed, semen collection, and basic extender are displayed in Tables 5-8. The statistical analysis's findings demonstrated that the addition of trehalose to semen characteristics was unaffected by animals, semen collection, or basic extenders ($p > 0.05$). The addition of trehalose on semen characteristics affected by animal breed showed the significance, namely in motility, acrosome integrity, and hypoosmotic swelling test ($p < 0.001$, from one-way ANOVA), however, the findings of the post hoc tests were not significant at 5% level using least-square means.

Even though semen obtained from an artificial vagina (AV) has higher characteristics than an electro ejaculator (EE), statistical analysis did not identify any differences. The two most popular techniques for collecting semen are electroejaculation and the use of an artificial vagina. According to Marco-Jimenez [53], EE samples may contain more sperm overall but less of them per volume than AV samples. Furthermore, Rego [54] noted that although the technique of semen collection had no impact on the morphology or motility of sperm, samples obtained using EE had a reduced sperm concentration. According to additional research, the artificial vaginal semen samples collected by EE had different sperm concentrations but not variations in sperm motility or morphology [55].

Table 5. Addition of trehalose on semen characteristics affected by animal.

Parameter	***p*-value**	**Buffalo**	**Bull**	**Goat**	**Ram**
Motility, %	0.634	56.9	42.3	47.5	45.1
Progressive motility, %	0.333	12.5	33.9	35.7	23.6
Path velocity, µm/s	0.825	69.3	69.4	87.9	99.4
Acrosome integrity, %	0.841	53.6	53.4	31	32.4
HOST, %	0.752	33.5	45.9	38	42.7
Viability, %	0.310	81.9	60.6	51.9	60.5
Abnormality, %	0.943		10.5	9.07	9.42

HOST = hypoosmotic swelling test; p-value = significant value if the *p*-value is less than 0.05.

Table 6. Addition of trehalose on semen characteristics affected by semen collection.

Parameter	*p*-value	Artificial Vagina	Electro Ejaculator	Testicular Tissue
Motility, %	0.476	48.1	40.1	
Progressive motility, %	0.306	29.3	17.3	
Path velocity, µm/s	0.429	88.6	68.1	
Acrosome integrity, %	0.103	45.8	7.33	
HOST, %	0.820	40.1	41.8	
Viability, %	0.637	59.0	58.9	77.8

HOST = hypoosmotic swelling test; p-value = significant value if the *p*-value is less than 0.05.

Table 7. Addition of trehalose on semen characteristics affected by the basic extender.

Parameter	*p*-value	DMEM	Soybean	Tris	Tris Citrate	Tris Glucose	Citrate
Motility, %	0.360		52	46	44.9	69.6	
Progressive motility, %	0.391		22.3	25.2	22.6	55.8	
Path velocity, µm/s	0.007		162[b]	76.9[a]	69.3[a]	89.8[a]	
Acrosome integrity, %	0.113			31.8	58.1		
HOST, %	0.386			44.7	36.5		
Viability, %	0.617	77.8		58	60.4		
Abnormality, %	0.387			7.6	10.2		

DMEM = Dulbecco's modified Eagle's medium; HOST = hypoosmotic swelling test; *p*-value = significant value if the *p*-value is less than 0.05.

Table 8. Addition of trehalose on semen characteristics affected by animal breed.

Breed	Motility, %	Progressive motility, %	Path velocity, µm/s	Acrosome integrity, %	HOST, %	Viability, %	Abnormality, %
p-value	<0.001	0.318	0.89	0.52	<0.001	<0.001	0.477
Akkaraman	53.2				48.1	68	7.1
Angora	43.6	12.4	86.5	3.11	50.1		
Black Bengal	13.9				8.55		
Boer	59.5	49.1		70			7.64
Dorset	28.7	16.2	78	7.33	46.7		
Holstein	60.1	33.9	69.4	45.9		37.9	
Karan Fries	41.9				44.3	47.8	
Markhoz	45.4	34.5			46.3	51.9	10.7
Merino	35.2			41		43	
Merino Sakiz	51.5	18.4	58.2		36.9	58.9	
Murrah	46.8				44	59.2	
Nili Ravi	57	11.5	68.2	62	30.8		
Qinchuan						77.8	
Shiba	69.6	55.8	89.8				
Suffolk		44.4				63.3	12.9
Zandi	52	22.3	162				

HOST = hypoosmotic swelling test; *p*-value = significant value if the *p*-value is less than 0.05.

Different types of basic extenders were utilized, according to the data sources in this meta-analysis study, but statistical findings indicate that the addition of trehalose on semen characteristics was unaffected by basic extenders. Furthermore, extenders protect sperm against osmotic stress, cold shock, and changes in membrane fluidity and permeability, while also giving them energy substrates for glucose metabolism [56]. Improved semen extenders and additives should be utilized to increase the quality of the semen and accelerate the rate of sperm fertilization [57]. Due to its ability for buffering, diuretic, and osmotic activities, as well as its low toxicity at high concentrations, tris (hydroxymethyl aminomethane) is a common organic buffer found in spermatozoa preservation extenders [58]. The most prevalent components in extenders used to freeze semen are tris and citrate [59]. To maintain the osmotic pressure of the diluents, carbohydrates such as glucose are essential because they cause cell dehydration and reduced ice crystal formation in the spermatozoa [60]. Furthermore, the membranes are stabilized because of its interaction with membrane phospholipid at low temperatures [61]. In addition to being used successfully to supplement sperm cryopreservation media in livestock species, soybean may be a good replacement for egg yolk [62].

4 Conclusion

It can be concluded that the study recommended using trehalose in semen diluent as a protective material for their success in the cryopreservation process of ruminant spermatozoa. The trehalose expansion in the extender reduces the percentage of abnormality while improving the post-thaw characteristics of bovine spermatozoa, such as the percentage of motility and the hypoosmotic swelling test. The recommended optimal level of trehalose addition to maximize sperm motility is around 98.2 mM, or to optimize the hypoosmotic swelling test value, it is about 74 mM.

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Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution statement
FA Pamungkas: Conceptualization, Methodology, Investigation, Writing- Original draft preparation. MM Sholikin: Data curation, Writing- Reviewing and Editing. TP Priyatno: Visualization, Investigation, Writing- Reviewing and Editing. Herdis: Software, Validation, Writing- Reviewing and Editing. T Kostaman: Software, Validation, Writing- Reviewing and Editing. Santoso: Software, Validation, Writing- Reviewing and Editing. DA Kusumaningrum: Software, Validation, Writing- Reviewing and Editing. SY Hayanti: Software, Validation, Writing- Reviewing and Editing.

References

1. N. Prieto-Martínez, I. Vilagran, R. Morató, et al. Reprod Fertil Dev. **28(6):**663 (2016).
2. A.E. Öztürk, M. Bodu, M.N. Bucak, et al. "Cryobiology and cryopreservation of sperm," in *Cryopreservation - Current Advances and Evaluations*. (IntechOpen, 2020).
3. H.D. Guthrie and G.R. Welch, Theriogenology **78(8):**1700-1708 (21012).
4. J.M. Sztein, T. Takeo, N. Nakagata. Cryobiology **82**:57-63 (2018).
5. D.R. Câmara, S.V. Silva, F.C. Almeida, et al. Theriogenology **76(2):**342-350 (2011).
6. A.S. Oude Vrielink, A. Aloi, L.L.C. Olijve, et al. Biointerphases **11(1):**018906 (2016).
7. A. Najafi, H. Daghigh-Kia, H.V. Dodaran, et al. Anim Reprod Sci. **177:** 35-41(2017).
8. M. Karunakaran, P. Konyak, A. Mandal, et al. Indian J Anim Res. **53(1)**:37-40 (2019).
9. E. Aisen, M. Quintana, V. Medina, et al. Cryobiology. **50(3)**:239-49 (2005).
10. A. Najafi, M. Zhandi, A. Towhidi, et al. Cryobiology. **66(3)**:275-282 (2013).
11. A. Patist, H. Zoerb. Colloids Surfaces B Biointerfaces. **40(2)**:107-113 (2005).
12. S. Chhillar, V.K. Singh, R. Kumar, et al. Anim Reprod Sci. **135(1-4)**:1-7 (2012).
13. E. Ahmad, M. Aksoy. Anim Heal Prod Hyg. **1**:123-129 (2012).
14. D. Sauvant, P. Schmidely, J.J. Daudin, et al. Animal. **2(8)**:1203-1214 (2008).
15. O. Sjofjan, D.N. Adli, R.P. Harahap, et al. F1000Research. **10**:183 (2021).
16. A.A. Selcuk. Turkish Archives of Otorhinolaryngology. **57(1)**: 57-58 (2019).
17. E.M.E. Aboagla, Biol Reprod. **69(4)**:1245-1250 (2003).
18. T. Matsuoka, H. Imai, H. Kohno, et al. J. Reprod. Dev. **52(5)**:675-683 (2006).
19. M.N. Bucak, A. Ateşşahin, O. Varışlı, et al. Theriogenology. **67(5)**:1060-1067 (2007).
20. B. Khalili, A. Farshad, M.J. Zamiri, et al. J Anim Sci. **22(12)**:1614-1619 (2009).
21. O. Uysal and M.N. Bucak, Ankara Üniversitesi Vet. Fakültesi Derg. **56(2)**:99-103 (2009).
22. J.H. Hu, L.S. Zan, X.L. Zhao, et al. J Anim Sci. **88(5)**:1657-1662 (2010).
23. S.W. Naing, H. Wahid, K. Mohd Azam, et al. Anim Reprod Sci. **122(1-2)**:23-28 (2010).
24. R.A. Tonieto, K.L. Goularte, G.D.A. Gastal, et al. Small Rumin. Res. **93(2-3)**:206-209 (2010).
25. U. Cirit, H. Bağış, K. Demir, et al. Anim Reprod.Sci. **139(1-4)**:38-44 (2013).
26. R. Kumar, V. Singh, S. Chhillar, et al. Reprod Domest Anim. **48(3)**:407-415 (2013).
27. P.B. Tuncer, U. Taşdemir, S. Büyükleblebici, et al. Small Rumin. Res. **113(2-3)**:383-389 (2013).
28. M.R. Badr, A.M.S Azab and Z.M. Rawash, Assiut Vet Med J. **60(142)**:38-45 (2014).
29. R.I. El-Sheshtawy, G.A. Sisy and W.S. El-Nattat, Asian Pacific J Reprod. **4(1)**:26-31 (2015).
30. X.G. Zhang, Y.H. Wang, C. Han, et al. Cryobiology. **70(3)**:246-252 (2015).
31. S. Iqbal, S.M.H. Andrabi, A. Riaz, et al. Theriogenology. **85(5)**:954-959 (2016).
32. S. Iqbal, S. Naz, H. Ahmed, S.M.H. Andrabi, Andrologia. **50(1)**:1-6 (2018).
33. C. Öztürk, S. Güngör, M.B. Ataman, et al. Acta Vet Hung. **65(3)**:429-439 (2017).
34. S. Gungor, A. Ata and M.E. Inanc, Acta Sci Vet. **46(1)**:7 (2018).
35. C. Hidayat, A. Irawan, A. Jayanegara, et al. Vet World. **14(6)**:1405-1411 (2021).

36. D. Sauvant, P. Schmidely, J.J. Daudin, et al. Animal. **2(8)**:1203-1214 (2008).
37. N.R. St-Pierre, J. Dairy Sci. **84(4)**:741-755 (2001).
38. S. Nakagawa and H. Schielzeth, Methods Ecol. Evol. **4(2)**:133-142 (2013).
39. R Core Team, *R: A language and environment for statistical computing* (R Found, Stat. Comput. Vienna, Austria, 2022)
40. N.S.S. Reddy, G.J. Mohanarao and S.K. Atreja, Anim Reprod Sci. **119(3-4)**:183-190 (2010).
41. G.I. Olgenblum, L. Sapir and D. Harries, J Chem Theory Comput. **16(2)**:1249-1262 (2020).
42. S.M. Andrabi, M. Ansari and N. Afzal, Pak Vet J. **28(4)**:159-162 (2008).
43. E.G. Aisen, H.L. Alvarez, A. Venturino, et al., Theriogenology. **53(5)**:1053-1061 (2000).
44. V. Singh, S. Atreja, R. Kumar, et al. Reprod Domest Anim. **47(4)**:584-590 (2012).
45. E. Zuther, K. Büchel, M. Hundertmark, et al. FEBS Lett. **576(1-2)**:169-173 (2004).
46. Q. Chen and G.G. Haddad, J Exp.Biol. **207(18)**:3125-3129 (2004).
47. M.N. Bucak, P.B. Tuncer, S. Sarıözkan, et al. Small Rumin Res. **81(1)**:13-17 (2009).
48. J.M. Blanco, J.A. Long, G. Gee, et al. Anim Reprod Sci. **123(3-4)**:242-248 (2011).
49. Y. Sato, A. Tajima, M. Kiguchi, et al. J Hum Genet. **65(8)**:683-691 (2020).
50. P. Sonseeda, T. Vongpralub and B. Laopaiboon, Thai J Vet Med. **43(3)**:347-352 (2013).
51. J. Adamu, A. Dauda and H. Abbaya, Int J Avian Wildl Biol. **4(3)**:90-94 (2019).
52. T. Greiser, H. Sieme, G. Martinsson, et al. Theriogenology. **142**:8-14 (2020).
53. F. Marco-Jiménez, J. Vicente and M. Viudes-de-Castro, Reprod Domest Anim. **43(4)**:403-408 (2008).
54. J.P.A. Rego, A.A. Moura, A.S. Nouwens, et al. Anim Reprod Sci. **160**:126-137 (2015).
55. A.D. Barth, A.A. Arteaga, L.F. Brito, et al. Anim Reprod Sci. **84(3–4)**:315-325 (2004).
56. C.M.O Medeiros, F. Forell, A.T.D. Oliveira, et al. Theriogenology. **57(1)**:327-344 (2002).
57. F. Baiee, W. Haron, R. Yusoff, et al. Pak J Zool. **50(1)** (2017).
58. A. Niasari-Naslaji, S. Mosaferi, N. Bahmani, et al. Cryobiology. **53(1)**:12-21 (2006).
59. N. Raheja, S. Grewal, N. Sharma, et al. Journal of Entomology and Zoology Studies. **6(3)**:239-245 (2018).
60. P.H. Purdy, Small Rumin Res. **63(3)**:215-225 (2006).
61. C. Gangwar, D. Jena, S.D. Kharche, et al.Indian J Small Ruminants. **26(1)**:125 (2020).
62. S. Chelucci, V. Pasciu, S. Succu, et al. Theriogenology. **83(6)**:1064-1074 (2015).