

Cryopreservation Efficiency Of Holstein Bulls: the Effect of Adding Relaxin and Type of Extenders on Semen Quality

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ABSTRACT

This study examined the effect of adding relaxin on cryopreserved sperm quality using tris and soybean lecithin extenders. After pooling of semen, the following six groups were evenly divided as following: (TC) tris extender only, (T1): tris extender with 60 ng/ml relaxin, (T2): tris extender with 90 ng/ml relaxin, (SC): Soybean lecithin extender only, (S1): soybean lecithin extender with 60 ng/ml relaxin; (S2): Soybean lecithin extender with 90 ng/ml relaxin. The quality of the sperm was evaluated at cooling (5°C), 48 hours, one- and two-months post cryopreservation. The parameters studied include the percentage of sperm individual motility, viability, total abnormalities, acrosome integrity, plasma membrane integrity, antioxidant capacity, levels of malondialdehyde (MDA), and DNA fragmentation. The percentage of sperm motility and viability were significantly increased for the T1 and S1 treatments compared with other treatments for all periods. The percentage of plasma membrane and acrosome integrity enhanced significantly in the T1, and S1 treatments compared with others for all periods. The DNA fragmentation and MDA levels were also reduced in treatment T1, indicating better defense against oxidative stress and DNA damage. In conclusion, adding 60 ng/ml of relaxin to tris semen extenders improved the post cryopreservation sperm quality in Holstein bulls.

Key words: Cryoprotectant enhancement, DNA integrity, Malondialdehyde, Oxidative stress, Relaxin, soybean lecithin extender, Viability analysis.



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INTRODUCTION

Cryopreserve of sperm has been an effective approach for preserving sperm fertility over an extended period of time (Mahdi, 2023). Spermatozoa are prone to extreme stress during the cryopreservation and thawing processes, which frequently results in reduces motility, viability, and general quality (Santonastaso et al., 2021, Al-Hadithi et al., 2024). The essential choice of extender solutions and spermatozoa during cryopreservation is a hard decision that influence cellular damage reduction, (Ozimic, et al., 2023). According to specific cell surface receptors in spermatozoa, relaxin, is an insulin superfamily regulatory peptide, which could have a physiological effect in sperm motility and fertility (Kohsaka, et al., 2003, Kandil et al., 2024). Spermatozoa cryopreservation was practiced for decades and is a very useful technique for long-term preservation of sperm fertility. The process of cryopreservation and

thawing can induce major stress on spermatozoa, often leading to decreased motility, viability, and overall quality (Medeiros, et al., 2002). The preservation of spermatozoa while cryopreservation relies heavily on the choice of extender solutions, which plays a role in minimizing cellular damages (Layek, et al., 2016). Relaxin ways of influence sperm quality are very intriguing because it could enhance the results of ART procedures (Miah, et al., 2015d). Relaxin, an insulin superfamily regulatory peptide It has recommended to have physiological influence on sperm motility and fertility by specific cell surface receptors in spermatozoa (Kohsaka, T et al., 2003). Relaxin has been suggested to improve motility of humans (Ferlin, et al., 2012), bulls (Miah, et al., 2007a, Miah, et al., 2011c) and porcine spermatozoa (Miah, et al., 2008b). In previous studies, conducted by other species, the effect of relaxin supplement in the sperm-thawing media has been

examined at the end of the cryopreservation process (Elkhawagah, et al., 2013a, Elkhawagah, et al., 2015b). Tris and soybean lecithin are popular extender choices in the field of sperm cryopreservation (Niu et al., 2024). While soybean lecithin extenders are recognized for their nutrient content and potential for enhanced sperm preservation (Bustani, and Baiee. 2021). Tris's extenders are preferred for their buffering capacity and ability to preserve sperm viability (Hameed, et al., 2023, Kose, et al., 2024). Enhancing cryopreservation techniques requires an understanding of how relaxin interacts with extenders and impacts sperm quality. Therefore, the current study aims to find out the effect of adding different concentrations of relaxin to two types of semen extender which include tris and soybean lecithin on semen characteristics stored at freezing of Holstein bulls.

MATERIALS AND METHODS

Semen collection and extenders: The semen was collected from the bulls using the artificial vagina, with temperature of 40-41 °C at the early morning hours (6 am), semen was pooled by collecting one ejaculate per bull weekly over seven weeks, with 1ml taken from each ejaculate, for the purpose of removing the individual differences between the bulls, and ejaculate was taken from bulls after a false mount of the bull to increase their sexual desire. To avoid exposure to environmental factors like light and heat, the sample was placed in a 37°C-water bath until rectification and extender addition (Afzali Chegeni, et al., 2020). The tests conducted to explore fresh semen included (ejaculate volume, pH, sperm concentration, and the percentage of collective and individual movement of sperm). It was divided equally among the six treatments and transferred directly to refrigerator at 5 °C, and then filling the special tubes for each treatment for the purpose of preserving them with liquid nitrogen at a temperature of -196°C. The best concentration of relaxin was added to the Tris buffer and soybean extenders. All samples that had a concentration of $\geq 800 \times 10^6$ /ml, and motility $\geq 70\%$ were used as semen source. Semen samples were pooled and diluted with Tris's extender and soybean lecithin extender,

according to instructions (Centrifuge the vial before opening. Reconstitute in water to a concentration of 0.1-1.0 mg/ml. Do not vortex. This solution can be stored at 2-8° C for up to 1 week. For extended storage, it is recommended to further dilute in a buffer containing a carrier protein (example 0.1% BSA) and store in working aliquots at -20° C to -80° C.), and supplemented with relaxin (SRP3147, Sigma-Aldrich, Italy) at different concentrations: 0 (control), 60 and 90ng/ml. Diluted semen with a final concentration of 30×10^6 spermatozoa/ml was slowly cooled to 5°C, packed into 0.25 ml polyvinyl straws (Minitub, Germany), equilibrated for 4 hours and frozen. The tests were performed at different preservation periods (cooling at a temperature of 5 °C and after 48 hours and one month and two months of cryopreservation).

Sperm individual motility: The individual motility of sperm was estimated according to Timourian (Timourian, H. and G. Watchmaker 1970), by placing a drop of semen on a glass slide with a small drop of sodium citrate at a concentration of 2.9%, and placing the cover slide under a light microscope and examining it at a magnification of 40×. Individual motility based on the proportion of motile sperms is forward motility, while sperms with abnormal motility (circular, backward, pendulum) are considered non-motile.

Plasma membrane integrity: According to Jeyendran (Jeyendran, R et al., 1984), the percentage of sperm with an intact plasma membrane was estimated by placing two drops of the semen sample to be examined in test tubes containing 1 ml of (HOST-hypo-osmotic solution) consisting of (8.72 g/L fructose) and (4.74 g / sodium citrate), as its osmotic pressure is 100 mm osmol / liter, and the pH = 8. The tubes were placed in a water bath at a temperature of 37 °C for a full hour, after which a drop was taken, and a smear was made on a clean glass slide. 200 sperms were counted in the fields. Separate, coiled-tailed, swollen spermatozoa were counted as intact under a microscope at 40× magnification.

Acrosome integrity: Pigmentation is based on the method developed by Kovacs and Foote (Kovacs, A. and R. Foote. 1992, Nsaif, Z. and S. Eidan. 2024). Giemsa stain was prepared by

mixing the following materials and in the specified quantities for each: Giemsa powder 3.8 g, Methanol 375 ml, Glycerol 125 ml. It was left for two weeks, then filtered it. Take 3ml of the dye and mix it with 2ml of Sorenson buffer.

DNA integrity, MDA, and total antioxidant:

DNA integrity percentage as described by Tejada, (Tejada, et al., 1984), assessment of sperm abnormalities was assessed, For the purpose of estimating the total antioxidant activity in seminal plasma (Moskovtsev, and C. L 2013). The kit was prepared from the American company Sigma (1,1-Diphenyl-2-picryl-hydrazyl (DPPH), Sigma Aldrich Co., St.Louis, USA). The concentration of malondialdehyde in seminal plasma was estimated based on the method described by (Kumaresan, Ansari and Garg, 2005).

Statistical analysis: Statistical computations were done using the SAS software program to explore the influence of treatment and time (Cary, N.2012). Duncan's multiple range test for comparison between means (Duncan, D. B.1955, Cary, N.2012) Was used ($p < 0.05$).

RESULTS AND DISCUSSION

Sperm individual motility percentage: All treatments exhibit a decrease in motility over time. The decline is most pronounced between the post-thawing and the two-month

cryopreservation periods. Relaxin improves motility in both extenders, the T1 showed the best results. Soybean lecithin treatments benefit less from relaxin, maintaining lower motility throughout the experiment as shown in (Table 1). The addition of relaxin, particularly the T1, significantly ($P \leq 0.01$) enhances sperm motility during cryopreservation. Soybean lecithin treatments, even with relaxin, exhibit lower motility compared to their Tris counterparts.

Sperm viability percentage: All treatments show an increase in viability over time, with the most significant increases ($P \leq 0.01$) typically occurring after one and two months of cryopreservation. T1 and T2 had the highest viability rates, indicating that these treatments are more effective at maintaining sperm viability during extended storage periods (Table 2). SC, S1 and S2 showed lower viability rates compared to the Tris treatments. At each time point, the TC and SC generally show lowest compared to treatments with relaxin. T2 and S2 generally exhibit lower abnormalities, indicating better viability. This trend is consistent across all time points. Overall, the T2 and S2 significantly enhances ($P \leq 0.01$) the viability of spermatozoa during cryopreservation and being the most effective treatments.

Table 1. Effect of adding relaxin to tris and soybean lecithin extenders on individual sperm motility (%) at Holstein bulls after different periods of preservation (mean $\pm$ SE).

Treatments	Time				Significance Level
	Cooling 5°C	48h Post-Cryopreservation	One month Post-Cryopreservation	Two months Post-Cryopreservation	
TC	27.50 $\pm$ 2.11 a A	23.12 $\pm$ 1.87 ab A	17.50 $\pm$ 2.31 bc AB	15.00 $\pm$ 2.67 c AB	**
T1	28.12 $\pm$ 2.30 a A	23.75 $\pm$ 2.05 ab A	20.00 $\pm$ 2.83 ab A	19.37 $\pm$ 3.33 b A	*
T2	26.25 $\pm$ 2.05 a A	18.12 $\pm$ 2.30 b AB	15.62 $\pm$ 2.57 b ABC	15.00 $\pm$ 3.13 b AB	**
SC	18.12 $\pm$ 3.12 a B	13.12 $\pm$ 2.48 ab BC	9.37 $\pm$ 1.75 b C	8.75 $\pm$ 1.82 b B	*
S1	18.12 $\pm$ 2.66 a B	13.75 $\pm$ 1.82 ab BC	11.25 $\pm$ 1.82 b BC	10.62 $\pm$ 2.20 b B	**
S2	15.62 $\pm$ 2.39 a B	10.00 $\pm$ 1.63 a C	10.00 $\pm$ 2.3 a C	9.37 $\pm$ 1.75 a B	NS
Significance Level	**	**	**	*	

N.S Non-significant * ($P \leq 0.05$) ** ($P \leq 0.01$) Averages are in capital letters to compare treatments, and averages are in lower case letters to compare durations. TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender +90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin

Table 2. Effect of adding relaxin to tris and soybean lecithin extenders on the sperm viability (%) at Holstein bulls after different periods of preservation (mean± SE)

Treatments	Time				Significance Level
	Cooling 5°C	48h Post-Cryopreservation	One month Post-Cryopreservation	Two months Post-Cryopreservation	
TC	2.09±78.31 a AB	3.43±71.08 ab BC	2.31±66.59 b BC	4.19±61.88 b BC	**
T1	2.09±83.60 ab A	1.98±79.92 a A	2.13±78.26 ab A	2.90±74.73 b A	*
T2	1.50±78.88 a A	3.34±76.69 ab AB	2.79±70.00 bc B	2.79±67.18 c AB	**
SC	1.61±72.55 a C	3.57±65.91 ab C	3.15±60.10 bc C	3.32±56.51 c C	**
S1	1.91±78.45 a ABC	1.27±76.72 a AB	1.73±72.89 ab AB	2.79±69.36 b AB	**
S2	1.37±73.62 a BC	2.72±71.45 ab BC	1.99±64.94 bc BC	2.67±60.58 c BC	**
Significance Level	*	**	**	**	

N.S Non-significant * (P≤0.05) ** (P≤0.01) Averages are in capital letters to compare treatments, and averages are in lower case letters to compare durations. TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender +90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin.

Percentage of sperm Total Abnormality:

Sperm abnormalities increased over time, with the most substantial changes after one and two months of cryopreservation. At the cooling stage and SC exhibited higher abnormalities compared to the relaxin treatments, with T2 showing the lowest abnormalities. After 48h,

the abnormalities remained elevated in SC and S2, while T1 and T2 showed the fewest abnormalities. After one month, SC had the highest abnormalities among treatments with relaxin, by two months, TC and SC showed the highest abnormalities, while S2 maintained the lowest abnormalities (Table 3).

Table 3. Effect of adding relaxin to tris and soybean lecithin extenders on sperm abnormalities (%) at Holstein bulls after different periods of preservation (mean± SE).

Treatments	Time				Significance Level
	Cooling 5°C	48h Post-Cryopreservation	One month Post-Cryopreservation	Two months Post-Cryopreservation	
TC	0.38±13.57 c B	0.33±16.68 b BC	0.47±19.75 a AB	1.41±20.60 a B	**
T1	0.23±12.81 c B	0.30±14.78 b D	0.36±16.71 a C	0.78±17.51 a C	**
T2	0.07±13.08 d B	0.17±15.76 c CD	0.26±18.86 b B	0.85±21.12 a B	**
SC	0.33±15.20 d A	0.34±18.18 c A	0.40±20.70 b A	1.00±24.30 a A	**
S1	0.35±13.33 d B	0.47±15.36 c D	0.44±17.17 b C	0.60±19.12 a BC	**
S2	0.40±14.67 d A	0.46±16.93 c B	0.46±19.50 b AB	0.55±21.36 a B	**
Significance Level	**	**	**	**	

N.S Non-significant * (P≤0.05) ** (P≤0.01) Averages are in capital letters to compare treatments, and averages are in lower case letters to compare durations. TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender +90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin

Sperm plasma membrane integrity: When comparing the different treatments across both tris and soybean extenders, it is evident that the addition of relaxin significantly improved ($P \leq 0.01$) plasma membrane integrity compared to the control groups. T1, T2, S1, and S2 showed better results than the control, with the S1 and T1 treatment generally outperforming the T2 and S2 treatment in maintaining membrane integrity over time. In both T1 and S1 relaxin treatment demonstrated the highest initial integrity cooling and maintained superior levels throughout the cryopreservation periods. The T2 and S2

relaxin treatment, were effective, exhibited a slightly greater decline in integrity over time compared to the 60 ng/ml treatment. This indicates a lower concentration of relaxin might be more beneficial for preserving spermatozoa plasma membrane integrity during the cryopreservation process as shown in (Table 4). Immediately cooling, the integrity was at its highest for all groups. After 48h of cryopreservation, there was a noticeable decline in integrity, although relaxin-treated samples still maintained higher levels compared to the controls.

Table 4. Effect of adding relaxin to tris and soybean lecithin extenders on sperm plasma membrane integrity (%) at Holstein bulls after different periods of preservation (mean $\pm$ SE).

Treatments	Time				Significance Level
	Cooling 5°C	48h Post-Cryopreservation	One month Post-Cryopreservation	Two months Post-Cryopreservation	
TC	1.20 $\pm$ 81.57 a C	2.19 $\pm$ 72.48 b D	1.95 $\pm$ 69.81 bc C	2.93 $\pm$ 63.87 c D	**
T1	0.86 $\pm$ 90.76 a A	0.75 $\pm$ 87.21 b A	0.97 $\pm$ 83.86 b A	1.91 $\pm$ 77.91 c A	**
T2	1.79 $\pm$ 89.36 a A	1.88 $\pm$ 83.61 b AB	1.36 $\pm$ 81.75 b A	1.23 $\pm$ 75.28 c AB	**
SC	1.77 $\pm$ 84.16 a BC	1.33 $\pm$ 77.71 b C	1.26 $\pm$ 74.06 B b	1.21 $\pm$ 67.53 c CD	**
S1	1.45 $\pm$ 86.96 a AB	1.52 $\pm$ 84.51 ab AB	1.23 $\pm$ 80.71 b A	1.56 $\pm$ 75.28 c AB	**
S2	1.76 $\pm$ 87.16 a AB	1.10 $\pm$ 81.73 b BC	0.96 $\pm$ 76.62 c B	1.88 $\pm$ 70.78 d BC	**
Significance Level	**	**	**	**	

N.S Non-significant * ($P \leq 0.05$) ** ($P \leq 0.01$) Averages are in capital letters to compare treatments, and averages are in lower case letters to compare durations. TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender +90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin.

Percentage of sperm acrosome integrity

In examining the acrosome integrity of spermatozoa across various treatments, both the tris and soybean lecithin extenders showed distinct patterns. The TC and SC in both extenders demonstrated an initial high level of acrosome integrity, which decreased over time. The addition of relaxin T1, S1 and T2, S2 helped maintain higher levels of acrosome integrity compared to controls. Among the relaxin treatments, the T1 and S1 concentration generally exhibited better preservation of acrosome integrity across all time points than the T2 and S2. This trend was observed in both tris and soybean lecithin

extenders, indicating that the lower relaxin concentration is more effective in maintaining acrosome integrity during the cooling and freezing process. Acrosome integrity showed a consistent decline over time across all treatments and extenders. During the post-thawing phase, acrosome integrity was highest for all groups. After 48h cryopreservation, there was a noticeable reduction in integrity across the board, though the relaxin-treated samples still maintained better levels than the controls. One month cryopreservation, the decline continued, with all treatments showing reduced acrosome integrity. By two months, the integrity levels were the lowest for all

treatments, with the control groups showing the most significant ($P \leq 0.01$) deterioration. Despite the overall decrease over time, the relaxin treatments helped in maintaining higher acrosome integrity compared to the control groups, emphasizing the protective role

of relaxin during extended cryopreservation. The data highlights the importance of both treatment type and cryopreservation duration in preserving acrosome integrity in spermatozoa shown in (Table 5).

Table 5. Effect of adding relaxin to tris and soybean lecithin extenders on sperm acrosome integrity (%) at Holstein bulls after different periods of preservation (mean $\pm$ SE).

Treatments	Time				Significance Level
	Cooling 5°C	48h Post-Cryopreservation	One month Post-Cryopreservation	Two months Post-Cryopreservation	
TC	0.74 $\pm$ 83.53 a A	1.12 $\pm$ 77.18 b AB	1.74 $\pm$ 74.25 b A	1.26 $\pm$ 73.81 c AB	**
T1	0.81 $\pm$ 83.57 a A	0.75 $\pm$ 78.84 b A	0.80 $\pm$ 74.34 c A	1.74 $\pm$ 74.25 c A	**
T2	0.51 $\pm$ 81.37 a A	0.72 $\pm$ 78.62 b A	0.99 $\pm$ 75.21 c A	1.01 $\pm$ 70.82 d AB	**
SC	0.84 $\pm$ 81.79 a A	1.13 $\pm$ 75.53 b B	1.30 $\pm$ 72.35 b A	1.59 $\pm$ 67.46 c B	**
S1	0.75 $\pm$ 82.34 a A	0.58 $\pm$ 78.14 b AB	0.84 $\pm$ 73.83 c A	2.36 $\pm$ 73.43 c A	**
S2	0.79 $\pm$ 81.90 a A	0.71 $\pm$ 76.58 b AB	1.15 $\pm$ 73.35 bc A	2.05 $\pm$ 70.66 c AB	**
Significance Level	NS	NS	NS	NS	

N.S Non-significant * ($P \leq 0.05$) ** ($P \leq 0.01$) Averages are in capital letters to compare treatments, and averages are in lower case letters to compare durations. TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender +90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin

Seminal plasma total antioxidants capacity, Concentration of MDA and Sperm's DNA integrity percentage: The levels of antioxidants across the six treatments (TC, T1, T2, SC, S1, and S2) showed no statistically significant differences, indicating that the treatments did not markedly affect the antioxidant capacity of the samples. T1 exhibited the highest antioxidant levels, followed by T2, S2, SC, S1, and TC respectively. The fairly consistent antioxidant levels across all treatments suggest that the antioxidant presence alone might not be a decisive factor in determining the overall sperm quality preservation. Significant differences ($P \leq 0.05$) were observed in the levels of malondialdehyde (MDA) among the treatments. TC and SC had the highest MDA levels, indicating the greatest oxidative stress, while T1, T2 and S1 showed moderate levels of MDA, suggesting intermediate oxidative stress. T1 exhibited the lowest MDA levels, signifying the least oxidative stress. The

analysis of DNA integrity revealed significant differences ($P \leq 0.01$) between the treatments. S1 and S2 displayed the highest levels of DNA integrity, and the lowest preservation of sperm DNA integrity T2 and SC. T1 showed moderate levels of DNA integrity, suggesting intermediate DNA integrity preservation. In contrast, TC demonstrated the lowest DNA integrity, indicating the best preservation of sperm DNA integrity. T1 emerged as the most effective treatment for preserving sperm quality, characterized by the lowest levels of oxidative stress and the highest level of DNA integrity. Despite higher antioxidant levels, the S2, S1 and S2 had the poorest outcomes in terms of DNA integrity, highlighting the limitations of these treatments in maintaining sperm quality. This analysis underscores the importance of selecting treatments that balance oxidative stress reduction and DNA integrity preservation to ensure optimal sperm quality (Table 6).

Table 6. Effect of adding relaxin to tris and soybean lecithin extenders on TAC, MDA, DNA Integrity in seminal plasma of Holstein bulls post cryopreservation for two months (mean $\pm$ SE)

Treatments	TAC mg/dl Post-Cryopreservation	MDA μ mol Post-Cryopreservation	DNA Integrity (%)
TC	0.00015 $\pm$ 0.0035 B	18.60 $\pm$ 89.18 A	0.83 $\pm$ 87.49 B
T1	0.00056 $\pm$ 0.0058 A	5.68 $\pm$ 48.50 B	0.16 $\pm$ 90.83 AB
T2	0.00072 $\pm$ 0.0045 AB	1.47 $\pm$ 54.80 B	1.67 $\pm$ 91.33 AB
SC	0.00030 $\pm$ 0.0057 A	8.61 $\pm$ 96.22 A	1.00 $\pm$ 91.00 AB
S1	0.00026 $\pm$ 0.0052 A	9.27 $\pm$ 64.11 AB	1.33 $\pm$ 92.00 A
S2	0.00052 $\pm$ 0.0056 A	11.04 $\pm$ 91.04 A	0.83 $\pm$ 93.16 A
Significance Level	NS	*	**

N.S Non-significant * ($P \leq 0.05$) ** ($P \leq 0.01$) Averages are in capital letters to compare coefficients

TC: Control with Tris Extender only. T1: Tris Extender +60ng/ml Relaxin. T2: Tris Extender

+90ng/ml Relaxin. SC: Control treatment with Soybean lecithin Extender only. S1: Soybean lecithin Extender +60ng/ml Relaxin. S2: Soybean lecithin Extender +90ng/ml Relaxin

Results and Discussions: The data showed a general decline in sperm motility over a period of time, with a notable drop between the cooling and two-month cryopreservation stages (Ivell, R. et al., 2017). The adding of relaxin, especially T1, resulted in a significant improvement in motility compared to controls and other relaxin treatments. This result confirms previous research (Elkhawagah, A. R et al., 2020c) showing relaxin can improve sperm motility and viability during cryopreservation (Rastegarnia, A. et al., 2014). The better results that Tris's extenders achieve when combined with relaxin point to a mutually beneficial relationship that maintains sperm motility. According to well-established research, cryopreservation can result in increased sperm damage and reduce viability. This is supported by the observed increase in sperm abnormalities with time across all treatments (Elkhawagah, A. R. et al., 2020d). T2 and S2 were more successful in preserving sperm viability following long-term freezing, as they consistently displayed the least rise in abnormalities (Ivell, R. et al., 2017). Relative to the relaxin-treatments, the TC and SC showed more anomalies, suggesting that relaxin is helpful in reducing damage during the freezing process. TC and SC showed more overall abnormalities than relaxin-treatments (especially T2) at all time periods. This confirms the finding by (Ivell, R. et al., 2017)

that relaxin reduces sperm abnormalities during cryopreservation. Although there was an initial increase in cooling, the trend of fewer abnormalities in T2 found displays that higher relaxin concentrations offer better long-term protection against sperm destruction. Furthermore, of the necessity for optimal cryopreservation strategies containing efficient cryoprotectants and extenders in the notable increase in anomalies in TC and SC over a period of time. When contrast to the control, relaxin treatments greatly improved plasma membrane integrity; T1 and S1 generally fared greater than T1 and S2. This result is in line with studies displaying relaxin maintains the integrity of the plasma membrane after cryopreservation (Feugang, J. M. et al., 2015). Relaxin concentrations that are less than 60ng/ml may be more successful in maintaining sperm membrane integrity during cryopreservation, has seen a higher reduction in membrane integrity in T2 and S2 over a long period. The general pattern emphasizes relaxin protective function in maintaining membrane integrity, even if the optimal dose varies. Relaxin treatment samples displayed generally higher acrosome integrity than TC and SC, with T1 and S1 showing the best preservation at all time points. Research suggesting that relaxin can help contribute to keeping the integrity of the acrosome during cryopreservation (Elkhawagah, A. R et

al.,2020c). The fact that acrosome integrity consistently reduces with time in all treatment's controls displaying the worst degradation confirms the role that relaxin plays in maintaining acrosome performance. Since T1 and S1 function more accurately than other proteins, relaxin concentrations possibly need to be lowered in order to preserve acrosome integrity (Elkhawagah, A. et al.,2023d). The lack of substantial changes in antioxidant levels between treatments recommend that the availability of antioxidants could not be the only factor that determines whether or not sperm quality is preserved. Antioxidants are important, but other factors that including oxidative stress and treatment settings also have an impact on how well sperm are preserved (Saieed, A. et al., 2018, Ahmed, H. et al.,2023). The relatively constant amounts of antioxidants imply that other mechanisms may have a more significant role in sperm quality throughout cryopreservation. Malondialdehyde (MDA), a biomarker of oxidative stress, showed considerable variations in levels even when antioxidant levels were similar throughout treatments. During cryopreservation, T1 displayed the highest oxidative stress and the lowest MDA levels (Kose, H. et al.,2024). Relaxin's protective role is further supported by DNA integrity, the T1 showed the lowest quantity of damage. DNA sperm reproductive potential is highest determined by fragmentation, with reduced fragmentation indicating greater preservation of sperm DNA integrity. According to (Feugang, J. M. et al., 2015), the obstacles of choosing soybean lecithin extenders, which wouldn't be successful as the Tris extender in preventing DNA integrity during cryopreservation, are reflected in this study's greater DNA integrity in S1 and S2. The superior efficiency of T1 in maintaining sperm motility, viability, membrane integrity, and DNA integrity suggests that relaxin can greatly enhances sperm cryopreservation results when applied to the right quantities. During cryopreservation, relaxin considerably improved sperm motility, viability, and plasma membrane integrity. While relaxin worked best at higher doses, it gradually lost some of

its effect. When comparing the extenders, the soybean lecithin extender showed lower sperm quality.

CONCLUSION

The results of this study suggest that the use of relaxin during sperm cryopreservation improved the storage conditions, as evidence by the significant improvement in sperm motility, viability and the integrity of their acrosome and plasma membrane, supporting the hypothesis of the antioxidant role of relaxin.

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CONFLICT OF INTEREST

I declare that there are no conflicts of interest regarding the publication of this manuscript.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

Author/s signature on Ethical Approval Statement

Ethical Clearance and Animal welfare Funds:

AUTHOR'S CONTRIBUTION STATEMENT

Rania Dhyaa Hussein conducted the experimental work, data analysis, and manuscript writing, while Dr. Husam Jasim Banana and Omar Adel Mohammad supervised the study, reviewed the manuscript, and contributed to the research design.

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كفاءة الحفظ بالتجميد في ثيران الهولشتاين: تأثير إضافة الريلاكسين ونوع المخفف في نوعية السائل المنوي

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المستخلص

هدفت هذه الدراسة لمعرفة تأثير الريلاكسين في جودة النطف أثناء الحفظ بالتجميد وذلك باستخدام مخففي tris و soybean lecithin. بعد جمع السائل المنوي تم تقسيمه الى 6 مجاميع بالتساوي كما يلي: TC: مجموعة السيطرة باستخدام مخفف tris فقط؛ 1T: 60 نانوغرام/مل ريلاكسين مع مخفف tris؛ 2T: 90 نانوغرام/مل ريلاكسين مع مخفف tris؛ SC: مخفف soybean lecithin فقط؛ 1S: 60 نانوغرام/مل ريلاكسين مع مخفف soybean lecithin؛ S: 290 نانوغرام/مل ريلاكسين مع مخفف soybean lecithin. تم تقييم جودة النطف عند التبريد 5° م و 48 ساعة و 1 و 2 شهر بعد التجميد. تم حساب النسبة المئوية للحركة الفردية والنطف الحية والتشوهات الكلية وسلامة الأغشاء البلازمية والقدرة المضادة للأكسدة ومستويات المألون داي الدهايد وتحطيم الحمض النووي. أدت المعاملات 1T و 1S إلى زيادة ملحوظة في النسبة المئوية لحركة النطف ونسبة النطف الحية وانخفاض نسبة تشوهات النطف مقارنة مع بقية المعاملات ولجميع فترات الحفظ. كما أظهرت معاملات الريلاكسين تحسن في سلامة الأغشاء البلازمية وسلامة الأكروسوم للنطف، وخاصة في المعاملات 1T و 1S مقارنة مع بقية المعاملات ولجميع الفترات. أظهرت المعاملة 1T أقل نسبة ضرر الحامض النووي من بين جميع المعاملات. يمكن الاستنتاج من هذا البحث ان اضافته تراكيز 60 نانوغرام/مل من الريلاكسين الى مخففات السائل المنوي أدت الى تحسين صفات النطف المحفوظة بالتجميد لثيران الهولشتاين.

الكلمات المفتاحية: تعزيز الحماية من التجميد، سلامة الحامض النووي، المألوندايديهايد، الاجهاد التأكسدي، الريلاكسين، مخفف الصويا ليسيثين.