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Effect of Relaxin on the Biological Activities of Cryopreserved Holstein Bull Semen after Different Periods of Incubation

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Abstract

This study evaluated the effects of two relaxin concentrations (60ng/ml and 90ng/ml) combined with Tris (TC: control, T1: 60ng/ml, T2:90ng/ml) and soybean lecithin extender (SC: control, S1: 60ng/ml, S2:90ng/ml) on post-thaw sperm quality in Holstein bulls. Sperm parameters, including motility, viability, abnormalities, plasma membrane integrity, and acrosome integrity, were assessed over cryopreservation periods of 48 hours, one month, and two months, with post-thaw incubation for one and two hours. All treatments experienced a decline in motility and viability over time, but T1(60ng/ml in Tris) maintained significantly the highest motility compared to the control (TC) group ($p \leq 0.05$). Soybean lecithin extenders generally showed lower motility and viability, with S2(90ng/ml) performing the worst. One hour of incubation preserved sperm quality more effectively than two hours. Relaxin treatments improved the plasma membrane integrity, especially with soybean lecithin, and enhanced acrosome integrity, particularly in T2 and S2. However, higher relaxin concentration was associated with reduced motility and viability. Overall, T1 showed the best results, emphasizing the critical role of relaxin concentration optimization for improving post-thaw sperm quality and incubation time to enhance post-thaw sperm preservation.

Keywords: Relaxin, Holstein bulls, Cryopreservation, Sperm Biological Activities.

تأثير الريلاكسين على الانشطة البيولوجية للسائل المنوي المجمد لثيران الهولشتاين بعد فترات مختلفه من الحضن

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الخلاصه

تم في هذه الدراسة تقييم تأثير تركيزين من الريلاكسين (60 نانوغرام/مل و90 نانوغرام/مل) مع المحفف تريس :TC) التحكم، T1: 60نانوغرام/مل، T2: 90نانوغرام/مل) ومحفف الليسيثين الصويا (SC: التحكم، S1:60نانوغرام/مل، S2: 90 نانوغرام/مل)على جودة الحيوانات المنوية المجمدة المذابة في ثيران الهولشتاين. تم تقييم معايير الحيوانات المنوية، بما في ذلك الحركة، والحيوية، والتشوهات، وسلامة غشاء البلازما، وسلامة

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الأكروسوم، على مدار فترات التجميد التي استمرت 48 ساعة، وشهر واحد، وشهرين، مع حضانة ما بعد الإذابة لمدة ساعة وساعتين. شهدت جميع المعالجات انخفاضًا في الحركة والحيوية بمرور الوقت، لكن T1 (60 نانوغرام/مل في تريس) حافظ على أعلى حركة بشكل ملحوظ مقارنة بمجموعة التحكم (TC) ($p \leq 0.05$) . بشكل عام، أظهرت المحفظات التي تحتوي على الليسيثين الصويا حركة وحيوية أقل، حيث كان أداء S2 (90 نانوغرام/مل) هو الأسوأ. كانت الحضانة لمدة ساعة أكثر فعالية في الحفاظ على جودة الحيوانات المنوية مقارنة بساعتين. حسّنت معالجات الريلاكسين من سلامة غشاء البلازما، خاصة مع الليسيثين الصويا، وعزّزت سلامة الأكروسوم، وخصوصًا في T2 و .S2 ومع ذلك، ارتبطت التركيزات الأعلى من الريلاكسين بانخفاض في الحركة والحيوية. بشكل عام، أظهر T1 أفضل النتائج، مما يبرز أهمية تحسين تركيز الريلاكسين ومدة الحضانة لتحسين الحفاظ على الحيوانات المنوية بعد التجميد.

Introduction

Sperm cryopreservation is a crucial biological process that offers several advantages, such as preserving genetic variety, improving breeding initiatives; and boosting fertility. The possibility of cryoinjury; which lowers sperm quality and fertility, makes cryopreservation a double edge sword despite its widespread usage [1]. Osmatic stress, ice crystal formation and oxidative damage can all result from the freezing and thawing process, sperm motility, viability, and functional integrity are all lowered as a result[2]. To lessen this issue; a number of cryoprotective substances and extenders have been created to enhance sperm quality and following cryopreservation [3, 4].

Soybean lecithin extenders, one of the often utilized compounds, have demonstrated tremendous potential as a natural alternative, because of their identical freezing- protective qualities and minimal risk of contamination[5]. By simulating the natural lipid environments of sperm, soybean lecithin phospholipid diluents preserve membrane integrity throughout the freezing and thawing process [5]. They supply energy and shield the sperm membrane because of their insulting properties. There has been extensive usage of tris diluents, whish are made up of tris; fructose and citric acid [6]. The selection of diluents is crucial because of their impact on sperm motility, viability, and capacity to fertilize after cryopreservation [7, 8].

Sperm quality characteristics are greatly impacted by the incubation time following cryopreservation. Sperm have metabolic alternation during incubation possible oxidative stress, which can impact acrosome integrity, motility and viability while short incubation, time have conflicting effects, long incubation may worsen oxidative damage and result in lower quality, according to some research, motility recovery gets better within the first hour, but beyond two hours oxidative stress may cause a deterioration [9]. Thus, it is essential to investigate how incubation time and cryoprotectants interact in order to enhance post-thaw performance[10].

The peptide hormone relaxin, which is a member of the insulin like factor, has garnered interest due to its possible role in enhancing sperm motility and viability. It is well recognized that relaxin plays a function in parturition, pregnancy, sperm activity and the positive process [11]. Relaxin has been demonstrated to promote sperm motility, in spermatozoa by rising intracellular cyclic AMP levels and augmenting calcium influx, which are important for activating the motility machinery [12]. Furthermore, relaxin antioxidant qualities might aid in decreasing oxidative stress; which is valid reason for cryoinjury in sperm cells [13]. Prior researches had claimed that incorporation of relaxin into spermatozoa extenders can increase post-thaw motility and viability; however, the exact effects depend on the relaxin concentration, with 12.5ng/mL and 25ng/mL showing the greatest improvement in motility; mitochondrial membrane potential. Reduced apoptosis [14]. For instance; [15]discovered that

the motility, and acrosome integrity of swine spermatozoa following cryopreservation are considerably enhanced by; relaxin supplementations in a Tris extender. Likewise; relaxin is found to enhance sperm quality in semen [16], indicating that it can be used in all various animal species. Furthermore, research is necessary since the ideal concentration of relaxin, and its interaction with different extenders is still unclear.

The study aimed to evaluate the effects of relaxin on spermatozoa cryopreserved in soybean lecithin; and tris extenders at concentrations of 60ng/mL and 90ng/ml. spermatozoa quality; was assessed after cryopreservation for 48 hours, one month, and two months, as well as after incubation at 37°C for one, and two hours to assess the stability of relaxin protective effects. This study aims to improve sperm cryopreservation outcomes, as well as enhance reproductive success in animal breeding and assisted reproductive technology.

2. Material and methods

2.1. Semen collection and dilution

The study was conducted at the Department of Artificial Insemination, Department of Animal Resources, Ministry of Agriculture. The bulls used in this study were Holstein bulls, a breed known for their high reproductive performance and excellent fertility traits. Four well trained Holstein bulls, aged 2.5-3 years and weighting 500-700kg were used for semen collection. The semen was collected from Holstein bulls using an artificial vagina at 40-41°C during early morning hours with one ejaculate per bull weekly for seven weeks. Relaxine concentration (60ng/ml and 90ng/ml) was added to the tris and soyabean lecithin extenders. To minimize individual differences, 1ml from each ejaculation was pooled after a false mount to enhance sexual stimulation. Relaxin concentration (60ng/ml and 90ng/ml) was added to the tris extender and soybean lecithin extenders. All samples that had a sperm concentration of $\geq 800\times10^6$/ml and motility≥70% were used in the experiment.

Semen samples were pooled and diluted with tris and soybean lecithin extenders (IMV, France), according to instructions, and supplemented with relaxin (SRP3147, Sigma-Aldrich, Italy) at different concentrations: 0 (control), 60 and 90ng/ml for both extenders. Diluted semen with a final concentration of 30 × 106 spermatozoa/ml was slowly cooled to 5°C, packed into 0.25 ml polyvinyl straws (IMV, France), equilibrated for 4 hours, and frozen using a controlled-rate freezer (SY LAB Gerate GmbH, Neupurkersdorf, Austria) following the standard procedure[17]. Individual motility, plasma membrane integrity, and acrosome integrity were assessed at multiple time points: 48 hours, 1 month, and 2 months post-cryopreservation. Additionally, these parameters were measured after post-thaw incubation at 1 and 2 hours.

2.2 Ethical approval

Permission was granted by the local ethics of the faculty of the College of Agricultural Engineering Sciences / University of Baghdad (1684).

2.3 Assessment of sperm concentration

The concentration of sperm count/ml in fresh semen before dilution was estimated by using a device measuring the concentration of sperm of French origin and belonging to the Department of IVF Hamilton Technologies (IMV, France) by calculating the number of sperm and giving the appropriate dilution ratio for each ejaculate.

2.4 Assessment of sperm individual motility

The individual motility of sperm was estimated according to what was explained previously [18] by placing 20 µL of semen on a glass slide with a small drop of sodium citrate at a concentration of 2.9% and covered with a cover slide and examined under a light microscope at a magnification of 40×. Individual motility, based on the proportion of motile sperms, is defined as forward motility, while sperms with abnormal motility (circular, backward,

pendulum) are considered non-motile. A total of four sperm samples were analyzed, with 200 individual sperm assessed per sample.

2.5 Assessment percentage of live sperms

The percentage of live sperm was calculated by the method of [19] by taking 20 µL of fresh, cooled, or frozen semen and placing it on a glass slide on a hot plate at 37 °C and adding 20 µL of eosin - nigrosine were mixed, a smear of the mixture is made on a second glass slide at an angle of 45 degrees and examined under a light microscope with 40× magnification as follow: live sperm appear transparent because they do not accept dyeing while dead sperm appear pink due to dye entering.

2.6 Assessment percentage of abnormal sperms

The percentage of deformed sperm was estimated according to the method of [20] based on the same slide used in calculating the percentage of live and dead sperm, as eosin-nigrosine was used by examining 200 sperm in separate microscopic fields, and the abnormalities were classified according to what was stated by [21, 22] to the following: head abnormalities, tail´s mid piece abnormalities, tail's principal and terminal abnormalities.

2.7 Assessment of sperm plasma membrane integrity

The percentage of sperm with a healthy plasma membrane was estimated by placing two drops of the semen sample in test tubes containing 1 ml of (HOST-hypo-osmotic solution, which consists of 8.72 g/L fructose and 4.74 g/ sodium citrate), as its osmotic pressure is 150 m osmol/liter, and the pH = 8. The tubes were placed in a water bath at a temperature of 37 °C for 15min, after which 20 µL was taken, a smear was made on a clean glass slide, and 200 sperms were counted in the fields separate, spermatozoa with coiled tails or swollen morphology were considered to have intact plasma membranes under a microscope at 40× magnification. In contrast, sperm exhibiting signs of membrane damage, such as broken or detached tails or irregular acrosomal morphology, were classified as lacking integrity.

2.8 Assessment of acrosome integrity

A sperm smear was prepared on a glass slide, air-dried, and then stained using Giemsa stain to evaluate acrosome integrity, following the method developed by [18, 23]. Giemsa was prepared by mixing the following materials in the specified quantities for each: Giemsa powder 3.8 gm, methanol 375 ml, glycerol 125 ml. It was left for two weeks and then filtered. 3ml of the dye was taken and mixed with 2ml of Sorenson buffer.

2.9 Thawing and Incubation

After 48 hours, one month, and two months of sperm cryopreservation for all treatments, the straws were removed from liquid nitrogen and thawed by placing them in a water bath at 37°C. Thawing was done after 1 and 2 hours to ensure complete and consistent warming of the sperm samples. Following thawing, the samples were incubated at 37°C to assess whether the relaxin in the extenders could maintain the sperm's ability to maintain the sperm quality at the best levels. The incubation was specifically designed to simulate the conditions sperm would encounter post-thaw, providing insights into the potential of relaxin to preserve sperm quality, such as motility, viability, and membrane integrity, during extended storage periods [24].

2.10 Statistical analysis

The statistical computations were done using the SAS software to explore the influence of treatment and time [25]. Duncan's multiple range test for compassion between means [25, 26] was used ($p< 0.05$).

Results

3.1 Sperm individual motility percentage

At 48 hours post-thaw, significant differences (P≤0.01) in motility were observed. T1 showed the highest individual motility, remaining stable from post-thaw through 1 hour of incubation, significantly decreasing by 2 hours. T2 and SC showed lower motility. The significance levels (P≤0.01) confirm that relaxin improves motility in tris extender treatments compared to soybean lecithin-based treatments.

After one month of cryopreservation, T1 continues to exhibit superior sperm motility compared to other treatments. Post-thaw motility for T1 was significantly higher than T2 and SC with a significance level of (P≤0.01). The motility for T1 remains stable during the 1-hour incubation period, while other treatments exhibit sharp declines after 1 hour, especially SC and S2. The performance of soybean lecithin-based treatments shows no significant improvement in motility, suggesting that tris-based extenders are more effective in preserving sperm quality with relaxin as shown in Table 1.

After two months of storage, sperm motility declines across all treatments, with no statistical significance detected in most comparisons. However, T1 still exhibits the best motility results, though the improvement is no longer significant compared to other treatments. S1 and S2 showed the lowest motility throughout this period. The trend indicates that tris extenders, particularly T1, provide better cryoprotection over longer storage durations, but the protective effects diminish over time, as shown in Figure 1.

Overall, the experiment shows that T1 consistently results in better post-thaw motility at 48 hours and one month, with significance at P≤0.01. However, sperm motility declines over time, especially after two months, and the significance diminishes, highlighting the challenges of long-term sperm cryopreservation.

Table 1: Effect of Relaxin on cryopreserved individual sperm motility in Holstein bulls after different periods of cryopreservation after post-thawing, 1h and 2h of incubation (mean± standard error).

Treatments	48h%			Sig.	One Month%			Sig.	Two Month%			Sig.
	Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h	
TC	23.12±1.87 A a	13.75±1.82 A b	10.00±1.88 A b	*	17.50±2.31 AB a	6.66±1.66 BC b	5.00±0 A b	*	15.00±2.67 AB a	6.66±1.66 B a	5.00±0 A a	NS
T1	23.75±2.05 A a	13.75±1.25 A b	10.62±1.47 A b	*	20.00±2.83 A a	11.66±1.66 A ab	6.66±1.66 A b	*	19.37±3.33 A a	10.00±0 AB a	10.00±0 A a	NS
T2	18.12±2.30 AB a	6.87±0.91 B a	5.62±0.62 B a	*	15.62±2.57 ABC a	5.00±0 C b	5.00±0 A b	*	15.00±3.13 AB a	11.66±1.66 AB a	5.00±0 A a	NS
SC	13.12±2.48 BC a	8.75±1.25 B ab	6.25±0.81 B b	*	9.37±1.75 C a	5.00±0 C a	5.00±0 A a	NS	8.75±1.82 B a	6.66±1.66 B a	8.33±3.33 A a	NS
S1	13.75±1.82 BC a	7.50±0.94 B b	6.25±0.81 B b	*	11.25±1.82 BC a	8.33±1.66 AB a	6.66±1.66 A a	NS	13.33±1.66 B a	10.62±2.20 A a	6.66±1.66 A a	NS
S2	10.00±1.63 C a	6.25±0.81 B b	5.62±0.62 B b	*	9.37±1.75 C a	5.00±0 C a	6.66±1.66 A a	NS	10.00±2.31 B a	8.33±1.66 AB a	8.33±3.33 A a	NS
Sig.	**	**	**		**	**	NS		*	*	NS	

N.S Non-significant * (P≤0.05) **(P≤0.01) Averages are in capital letters to compare coefficients, and averages are in lowercase 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.

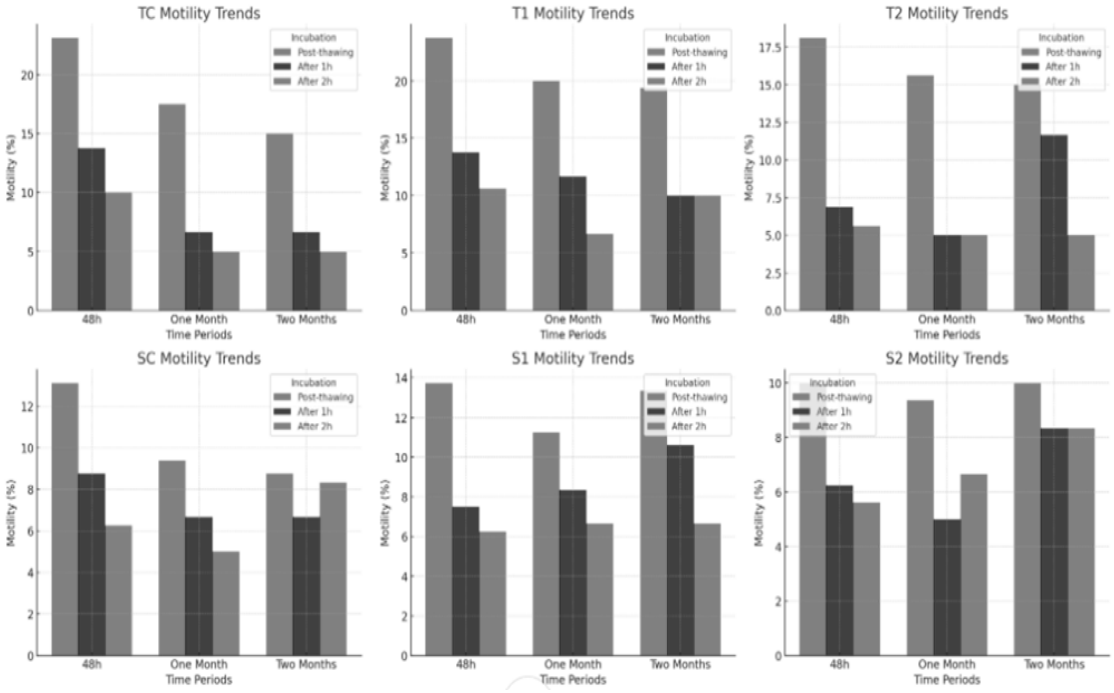

Figure 1: Motility (%) for six treatments (TC, T1, T2, SC, S1, S2) across incubation periods (post-thaw, 1 hour, 2 hours) over three time points (48h, 1 month, 2 months).

3.2 Sperm viability percentage

After 48h, the highest viability was observed in T1 and T2, with statistical significance ($P \leq 0.01$), showing that the relaxin treatments, particularly T1, were effective in maintaining sperm viability. The control groups, TC and SC, had lower values, respectively. This suggests that relaxin had a protective effect on sperm viability immediately after thawing.

After one month, T1 continued to show the highest viability, though this result was not statistically significant (NS) in duration. Meanwhile, the S1 treatments also performed well, showing a moderate level of significance ($P \leq 0.05$), suggesting that relaxin still played a beneficial role at this stage of preservation.

By two months, T1 maintained the best viability with statistical significance ($P \leq 0.05$), indicating that relaxin continued to protect the sperm during extended cryopreservation. The S1 treatment remained high with similar significance, highlighting relaxin long-term benefits. Overall, the relaxin-treated samples, especially T1, consistently exhibited better viability over the control treatments, demonstrating its potential to improve sperm preservation (Table 2).

Table 2: Effect of adding relaxin on sperm viability percentage in Holstein bulls after different periods of cryopreservation after post-thawing 1h and 2h of incubation (mean± standard error).

Treatments	48h%			Sig.	One Month%			Sig.	Two Month%			Sig.
	Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h	
TC	71.08±3.43 BC a	62.05±5.10 D b	54.83±2.42 D c	**	66.59±2.31 BC a	60.38±3.10 A a	49.38±3.99 A a	NS	61.88±4.19 BC a	59.83±3.71 B a	48.30±3.25 B a	NS
T1	83.60±2.09 A a	80.10±1.73 A a	72.77±2.93 A b	*	78.26±2.13 A a	72.96±1.76 A a	64.06±2.18 A a	NS	74.73±2.90 A a	66.48±4.75 A ab	59.76±4.36 A b	*
T2	76.69±3.34 AB a	70.43±3.20 BC a	61.63±3.03 BC b	*	70.28±1.19 B a	70.00±2.79 A ab	59.55±1.07 A b	*	67.18±2.79 AB a	64.10±2.95 A a	55.45±3.64 A a	NS
SC	65.91±3.57 C a	62.08±2.10 D a	59.20±3.25 D b	*	60.10±3.15 C a	61.70±3.62 B a	56.40±3.22 A a	NS	56.51±3.32 C a	56.46±3.21 B a	45.40±4.24 A a	NS
S1	78.45±1.27 AB a	74.50±2.49 AB ab	69.16±2.60 AB b	*	72.89±1.73 AB a	74.01±2.55 A ab	62.33±1.88 A b	*	69.36±2.79 AB ab	69.05±4.43 A a	59.21±2.57 A b	*
S2	71.45±2.72 BC a	64.14±2.74 CD a	55.18±3.64 CD b	*	64.94±1.99 BC a	61.55±1.54 B ab	54.71±2.37 A b	*	60.58±2.67 BC a	59.98±1.31 B a	53.04±3.57 AB a	NS
Sig.	**	**	**		**	NS	NS		**	**	NS	

N.S Non-significant * (P≤0.05) **(P≤0.01) Averages are in capital letters to compare coefficients, and averages are in lowercase 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

3.3 Percentage of sperm total abnormality

At 48 hours, significant differences were observed ($P \leq 0.01$) between the treatments. T1 exhibited the highest total abnormalities, followed by TC. SC had significantly lower abnormalities compared to the others. After 1 hour, abnormalities increased significantly in most groups, with SC showing the smallest increase, indicating its protective effect. After 2 hours, the abnormalities further increased in all groups, with SC still maintaining significantly lower values.

After one month, abnormalities remained significant ($P \leq 0.05$), especially in the T1, which had the highest abnormalities compared to others. SC again showed better resistance to the increase in abnormalities after post-thawing. After 1 hour, abnormalities in SC increased slightly, but it still performed better than T1 and T2. After 2 hours, SC had a significantly lower abnormality rate compared to the other treatments, as shown in Table 3.

By two months, the abnormality levels were higher across all groups, with significant differences ($P \leq 0.01$) among the treatments. T1 showed the highest abnormality, and SC again performed well in maintaining lower abnormalities. After 1 hour, abnormalities in SC were significantly lower than in T1. After 2 hours, the pattern remained, with SC maintaining significantly lower abnormalities than other treatments. In conclusion, SC consistently showed lower sperm abnormalities over time and incubation periods compared to the other treatment groups. However, in T1, sperm abnormalities were lower at specific time points. Overall, the soybean lecithin extender (SC) was more effective in reducing sperm abnormalities than the Tris extender (T1 and TC) across most time points.

Table 3: Effect of adding relaxin on sperm Total Abnormalities in Holstein bulls after different periods of cryopreservation after post-thawing, 1h and 2h of incubation (mean± standard error).

Treatments	48h%			Sig.	One Month%			Sig.	Two Month%			Sig.
	Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h	
TC	16.68±0.33 BC a	18.03±0.46 B b	19.32±0.47 A c	**	22.90±1.70 AB a	21.33±1.07 A ab	19.75±0.47 AB b	*	20.60±1.41 B a	24.03±0.60 AB a	24.36±0.58 A a	NS
T1	14.78±0.30 D b	15.38±0.35 D ab	16.20±0.47 B a	*	16.71±0.36 C b	18.53±1.11 B ab	19.10±0.55 B a	*	17.51±0.78 C b	19.86±0.13 C ab	20.76±0.26 C a	*
T2	15.76±0.17 CD a	10.16±0.61 C a	16.66±0.38 B a	NS	17.78±0.40 B b	18.86±0.26 A b	19.50±0.28 AB a	*	21.12±0.85 B a	22.63±0.31 B a	21.66±0.37 BC a	NS
SC	18.18±0.34 A a	20.77±0.22 A b	19.17±0.40 A c	**	20.70±0.40 A b	21.93±1.03 A ab	23.40±1.01 A a	*	24.30±1.00 A a	25.60±0.30 A a	24.33±0.60 A a	NS
S1	15.36±0.47 D b	16.97±0.44 CD ab	16.13±0.46 B a	*	17.17±0.44 C a	18.93±1.08 B a	18.66±0.35 B a	NS	19.12±0.60 BC a	20.56±0.80 C a	19.53±1.38 C a	NS
S2	16.93±0.46 B b	19.32±0.59 B ab	18.13±0.49 A a	*	19.50±0.46 AB b	21.40±0.88 AB b	20.66±0.86 AB b	*	21.36±0.55 B b	24.10±0.70 AB ab	23.03±0.54 AB a	*
Sig.	**	**	**		**	*	*		**	**	**	

N.S Non-significant * (P≤0.05) **(P≤0.01) Averages are in capital letters to compare coefficients, and averages are in lowercase 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

3.4 Sperm plasma membrane integrity

After 48 hours of freezing, T1 exhibited the highest plasma membrane integrity post-thaw, significantly higher than all other treatments ($p < 0.01$). T2 followed closely, while TC had the lowest plasma membrane integrity, significantly lower than both T1 and T2 ($p < 0.05$). After 1 hour of incubation, T1 still showed the highest plasma membrane integrity, maintaining a significant advantage over SC and TC, with differences between these groups being highly significant ($p < 0.01$). At the 2-hour mark, T1 and T2 continued to have the highest plasma membrane integrity, while SC and TC experienced a significant decline ($p < 0.01$).

After one month of freezing, T1 again demonstrated the best plasma membrane integrity post-thaw, significantly higher than SC and S2 ($p < 0.01$). T2 also maintained high integrity, although not significantly different from T1. After 1 hour of incubation, S1 and T2 showed the highest values, with SC and TC significantly lower ($p < 0.01$). After 2 hours, T1 still retained superior plasma membrane integrity, significantly higher than SC and TC ($p < 0.01$).

After two months of freezing, T1 remained the best post-thaw treatment, followed by T2, showing significantly better plasma membrane integrity than SC and TC ($p < 0.01$). After 1 hour of incubation, T1 showed the highest integrity, significantly higher than SC and TC ($p < 0.01$). Even after 2 hours, T1 retained the best results, significantly better than SC and TC ($p < 0.01$). This indicates that T1 is consistently the most effective treatment in preserving plasma membrane integrity over time and with extended incubation, as shown in Table 4.

Table 4: Effect of adding relaxin on sperm plasma membrane integrity in Holstein bulls after different periods of cryopreservation after post-thawing, 1h, and 2h of incubation (mean± standard error).

Treatments	48h%			Sig.	One Month%			Sig.	Two Month%			Sig.
	Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h	
TC	72.48±2.19 D a	68.66±0.72 C a	66.20±2.13 D b	*	69.81±1.95 C a	66.45±2.32 C a	58.53±1.77 B a	NS	63.87±2.93 D a	64.20±1.41 C a	53.48±4.60 D a	NS
T1	87.21±0.75 A a	84.48±0.91 A b	80.25±0.99 A c	**	83.86±0.97 A a	81.66±2.24 A a	78.50±3.01 A a	NS	77.91±1.91 A a	78.56±1.82 AB a	72.15±1.78 A a	NS
T2	83.61±1.88 AB a	77.83±2.02 B b	77.41±0.48 BC c	**	81.75±1.36 A a	77.83±1.31 AB a	71.55±2.00 A a	NS	75.28±1.23 AB a	75.85±3.35 AB a	71.03±1.03 A a	NS
SC	77.71±1.33 C a	70.24±1.25 C b	68.03±1.22 D c	**	74.06±1.26 B a	68.75±1.14 C b	61.86±1.44 B b	*	67.53±1.21 CD a	66.68±3.23 C a	53.02±3.48 CD a	NS
S1	84.51±1.52 AB a	81.10±1.97 AB ab	77.22±1.97 AB b	*	80.71±1.23 A a	79.51±4.30 AB a	75.41±1.73 A a	NS	75.28±1.56 AB ab	76.55±1.76 A a	68.28±1.65 AB b	*
S2	81.73±1.10 BC a	76.65±1.50 B b	72.25±3.57 C c	**	76.62±0.96 B c	74.18±3.76 BC a	68.18±1.22 AB a	NS	70.78±1.88 BC a	69.95±1.67 BC a	2.12±64.36±2.12 BC a	NS
Sig.	**	**	**		**	**	**		**	**	**	

N.S Non-significant * (P≤0.05) **(P≤0.01) Averages are in capital letters to compare coefficients, and averages are in lowercase 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.

3.5 Percentage of sperm acrosome integrity

After 48 hours of freezing, T1 and T2 had the highest post-thaw motility, both significantly better than SC ($p < 0.05$). While S1 showed good motility outcomes, they were slightly lower than those of T1 and T2, although no significant differences were observed. After 1 hour of incubation, T1 maintained the highest motility, which was significantly higher than SC ($p < 0.01$). S1 also showed comparable motility levels to T1 but did not reach statistical significance. At the 2-hour mark, T2 continued to show better motility than SC, with this difference being highly significant ($p < 0.01$), while S2 demonstrated moderate preservation of motility, slightly higher than SC but lower than T2. This result shows that relaxin treatments (T1 and T2) are more effective at preserving motility during short-term freezing compared to the soybean lecithin extender (SC), although S1 provided a viable alternative with relatively good outcomes.

After one month of freezing, T2 showed the highest post-thaw motility, though the differences between T2, T1, and TC was not statistically significant. S1 demonstrated motility comparable to T1 and T2, indicating its effectiveness in cryopreservation, while S2 showed lower motility than S1 and the tris-based treatments. After 1 hour of incubation, T2 maintained the highest motility, though no significant differences were observed between treatments. After 2 hours, T1 and T2 retained higher motility compared to SC, with the difference between T1 and SC being statistically significant ($p < 0.05$). S1 exhibited motility levels similar to T1, showing that soybean lecithin with relaxin at 60 ng/ml (S1) can preserve motility effectively over this period.

After two months of freezing, T1 had the highest post-thaw motility, followed by T2, while SC showed significantly lower motility than T1 ($p < 0.05$). S1 demonstrated comparable post-thaw motility to T2, outperforming SC, while S2 showed moderate motility preservation but lower results than S1. After 1 hour of incubation, T1 still performed the best, with significantly higher motility than SC ($p < 0.01$). S1 showed similar performance to T1, while S2 remained comparable to T2. After 2 hours of incubation, T1 continued to exhibit significantly better motility compared to SC ($p < 0.01$), and S1 showed favorable motility retention comparable to the tris-based relaxin treatments.

Overall, T1 consistently showed the best preservation of motility across all freezing periods and incubation times, with S1 emerging as a strong alternative, demonstrating the effectiveness of soybean lecithin combined with moderate relaxin concentrations in maintaining sperm quality during cryopreservation.

Table 5: Effect of adding relaxin on sperm acrosome integrity in Holstein bulls after different periods of cryopreservation after post-thawing, 1h, and 2h of incubation (mean± standard error).

Treatments	48h%			Sig.	One Month%			Sig.	Two Month%			Sig.
	Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h		Post-thawing	After 1h	After 2h	
TC	77.18±1.12 AB a	71.86±2.07 AB a	65.56±0.69 B b	*	73.81±1.74 A a	69.13±0.53 A b	61.30±3.02 A b	*	70.11±1.26 AB a	63.06±1.96 C b	58.83±1.83 C c	**
T1	78.84±0.75 A a	75.25±0.75 A b	71.93±1.23 A c	**	74.34±0.80 A a	70.26±1.50 A b	68.13±0.24 A b	*	74.25±1.74 A c	70.16±0.60 AB b	67.45±0.57 AB a	*
T2	78.62±0.72 A b	74.86±0.82 A a	69.33±3.26 A a	*	75.21±0.99 A a	72.48±2.35 A a	69.30±1.28 A a	NS	70.82±1.01 A a	67.03±1.46 BC ab	63.93±1.58 BC b	*
SC	75.53±1.13 B b	69.28±1.40 B a	59.05±1.90 B a	*	72.35±1.30 A a	69.61±1.72 A ab	65.76±3.01 A b	*	67.46±1.59 B a	64.73±2.26 BC a	61.70±2.51 C a	NS
S1	78.14±0.58 AB a	74.90±1.87 A b	69.18±2.35 A c	**	73.83±0.84 A ab	74.75±2.50 A a	69.53±1.34 A b	*	73.43±2.36 A a	74.58±0.64 A a	69.76±1.67 A a	NS
S2	76.58±0.71 AB a	72.10±0.86 AB b	69.70±2.90 A c	**	73.35±1.15 A a	70.90±3.08 A a	67.28±1.67 A a	NS	70.66±2.05 AB a	65.66±1.20 BC ab	60.38±1.44 C b	*
Sig.	*	**	**		NS	NS	NS		NS	**	**	

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

Discussion

This study, explored the effects of different concentrations of relaxine combined with tris and soyabean lecithin extenders on spermatozoa quality parameters; during cryopreservation, and post-thaw incubation. A steady decline in sperm motility was observed overtime for all treatments; as well documented phenomena in cryopreservation; due to cumulative cellular stress and cryodamage. While the control group TC show the expected gradual decrees in motility; relaxine treatments T1 and T2, showed the potential to reduce this effect; particularly T1, which consistently maintained higher motility compared to T2. This suggests that the less relaxine concentration (60ng/ml); is more effective in maintaining motility, likely due to a balance between enhancing mitochondrial activity. Avoid, overstimulation; or osmotic

imbalance, which occur on higher concentrations. The superior performance of tris extenders; T1 and T2, can attributed their strong buffer ability, which helps stabilize PH, and osmotic conditions during cryopreservation and thawing. Additionally; tris extenders, are known for their antioxidant properties; which reduce oxidative damage, further, protecting spermatozoa integrity. Relaxin, at T1 concentration; complements those effects by enhancing mitochondrial, function and maintaining energy production, which is critical for sperm motility and survival.

In contrast, soyabean lecithin extenders (S1 and S2) showed comparatively poor motility, with S2 performing the worst. While soyabean lecithin extender; provide phospholipids that stabilize the sperm membrane, and reduce cold shock damage, higher relaxine concentrations (as in S2); may disrupt membrane integrity or increase osmotic stress, contributing to faster motility declines. These finding suggests that combination of relaxin and extenders type must be carefully optimize, with less relaxin concentration (T1 and S1) demonstrated greater efficiency on preserving motility and overall sperm quality.

In comparison to tris extender, soybean lecithin treatments SC, S1, and S2 generally displayed a decreased in motility, with S2 exhibiting the lowest motility. This implies that the combination of elevated relaxin concentrations with soybean lecithin may be deleterious to motility. However, there were marginal increases in motility at the two-month point for certain treatments, which may indicate a delayed sperm recovery after thawing. Similar trends for sperm viability were noted[17], and it was reported that the control vitality gradually decreased with time, whereas the concentrations 50ng/ml and 100ng/ml showed better viability an hour after thawing, especially in the first two periods of 48 hours and 1 month. However, after two hours; viability was decreased, indicating that the relaxin's protective benefits, might wear off over time. Compared to tris treatments. Soybean lecithin therapies, particularly SC, exhibited higher viability. An hour after thawing; SC showed a noticeable increase in viability; however, no more changes were; seen after two hours. This implies that even if soybean lecithin offers protection right away; its benefits cannot last for long incubation times [9]. S1's viability showed consistent recovery. Suggesting that S1, with its modest relaxin dose, effectively preserved, sperm viability during the freezing and thawing processes.

According to [17], the TC groups total abnormalities; gradually increase over time, especially after an hour at the one-month mark. This suggest that, sperm preserved in tris without relaxin become more susceptible to structural damage, due to the lack of protective agents. In comparison, T1 consistently demonstrated; modest rate of abnormality across all phases. With significant reductions over time. Indicating that the relaxine at dose of 60ng/ml; effectively protects against structural damage. This protection can be attributed, to the relaxin role in membrane, thereby maintaining sperm integrity. The buffering capacity of tris, further support, those protective effects by maintaining optimal pH and osmatic environment during freezing and thawing.

T2, initially had the lowest abnormality rates, its performance was varied over time. This suggest that a higher dose of relaxin (90 ng/ml); may have a lower consistent protective effect; possibly due to osmotic stress or overstimulation of cellular process. SC shoed significant improvement in the first hour of incubation; likely due to stabilized effects on the soyabean lecithin phospholipids content, on the sperm membrane. Two months, S1 shows a significant reduction in abnormality; further supporting the effectiveness of moderate dose of relaxine (60 ng/ml); with soyabean lecithin extender. S2; showed variation overtime but maintained less abnormality rate compared to TC; suggesting its ability to maintained sperm integrity, under certain conditions. In summary, T1 outperformed other treatments due to the optimal balance, between tris and relaxin (60ng/ml); which synergistically reduced, oxidative stress; stabilized

the membrane, and preserved mitochondrial function. The balance approach resulted in consistently less rate of abnormalities compared to treatments with higher concentration; of relaxin (T2andS2); or treatment that rely on soybean lecithin (SC, S1, S2).

In term of plasma membrane integrity, TC shows variable membrane stability, with a reduction shortly after thawing followed by recovery over time. Particularly by the two-month mark. T1 show consistent membrane integrity over time; with significant improvement in 2 months, demonstrating that 60ng/ml relaxin; can provide sustained plasma membrane protection. In conversely, T2 show a lot more viability, higher initial integrity, follow by a sharp decline. Soybean lecithin, treatments showed; generally higher initial membrane integrity then tris treatments, with S1 exhibiting a notable maximum in 1 month. This suggests; that soybean lecithin with relaxin concentration enhance membrane stability. S2, although it variable; show recovery at two months; demonstrated higher relaxin concentrations may stabilize, the membrane over time[27].

Furthermore, this study examined acrosome integrity, critical parameter for spermatozoa fertilization potential. TC show an initial improvement in acrosome integrity; after one hour of thawing, follow by a decline; reveals that tris alone may not sustain acrosome integrity; over time. T1 and T2 show the highest acrosome integrity, at one and two months, suggesting that relaxin plays a critical role, in protecting the acrosome during extended storage. Soybean lecithin treatments SC, S1, and S2 show, higher acrosome integrity showed higher acrosome integrity than tris, with SC showing the highest stability, after two months. With increased doses; of relaxin mixed; with soybean lecithin; the acrosome integrity, was sufficiently maintained; as demonstrated by the considerable, improvement in acrosome integrity over time(S2)

Conclusion

This study highlights the importance of selecting appropriate extenders and additives; such as relaxine, to improves spermatozoa cryopreservation. Tris extenders better in long-term stability; while soyabean lecithin was superior, for short-term preservation. Relaxine improved sperm quality; depended on concentration and incubation duration. Those results could help guide, improved cryopreservation protocols for reproductive technologies.

Conflict of Interest: “The authors declare that they have no conflict of interest.”

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