EFFECT OF SPERMS FREEZABILITY ON SOME SEMEN ATTRIBUTES AND AMINO ACID CONCENTRATIONS IN SEMINAL PLASMA OF HOLSTEIN BULLS

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ABSTRACT

This study investigated the effect of different sperm freezability on some semen characteristics and amino acid concentrations in the seminal plasma of Holstein bulls cryopreserved for two years. The semen straws (0.25 ml) that cryopreserved for two years were divided into three equal groups (7 bulls per group) according to the sperm's freezability: medium (73.02%; F1), good (83.76%; F2), and high (91.20%; F3). The F1 bulls were superior (P≤ 0.05) in sperm's cell individual motility (SCIM) for fresh semen as compared with F2 and F3 bulls. The F2 and F3 groups were superior (P≤ 0.01) to the F1 group in live sperm percentage at cryopreservation. The sperm's DNA damage percentage was lesser (P≤ 0.05) in the F1 and F3 groups as compared with the F2 group. The differences among groups in normal sperm percentage, sperm's plasma membrane and acrosome integrity percentage, malondialdehyde, and total antioxidant concentrations lacked significance. The essential and non-essential amino acid concentrations in seminal plasma did not differ among groups. In conclusion, sperm freezability influenced some semen attributes but did not affect amino acid concentrations in the seminal plasma of Holstein bulls.

Keywords: Sperm characteristics, metabolic markers.

تأثير قابلية تجميد النطف في بعض خصائص السائل المنوي وتركيز الأحماض الأمينية في البلازما المنوية لثيران الهولشتاين

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المستخلص

اجريت هذه الدراسة لبيان تأثير قابليات تجميد نطف مختلفة في بعض صفات السائل المنوي وتركيز الأحماض الأمينية في البلازما المنوية لثيران الهولشتاين المحفوظة بالتجميد لمة سنتين. تم استعمال قصبات السائل المنوي المحفوظة بالتجميد لمدة سنتين بسعة 0.25 مل، قسمت الى ثلاث مجاميع متساوية (7 ثيران لكل مجموعة) حسب قابلية التجميد: متوسطة (73.02 %; F1)، جيدة (83.76 %; F2) وعالية (91.20 %; F3). تفوقت (P≤0.05) لثيران المجموعة F1 على المجموعتين F2 وF3 في النسبة المئوية لحركة النطف الفردية للسائل المنوي الطازج، في الوقت الذي تفوقت (P≤0.01) فيه ثيران المجموعتين F2 وF3 على المجموعة F1 في النسبة المئوية للنطف الحية بعد الحفظ بالتجميد، مع انخفاض (P≤0.05) النسبة المئوية لضرر المادة الوراثية لدى مجموعتي الثيران F1 وF3 مقارنةً بالمجموعة F2. انعدمت الفروق المعنوية بين مجاميع الثيران الثلاثة في كل النسبة المئوية للنطف الطبيعية والسليمة الغشاء البلازمي والأكروسوم وتركيز المالون ثنائي الألديهايد ومضادات الأكسدة الكلية في البلازما المنوية. وفي الوقت نفسه، لم يختلف تركيز الأحماض الأمينية الأساسية وغير الأساسية في البلازما المنوية بين المجاميع المختلفة. يمكن الاستنتاج ان قابلية تجميد النطف كان لها تأثير في بعض صفات السائل المنوي لكنها لم تؤثر في تركيز الأحماض الأمينية في البلازما المنوية لثيران الهولشتاين.

الكلمات المفتاحية: صفات النطف، دلائل ايضية.

Received: 14/2/2022, Accepted:8/5/2022

INTRODUCTION

Nowadays, artificial insemination has become a stable technology and has been commercially available for dairy cattle owners worldwide for more than 60 years, enabling them to use elite bulls efficiently and making a rapid improvement for the genotypes and the productive characteristics of the cows without purchasing elite bulls (38). Moreover, it has become necessary to see more development in semen analysis procedures, semen extenders, adding antioxidants, sperm selection, sperm sexing, and storage, accompanied by early prediction of bull fertility by determining some genes and biomarkers in seminal plasma (3, 4, 8, 9, 19, 27, 38). The seminal plasma of bulls is composed of testicular, epididymal, and accessory sex glands secretions. These contain proteins, ions, and metabolites like amino acids, lipids, monosaccharides, fatty acids, nucleotides, minerals, and steroid hormones (2, 11, 27, 28). The metabolites are the final products of metabolic pathways that play principal role in sperm physiology and metabolic activities (14). Some seminal plasma components have positive effects in resisting the conditions of sperms cryopreservation, and others have harmful effects (23, 26, 30). Sixty-three metabolized compounds were identified, including 21 amino acids, in the seminal plasma of bulls with different fertility rates (37), explaining the importance of estimating the metabolites in seminal plasma between high and low-fertile bulls. The sperm cryopreservation technique is of great importance in increasing the production of cows and improving the quality of their products, as it accelerates genetic diversity by inseminating a large number of cows and facilitates the spread of genetically superior bulls worldwide (35). Bull's sperms are greatly affected by decreasing temperature, especially the sperm's plasma membrane, as cryopreservation leads to significant changes in the activity of membrane proteins and the consequent change in the permeability of water and solutes, which in turn will lead to the loss of sperm's viability (10). Excluding data from Ugur et al. (36), who studied the relationship of amino acid concentration in the seminal plasma of bulls with different sperm freezability of these bulls, no previous trial studied the relationship between the concentration of amino acids in the seminal plasma of Holstein bulls of different freezability and post-cryopreserved semen characteristics for two years. Therefore, this study explored the influence of different sperm freezability on some semen characteristics and amino acid concentrations in the seminal plasma of Holstein bulls cryopreserved for two years.

MATERIALS AND METHODS

Experimental animals and design

The study was conducted at the Artificial Insemination Department (AID) in the Iraqi Ministry of Agriculture. Twenty-one Holstein bulls of 2-7.5 years old and 650-1000 kg body weight used currently. The semen straws (0.25 ml) that cryopreserved for two years were divided into three equal groups (7 bulls per group) according to the sperm's freezability: medium (73.02%; F1), good (83.76%; F2), and high (91.20%; F3). All bulls were healthy, disease-free, and under constant veterinary supervision. All bulls were allocated on a daily concentrate ration (4-6 kg/bull). The ration consisted of 35% barley, 33% wheat bran, 10% maize, 20% soybean meal, 0.5% CaCl2, 0.5% salt, 1% vitamins, and minerals. Roughage consisted of alfalfa hay (7-9 kg/animal per day), with the green forage at a rate of 50-60 kg/animal per day. Salt blocks and freshwater were available *ad libitum* to the animals.

Semen evaluation

Percentages of sperm cell individual motility, livability, and normality were assessed according to Al-Nuaimi (5). Also, percentages of the sperm plasma membrane and acrosome integrity were tested based on Sultan (34). Moreover, Sperm DNA damage percentage, malondialdehyde, and total antioxidant concentrations were determined currently according to Sultan (34) and Al-Nuaimi (5).

Determination of amino acids concentrations in seminal plasma: The concentration of amino acids in the seminal plasma was estimated using high-performance liquid chromatography HPLC). Concurrently, the concentration of essential amino acids (tryptophan, lysine, threonine, valine, methionine) and non-essential amino acids (serine, glutamine, glycine, arginine, and

alanine and cysteine and aspartic) were calculated based on the method of Mohammad et al. (25).

Statistical analyses

Statistical computations were carried out using General Linear Model (GLM) procedure in the SAS program (32), using CRD to examine the influence of sperm freezability on semen immediate and microscopic characteristics and amino acid concentrations in seminal plasma. The statistical model for the analysis of variance was as follows:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where:

Y_{ij} = dependent variable (semen immediate and microscopic characteristics)

μ= Overall mean

G= Effect of sperm freezability (F1, F2, and F3 groups).

e_{ij}= error term

Differences among means were compared using Duncan multiple range test

RESULTS AND DISCUSSION

Sperm cell individual motility: The F1 group exhibited a higher ($P \leq 0.05$) sperm cell individual motility (SCIM) of the fresh semen (67.85 ± 1.48 %) as compared with the F2 (62.85 ± 1.48 %) and F3 (57.14 ± 1.01%) groups however, the differences among groups in SCIM at 48 hours, and two years' post-cryopreservation (PC) lacked significance (Table 1). The absence of a significant effect of freezability on SCIM percentage, 48 hours and two years PC may return to the selection of good ejaculates in terms of SCIM by AID staff with no less than 50 % that are cryopreserved and neglect the others of poor quality. The results of SCIM for the fresh semen ranged between 57.14 ± 1.01 - 67.85 ± 1.48 % (Table 1), confirming this notion. The careful selection of good-quality sperms increased pregnancy rates for cows inseminated with these straws. The pregnancy rate results (62%) of cows inseminated with these bulls (5) confirmed the current results. The study involved 1014 owners in 12 Iraqi provinces. The SCIM percentage of fresh semen for the current three groups of bulls were higher than that of Al-Saedy and Abdulkareem (6) for Holstein bulls (22.09-48.37%) and similar to what Eidan (12) obtained for these bulls (59.87 ± 0.69%). The differences among studies may be due to the various management practices and feeding levels provided to animals in the different studies. The SCIM percentage is one of the most common criteria for assessing semen quality after the freezing and thawing protocols (17). However, it is not an accurate predictor of fertility when artificially inseminating cows (22). Methods for evaluating sperm motility have developed after using a computer-assisted sperm analysis (CASA) technique, which measures different types of sperm motility closely related to fertility compared to the traditional microscopic evaluation (19). It is well-known that the semen of bulls are not resistant to stressors resulting from long-term cryopreservation like cold shock, osmotic disturbance, and ice crystal formation. These will consequently affect the sperm function resulting from high membrane fluidity and its reflection on its physiology and male fertility (29). The presence of good-quality semen extenders that provide a suitable pH, cryoprotectants, and appropriate antioxidants will reduce these stressors, positively reflecting an increase in sperm freezability (13). The absence of differences in SCIM percentages between the 48 hours and two years PC for the F2 (52.85 ± 1.48 vs. 52.85 ± 1.48%) and F3 (52.15 ± 1.01 vs. 50.71 ± 1.30%; Table 1) groups confirms the presence of superior semen extenders and cryopreservation steps at the AID for maintenance of the long-term freezability of sperms.

Table 1. Effect of sperm freezability on sperm cell individual motility in fresh and cryopreserved semen of Holstein bulls for 48 hours and two years PC (Mean ± SE).

Trait / Bulls with different freezability	Sperm cell individual motility percentage in fresh semen	Sperm cell individual motility percentage 48 hours post-cryopreservation	Sperm cell individual motility percentage two years hours post-cryopreservation
F1 (n=7)	67.85±1.48 A	51.42±0.92	49.28±1.30
F2 (n=7)	62.85±1.48 B	52.85±1.48	52.85±1.48
F3 (n=7)	57.14±1.01 C	52.14±1.01	50.71±1.30
Level of significance	P≤0.05	N.S	N.S

Means with different superscripts within each column differ significantly (P≤0.05).
F1: Medium sperm freezability (73.02 %), F2: Good sperm freezability (83.76 %).
F3: High sperm freezability (91.20 %).

Percentages of live, normal, plasma membrane integrity and acrosome integrity of sperms: Greater (P≤0.05) live sperm percentage was observed for the F2 (81.78 ± 0.63 %) and F2 (82.85 ± 1.07 %) groups as compared with the F1 group (77.35 ± 0.63 %; Table 2). The differences among groups in the percentages of the normality, plasma membrane integrity, and acrosome integrity of sperms lacked significance; however, they tended to be higher in the F2 group than in the F1 and F3 groups (Table 2). To the best of our knowledge, this is the first study in Iraq and internationally that deals with the influence of freezability on semen attributes of Holstein bulls. Studying the relationship of freezability with semen characteristics has become necessary to improve bull fertility, assisted reproductive technologies, and artificial insemination success. These will enhance the bull selection programs and improve the dairy cattle industry (35). The freezability percentages under the current study (73.02 - 91.20%) for two years PC were higher than that recorded by Eidan et al. (17), ranging between 43.58 - 68.36% after 48 hours and three months PC. The current study demonstrated a clear relationship between sperm freezability and semen characteristics like live sperm, sperm plasma membrane, and acrosome integrity percentages with varying degrees of significance and numerical effect. These will indicate the possibility of using it as a criterion for the fertility of bulls. Moreover, the sperm's plasma membrane integrity is necessary for good motility and a high sperm fertilization rate. Semen samples with low sperm progressive motility often suffer from a defect in the plasma membrane. The current results agreed with those reported by Hitit et al. (18), who did not find significant differences in acrosome integrity percentage between the two groups of bulls with high and low freezability, with a tendency to increase this percentage in bulls with high freezability. The current results of live sperm percentage at 48 hours PC (77.35 - 82.85%) were higher than those obtained by Al-Nuaimi et al. (6; 44.28 ± 3.62%). On the other hand, the current plasma membrane integrity percentage (81.42 - 83.28%) is higher than what was obtained by Eidan (15; 48.17 ± 2.94%).

Table 2. Effect of sperm freezability on Percentages of live, normal, plasma membrane integrity and acrosome integrity of sperms of Holstein bulls for two years PC (Mean ± SE).

Trait / Bulls with different freezability	Live sperm percentage	Normal sperm percentage	Sperm plasma membrane integrity percentage	Sperm acrosome integrity percentage
F1 (n=7)	77.35±0.63 B	82.01±1.10	81.42±0.66	91.92±0.36
F2 (n=7)	1.07±81.78 A	82.10±1.58	83.28±1.37	92.41±0.64
F3 (n=7)	82.85±0.66 A	82.93±1.33	82.14±0.25	91.07±0.57
Level of significance	P≤0.05	N.S	N.S	N.S

Means with different superscripts within each column differ significantly (P≤0.05).
F1: Medium sperm freezability (73.02 %), F2: Good sperm freezability (83.76 %).
F3: High sperm freezability (91.20 %).

Malondialdehyde and total antioxidants concentrations in seminal plasma and sperm DNA damage percentage: Non-significant differences were observed among groups in malondialdehyde concentrations in seminal plasma, despite a tendency to decrease in the F2 group (15.30 ±2.00 µmol/10^9 sperm) by 26.7 and 4.38 % compared to the F3 (19.39 ± 1.83 µmol/109 sperm) and F1 (15.97 ± 2.29 µmol/10^9 sperm) groups (Table 3). Concomitantly, the differences among groups in total antioxidant concentrations in seminal plasma lacked significance, with the trend of increase in the F2 group (144.278 ± 6.39 µg/dl) by 21 and 5 % compared to the F3 (119.16 ± 6.27 µg/dl) and F1 (137.26 ± 9.44 µg/dl) groups (Table 3). On the other hand, the F3 and F1 groups recorded lesser (P≤0.01) sperm DNA damage percentages (7.02 ± 0.65 and 8.44 ± 0.99 %, respectively) compared to the F2 group (12.42 ± 1.78 %; Table 3). The absence of a significant effect among the three bull groups in the malondialdehyde concentrations confirms that the semen straws with different freezability do not suffer from oxidative stress because this compound is considered a by-product for lipid peroxidation, generation of free radicals, and oxidative stress (30). The non-significant differences among groups in the percentages of normality, the plasma membrane, and acrosome integrity of sperms (Table 2) confirmed this notion. The non-significant differences among bull groups in the total antioxidant concentrations indicate adequate amounts of natural antioxidants in the seminal plasma, like catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase (SOD). These will prevent the oxidative stress of sperms (31). Mahmood et al. (24) recorded values of some natural antioxidants in frozen seminal plasma for Holstein bulls, being 0.84 ± 0.24 unit/mol for catalase and 30.87 ± 11.11 unit/mol for SOD enzymes. Concomitantly, Sultan (34) recoded values of these two enzymes in seminal plasma of frozen straws for Holstein bulls, namely 34.48-48.29 µmol/ml for catalase and 449.42-790.64 µmol/ml for SOD enzymes. The current total antioxidant concentrations in seminal plasma (Table 3) were higher than those obtained by Sultan (34; 1.72 - 1.80 mg/dl) for Holstein bulls. These differences may be due to the variation in the duration of cryopreserved straws and the kit used for analysis. Ahmed et al. (1) found that prolonging the cryopreservation process increases the lipid peroxidation and malondialdehyde concentrations in Nili Ravi buffalo bulls. On the other hand, the current malondialdehyde concentrations in seminal plasma (15.30-19.39 µmol/10^9 sperm; Table 3) were higher than those obtained by Sultan (40; 8.46-12.18 µmol/10^9 sperm) and Mahmood et al (24; 11.73 ± 2.68 µmol/10^9 sperm), and approach to what was obtained by Al-Nuaimi (6; 15.25 ± 2.18 µmol/10^9 sperm) in Holstein bulls. The current sperm DNA damage percentage (7.02-12.42 %; Table 3) is within the normal range for bull sperms. It is well-known that this percentage is 15% or less in highly fertile bulls, 15-25% in medium-fertility bulls, and 25– 50% in low-fertility bulls. The intact and undamaged DNA indicates its capability to synthesize cellular proteins and fats. The damaged DNA stimulates factors that modify manufacturing proteins and lipids inside the cells and produce substances that stimulate the occurrence of pro-inflammatory, antioxidants, and cytokines (12).

Table 3. Effect of sperm freezability on the concentrations of malondialdehyde and total antioxidants and sperm DNA damage of Holstein bulls for two years PC (Mean ± SE).

Trait / Bulls with different freezability	Malondialdehyde concentration in seminal plasma (µmol/10^9sperm)	Total antioxidant concentration (µg /dl)	Sperm DNA damage percentage
F1 (n=7)	15.97±2.29	137±9.44	8.44±0.99 A
F2 (n=7)	15.30±2.00	144.29±16.39	12.42±1.78 B
F3 (n=7)	19.39±183	119.16±6.27	7.02±0.65 A
Level of significance	N.S	N.S	P≤0.05

Means with different superscripts within each column differ significantly (P≤0.05).
F1: Medium sperm freezability (73.02 %), F2: Good sperm freezability (83.76 %).
F3: High sperm freezability (91.20 %).

Essential amino acids concentrations in seminal plasma: The differences among groups in tryptophan concentrations in seminal plasma lacked significance, despite a tendency to be higher in the F2 group (149.17 ± 6.07 ppm) by 8 and 3 % than in F1 (136.51 ± 4.5 ppm) and F3 (144.00 ± 9.93 ppm) groups (Table 4). Similarly, non-significant differences were observed among groups in lysine concentration. However, it tended to be higher in the F2 group (131.87 ± 14.43 ppm) by 7 and 1% than in the F1 (122.27 ± 38.36 ppm) and F3 (130.44 ± 43.12 ppm) groups (Table 4). Concomitantly, the differences among groups in threonine, valine, and methionine concentrations in seminal plasma lacked significance despite a tendency to be higher in the F2 group compared with F1 and F3 groups (Table 4).

Table 4. Effect of sperm freezability on the essential amino acid concentrations in seminal plasma of Holstein bulls for two years PC (Mean ± SE).

Essential amino acid / Bulls with different freezability	Tryptophan	Lysine	Threonine	Valine	Methionine
F1 (n=7)	136.51±4.5	122.27±38.36	651.62±114.75	427.50±11.18	256.42±17.58
F2 (n=7)	149.17±6.07	131.87±14.43	116.63±631.22	426.00±20.06	248.71±9.73
F3 (n=7)	144.00±9.93	130.44±43.12	110.68±636.24	434.55±9.22	252.14±14.86
Level of significance	N.S	N.S	N.S	N.S	N.S

F1: Medium sperm freezability (73.02 %), F2: Good sperm freezability (83.76 %).
F3: High sperm freezability (91.20 %).

Non-essential amino acids concentrations in seminal plasma: Non-significant differences were noticed among groups in serine concentrations in seminal plasma, despite a tendency to be higher in the F3 group (309.38 ± 7.68 ppm) by 56 % than in F1 (136.84 ± 6.56 ppm) and F2 (135.57 ± 5.56 ppm) groups (Table 5). Similarly, the differences among groups in glutamic acid concentrations in seminal plasma lacked significance. However, it tended to be higher in the F2 group (1156.44 ± 112.70 ppm) by 20 % compared with the F1 (925.27 ± 132.02 ppm) and F3 (922.82 ± 64.62 ppm) groups (Table 5). Also, non-significant differences were shown among groups in glycine concentrations in seminal plasma, despite a tendency to be higher in the F1 group (541.75 ± 39.54 ppm) by 20 and 3 % than in F3 (451.74 ± 73.93 ppm) and F2 (527.80 ± 23.26 ppm) groups (Table 5). The arginine concentrations in seminal plasma did not differ among groups, despite a tendency to be higher in the F2 group (274.50 ± 42.36 ppm) by 19 and 15 % compared to the F1 group (221.51 ± 5.93 ppm) and F3 (232.40 ± 8.01 ppm) groups (Table 5). The differences among alanine, cysteine, and aspartic acid groups lacked significance (Table 5).

Table 5. Effect of sperm freezability on the non-essential amino acid concentrations in seminal plasma of Holstein bulls for two years PC (Mean ± SE).

Non- essential Amino acids / Bulls with different freezability	Serine	Glutamic acid	Glycine	Arginine	Alanine	Aspartic acid
F1 (n=7)	136.84 ± 6.56	925.27 ± 132.02	541.75 ± 39.45	221.51 ± 5.93	256.42 ± 17.58	225.14 ± 7.18
F2 (n=7)	135.57 ± 5.56	1156.44 ± 112.70	527.80 ± 23.26	274.50 ± 42.36	248.71 ± 9.73	232.48 ± 15.26
F3 (n=7)	309.38 ± 7.68	922.82 ± 164.62	451.74 ± 73.93	232.40 ± 8.01	252.14 ± 14.86	229.40 ± 10.07
Level of significance	N.S	N.S	N.S	N.S	N.S	N.S

F1: Medium sperm freezability (73.02 %), F2: Good sperm freezability (83.76 %).
F3: High sperm freezability (91.20 %).

The non-significant differences among bull groups in essential and non-essential amino acids were consistent with the current semen characteristics, which did not differ significantly among the three bull groups**.** Two reasons behind non-significance. The current Holstein bulls were imported from well-known global cattle stations in Australia and obtained form the embryo transfer technology of pure Holstein-Friesian cows imported from New Zealand. No random cross-breeding trials conducted on these bulls, and no genetic variations occurred over the past period. The second reason is that the superior ejaculates are cryopreserved only by the AID staff. The poor-quality ejaculates were neglected to preserve the quality of the straws produced for artificial insemination. Therefore, it may be difficult to find differences in the quality of ejaculates among bulls. The current results did not agree with those reported by Ugur et al. (36), who found a relationship between the percentage of sperm freezability and the concentration of amino acids in the seminal plasma of bulls. They investigated that the phenylalanine concentration was higher in bulls with high sperm freezability than those with low sperm freezability. Concurrently, similar authors found non-significant differences in amino acid concentrations between high and low sperm freezability, which were in line the current results. The numeric increase (+56%) in the concentration of serine in the seminal plasma of bulls with high freezability (F3) compared to the remaining two groups indicates the possibility of its role in maintaining the sperm quality during cryopreservation. It is well-known that the electrostatic interactions among the phospholipids in the sperm cell membrane and most of the amino acids in the seminal plasma, including serine, help form a layer on the surface of the sperm that protects them from cryopreservation damage (21). On the other hand, some inhibitors of serine proteases secreted in bull semen, including anti-pain and plasminogen activator inhibitors-1 (PAI-1), increased the sperm acrosome integrity percentage during cryopreservation (7, 33). The numeric increase (+20%) in the concentration of glutamic acid in the seminal plasma of bulls of the good freezability (F2) explains the role of glutamic acid in maintaining good sperm freezability. Glutamic acid has a relationship with some genes and enzymes that occur in the mitochondria, which confirms its oxidative and reductive activity, regulation of cell death, and the metabolism of oxygen-containing amino acids (Oxoacids metabolic process). These compounds react with water to form acid oxides. Moreover, glutamic acid has a role in cell energy production (36). On the other hand, glutamic acid is the main compound of glutathione that inhibits sperm cellular damage from lipid peroxidation and the production of reactive oxygen species (Ugur et al., 36).

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