Influence of thawing period on some post-thaw semen characteristics of Holstein bulls following catalase addition to Tris extender

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Abstract

This study was undertaken to investigate the influence of thawing period on some post-thaw semen characteristics of Holstein bulls following adding catalase to Tris extender. Five Holstein bulls of 2.5-3 years old were used in this experiment. Semen was collected via artificial vagina at once ejaculate per bull per week for seven weeks experimental period. Pooled semen was equally divided into two groups using Tris extender. Catalase (100 IU/ml) was added to Tris extender as compared with control group (Tris extender). Following one year cryopreservation period, straws were thawed at 37˚C and examined after different thawing time (15, 30, 60 and 120 minutes). Sperm individual motility, live sperm percentage and total sperm abnormality were investigated. Sperm individual motility were superior ($P\leq 0.04$-$P\leq 0.08$) in catalase as compared with the control group during all thawing time. However, 15 and 30 minutes exhibited the highest ($P\leq 0.002$) motility percentage in catalase group. Similarly, higher live sperm percentage was noticed in catalase as compared with control group. The 15 and 30 minutes being the better live percentage for both groups. In contrast, the differences between groups in sperm abnormality percentage lacked significance. The least abnormality percentage was observed following 15 minutes and the highest at 120 minutes thawing time for both groups. In conclusion, the addition of catalase to Tris extender led to improved post-thaw sperm individual motility and live percentage of Holstein bulls following 15 and 30 minutes thawing period. This will in turn enhance fertility rate of artificially-inseminated cows, and owner's economic income consequently.

تاثير وقت الاسالة على بعض خصائص السائل المنوي بعد الاسالة لدى ثيران الهولشتاين بعد اضافة الكاتليز إلى مخفف ترس

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الخلاصة

أجريت هذه الدراسة لبيان تاثير وقت الاسالة على بعض خصائص السائل المنوي بعد الاسالة لدى ثيران الهولشتاين بعد اضافة انزيم الكاتليز الى مخفف ترس. استخدم في هذه التجربة خمسة ثيران هولشتاين بعمر 2.5−3 سنوات. تم جمع السائل المنوي بمعدل قذفة واحدة اسبوعياً لكل ثور ولمدة سبعة اسابيع. تم تجميع السائل المنوي للثيران جميعها وتقسيمه بالتساوي الى مجموعتين باستخدام مخفف ترس. تم اضافة انزيم الكاتليز (100 وحدة دولية/ مللتر) الى مخفف ترس ومقارنتها مع مجموعة السيطرة (مخفف ترس لوحده). وبعد فترة حفظ بالتجميد لمدة سنة، تمت اسالة القصبات بدرجة حرارة 37 درجة مئوية وعلى مدد اسالة مختلفة (15، 30، 60 و120 دقيقة). تم دراسة كل النسبة المئوية للحركة الفردية للنطف والنطف الحية وكذلك النطف المشوهة. تفوقت الحركة الفردية للنطف ($P\leq 0.04$−$P\leq 0.08$) في المجموعة المضاف إليها الكاتليز مقارنة بمجموعة السيطرة ولجميع مدد الإسالة المدروسة، في الوقت الذي أظهرت فيه مدتا الإسالة 15 و30 دقيقة اعلى ($P\leq 0.002$) نسبة للحركة الفردية

للنطف لدى مجموعة الكاتليز . وبشكل مماثل، فقد اظهرت النتائج اعلى نسبة للنطف الحية لدى مجموعة الكاتليز مقارنةً بمجموعة السيطرة، مع تميز المدتان 15 و30 دقيقة بتحقيق افضل نسبة للنطف الحية لكلا المجموعتين. وعلى العكس من الصفتين المذكورتين اعلاه، انعدمت الفروق المعنوية بين المجموعتين في النسبة المئوية للنطف المشوهة. وقد بلغت اقل نسبة للتشوهات عند المدة 15 دقيقة واعلاها لدى المدة 120 دقيقة ولكلا المجموعتين. يمكن الاستنتاج، بان اضافة انزيم الكاتليز الى مخفف ترس ادى الى تحسن كل من النسبة المئوية للحركة الفردية والنطف الحية لثيران الهولشتاين بعد 15 و30 دقيقة من الاسالة. ان هذا سيؤدي بالتأكيد الى تحسن نسبة الخصوبة لدى الابقار الملقحة اصطناعياً بهذا السائل المنوي وبالتالي زيادة العائد الاقتصادي لمربي الأبقار.

Introduction

Bovine semen has been cryopreserved since more than a half century for artificial insemination and nowadays it is being widely used all over the world (1). It is well known that the cryopreservation procedure produced reactive oxygen species (ROS) (2). ROS induced lipid peroxidation (LPO) for sperm membrane, DNA damage and enzyme inactivation which reflected negatively on the decline of sperm motility, viability and fertilizing ability in bull (2, 3, 4). The continuous liberation of ROS from sperm metabolism, abnormal and immature sperms, as well as the output of freezing-thawing processes of semen which is often accompanied by a low concentration of antioxidants in sperm and seminal plasma, causing sperm oxidative stress (5). Moreover, sperm and seminal plasma have low endogenous antioxidants (6). Antioxidants in the semen includes enzymatic and non-enzymatic antioxidants like superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase as well as, vitamin C, E, A and glutathione (5, 6). Catalase is one of the enzymatic antioxidants defense existed in both sperm cytoplasm and seminal plasma, playing an important role in protection of sperm against ROS. Catalase removes or minimizes both intracellular and extracellular H_2O_2 to water and oxygen (7, 8). Adding of catalase to semen extender improved viability and decreased malondialdehyde (MDA) concentrations (9, 10). The post-thaw semen quality is thought to be affected by numerous factors in the cryopreservation procedure such as type of extender, glycerol concentration, packaging method, freezing rate and thawing period (11, 12). A practical thaw for bull spermatozoa, recommended by most AI organizations, is as 35-37˚C water bath for at least 30 seconds (13, 14, 15). It has been shown that an increase in post-thaw viability will result in increased fertility of the semen (16). Very limited trials have been carried out to investigate the influence of thawing period on post-thaw semen characteristics in Holstein (15) and buffalo (12) bulls. However, the interaction between different thawing periods and catalase addition to Tris extender and its effect of post-thaw semen attributes in Holstein bulls was not previously investigated. This prompted us to explore these effects currently.

Material and Methods

- **Animals and semen collection:** Five Holstein bulls of 2.5-3 years old with good quality semen characteristics (>70% forward individual motility and concentrations of at least 1.0×10^9 spermatozoa/ml) were selected to be the semen source. The bulls were clinically proven to be free from any general or genital diseases and were maintained at the Livestock Central Artificial Insemination Department pertaining to the Ministry of Agriculture (Baghdad, Iraq). Ejaculates were collected from the bulls using an artificial vagina at once a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the evaluated samples.

- **Semen processing and groups:** Following one year cryopreservation period, straws were thawed at 37˚C and examined after different thawing time (15, 30, 60 and 120 minutes). Tris-based extender (24.2 g of Tris, 13.4 g of citric acid, 10 g of fructose, 19.2% v/v egg yolk, 64 ml glycerol and 1000 ml of distilled water at a pH of 6.8) was used experimental extender. The extender with pooled semen were divided into two parts. Catalase (100 IU/ml), Sigma-Aldrich, USA) was added as compared with control group (Tris extender). A drop of semen was placed on a pre-warmed microscope slide and was subjectively assessed at 37˚C for its percentage of individual motility (17). Live sperm percentage was estimated using eosin-nigrosin stain. Following smearing, 200 sperms were counted by 400 × microscope (18). Abnormal spermatozoa were identified following staining with eosin-nigrosine (19) using similar slide for live sperm determination. The percentage head (giant, narrow, pyriform, twin and detached), tail midpiece (Swollen, twin and proximal and distal protoplasmic droplets), tail principal and terminal (bent, coiled, and twin) as well as sperm abnormalities were determined (20).
- **Statistical analyses:** Statistical computations were performed using a general liner model (GLM) procedure in the SAS program (21) to investigate effects the thawing period and addition of catalase to Tris extender on some semen characteristics. The statistical model for analysis of variance was:

$Y_{ijk} = \mu + Ti + P_j + e_{ijk}$

Where:

Y_{ijk} = Dependent variable (individual motility, live sperm percentage and sperm abnormality percentage).

μ = Overall mean.

T_i = Effect of addition (Control and catalase).

P_j = Effect of thawing periods

e_{ijk} = Error term.

Differences among means were computed using the Duncan multiple range test (22)

Results

- **Sperm individual motility:** The catalase group exhibited higher ($P\leq 0.04$-$P\leq 0.002$) sperm individual motility percentage in comparison with the control group during the whole thawing periods (Table 1). Within catalase group, 15 (36.66 ± 4.41 %) and 30 (45.00 ± 5.00 %) minutes post-thawing periods recorded greater ($P \leq 0.002$) individual motility than other periods (Table 1). Concomitantly, higher individual motility was noticed at 15 minutes post-thawing period (18.33 ± 4.41%) as compared with its counterpart periods within control group (Table 1). Furthermore, lesser sperm motility was showed at 120 minutes post-thaw either in catalase (16.66± 1.66 %) or control (5.00 ± 0.00 %) groups (Table 1).

Table (1) Effect of different thawing periods on sperm individual motility percentage of Holstein bulls following adding catalase to Tris extender (Mean ± S.E.)

Thawing period (Minutes) / Groups	15	30	60	120	Level of Significance
Catalase	36.66 ± 4.41a A	45.00 ± 5.00a A	23.33 ± 1.66a B	16.66 ± 1.66a B	P≤ 0.002
Control	18.33 ± 4.41b A	13.33 ± 1.66b AB	11.66 ± 1.66b AB	5.00 ± 0.00b B	P≤ 0.03
Level of Significance	P≤ 0.04	P≤ 0.004	P≤ 0.008	P≤ 0.002	-

Means with capital superscripts within each row indicate comparison among thawing times and small superscripts within each column indicate comparison among groups within each time.

- **Live sperm percentage:** Greater (P<0.02- P<0.0001) live sperm percentage was observed in catalase group in comparison with control one during the whole thawing periods (Table 2). Within catalase group, the 15 and 30 minutes achieved higher (P≤0.05) live sperm percentage (71.63 ± 3.47 and 72.03 ± 6.30 % respectively), as compared with the 120 minutes thawing period (59.06 ± 0.43%) (Table 2). Similarly, the greatest (P≤0.08) live percentage was observed within the control group at 15 (56.33 ± 2.47 %) and 30 (53.33 ± 2.58%) minutes than 60 (47.20 ± 1.13%) and 120 (44.83 ± 0.66%) minutes thawing period (Table 2).

Table (2) Effect of different thawing time on live sperm percentage of Holstein bulls following adding catalase to Tris extender (Mean ± S.E.)

Thawing period (Minutes) / Groups	15	30	60	120	Level of Significance
Catalase	71.63 ± 3.471a A	72.03 ± 6.30a A	62.30 ± 1.13a AB	59.06 ± 0.43a B	P ≤ 0.05
Control	56.33 ± 2.47b A	53.33 ± 2.58b A	47.20 ± 1.13b B	44.83 ± 0.66b B	P ≤ 0.008
Level of Significance	P≤ 0.02	P≤ 0.05	P≤ 0.0007	P≤ 0.0001	

Means with capital superscripts within each row indicate comparison among thawing times and small superscripts within each column indicate comparison among groups within each time.

- **Sperm abnormality percentage:** The differences in sperm abnormality percentage between catalase and control groups during the whole thawing periods lacked significance, however, it tended to be numerically higher in catalase than control group (Table 3). Greater (P≤0.05) sperm abnormality percentage was noticed at 120 minutes thawing period (21.00 ± 2.08%) than those at 15 minutes (12.03 ± 2.27%) within catalase group (Table 3). Concomitantly, similar trend was observed within control group, being higher (P≤0.03) abnormality percentage at 60 (22.76 ± 0.89%) and 120 (24.50 ± 0.55%) minutes as compared with 15 minutes (18.76 ± 1.70%) (Table 3).

Table (3) Effect of different thawing time on sperm abnormality percentage of Holstein bulls following adding catalase to Tris extender (Mean ± S.E.)

Thawing time (Minutes) / Groups	15	30	60	120	Level of Significance
Catalase	12.03 ± 2.27a B	16.53 ± 2,64a AB	18.73 ± 2.08a AB	21.00 ± 2.08a A	P≤ 0.05
Control	18.76 ± 1.70a B	21.46 ± 0.99a AB	22.76 ± 0.89a A	24.50 ± 0.55a A	P≤ 0.03
Level of Significance	NS	NS	NS	NS	

Means with capital superscripts within each row indicate comparison among thawing times and small superscripts within each column indicate comparison among groups within each time. NS= Non-significant.

Discussion

High viability and motility of spermatozoa are important factors for successful artificial insemination, due to the pronounced correlation between post-thawing sperm viability and subsequent conception rate has been documented (23, 24). Motility is one of the most important factors in determining bull sperm because it obtains an indicative information about the sperm cell's energy sources (25). Higher sperm individual

motility of catalase group in comparison with control group is in line with those obtained by (26)(20.8 ± 2.9 vs. 11.6 ± 7.6%) and (10) (44.28 ± 2.76 vs. 21.43 ± 3.03%) who add 200 and 100 IU/ ml catalase to Tris extender in Holstein bulls respectively. These improvements may returns to the antioxidant role of catalase as the first cellular defense against ROS (27). One molecule of catalase has an ability to dissociate two million molecules of H_2O_2 per minute, in addition to its role as NADPH oxidase inhibitor, that may collectively reduce the superoxide O_2^- (28, 29). The catalase activity is dependent on NADPH activity within the sperm plasma membrane, in which the enzyme binds to protect itself from inactivation, consequently increasing its activity (30). On the other hand, these data are disagreed with those reported by Asadpour *et al* (2011) who did not found enhancement of sperm motility after addition of 100 and 200 IU/ml to Tris extender in Holstein bulls. Higher sperm individual motility after 15 and 30 minutes post-thawing period within catalase group explained that slow rate of thawing is beneficial tool to improve Holstein bulls frozen semen characteristics when added catalase to Tris extender. The sperm motility pattern reflects the biochemical environment and physical conditions imposed on bull spermatozoa. These results were contradict with those of (15) who demonstrated that thawing at 37˚C for 30 seconds yielded a higher sperm motility as compared with the other protocols in Holstein bulls. The current data were also disagreed with (12) who recorded recommended 70˚C for 6 seconds thawing rate for buffalo semen to harvest a good sperm motility. Decreasing sperm motility following 60 and 120 minutes may clarifying the negative influence of long thawing rate on sperm DNA integrity and sperm motility consequently (31).

Greater live sperm percentage in catalase as compared with control groups might reflects the role of catalase in the enzymatic metabolism of H_2O_2 and prevent the formation of OH, thus reducing the oxidative stress (32) or improved the plasma membrane integrity and thus increasing survival rate (10). These results were in accordance with those reported by (33), who yielded a good live sperm percentage (86.67 ± 4.41%) of bulls by adding 100 IU/ ml of catalase. The pronounced live sperm percentage following 15 and 30 minutes post-thawing periods currently in catalase group explained that these periods could be safely used to improve survival rate following cryopreservation in Holstein bulls when added catalase enzyme. Declining live sperm percentage after 60 and 120 minutes post-thawing period, explained that leaves straws for a long time may lead to pH fluctuations and consequently protein denaturation and cell death (34).

The lacked significance between catalase and control groups in sperm abnormality percentage confirmed the results of (10) who did not find an obvious effect of adding 100 IU/ml of catalase on total sperm abnormality percentage in Holstein bulls as compared with control group, three months post-cryopreservation (20.60 ± 1.05 vs. 21.65 ± 0.64%). The sperm abnormality percentage was reduced significantly after 15 and 30 minutes post-thawing period as compared with other periods, confirming that these thawing periods are appropriate to prevent sperm DNA damage (35) concomitantly with the protective antioxidant effect of catalase to maintain normal sperm morphology. The higher sperm abnormality percentage at 15 and 30 minutes post-thawing periods in control group (18.76-24.50%) may confirm the protective role of catalase from ROS.

In conclusion, the addition of 100 IU/ml of catalase to Tris extender has improved sperm motility and livability percentages in Holstein bulls following 15 and 30 minutes post-thawing periods.

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