Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Comparative Study of Taurine Extenders on the Cryosurvivability and Fertility in Buffalo Bull Semen.

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Abstract

THE objective of this investigation was to explore the cryosurvivability and in-vivo fertility in buffalo bull semen extended in Tris taurine, Tris taurine BHT and Tris taurine CLC. The basic extender was Tris-citric acid- fructose egg yolk glycerol (TCFYG). Tris extender with zero taurine, zero BHT and zero CLC was kept as a control. The other extenders were Tris containing taurine (50Mm/l), Tris containing taurine and Butylated Hydroxytoluene [BHT] (1Mm BHT+60 Mm/L taurine) and Tris containing Taurine and cholesterol-loaded cyclodextrin [CLC](1mg/ml CLC+60 Mm/L taurine). We exposed the extended semen to the freezing protocol. Taurine supplement to the preservation diluent ameliorated sperm quality as indicated by post freeze-thaw spermatozoal motility, normal acrosome , viability index and significant decrease of percent of premature capacitation. Sperm motility, viability index and sperm cell membrane fluidity (HOST) were obviously enhanced accompanied by the lowest significant percent of premature capacitation upon on adding taurine BHT in the freezing diluent. Significant decrease in post-thawing sperm motility with the advance of time in all the extenders was observed. Non-significant elevation of the total antioxidants (TAC) in TrisTaurine BHT and non-significant increase of malondialdehyde (MDA) in TrisTaurine CLC relative to the control were recorded. The superior conception rate (CR) was recorded in Tris Taurine BHT followed by Tris Taurine and Tris Taurine CLC if compared to the control. It could be concluded that, TrisTaurine BHT is considered the best ameliorating for sperm cryosurvivability and fertility followed by Tris Taurine and Tris Taurine CLC.

Key words: buffalo, freezing, semen, Taurine.

Introduction

Artificial insemination (AI) is the actual principal technological technique for the wide spreading of the desirable super genetic characteristics to enhance the genetic structure of our livestock [1, 2].

The subfertility of bulls used in AI programs is a causative factor for great economic losses especially when the subfertile bulls are genetically superior [3]. The normal capacitation and capability to fertilize the oocytes occurs during the journey of the spermatozoa in the female genital tract after various alterations including rearrangement of the spermatozoal membrane and changes in sperm motility and metabolic activities [4]. Capacitation is promoted in the female reproductive tract by the effect of bicarbonate and calcium ions [5]. The premature capacitation and spontaneous acrosome reaction occurring during cryopreservation is related to protein and lipid changes of the sperm membrane resulting from an uncontrolled influx of calcium ions with consequent lower in-vitro fertilizing capacity [6]. The laboratory evaluation for sperm capacitation is of a great importance for the detection of the normality of spermatozoa after cryopreservation [3].

The freezing process results in the deterioration of approximately fifty percent of the preserved sperms [7], principally from the ice crystals formation inside the sperm cells during the freezing procedures [7, 8]. The freeze-thawing steps cause morphological and physiological damages resulting from the excessive production of oxygen-free radicals [9]. Because of its high unsaturated fatty acid content, the sperm cell membrane is susceptible to lipid peroxidation, which can cause oxidative damage and reduce sperm motility, vitality, and DNA status [10, 11]. So, the composition of the freezing diluent is almost important to reduce such deterioration [12, 11]. The freezing diluent applied for sperm storage of animal species must have

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(Received 04 July 2024, accepted 02 July 2025)
DOI: 10.21608/ejvs.2025.301641.2226

appropriate pH, normal osmolality and buffering capacity to preserve spermatozoa from damage during freezing [13, 14].

Enhancement of sperm freezability in buffaloes is considered a top target, this could be attained by enrichment of the extended semen by antioxidant ingredients [15].

Taurine cryopreservatives were used in the preservative diluents of bull [16-18]; boar [19-21]; ram [22]; goat [23] and dog spermatozoa [24, 25] to ameliorate the sperm value after freeze-thawing. As a sulfonic essential amino acid, taurine can penetrate the sperm membrane and has an antioxidant impact and inhibit fatty acid peroxidation and preserve the cells from the hazard of the oxygen anions [16, 26], interacting with the spermatozoal membrane fatty acids, producing hyperosmotic media, accompanied with intracellular withdrawal of water and so minimizing the extent of sperm cell damage by ice crystallization [27, 28]. The impact of semen diluent supplements like taurine on sperm characteristics in frozen buffalo semen has not been adequately discussed. So, the current investigation was implemented to explore the impact of these supplements on sperm characteristics.

Ameliorating the post freeze-thawing sperm quality in bulls could be achieved through the application of BHT as it is the synthetic analogue of vitamin E [29]; in goat [30]; in boars [31] and rams [32].

The cholesterol / phospholipids ratio of the plasma membrane is a major cause of loss of sperm membrane flexibility and vitality through the freezing process [33, 34]. Cholesterol minimizes the lipid phase transition temperature of the sperm membranes keeping them in a fluid condition at lower temperatures, thus minimizing the membrane injury [35]. Cholesterol added to the extender during the freezing process is incorporated into the sperm membrane improving its fluidity [36, 37]. Cyclodextrins are cyclic hepatosaccharides having a hydrophobic centre [38] and can transport cholesterol into the sperm membrane [39]. cholesterol loaded into cyclodextrin and added to bovine semen before the freezing process ameliorates sperm membrane integrity and sperm motility [37]. So, this investigation aimed at assessing the cryosurvivability and in-vivo fertility in buffalo bull semen extended in Tris taurine, Tris taurine BHT and Tris taurine CLC.

Material and Methods

Semen Gathering and Initial Assessment

Five mature local breed buffalo bulls (weighing between 435 and 500 kg and ages 3-5years) were used to collect semen from the Semen Freezing Center of the General Organization for Veterinary Services. Using a prepared artificial vagina, semen samples were taken once a week for eighteen weeks. The two main parameters used to assess sperm samples were concentration and sperm forward motility. A sufficient amount of semen was obtained by pooling samples of semen with (70%) minimum sperm motility and normal morphological sperm percent, in order to eradicate the bull individual variance. Before being extended, the semen was held in the water bath at 37°C for 10 minutes.

Processing of semen

20% whole egg yolk (TCFY) was added to the basic extender, Tris-citric acid-fructose (TCF), which was adjusted in accordance with de Paz *et al.* [40] and Roof et al. [41]. As a control, Tris extender with zero taurine, zero BHT, and zero CLC was maintained. Tris with taurine (50 Mm/l), Tris with taurine and BHT (1 Mm BHT+60 Mm/L taurine), and Tris with taurine and CLC (1 mg/ml CLC+60 Mm/L taurine) were the other extenders. Supplementary semen specimens were acquired, yielding a neat sperm concentration of 60×10^6/mL. After being gradually chilled to 5°C for around two hours, diluted semen was allowed to equilibrate for an additional two hours. After the 0.25 ml polyvinyl French straws were loaded with semen, they were placed horizontally on a designated rack and submerged in liquid nitrogen right away after being exposed to vapour for ten minutes at a height of four centimetres on top of the surface [1].

Assessment of Sperm Criteria

After the buffalo bull spermatozoa were refrigerated and frozen, the assessment was carried out. For 60 seconds, the frozen straws were thawed at 37°C. Sperm motility, liveability, morphological anomalies, sperm membrane integrity, and acrosome integrity were the traits that were investigated.

Progressive motility

was measured by adding a 2.9% sodium citrate dehydrate solution to a drop of diluted semen that had been slightly heated beforehand. The drop was placed on a glass slide, covered with a sanitized cover slip, and viewed at 400X magnification. A minimum of 200 spermatozoa from various microscopic fields were investigated. A defined range of 0 to 100% was used to calculate sperm motility [42].

Abnormality and live sperm cell percentage

Eosin-nigrosine stain was used in fragile smears at 400X magnification to assess the percentage of live sperm cells. At least 200 sperm were counted in five microscopic areas, and morphologically aberrant spermatozoa were calculated in the same smears [43].

Test for hypo-osmotic swelling (HOST)

By dissolving 6.25 grams of sodium citrate dihydrate and 11.25 grams of fructose in 1000 millilitres of distilled water, the hypo-osmotic solution (125 mOsm/1) was established. After gradually combining 10µl of semen with 1 ml of solution, the mixture was incubated at 37°C for 60 minutes. Following incubation, a single drop of the semen-containing solution was placed on a glass slide, covered with a cover slip, and seen at 400X magnification. Two hundred sperms in all were determined; it was estimated [44] what percentage of spermatozoa were positive for HOST (had a swollen or curled tail).

Acrosome morphology

Trypan blue/Giemsa staining was used to analyse semen samples with minimal changes [45]. Trypan blue staining was used at a concentration of 0.27%. Two smears were made using a single droplet of diluted semen and one drop (5 µl) of Trypan blue combined on a slide. Slides were allowed to air dry in a vertical position before being fixed for 30 minutes at 370C in 10% buffered formol saline (9 gm NaCl, 6.5 gm Na2H PO4, and 4 gm NAH2PO4). Slides were placed in jars with the Giemsa solution and kept there for the night. Fresh Giemsa staining solution was prepared by mixing distilled water with 14.3% (v/v) Giemsa stock solution (3 ml Giemsa+2ml phosphate buffer+45 ml distilled water (Sigma GS-500). Slides were cover-slipped, allowed to air dry in a vertical position, and then rinsed one more in distilled water.

Viability index

The post- thawing viability indices were computed as recorded by Milovanov [46]and computed as half of the post-thawing sperm forward motility plus the sum of sperm motilities post first, second-, and third-hours post- thawing.

In vivo conception rate (CR)

Buffalo females (number=227) were inseminated artificially with Tris Taurine-supplemented frozen post-thawed semen. The conception rate was recorded through rectal palpation after two months of artificial insemination. The inseminated animals were used through the cooperation with Beni-Suef Governorate. The insemination pistol was used to artificially inseminate females, depositing semen inside the uterus. Two months after insemination, the females who had been inseminated were checked by rectal palpation. CR was computed using the formula:

CR =

Statistical analysis

The computer application SPSS [47] version 14.0 was used to compute statistical analysis data and perform an analysis of variance (ANOVA) for the various criteria comparing the control and experimental replications. The Duncan test was used to calculate the significant difference between means at $P<0.05$.

Results

Table (1) explored that, taurine supplement to the freezing diluent ameliorated sperm quality as indicated by post freeze- thaw sperm forward motility, acrosome normality and viability index with significant decrease of percent of premature capacitation. Sperm motility, viability index, sperm membrane flexibility (HOST) enhanced by taurine BHT after freezing accompanied by the lowest significant percent of premature capacitation.

Table (2) exhibited significant decrease in post-thawing sperm motility with the advance of time in all the extenders used.

Table (3) showed non-significant elevation of the total antioxidants (TAC) in Tris

Taurine BHT and non- significant increase of malondialdehyde (MDA) in Tris Taurine CLC relative to the control.

Table (4) showed the superior conception rate (CR) in Tris Taurine BHT (66%) followed by Tris Taurine (65.5%) and Tris Taurine CLC (61.2%) if compared to the control (47.3%).

Discussion

Multiple antioxidant biochemicals exist in the sperm cells and seminal fluid mainly the enzymes having antioxidant effects (CAT, SOD, GSH). Their antioxidation activity is inadequate and stepwise decreases with the advance of the freeze-thawing procedures, therefore, antioxidation additives have to be applied in the semen diluents [48]. Taurine has a positive act in increasing CAT levels and thus improving the antioxidant status [22].

In the present study, the addition of taurine in the preservative diluents upregulated sperm quality that was manifested among the after freeze-thaw sperm forward motility, spermatozoal membrane status, acrosomal integrity, and liveability. The present findings are compatible with those of Reddy *et al.* [49] who documented enhanced sperm value by the impact of taurine.

The conception rate was the best in Tris Taurine BHT followed by Tris Taurine and Tris Taurine CLC and these finding is consistent with the best spermatozoal motility in these extenders, the higher sperm resistance as indicated by the higher viability index, the lowest percent of premature capacitation with the higher post freezing total antioxidant levels.

These results are consistent with those of Bucak *et al.* [22] in the semen of bovines and with the findings recorded by Mahmoud *et al.* [50] who explored that motility is a strong marker for the sperm criteria, and added that clear relations were

obvious between motility and each of the membrane integrity and spermatozoal anomalies. In this regard, Li *et al.* [51] documented a significant correlation between sperm motility and IVF. Vale [52] established a conception rate of more than fifty percent as a satisfactory finding post AI with frozen-thawed buffalo spermatozoa. Al Naib *et al.* [53], classified bulls with a conception rate of nearly fifty percent as having high fertilizing potential, and the sperms of super-fertility animals are capable of going through the artificial mucus with promoted efficiency to fertilize oocytes in vitro.

Thawing is more deleterious than freezing, where the spermatozoa are exposed to hypotonic medium [53]. Swelling of sperm due to water uptake upon a hypotonic osmotic stress is more hazardous than shrinkage upon hypertonic osmotic stress, particularly post- cryopreservation due to the increased levels of reactive oxygen species. Shibahara *et al.* [54] stated a significant correlation between viability index and sperm motility in human. So, the preservation of spermatozoal motility during thawing is positively correlated to the viability index which is considered as a good indicator for sperm resistance.

Our results were similar to that obtained in buffalo [55, 48]; ram [56]; rabbits [22] and boar spermatozoa [21]. These results are compatible with Shoae and Zamiri [29] who observed enhanced post-freeze-thawing sperm value in bulls upon using the concentration 0.5 to 1 Mm BHT and that over concentrations had deleterious effects.

The freeze- thaw process led to excessive production of oxygen free anions causing oxidative sperm stress leading to inferior motility, membrane integrity and fertilizing capacity [57].

Multiple research explored the enhancing impact of taurine on post freeze-thaw sperm quality [22, 17]. During the cryopreservation process, taurine, a sulfuric amino acid, protects sperm from oxidative damage by acting as a non-enzymatic scavenger of oxygen free radicals with consequent reduction of sperm stress [17, 58].

The Taurine supplement may induce cryoprotective after-freezing output on the physiological function of mitochondria and acrosome with consequent energy production from the ATP intracellular enzyme leading to improved spermatozoal motility. The assessment of vitality, acrosomal and sperm membrane integrities have to be involved as motility only is inadequate for sperm evaluation after freezing.

Cholesterol/phospholipid ratio is the principal factor influencing sperm membrane fluidity and integrity, especially during cryopreservation and taurine lowers the cholesterol efflux action from the sperm membrane with consequent conservation of its integrity [33, 34].

The inclusion of Butylated Hydroxytoluene antioxidant fraction in semen diluents of buffaloes enhanced after the freeze-thawing sperm value [55]. Also, this improvement was recorded in rams [59]; goats [42] and canines [60]. Patel et al. [61] recorded that, supplementary Butylated Hydroxytoluene has promoted the value of Hariana bull semen post extension, cooling, and freeze-thawing with a prominent amelioration in the sperm value in all these steps with the enrichment of 0.5 mM and 1 mM BHT.

The enhanced after freeze-thawing sperm quality in the current investigation is due to the antioxidant potential of BHT [62-64, 7], the permeation into the cell membrane improving its integrity [29]. As a phenolic antioxidant chemical Butylated hydroxytoluene when added to the semen diluent lowers the spermatozoal membrane fluidity deterioration through the freezing process [60]. BHT has an antioxidant effect by reducing lipid peroxidation [29, 65]. Butylated hydroxytoluene is the Vitamin E synthetic analogue, and is beneficial in oxidation reactions via transforming the anion radicals to water molecules [66]. BHT also has a strong antiviral impact and has been involved in the inactivation of lipid-containing viruses [67]. Our results are opposite to the findings recorded by Ball *et al.* [68] who found a hazardous impact of Butylated hydroxytoluene in stallion sperm storage. This contradictory finding can be related to susceptibility species differences.

The results of the current investigation come along with those recorded by Rajoriya *et al.* [69] who postulated that buffalo bull spermatozoa incubated with CLC (3mg for120×10^6) sperm before the freezing process improved sperm cryosurvival. Moore *et al.* [70] reported an improved percentage of post-thaw viability and an increased number of sperm cells that come in contact with zona pellucid in CLC enrichment to sperm freezing in equines.

The present improvement in sperm quality post-freezing due to the addition of CLC to the extender before freezing may be due to the protection of the sperm membrane from damage that occurs during cryopreservation [70]. Cholesterol added to the bull or equine semen prefreezing is incorporated into the sperm membrane preserving its fluidity and integrity [36, 37].

The total cholesterol fraction of the sperm which promotes maximum freezability, is 2-3 times more than the control sperm and when cholesterol elevated to four to five times than that in the control, it becomes hazardous to the sperm cells [37]. It could be concluded that, Tris Taurine BHT is considered the best ameliorating for sperm cryosurvivability and

fertility followed by Tris Taurine and Tris Taurine CLC.

Acknowledgement

The authors are extremely grateful to the National Research Centre and the Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture, Abbasia, Egypt, for sponsoring and supporting this work. This work was supported by the National Research Centre.

Conflict of interest

No competing interests

Ethical Approval

The experimental plan was approved by the Medical Research Ethics Committee of the National Research Centre, Dokki, Egypt and its registration number is 19/104 and its date is 10/10/2019.

TABLE 1. Comparative study of Tris taurine , Tris taurine BHT and Tris taurine CLC Extenders on frozen post-thawed extended buffalo bull Semen (Mean±SE).

Diluent	Motility	Alive	Abnormalities	Host	Acrosome	Viability index	Capacitation
Control (Tris extender)	35.40±7.90^{a}	80.40±.400^{a}	10.20±.20^{c}	57.60±.21^{a}	85.00±1.58^{ab}	82.56±0.57^{a}	22.67±1.20^{c}
Tris Taurine (50Mmol/L)	63.00±1.22^{b}	91.20±.800^{b}	6.6000±.40^{a}	58.80±1.77^{a}	88.00±.94^{b}	109.50±1.04^{d}	18.33±.88^{b}
Tris Taurine BHT (1MmBHT+60Mmol/Ltaurine)	62.00±1.22^{b}	82.60±1.24^{a}	10.8000±.48^{c}	72.30±.70^{c}	84.00±.83^{a}	106.33±0.88^{c}	7.33±.67^{a}
Tris Taurine CLC (1mg/ml+60Mmol/Ltaurine)	57.60±1.12^{b}	89.60±1.28^{b}	8.2000±.200^{b}	63.14±.96^{b}	83.80±1.35^{a}	98.25±0.25^{b}	19.50±.50b
p-value	0.0001	0.0001	0.0001	0.000	.094 (NS)	0.000	0.000

Means different superscripts (a, b, c, d) within column differ at P<0.05, non-significant (NS). Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG).

TABLE 2. Comparative study of Tris taurine , Tris taurine BHT and Tris taurine CLC Extenders on post frozen-thawed diluted buffalo bull Semen (Mean±SE).

Hours	Control (Tris extender)	Tris Taurine	Tris Taurine BHT	Tris Taurine CLC	p-value
0	35.40±7.90^{a}	63.00±1.22^{b}	62.00±1.22^{b}	57.60±1.12^{b}	0.0001
1	27.00±1.20^{a}	34.00±6.00^{a}	31.00±5.60^{a}	30.00±3.50^{a}	0.748
2	23.00±1.20^{a}	28.00±3.70^{a}	28.00±3.70^{a}	23.75±2.39^{a}	0.517
3	16.00±2.45^{a}	18.00±3.74^{a}	18.00±3.74^{a}	16.25±3.75^{a}	0.959

Means different superscripts (a, b) within column differ at P<0.05, non significant (NS). Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG).

TABLE 3. Effect of different extenders on Antioxidant concentration-TAC (mM) and MDA concentration (µM)

Diluent	TAC	MDA
Control (tris extender)	0.22±.020	8.60±.06^{ab}
Tris Taurine	0.21±.06	7.15±.15^{a}
Tris Taurine BHT	0.34±.01	8.03±1.04^{ab}
Tris Taurine CLC	0.25±.06	9.72±0.72^{b}
p-value	.197 (NS)	.104 (NS)

(NS) denotes non significant.

TABLE 4. Effect of Tris extender enriched with Taurine, Taurine BHT and Taurine CLC on a field conception rate test in buffalo.

Experimental	Number of artificially inseminated animals	Number of pregnant animals	Conception ratio (CR, %)
Tris Taurine (50Mmol/L)	55	36	65.5 %
Tris Taurine BHT (1MmBHT+60Mmol/Ltaurine)	50	33	66%
Tris Taurine CLC (1mg/ml+60Mmol/Ltaurine)	67	40	61.2%
Control (TCFYG)	55	26	47.3%

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دراسة مقارنة على كفاءة حفظ الحيوانات المنوية بالتجميد ونسبة الخصوبة لطلائق الجاموس باستخدام مخففات التورين

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الملخص

استهدفت الدراسة الحالية تقييم كفاءة حفظ الحيوانات المنوية لطلائق الجاموس بالتجميد ونسبة الخصوبة باستخدام مخففات التورين، تم تخفيف السائل المنوى باستخدام مخفف التريس المحتوى على تركيزات صفر تورين وصفر بيتاهيدروكسى تولوين وصفر كولسترول لوديد سيكلودكسترين (مجموعة الكنترول).واحتوى التريس على تورين 50 مل مول/لتر ،تريس محتوى على 1مل بيتاهيدروكسى تولوين+60مل مول تورين/لتر،تريس محتوى على كولسترول لوديد سيكلودكسترين 1 ملجم/مل+60مل مول تورين/لتر. تم تعريض السائل المنوى المخفف لبرنامج التجميد . تم التقييم .أظهرت النتائج تحسن فى صفات السائل المنوى بعد التجميد والاذابة. وكانت أحسن النتائج وكذلك نسبة العشار مع مجموعة الترس تورين بيتاهيدروكسى تولوين يليها مجموعة التريس تورين ثم الترس تورين كولسترول لوديد سيكلودكسترين مقارنة بالكنترولزالخلاصة أن مجموعة الترس تورين بيتاهيدروكسى تولوين أعطت أحسن النتائج فيما يخص نوعية السائل المنوى وكذلك نسبة العشار.

الكلمات الدالة: تورين، الجاموس، السائل المنوى، تجميد.