# ROLE OF ANTIOXIDANTS CYSTEINE AND TAURINE IN TRIS EGG YOLK BASED EXTENDER FOR CRYOPRESERVATION OF SURTI BUFFALO SEMEN

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Received : 07.04.15 Accepted : 14.11.15

**ABSTRACT**

The study was undertaken on 40 semen ejaculates collected from 5 Surti bulls (8 ejaculates per bull). Ejaculates with >70% initial motility were split into 5 equal fractions and were diluted with standard Tris citrate fructose egg yolk glycerol (TFYG) extender as control and TFYG having 2 additives Cysteine HCl (0.5 & 1.0 mg/ml) and Taurine (4.0 & 6.0 mg/ml) each at 2 levels to study their comparative efficacy for cryopreservation of buffalo spermatozoa. After 12-24 hrs of cryopreservation, each of the 5 split-samples were thawed in water bath at 37°C for 30 seconds and observed for post-thaw motility, viability, morphology, acrosomal integrity and plasma membrane integrity. The post-thaw longevity of sperm at 37°C incubation was also studied for 3 hrs. Highly significant (P<0.01) variations among five extender-additive formulations, viz., control TFYG extender, TFYG + cysteine @ 0.5 mg/ml, TFYG + cysteine @ 1 mg/ml, TFYG + taurine 4 mg/ml and TFYG + taurine 6 mg/ml were observed at pre-freeze stage in sperm motility (65.37±0.84, 68.37±0.81, 72.62±0.69, 73.25±0.81 and 71.12±1.02 %), viability (72.75±0.99, 70.62±0.81, 78.97±0.93, 79.37±0.88 and 76.32±1.14 %), total sperm abnormalities (6.35±0.27, 5.73±0.28, 5.45±0.25, 5.80±0.28 and 6.38±0.29 %), acrosomal integrity (89.70±0.39, 90.58±0.34, 90.90±0.35, 88.53±0.46 and 86.28± 0.45 %) and HOS reactive sperm (70.82±0.92, 74.17± 0.93, 78.12±0.79, 78.08±0.95 and 75.03±1.08 %); and so also in post-thaw sperm motility (38.37±0.95, 39.50±0.80, 46.50± 0.72, 50.00±0.62 and 34.25±0.71 %), viability (44.82±1.02, 46.37±0.99, 52.97±0.79, 55.02±0.80 and 41.07±0.99 %), total sperm abnormalities (9.10±0.22, 8.15±0.26, 7.10±0.26, 7.90±0.29 and 8.90±0.31 %), acrosomal integrity (82.43±0.32, 84.40±0.18, 85.73±0.18, 82.75±0.30 and 79.98±0.35 %) and HOS reactivity (42.52±1.04, 45.10±1.04, 51.62±0.82, 53.73±0.69 and 39.10±0.91 %). At all the stages of cryopreservation process, post-thaw stage and after incubation in particular, semen diluted with TFYG + taurine 4 mg/ml and TFYG + cysteine @ 1 mg/ml showed significantly better preservation of all sperm quality parameters than other extender additives. Thus TFYG + taurine 4 mg/ml or TFYG + cysteine @ 1 mg/ml can be recommended as better extender-additive for cryopreservation of buffalo semen.

**Key Words**: Buffalo semen, Cryopreservation, Semen extender, Antioxidants, Cysteine HCl, Taurine, Sperm quality

## INTRODUCTION

The discovery of biological role of amino acids in prevention of cell damage during freezing stress has come from the observation that a variety of plants are able to accumulate the amino acid proline in response to cold temperature and it has also been reported that some amino acids protect several type of animal cells against freezing stress including sperm (El-Sheshtawy *et al.*, 2008). Buffalo semen is known for its relatively poor freezability in comparison with cattle semen (Shelke and Dhami, 2001). Many attempts have been made to improve the basic buffers developed in the early 1950s by inclusion of additives such as vitamins, amino acids, chelating agents, enzymes, antioxidants, metabolic

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stimulants and others. Seminal plasma of domestic animals contains mainly water soluble vitamins C and B (Sansone *et al*., 2000). Although bovine semen has natural defense system against the oxidative stress, it is considered insufficient under cryopreservation-mediated stress. Hence, reinforcement of semen extender with suitable antioxidant is suggested to reduce oxidative damage during freeze-thawing of bull and buffalo spermatozoa (Ansari *et al*., 2011[a,b]).

Cysteine, a precursor of intracellular glutathione, has been shown to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both *in vivo* and *in vitro* and protecting the membrane lipids and proteins due to indirect radical scavenging properties. Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post-thawed sperm motility in many species (Memon *et.al*., 2011). In recent years, taurine has been used as antioxidant in semen extenders and in the cryopreservation of boar, bull, human, ram, and goat sperm to improve motility, viability, membrane integrity, and fertility of spermatozoa (Perumal *et al.,* 2013). Cysteine 1 mg/ml and taurine 4 mg/ml separately in Tris extender were found to be significantly beneficial in improving keeping quality of buffalo semen at 5°C storage (Orin *et al.,* 2015). Keeping these facts in view, the present study was aimed to find the suitability of amino acids cysteine and taurine in tris egg yolk extender for cryopreservation of buffalo semen.

## MATERIALS AND METHODS

The study was undertaken on five healthy Surti buffalo bulls, aged 4 to 6 years, during the favourable breeding season at Central Sperm Station of the College of Veterinary Science & Animal Husbandry, Anand, Gujarat (India). All these bulls were maintained under standardized managemental conditions and were in regular twice a week semen collection schedule using artificial vagina. Eight ejaculates with >70% initial motility were studied from each bull (total 8 x 5 = 40 ejaculates) at weekly interval following split-sample technique.

Just before semen collection, tris citrate fructose yolk glycerol (TFYG) extender with strepto-penicillin was prepared as per Davis *et al.* (1963) formula. Out of the 100 ml standard TFYG extender, 5 aliquots of 20 ml each were made. Cysteine powder @ 10 mg, and 20 mg was added in two different cylinders containing 20 ml TFYG extender to give final concentration of 0.5 and 1.0 mg/ml, respectively. Similarly, taurine was added @ 80 mg and 120 mg to the 20 ml extender to give final concentration of 4.0 and 6.0 mg/ml, respectively. The fifth aliquot of 20 ml TFYG was kept as control. Finally, prepared extenders were stirred with magnetic stirrer and kept in thermo-regulatory water bath at 34°C until used for extension.

Immediately after collection the ejaculates were placed in water bath at 34°C and evaluated for semen quality. Sperm concentration and dilution rate of semen were estimated on Hamilton dilutor (Accucell, IMV, France). The accepted ejaculates were immediately diluted with different extender-additive preparations @ 100 million sperm/ml and evaluated for sperm quality parameters such as motility, viability and morphology (eosin-nigrosin stain), acrosomal abnormalities (Giemsa stain) and plasma membrane integrity (HOS test, with hypo-osmotic media having 150 mOsm/l osmolality) as per standard procedures.

The French mini straws (at least 10 Nos) were filled with each extended aliquot using automatic filling and sealing machine (IS4 System, IMV Technologies, France). Details regarding bull number, name and level of additive used, date etc. were printed using jet printer (Domino printer) on the filled straws for identification. The straws were racked and equilibrated at 5°C temperature for 4 h in cold handling cabinet (IMV Technologies, France). After equilibration, the samples were evaluated for pre-freeze sperm motility, viability, morphology, acrosome integrity and plasma membrane integrity. Freezing of the straws of all split-samples was carried out in a programmable bio-freezer (Digitcool 5300 CE ZH 350, IMV Technologies, France) using a previously tested freezing curve for bovine semen (from 4°C to -10°C at 5°C/min; from -10°C to -100°C at 40°C /min; and from -100°C to

-140°C at 20°C/min) and holding the straws in $LN_2$ vapour for 8-9 minutes in the freezer. The straws were then plunged into liquid nitrogen (-196°C) for storage. After 18-24 h, thawing of semen was done in water bath at 37°C for 30 sec and evaluated for semen quality. The thawed samples were also taken into sugar tubes and incubated at 37°C temperature in water bath for another 3 h to study the post-thaw longevity of spermatozoa.

The values of all sperm quality parameters were expressed as percentages of total sperm examined at each stage of processing and were analyzed statistically using ANOVA and Duncan's NMRT by employing online SPSS software version 20.00 to know the difference between treatments at various steps of cryopreservation.

## RESULTS AND DISCUSSION

Our results regarding the effect of antioxidant cysteine and taurine in tris based extender for cryopreservation of Surti buffalo semen evaluated at initial, pre-freeze and post-thaw stages in term of sperm quality parameters such as motility, viability, morphology, acrosomal integrity and plasma membrane integrity are presented in Tables 1-4.

In all the five treatments, all the sperm quality parameters studied declined gradually and significantly (P<0.01) at each step of cryopreservation, and they also varied significantly (P<0.01) between treatments. Spermatozoa in TFYG extender with taurine 4 mg/ml and cysteine 1 mg/ml showed significantly higher progressive motility than other levels, and the pattern was mostly similar at each stage of cryopreservation. Taurine @ 6 mg/ ml concentration in TFYG extender was found to have the worst effect on all sperm parameters, particularly sperm motility and acrosome integrity, which deteriorated greatly after cryopreservation. The proportion of live sperm followed the trend of motile sperm at each stage of cryopreservation and the values in extender having cysteine 1 mg/ml and taurine 4 mg/ml were significantly (P<0.01) higher than with other the extenders (Table 1).

Spermatozoa with intact acrosome were more in extender containing cysteine 1 mg/ml and least in extender containing taurine at 6 mg/ml at all stages of cryopreservation. The percentage of HOS reactive spermatozoa followed the trend similar to that of intact acrosome in all treatments at all stages of cryopreservation process. The highest HOS reactive sperm percentages were observed in TFYG having taurine 4 mg/ml and cysteine 1 mg/ml, while other levels of antioxidants showed statistically lower values at all stages of processing and freezing (Table 1).

Further, TFYG extender having 1.0 and 0.5 mg/ml cysteine and 4 mg/ml taurine showed lesser segment wise as well as overall sperm abnormalities and different forms of acrosomal defects than other treatments and control, particularly at post-thaw stage. The total sperm and acrosome abnormalities were also lowest in TFYG extender with inclusion of cysteine @ 1 mg/ml and the highest in TFYG with inclusion of taurine @ 6 mg/ml concentration, statistically similar to control TFYG extender (Table 2 & 3).

The post-thaw incubation survival was significantly (P<0.05) better in TFYG extender with taurine @ 4 mg/ml, followed by TFYG with cysteine @ 1 mg/ml and cysteine @ 0.5 mg/ml as compared to control TFYG extender at all the intervals of incubation study (Table 4). However, taurine at 6 mg/ml concentration in TFYG diluent was found to have the most adverse effect on post-thaw survival of buffalo spermatozoa as the post-thaw motility was the lowest among all combinations and it could sustain good motility only up to 1 hr of incubation. The results in general show that TFYG extender with taurine 4 mg/ml and TFYG with cysteine 1 mg/ml could sustain better sperm survival at least for 2 hrs after thawing.

The present findings on the beneficial effect of cysteine @ 1 mg/ml in tris based extender in freezing of semen is consistent with the earlier reports (Uysal *et al.,* 2007). Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post-thawed sperm motility. It has been proved that thiols such as glutathione and cysteine prevented the loss of sperm motility in frozen thawed bull semen. Cysteine has been shown to prevent the loss in motility of freeze-thawed bull semen (Ansari *et al.*, 2011[a]). Study of Ansari *et al.* (2011[a,b]) also proved

that supplementation of cysteine @ 0.5 mM was significantly better and levels of 1.0, 2.0 and 3.0 mM in extender deteriorated the post-thaw quality of bull and buffalo semen. The same trend was also noted in our pilot trials (Orin *et al.*, 2015) with 1.5 mg/ml cysteine and 8 mg/ml taurine, compared to the two levels each used in this study.

Our observation of higher acrosomal integrity in tris extender with inclusion of cysteine as additive compared to control or taurine is in agreement with the findings of Kumar and Atreja (2012). They reported significantly higher post-thawed percentage of intact acrosome (80.60 ± 0.70 %) in cysteine added Tris diluent for buffalo semen. Raval and Dhami (2010) also reported higher levels of intact acrosomes in crossbred bull semen cryopreserved in TFYG extender having cysteine 1 mg/ml than control Tris extender.

The present findings of taurine compared with the observations of Ae Oh *et al.* (2012), who evaluated the effect of adding taurine to a tris based egg yolk extender on Korean Jeju Black bull sperm quality following cryopreservation. The addition of taurine significantly improved ($p<0.05$) the motility and viability of spermatozoa compared to control extender. Moreover, membrane integrity to swollen sperm ratio was also significantly increased ($p<0.05$) in presence of taurine compared to control extender in KJ black bulls semen. Kishore *et al.* (2011) had shown that semen fortification with taurine 20 mM resulted in significant and better quality preservation of most of the sperm parameters of bull semen, but higher levels were detrimental. Our findings are in accordance with their observations that supplementation of taurine at certain level (4 mg/ml) resulted in significantly better semen quality parameters after cryopreservation. Taurine is an intracellular amino acid found in majority of the mammalian tissues and plays its role in cell proliferation, viability, osmo-regulation and prevents injuries induced by oxidants in many tissues. It also maintains the stability of bio-membranes, scavenges ROS, minimizes the end products of lipid peroxidation, modulates $Ca^{2+}$ uptake and inhibits protein phosphorylation (Kumar and Atreja, 2012). Hence, the improved semen preservability observed after addition of taurine at lower level in semen extender can be attributed to these functions of taurine, which is mainly through scavenging of reactive oxygen species.

## CONCLUSIONS

The inclusion of taurine @ 4 mg/ml or cysteine @ 1 mg/ml in tris citrate fructose yolk glycerol (TFYG) extender significantly ($P<0.01$) enhanced progressive sperm motility, viability, and membrane integrity with reduced sperm/acrosome abnormalities at all stages of cryopreservation of buffalo semen including acceptable post-thaw longevity of sperm up to at least 2 hrs when compared with other levels and control. Therefore, taurine @ 4 mg/ml or cysteine @ 1 mg/ml in TFYG extender can be recommended as a suitable additive for improvement in cryopreservation of buffalo sperm. However *in vivo* fertility trials need to be carried out to validate the recommendation.

**TABLE 1: MEAN (±SE) PERCENTAGES OF PROGRESSIVELY MOTILE, LIVE, INTACT ACROSOME AND HOS REACTIVE SPERMATOZOA IN SURTI BUFFALO SEMEN AT DIFFERENT STAGES OF CRYOPRESERVATION IN CONTROL AND TREATMENT GROUPS**

| Freezing Stage | Extender-additives | Progressive motility % | Live sperm % | Intact acrosome % | HOS reactive sperm % |
|---|---|---|---|---|---|
| On dilution | TFYG Control | 75.12$^{a}$±0.77 | 82.75$^{a}$±0.76 | 92.85$^{b}$±0.34 | 82.72$^{a}$±0.91 |
| | Cysteine 0.5 mg/ml | 77.75$^{b}$±0.69 | 84.27$^{ab}$±0.66 | 93.08$^{b}$±0.32 | 83.40$^{a}$±0.70 |
| | Cysteine 1 mg/ml | 80.62$^{c}$±0.65 | 86.00$^{b}$±0.49 | 93.28$^{b}$±0.33 | 85.85$^{b}$±0.67 |
| | Taurine 4 mg/ml | 81.75$^{c}$±0.79 | 86.17$^{b}$±0.65 | 92.23$^{b}$±0.39 | 86.25$^{b}$±0.64 |
| | Taurine 6 mg/ml | 81.00$^{c}$±0.82 | 84.80$^{b}$±0.83 | 90.55$^{a}$±0.44 | 85.63$^{b}$±0.86 |
| | Pooled | 79.25$^{r}$±0.37 | 84.80$^{r}$±0.32 | 92.40$^{r}$±0.18 | 84.77$^{r}$±0.35 |
| Pre- freeze | TFYG Control | 65.37$^{a}$±0.84 | 72.75$^{a}$±0.99 | 89.70$^{c}$±0.39 | 70.82$^{a}$±0.92 |
| | Cysteine 0.5 mg/ml | 68.37$^{b}$±0.81 | 70.62$^{b}$±0.81 | 90.58$^{d}$±0.34 | 74.17$^{b}$±0.93 |
| | Cysteine 1 mg/ml | 72.62$^{c}$±0.69 | 78.97$^{cd}$±0.93 | 90.90$^{d}$±0.35 | 78.12$^{c}$±0.79 |
| | Taurine 4 mg/ml | 73.25$^{c}$±0.81 | 79.37$^{d}$±0.88 | 88.53$^{b}$±0.46 | 78.08$^{c}$±0.95 |
| | Taurine 6 mg/ml | 71.12$^{c}$±1.02 | 76.32$^{bc}$±1.14 | 86.28$^{a}$±0.45 | 75.03$^{b}$±1.08 |
| | Pooled | 70.15$^{q}$±0.43 | 76.61$^{q}$±0.45 | 89.20$^{q}$±0.21 | 75.25$^{q}$±0.46 |
| Post- thaw | TFYG Control | 38.37$^{b}$±0.95 | 44.82$^{b}$±1.02 | 82.43$^{b}$±0.32 | 42.52$^{b}$±1.04 |
| | Cysteine 0.5 mg/ml | 39.50$^{b}$±0.80 | 46.37$^{b}$±0.99 | 84.40$^{c}$±0.18 | 45.10c±1.04 |
| | Cysteine 1 mg/ml | 46.50$^{c}$±0.72 | 52.97$^{c}$±0.79 | 85.73$^{d}$±0.18 | 51.62$^{b}$±0.82 |
| | Taurine 4 mg/ml | 50.00$^{d}$±0.62 | 55.02$^{c}$±0.80 | 82.75$^{b}$±0.30 | 53.73$^{d}$±0.69 |
| | Taurine 6 mg/ml | 34.25$^{a}$±0.71 | 41.07$^{a}$±0.99 | 79.98$^{a}$±0.35 | 39.10$^{a}$±0.91 |
| | Pooled | 41.72$^{p}$±0.53 | 48.05$^{p}$±0.55 | 83.06$^{p}$±0.18 | 46.42$^{p}$±0.56 |

TFYG=Tris fructose yolk glycerol; Means bearing different superscripts between additives (a,b,c,d,e) and between stages of cryopreservation (p,q,r) differ significantly (P<0.05).

**TABLE 2: MEAN (±SE) PERCENTAGES OF SEGMENT WISE AND TOTAL SPERM ABNORMALITIES IN SURTI BUFFALO BULL SEMEN AT DIFFERENT STAGES OF CRYOPRESERVATION IN CONTROL AND TREATMENT GROUPS**

| Freezing Stage | Extender-additives | Sperm segments | | | Overall |
|---|---|---|---|---|---|
| | | Head | Midpiece | Tail | |
| On dilution | TFYG Control | 1.20±0.09 | 1.60±0.10 | 2.13±0.19 | 4.93±0.25 |
| | Cysteine 0.5 mg/ml | 1.20±0.09 | 1.55±0.10 | 1.90±0.19 | 4.68±0.26 |
| | Cysteine 1 mg/ml | 1.15±0.09 | 1.43±0.09 | 1.95±0.19 | 4.53±0..25 |
| | Taurine 4 mg/ml | 1.18±0.09 | 1.48±0.11 | 1.95±0.19 | 4.60±0.24 |
| | Taurine 6 mg/ml | 1.20±0.07 | 1.63±0.09 | 2.10±0.17 | 4.93±0.20 |
| | Pooled | 1.19$^{q}$±0.04 | 1.54$^{p}$±0.04 | 2.01$^{p}$±0.08 | 4.73$^{p}$±0.11 |
| Pre-freeze | TFYG Control | 1.50$^{b}$±0.11 | 1.85±0.10 | 3.00$^{b}$±0.19 | 6.35$^{b}$±0.27 |
| | Cysteine 0.5 mg/ml | 1.20$^{a}$±0.09 | 1.90±0.11 | 2.60$^{ab}$±0.21 | 5.73$^{a}$±0.28 |
| | Cysteine 1 mg/ml | 1.30$^{ab}$±0.08 | 1.83±0.09 | 2.33$^{a}$±0.18 | 5.45$^{a}$±0.25 |
| | Taurine 4 mg/ml | 1.28$^{ab}$±0.09 | 1.83±0.12 | 2.70$^{ab}$±0.21 | 5.80$^{a}$±0.28 |
| | Taurine 6 mg/ml | 1.38$^{ab}$±0.11 | 2.13±0.11 | 2.88$^{ab}$±0.19 | 6.38$^{b}$±0.29 |
| | Pooled | 1.33$^{q}$±0.04 | 1.99$^{q}$±0.05 | 2.70$^{q}$±0.09 | 5.94$^{q}$±0.12 |
| Post-thaw | TFYG Control | 1.73±0.09 | 2.88$^{c}$±0.14 | 4.50$^{c}$±0.17 | 9.10$^{c}$±0.22 |
| | Cysteine 0.5 mg/ml | 1.55±0.09 | 2.60$^{bc}$±0.12 | 4.00$^{bc}$±0.18 | 8.15$^{b}$±0.26 |
| | Cysteine 1 mg/ml | 1.50±0.09 | 2.30$^{a}$±0.12 | 3.40$^{a}$±0.17 | 7.10$^{a}$±0.26 |
| | Taurine 4 mg/ml | 1.58±0.09 | 2.45$^{ab}$±0.11 | 3.88$^{ab}$±0.21 | 7.90$^{b}$±0.29 |
| | Taurine 6 mg/ml | 1.75±0.11 | 2.75$^{bc}$±0.14 | 4.35$^{bc}$±0.20 | 8.90$^{c}$±0.31 |
| | Pooled | 1.62$^{r}$±0.04 | 2.58$^{r}$±0.06 | 4.03$^{r}$±0.09 | 8.23$^{r}$±0.13 |

TFYG=Tris fructose yolk glycerol; Means bearing different superscripts between additives (a,b,c,d,e) and between stages of cryopreservation (p,q,r) differ significantly (P<0.05).

**TABLE 3: MEAN (± SE) PERCENTAGES OF VARIOUS TYPES OF SPERM ACROSOMAL ABNORMALITIES IN SURTI BUFFALO BULL SEMEN AT DIFFERENT STAGES OF CRYOPRESERVATION IN CONTROL AND TREATMENT GROUPS**

| Freezing Stage | Extender-additives | Sperm Acrosomal Abnormalities | | | | Overall |
|---|---|---|---|---|---|---|
| | | Swollen | Ruffled | Detached | Denuded | |
| On dilution | TFYG Control | $3.03^{a}\pm0.015$ | $2.18^{a}\pm0.12$ | $1.33^{ab}\pm0.08$ | $0.68^{a}\pm0.08$ | $7.15^{a}\pm0.34$ |
| | Cysteine 0.5 mg/ml | $2.95^{a}\pm0.16$ | $2.15^{a}\pm0.11$ | $1.18^{a}\pm0.07$ | $0.70^{a}\pm0.09$ | $6.93^{a}\pm0.32$ |
| | Cysteine 1 mg/ml | $2.88^{a}\pm0.16$ | $2.03\pm0.12$ | $1.18^{a}\pm0.107$ | $0.65^{a}\pm0.08$ | $6.73^{a}\pm0.33$ |
| | Taurine 4 mg/ml | $3.20^{a}\pm0.17$ | $2.30^{a}\pm0.13$ | $1.45^{bc}\pm0.09$ | $0.80^{b}\pm0.08$ | $7.78^{a}\pm0.39$ |
| | Taurine 6 mg/ml | $3.83^{b}\pm0.18$ | $2.85^{b}\pm0.16$ | $1.68^{c}\pm0.10$ | $0.98^{b}\pm0.10$ | $9.33^{b}\pm0.45$ |
| | Pooled | $3.18^{p}\pm0.08$ | $2.30^{p}\pm0.06$ | $1.36^{p}\pm0.04$ | $0.76^{p}\pm0.04$ | $7.58^{p}\pm0.18$ |
| Pre-Freeze | TFYG Control | $4.40^{ab}\pm0.18$ | $3.10^{ab}\pm0.15$ | $1.78^{ab}\pm0.09$ | $1.03^{ab}\pm0.08$ | $10.30^{b}\pm0.39$ |
| | Cysteine 0.5 mg/ml | $3.95^{a}\pm0.15$ | $2.90^{a}\pm0.13$ | $1.63^{a}\pm0.08$ | $0.98^{ab}\pm0.06$ | $9.40^{a}\pm0.35$ |
| | Cysteine 1 mg/ml | $3.93^{a}\pm0.16$ | $2.75^{a}\pm0.14$ | $1.53^{a}\pm0.10$ | $0.90^{a}\pm0.07$ | $9.10^{a}\pm0.35$ |
| | Taurine 4 mg/ml | $4.80^{b}\pm0.21$ | $3.48^{b}\pm0.17$ | $1.98^{b}\pm0.09$ | $1.23^{bc}\pm0.12$ | $11.48^{c}\pm0.46$ |
| | Taurine 6 mg/ml | $5.65^{c}\pm0.19$ | $4.28^{c}\pm0.16$ | $2.35^{c}\pm0.12$ | $1.43^{c}\pm0.09$ | $13.73^{d}\pm0.45$ |
| | Pooled | $4.55^{q}\pm0.09$ | $3.30^{q}\pm0.08$ | $1.85^{q}\pm0.05$ | $1.11^{q}\pm0.04$ | $10.80^{q}\pm0.21$ |
| Post-thaw | TFYG Control | $6.88^{c}\pm0.14$ | $5.60^{c}\pm0.13$ | $3.03^{b}\pm0.12$ | $2.03^{d}\pm0.12$ | $17.53^{c}\pm0.32$ |
| | Cysteine 0.5 mg/ml | $6.50^{b}\pm0.10$ | $4.80^{b}\pm0.11$ | $2.68^{a}\pm0.10$ | $1.53^{a}\pm0.10$ | $15.60^{ab}\pm0.18$ |
| | Cysteine 1 mg/ml | $5.75^{a}\pm0.09$ | $4.33^{a}\pm0.10$ | $2.55^{a}\pm0.09$ | $1.68^{a}\pm0.08$ | $14.28^{a}\pm0.18$ |
| | Taurine 4 mg/ml | $6.75^{bc}\pm0.15$ | $5.28^{c}\pm0.016$ | $3.03^{b}\pm0.12$ | $2.10^{bc}\pm0.10$ | $17.25^{c}\pm0.30$ |
| | Taurine 6 mg/ml | $7.75^{d}\pm0.15$ | $6.23^{d}\pm0.14$ | $3.68^{c}\pm0.14$ | $2.38^{c}\pm0.09$ | $20.03^{d}\pm0.35$ |
| | Pooled | $6.73^{r}\pm0.07$ | $5.25^{r}\pm0.07$ | $2.99^{r}\pm0.06$ | $1.94^{r}\pm0.05$ | $16.94^{r}\pm0.18$ |

TFYG=Tris fructose yolk glycerol; Means bearing different superscripts between additives (a,b,c,d,e) and between stages of cryopreservation (p,q,r) differ significantly ($P<0.05$).

**TABLE 4: MEAN (± SE) PERCENTAGES OF PROGRESSIVELY MOTILE SPERMATOZOA IN SURTI BUFFALO BULLS SEMEN AT DIFFERENT INTERVALS OF POST-THAW INCUBATION (37°C) IN CONTROL AND TREATMENT GROUPS**

| Extender-additives | Freezing Stage | | | |
|---|---|---|---|---|
| | 0-hr | 1-hr | 2-hr | 3-hr |
| TFYG Control | $37.75^{b}\pm1.00$ | $24.13^{b}\pm0.88$ | $12.38^{b}\pm0.86$ | $3.25^{b}\pm0.58$ |
| Cysteine 0.5 mg/ml | $39.38^{b}\pm0.80$ | $27.18^{c}\pm1.02$ | $14.38^{b}\pm0.84$ | $4.63^{b}\pm0.58$ |
| Cysteine 1 mg/ml | $46.75^{c}\pm0.71$ | $32.75^{d}\pm0.82$ | $18.88^{c}\pm0.70$ | $8.88^{c}\pm0.66$ |
| Taurine 4 mg/ml | $50.88^{d}\pm0.59$ | $36.13^{e}\pm0.92$ | $21.50^{d}\pm0.88$ | $10.00^{c}\pm0.65$ |
| Taurine 6 mg/ml | $32.88^{a}\pm0.74$ | $19.25^{a}\pm0.88$ | $8.13^{a}\pm0.64$ | $0.63^{a}\pm0.26$ |

TFYG=Tris fructose yolk glycerol; Means bearing different superscripts within the column differ significantly ($P<0.05$).

**ACKNOWLEDGEMENT**

We thank the Dean of Veterinary College, Anand Agricultural University, Anand, Gujarat for the facilities and funds provided for this research work.

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