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Effect of Antioxidant Preservative on Cold Protection Ability of Low Grade Riverine Buffalo (*Bubalus bubalis*) Bull Spermatozoa

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ABSTRACT : An experiment was conducted to investigate the effect of Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT), Pentoxifylline (PTX), Theophylline (TPY) and Theobromine (TBR) on cold protection ability of Murrah buffalo semen at room (22-25°C) and refrigerated temperature (4-7°C). Each semen sample was divided into six parts of equal volume and sperm concentration; the first was kept as a control and the remaining five were treated with BHA, BHT, PTX, TPY or TBR. Sperm motility, abnormal spermatozoa, live-dead count, hypo-osmotic swelling and acrosomal integrity were studied at room and refrigerated temperature for various incubation periods viz.; 0, 4, 8, 12 and 24 h at room and 0, 12, 24, 36, 48, 60 and 72 h at refrigerated temperature. Significant improvement in sperm motility, live-dead count, hypo-osmotic swelling and acrosomal integrity were observed in BHT, PTX and TPY fortified extender at room and refrigerated temperature for various incubation periods. From the present study it could be concluded that cold protection ability of buffalo semen can be improved through the addition of BHT followed by PTX and TPY. (**Key Words** : Buffalo Semen, Sperm Motility, Non-Eosinohilic, Butylated Hydroxy Anisole, Butylated Hydroxy Toluene, Pentoxifylline, Theophylline, Theobromine)

INTRODUCTION

Buffaloes in the Asian continent play an important role as a producer of milk, draught power, dung and other value added products. Their contribution to the national milk grid in India is around 50% in spite of the fact that the population of buffaloes is less than half of the total cattle population. Reproduction is the central trait in animal production throughout the world. High reproduction capacity increases economic efficiency in milk and meat production. Buffalo semen is known for its poor quality and freezability which is well documented in the literature (Roy et al., 1962; Sengupta, 1963). Motility as well as fertility of spermatozoa can be improved by incorporating various motility enhancing agents like pentoxifylline (PTX) (Yovich et al., 1984; Sikka et al., 1995), or antioxidants (Irvine, 1996; Perinaud et al., 1997). PTX increases cAMP level by a methylxanthine inhibition of phosphodiesterase and thus improves motility, capacitation and acrosome reaction (Yovich et al., 1984; Sikka et al., 1995). This increase in cAMP causes activation of protein kinase and phosphorylation of endogenous protein. Motility of spermatozoa is increased due to utilization of increased energy production by accelerating glycolysis and TCA cycle activity (Haesungcharern and Chulavatnatol, 1973).

The antioxidants check the chemical breakdown of substrate resulting from oxidation. Antioxidant preservatives neutralize the free radicals that initiate and help propagate these reactions. The maintenance of sperm membrane phospholipids together with the susceptibility to peroxidation depends on adequate antioxidant properties, which reduce the risk of damage to spermatozoa and probably their lack of survival during storage (Strzeżek et al., 1999; Strzeżek, 2002). Thus, a deficiency of these fractions can affect the overall protection of the spermatozoa from oxidative damage, which can have a negative effect on sperm motility and fertilization.

Among methyl xanthines, caffeine, PTX, Theobromine (TBR) and Theophylline (TPY) have been used. Methyl xanthine supplementation has resulted in better seminal characteristics in fresh and cryopreserved spermatozoa viz.,

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motility and curvilinear velocity (Chauhan et al., 1983). The addition of methyl xanthine to sperm suspension seems to improve sperm function leading to better sperm fertilizing ability (Chauhan et al., 1983).

Therefore, this study was an endeavour towards the improvement of chilled buffalo semen by using Butylated hydroxy anisole (BHA), Butylated hydroxy toluene (BHT), PTX, TPY and TBR as sperm motility promoting factors.

MATERIALS AND METHODS

Four healthy Murrah buffalo bulls, 2.5 to 5.0 years of age with body weight varying from 300 to 700 kg and maintained under identical nutrition and management conditions at the National Dairy Research Institute, Karnal, India, were selected randomly from the herd for the study. Vaccination and deworming were done regularly as per the farm schedule.

Semen collection and evaluation

The bulls were washed before taking to the site of collection. Semen was collected (Walton, 1945) using an artificial vagina (AV) of 12 inches size "Danish Model" with smooth lining, over a male dummy bull once a week. On each collection, two ejaculates were taken in succession, (Foster et al., 1970) and each ejaculate was preceded by a period of sexual preparation consisting of at least two false mounts separated by about one minute restraint. The second ejaculate was collected about 15 minutes after the first. Immediately after collection, each ejaculate was placed in a water bath at 30°C and examined for various physical attributes viz. volume, mass activity, sperm motility and sperm concentration.

Semen volume was collected in a 15 ml graduated metal free glass tube (0.1 ml accuracy). Mass motility was assessed just after the semen collection. Gross swirl rating (GSR) of undiluted semen was performed within one min of collection. Two 10 µl aliquots of undiluted semen were placed separately on a warmed slide located on a stage warmer (37°C) and scored on a scale of 0-4 using a 10X objective lens on a phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan). Motility was expressed qualitatively on a motility scale (0-4) as described by Matharoo et al. (1985). Semen with +3 or below grade was used for further processing. Manual progressive motility and percentage motile spermatozoa were determined by placing 100 µl of undiluted semen into pre-warmed tubes containing 900 µl of Tris buffer and mixing. Twenty microliters of diluted semen was placed on a warmed glass slide (37°C) and allowed to spread uniformly under the cover slip. Strength of motility rating was scored using 200× magnification with a phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a 37°C-heated stage. Percent progressive motility (0-100%) was measured at five representative areas of the slide. The average of the five scores for each category was recorded. If the difference between two consecutive counts exceeds 10 percent, two new counts were performed.

Experimental design

A total of 9 double ejaculates was taken from 4 Murrah buffalo bulls (MU) making a total of 72 ejaculates. Every ejaculate was divided into six parts, each containing an equal volume and number of sperm, before room and refrigerated temperature incubation, and subjected to the following treatments:

Group	Part of semen	Treatment
Control	1st	Plant Based Extender (PBE) developed in our laboratory was used as extender which contained Tris buffer- 71 parts, soymilk- 29 parts, BHT 1.5 mM, PTX-3.6 mM, TPY-10 mM and glycerol (vol/vol)- 6.4 percent
BHA	2nd	Fortified with 1.5 mM BHA (Cat.no. B 1253, Sigma-Aldrich, Germany) immediately after initial dilution (1:15) of the semen.
BHT	3rd	Fortified with 1.5 mM BHT (Cat.no. B 1378, Sigma-Aldrich, Germany) immediately after initial dilution (1:15) of the semen
PTX	4th	Fortified with 3.6 mM PTX (Cat.no. P 1784, Sigma-Aldrich, Germany) immediately after initial dilution (1:15) of the semen
TPY	5th	Fortified with 10 mM TPY (Cat.no. T 1633, Sigma-Aldrich, Germany) immediately after initial dilution (1:15) of the semen
TBR	6th	Fortified with 10 mM TBR (Cat.no. T 4500, Sigma-Aldrich, Germany) immediately after initial dilution (1:15) of the semen

Each semen sample was diluted with PBE at 30 million sperm/ml after adding the aforementioned sperm motility enhancers.

The extended semen was stored at room temperature (22 to 25°C) for 0, 4, 8, 12, and 24 h and at refrigerated temperature (4 to 7°C) for 0, 12, 24, 36, 48, 60 and 72 h as per Raina (1999) and evaluated for morphological attributes.

Live-dead count : Semen samples were kept at 37°C for 30 min before analysis. Forty microlitre (µl) of neat semen was mixed in a micro-centrifuge tube with 400 µl eosin-nigrosine staining solution. The suspension was kept for one minute at room temperature (27°C). Then, a 12 µl droplet was transferred by pipette to a labelled microscope slide (pre-warmed to 37°C) where it was smeared. Duplicate smears were made from each sample, allowed to air dry at room temperature and examined directly. About 200 sperm were assessed at a magnification of 1,000× under oil immersion with a high-resolution 100× bright field objective (not phase contrast). Sperm that were white (unstained) were classified as non-eosinophilic and those

that showed any pink or red colouration were classified as dead, with the sole exception of sperm with a slight pink or red appearance restricted to the neck region ('leaky necks'), which were assessed as eosinophilic.

Morphological abnormality : The same slide which was made for eosin-nigrosine staining was used for calculating morphological abnormality. A drop of oil was applied to the cover-slip and the semen was examined at 1,000× under a DIC (differential interference contrast) microscope. If the preparation was too thick, examination was difficult because many sperm heads were laid on their edges rather than flat. Each cell, even in thin preparations, was usually not totally in one focal plane and it was necessary therefore to focus up and down slightly on each cell. About 200 spermatozoa were counted in different fields and the percentage of abnormal spermatozoa was calculated as follows:

$$\text{Head/midpiece/tail abnormalities (\%)} = \frac{\text{No. of abnormal head/midpiece/tail}}{\text{Total spermatozoa counted}} \times 100$$

Hypo-osmotic swelling test (HOST) : The hypo-osmotic swelling test was performed according to the methods described by Correa and Zavos (1994). Sperm tail curling was recorded as an effect of swelling due to influx of water. A total of about 200 spermatozoa were counted in different fields at 400× magnification under a DIC microscope. The total proportion of swollen spermatozoa was calculated by dividing the number of reacted cells by the total spermatozoa counted in the same area and multiplying the figure by 100. The proportion of swollen spermatozoa from a control sample was subtracted from this value.

These spermatozoa were classified into four different classes according to the presence of the following swelling patterns (Takahashi et al., 1990), namely, A, No swelling, no membrane reaction; B, Swelling of tip of tail; C, Different type of hairpin-like swelling pattern or swelling of mid-piece and D, Complete tail swelling. Spermatozoa displaying B, C or D were considered positive for the HOST test.

Acrosome integrity : Staining was carried out as described by Hancock (1952). A thin smear of extended semen was prepared on a non-greasy, clean and dry slide. The smear was air-dried at room temperature for at least 10 minutes in a current of warm air. The smear was fixed by immersion in buffered formal saline (10 percent) for 15 minutes, then washed in running tap water for 15-20 minutes and dried. Again the slide was immersed in buffered Giemsa solution for 90 minutes, rinsed briefly in distilled water and dried. The dried smears were studied at 1,000× under a light microscope using oil immersion without a cover glass. Each time about 200 spermatozoa were counted for acrosomal status after staining.

All the physical and morphological attributes viz, sperm motility, live-dead count, abnormal sperms, acrosomal integrity and hypo-osmotic swelling were recorded after dilution at room temperature (25°C) for 0, 4, 8, 12, and 24 h of storage and at refrigerated temperature (4 to 7°C) for 0, 12, 24, 36, 48, 60 and 72 h of storage.

Statistical analysis

The data were subjected to analysis of variance to study the effect of treatments on different physical and morphological attributes of semen during various intervals of storage. The following statistical models were used for ANOVA as described by Snedecor and Cochran (1989) and the significant difference between two parameters was evaluated through LSD.

$$Y_{ijk} = \mu + E_i + I_j + (EI)_{ij} + e_{ijk}$$

Where, Y_{ijk} = k^{th} observation of j^{th} stage or interval of preservation,

μ = Overall mean,

I_j = the effect of j^{th} stage or interval of preservation,

$(EI)_{ij}$ = The effect of (ij^{th}) additive-interval or stage interaction,

e_{ijk} = The Random Error, NID (0, $\sigma^2 e$)

Descriptive statistics (SAS 8.2) were performed on the data to determine normality.

RESULTS

Performance of additives at room temperature (22-25°C)

A comparative study of performance of various additives at room temperature was carried out and the data are shown in Table 1 to 3.

Sperm motility (per cent) : Analysis of data revealed significant ($p<0.05$) differences in individual motility between additives at various hours of incubation in buffalo bull semen.

Sperm motility (%) was higher in BHT, PTX, TPY and TBR than in BHA and the control group at 0 hour in MU bull semen (Table 1). For the remaining periods, motility (%) was significantly ($p<0.01$) greater in BHT, PTX and TPY than in the Control (C), BHA and TBR for MU bull semen. Overall, results showed a significant deterioration in motility after each preservation stage. Motility was found to be better preserved in extender fortified with BHT, PTX and TPY.

Non-eosinophilic count (per cent) : Non-eosinophilic count was significantly ($p<0.05$) higher in BHT, PTX and TPY than in TBR, BHA and C groups at 0 h in cattle bull semen (Table 1).

Table 1. Effect of additives on sperm motility* (%) and non-eosinophilic count* (%) of Murrah bull semen at different hours of preservation at room temperature

Item	Hours	C	BHA	BHT	PTX	TPY	TBR
Sperm motility	0	57.92±3.96	59.58±3.87	65.42±3.56	65.42±3.56	65.42±3.56	59.17±3.98
	4	47.50^{a}±3.97	50.00^{ab}±3.69	59.17^{b}±3.58	58.75^{b}±3.54	59.17^{b}±3.58	48.33^{a}±3.96
	8	34.17^{a}±4.43	36.25^{a}±4.18	50.00^{b}±3.43	48.33^{b}±3.66	47.92^{b}±4.01	35.00^{a}±4.65
	12	22.08^{a}±4.71	24.17^{a}±4.43	39.58^{b}±3.61	37.50^{b}±3.87	37.50^{b}±4.06	23.75^{a}±5.08
	24	1.25^{A}±1.25	1.67^{A}±1.67	15.42^{B}±2.64	12.92^{B}±2.57	12.92^{B}±2.34	1.67^{A}±1.28
	Overall	29.28^{A}±1.66	30.50^{A}±1.67	44.06^{B}±1.48	43.08^{B}±1.52	41.61^{B}±1.58	30.78^{A}±1.71
Non-eosinophilic count	0	71.00^{a}±3.72	71.92^{a}±3.81	81.08^{bc}±2.86	81.58^{bc}±3.24	82.50^{c}±2.80	72.58^{ab}±3.28
	4	61.17^{a}±4.07	62.50^{ac}±3.61	72.17^{b}±3.49	71.17^{bc}±3.28	71.67^{bc}±2.93	59.75^{a}±3.21
	8	43.42^{a}±4.73	47.92^{ac}±4.50	61.00^{b}±4.19	59.83^{bc}±4.46	58.42^{b}±4.32	44.42^{a}±4.69
	12	31.83^{a}±4.93	35.58^{ac}±4.73	50.83^{b}±4.29	50.00^{b}±4.59	48.00^{bc}±4.47	31.25^{a}±6.07
	24	6.33^{a}±2.62	11.92^{ac}±2.59	24.33^{b}±3.96	21.17^{b}±3.22	19.92^{bc}±2.77	6.42^{a}±1.90
	Overall	38.67^{A}±1.87	41.25^{A}±1.76	55.35^{B}±1.69	54.80^{B}±1.72	53.04^{B}±1.79	39.81^{A}±1.92

* Mean±SE. Means bearing different superscripts within same row differ significantly ($^{abc}p<0.05$, $^{ABC}p<0.01$).
C = Control; BHA = Butylated hydroxy anisole; BHT = Butylated hydroxy toluene; PTX = Pentoxifylline; TPY = Theophylline; TBR = Theobromine.

Table 2. Effect of additives on various types of abnormalities* (%) at different hours of preservation at room temperature of Murrah bull semen

Abnormalities	Hours	C	BHA	BHT	PTX	TPY	TBR
Head	0	3.33±0.43	3.42±0.51	2.75±0.28	2.83±0.32	2.92±0.29	2.42±0.42
	4	3.17±0.44	3.58±0.40	3.58±0.48	3.58±0.40	4.00±0.55	3.75±0.43
	8	3.75±0.39	3.17±0.46	4.00±0.39	3.58±0.40	3.92±0.40	3.17±0.41
	12	2.50±0.47	2.92±0.45	3.33±0.47	3.25±0.37	3.17±0.27	3.17±0.39
	24	4.83^{A}±0.37	4.83^{A}±0.47	2.75^{B}±0.31	3.25^{B}±0.35	3.42^{B}±0.29	4.83^{A}±0.44
Mid piece	0	2.33^{a}±0.26	3.58^{ab}±0.51	3.42^{ab}±0.54	3.75^{b}±0.49	3.75^{b}±0.49	3.75^{b}±0.49
	4	2.83±0.44	2.17±0.17	3.00±0.58	3.00±0.51	3.08±0.47	2.42±0.26
	8	2.50±0.34	2.25±0.33	2.42±0.42	2.33±0.36	2.67±0.40	2.42±0.31
	12	2.58±0.26	2.92±0.31	2.75±0.41	2.67±0.38	2.75±0.41	2.75±0.37
	24	3.58^{a}±0.45	3.58^{a}±0.36	2.33^{bc}±0.48	2.25^{bc}±0.43	2.75^{ac}±0.37	3.67^{a}±0.40
Tail	0	10.92^{a}±1.58	8.92^{ab}±1.56	7.58^{b}±0.98	6.33^{b}±0.54	6.75^{b}±0.54	7.25^{b}±0.81
	4	15.92^{a}±1.65	14.42^{ac}±1.25	10.75^{b}±1.17	10.92^{bc}±1.10	10.75^{b}±0.92	16.42^{a}±1.49
	8	23.42^{a}±2.60	22.25^{ac}±2.32	14.67^{b}±1.01	15.67^{b}±1.26	16.92^{bc}±1.45	23.00^{a}±2.68
	12	33.50^{a}±3.37	32.08^{a}±3.52	21.25^{b}±2.30	22.17^{b}±2.68	21.83^{b}±2.62	32.33^{a}±3.44
	24	48.00^{A}±1.30	48.83^{A}±1.20	37.17^{B}±2.23	39.75^{B}±2.16	40.58^{B}±2.02	48.42^{A}±1.19
Total	0	16.58±1.89	15.92±1.78	13.75±1.50	12.92±1.02	13.42±0.87	13.42±1.20
	4	21.92^{ab}±2.09	20.17^{ab}±1.33	17.33^{a}±1.79	17.50^{a}±1.46	17.83^{a}±1.53	22.58^{b}±1.71
	8	29.67^{a}±2.43	27.67^{ac}±2.33	21.08^{b}±1.34	21.58^{b}±1.66	23.50^{bcd}±1.73	28.58^{ad}±2.57
	12	38.58^{a}±3.50	37.92^{a}±3.30	27.33^{b}±2.05	28.08^{b}±2.52	27.75^{b}±2.56	38.25^{a}±3.76
	24	56.42^{A}±1.79	57.25^{A}±1.68	42.25^{B}±2.32	45.25^{B}±2.49	46.75^{B}±1.92	56.92^{A}±1.58

* Mean±SE. Means bearing different superscripts within same row differ significantly ($^{abcd}p<0.05$, $^{AB}p<0.01$).
C = Control; BHA = Butylated hydroxy anisole; BHT = Butylated hydroxy toluene; PTX = Pentoxifylline; TPY = Theophylline; TBR = Theobromine.

TPY gave a significantly ($p<0.05$) better result compared to BHA, PTX and TBR in terms of non-eosinophilic count (%) at 0 h in MU bull semen (Table 1). BHT, PTX and TPY supported the sperm non-eosinophilic count significantly ($p<0.05$) better than all other additives in MU bull semen after 24 h of incubation.

Overall, results showed a significant deterioration in non-eosinophilic count after each preservation stage. Damage to spermatozoa was found to be least in extender fortified with BHT, PTX and TPY.

Sperm abnormalities (per cent) : Tail abnormality (per cent) was the most prominent out of various types of abnormalities in Murrah bull semen (Table 2). There was no difference in various additives in head abnormality (per cent) for most of the periods, except for 24 h in MU bull semen. All additives resulted in significantly ($p>0.05$) less head abnormality (per cent) than the control group at 0 h. Head abnormality in BHT, PTX and TPY was significantly ($p<0.01$) less than the control in MU bull semen after 24 hours of incubation. No difference in mid-piece abnormality (per cent) was observed between additives at any incubation period, except for BHT and TPX in MU which were significantly ($p<0.05$) less than the control after 24 h of incubation.

Table 3. Effect of additives on acrosome integrity* (%) and HOST* (%) of bovine semen (N = 12) at different hours of preservation at room temperature

Item	Hours	C	BHA	BHT	PTX	TPY	TBR
Acrosome integrity	0	67.83^{a}±5.14	82.17^{bc}±3.45	88.67^{bc}±1.98	89.50^{b}±1.47	89.50^{b}±1.62	80.67^{c}±3.68
	4	57.67^{a}±4.60	62.25^{ab}±4.74	70.17^{b}±4.00	67.67^{ab}±4.69	68.67^{ab}±4.59	61.00^{ab}±4.29
	8	46.33^{a}±3.27	47.17^{ac}±3.51	62.00^{b}±3.64	60.08^{b}±4.00	57.33^{bc}±4.38	47.50^{a}±3.93
	12	36.42^{a}±5.07	37.75^{a}±4.19	51.08^{b}±3.61	50.17^{b}±3.28	49.25^{b}±3.70	36.42^{a}±4.58
	24	14.17^{A}±1.78	14.58^{A}±1.92	30.17^{B}±2.43	25.92^{B}±2.88	25.83^{B}±2.45	14.42^{A}±1.49
	Overall	42.02^{A}±1.65	45.72^{A}±1.91	58.86^{B}±1.63	57.81^{B}±1.71	56.26^{B}±1.76	46.44^{A}±1.95
HOST	0	59.17±4.05	61.00±4.47	60.33±4.62	60.25±4.64	60.25±4.64	60.25±4.64
	4	45.09^{a}±4.34	48.27^{ab}±3.27	60.25^{b}±4.06	59.75^{ab}±4.24	60.00^{b}±4.38	48.75^{ab}±4.32
	8	35.67^{a}±3.80	35.33^{a}±3.69	51.75^{b}±4.33	50.42^{b}±4.04	47.17^{b}±4.71	35.25^{a}±4.06
	12	22.83^{a}±4.60	26.42^{ac}±4.78	40.08^{b}±4.27	39.42^{b}±4.21	38.92^{bc}±4.59	24.67^{a}±5.10
	24	2.58^{A}±1.17	3.08^{A}±1.74	17.50^{B}±3.00	13.42^{B}±3.16	12.50^{B}±2.14	3.08^{A}±1.10
	Overall	30.16^{A}±1.70	31.52^{A}±1.71	44.94^{B}±1.52	43.23^{B}±1.57	41.89^{B}±1.61	31.60^{A}±1.74

* Mean±SE. Means bearing different superscripts within same row differ significantly (^{abc} $p<0.05$, ^{AB} $p<0.01$).
C = Control; BHA = Butylated hydroxy anisole; BHT = Butylated hydroxy toluene; PTX = Pentoxifylline; TPY = Theophylline; TBR = Theobromine.

The tail abnormality (per cent) was significantly ($p<0.01$) less in BHT, PTX and TPY in MU bull semen. As tail abnormality (per cent) constituted the major portion of total abnormality (per cent), so the same trend was visible in total abnormality (per cent) as in the tail abnormality (per cent).

Overall, the results showed a significant increase in spermatozoal tail and total abnormality after each preservation stage, however, the head and mid-piece abnormalities were largely unaffected. Tail and total abnormality was found to be least in extender fortified with BHT, PTX and TPY.

Acrosomal Integrity (per cent) : Initially, acrosomal integrity (per cent) in BHA, BHT, PTX, TPY and TBR was significantly ($p<0.01$) higher than the control when used as semen additive in PBE for the extension of SW, KF and MU semen (Table 3).

BHT, PTX and TPY addition resulted in significantly ($p<0.05$) better acrosome integrity than for the control, BHA and TBR addition in bovine bull semen after 24 h of incubation. Overall, results showed a significant deterioration in intact acrosome (per cent) after each preservation stage. From the results, it was evident that spermatozoa acrosome integrity was best preserved in extender fortified with BHT, PTX and TPY after 24 h of storage.

Hypo osmotic swelling test (per cent) : There was no difference in HOST (%) value of different additives at 0 h in MU bull semen (Table 3). BHT, PTX and TPY were significantly ($p<0.01$) better than C, BHA and TBR in MU bull semen.

Overall, results showed a significant deterioration in plasma membrane integrity (per cent) after each preservation stage. The plasma membrane integrity as measured by HOST was found to be better preserved in extender fortified with BHT, PTX and TPY.

Performance of additives at refrigerator temperature (4-7°C)

A comparative study of performance of various additives at refrigerator temperature was carried out and the data are shown in Table 4 to 6.

Sperm motility (per cent) : Initially sperm motility (per cent) of MU bull semen was significantly ($p<0.05$) higher in PBE fortified with BHT, PTX and TPY than in BHA, TBR and control groups at refrigerator temperature (Table 4). BHT, PTX and TPY were significantly ($p<0.05$) better than BHA, TBR and control groups in MU bull semen after 72 h of incubation.

Overall, the results showed a significant deterioration in motility after each preservation stage. Motility was found to be better preserved in extender fortified with BHT, PTX and TPY.

Non-eosinophilic count (per cent) : No difference in any of the additives for non-eosinophilic count was manifested in MU bull semen at 0 h of refrigerator temperature (Table 4). BHT, PTX and TPY supplemented groups were significantly ($p<0.05$) superior to the control group in MU bull semen after 12 h of incubation at refrigerator temperature. Subsequently for all periods of incubation, non-eosinophilic count was significantly ($p<0.01$) higher in BHT, PTX and TPY supplemented groups than in BHA, TBR and control groups in MU bull semen.

Overall the results indicated that PBE fortified with BHT, PTX and TPY could support the preservation of livability of spermatozoa to the maximum extent in storage at refrigerator temperature (4 to 7°C).

Sperm abnormalities (per cent) : Tail abnormality was the most prominent in MU bull semen (Table 5).

There was no significant ($p>0.05$) difference between head and mid-piece abnormality of any of the additives for any period of incubation at the refrigerator temperature under study. The tail abnormality was significantly ($p<0.05$)

Table 4. Effect of additives on sperm motility* (%) and non-eosinophilic count (%) of Murrah bull semen at different hours of preservation at refrigerator temperature (4-7°C)

Item	Hours	C	BHA	BHT	PTX	TPY	TBR
Sperm motility	0	$47.50^{a}\pm4.33$	$52.08^{ac}\pm3.96$	$60.00^{bc}\pm3.79$	$59.58^{bc}\pm3.72$	$59.58^{bc}\pm3.72$	$47.92^{a}\pm4.24$
	12	$37.08^{a}\pm4.15$	$40.83^{ac}\pm4.26$	$50.83^{bc}\pm3.63$	$50.00^{bc}\pm3.64$	$49.17^{bc}\pm3.68$	$37.92^{a}\pm4.01$
	24	$26.67^{a}\pm4.00$	$29.58^{ac}\pm4.42$	$41.67^{b}\pm3.71$	$40.83^{b}\pm3.47$	$39.58^{bc}\pm3.72$	$27.50^{a}\pm3.87$
	36	$15.00^{a}\pm3.48$	$17.50^{a}\pm3.77$	$33.33^{b}\pm3.71$	$30.83^{b}\pm3.53$	$29.58^{b}\pm3.34$	$17.08^{a}\pm3.61$
	48	$6.67^{a}\pm2.56$	$8.33^{a}\pm2.56$	$24.58^{b}\pm3.51$	$21.25^{b}\pm3.49$	$20.00^{b}\pm3.43$	$7.92^{a}\pm2.98$
	60	$2.08^{a}\pm1.15$	$2.50^{a}\pm1.31$	$16.25^{b}\pm3.38$	$12.50^{b}\pm3.34$	$11.67^{b}\pm3.10$	$2.92^{a}\pm1.68$
	72	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$9.17^{b}\pm2.94$	$5.83^{b}\pm2.21$	$6.25^{b}\pm2.05$	$0.00^{a}\pm0.00$
	Overall	$15.77^{A}\pm1.12$	$17.44^{A}\pm1.20$	$30.12^{B}\pm1.28$	$28.53^{B}\pm1.30$	$28.10^{B}\pm1.28$	$16.77^{A}\pm1.14$
Non-eosinophilic count	0	$58.42^{ab}\pm4.32$	$61.08^{ab}\pm4.15$	$68.92^{a}\pm4.28$	$69.50^{a}\pm4.58$	$68.92^{a}\pm3.75$	$56.17^{b}\pm5.04$
	12	$46.42^{ac}\pm4.29$	$49.75^{ad}\pm4.47$	$59.42^{d}\pm4.49$	$57.75^{bcd}\pm4.16$	$58.50^{b}\pm4.43$	$44.58^{a}\pm4.21$
	24	$34.58^{a}\pm4.10$	$35.50^{a}\pm4.62$	$49.00^{b}\pm4.04$	$48.92^{b}\pm3.55$	$47.17^{b}\pm3.91$	$35.42^{a}\pm4.01$
	36	$23.25^{a}\pm3.25$	$24.50^{a}\pm4.01$	$41.75^{b}\pm3.64$	$38.00^{b}\pm3.40$	$35.75^{b}\pm3.54$	$23.42^{a}\pm3.93$
	48	$14.58^{a}\pm2.60$	$14.08^{a}\pm3.06$	$33.25^{b}\pm4.14$	$26.67^{b}\pm3.48$	$25.00^{b}\pm3.56$	$13.58^{a}\pm3.45$
	60	$6.92^{A}\pm1.75$	$7.17^{A}\pm2.21$	$21.17^{B}\pm3.68$	$18.25^{B}\pm3.27$	$18.17^{B}\pm2.94$	$5.83^{A}\pm1.80$
	72	$1.75^{A}\pm0.45$	$1.58^{A}\pm0.23$	$13.08^{B}\pm3.03$	$9.25^{B}\pm2.13$	$10.75^{B}\pm2.15$	$1.25^{A}\pm0.18$
	Overall	$22.80^{A}\pm1.28$	$23.68^{A}\pm1.35$	$37.92^{B}\pm1.41$	$35.26^{b}\pm1.41$	$35.14^{B}\pm1.36$	$22.14^{A}\pm1.30$

* Mean±SE. Means bearing different superscripts within same row differ significantly ($^{abc}p<0.05$, $^{ABC}p<0.01$).
C = Control; BHA = Butylated hydroxy anisole; BHT = Butylated hydroxy toluene; PTX = Pentoxifylline; TPY = Theophylline; TBR = Theobromine.

Table 5. Effect of additives on various types of abnormalities (%) at different hours of preservation at refrigerator temperature (4-7°C) of Murrah bull semen

Abnormalities	Hours	C	BHA	BHT	PTX	TPY	TBR
Head	0	3.50±0.31	3.75±0.41	3.50±0.58	3.00±0.41	3.42±0.38	3.75±0.31
	12	3.17±0.49	3.25±0.39	3.50±0.40	3.27±0.36	2.92±0.42	3.50±0.34
	24	3.50±0.42	3.50±0.38	3.67±0.28	3.33±0.23	3.58±0.38	3.67±0.36
	36	3.42±0.40	3.92±0.42	3.25±0.61	2.75±0.54	2.67±0.64	3.75±0.37
	48	4.33±0.56	3.67±0.43	3.42±0.42	3.67±0.43	3.83±0.32	3.92±0.38
	60	3.92±0.48	3.75±0.57	3.08±0.43	3.00±0.43	3.50±0.54	4.17±0.42
	72	4.92±0.51	4.67±0.54	3.83±0.60	3.83±0.35	3.42±0.58	4.75±0.25
Mid piece	0	2.75±0.31	2.58±0.42	2.75±0.46	3.33±0.47	3.17±0.47	2.33±0.33
	12	2.33±0.36	3.42±0.26	2.42±0.29	2.42±0.23	3.08±0.40	2.67±0.33
	24	2.33±0.28	3.17±0.41	3.17±0.53	3.50±0.26	2.92±0.34	2.83±0.35
	36	2.83±0.35	3.42±0.34	2.92±0.31	2.75±0.37	2.83±0.35	2.58±0.29
	48	4.08±0.45	3.33±0.47	3.42±0.36	2.75±0.31	2.75±0.31	2.75±0.39
	60	3.67±0.38	3.50±0.52	2.00±0.37	2.58±0.38	2.75±0.37	3.00±0.44
	72	3.17±0.44	3.33±0.31	3.25±0.22	3.25±0.39	3.00±0.28	2.75±0.31
Tail	0	$17.42^{a}\pm1.66$	$14.58^{ac}\pm1.43$	$11.42^{bc}\pm0.93$	$12.00^{bc}\pm1.13$	$12.50^{bc}\pm1.17$	$16.50^{a}\pm1.74$
	12	$23.08^{a}\pm2.42$	$20.67^{a}\pm2.65$	$14.25^{b}\pm1.38$	$15.00^{b}\pm1.46$	$14.83^{b}\pm1.54$	$22.67^{a}\pm2.17$
	24	$29.50^{a}\pm2.87$	$28.83^{a}\pm2.76$	$19.83^{b}\pm2.27$	$20.17^{b}\pm1.88$	$19.58^{b}\pm1.96$	$28.83^{a}\pm2.91$
	36	$39.00^{a}\pm3.22$	$36.00^{a}\pm3.53$	$25.67^{b}\pm2.51$	$27.08^{b}\pm2.87$	$28.58^{b}\pm2.47$	$37.50^{a}\pm3.11$
	48	$43.83^{A}\pm2.12$	$43.67^{A}\pm2.05$	$29.75^{B}\pm2.53$	$32.67^{B}\pm2.97$	$33.67^{B}\pm2.83$	$42.92^{A}\pm2.58$
	60	$48.67^{a}\pm0.96$	$49.33^{a}\pm1.02$	$37.75^{b}\pm3.00$	$40.67^{b}\pm2.78$	$41.42^{bc}\pm2.54$	$47.25^{ac}\pm1.94$
	72	$49.75^{a}\pm0.55$	$49.67^{a}\pm0.54$	$43.50^{bc}\pm2.46$	$46.25^{ac}\pm1.85$	$44.08^{bc}\pm1.73$	$49.50^{a}\pm0.58$
Total	0	$23.67^{a}\pm1.86$	$20.92^{ad}\pm1.78$	$17.67^{bd}\pm1.58$	$18.33^{bcd}\pm1.66$	$19.08^{ad}\pm1.55$	$22.58^{ac}\pm1.96$
	12	$28.58^{a}\pm2.44$	$27.33^{a}\pm2.56$	$20.17^{b}\pm1.67$	$20.42^{b}\pm1.78$	$20.83^{b}\pm1.95$	$28.83^{a}\pm2.16$
	24	$35.33^{a}\pm2.74$	$35.50^{a}\pm2.71$	$26.67^{b}\pm2.13$	$27.00^{b}\pm1.81$	$26.08^{b}\pm2.00$	$35.33^{a}\pm2.95$
	36	$45.25^{a}\pm3.66$	$43.33^{a}\pm3.72$	$31.83^{b}\pm2.21$	$32.58^{b}\pm2.55$	$34.08^{b}\pm2.04$	$43.83^{a}\pm2.91$
	48	$52.25^{A}\pm2.67$	$50.67^{A}\pm2.49$	$36.58^{B}\pm2.48$	$39.08^{B}\pm2.96$	$40.25^{B}\pm2.89$	$49.58^{A}\pm2.87$
	60	$56.25^{A}\pm1.34$	$56.58^{A}\pm1.58$	$42.83^{B}\pm3.13$	$46.25^{B}\pm3.11$	$47.67^{B}\pm2.99$	$54.42^{A}\pm2.25$
	72	$57.83^{a}\pm0.73$	$57.67^{a}\pm0.84$	$50.58^{bc}\pm2.69$	$53.33^{ac}\pm2.24$	$50.50^{bc}\pm1.93$	$57.00^{a}\pm0.73$

* Mean±SE. Means bearing different superscripts within same row differ significantly ($^{abcd}p<0.05$, $^{AB}p<0.01$).

Table 6. Effect of additives on acrosome integrity* (%) and HOST (%) of Murrah bull semen (N = 12) at different hours of preservation at refrigerator temperature (4-7°C)

Items	Hours	C	BHA	BHT	PTX	TPY	TBR
Acrosome integrity	0	60.50±5.25	65.75±4.38	69.50±4.25	69.75±4.26	68.83±4.20	61.75±4.57
	12	47.92^{a}±3.03	52.08^{a}±3.76	65.33^{b}±4.21	65.00^{b}±4.45	64.08^{b}±4.30	49.08^{a}±3.89
	24	42.67^{a}±4.14	42.00^{a}±3.36	53.08^{b}±3.85	53.08^{b}±3.34	52.50^{b}±3.22	40.33^{a}±3.13
	36	26.08^{a}±4.20	31.00^{a}±4.47	48.08^{c}±3.55	44.67^{c}±2.64	41.17^{bc}±3.12	31.33^{ab}±3.63
	48	19.58^{A}±2.84	21.25^{A}±2.99	39.00^{B}±2.62	34.00^{B}±3.27	34.50^{B}±3.20	21.50^{A}±3.46
	60	15.25^{a}±1.52	15.92^{a}±1.76	29.42^{b}±3.86	24.58^{bc}±3.20	24.25^{b}±2.88	17.08^{ac}±2.44
	72	12.83^{a}±0.55	12.75^{a}±0.59	22.25^{b}±3.31	18.83^{b}±2.55	20.50^{b}±2.66	12.50^{a}±0.57
	Overall	28.65^{A}±1.13	30.51^{A}±1.20	43.02^{B}±1.26	41.31^{B}±1.29	41.20^{B}±1.28	30.01^{A}±1.18
HOST	0	49.08±4.96	53.75±4.93	59.75±4.27	59.83±3.50	59.08±4.08	48.67±4.82
	12	36.75^{a}±3.53	38.75^{a}±3.85	51.50^{b}±4.35	51.67^{b}±4.86	51.75^{b}±4.68	36.83^{a}±3.72
	24	28.33^{a}±4.00	30.67^{a}±4.67	41.75^{b}±4.28	40.42^{b}±3.66	40.67^{b}±3.34	27.92^{a}±3.92
	36	14.33^{a}±4.54	17.67^{ac}±4.28	33.25^{b}±2.98	33.67^{b}±3.52	28.42^{bcd}±3.09	18.25^{ad}±3.89
	48	7.75^{A}±2.45	9.50^{A}±2.65	25.50^{B}±3.14	21.50^{B}±4.11	22.50^{B}±3.64	8.67^{A}±3.11
	60	3.08^{a}±1.06	3.17^{a}±1.11	16.75^{b}±3.51	12.25^{bc}±2.91	11.25^{bd}±2.91	5.08^{acd}±2.06
	72	1.67^{a}±0.31	1.42^{a}±0.26	10.83^{b}±2.98	7.08^{b}±2.29	8.92^{b}±2.14	1.50^{a}±0.38
	Overall	16.88^{A}±1.16	18.53^{A}±1.24	30.75^{B}±1.30	29.75^{B}±1.35	28.94^{B}±1.29	17.79^{A}±1.16

* Mean±SE. Means bearing different superscripts within same row differ significantly ($^{abcd}p<0.05$, $^{AB}p<0.01$).
C = Control; BHA = Butylated hydroxy anisole; BHT = Butylated hydroxy toluene; PTX = Pentoxifylline; TPY = Theophylline; TBR = Theobromine.

lower in PBE fortified with BHT, PTX and TPY than in control groups up to 72 h of incubation at refrigerator temperature in MU bull semen. As the tail abnormality constituted the major portion of total abnormality, so the same trend was found in total abnormality.

Overall, the results showed a significant increase in spermatozoal tail and total abnormality after each preservation stage at refrigerator temperature (4 to 7°C), however, the head and mid-piece abnormalities were largely unaffected. Tail and total abnormality was found to be significantly ($p<0.05$) less in extender fortified with BHT, PTX and TPY.

Acrosomal Integrity (per cent) : Initially there was no significant difference among any of the additives in acrosomal integrity for MU bull semen (Table 6). But this trend was changed subsequently when BHT, PTX and TPY were significantly ($p<0.05$) better than control, BHA and TBR groups in MU bull semen after 72 h of incubation at refrigerated temperature.

Overall, the results showed a significant deterioration in the acrosome integrity up to 48 h, thereafter there was not much degradation of integrity. From the results, it was evident that spermatozoal acrosome integrity was best preserved in PBE fortified with BHT, PTX and TPY in MU bull semen after 72 h of storage.

Hypo osmotic swelling test (per cent) : There was no difference for HOST (per cent) among the various additives initially in MU bull semen (Table 6).

But this trend was changed between periods of incubation where reaction of sperm to hypotonic medium was significantly higher in PBE fortified with BHT, PTX and TPY than control, BHA or TBR fortified groups with variable degree of significance ($p<0.01$ to 0.05).

Overall, the results showed a significant deterioration in the plasma membrane integrity up to 48 h, thereafter there was not much degradation of integrity. After 72 h of storage, it was evident that the plasma membrane integrity as measured by HOST was best preserved in PBE fortified with BHT, PTX or TPY in MU bull semen.

DISCUSSION

Mammalian spermatozoa are extremely sensitive to oxidative damage (Lucy, 1972). Lipid peroxidation plays an important role in spermatozoon ageing, shortening life-span and affecting the preservation of semen for artificial insemination (Alvarez and Storey, 1982). Maintenance of sperm membrane phospholipids and susceptibility to peroxidation depends on adequate antioxidant, which reduces the risk of damage to spermatozoa and increases their survival chances during storage (Strzeżek et al., 1999; Strzeżek, 2002). Thus, a deficiency of these fractions can affect the overall protection of the spermatozoa from oxidative damage, which can have a negative effect on sperm motility and fertilizing ability. The process of peroxidation induces structural changes, particularly in the acrosomal region of the sperm cell, a fast irreversible loss of motility, deep change in metabolism and a high rate of intracellular component release (John and Mann, 1977a). *In vitro* studies have shown BHA and BHT to act as free radical scavengers which protect cell membrane against lipid peroxidation (Beconi et al., 1993). During the process of freezing, spermatozoa have to undergo cold shock which increases their susceptibility to lipid peroxidation (John and Mann, 1977b; John et al., 1979; Pursel and Park, 1985).

Stimulatory effects of methyl xanthine on capacitation and acrosome reaction have also been demonstrated. Overall, the addition of methyl xanthine to sperm suspension seems to improve sperm function leading to better sperm fertilizing ability (Chauhan et al., 1983). Similar results were also observed in the present study while fortifying the PBS for dilution of bovine semen with PTX and TPY. However, desirable results could not be achieved by fortification of PBE with BHA and TBR.

Killian et al. (1989) hypothesized that BHT serves as a scavenger of free oxygen radicals, associated with the diluent and sperm, to minimize damage to the sperm motility apparatus and membranes, and which also may affect motility indirectly. Graham and Hammerstedt (1992) reported that BHT with no egg yolk present reduced sperm motility, but addition of egg yolk in BHT-treated sperm improved motility, and lipid vesicles in milk and egg yolk (Hu et al., 2006) interacted synergistically with BHT to protect spermatozoa from cold shock. Anderson et al. (1994) reported that the use of BHT improved viability of frozen and thawed sperm and inactivated lipid containing viruses. In the present study also, BHT fortification in PBE for extension of bovine bull semen has improved the viability of sperm.

Methyl xanthine supplementation resulted in better seminal characteristics in fresh and cryopreserved spermatozoa viz., motility and curvilinear velocity (Chauhan et al., 1983). Fayed and Hattab (1991) observed that supplementation of TPY (0.5 ng/ml) improved the keeping quality of chilled semen up to 5 days. PTX may be added to boost the motility of the sperm (Aitken et al., 1993). PTX increases the duration of activity of spermatozoa by increasing the level of cyclic adenosine monophosphate (cAMP) or by reducing the decomposition of cAMP (Perry and Higgs, 1998) by stimulating the enzyme adenylate cyclase (AC stimulator). Thus, in the present study, improved motility was observed in bovine bull semen after fortification of PBE with BHT, PTX and TPY.

Sperm abnormalities (per cent) are one of the most significant indicators of subsequent fertility in a bull (Saacke, 1990). Disturbance in spermatogenesis gives rise to morphological abnormalities. The relationship between sperm morphology and fertility has been evaluated in several studies. Abnormal sperm morphology has been correlated with reduced fertility in cattle (Sekoni and Gustafsson, 1987; Barth and Oko, 1989; Correa et al., 1997; Thundathil et al., 2000) and buffalo (Sengupta and Bhela, 1988). Bull fertility depends upon morphologically normal spermatozoa being present in the ejaculate (Tharwat, 1998) and is hardly affected if abnormal spermatozoa do not exceed 15-20 per cent (Pant et al., 2002). In particular, the occurrence of abnormal sperm head morphology is associated with lower fertility in the bull (Saacke and White, 1972; Sekoni and Gustafsson, 1987). However, a number of other studies have shown no correlation between sperm morphology and fertility (Bratton et al., 1956; Linford et al., 1976) with clear associations between normal bull sperm morphology and fertility continuing to remain elusive (Johnson, 1997). In the present study the increase of abnormality (per cent) is mainly in the tail which suggests that osmolality change in the media may be responsible for this increase as suggested by Joshi et al. (2006). The additives responsible for resisting change in the morphology of spermatozoa are BHT, PTX and TPY.

The addition of methyl xanthine to sperm suspension seems to improve sperm function leading to better sperm fertility (Chauhan et al., 1983). These authors found that when BHT-treated ram sperm were subjected to cold shock, acrosome damage was reduced, and the percentage of motile sperm was higher than for untreated sperm. Similarly, in the present study, methyl xanthine inclusion in the PBE for extension of bovine bull semen resulted in reduced acrosome damage.

Antioxidant preservatives (like BHA, BHT and methyl xanthines) are used to stop auto-oxidation that causes a chain reaction in the unsaturated fatty acids in oils and lipid, and help in slowing down the oxidation of fats and oils. Oxygen reacts preferentially with antioxidants rather than oxidizing fats or oils, thereby protecting them from spoilage. BHA is a synthetic analogue of vitamin E and acts by reducing oxygen radicals and interrupting the propagation of oxidation processes. However, in the present study, BHA has not shown its motility enhancer role.

BHT is an organic soluble molecule which modifies the properties of lipid bilayers and membrane of sperm cells (Hammerstedt et al., 1978). BHT readily incorporates into sperm membranes and prevents membrane damage after exposure to cold (Anderson et al., 1994). Use of spin labels and electron spin resonance techniques suggests that BHT acts on membranes to increase fluidity and to render them less susceptible to cold shock (Anderson et al., 1994). These reports explain the superiority of BHT as an additive over others as evidenced from the present results. Thus, future prospects for use of fortified PBE for the preservation of MU bull semen could be recommended as also supported from the results of acrosome integrity testing. Similarly, Hammerstedt et al. (1978) found that sperm from bulls and rams treated with 0.5 mM BHT were protected from membrane damage during cold shock. Addition of 0.5 mM BHT to whole milk extender during semen processing did not affect bull non-return rates (Anderson et al., 1994).

PTX, TPY and TBR are methylxanthine phosphodiesterase inhibitors which reduce super oxide

anions responsible for DNA apoptosis when used in a concentration of 3.6 mM (Maxwell et al., 2002). In the present study, TBR was not able to perform well as a semen additive, unlike PTX and TPY. Similarly, Vega (1997) reported that addition of PTX (6.0 mM) prolonged the viability of post-thaw bovine semen. Apart from modulation of sperm function, a protective effect on sperm membrane by PTX has been demonstrated (Vega, 1997). This effect may be ascribed to neutralization of reactive oxygen species (ROS) and reduction of lipid peroxidation (Vega, 1997). The use of BHT improved viability of frozen and thawed sperm and inactivated lipid containing viruses (Anderson et al., 1994). In the present study, BHT, PTX and TPY being oxygen radical scavengers, supported the preservation of viability of spermatozoa, in agreement with other studies. So, further studies should be carried out to standardize the optimum concentration of TBR to be incorporated in PBE for extension of Murrah bull semen in order to obtain its maximum benefit.

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