Asian-Aust. J. Anim. Sci.
Vol. 24, No. 10 : 1348 - 1357
October 2011

www.ajas.info
http://dx.doi.org/10.5713/ajas.2011.10243

Study on Suitable Semen Additives Incorporation into the Extender Stored at Refrigerated Temperature

M. Bhakat[1,*], T. K. Mohanty, V. S. Raina, A. K. Gupta, P. K. Pankaj[2], R. K. Mahapatra[3] and M. Sarkar[3]
Artificial Breeding Complex, National Dairy Research Institute, Karnal-132001, Haryana, India

ABSTRACT : The objective of this study was to compare the effect of Butylated Hydroxy Toluene (BHT), Pentoxifylline (PTX) and α-tocopherol (Vit E) on semen quality parameters of Karan Fries bulls. The fortification of extender by various semen additives improves motility as well as fertility of spermatozoa. Split samples of 24 ejaculates of four Karan Fries bulls were extended in extender with or without various additives such as BHT, PTX and Vit E, and performance was evaluated at an interval of 0, 24, 48 and 72 h at refrigerated temperature (4-7°C). Results of the present study revealed that addition of BHT, PTX and Vit E in extender improved sperm cell function, such as motility, viability, HOST, and acrosome integrity, as compared to the control during liquid storage up to 48 h of preservation at refrigerated temperature. There was no significant ($p<0.05$) difference between any of the additives up to 48 h of preservation. Overall, the results showed a significant ($p<0.05$) deterioration in motility after each storage interval. The results showed a significant deterioration in the acrosome integrity and plasma membrane integrity up to 48 h; subsequently, there was not much degradation of both the semen quality parameters. There was a significant increase in spermatozoal tail and total abnormality after each storage interval at refrigerator temperature (4 to 7°C); however, the head and mid-piece abnormalities were almost unaffected. Tail and total abnormality were least in extender fortified with BHT, PTX and Vit E at different hours of incubation as compared to the control. The addition of 1.5 mM BHT, 3.6 mM PTX and 1 mg/ml Vit E in the semen extender has more beneficial effect in terms of semen quality and preservability of spermatozoa. (**Key Words :** Butylated Hydroxy Toluen, Pentoxifylline, Vit E, Semen Quality of Karan Fries, Refrigerated Temperature)

INTRODUCTION

Mammalian spermatozoa are extremely sensitive to oxidative damage. Lipid peroxidation plays a leading role in aging of spermatozoa, shortening its life-span *in vivo* and affecting the preservation of semen for artificial insemination. The process of peroxidation induces structural alterations; particularly in the acrosomal region of the sperm cell, a fast and irreversible loss of motility, a deep change in metabolism and a high rate of release of intracellular components. Lipid peroxidation has been defined as an important aspect of oxidative stress in mammalian spermatozoa for many years (Jones and Mann, 1973; Jones et al., 1979). The susceptibility of spermatozoa to oxidative damage is attributed to the high concentration of unsaturated fatty acids in membrane phospholipids, and limited antioxidant capacity of spermatozoa as well as the ability of spermatozoa to generate reactive oxygen species (ROS) (Aitken, 1995; storey, 1997). Spermatozoa are capable of generating ROS (Aitken et al., 1997; Balla et al., 2001), and the controlled generation of ROS may have physiological functions in signaling events controlling sperm capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion. The uncontrolled production of ROS by defective spermatozoa can have a detrimental effect on sperm function (Baumber et al., 2000). Oxidative stress appears as a consequence of this extreme ROS production or from alteration in the antioxidative mechanisms of defense. Oxidative stress results in a fall in intracellular ATP levels which decreases sperm motility and also initiates lipid peroxidation in the polyunsaturated fatty acid-rich sperm plasma membrane. Such events have been associated with increased permeability, enzyme inactivation and production of spermicidal end products. Lipid peroxidation has been suggested as the cause of abnormal acrosome reaction and loss of membrane fluidity,

* Corresponding Author : M. Bhakat. Tel: +91-9286299365, E-mail: bhakat.mukesh@gmail.com
[1] Deptt. of LPM, DUVASU, Mathura, UP, India.
[2] NRC., Pig, Gowhati, Assam, India.
[3] IVRI, Izzatnagar, UP, India.
Received July 3, 2010; Accepted October 29, 2010

culminating in a loss of fertilizing potential of spermatozoa (Verma and Kanwar, 1998). Production of ROS cannot be prevented completely; but several measures may be taken to minimize it to improve motility as well as fertility of spermatozoa by incorporating various motility-enhancing agents like antioxidants, antioxidant preservatives and methyl xanthines.

The important cellular systems of protection against oxidative damage are BHT, PTX and Vit E. Although numerous studies have examined the addition of BHT, PTX and Vit E (Aguero et al., 1995; Donoghue and Donoghue, 1997) to semen in order to improve sperm preservation during storage of liquid semen, there has not been any consistent improvement in the maintenance of sperm motility or fertility during liquid semen storage with these additives. Beneficial effects of BHT (Bamba and Cran, 1992; Pankaj, 2006), PTX (Pankaj, 2006) and Vit E addition (Aguero et al., 1995; Donoghue and Donoghue, 1997; Cerolini et al., 2000; Douard et al., 2004) have been observed; however, addition of BHT, PTX and Vit E during liquid storage showed no improvement (Balla et al., 2001; Raina et al., 2002; Shukla and Misra, 2005). The objective of this study was to compare the effect of BHT, PTX and Vit E on semen quality parameters of Karan Fries bulls.

MATERIALS AND METHODS

The present investigation was conducted on four Karan Fries bulls aged 3 to 4 years (Tharparkar×Holstein Frisien crosses between 50 to 75% exotic inheritance) and maintained at the Artificial Breeding Complex, National Dairy Research Institute, Karnal, Haryana, India, which is located at latitude 29.43°N and longitude 72.2°E in a semi-arid tract of the country.

Semen collection and initial evaluation

Semen was collected in a pre-warmed (42-45°C) bovine artificial vagina (IMV model-005417) with smooth neoprene liner (IMV-005331). On each collection, two ejaculates were taken at an interval of 20 to 30 min between successive ejaculates and each ejaculate was preceded by a period of sexual preparation consisting of at least two false mounts separated by about a one minute restraint. A total of 24 ejaculates were taken from four bulls to identify the best additive at different hours of preservation (0, 24, 48 and 72) at refrigerated temperature (4-7°C). Each ejaculate was evaluated for volume and mass activity. Sperm concentration was determined using a haemocytometer (Improved Neubauer, HBG, Germany). The ejaculates were split into four parts. Each part was diluted separately by Tris-based extender (Tris 3.311% w/v, Citric acid 1.831% w/v, Dextrose 0.781% w/v, L-cystine hydrochloride 0.156%, Glycerol 6.9% v/v, fresh egg yolk 20% v/v, penicillin 1,000 IU/ml, streptomycin 0.01% w/v, OP 325 mOsmol/kg, pH 6.8, Millipore water) fortified with BHT (1.5 mM), PTX (3.6 mM) and Vit E (1 mg/ml) (Sigma-Aldrich, Germany) semen additives.

Semen analyses

Semen analyses (motility, non-eosinophilic count, HOST and acrosome integrity) were performed for control and semen samples fortified with additives at different hours of preservation (0, 24, 48 and 72) at refrigerated temperature (4-7°C).

Sperm motility percentage : The manual progressive motility and percentage of motile spermatozoa were determined by placing 100 µl of undiluted semen into pre-warmed tubes in digitally controlled heat blocks containing 900 µl of Tris buffer and mixed thoroughly. Six to eight microliters of diluted semen was placed on a warmed glass slide (37°C) and allowed to spread uniformly under the cover slip (18×18 mm). Initial progressive motility rating was scored using 200× magnifications with a phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a Tokoiheat-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 exceeded 10 percent, two new counts were performed.

Non-eosinophilic (live) spermatozoa (%) : Forty microlitre (µl) of neat semen was mixed in a micro-centrifuge tube with 400 µl eosin-nigrosine staining solution (0.67 g of eosin-Y yellow, 0.9 g sodium chloride, 100 ml distilled water and 10 g of nigrosine). The suspension was kept for one minute at room temperature (27°C). Subsequently, a 12 µl droplet was transferred by pipette to a labeled microscope slide (pre-warmed to 37°C) for preparing a smear. Duplicate smears were made from each sample and allowed to air-dry at room temperature. About 200 sperms were assessed under bright field 100× oil immersion objectives.

Morphological abnormality : The same slide made for eosin-nigrosine staining was used for screening morphological abnormalities. A drop of oil was applied to the cover-slip and the semen was examined with 100× objectives under bright field. About 200 spermatozoa were counted in different fields and the percentage of abnormal spermatozoa was calculated by dividing the number of head, mid-piece, tail and total abnormalities by the total spermatozoa counted and multiplying the figure by 100.

Hypo-osmotic swelling test (HOST) : Sperm membrane integrity was assessed using the hypo-osmotic swelling test according to the methods described by Correa and Zavos

(1994). Hypo-osmotic solution (Sodium citrate- 0.735 g; Fructose- 1.351 g; Millipore water- 100 ml and Osmolality- 150 mOsm kg^{-1}) was mixed with 0.1 ml of semen and incubated at 37°C for one hour. Following incubation, a drop of well-mixed solution was placed on a clean dry glass slide and covered with a cover-slip. 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 with 40× objectives under DIC phase contrast. 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.

Acrosome integrity : Staining was carried out as described by Hancock (1952). A thin smear of extended semen was prepared on a grease-free, clean and dry slide. The smear was air-dried at room temperature for at least 10 minutes in a current of warm air and was fixed by immersion in buffered formal saline (10 percent) for 15 minutes. Then it was washed in running tap water for 15-20 minutes and dried. The slide was immersed again in buffered Giemsa solution for 90 minutes, rinsed briefly in distilled water and dried. The dried smears were studied under 100× oil immersion objectives. About 200 spermatozoa were counted for acrosomal status after staining.

Statistical analysis

The effects of various additives on different sperm variables at different hours of storage at refrigerated temperature were examined by analysis of variance (Snedecor and Cochran, 1967). Prior to the analysis, proportionality data (motility, percent non-eosinophilic count, HOST, acrosome integrity and abnormality data) were transformed using arcsine [asin (sqrt (percent/100))] (Snedecor and Cochran, 1994) with adjustment to allow for zero values. Comparison between different treatment groups was done by Fisher's Least Significant Difference (LSD) test. The differences at $p \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The semen of the Karan Fries bulls under study was extended in extender with or without various additives such as Butylated Hydroxy Toluene (BHT), Pentoxifylline (PTX) and α-tocopherol (Vit E) and performance was evaluated at an interval of 0, 24, 48 and 72 h at refrigerated temperature (4-7°C).

Initial motility (percent)

The estimate of mass motility is not very precise. Progressive individual motility is one of the major criterions of semen quality (Lasley, 1942) and is an important determinant of the success rate of the fertilization. The mean percentages of sperm motility under various additives are presented in Table 1. These results show that from 0 to 48 h of incubation, motility was significantly ($p<0.05$) greater in BHT, PTX and Vit E as compared to the control, but after 72 h of incubation, only BHT and Vit E were significantly ($p<0.05$) better than the control group (11.26, 10.91 vs. 4.53). There was no significant ($p<0.05$) difference between any of the additives at all intervals of refrigeration.

Table 1. Effect of additives (C: control; BHT: Butylated Hydroxy Toluene; PTX: Pentoxifylline; Vit. E: α-tocopherol) on motility (percent) at different hours of preservation at refrigerated temperature (4-7°C) of Karan Fries semen (N = 24)

Hours	C		BHT		PTX		Vit. E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	53.61^{a}	1.63	66.76^{b}	2.01	64.34^{b}	1.75	65.45^{b}	1.82
24	30.13^{A}	1.85	46.05^{B}	2.02	43.74^{B}	1.91	45.22^{B}	1.93
48	12.06^{a}	2.06	22.67^{b}	2.25	20.16^{b}	2.15	21.27^{b}	2.21
72	4.53^{a}	1.50	11.26^{b}	2.05	8.71^{ac}	2.10	10.91^{bc}	2.04

Means bearing different superscripts within same row differ significantly (^{abc} $p<0.05$; ^{AB} $p<0.01$).

Table 2. Effect of additives (C: control; BHT: Butylated Hydroxy Toluene; PTX: Pentoxifylline; Vit. E: α-tocopherol) on Non-eosinophilic count (percent) at different hours of preservation at refrigerated temperature (4-7°C) of Karan Fries semen (N = 24)

Hours	C		BHT		PTX		Vit. E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	62.42^{a}	1.98	74.57^{b}	2.19	71.83^{b}	2.05	73.53^{b}	2.09
24	34.39^{A}	2.17	55.71^{B}	2.13	49.86^{B}	1.99	50.43^{B}	2.11
48	15.64^{a}	2.31	29.56^{b}	2.30	24.82^{b}	2.30	25.76^{b}	2.39
72	6.27^{a}	1.79	13.59^{b}	1.86	12.59^{b}	2.14	13.89^{b}	2.17

Means bearing different superscripts within same row differ significantly (^{ab} $p<0.05$; ^{AB} $p<0.01$).

Non- eosinophilic count (percent)

The viability (live/dead) stain was used to measure the ability of dyes (eosin) to pass through the sperm membrane. If the membrane is intact, as in the case of viable spermatozoa, passage of dye is prevented. If the membrane is broken, as is often the case with dead or dying spermatozoa, the dye will pass into the sperm cytoplasm. The live percent of spermatozoa can be predicted by initial motility of semen (Tomar, 1984), but the actual evaluation of live percentage is desirable. Semen samples having less than 50 percent live spermatozoa are of questionable fertilizing capacity, whereas samples containing 50-90 percent live spermatozoa show no difference in fertilizing capacity. Semen with more than 30 percent initial dead spermatozoa is not good for preservation. The mean percentage of non-eosinophilic counts under various additives are presented in Table 2. In Karan Fries bull semen, for all periods of incubation, non-eosinophilic count was significantly ($p<0.05$) higher with all three additives (BHT, PTX and Vit E) as compared to the control group, but there was no significant ($p<0.05$) difference between any of the additives for non-eosinophilic count.

Hypo osmotic swelling test (percent)

An important property of the sperm cell membrane is its ability to permit selective transport of molecules. HOST is very important to analyze the functional integrity of the sperm membrane because these characteristics are crucial for the viability and fertilizing ability of spermatozoa. The HOST is a simple, sensitive, inexpensive and easily reproducible test for assessing the functional integrity of sperm membranes after incubation under hypo-osmotic stress conditions. During the HOST, normal spermatozoa after exposure to hypo-osmotic stress undergo swelling, and subsequently increase in volume due to influx of water to establish equilibrium (Jeyendran et al., 1984). To identify the hypo-osmotic solution that causes maximum swelling of the spermatozoa, the hypo-osmotic medium should exert an osmotic stress large enough to cause an observable increase in volume, but small enough to prevent lysis of the sperm membrane (Zavos, 1990). This fact is more easily observable in the tail than in the head of the individual sperm (Takahashi et al., 1990) because the plasma membrane surrounding the tail appears to be more loosely attached than the membrane surrounding the head (Jeyendran et al., 1984). Sperm membrane integrity was assessed using the HOST. The mean percentages of HOST under various additives are presented in Table 3. In the Karan Fries bull semen, for all periods of incubation except after 72 h, BHT, PTX and Vit E were significantly ($p<0.05$) better than the control. After 72 h, there was no significant ($p>0.05$) difference among any of the additives.

Acrosome integrity (percent)

Acrosome reaction dysfunction has been proposed as one of the causes of fertilization failure. Therefore, assessment of acrosomal integrity is always a major part of the assessment of semen quality or spermaotozoal viability. Table 4 presents the mean percentages of acrosome integrity (Plate 17) under various additives. All three additives BHT, PTX and Vit. E, had similar effect on acrosome integrity up to 48 h of refrigeration; acrosomal integrity was significantly ($p<0.05$) better in BHT, PTX and Vit E than the control from 0-48 h with variable degree of significance

Table 3. Effect of additives (C: control; BHT: Butylated Hydroxy Toluene; PTX: Pentoxifylline; Vit. E: α-tocopherol) on HOST (percent) at different hours of preservation at refrigerated temperature (4-7°C) of Karan Fries semen (N = 24)

Hours	C		BHT		PTX		Vit. E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	45.05^{a}	1.62	55.95^{b}	1.82	55.50^{b}	1.72	57.98^{b}	1.65
24	20.73^{A}	1.58	35.31^{B}	2.07	36.60^{B}	1.89	37.41^{B}	1.96
48	9.89^{a}	1.87	18.86^{b}	1.84	16.22^{b}	1.39	17.28^{b}	1.50
72	2.14	1.59	3.87	1.85	3.66	1.79	5.05	1.80

Means bearing different superscripts within same row differ significantly (^{ab} $p<0.05$, ^{AB} $p<0.01$).

Table 4. Effect of additives (C: control; BHT: Butylated Hydroxy Toluene; PTX: Pentoxifylline; VIT E: α-tocopherol) on acrosome integrity (percent) at different hours of preservation at refrigerated temperature (4-7°C) of Karan Fries semen (N = 24)

Hours	C		BHT		PTX		Vit. E	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	63.88^{a}	1.87	77.37^{b}	1.97	75.94^{b}	1.88	73.89^{b}	1.89
24	44.96^{A}	1.96	59.63^{B}	2.03	59.04^{B}	1.93	58.58^{B}	2.04
48	22.52^{a}	2.41	36.6^{b}	2.02	32.53^{b}	2.28	33.83^{b}	2.23
72	16.17	1.78	22.3	1.71	21.85	1.6	21.15	1.59

Means bearing different superscripts within same row differ significantly (^{ab} $p<0.05$, ^{AB} $p<0.01$).

($p<0.01$ to 0.05). However, this trend was modified after 72 hours when there was no difference ($p>0.05$) for HOST (%) among the various additives and the control.

Sperm abnormalities (percent)

The most significant indicators of bull fertility are the percentages of sperm abnormalities (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; Barth and Bowman, 1994; Correa et al., 1997) and buffalo (Sengupta and Bhela, 1988). 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). The mean percentages of various types of sperm abnormalities under various additives are presented in Table 5.

Table 5 indicates that, compared to all the various sperm abnormalities, tail abnormality was the most prominent. There was no significant ($p<0.05$) difference between head and mid-piece abnormality for any of the additives at any period of incubation under study. Initially, tail abnormality was significantly ($p<0.05$) less in BHT, PTX and Vit E than the control. This was evident until the last period of incubation. A similar trend was portrayed by total abnormality as the tail abnormality constituted the major portion of total abnormality.

In the present study, the addition of BHT, PTX and VIT E during liquid storage improved sperm cell function (motility, viability, HOST, acrosome integrity). Incorporation of BHT, PTX with PBE (Pankaj, 2006) in Karan Fries, Murrah and Sahiwal bull semen, and Vit E (Raina et al., 2002) in Murrah buffalo bull semen at 4-7°C showed better effect on sperm cell function.

Antioxidant preservative (BHT) is used to stop auto-oxidation that causes a chain reaction in the unsaturated fatty acids in oils and lipid, and they help to slow down rancidity in fats and oils. BHT may also have antiviral and antimicrobial activities. Oxygen reacts preferentially with BHT rather than oxidizing fats or oils, thereby protecting them from spoilage. BHT is an organic soluble molecule which modifies the properties of the lipid bilayer and membrane of sperm cells (Hammerstedt et al., 1978). It is an antioxidant that has antiviral properties and sustains sperm viability during freezing and thawing. Addition of 0.5 mM BHT to whole milk extender during semen processing did not affect bull non-return rates (Anderson et al., 1994). BHT readily incorporates into sperm membranes and prevents damage after exposure to cold (Anderson et al., 1994). Killian et al. (1989) hypothesized that BHT serves as a scavenger of oxygen free radicals, which are associated with the diluent and sperm, to minimize damage to the sperm motility apparatus and membranes, and which also

Table 5. Effect of additives (C: control; BHT: Butylated Hydroxy Toluene; PTX: Pentoxifylline; Vit. E: α-tocopherol) on various types of abnormalities (percent) at different hours of preservation at refrigerated temperature (4-7°C) of Karan Fries semen (N = 24)

Abnormalities	Hours	C		BHT		PTX		Vit. E	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Head	0	3.48	0.56	3.87	0.79	3.96	0.77	3.75	0.82
	24	2.49	0.73	3.37	0.68	2.88	0.68	2.82	0.72
	48	3.52	0.85	2.13	0.82	2.64	0.76	2.78	0.69
	72	4.70	0.71	3.72	0.78	4.35	0.53	4.30	0.73
Mid-piece	0	2.86	0.56	3.24	0.89	2.95	0.88	3.00	0.85
	24	2.42	0.68	2.90	0.61	2.67	0.66	2.22	0.73
	48	2.77	0.68	2.00	0.76	2.27	0.73	2.05	0.73
	72	3.57	0.63	3.05	0.73	3.51	0.53	2.53	0.75
Tail	0	15.56^{A}	0.75	6.80^{B}	1.35	7.81^{B}	1.26	7.42^{B}	1.24
	24	32.43^{A}	0.86	18.44^{B}	1.11	19.59^{B}	0.87	19.70^{B}	0.96
	48	38.22^{a}	0.94	30.92^{b}	0.87	33.32^{b}	0.66	33.47^{b}	0.77
	72	48.67^{A}	0.90	40.35^{B}	0.82	41.41^{B}	0.95	41.04^{B}	0.80
Total	0	22.10^{a}	1.11	14.25^{b}	1.83	15.02	1.76	14.45	1.76
	24	37.91^{A}	1.26	25.02^{B}	1.50	25.50^{B}	1.27	25.17^{B}	1.37
	48	45.31^{a}	1.36	35.76^{b}	1.31	38.88^{b}	1.10	38.90^{b}	1.16
	72	57.79^{A}	1.45	47.90^{B}	1.33	49.73^{B}	1.29	48.63^{B}	1.31

Means bearing different superscripts within same row differ significantly (^{ab} $p<0.05$, ^{AB} $p<0.01$).

may affect motility indirectly. 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). The use of BHT improved viability of frozen and thawed sperm (Anderson et al., 1994) and inactivated lipid-containing viruses (Anderson et al., 1994). Addition of BHT to semen extenders prior to freezing may improve the fertility of bull semen and reduce the risk of transmitting viral diseases to cows during AI.

Previous studies suggested that BHT effectively prevents cold shock of sperm during rapid cooling. 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. Anderson et al. (1994) 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. However, Anderson et al. (1994) also concluded that BHT added to semen during extension did not affect bull fertility. 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 interacted synergistically with BHT to protect spermatozoa from cold shock.

Bamba and Cran (1992) reported that addition of 0.05-0.1 mmol L^{-1} BHT improved progressive motility and acrosome integrity. On the contrary, Balla et al. (2001) found no significant improvement in the maintenance of motility during liquid semen storage at 5°C, whereas Killian et al. (1989) observed that treatment of bull spermatozoa with 0.5 and 0.75 mM BHT L^{-1} had no effect on motility improvement. Findings of the present study are in agreement with the findings of Pankaj (2006), who reported significant improvement of motility, acrosome integrity, plasma membrane integrity and preservation of liveability of spermatozoa preserved in PBE fortified with BHT on storage at refrigerator temperature (4 to 7°C). During semen processing, addition of 0.5 mM BHT to whole milk extender did not affect non-return rates of bulls (Anderson et al., 1994). All these workers did their experiments using only good quality semen and may account for the observation that, in most cases, addition of BHT did not improve bull fertility. 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, Pentoxifylline has been used. Methyl xanthine supplementation resulted in better seminal characteristics in fresh and cryopreserved spermatozoa viz., motility and curvilinear velocity. A stimulatory effect of methyl xanthine on capacitation and acrosome reaction has also been demonstrated (Aitken et al., 1993). Overall, the addition of methyl xanthine to sperm suspension seems to improve sperm function leading to better sperm fertilizing ability (Esteves et al., 2007). Pentoxifylline (PTX) is a methylxanthine phosphodiesterase inhibitor which reduces superoxide anions responsible for DNA apoptosis when used at a concentration of 3.6 mM (Maxwell et al., 2002). In contrast to *in-vitro* exercises with higher concentrations, no essential influence may be expected on mobility of spermatozoa on oral administration of Pentoxifylline because of the low concentration in seminal fluid.

Pentoxifylline and cAMP (cyclic adenosine mono-phosphate) seemed to increase the duration of activity of spermatozoa. The effects of adding cAMP seem to confirm that an increase of the intracellular content of this compound could determine a longer-lasting activity of ejaculated human spermatozoa. Pentoxifylline may be added to boost the motility of sperm. The use of Pentoxifylline or related compounds for stimulation of motility of spermatozoa has been reported earlier (Aitken et al., 1997), but not for increasing the life span of the sperm or for reducing PMN recruitment. It has been suggested that PDEs reduce the decomposition of cAMP. Increasing the concentration of cAMP may also be achieved by its addition or by using compounds that stimulate the formation of cAMP and tyrosine-phosphorylation at the tail level. cAMP has a role in sperm kinematics and in the acrosome reaction second-messenger system. Treatments that increase intracellular cAMP concentrations often cause an increase in sperm motility and kinematics as well as in the agonist-induced acrosome reaction and fertilization rates. It has been postulated also that PTX has the ability to scavenge reactive oxygen radicals (Esteves et al., 2007). Compounds such as dibutyryl cAMP that stimulate the formation of cAMP may do so by stimulating the enzyme adenylate cyclase (AC stimulator). PDEs and cAMP-increasing compounds or compositions thereof are considered as functional equivalents. Accordingly, PDEs can be replaced wholly or partially by other compounds or compositions that increase the amount of cAMP (Perry and Higgs, 1998).

The phosphodiesterase inhibitors, or their functional equivalents, can be added to the composition comprising sperm at any moment prior to insemination. Suitable moments for adding the phosphodiesterase inhibitor include directly after collecting the ejaculate or during the processing of the ejaculate to dosages in a form and amount suitable for artificial insemination. The phosphodiesterase inhibitor (PDE), or functional equivalent thereof, used when

the animal is inseminated with semen enables a lower dose of sperm to be used, wherein insemination performed using the techniques of the present study provide a similar fertilization rate as if a larger dose of sperm were used. Also, a longer interval of time between insemination and ovulation becomes permissible without significantly affecting the fertilization rates. They prolonged fertility arises since phagocytosis of the sperm is strongly reduced. This maintains the population of sperm in the uterus at a higher level than what is found with conventional insemination. Thus, the population of sperm in the oviducts is maintained or sustained for a prolonged period of time and strongly increases the chances of fertilization. It also pertains to a method for reducing the recruitment of leukocytes within an artificially inseminated mammal by adding a phosphodiesterase inhibitor or functional equivalent thereof.

Enhancement of the antioxidant capacity of semen could present a major opportunity for improving male fertility. The beneficial consequences of an effective protection against lipid peroxidation are likely to result from two related mechanisms. First, defence against peroxidative damage is essential to maintain the structural integrity of the spermatozoa. Second, minimization of lipid peroxidation will prevent any reduction in the concentrations of the functionally important polyunsaturated fatty acids of the sperm phospholipids. Additional possibilities may also be envisaged for the use of antioxidants in improving the viability of semen during preservation.

Pentoxifylline improves the movement characteristics in most asthenozoospermic individuals (Tesariki et al., 1992; Yunes et al., 2005). Addition of Pentoxifylline increased the post-thaw motility in human semen (Bell et al., 1993; Aribarg et al., 1994), in cat semen (James et al., 1994), as well as post-thaw sperm fertilizing ability in human semen (Esteves et al., 2007), whereas Stanic et al. (2002) reported a detrimental effect on human semen. Vega (1997) reported that addition of Pentoxifylline (6.0 mM) prolonged the viability of post-thaw bovine semen. Apart from modulation of sperm function, a protective effect on the sperm membrane by Pentoxifylline has been demonstrated. This effect may be ascribed to neutralization of reactive oxygen species (ROS) and reduction of lipid peroxidation (Bell et al., 1993). Glogowski et al. (2002) also reported alkaline phosphatase inhibition was 27% for seminal plasma and 35% for spermatozoa by addition of 20 mM pentoxyfylline. No significant effect on sperm mobility was found when pentoxifylline was added to semen at 2.5, 5, or 10 mM at 5°C (Parkhurst et al., 2000). Rizk et al. (2005) found that fertilization rate was significantly higher in oocytes which were inseminated with spermatozoa treated with PF compared with controls (56.3 versus 30.7%; $p<0.05$). Findings of the present study compare well with the findings of Pankaj (2006) who reported significant improvement of motility, acrosome integrity, plasma membrane integrity and preservation of liveability of spermatozoa preserved in PBE fortified with BHT on storage at refrigerator temperature (4 to 7°C).

An important cellular system of protection against oxidative damage is α-tocopherol (Vit E), an important natural antioxidant in both feeds and biological systems. α-Tocopherol, a lipophilic component, not only scavenges oxygen radicals from within the membrane but also intercepts lipid peroxyl radicals which appear to be important in the propagation of the chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1999). α-Tocopherol is a powerful antioxidant and has been shown to afford mammalian cells some protection from oxidative attack both *in vivo* (Mickle et al., 1989) and *in vitro* (Wu et al., 1990). This chain-breaking antioxidant is not synthesized by mammalian cells and once membrane tocopherol is consumed during periods of oxidative stress, cellular lipids are subjected to peroxidation, which can result in toxic damage (Zhang et al., 2001). Through inhibition of lipid peroxidation Vit E protects the sperm plasma membrane (Pena et al., 2003). As the intracellular ATP level increases there is a decline in abnormal acrosome reaction and sperm motility thereby improves (Breininger et al., 2005). It also helps in preserving metabolic activity and cellular viability since cell permeability and enzyme inactivation declines (Verma and Kanwar, 1998; Almedia and Ball, 2005).

Although numerous studies have examined the addition of vitamin E or its derivatives to semen in order to improve sperm preservation during storage of liquid semen (Aguero et al., 1995; Donoghue and Donoghue, 1997; Upreti et al., 1997; Donnelly et al., 1999; Cerolini et al., 2000; Balla et al., 2001), there have not been consistent improvements in the maintenance of sperm motility or fertility during liquid semen storage with addition of vitamin E or its analogs. Addition of α-tocopherol did not improve maintenance of motility during liquid semen storage in equine (Balla et al., 2001) and in ram semen (Upreti et al., 1997), although other reports suggested a positive response to addition of α-tocopherol during storage of equine (Aguero et al., 1995), ram (Sarlos et al., 2002), turkey (Douard et al., 2004) and boar semen (Breininger et al., 2005). During storage of boar semen at 19°C, the addition of α-tocopherol reduced lipid peroxidation as measured by thiobarbituric acid reactive substance (TBARS) production and improved maintenance of sperm viability during liquid storage for a period of 5 days (Cerolini et al., 2000). Likewise, in poultry, some studies demonstrated a positive effect of addition of α-tocopherol on maintenance of sperm viability (Donoghue and Donoghue, 1997) whereas other studies have

demonstrated an increase in lipid peroxidation during storage that was unaffected by addition of vitamin E (Long and Kramer, 2003; Douard et al., 2004). There were reports on improvement of post-thaw quality from α-tocopherol addition in bull (Al-Khanak, 1989), human (Askari et al., 1994), sheep (Herdis et al., 2002) and buffalo bull semen (Raina et al., 2002; Metwelly, 2004). However, Shukla and Misra (2005) found no significant effect of α-tocopherol addition in Murrah buffalo bull semen. Herdis et al. (2002) reported that alpha-tocopherol treated sperm had significantly higher post-thaw live percentage (75% vs. 64.8%) and membrane-intact percentage (65.8% vs. 55.2%) than controls ($p<0.05$), but not significantly different from that with BHT addition. The presence of alpha-tocopherol resulted in higher ($p<0.05$) motility percentage (45.8%) than BHT addition (40%), but not different from the control (41.7%). Acrosomal intact percentage after alpha-tocopherol addition (54.8%) was higher ($p<0.05$) compared to BHT addition (49.7%) or the control (49.8%). However, in the present study there was no significant ($p>0.05$) difference of semen quality parameters among Vit E and BHT during any hour of preservation at refrigerated temperature.

Although BHT, PTX and Vit E have been added during short-term storage of liquid semen from a variety of species, the outcome relative to improving motility parameters during storage has varied with species and study. It may be concluded that when poor quality semen was treated with additives (BHT, PTX and Vit E) there is more beneficial effect in terms of semen quality and preservability of spermatozoa. It has also been found that sperm treatment with additives improved acrosome integrity and HOST percentage during storage at refrigerated temperature. This strategy may be useful because it can improve the fertilizing potential of liquid semen from low-grade ejaculates. Increasing demand for artificial insemination and the aim of sperm banking to preserve more semen with better fertility can be achieved by using this strategy in poor quality semen, which is generally discarded at semen stations.

REFERENCES

Aguero, A., M. H. Miragaya, N. G. Mora, M. G. Chaves, D. M. Neild and M. T. Beconi. 1995. Effect of vitamin E addition on equine sperm preservation. Comunicaciones Biologicas 13:343-356.

Aitken, R. J. 1995. Free radicals, lipid peroxidation and sperm function. Reprod. Fertil. Dev. 7:659-668.

Aitken, R. J., F. Best, D. W. Richardson, R. Schats and G. Simm. 1993. Influence of caffeine on movement characteristics, fertility capacity and ability to penetrate cervical mucus of human spermatozoa. J. Reprod. Fertil. 67:19-27.

Aitken, R. J., H. M. Fisher, N. Fulton, E. Gomez, W. Knox, B. Lewis and S. Irvine. 1997. Reactive oxygen species generated by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. Mol. Reprod. Dev. 47:468-482.

Al Khanak, K. 1989. The protective action of tocopherol during cryopreservation of bull semen. Zhivotnov dni Nauki 26(8):70-74.

Almeida, J. and A. Ball. 2005. Effect of α-tocopherol and tocopherol succinate on lipid peroxidation in equine spermatozoa. Anim. Reprod. Sci. 87:321-327.

Anderson, S., W. Harkness, Y. Akin, M. Kaproth and G. Killian. 1994. Categorial data analysis of the effect on bull fertility of butylated hydroxytoluene addition to semen extenders prior to freezing. J. Dairy Sci. 77(8):2302-2307.

Aribarg, A., N. Sukcharoen, U. Jetsawangsri, Y. Chanprasit and J. Ngeamvijawat. 1994. Effects of pentoxifylline on sperm motility characteristics and motility longevity of postthaw cryopreserved semen using computer-assisted semen analysis (CASA). J. Med. Assoc. Thai. 77(2):71-75.

Askari, H. A., J. H. Check, N. Peymer and A. Bollendorf. 1994. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Argiives of Andrology 33:11-15.

Balla, B. A., V. Medinab, C. G. Gravancea and J. Baumbera. 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. Theriogenology 56(4):577-589.

Bamba, K. and D. G. Cran. 1992. Effects of treatment with butylated hydroxytoluene on the susceptibility of boar spermatozoa to cold stress and dilution. J. Reprod. Fertil. 95:65-77.

Barth, A. D. and P. A. Bowman. 1994. The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. Can. Vet. J. 35:93-102.

Barth, A. D. and R. J. Oko. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University press, Ames. pp. 17-37.

Baumber, J., B. A. Ball, C. G. Gravance, V. Medina and M. C. G. Davies-morel. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosome integrity, mitochondrial membrane potential and membrane lipid peroxidation. J. Androl. 21:895-902.

Bell, M. R. Wang, W. J. G. Hellstrom and S. C. Sikkaf. 1993. Effect of cryoprotective additives and cryopreservation protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. J. Androl. 14:472-478.

Bratton, R. W., R. H. Foote, C. R. Henderson, S. D. Musgrave, H. O. Dunbar and J. P. Beardsly. 1956. The relative usefulness of combinations of laboratory tests for predicting the fertility of bovine semen. J. Dairy Sci. 39:1542-1549.

Breininger, E., B. Beorlegui, M. Cristian and T. Beconi. 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. Theriogenology 63: 2126-2135.

Cerolini, S., A. Maldjian, P. Surai and R. Nobel. 2000. Viability and susceptibility to lipid peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci. 58: 99-11.

Correa, J. R., M. M. Pace and P. M. Zavos. 1997. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of

bull in an artificial inseminations program. Theriogenology 48: 721-731.

Correa, J. R. and P. M. Zavos. 1994. The hypo-osmotic swelling test; its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology 42:351-360.

Donnelly, E. T., N. McClure and S. E. Lewis. 1999. Antioxidant supplementation *in vitro* does not improve human sperm motility. Fertil. Steril. 72:484-495.

Douard, V., D. Hermier, M. Magistrini, C. Labbe and E. Blesbois. 2004. Impact of changes in composition of storage medium on lipid content and quality of turkey spermatozoa. Theriogenology 61(1):1-13.

Esteves, S. C., D. M. Spaine and A. P. Cedenho. 2007. Effects of pentoxyfylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopreserved by the liquid nitrogen vapor method. Braz. J. Med. Biol. Res. 40(7):985-992.

Glogowski, J., R. D. Douglas and A. Ciereszko. 2002. Inhibition of alkaline phosphatase activity of boar semen by pentoxifylline, caffeine, and theophylline. J. Androl. 23(6):772-780.

Graham, J. K. and R. H. Hammerstedt. 1992. Differential effects of butylated hydroxytoluene analogs on bull sperm subjected to cold-induced membrane stress. Cryo. 29(1):106-117.

Hammerstedt, R. H., A. D. Keith, W. Snipes, R. P. Amann, D. Arruda and L. C. Grief. 1978. Use of spin labels to evaluate effects of cold shock and osmolarity on sperm. Biol. Reprod. 18(4):686-696.

Hancock, J. L. 1952. The morphology of bull spermatozoa. J. Exp. Biol. 29:445-453.

Herdis, I. Kusuma, M. Surachman, M. Riza, I. K. Sutama, I. Inounu, B. Purwantara and I. Arifiantini. 2002. Improvement of frozen semen quality of Garut Sheep through the addition of alpha -tocopherol into yolk egg-skim milk diluent. Jurnal Ilmu Ternak dan Veteriner 7(1):12-17.

James, J. S., A. G. Kenneth and D. R. Armant. 1994. Stimulation of cryopreserved epididymal spermatozoa ofthe domestic cat using the motility stimulants caffeine, pentoxifylline, and 2'-deoxyadenosine. J. Androl. 15:157-164.

Jeyendran, R. S., H. H. van der Ven, M. Perez Pelaez, B. G. Crabo and L. J. D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70:219-228.

Johnson, W. H. 1997. The significance of bull fertility of morphologically abnormal sperm. In: Bull fertility (Ed. S. D. Van Camp). Veterinary clinics of North America. Food Anim. Pract. 13:255-270.

Jones, R. and T. Mann. 1973. Lipid peroxidation in spermatozoa. Proc. Roy. Soc. London B. 184:103-107.

Jones, R., T. Mann and R. J. Sherins. 1979. Peroxidative breakdown of phospholipids in human spermatozoa: Spermicidal effect of fatty acid peroxides and protective action of seminal plasma. Fertil. Steril. 31:531-537.

Killian, G., T. Honadel, T. Mcnutt, M. Henault, C. Wegner and D. Dunlap. 1989. Evaluation of BHT as a cryopreservative added to whole or skim milk diluents for bull semen. J. Dairy Sci. 72(5):1291-1295.

Lasley, J. F. and R. Bogart. 1942. Some factors influencing the resistance of bull sperm to unfavourable environmental conditions. J. Anim. Sci. 1:79.

Linford, E., F. A. Glover, C. Bishop and D. L. Stewart. 1976. The relationship between semen evaluation methods and fertility in bull. J. Reprod. Fertil. 47:283-291.

Long, J. A. and M. Kramer. 2003. Effect of vitamin E on lipid peroxidation and fertility after artificial insemination with liquid-stored turkey semen. Poult. Sci. 82(11):1802-1807.

Maxwell, D. T., J. D. Jacobson, A. King and P. J. Chan. 2002. Effect of pentoxifylline on tumor suppressor and proto-oncogene apoptosis in sperm. J. Assist. Reprod. Genet. 19(6): 279-283.

Metwelly, K. K. 2004. Fertilizing capacity of frozen buffalo-bull semen treated by calcium channel blocker (Verapamil) and/or antioxidant (alpha-tocopherol). Assiut Veterinary Medical Journal 50(100):217-227.

Mickle, D. A., R. K. Li, R. D. Weisel, P. L. Birnbaum, T. W. Wu, G. Jackowski, M. M. Mandonik, G. W. Burton and K. U. Ingold. 1989. Myocardial salvage with trolox and ascorbic acid for an acute evolving infarction. Ann. Thorac. Surg. 47:553-557.

Pankaj, P. K. 2006. Study of critical control points associated with cryopreservation of bovine semen. Ph.D. Thesis, NDRI Deemed University, Karnal, Haryana.

Parkhurst, A. M., N. Korn and R. J. Thurston. 2000. The effects of methylxanthines on the mobility of stored turkey sperm. Poult. Sci. 79(12):1803-1809.

Pena, A. I., L. L. Lugilde, M. Barrio, P. G. Herradon and L. A. Quintela. 2003. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca^{2+} concentration of dog spermatozoa. Theriogenology 59(8):1725-1739.

Perry, M. J. and G. A. Higgs. 1998. Chemotherapeutic potential of phosphodiesterase inhibitors. Curr. Opin. Chem. Biol. 2:472-481.

Raina, V. S., A. K. Gupta and K. Singh. 2002. Effect of antioxidant fortification on preservability of buffalo semen. Asian-Aust. J. Anim. Sci. 15(1):16-18.

Rizk, B., S. Fountain, S. Avery, C. Palmer, M. Blayney, M. Macnamee, C. Mills and P. Brinsden. 2005. Successful use of pentoxifylline in male-factor infertility and previous failure of *in vitro* fertilization: A prospective randomized study. J. Assisted Reprodn. Genet. 12(10):710-714.

Saacke, R. G. 1990. What is abnormal? And is abnormal dependent upon the animal? In Proc. 13th Tech. Conf. AI Reprod., Milwaukee, WI. Pp 67. Natl. Assoc. Anim. Breeders, Columbia, MO.

Saacke, R. G. and J. M. White. 1972. Semen quality tests and their relationship to fertility. In: Proceedings of the fourth NAAB Technical Conference on Artificial Insemination in Reproduction. Madison, WI; p. 22-27.

Sarlos, P., A. Molnar, M. Kokai, G. Gabor and J. Ratky. 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Vet. Hung. 50(2):235-245.

Sekoni, V. O. and B. K. Gustafsson. 1987. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. Br. Vet. J. 143: 312-317.

Sengupta, B. P. and S. L. Bhela. 1988. Current status of male

fertility research in buffaloes. An overview. Proc.II. World Buffalo Congr. New Delhi, India. p. 63.

Shukla, M. S. and A. K. Misra. 2005. Effect of antioxidants alpha-tocopherol, ascorbic acid and n-propyl gallate on Murrah semen cryopreservation. Buffalo J. 21(1):27-38.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical methods. Iowa State University Press, Ames.

Snedecor, G. W. and W. G. Cochran. 1994. Statistical methods. 6th edn., Oxford and IBH publ. co., New Delhi.

Stanic, P., Z. Sonicki and E. Suchanek. 2002. Effect of pentoxifylline on motility and membrane integrity of cryopreserved human spermatozoa. Int. J. Androl. 25(3):186-190.

Storey, B. T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol. Hum. Reprod. 3:203-213.

Strzezek, J. 2002. Secretory activity of boar seminal vesicle glands. Reprod. Biol. 2:243-266.

Strzezek, J., S. Lapkiewicz and M. Lecewicz. 1999. A note on antioxidant capacity of boar seminal plasma. Anim. Sci. Pap. Rep. 17:181-188.

Takahasi, K., A. Vehida and M. Kitao. 1990. Hypo-osmotic swelling test of sperm. Arch. Androl. 25:225-242.

Tesariki, J., A. Thébault and J. Testart. 1992. Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. Hum. Reprod. 7(9):1257-1263.

Tomar, N. S. 1984. Artificial insemination and reproduction of cattle and buffaloes, 3rd edn. Saroj Prakashan Publishers Allahabad, UP, India.

Upreti, G., K. Jensen, J. Oliver, D. Duganzich, R. Munday and J. Smith. 1997. Motility of ram spermatozoa during storage in a chemically defined diluent containing antioxidants. Anim. Reprod. Sci. 48:269-278.

Vega, W. G. 1997. Proc. 5th world buffalo congress, Caserta, Italy. Vol.1:103-123.

Verma, A. and K. C. Kanwar. 1998. Human sperm motility and lipid peroxidation in different ascorbic acid concentrations: An *in vitro* analysis. Androlgia 30:325-329.

Wu, T. W., N. Hashimoto, J. Wu, D. Carey, R. K. Li, D. A. Mickle and R. D. Weisel. 1990. The cryoprotective effect of trolox demonstrated with three types of human cells. Biochem. Cell Biol. 68:1189-1194.

Yunes, R., P. Fernández, G. F. Doncel and A. A. Acosta. 2005. Cyclic nucleotide phosphodiesterase inhibition increases tyrosine phosphorylation and hyper motility in normal and pathological human spermatozoa. Biocell 29(3):287-293.

Zavos, P. M. 1990. Hypo-osmotic swelling test (HOS) functional integrity of sperm membrane. J. Assit. Reprod. Tech. Androl. 2:215-216.

Zhang, J. G., F. A. Nicholls-Grzemski, M. A. Tirmenstein and M. W. Fariss. 2001. Vitamin E succinate protects hepatocytes against the toxic effect of reactive oxygen species generated at mitochondrial complexes I and III by alkylating agents. Chem. Biol. Interact. 138:267-284.