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Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage [1]

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Abstract

The changes in viability, susceptibility to peroxidation and fatty acid composition of total phospholipid were studied in boar spermatozoa during 5 day liquid storage in a standard or α-tocopherol (αT) enriched diluent. The sperm rich fraction of the ejaculates was collected from 6-month old boars. Sperm viability progressively decreased during storage and αT inclusion into the diluent significantly inhibited this trend. αT inclusion also decreased significantly peroxidation (TBARS production of spermatozoa). Spermatozoa stored in the treatment diluent became rapidly enriched in αT with a concomitant decrease of αT content in the medium. The proportion of polyunsaturates, mainly 22:6$n-3$, decreased with a complementary increase in the content of the saturates, mainly 18:0. The inclusion of αT into the diluent was effective in totally preventing the significant decrease of 22:6$n-3$ observed in sperm phospholipid in the control samples during the storage period. It is concluded that the αT inclusion in the boar semen diluent increased cell viability through its prevention of an oxidative reduction in the levels of the major polyunsaturated fatty acids, namely 22:6$n-3$.

Keywords: Boar semen; Liquid storage; Viability; α-Tocopherol; Fatty acids

1. Introduction

In commercial practice boar semen used for artificial insemination is stored at 17–19°C following the addition of an appropriate diluent. The success of a selection of

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boar semen extenders has been studied over storage periods of 5 to 7 days (Revell and Glossop, 1989; Machaty et al., 1992; Waberski et al., 1994; Alexopoulos et al., 1996; Korniewicz et al., 1996; Laforest and Allard, 1996; Lyczynski and Kolat, 1996). Nevertheless, the diluents are not able to completely prevent changes to a range of essential features for sperm functions which rapidly occur under in vitro conditions (Revell and Glossop, 1989; Alexopoulos et al., 1996; Laforest and Allard, 1996).

As a result of such structural and biochemical changes a significant reduction in fertilising capacity and insemination efficiency has been routinely observed (Waberski et al., 1994; Alexopoulos et al., 1996; Korniewicz et al., 1996; Laforest and Allard, 1996; Lyczynski and Kolat, 1996).

An increased concentration of the insemination dose has therefore been used in order to compensate for the reduced fertilising ability of stored vs. fresh spermatozoa but the results reported so far show wide inconsistencies and discrepancies (Machaty et al., 1992; Alexopoulos et al., 1996).

The molecular mechanisms responsible for the sperm deterioration during in vitro storage are not clear. Evidence is accumulating that changes in the sperm membrane structure and thus in membrane properties are responsible for the decreased fertilising ability of stored spermatozoa. For example, the ability of the molecular organisation of the sperm membrane to respond to cooling may be impaired through an inability to change fluidity (Buhr et al., 1989). Fluidity is linked to the integrity of the membrane lipids (Stubbs and Smith, 1984) and changes in the lipid composition of the plasma membrane may therefore be associated with the cooling and storage effects. As spermatozoan cells are characterised by a high level of polyunsaturated fatty acids, lipid peroxidation may be one of the mechanisms responsible for the negative biochemical and physiological changes during sperm storage. However, data on the changes in sperm lipid composition and their susceptibility to peroxidation during storage are limited.

The aim of the present work was to study the changes in viability, susceptibility to peroxidation and fatty acid composition of total phospholipid in boar spermatozoa during 5 day storage at 19°C in a standard or α-tocopherol enriched diluent.

2. Materials and methods

2.1. Semen collection and dilution

The sperm rich fraction of the ejaculate was collected from 6-month old boars ($n = 3$). Semen samples were divided into 2 aliquots which were then diluted with a BTS (Beltsville Thaw Solution) standard extender (D + glucose 41 g, sodium hydrogen carbonate 1.25 g, EDTA 1.25 g, trisodium citrate 6 g, potassium chloride 0.75 g, Lyncaspectin 4.4 ml/l, all diluted to 1 l in distilled water) (Machaty et al., 1992) and BTS-αT (DL α-tocopherol, Fluka, UK; 0.2 mg/ml) extender. All samples were diluted to 70×10^6 cells/ml and stored for 5 days at 19°C with gentle shaking. Each day over a 5 day period, sperm viability by the SYBR14/propidium iodide procedure (Garner and

Johnson, 1995) was measured and 10 ml (7×10^8 total sperm) were taken from each bottle and centrifuged at 700 g at 20°C for 20 min; following washing twice with BTS, the cell pellet was used for subsequent analysis. Sperm viability was assessed by the SYBR14 and propidium iodide (PI) procedure (Garner and Johnson, 1995); both fluorescent dies, SYBR14 and PI, were supplied in the LIVE/DEAD sperm viability kit (Molecular Probe, Eugene, OR, USA); 0.4 ml diluted semen was incubated with 4 μl SYBR14 (28.25 μg/ml) for 10′ and then with 5 μl PI (1.60 mg/ml) for 5′ at 36°C, at least 200 cells were counted at the fluorescent microscope and classified as follow: live cells fluoresced bright green, dead cells fluoresced red and damaged cells fluoresced both green and red.

2.2. Lipid analysis

Total lipid was extracted from the cell pellet following homogenisation in a suitable excess of chloroform:methanol (2:1 v:v) (Christie, 1982). Phospholipid (PL) was separated by thin layer chromatography on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1 v:v:v). After visualisation under UV light following spraying with 0.1% (w:v) 2,7-dichlorofluorescein in methanol, the PL band was scraped from the plate and the PL eluted from the silica by washing twice with methanol. The fatty acids were *trans*-methylated by refluxing with methanol:toluene:sulphuric acid (20:10:1 v:v:v) in the presence of a pentadecanoic acid standard (Hamilton et al., 1992) and quantified by gas chromatography by a standard procedure using a capillary column (Carbowax, 30 m × 0.25 mm, film thickness 0.25 μm; Altech, Carnforth, UK) in a CP9001 instrument (Chrompack, Middleburg, The Netherlands) connected to an EZ Chrom data system (Scientific Software, San Ramon, CA, USA). The data processing system enabled the expression of the fatty acid composition in terms of proportion by weight. The identification of the peaks was confirmed by comparison with the retention times of standard fatty acid methyl ester mixtures (Sigma, Poole, UK).

2.3. Quantification of lipid peroxidation

The susceptibility of the spermatozoa to peroxidation by thiobarbituric acid reactive substance (TBARS) production and the α-tocopherol (αT) contents were measured on day 0, 3 and 5 of storage. Semen samples were mixed with 0.01 M sodium phosphate buffer (pH 7.4) containing 1.15% (w:v) KCl. The homogenates were incubated for 30 min at 37°C in the presence of $FeSO_4$ (0.1 mM), butylated hydroxytoluene was added to a final concentration of 0.01% (v:v) and the concentration of TBARS determined by the spectrophotometric method of Ohkawa et al. (1979). The amount of malonaldehyde (MDA) produced was quantified against a standard curve at 525 nm wavelength. αT was measured by High Performance Liquid Chromatography method of McMurray et al. (1980) involving saponification with alcoholic potassium hydroxide in the presence of pyrogallol for 30 min at 70°C and extraction with light petroleum spirit. The mobile

phase was methanol:water (97:3) and the flow rate was of 1.05 ml/min. Calibration was performed using standard solutions of α-tocopherol in methanol.

2.4. Statistical analysis

Analysis of variance was performed using the SAS® GLM procedure (SAS, 1997). A nested model was used; the treatment(sample), the day of storage and the interaction (treatment*day of storage) were considered as sources of variation. The difference between least square means was tested by the Student's *t*-test.

3. Results

3.1. Sperm viability

The effects of storage, treatment and their interaction on the proportions of live and dead cells and damaged cells are shown in Figs. 1–3 and the analysis of variance in Table 1. The day of storage, the treatment and their interaction had a significant effect on the proportion of live and dead cells, whereas only the day of storage had a significant effect on the proportion of damaged cells. The proportion of live cells progressively decreased from the 2nd day of incubation to reach a minimum value at day 4. The change in the proportion of dead spermatozoa was complementary to that

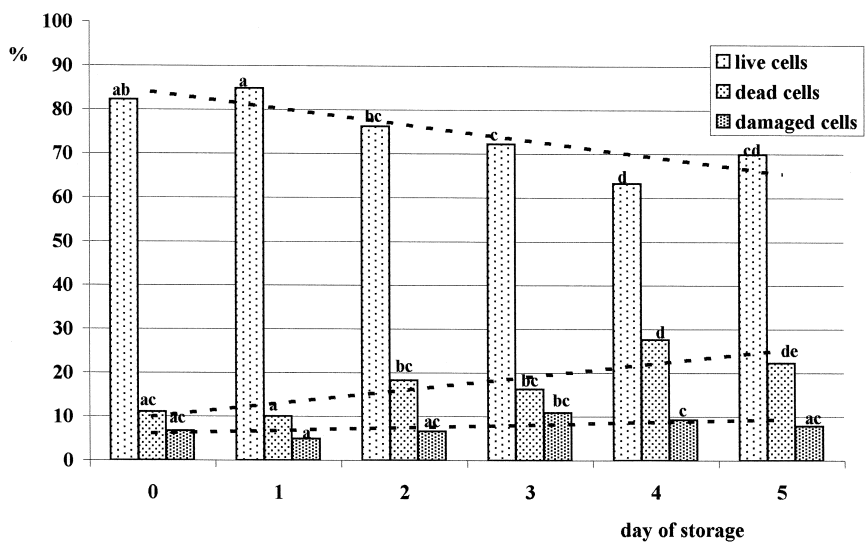

Fig. 1. Percentage of live (SE < 3% of the mean), dead and damaged boar spermatozoa during 5 day storage at 19°C (bars with different letters are significantly different between days within group) (standard errors are lower than 3% of the mean value for live cells and 20% of the mean value for dead and damaged cells).

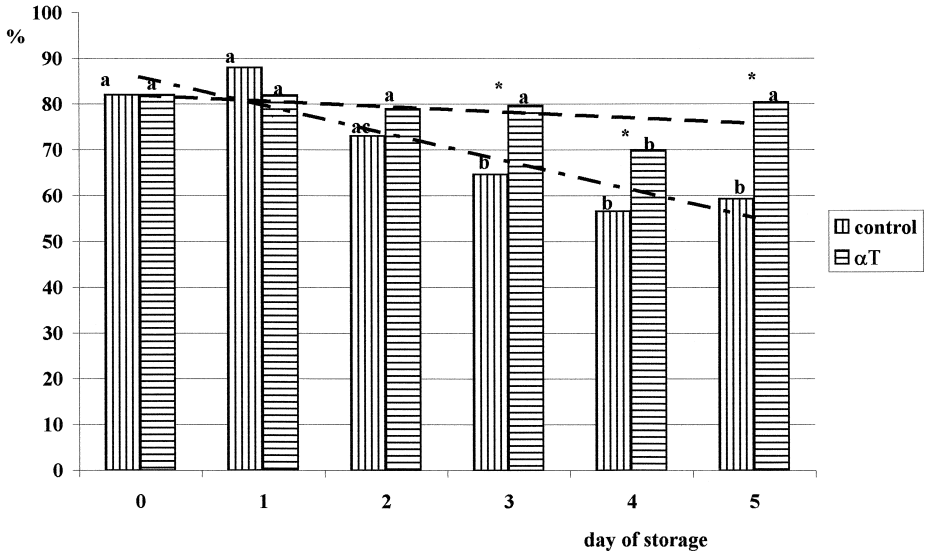

Fig. 2. Percentage of live boar spermatozoa during 5 day storage in a control or αT enriched diluent at 19°C (bars with different letters are significantly different between days within group; * shows a significant difference between groups within day) (standard errors are lower than 6% of the mean value).

observed for the live cells, whereas the proportion of damaged cells showed no apparent change over the entire 5 days of storage. The inclusion of αT into the diluent prevented the significant decrease in cell viability and, as a result, there was a significantly higher

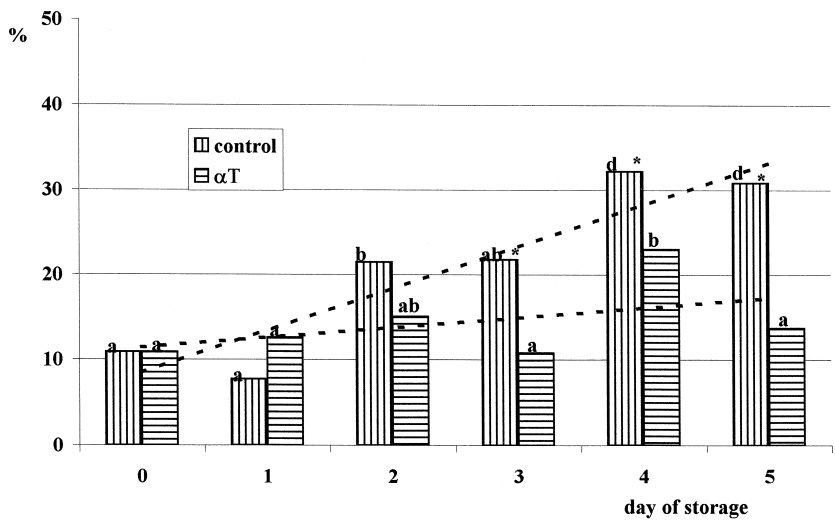

Fig. 3. Percentage of dead boar spermatozoa during 5 day storage in a control or αT enriched diluent at 19°C (bars with different letters are significantly different between days within group; * shows a significant difference between groups within day) (standard errors are lower than 25% of the mean value).

Table 1
Results of the analysis of variance: probability (Pr > F) values of the sources of variations considered (ns = not significant)

Variable	Sources of variations		
	Treatment	Day of storage	Interaction
Live cells	0.012	0.001	0.005
Dead cells	0.015	0.001	0.010
Damaged cells	ns	0.046	ns
PL saturates	ns	0.002	ns
PL PUFA	ns	0.037	ns
C14:0	ns	ns	ns
C16:0	ns	ns	ns
C18:0	ns	0.006	ns
C18:1	ns	0.003	ns
C18:2$n-6$	ns	0.002	ns
C20:4$n-6$	ns	0.005	ns
C22:4$n-6$	ns	ns	ns
C22:5$n-6$	ns	ns	ns
C22:6$n-3$	ns	ns	ns

proportion of live cells compared to the control at days 3, 4 and 5. As a result spermatozoa viability did not change between day 0 and day 5 in the αT group whereas it significantly decreased in the control group.

3.2. Susceptibility to peroxidation

The effect of storage and treatments on αT and TBARS concentrations in the spermatozoa are given in Table 2. No statistical differences were found in the level of αT in the control group between days 0 and 5 of storage. The level of αT in the spermatozoa of the treatment group dramatically increased after 24 h of incubation, further significantly increased by days 3 and 4, to finally decrease at day 5. The content

Table 2
The effect of storage and treatments on the αT content and the susceptibility to peroxidation of spermatozoa (spz). (na = not assessed; MDA = malonaldehyde)

αT content of spz ($\mu g/10^9$ cells)	Day 0	Day 1	Day 3	Day 4	Day 5
Control	1.33	na	na	na	1.03
αT treatment	1.33[a]	344[b]	548[c]	553[c]	481[d]
μg MDA / 10^9 cells					
Control	1.5	na	18.9	na	28.6
αT treatment	1.5	na	7.4	na	6.5

[a,b,c,d] Means with different superscript are significantly different $P < 0.05$.

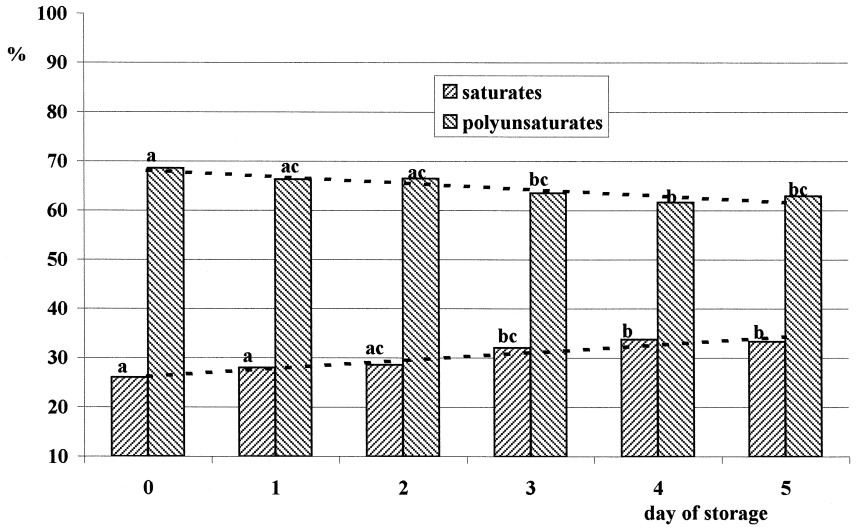

Fig. 4. Proportion of total saturates and polyunsaturates in boar sperm phospholipid during 5 day storage at 19°C (bars with different letters are significantly different between days) (standard errors are lower than 7% of the mean value).

of αT in the treatment diluent decreased from 233 to 205 μg/ml from day 0 to day 5 of storage. A significant biological incorporation of αT from the diluent into the spermato-

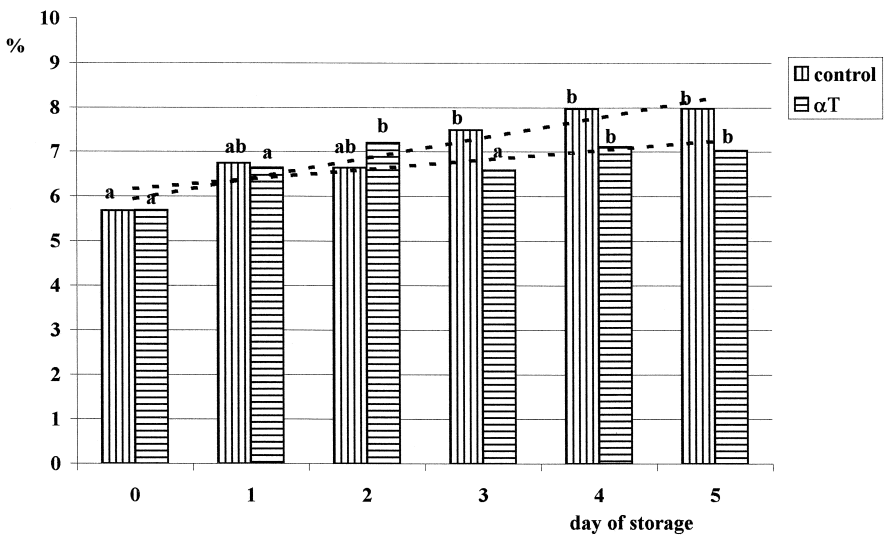

Fig. 5. Proportion of stearic acid (18:0) in boar sperm phospholipid during 5 day storage in a control or αT enriched diluent at 19°C (bars with different letters are significantly different between days within group) (standard errors are lower than 7% of the mean value).

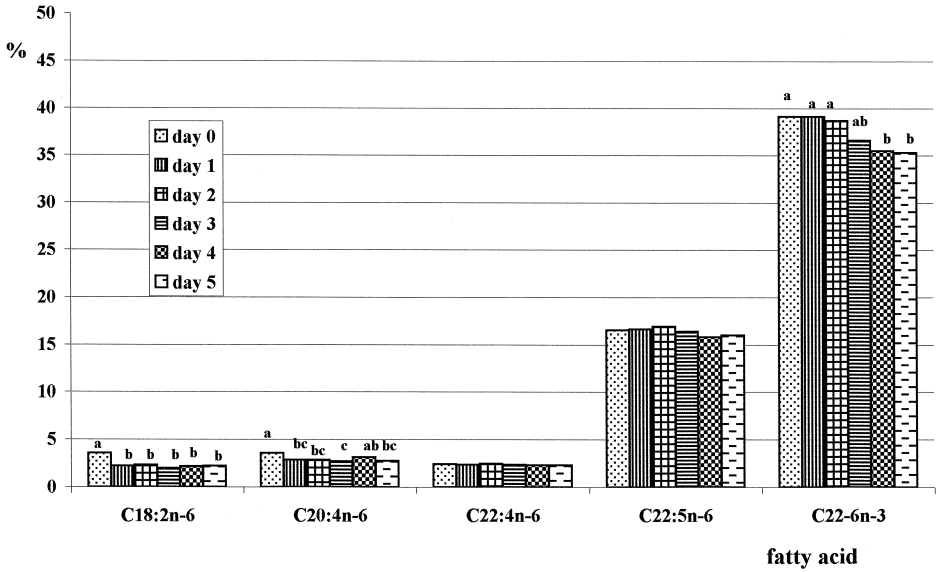

Fig. 6. Proportion of the major polyunsaturates of boar sperm phospholipid during 5 day storage at 19°C (bars with different letters are significantly different between days) (standard errors are lower than 9% of the mean value).

zoa during the storage period was indicated by the fact that even repeated washings of the cells with the diluent failed to change the αT content of the sperm. The uptake of

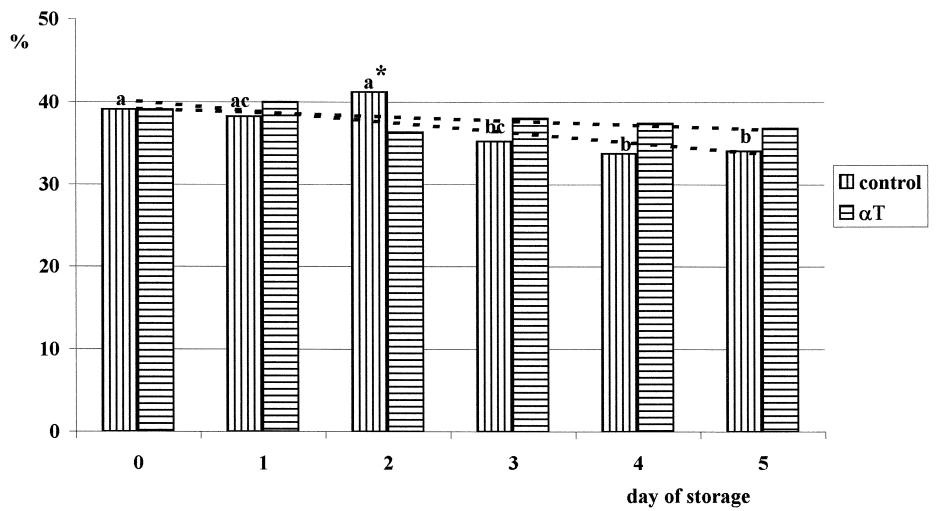

Fig. 7. Proportion of docosahexaenoic acid (22:6$n-3$) in boar sperm phospholipid during 5 day storage in a control or αT enriched diluent at 19°C (bars with different letters are significantly different between days within group; * shows a significant difference between groups within day) (standard errors are lower than 5% of the mean value).

αT from the diluent and its concomitant increased association with the cells was relatable with a large reduction in susceptibility to peroxidation, whereas the TBARS production after in vitro induction of peroxidation increased dramatically over the 5 days of storage in the control group. This equated to a reduction of 61 and 77% respectively in the susceptibility to peroxidation on days 3 and 5 as a result of αT inclusion into the diluent.

3.3. Sperm fatty acid composition

The fatty acid composition of the PL fraction of the spermatozoa and appropriate statistical analyses are shown in Figs. 4–7 and Table 1 respectively. The proportion of total polyunsaturated fatty acids decreased over the period of storage and was balanced by a complementary increase in the proportion of total saturated fatty acids. The increase of saturates was mainly due to an increase in the level of 18:0. The decrease in the proportion of PUFA was due to changes in the proportions of all the major PUFA of both $n-6$ and $n-3$ series. No change occurred in the 22:5$n-6$ and 22:4$n-6$ contents of sperm phospholipid during storage. αT inclusion totally prevented the decrease of 22:6$n-3$. The treatment did not have any effect on the proportions of 18:2$n-6$ and 20:4$n-6$.

4. Discussion and conclusion

Lipid peroxidation is recognised as a damaging process to spermatozoa leading to motility loss and reduced fertilising ability in spermatozoa of many species including man (Alvarez and Storey, 1984; Whishart, 1984; Aitken et al., 1989; Gagnon et al., 1991; De Lamirande and Gagnon, 1992; Hammerstedt, 1993; Aitken, 1994; Lenzi et al., 1996). Lipid peroxidation occurs spontaneously in mammalian spermatozoa (Alvarez and Storey, 1989) and is greatly enhanced in human sub-fertile ejaculates (Aitken et al., 1993a) or fowl stored semen (Wishart, 1989; Cecil and Bakst, 1993).

The mechanisms by which reactive oxygen species (ROS) disrupt the sperm functions is believed to involve the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane (Aitken, 1994) and this process plays an important role in the pathophysiology of male infertility (Aitken et al., 1993b). ROS increases DNA fragmentations (Lopes et al., 1998), modify the cytoskeleton (Hindshaw et al., 1986), affect the sperm axoneme development (De Lamirande and Gagnon, 1992), and inhibit sperm-oocyte fusion (Aitken et al., 1993a).

A complex antioxidant system is present in spermatozoa and seminal plasma to scavenge the oxygen radicals and prevent their damaging action under normal physiological conditions. Such a system embraces enzymatic activities, such as superoxide dismutase, catalase and glutathione peroxidase, and water- (ascorbic acid, glutathione and uric acid) or fat- (vitamin E, carotenoids and ubiquinones) soluble natural compounds (Surai et al., 1998a). Nevertheless, the antioxidant system of the cells is not potent enough to prevent lipid peroxidation completely, especially during in vitro

storage when the production of free radicals could be significantly enhanced as a result of metabolic changes (Hammerstedt, 1993).

The present results show that liquid storage of boar semen was characterised by a significant decrease in cell viability which occurred following the 2nd day of incubation and thereafter. At the same time, a significant decrease in the relative proportion of 22:6$n-3$ in spermatozoa phospholipid was observed. 22:6$n-3$ is the major polyunsaturate of boar spermatozoa (Poulos et al., 1973) and plays an essential role in membrane fluidity and cell functions (Salem et al., 1986). Moreover, it has been shown that in mammalian spermatozoa 22:6$n-3$ and 20:4$n-6$ are preferentially oxidised during sperm storage (Jones and Mann, 1976; Griveau et al., 1995).

Also from the present results, it is clear that αT is made available via the storage diluent, the spermatozoa become highly enriched in such an active antioxidant compound and significantly increase their resistance to lipid peroxidation. As a result, the αT enriched spermatozoa are able to efficiently protect 22:6$n-3$ and prevents its subsequent loss. By contrast, during storage under the control conditions the endogenous level of αT was wholly insufficient to protect the lipid against peroxidation and as a result the level of 22:6$n-3$ in the sperm phospholipid decreased significantly. Based on the observations that the αT content of the spermatozoa increased during the first 4 days of semen storage and was strongly bound to the spermatozoa, it can be suggested that αT, through its lipophilic properties, could be incorporated into the lipid rich structure of the sperm plasma membrane. Consequential upon these improved biochemical features, the spermatozoa maintained integrity to a far greater extent throughout the storage period and there was a prevention of the progressive decline in viability observed under the control conditions.

From evidence with avian semen (Surai et al., 1997a,b) under induced conditions of peroxidation in vitro, the inclusion of αT prevented the loss of phosphatidylserine, phosphatidylethanolamine and phospholipid bound polyunsaturated fatty acids from occurring in spermatozoa (Surai et al., 1998b). Furthermore from studies with other species, the extent and manner of the protective effect of αT or other antioxidant systems shows distinct variations (Hammerstedt et al., 1976; Pursuel, 1979; Watson and Anderson, 1983; Aitken et al., 1989; Blesbois et al., 1993; Griveau and Le Lannou, 1994; Donoghue and Donoghue, 1997).

Since free radical production is taking place during normal physiological metabolism in the cells (Halliwell, 1994), total elimination of lipid peroxidation is an impossible aim and a continuum of irreversible negative effects on sperm functions of some level have to be accepted over a period of storage (Hammerstedt, 1993). Although excessive production of ROS are notably detrimental to spermatozoa, there is a growing body of evidence which suggests that low levels of ROS are involved in the physiological control of a range of mammalian sperm functions (Kumar et al., 1991; Bondin et al., 1997; De Lamirande et al., 1997; Griveau and Le Lannou, 1997; Lander, 1997; O'Flaherty et al., 1998). However, the present results clearly show that in the boar the inclusion of αT into the storage diluent completely prevents any deterioration in the parameters of sperm quality that were measured and provides protection to the viability of the cells up to 5 days of storage. As a consequence a considerable enhancement of the AI fertilising capacities of the stored semen could be expected.

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