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The relationship between the dietary provision of α-tocopherol and the concentration of this vitamin in the semen of chicken: effects on lipid composition and susceptibility to peroxidation

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This study is an attempt to enhance the resistance of chicken semen to peroxidative damage by supplementing the diet of cockerels with the major lipid-soluble antioxidant α-tocopherol. Cockerels at 6 months of age were fed for 8 weeks with feed containing 0, 20, 200 or 1000 mg α-tocopherol kg^{-1}. Semen was collected during the final 2 weeks of the supplementation period and the concentrations of α-tocopherol in the spermatozoa and the seminal plasma were determined. The concentrations of α-tocopherol in whole semen, spermatozoa and seminal plasma were approximately twice as high when the supplementation was 200 mg kg^{-1} compared with when supplementation was 20 mg kg^{-1}; however, supplementation at 1000 mg kg^{-1} did not achieve any further increase in these concentrations of α-tocopherol. Thus, the concentration of α-tocopherol in semen displays only a limited responsiveness to manipulation by dietary means. In contrast, the concentrations of the vitamin in the testes and liver were found to be much more amenable to dietary manipulation, exhibiting increases of six–seven-fold over the whole range of supplementation. However, the dietary-induced increase in the α-tocopherol content of semen did result in a significant reduction in the susceptibility of the semen to lipid peroxidation. A further effect of enhancing the concentration of this vitamin in the semen was a significant increase in the proportions of C_{20-22} polyunsaturated fatty acids in the sperm phospholipids. In addition, the proportion of phosphatidylethanolamine in the phospholipid was increased whereas that of sphingomyelin was reduced at the higher concentrations of α-tocopherol supplementation. Thus, an increased dietary intake of α-tocopherol does produce beneficial changes in the antioxidant capacity and lipid profile of semen, albeit to a relatively limited extent.

Introduction

The phospholipids of spermatozoa are characterized by extremely high proportions of long-chain, highly polyunsaturated fatty acids. In mammals, polyunsaturates of the n-3 series (particularly 22:6n-3) are the major acyl components of these cells (Neill and Masters, 1972; Poulos *et al.*, 1973; Scott, 1973; Jain and Anand, 1976; Lin *et al.*, 1993), whereas in birds fatty acids of the n-6 series (mainly 20:4n-6 and 22:4n-6) predominate (Darin-Bennett *et al.*, 1974; Howarth *et al.*, 1977; Ravie and Lake, 1985; Kelso *et al.*, 1996). There is considerable evidence that such fatty acids play an important, although as yet unspecified, role in sperm function since cases of impaired fertility in both mammals (Nissen *et al.*, 1981; Nissen and Kreysel, 1983; Sebastian *et al.*, 1987) and birds (Kelso *et al.*, 1996) have been associated with reduced amounts of these polyunsaturates in spermatozoa. However, the high degree of polyunsaturation typical of sperm lipids renders these gametes highly susceptible to lipid peroxidation, with the consequent risk of damage to cellular structures (Niki *et al.*, 1993). In fact, peroxidative damage to spermatozoa is believed to be a major cause of male subfertility (Jones *et al.*, 1979; Wishart, 1984; Aitken *et al.*, 1989; Alvarez and Storey, 1989; Selley *et al.*, 1991; Cecil and Bakst, 1993; Hammerstedt, 1993; Aitken, 1994; Sikka *et al.*, 1995). Thus, the viability and fertilizing ability of spermatozoa are highly dependent on the expression of an effective antioxidant capacity by these cells and in the surrounding seminal plasma.

α-Tocopherol (vitamin E) is the major lipid-soluble antioxidant present in cell membranes and plays a crucial role in breaking the chain reaction of peroxidation (Freisleben and Packer, 1993). Dietary supplementation with α-tocopherol has been demonstrated to reduce the susceptibility to lipid

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peroxidation and improve the fertility of human (Kessopoulou *et al.*, 1995, Geva *et al.*, 1996) and boar (Brzezinska-Slebodzinska *et al.*, 1995) semen. The study described here attempted to enhance the resistance of avian semen to lipid peroxidation by supplementing the diet of cockerels with α-tocopherol.

Materials and Methods

Supplementation and sampling

Cockerels (Rhode Island Red strain, 6 months of age) were allocated to four dietary groups with 15 birds in each group. The birds were housed individually and fed a standard wheat–barley-based ration balanced in nutrients. The concentration of feed-derived α-tocopherol in the basal diet was 12 mg kg^{-1} feed. One of the groups was supplied with this basal diet with no additional α-tocopherol supplementation. The diets of the other three groups were supplemented with 20, 200 and 1000 mg α-tocopherol acetate kg^{-1} feed. Semen was obtained three or four times a week, as described previously by Kelso *et al.* (1996). The birds were maintained on the diets for 8 weeks. Semen collected during the final 2 weeks of the trial was used for the measurements. Five replicate samples of semen, each consisting of pooled material from three birds, were obtained for each dietary group. Determination of number of sperm cells and the separation of semen into cells and seminal plasma were performed as described by Kelso *et al.* (1996). At the end of the 8 week period, the birds were killed by cervical dislocation. The testes and liver from five of the birds in each group were collected. The samples were stored in liquid nitrogen for up to 2 weeks before analysis.

Determination of α-tocopherol

α-Tocopherol was determined by the method of McMurray *et al.* (1980) as modified by Gaal *et al.* (1995). In brief, the samples were saponified with ethanolic KOH in the presence of pyrogallol; α-tocopherol was then extracted from the mixture with petroleum spirit. The extract was dried under nitrogen, redissolved in methanol and injected on to a Spherisorb type S30DS2, 3 μ C_{18} reverse phase HPLC column, 15 cm × 4.6 mm (Phase Separations, Clwyd). Chromatography was performed using a flow rate of 1.1 ml min^{-1}. Fluorescence detection and quantification of α-tocopherol made use of excitation and emission wavelengths of 295 and 330 nm, respectively. Calibration was performed using standard solutions of α-tocopherol in methanol.

Susceptibility to lipid peroxidation

Semen samples were mixed with 9 volumes of sodium phosphate buffer (0.01 mol l^{-1}, pH 7.4) containing 1.15% (w/v) KCl. The homogenates were incubated for 20 min at 37°C in the presence of $FeSO_4$ (0.1 mmol l^{-1}). At the end of the incubation, butylated hydroxytoluene was added so that its concentration was 0.01% (v/v), and the concentration of thiobarbituric acid-reactive substances (TBARS) was determined by the method of Ohkawa *et al.* (1979).

Lipid analysis

Samples were homogenized in a suitable excess of chloroform:methanol (2:1, v:v) and extracts of total lipid were prepared. The extracts were subjected to thin-layer chromatography on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1, v:v) and the band corresponding to phospholipid was eluted from the silica with methanol. The isolated phospholipid fraction was transmethylated (Kelso *et al.*, 1996) and the fatty acid composition was determined by gas–liquid chromatography using a capillary column system (Carbowax, 30 m × 0.25 mm, film thickness 0.25 μm; Alltech, Carnforth) in a CP9001 instrument (Chrompack, Middleburg, The Netherlands). Integration of the peaks and subsequent data handling were performed using an EZ Chrom Data System (Scientific Software Inc., San Jose, CA). The identities of the peaks were verified by comparing them with the retention times of standard fatty acid methyl esters (Sigma Chemical Co., Poole).

The proportions of the individual phospholipid classes (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SP) and cardiolipin (CL)) within the total phospholipid were determined by high-performance, thin-layer chromatography (HPTLC) using a solvent system of methyl acetate:isopropanol:chloroform:methanol:KCl (0.25%, w/v, in H_2O) (25:25:25:10:9, v:v), as described by Olsen and Henderson (1989). After charring (plates were sprayed with 3% (w/v) cupric acetate in 8% (v/v) orthophosphoric acid and then heated at 160°C for 20 min), quantification of the separated phospholipid classes was performed by densitometry using a Shimadzu CS-9001 PC dual-wavelength, flying-spot, thin-layer scanner (Dyson Instruments, Houghton-le-Spring).

Total phospholipid was subjected to HPLC coupled to a light-scattering mass detector system to purify the individual phospholipid classes before determining their fatty acid compositions, as described in detail by Christie (1985).

Expression of results

All results are presented as means ± SEM of measurements from five replicate samples. Statistical analysis was by Student's *t* test.

Results

The concentration of α-tocopherol in the liver and testes was very responsive to dietary manipulation, increasing by a factor of 7.3 in the liver and by a factor of 5.8 in the testes over the range of supplementation used (Table 1). In marked contrast, the concentration of α-tocopherol in whole semen and its constituent spermatozoa and seminal plasma displayed considerable resistance to dietary enhancement. For instance, increasing the dietary supplementation with α-tocopherol from 20 to 1000 mg kg^{-1} feed produced, at best, a twofold increase in the concentration of the vitamin in the semen and its

Table 1. Effect of dietary supplementation with α-tocopherol on the concentration of α-tocopherol in the semen, testes and liver of cockerels

Supplement (mg kg^{-1} feed)	Concentration of α-tocopherol				
	Whole semen (ng ml^{-1})	Seminal plasma (ng ml^{-1})	Spermatozoa (ng per 10^9 cells)	Testes (μg g^{-1})	Liver (μg g^{-1})
0	456 ± 22	193 ± 11	76.4 ± 4.1	4.2 ± 0.2	7.1 ± 0.5
20	535 ± 36	213 ± 15	86.9 ± 7.1	5.9 ± 0.3[b]	9.0 ± 0.5[c]
200	1043 ± 68[a]	402 ± 19[a]	174.3 ± 9.2[a]	14.7 ± 0.8[a]	26.4 ± 1.7[a]
1000	1019 ± 55[a]	356 ± 23[a]	174.1 ± 8.2[a]	24.2 ± 1.1[a]	51.9 ± 4.1[a]

Values are means ± SD of measurements from five semen samples or from tissue from five birds.
Significance of difference from zero supplementation: [a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.02$.

Table 2. Effect of dietary supplementation with α-tocopherol on the susceptibility of cockerel semen to lipid peroxidation *in vitro*

Supplementation (mg kg^{-1} feed)	TBARS (nmol ml^{-1} semen)
0	22.4 ± 1.1
20	19.9 ± 1.3
200	12.5 ± 1.1[a]
1000	11.6 ± 1.1[a]

Values are means ± SD of measurements from five semen samples.
Significance of difference from zero supplementation: [a]$P < 0.001$.
TBARS, thiobarbituric, acid-reactive substances.

Table 3. Effect of dietary supplementation with α-tocopherol on the fatty acid composition of sperm phospholipid in cockerels

Fatty acid	α-Tocopherol supplementation (mg kg^{-1} feed)			
	0	20	200	1000
16:0	18.4 ± 0.3	16.9 ± 0.3	15.5 ± 0.4[c]	15.6 ± 0.3[c]
18:0	17.8 ± 0.4	17.4 ± 0.4	18.0 ± 0.2	17.9 ± 0.6
18:1n-9	20.6 ± 1.1	15.6 ± 0.4[b]	15.0 ± 0.3[b]	15.5 ± 0.2[b]
18:2n-6	4.9 ± 0.3	2.1 ± 0.1[a]	2.3 ± 0.1[b]	3.2 ± 0.1[b]
20:1n-9	3.1 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.3 ± 0.1
20:4n-6	7.5 ± 0.4	10.7 ± 0.6[c]	11.5 ± 0.4[b]	11.2 ± 0.1[b]
22:4n-6	14.4 ± 0.3	16.6 ± 0.2[c]	19.0 ± 0.3[b]	18.8 ± 0.6[b]
22:6n-3	2.6 ± 0.1	2.9 ± 0.1	2.5 ± 0.1	3.2 ± 0.1

Values are means ± SD of measurements from five semen samples.
Data represent the per cent amounts (w/w) of fatty acids; only major fatty acids (> 1%) are shown.
Significance of difference from zero supplementation: [a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.02$.

constituents. In addition, the accumulation of the vitamin in the semen appeared to demonstrate a saturation profile since an increase in the dietary supplementation from 200 to 1000 mg kg^{-1} feed produced no further increase in the concentration of α-tocopherol in the semen samples.

In spite of the attenuated response of the content of α-tocopherol in semen to dietary manipulation, the twofold increase that was achieved in the concentration of this vitamin in the semen did produce a significant improvement in the resistance of the samples to lipid peroxidation. Thus, the accumulation of TBARS during the incubation of semen *in vitro* under pro-oxidant conditions was reduced by approximately 50% by dietary supplementation with the vitamin (Table 2). In fact, the susceptibilty of semen to peroxidation displayed a very high negative correlation ($r = -0.998$) with the α-tocopherol content of the semen. The susceptibility of testes homogenates to peroxidation *in vitro* was also reduced by dietary supplementation with α-tocopherol. For example, the production of TBARS (nmol g^{-1} of testis) was reduced from 12.0 ± 1.1 at zero supplementation to 5.3 ± 0.3 at the highest (1000 mg α-tocopherol kg^{-1} feed) concentration of supplement.

The beneficial consequences of this enhanced resistance to peroxidation are exemplified in Table 3, which shows that dietary supplementation with α-tocopherol resulted in a promotion of the polyunsaturation status of the sperm phospholipid. Thus, increasing the concentration of the vitamin in the diet resulted in significant increases in the proportions of the characteristic C_{20-22} polyunsaturated components of the spermatozoa; namely, 20:4n-6 and 22:4n-6. The proportions of both 20:4n-6 and 22:4n-6 in the sperm phospholipid were very highly correlated ($r = 0.773$ and 0.943, respectively) with the concentration of α-tocopherol in the semen. The proportions of C_{20-22} polyunsaturates in the phospholipids of the testes and liver were also significantly increased by dietary supplementation with this vitamin (data not shown).

The effects of α-tocopherol supplementation on sperm lipid parameters were not confined simply to changes in fatty acid composition. Such supplementation also results in a subtle but significant rearrangement in the proportion of the different phospholipid classes of the spermatozoa (Table 4). The proportion of PE within the total phospholipid was increased by α-tocopherol supplementation in parallel with a decrease in the proportion of SP. The fatty acid composition of the individual phospholipid classes of spermatozoa from the group receiving supplementation of 20 mg α-tocopherol kg^{-1} feed are shown (Table 5). The results indicate that both PE and PC contain considerable proportions of 20:4n-6 and 22:4n-6 in agreement

Table 4. Effect of dietary supplementation with α-tocopherol on the proportion of the phospholipid classes of cockerel spermatozoa

Phospholipid	α-Tocopherol supplementation (mg kg^{-1} feed)			
	0	20	200	1000
PE	30.5 ± 0.3	31.1 ± 0.1	32.8 ± 0.2[b]	33.6 ± 0.2[b]
PC	31.5 ± 1.1	32.2 ± 1.2	32.9 ± 2.1	32.6 ± 2.8
PS	21.7 ± 0.3	21.3 ± 0.4	21.7 ± 0.4	22.1 ± 1.1
CL	4.3 ± 0.1	4.1 ± 0.1	4.9 ± 0.1	4.4 ± 0.1
SP	11.0 ± 0.9	9.3 ± 0.2[b]	7.8 ± 0.4[a]	7.2 ± 0.6[a]

Values are means ± SD of measurements from five semen samples.
Results are the per cent amounts (w/w) of total phospholipid.
Significance of difference from zero supplementation: [a]$P < 0.01$, [b]$P < 0.02$.
PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; CL, cardiolipin; SP, sphingomyelin.

with previous work (Kelso *et al.*, 1996). Furthermore, PS was found to be highly enriched in 22:4n-6, whereas SP and CL were composed largely of saturated and monounsaturated fatty acids. Thus, increasing the dietary concentration of α-tocopherol enforces an increase in the proportion of the highly polyunsaturated PE at the expense of the highly saturated SP.

Discussion

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. (1) Defence against peroxidative damage is essential to maintain the structural integrity of the spermatozoa. (2) Minimization of lipid peroxidation will prevent any reduction in the concentrations of the functionally important C_{20-22} 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 cryopreservation.

The approach taken in the present study was to supplement the diet of cockerels with increasing concentrations of the major antioxidant α-tocopherol. The natural constituents of the unsupplemented diet contained 12 mg of this vitamin per kg of feed. The birds whose diet was supplemented with an additional 20 mg kg^{-1} feed of the vitamin can be considered as the control group, since this amount of supplementation is normally used for poultry production in the Ukraine where the trial was performed (although 40 mg kg^{-1} is the usual amount of supplementation in the UK). Increasing the amount of supplementation to 200 mg kg^{-1} feed resulted in marked increases in the concentrations of the vitamin in the liver and testes, and further increases occurred in these tissue concentrations when the diet was supplemented with 1000 mg kg^{-1} feed. Thus, these tissues are very responsive to the dietary manipulation of their content of α-tocopherol across the range of supplementation amounts used in this experiment. However, the main target of this study, the semen, was much less responsive to such dietary manipulation. A relatively minor increase in the concentration of the vitamin in semen was achieved by increasing the dietary provision from 20 to 200 mg kg^{-1} and no further increase was achieved beyond this, suggesting that the saturation level is attained at a fairly low concentration of α-tocopherol in the semen. A possible explanation for such a marked difference in the response between the liver and the spermatozoa may be that, although the liver can accumulate cytoplasmic lipid droplets (Mooney and Lane, 1981) and therefore lipid-soluble vitamins such as α-tocopherol, spermatozoa contain few such inclusions, with the consequence that the phospholipid bilayers of the cell membranes will represent the main site of accumulation of the vitamin. The maximal concentration of α-tocopherol that can be accommodated in cell membranes is usually very low (less than 1 mol of the vitamin per 1000 mol of phospholipid; Packer, 1992). Thus, there may be a natural biological limitation on the extent to which the α-tocopherol concentration of spermatozoa can be increased.

Table 5. Fatty acid composition of the phospholipid classes in cockerel spermatozoa when the cockerel diet was supplemented with 20 mg α-tocopherol kg^{-1} feed

Fatty acid	PE	PC	PS	CL	SP
16:0	14.6 ± 1.2	19.2 ± 1.1	13.3 ± 1.0	17.4 ± 1.1	23.8 ± 1.4
16:1n-7	3.9 ± 0.1	—	—	2.0 ± 0.1	1.7 ± 0.1
18:0	12.7 ± 1.1	24.7 ± 1.1	19.9 ± 1.0	14.8 ± 0.0	18.7 ± 1.4
18:1n-9	28.1 ± 1.7	17.7 ± 1.8	34.2 ± 3.6	45.6 ± 3.1	41.8 ± 3.1
18:2n-6	2.2 ± 0.1	1.5 ± 0.1	—	2.1 ± 0.1	2.4 ± 0.1
20:1n-9	2.5 ± 0.1	4.2 ± 0.1	—	2.6 ± 0.1	1.8 ± 0.1
20:4n-6	11.8 ± 1.1	9.3 ± 0.6	—	3.3 ± 0.6	1.3 ± 0.8
22:4n-6	18.5 ± 1.1	14.6 ± 0.8	32.5 ± 2.9	10.8 ± 0.8	1.8 ± 1.7
22:6n-3	2.9 ± 0.4	2.3 ± 0.3	—	—	1.4 ± 0.1

Values are means ± SD of measurements from five semen samples.
Results are the per cent amounts (w/w) of fatty acids; only major fatty acids (> 1%) are shown.
PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; CL, cardiolipin; SP, sphingomyelin.

However, even the relatively limited enhancement of the concentration of α-tocopherol in semen that was achieved by dietary means was found to produce significant benefits by reducing the susceptibility of the semen to lipid peroxidation. An inverse relationship between susceptibility to peroxidation and α-tocopherol content has previously been demonstrated for microsomal fractions from various mammalian tissues (Kornbrust and Mavis, 1980). The increase in the proportion of C_{20-22} polyunsaturated fatty acids in the sperm phospholipids that resulted from dietary supplementation of the birds with the vitamin may be a reflection of the reduced peroxidative susceptibility of the semen *in vivo*. This in itself may have beneficial consequences for fertility since it has been shown that age-related decreases in fertility in cockerels are associated with decreased concentrations of these polyunsaturates in the phospholipids of the spermatozoa (Kelso *et al.*, 1996). An important aim for future work would be to determine the effects of dietary supplementation with α-tocopherol on a range of parameters of sperm function and fertilizing ability *in vitro* and *in vivo*.

Two main points emerge from this study. (1) Increasing the antioxidant capacity of semen can produce real benefits in parameters related to sperm viability and function and this approach could be pursued as a means of improving male fertility. (2) Dietary supplementation with α-tocopherol, at least in chickens, is likely to be an inefficient means of achieving this improvement since very high dietary concentrations of this vitamin are needed to produce limited beneficial effects. However, it should be noted that because it uses the natural route, feeding this vitamin to birds is a more effective way of incorporating α-tocopherol into the sperm membranes than addition of the vitamin to semen diluents after collection (Surai, 1992). Alternative approaches to promote the antioxidant potential of semen are currently under investigation in this laboratory.

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