

Original article

**Oxidative status and semen characteristics  
of rabbit buck as affected by dietary  
vitamin E, C and n-3 fatty acids**

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**Abstract** — The aim of this study was to investigate the effect of dietary supplementation of long chain fatty acids ( $C \geq 20$  LCP) of the n-3 series, vitamin E and vitamin C on the antioxidant capacity of rabbit buck and on semen characteristics. Fifty male rabbits at 30 days were randomly assigned to five different diets: Control (50 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate), Vitamin E (200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate), n-3 (2% ROPUFA oil + 50 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate), n-3 + E (2% ROPUFA oil + 200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate) and n-3 + E C (2% ROPUFA oil + 200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate + 0.5 g·L<sup>-1</sup> vitamin C in the drinking water). The levels of vitamins E and C and reactive oxygen metabolites (ROMs) in the blood plasma were evaluated at different ages. The antioxidant capacity and ROMs of seminal plasma, the fatty acid profile of sperm phospholipids, the semen traits and the oxidative processes during storage (24 h at +4 °C) were carried out weekly for 5 wk starting from the 5th month of age. Vitamin E addition showed enough antioxidant protection only when associated with no lipid enriched diets. The n-3 supplementation modified the fatty acid profile of the spermatozoa membrane and simultaneously enhanced oxidative processes. Only the association with supranutritional levels of vitamins E and C inhibited the oxidative processes and improved the characteristics of fresh and stored rabbit semen.

**rabbit spermatozoa / oxidative status / vitamins C and E / n-3 fatty acids**

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## 1. INTRODUCTION

In the membrane of mammal spermatozoa, about 30–50% of the fatty acids are Long Chain Polyunsaturated ( $\geq 20$  C - LCP) of the n-3 series and namely docosahexaenoic acid (DHA, C22:6n-3) [1]. This particular lipid profile is correlated with some membrane properties and functions such as fluidity, ion exchange and motility [2].

These fatty acids originate from  $\alpha$ -linolenic acid, and mature rabbits are able to elongate and desaturate  $\alpha$ -linolenic acid efficiently [3]. However, the health status, stress condition and ageing of the animals [4, 5] can lower the conversion rate.

Many authors [6–9] have reported that dietary supplementation with LCP n-3 fatty acids in males of several species is fixed in the sperm membrane, resulting in improved spermatozoa characteristics. Such enrichment of the cell membrane simultaneously increases the susceptibility of spermatozoa to peroxidation, which in turn has been proposed as one of the major causes of male infertility [10, 11]. Thus, to ensure suitable sperm membrane function, it is crucial to maintain the equilibrium between the level of unsaturation and oxidative stability [12].

The fatty acid profile of spermatozoa and the Reactive Oxygen Metabolites (ROMs) production [13] greatly affect gametes interaction. ROMs are necessary to start capacitation and acrosome reaction even though their amount must remain low in order to avoid cell damage or inactivation of critical enzyme pathways and systems [14].

Certain major antioxidants added to the diets or directly to the storage medium delay the development of ROMs. Askari et al. [15] reported that supplementation with vitamin E and/or vitamin C reduced the generation of ROMs and prevented loss of motility, mainly during the storage of human spermatozoa.

The aim of the present paper was to evaluate the effect of dietary supplementation

with LCP n-3 fatty acids and antioxidants (vitamins E and C) on the oxidative status of rabbit bucks and on the characteristics of their semen.

## 2. MATERIAL AND METHODS

### 2.1. Animals, diets and sampling

The trial was carried out in the experimental rabbitry of the Department of Animal Science with a photoperiod of 16 h light-day<sup>-1</sup> and a temperature of  $18.5 \pm 1.2$  °C.

At weaning, (30 d), fifty New Zealand White male rabbits were divided into 5 homogeneous groups and fed ad libitum as follows:

- (1) control (50 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate);
- (2) vitamin E (200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate);
- (3) n-3 (2% ROPUFA oil + 50 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate);
- (4) n-3 +E (2% ROPUFA oil + 200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate);
- (5) n-3 + E C (2% ROPUFA oil + 200 mg·kg<sup>-1</sup> diet  $\alpha$ -tocopheryl acetate + 0.5 g·L<sup>-1</sup> vitamin C in the drinking water).

ROPUFA oil is a refined marine oil containing at least 30% of LCP n-3 (C20:5n-3 EPA, and C22:5n-3 > 25%, C22:6n-3 DHA 12.6%). It is stabilised by a mix of tocopherols, ascorbyl palmitate and rosemary extract (Istituto delle Vitamine, Milano, Italy).

The formulation and fatty acid profile of the diets are reported in Table I.

Blood samples, drawn from the marginal ear vein at 30, 50, 70, 90 and 150 days, were collected in heparinised vacutainers and centrifuged at  $5\,000 \times g$  for 10 min at +4 °C and stored at -80 °C until analysis for vitamins C and E and ROMs.

At 150 days of age, the rabbits were trained for semen collection with an

**Table I.** Formulation and fatty acid composition of the diets.

Ingredients (%)	Diet	
	Control	n-3
Dehydrated alfalfa meal	38	38
Barley meal	31	31
Soybean meal	20	20
Wheat straw	4	4
Soybean oil	2	–
ROPUFA oil	–	2
Molasses	0.8	0.8
Calcium carbonate	0.1	0.1
Dicalcium phosphate	1.1	1.1
Ligninsulfonate	0.5	0.5
Sodium chloride	0.5	0.5
Coccidiostatic	0.6	0.6
DL-methionine	0.4	0.4
Mineral vitamin – premix*	1	1
Major fatty acids (% of total fatty acids)		
Σ SFA <sup>1</sup>	23.87	20.12
Σ MUFA <sup>1</sup>	23.01	20.81
Σ PUFA <sup>1</sup>	53.12	59.07
C18:2n-6	44.25	35.12
C18:3n-3	8.54	14.25
C20:5n-3	0.35	1.52
C22:6n-3	0.58	2.01
Others <sup>2</sup>	0.58	2.01

\*Added per kg: Vit. A 11 000 UI; Vit. D3 2 000 UI; Vit. B1 2.5 mg; Vit. B2 4 mg; Vit. B6 1.25 mg; Vit. B12 0.01 mg;  $\alpha$ -tocopheryl acetate 50 mg; Biotine 0.06 mg; Vit. K 2.5 mg; Niacine 15 mg; Folic acid 0.30 mg; D-panthotenic acid 10 mg; Choline 600 mg; Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.

<sup>1</sup> SFA saturated fatty acids, MUFA monosaturated fatty acids, PUFA polyunsaturated fatty acids.

<sup>2</sup> C18:3n-6, C20:3n-6, C20:4n-6, C21:5n-3, C22:5n-3.

artificial vagina. Five rabbits per group were selected for the weekly collection of semen that was done for 5 consecutive times.

Immediately after collection semen samples were diluted 1:2 with TALP (Tyrode modified medium – 296 mOsm·g<sup>-1</sup>, pH 7.0). Kinetic and morpho-functional characteristics of spermatozoa were evaluated on an aliquot of individual semen samples, whereas chemical analysis was performed on the remaining pooled portions.

## 2.2. Chemical analyses

Chemical analyses of the diets were done according to AOAC methods [16]. Fatty acid composition was determined on lipids extracted from samples of about 5 g in a homogeniser with 20 mL of 2:1 chloroform: methanol [17], followed by filtration through Whatman No. 1 filter paper. Fatty acids were quantified as methyl esters with a Mega 2 Carlo Erba gas chromatograph, model HRGC (Milano, Italy), using a D-B wax capillary column (25 mm Ø, 30 m long). The fatty acid percentages and the sum of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) were calculated with the Chrom-Card.

The  $\alpha$ -tocopherol of the plasma [18] was determined by adding 500  $\mu$ L of distilled water and 1 mL of ethanol to 500  $\mu$ L of the sample, and then vortexing for 10 s. Then, 2 mL of hexane and BHT (0.01%) were added and the mixture was carefully shaken and centrifuged. An aliquot of the supernatant (0.8 mL) was dried, redissolved in 0.2 mL of ethanol/dioxane (1:1) and shaken for 10 min. After adding methanol (0.3 mL) and further shaking, 20  $\mu$ L were injected into the HPLC (CM 4000, Milton Roy, Riviera Beach, FL), using a silica column (Beckman, Fullerton, CA, USA).

Ascorbate was measured spectrophotometrically (524 nm) using the 2,4-dinitrophenylhydrazine method, corrected to

account for background interference according to the procedure of Dabrowski and Hintherleitner [19].

The assessment of the antioxidant capacity and ROMs in blood serum and seminal plasma was performed using, respectively, the Oxi-adsorbent and the d-ROMs tests produced by DIACRON® s.r.l. (Italy) [20].

Lipid peroxides in spermatozoa were measured using the kit D-cells on an aliquot of semen samples containing about  $30 \times 10^6$  cells·mL<sup>-1</sup>.

TBA-RS (Thiobarbituric Acid Reactive Substances) were evaluated on 1 mL of semen containing  $50 \times 10^6$  spermatozoa·mL<sup>-1</sup>, after induction of peroxidation with ferrous sulphate (0.2 mM) and sodium ascorbate (1 mM) at 37 °C for 1 h partially modifying the procedure of Aitken et al. [21].

Briefly, the TBA-RS value was determined by mixing 1 mL of this mixture with 2 mL of a stock solution containing 15% w/v trichloroacetic acid, 0.375% w/v TBA and 0.25N HCL. The solution was incubated at 90 °C for 15 min and, after cooling to room temperature, the precipitate was removed by centrifugation at  $1\,000 \times g$  for 10 min. The samples were read on a Jasko spectrofluorimeter (Model FP 1520) using excitation and emission wavelengths of 510 nm and 553 nm, respectively.

The TBA-RS and lipoperoxide levels of spermatozoa were determined immediately after semen collection and after 24 h of storage at 4 °C.

Lipid analysis of spermatozoa was performed on the polar fraction separated according to Juaneda and Rocquelin [22]. The lipids, extracted using the Folch et al. [17], were diluted in chloroform and injected on silica cartridges (Sep-pack, Waters S.A.). After adsorption of the sample, a syringe containing 20 mL of chloroform was connected to the top of the cartridge and the fraction containing non-phosphorus lipids was eliminated. The fraction containing the polar lipids was eluted with 30 mL of

methanol and then converted to methyl esters (FAME) which were quantified and calculated as previously shown.

### 2.3. Characteristics of semen and spermatozoa

The volume of ejaculate was determined using a graduated tube and the spermatozoa were counted with a haemocytometer.

The live cells were counted by fluorescent microscopy (Olympus CH<sub>2</sub>) with propidium iodide and carboxyfluorescein diacetate (Fluka) by counting 400 cells per sample.

The spontaneous acrosome reaction was assessed using the procedure of Mendoza et al. [23]. Spermatozoa were centrifuged at  $600 \times g$  for 5 min at 4 °C and the pellet was suspended in TALP to a final concentration of  $10^6$  spermatozoa·mL<sup>-1</sup>. After 30 min of incubation at 37 °C, in 5% CO<sub>2</sub>, 5 µL of sperm suspension were dropped on a slide with a chilled solution of FITC-PSA (25 mg·mL<sup>-1</sup>, Sigma). After washing with PBS to remove any excess stain and then drying. The slides were examined with an epifluorescence microscope (OLYMPUS – CH<sub>2</sub> excitation filter 335–425 nm).

Kinetic characteristics were evaluated immediately after semen collection (hour 0) and after 24 h of storage at 4 °C. Ten microlitres of the samples were diluted 1:30 and were laid over a pre-warmed Makler chamber at 37 °C. Six analyses/sample (2 drops  $\times$  3 fields) were done by Computer-Assisted Sperm Analysis (CASA - Version SCA 4.0, Microptic, Barcelona, Spain) using the parameters previously established for discriminating granules from static cells [24]. The sperm parameters recorded were: motile cells, track speed (VCL µm·s<sup>-1</sup>); linearity (LIN = progressive speed/track speed  $\times$  100), and amplitude of lateral head displacement (ALH µm).

## 2.4. Statistical analysis

Since a preliminary analysis showed that the buck effect was non-significant it was omitted. The data from 30 to 150 days ( $\alpha$ -tocopherol, ascorbic acid and ROMs in plasma) were statistically evaluated with a full factorial model comprising the interaction [25]. For the semen characteristics of adult bucks only the effect of dietary treatment was shown and the time of collection (n.s.) was absorbed. The results are presented as least square means and pooled standard error of the means (SEM). The significance of differences was performed by a t-test.

## 3. RESULTS

### 3.1. Plasma levels of $\alpha$ -tocopherol and ascorbic acid

The level of  $\alpha$ -tocopherol in the plasma increased with the age of the animals (Fig. 1). There were significant differences among the groups at 70 days of age and becoming more pronounced at 150 days.

In mature bucks the highest tocopherol value ( $10.8 \text{ mg}\cdot\text{L}^{-1}$ ) was found in the group supplemented with both vitamins whereas the lowest ( $2.8$  and  $3.2 \text{ mg}\cdot\text{L}^{-1}$  respectively

n-3 and the control) in animals with lower antioxidant addition. The rabbits fed supplemental vitamin E, with or without n-3, had the highest levels of plasma  $\alpha$ -tocopherol ( $8.2$  and  $7.9 \text{ mg}\cdot\text{L}^{-1}$ , respectively).

The level of ascorbic acid in the plasma showed the same trend (Fig. 2): the differences appeared at 70 days of age and reached the highest level at 150 days, mainly in the n-3+EC group ( $15.8 \text{ mg}\cdot\text{L}^{-1}$ ).

### 3.2. Plasma ROMs level

The ROMs level (Fig. 3) increased with age and was affected by dietary supplementation.

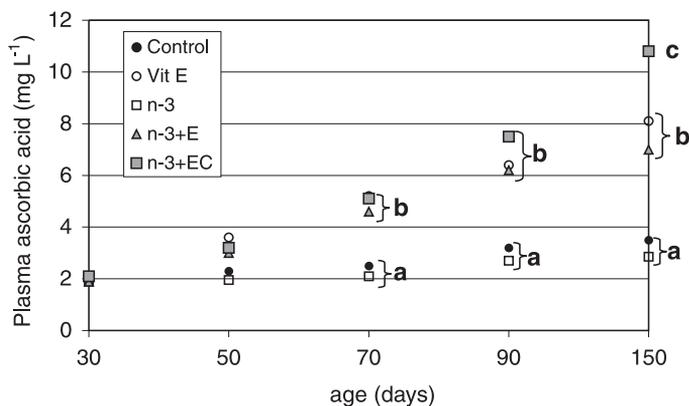
Feeding the standard amount of vitamin E ( $50 \text{ mg}\cdot\text{kg}^{-1}$ ), at 150 days the ROMs value was  $31.5 \text{ mg}\%$  of hydrogen peroxide and the supranutritional dose of vitamin E did not reduce this level in the diet enriched with n-3.

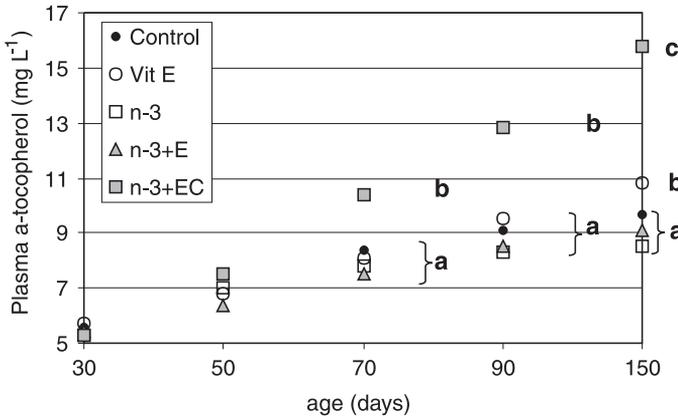
The n-3 group had the highest ROMs level and only the simultaneous ingestion of both vitamins reduced the reactive oxygen metabolites.

### 3.3. Semen characteristics

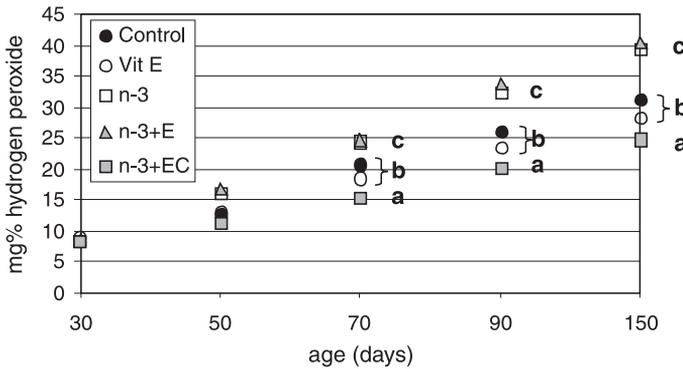
Dietary treatments did not modify the volume and the concentration of ejaculate or

**Figure 1.** Plasma  $\alpha$ -tocopherol levels at different ages (means not having a common letter (abc) at the same age are significantly different for  $P < 0.05$ ,  $n = 10$  for each group and age).





**Figure 2.** Plasma ascorbic acid levels at different ages (means not having a common letter (abc) at the same age are significantly different for  $P < 0.05$ ,  $n = 10$  for each group and age).



**Figure 3.** Plasma reactive oxygen metabolites (ROMs) production at different ages (means not having a common letter (abc) at the same age are significantly different for  $P < 0.05$ ,  $n = 10$  for each group and age).

the characteristics of fresh spermatozoa (Tab. II; live cells, motility, VCL, LIN and ALH).

On the contrary, the percentage of spontaneous acrosome-reacted sperm was significantly higher when the diet was enriched with n-3 and only the simultaneous addition of both vitamins compensated for this negative aspect.

Storage for 24 h at 4 °C worsened the qualitative characteristics of spermatozoa and the differences between the dietary treatment were enhanced. Dietary n-3 caused a decrease of live and motile cells and ALH. Vitamin E, especially if

combined with vitamin C, restored or in some cases (live and motile cells, VCL and ALH) improved the control values.

The spontaneous acrosome reaction was more pronounced after storage and showed the same ranking as fresh semen.

### 3.4. Phospholipid fatty acid profile of rabbit spermatozoa

Bucks supplemented with n-3 fatty acids, alone or in combination with vitamins, showed lower amounts of saturated fatty acids (SFA) (Tab. III) and higher percentages of polyunsaturated fatty acids (PUFA)

**Table II.** The main effects of dietary supplementation on fresh and stored semen characteristics.

Treatment	Control	Vit E	n-3	n-3+E	n-3+EC	n/group	SEM
Fresh semen							
Volume	mL	0.56	0.55	0.46	0.53	25	0.09
Concentration	no. $\times 10^6 \cdot \text{mL}^{-1}$	397.5	405.0	397.5	397.5	25	29.4
Live cells	%	91.0	90.0	87.5	85.5	25	16.8
Motile cells	%	87.2	85.0	78.2	77.5	150	15.0
VCL	$\mu\text{m} \cdot \text{s}^{-1}$	95.5	94.7	98.5	97.3	150	25.8
LIN	%	57.6	50.7	48.3	53.6	150	15.3
ALH	$\mu\text{m}$	3.5	3.6	3.5	3.4	150	1.9
Acrosome-reacted sperm	%	12.4 <sup>a</sup>	8.0 <sup>a</sup>	18.8 <sup>a</sup>	10.5 <sup>a</sup>	25	8.5
Stored semen							
Live cells	%	59.6 <sup>b</sup>	63.0 <sup>bc</sup>	55.6 <sup>a</sup>	67.5 <sup>c</sup>	25	20.2
Motile Cells	%	48.2 <sup>ab</sup>	51.4 <sup>b</sup>	49.6 <sup>ab</sup>	64.5 <sup>c</sup>	150	10.9
VCL	$\mu\text{m} \cdot \text{s}^{-1}$	71.3 <sup>ab</sup>	81.4 <sup>b</sup>	75.8 <sup>b</sup>	90.1 <sup>c</sup>	150	27.1
LIN	%	41.0	38.2	34.2	41.3	150	8.6
ALH	$\mu\text{m}$	2.8 <sup>a</sup>	3.7 <sup>b</sup>	3.1 <sup>a</sup>	3.8 <sup>b</sup>	150	1.9
Acrosome-reacted sperm	%	21.8 <sup>a</sup>	17.2 <sup>a</sup>	27.2 <sup>b</sup>	22.4 <sup>ab</sup>	25	9.3

Means not having a common letter (abc) in the same row are significantly different ( $P \leq 0.05$ ).

**Table III.** Main fatty acids of total phospholipids in rabbit spermatozoa (% of total fatty acids).

	Control	Vit E	n-3	n-3+E	n-3+EC	SEM
Σ SFA <sup>1</sup>	37.07 <sup>c</sup>	32.71 <sup>bc</sup>	30.60 <sup>ab</sup>	28.39 <sup>a</sup>	26.40 <sup>a</sup>	3.21
Σ MUFA <sup>1</sup>	15.90	16.50	15.80	15.45	15.35	1.10
Σ PUFA <sup>1</sup>	47.03 <sup>a</sup>	50.79 <sup>ab</sup>	53.60 <sup>b</sup>	56.16 <sup>bc</sup>	58.25 <sup>c</sup>	3.69
C18:2n-6	3.05	3.96	2.50	2.55	2.43	0.57
C18:3n-6	0.19	0.35	0.11	0.15	0.10	0.05
C20:3n-6	2.46	2.21	2.12	1.87	1.96	0.26
C20:4n-6	3.30 <sup>b</sup>	3.43 <sup>b</sup>	2.72 <sup>a</sup>	2.63 <sup>a</sup>	2.35 <sup>a</sup>	0.63
LCP n-6	5.76 <sup>b</sup>	5.64 <sup>b</sup>	4.50 <sup>a</sup>	4.50 <sup>a</sup>	4.31 <sup>a</sup>	1.02
C18:3n-3	0.71 <sup>a</sup>	1.14 <sup>ab</sup>	1.25 <sup>bc</sup>	1.68 <sup>c</sup>	1.76 <sup>cd</sup>	0.57
C20:3n-3	3.02	3.00	2.90	2.88	3.02	0.98
C20:5n-3	1.05 <sup>a</sup>	1.51 <sup>a</sup>	2.20 <sup>b</sup>	2.66 <sup>b</sup>	2.79 <sup>b</sup>	1.25
C21:5n-3	0.00 <sup>a</sup>	0.59 <sup>a</sup>	1.00 <sup>ab</sup>	1.59 <sup>b</sup>	1.67 <sup>b</sup>	0.95
C22:5n-3	10.51 <sup>a</sup>	11.05 <sup>b</sup>	12.30 <sup>b</sup>	12.84 <sup>b</sup>	13.48 <sup>c</sup>	1.56
C22:6n-3	22.74 <sup>a</sup>	23.55 <sup>a</sup>	26.50 <sup>b</sup>	27.31 <sup>b</sup>	28.68 <sup>b</sup>	2.45
LCP n-3 <sup>2</sup>	37.59 <sup>a</sup>	39.70 <sup>ab</sup>	44.90 <sup>c</sup>	47.28 <sup>c</sup>	49.64 <sup>d</sup>	2.87

<sup>1</sup> See footnotes in Table I.

<sup>2</sup> LCP long-chain polyunsaturated (≥ C20).

*n* = 5 for each group. Means not having a common letter (abcd) in the same row are significantly different (*P* ≤ 0.05).

compared to those fed non-supplemented diets (control and vitamin E). Furthermore, such dietary treatment produced a different partitioning of PUFA and reduced the level of n-6, mainly arachidonic acid, and increased the percentage of total n-3 (mainly α-linolenic, EPA and DHA). Mono-unsaturated fatty acids (MUFA) were not affected by the dietary treatment.

**3.5. Oxidative status in seminal plasma and sperm**

The production of ROMs in seminal plasma showed the same trend as in blood

plasma (Tab. IV). Vitamin E enhanced the antioxidant capacity (257.5 mmol HClO·mg<sup>-1</sup>) and TBARS during semen storage without affecting ROMs production.

The addition of n-3 fatty acids reduced the antioxidant capacity and increased the production of ROMs and TBARS, even when associated with vitamin E.

The n-3+EC showed the highest oxidative stability sustained by the highest antioxidant capacity (300.4 mmol HClO·mg<sup>-1</sup>), and the lowest TBARS and lipoperoxide values either in fresh or stored semen.

Storage for 24 h at 4 °C amplified the differences between the groups and favoured

**Table IV.** Oxidative status in seminal plasma and spermatozoa before and after 24 h of storage at 4 °C.

Treatment	Control	Vitamin E	n-3	n-3+E	n-3+EC	SEM
Traits						
Antioxidant capacity	mmol HClO.mg <sup>-1</sup>	175.9 <sup>b</sup>	110.0 <sup>a</sup>	150.2 <sup>b</sup>	300.4 <sup>c</sup>	12.6
ROMs	mg% of hydrogen peroxide	3.6 <sup>c</sup>	4.5 <sup>d</sup>	3.5 <sup>c</sup>	1.4 <sup>a</sup>	0.6
Liperoxides 0 h	ηmol hydrop 10 <sup>6</sup> spz	50.7 <sup>b</sup>	81.2 <sup>c</sup>	67.2 <sup>bc</sup>	32.9 <sup>a</sup>	7.0
Liperoxides 24 h	ηmol hydrop 10 <sup>9</sup> spz	38.5 <sup>bc</sup>	70.2 <sup>d</sup>	53.3 <sup>cd</sup>	15.8 <sup>a</sup>	6.6
TBARS 0h	nmol MDA/sperm <sup>8</sup>	19.8 <sup>b</sup>	23.2 <sup>c</sup>	18.4 <sup>b</sup>	13.1 <sup>a</sup>	2.5
TBARS 24h	nmol MDA/sperm <sup>8</sup>	30.0 <sup>b</sup>	45.8 <sup>d</sup>	37.9 <sup>c</sup>	19.5 <sup>a</sup>	3.0

*n* = 5 for each group.

Means not having a common letter (abcd) in the same row are significantly different (*P* ≤ 0.05).

the TBA-RS production. Stored semen had a lower amount of lipoperoxides but a higher level of aldehydes and ketones.

#### 4. DISCUSSION

The plasma  $\alpha$ -tocopherol level (Fig. 1) increased with the age of the rabbits. This is probably due to its continuous accumulation in the serum lipoproteins [26]. Mature rabbit bucks fed the n-3 diet showed the lowest plasma level of  $\alpha$ -tocopherol which was improved by its dietary supplementation ( $200 \text{ mg}\cdot\text{kg}^{-1}$ ) as reported by some authors in other species [27, 28]. The simultaneous ingestion of vitamins E and C determined a further accumulation of plasma  $\alpha$ -tocopherol probably due to a saving effect of ascorbate on tocopherol, which is able to repair the tocopheroxyl radicals [14]. Chen et al. [29] reported a synergistic effect between the two vitamins, since a moderate vitamin C addition to vitamin E-deficient rats enhanced the plasma  $\alpha$ -tocopherol concentration avoiding its oxidation.

Although the rabbit is able to synthesise vitamin C from glucose [30], stress conditions [31] due to intensive rearing systems impair this process and ascorbate supplementation has been shown to have a significant antioxidant effect.

The availability different vitamins of the resulting from dietary treatments had a marked effect on the oxidative status of the animals (Fig. 3) which is also affected by the age of the bucks. The increased production of ROMs with ageing is probably due to a physiological increase of the anabolic processes [32].

The supranutritional supplementation of vitamin E had a low effect on ROMs production. This trend was more pronounced when the diets were simultaneously fortified with n-3 and only the further addition of ascorbate strongly reduced the ROMs.

Such results suggest a different response of the two antioxidants. Vitamin C has a

scavenger and sparing effect on tocopherol [29], whereas  $\alpha$ -tocopherol mainly has a chain breaking effect [33]. The oxidative potential of the environment seems to affect the radical-trapping action of  $\alpha$ -tocopherol. When the system is "unstable" (n-3 groups) its effect is negligible whereas a less oxidative environment renders the action more evident.

The hypothesis of a response related to the oxidative potential of the system could also explain the scavenger effects of vitamin E reported by the other authors [34] in animals fed diets not enriched with lipids.

The depressive effect of a supra-nutritional level of antioxidants on some components of the endogenous antioxidant system, hypothesised by some authors [35], was not observed by us and supranutritional doses of both vitamins reduced the plasma ROMs [36]. Although the protective action can be better obtained by avoiding megadoses of antioxidants [37] and by the administration of physiological amounts, the optimum level of some key vitamins required for maintaining the health or enhancing some specific functions is higher than that needed for the prevention of deficiency [38].  $0.5 \text{ g}\cdot\text{L}^{-1}$  of ascorbate and amounts of vitamin E 4-fold higher than the requirement [30] did not show any negative response on the antioxidant capacity of the organism.

In addition to the effect on the whole organism, dietary treatments modified the balance between pro- and anti-oxidant factors even in the semen (Tab. IV).

Supplementation with LCP n-3 fatty acids largely increased the susceptibility of semen to peroxidation and only a supranutritional level of vitamins E and C counteracted this process [39].

Taking the spermatozoa of the control group as a reference,  $\alpha$ -tocopherol reduced TBA-RS, whereas n-3 fatty acids enhanced lipoperoxides and TBA-RS. In such conditions, vitamin E alone was not able to

control the first phase of the oxidative process (ROMs and lipoperoxide production) whereas it successively broke the lipid peroxidation cascade following the production of lipoperoxides by preventing the conversion of these compounds into their derivatives [33].

On the contrary, vitamin C, largely present in the aqueous phase of the rabbit seminal plasma (about 180–250  $\mu\text{mol}\cdot\text{L}^{-1}$  [37]), reduced ROMs and lipoperoxides, neutralising oxidants before they began to attack the fatty acids of spermatozoa.

During semen storage in all the groups the oxidative status of the spermatozoa worsened and there was a reduction in lipoperoxides and an increase in TBA-RS.

This was probably due to the conversion of lipoperoxides to aldehydes and ketones (TBA-RS). Halliwell and Gutterige [14] postulated that many membrane peroxides can be converted to alcohols by phospholipid hydroperoxide glutathione peroxidase enzymes. It is also possible that peroxides, in the presence of  $\text{Ca}^{++}$  ions, are cleaved from membranes by phospholipase  $\text{A}_2$ , converted to alcohols by glutathione peroxidase and restored to fatty acids through reacylation with fatty-acyl-coenzyme A [40].

The kinetic characteristics of fresh semen were not affected by dietary supplementation with vitamins and/or fatty acids but the deleterious effect of a high amount of lipid peroxides occurred only after storage in the n-3 group.

Peroxidative damage has been proposed as one of the major causes of defective sperm functioning and several authors have shown that a loss of motility in spermatozoa is often related to the accumulation of ROMs in cells.

However, the acrosome seemed to be the compartment of rabbit spermatozoa most sensitive to ROMs attack making this apparatus very unstable. It is widely known that peroxide accumulation impairs cell

membrane ion exchange, which is essential for maintaining normal sperm motility and disrupts the ionophore-induced acrosome reaction [41].

Recent studies have shown that spermatozoa damaged by highly spermatotoxic ROMs, such as hydrogen peroxide and hydroxyl radicals, can be reversed by the addition of vitamins E and C [15, 21].

As well as the increase in ROMs, n-3 fatty acid supplementation altered the membrane phospholipid fatty acid profile (Tab. III) reducing linoleic and arachidonic acid in the membrane, due to competition on the same metabolic pathway [42]. These fatty acid changes enhanced the susceptibility of spermatozoa to undergo the acrosome reaction.

This last event is crucial in the fertilisation process. The capacitated sperm activated by uterine fluid moves through the female's reproductive tract and reaches the *zona pellucida* (ZP). At this point the acrosomal reaction is essential for ZP crossing, membrane fusion and fertilisation of gametes. Therefore for artificial insemination, an earlier acrosome reacted spermatozoa reduces the number of sperms potentially capable of fertilizing oocytes [43].

## 5. CONCLUSION

This study showed that LCP n-3 fatty acid supplementation modified the fatty acid profile of the spermatozoa phospholipid and simultaneously reduced the oxidative stability of the rabbit semen. In such an oxidatively unbalanced system, vitamin E alone was not enough to restore stability and only the association with vitamin C reduced oxidative processes and improved the characteristics of fresh and stored semen.

Other studies on different strategies to "enrich" rabbit spermatozoa in LCP n-3 fatty acids for improving their fertilizing ability are necessary; furthermore it should

interesting to correlate the vitamin E and C concentrations in spermatozoa with their oxidative status and fatty acid composition.

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