Proceedings of the

4-7 july **2000** – Valencia Spain

These proceedings were printed as a special issue of WORLD RABBIT SCIENCE, the journal of the World Rabbit Science Association, Volume 8, supplement 1

ISSN reference of this on line version is 2308-1910
(ISSN for all the on-line versions of the proceedings of the successive World Rabbit Congresses)

CASTELLINI C., DAL BOSCO A., BERNARDINI M.

EFFECT OF DIETARY α-TOCOPHERYL ACETATE AND ASCORBIC ACID : VITAMIN CONTENT AND OXIDATION STATUS OF RABBIT SEMEN

Volume A, pages 105-110

EFFECT OF DIETARY α-TOCOPHERYL ACETATE AND ASCORBIC ACID: VITAMIN CONTENT AND OXIDATION STATUS OF RABBIT SEMEN

CASTELLINI C., DAL BOSCO A., BERNARDINI M.

Department of Animal Science, Borgo 20 Giugno, 74 - 06100 Perugia, Italy

ABSTRACT

The aim of the study was to verify the effect of α-tocopheryl acetate (50 vs 200 mg kg^{-1}) and ascorbic acid (0 vs 1 g L^{-1} water) on the oxidative status of rabbit bucks and semen. The research showed that supranutritional levels of the two vitamins reduced blood serum (26.24 vs 38.15 mg hydrogen peroxide 100 mL^{-1}) and seminal ROMs (1.98 vs 5.60 mg hydrogen peroxide 100 mL^{-1}) and oxidative products (32.91 vs 58.57 nmol hydroperoxide/10^9 sperm; TBA-RS 12.25 vs 20.90 ηmol MDA/10^8 sperm) and enhanced its antioxidant capacity (300.36 vs 175.95 ηmol HClO mL^{-1}). These positive effects increased in spermatozoa stored for 24 hours at 5° C.

INTRODUCTION

Radical Oxygen Molecules (ROMs) are normally generated during cell metabolism and support the phagocytosis of invading organisms. However, abnormal proliferation of ROMs increases structural damage of various cell components such us proteins, DNA, biomembranes (CHEN et al., 1997) since the natural antioxidant mechanisms have a limiting ability to protect tissues from oxidative injury. Excessive production of ROMs is particularly dangerous to spermatozoa (AITKEN et al., 1993); as a consequence of the high degree of unsaturation of membrane lipids (POULOS et al., 1973), peroxidation has been considered as one of the major causes of infertility (JONES et al., 1979).

The relationship between oxidative stress and certain major antioxidants is of great importance in delaying the development of ROMs which may damage membranes or modify critical enzyme pathways (HALIWELL, 1994).

In rabbit, some researchers have reported the effects of dietary antioxidants on fertilising ability and on the kinetic characteristics of spermatozoa (CASTELLINI et al., 1999; MINELLI et al., 1999), but no information is available on the oxidative status of bucks and semen.

The aim of the present study was to verify the effect of vitamins E and C on the oxidative status of rabbit bucks and on the characteristics of semen.

MATERIAL AND METHODS

Animals and diets

The trial was carried out from March to May 1999 in the experimental rabbitry of the Animal Science Department with a photoperiod of 16 hours light/day. Twenty New Zealand White rabbit bucks of proven fertility, reared in individual cages, were randomly assigned to the following four treatments: Control, basal diet with 50 mg α-tocopheryl acetate kg^{-1}; Vitamin E, 200 mg α-tocopheryl acetate kg^{-1}; Vitamin C, basal diet + 1 g ascorbic acid L^{-1} drinking water; Vitamin E+C, 200 mg α-tocopheryl acetate + 1 g ascorbic acid L^{-1}. The chemical composition of the diets was the following: 17.6% crude protein, 2.8 % ether extract, 17.7% crude fiber, and 10.8 Mj/kg of Digestible Energy. During the trial the feed and water intake was recorded.

Sample Collection and Semen Analysis

After a preliminary period of 1 month, semen was collected weekly (for 5 weeks) and samples were maintained at 5° C and analyzed within 1 hour. The number of live cells was assessed

by fluorescent microscopy (Olympus CH2) with Propidium Iodide and Carboxyfluorescein diacetate counting at least 500 cells per sample. Hypo-osmotic viability tests were done placing cells in media at 80 mOsm and counting the surviving of sperms after stress (CHAN et al., 1992). One hour before semen collection, blood samples were drawn from the marginal ear vein, collected in heparinized vacutainers and centrifuged at 10,000 x g for 10 min at + 5 °C.

Determination of Vitamin E and Vitamin C

The α-tocopherol of the plasma and semen was measured according to SCHUEP AND RETTENMEIER (1994) by HPLC (CM 4000, Milton Roy, Riviera Beach, FL), using a silica column (Beckman, Fullerton, CA, USA).

Ascorbate was determined spectrophometrically by the 2-4 dinitrophenylhydrazine method as described by DABROWSKI AND HINTHERLEITNER (1989).

Antioxidant status of blood plasma and semen

The assessment of the antioxidant capacity and ROMs in blood serum and seminal plasma was carried out using, respectively the Oxi-adsorbent kit and the d-ROMs test produced by DIACRON ® s.r.l. (Italy) (CESARONE et al., 1999). Lipoperoxides of spermatozoa were determined with the d-ROMs test kit 1 hour and 24 hours after semen collection and dilution (1:10) in two different medium: seminal plasma or Tris-glucose-citrate (300 mOsm g^{-1}, pH 7.1) maintained at + 5 °C.

Thiobarbituric Reactive Substances (TBA-RS) of spermatozoa were also measured 1 hour and 24 hours after collection, according to the procedure of AITKEN et al. (1993), inducing peroxidation with ferrous sulphate (0.2 mM) and sodium ascorbate (1 mM) at 37 °C for 1 hour. The results are expressed as nmol Malondialdeide (MDA)/10^8 sperm .

Statistical Analysis

Data were statistically evaluated with a linear model (GLM/SAS, 1990). The significance of differences was evaluated by t-tests.

RESULTS AND DISCUSSION

The average daily feed and water intake during the trial was respectively, 185 g and 345 mL.

The α-tocopherol level in the blood serum significantly increased according to the intake and also was affected by vitamin C supplementation (Table 1). Vitamin C combined with the basal diet reduced the α-tocopherol content by about 40% while, associated with the highest level of vitamin E, increased it about 3 times.

The α-tocopherol in the seminal plasma was less responsive to dietary supplementation: the highest increase (2.5 times the control value) was shown in vitamin E+C group.

Ascorbate was 2-4 times more concentrated in seminal plasma than in serum. The vitamin C group, had the highest level of ascorbate in both blood and semen; supplementation with 200 mg kg^{-1} of vitamin E reduced the ascorbate level in seminal plasma.

Ascorbate addition (vitamin C and vitamin E+C groups) significantly reduced ROMs in blood serum and seminal plasma, while vitamin E alone was less effective. The antioxidant capacity of seminal plasma was significantly affected by vitamin supplementation: bucks fed high vitamin E, associated or not with vitamin C, had the highest values and the same trend was observed for lipoperoxide and TBA-RS. The vitamin C group always gave the worst results.

These trends were even more pronounced after 24 hours of semen storage in different mediums and vitamin E, especially if supplemented simultaneously with ascorbate, gave the lowest level of lipoperoxides.

Table 1 – Vitamin level and oxidative status of serum and semen.

		Control	Vitamin E	Vitamin C	Vitamin E + C	Pooled SE
Blood serum						
α-tocopherol	μg mL^{-1}	3.67B	8.21C	2.16A	10.79D	1.72
ascorbate	"	9.85A	11.53A	19.73C	14.58B	1.89
ROMs	mg hydrogen peroxide 100 mL^{-1}	38.15C	35.81BC	34.92B	26.24A	3.52
Seminal plasma						
α-tocopherol	μg mL^{-1}	0.22A	0.34B	0.14A	0.55C	0.08
ascorbate	"	41.24B	30.39A	60.12C	44.42B	1.89
ROMs	mg hydrogen peroxide 100 mL^{-1}	5.60C	4.47BC	3.87B	1.98A	0.52
Antioxidant capacity	μmol HClO mL^{-1}	175.95Ab	257.57B	159.19Aa	300.36B	11.92
Lipoperoxides of sperm						
After 1 h	μmol hydrop/sperm 10^9	58.57B	50.73B	81.19C	32.91A	7.66
After 24 h in tris buffer	"	38.54C	27.78B	71.70D	19.84A	6.57
in seminal plasma	"	25.84B	19.33C	42.25D	9.97A	4.14
TBA-RS of sperms						
After 1 h	ηmol MDA/sperm 10^8	20.90B	15.88A	21.20B	12.25A	2.13
After 24 h	"	30.45BC	24.50AB	36.22C	18.81A	1.02
Live cells						
Standard medium	%	78.25	79.45	78.12	81.24	22.50
Hypo-osmotic medium	"	52.18	50.17	47.22	58.89A	11.92

N=25 for each group; A..D: P<0.01; a..b: P<0.05.

Therefore the antioxidant response of semen was affected by the relative amounts of the two vitamins which displayed an antagonistic-synergistic effect. When both molecules were at high levels they reduced the production of radicals, the oxidation products (lipoperoxides, TBA-RS), and gave the best antioxidant protection. On the contrary, when a megadose of vitamin C was added to the basal diet, the effect was absolutely negative. Also CHEN (1989) observed a similar tendency: a large dose of vitamin C given to rats, minimally adequately for vitamin E, promoted lipid peroxidation and significantly decreased the antioxidant potential of the animals.

Although the enrichment of ascorbate in the semen suggests a physiological role in limiting oxidation (ASKARI et al., 1994), ascorbic acid requires α-tocopherol within the membrane to scavenge radicals (CHEN, 1989).

These data confirm that the role of ascorbate is mainly related to a sparing effect on the active form of vitamin E (NIKI, 1984) and that a high concentration of ascorbate requires an adequate level of vitamin E otherwise it becomes a proxidant (VERNA et al., 1998). Regarding the mechanism of the two vitamins it appears that the main role of vitamin E is as a "chain-breaking" antioxidant with limited scavenger properties as shown by the limited reduction in ROMs, in contrast ascorbate mainly reduced ROMs. Thus the efficiency of vitamin E in controlling the oxidative stress depends on its level within the membranes; even reducing the percentage of seminal plasma by diluting semen with standard medium (Tris) its positive effect remained almost the same.

The number of live cells and the resistance to hypo-osmotic medium were similar in the control, vitamin E and vitamin C groups; only in the group E+C a significantly higher viability was obtained.
In conclusion it appears that the major antioxidant of rabbit semen is α-tocopherol and that vitamin C has a positive role only in association with the supranutritional level of vitamin E. The highest resistance to hypo-osmotic and oxidative stresses of groups supplemented with both vitamins, lead us to expect a better response to the freezing-thawing process. Further studies have to be carried out to verify of this hypothesis.

ACKNOWLEDGEMENT

Paper financed by MURST 40%. We thank Sister Mary F. Traynor for her valuable linguistic suggestions.

REFERENCES

AITKEN, R.J., HARKISS, D., BUCKINGHAM, D. 1993: Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil*. **98**: 257-65.

ASKARI, H.A., CHECK, J.H., PEYMER, N., BOLLENDORF, A. 1994: Effect of natural antioxidants tocopherols and ascorbic acids in maintenance of sperm activity during freeze-thaw process. *Arch Andrology*. **33**: 11-15.

CASTELLINI, C., LATTAIOLI, P., BERNARDINI, M. 1999: Effect of dietary supplementation with α-tocopheryl acetate and ascorbic acid on qualitative characteristics and fertilizing ability of rabbit semen. *World Rabbit Sci*. **7**: 217-220.

CESARONE, M.R., BELCARO, G., CARRATELLI, U., CORNELI, U., DE SANCTIS, M.T., INCANDELA, L., BARSOITI, A., TERRANOVA, R., NICOLAIDE, S.A. 1999: A simple test to monitor oxidative stress. *Int. Angiology*. **18**: 127-130.

CHAN, P.J., TREDWAY, D.R., PANG, S.C. 1992: Assessment of sperm for cryopreservation using the hypoosmotic viability test. *Fertil. Steril*. **58**: 841-844.

CHEN, C.S., CHAO, H.T., PAN, R.L., WEI, Y.H. 1997: Hydroxyl radical-induced decline in motility and increase in lipid peroxidation and DNA modification in human sperm. *Biochem. Mol. Biol. Int*. **43**: 291-303.

CHEN, L.H. 1989: Interaction of vitamin E and ascorbic acid. *In Vivo* **3**: 199-209.

DABROWSKI, K., HINTHERLEITNER, S. 1989: Applications of a simultaneous assay of ascorbic acid, dehydroascorbic acid and ascorbic sulphate in biological materials. *Analyst.* **114**: 83-87.

HALIWELL, B. 1994: Free radicals, antioxidants and human disease: curiosity, cause or consequence? *Lancet*. **334:** 721-724.

JONES, R., MANN, T., SHERINS, R. 1979: Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril.* **31**: 531-537.

MINELLI, G., ZUCCHI, P., LUZI, F., CAVANI, C. 1999. Addition de niveaux élevés des vitamines C et E seules ou en mélange dans l'aliment: influence sur les caractéristiques de la semence de lapin. 8èmes Jour. Rech. Cunicole Paris, 171-174.

NIKI, E. 1984: Interaction of ascorbate and α-tocopherol. *Ann. NY Acad. Sci.* **498**: 186-199.

POULOS A., DARIN-BENNET A., WHITE I.G. 1973: The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. *Comp Biochem*. **46**: 541-549.

SAS - SAS/STAT®. 1990: User's Guide, Version 6, Cary, NC, USA.

SCHUEP, W., RETTENMEIER, R. 1994: Analysis of vitamin E homologous in plasma and tissue: high-performance liquid chromatography. *Method. Enzymol.* **234**: 294-302.
VERNA, A., KANWAR, K.C. 1998: Human sperm motility and lipid peroxidation in different ascorbic acid concentrations: an in vitro analysis. *Andrologia*. **30**: 325-329.