FATTY ACID AND TOCOPHEROL COMPOSITION OF SEMEN COMPONENTS IN THE RABBIT

ZANIBONI L.[1], GLIOZZI T.[2], MALDJIAN A.[1], LUZI F.[3], CEROLINI S.[1]

[1]VSA Department, Università degli Studi, via Trentacoste 2, 20134 Milano. Italy.
[2]IBBA-CNR, via Bassini 15, 20133 Milano. Italy.
[3]Instituto di Zootecnica, Università degli Studi, via Celoria 10, 20133 Milano. Italy.
luisa.zaniboni@unimi.it

ABSTRACT

The aim of this study was to determine and compare the lipid composition and the tocopherol contents in the different components of rabbit semen: spermatozoa, seminal plasma and granules. Semen was collected via an artificial vagina from 16 rabbits. Semen collection was repeated in two subsequent weeks. Two ejaculates were obtained from each male on the same day. Individual ejaculates were pooled in 3 semen samples from 5-6 bucks each. Sperm concentration, viability and motility were measured. Granule concentration was also estimated. Seminal plasma was separated after centrifugation; spermatozoa and granules were separated by Percoll® density gradient centrifugation. Lipid were extracted from semen components and separated by TLC; fatty acid composition of phospholipid was determined by GC on capillary column. The content of tocopherols in semen components was determined by HPLC. Semen quality was good and the mean values recorded were in agreement with standard reproductive performance of bucks. Phospholipid was the most important lipid class found in the different components of rabbit semen. The characteristic fatty acid composition of rabbit spermatozoa has been confirmed: polyunsaturates accounted for the major proportion of fatty acids and were mainly represented by C22:5n-6 (36%); n-3 polyunsaturates were present in very low proportion, and also the proportion of monounsaturates was low. The fatty acid composition of phospholipid in seminal plasma and granules showed a lower level of unsaturation and a greater proportion of oleic acid in comparison with the gamete composition. Of interest was the presence of consistent proportion of different n-6 polyunsaturates, from C18:2 to C22:5, in seminal plasma. Two main forms of tocopherols, α and δ, have been found in all components of rabbit semen, and spermatozoa were the preferential site of accumulation.

Key words: rabbit, semen, fatty acids, tocopherols.

INTRODUCTION

Sperm cells contain very high proportions of polyunsaturated fatty acids (PUFAs); this characteristic composition confers to sperm plasma membranes the fluidity they require

to undergo the membrane fusion events that characterize fertilization. The proportion of sperm PUFAs has been directly related to semen quality in different species (NISSEN and KREYSEL, 1983; CEROLINI *et al*., 2002).

On the other hand, high level of PUFAs increases the susceptibility of the cells to free radical induced peroxidative damages, considered a significant cause of male infertility. Sperm cells possess an antioxidant protection, involving mechanisms to deal with free radicals which are constantly produced in cells and cause in turn lipid peroxidation. Vitamin E is one of the major natural lipid-soluble antioxidants present in cell membranes, and plays a crucial role in breaking the chain reaction of peroxidation.

Rabbit spermatozoa are rich in n-6 PUFAs, mainly C22:5, and lack in n-3 PUFAs, (POULOS *et al*., 1973) in contrast with spermatozoa of the majority of the domestic mammalian species, where C22:6n-3 predominates. Moreover, rabbit semen frequently contains many particles of various sizes originating from the accessory sex GLANDS (HOLTZ and FOOTE, 1978). These granules can interfere with the measurement of different semen quality parameters performed by non visual equipment (FARRELL *et al*., 1996; GLIOZZI *et al*., 2003). The aim of this study was to determine and compare the lipid composition and the tocopherol contents in the different components of rabbit semen: spermatozoa, seminal plasma and granules.

MATERIAL AND METHODS

Semen was collected via an artificial vagina (IMV, l'Aigle Cedex, France) from 16 mature commercial hybrid rabbits. Semen collection was repeated in two subsequent weeks. Rabbits were kept in an intensive rabbit farm in northwest Italy in accordance with the Italian and European Union legislation.

Two ejaculates were obtained from each male on the same day. The volume of each ejaculate was recorded for each collection before to dilute semen 1:2 with Tris extender and to pool the 1st and 2nd collection; individual ejaculates were then pooled in 3 semen samples from 5-6 bucks each. Pooled samples were immediately stored at 5°C and transferred to the laboratory. Sperm concentration was estimated microscopically using a Neubauer® counting chamber (Brand, Wertheim, Germany); on the same counting chamber, granule concentration was estimated. Sperm viability was measured by the nigrosin eosin staining method (PARENTE *et al*., 1994). Sperm motility was assessed subjectively using a microscope (x 400) with a hot plate (37°C, 5 min incubation). Semen samples were then centrifuged at 950 x *g* for 20 min at 5°C, the supernatant was centrifuged at 1900 x *g* for 20 min at 5°C and stored at -20°C for subsequent analysis on seminal plasma. Cell pellets from 1st and 2nd centrifugation were mixed together and diluted with Tris extender to obtain a cell concentration of 1.5 x 10^8 cells/ml. Diluted semen was then subjected to Percoll® density gradient centrifugation (GLIOZZI *et al*., 2003) in order to separate sperm cells from the granules. The rich granule fraction was collected and washed in Tris extender at 1900 x *g* for 20 min at 5°C and the granules harvested were quantified by weighing. The pellets obtained were then resuspended in

1 ml of Tris extender or in 0.35 ml of NaCl 5% according to the subsequent analysis to be performed (lipids or tocopherols respectively) and stored at -20°C. The cell rich loose pellets were washed in Tris extender at 1900 x *g* for 20 min at 5°C, re-suspended in 1 ml of Tris extender or in 0.35 ml of NaCl 5% as described for the granules and stored at -20°C.

Lipid composition of seminal plasma, granules and spermatozoa was determined. Total lipids were extracted in excess chloroform:methanol (2:1) (v:v) (FOLCH *et al*. 1957), and then separated by thin layer chromatography (TLC) in the following classes: phospholipids (PL), free cholesterol (Chol), free fatty acids (FFA), triacylglycerols (TG) and cholesterol ester (CE). The fatty acid profile of the major classes was determined by gas chromatography on capillary column (CHRISTIE *et al*., 1970; EVERSHED, 1992) after trans-methylation.

Vitamin E was measured in seminal plasma, granules and sperm cells by high performance liquid chromatography (DVORSKA *et al*, 2002). Alpha and δ tocopherol standards (Sigma Chemicals) were used for quantification of the amount detected.
Means and standard errors were calculated.

RESULTS AND DISCUSSION

The characteristics of the ejaculates collected for analyses are reported in Table 1. Mean values recorded for semen quality parameters were in agreement with standard reproductive performance of bucks. Granules, as expected, were present in semen samples and a large variability was observed between males.

Table 1. Characteristics of the ejaculates considered for analysis

Semen parameters	Mean	SE
Volume 1st jump (ml)	0.57	0.04
Volume 2nd jump (ml)	0.55	0.04
Concentration (10^6/ml)	352.1	35.0
Granule concentration (10^6/ml)	441.3	78
Total sperm number (10^6)	391.9	48.1
Motility (%)	55.8	3.75
Viable sperm (%)	59.6	4.50
Dead sperm (%)	40.4	4.50

Phospholipid was the most important lipid class found in the different components of rabbit semen. The average amount was 324.4 μg/10^9 in spermatozoa, 77.6 μg/ml in seminal plasma and 963.7 μg/g in granules. The fatty acid composition of total phospholipid showed some variations among spermatozoa, seminal plasma and granules (Table 1). The male gametes of the rabbit were characterised by a very high proportion of polyunsaturates, 49 %, represented by almost only n-6 fatty acids which is typical of rodent male gametes (BIERI and PRIVAL, 1965). The most important n-6 PUFA

was C22:5, as already reported in previous papers (POULOUS *et al*., 1973), and just itself accounted for the 36 % of total fatty acids. In contrast with the majority of other mammalian spermatozoa, n-3 polyunsaturates were present in very small amount and did not even accounted for 1% of total fatty acids (Table 2). The proportion of total polyunsaturates in seminal plasma phospholipid was 39 %, lower than in spermatozoa, still mainly represented by n-6 fatty acids. The most important n-6 PUFA in plasma phospholipid was C18:2, the essential precursor of the n-6 family, and also C22:4 and C22:5 accounted for significant proportions, as shown in Table 1. Monounsaturates, mainly 18:1n-9, were also well represented. The ratio saturates/polyunsaturates (S/P) was very close to 1 in both spermatozoa and seminal plasma. The proportion of polyunsaturates in granule phospholipid was 30 %, lower than in seminal plasma, and again n-6 PUFAs were the most represented. Total saturates accounted for the highest proportion (46%) of fatty acids in granules, therefore, the ratio S/P was increased to 1.57 compared to the values found in sperm and seminal plasma. Monounsaturates were also well represented in granule phospholipid, as found in seminal plasma.

Table 2. Fatty acid composition (mean ± SE of the proportion on total fatty acids) of total phospholipid in semen components of the rabbit.

Fatty acids (%)*	Spermatozoa	Granules	Seminal plasma
C14:0	3.16 ± 0.75	0.95 ± 0.11	1.19 ± 0.29
C16:0	19.8 ± 1.59	25.3 ± 2.45	20.9 ± 1.21
C16:1	<0.5	3.23 ± 2.04	0.95 ± 0.23
C18:0	23.0 ± 0.53	18.1 ± 0.84	18.1 ± 0.63
C18:1n9	3.65 ± 0.45	17.9 ± 0.89	16.8 ± 0.41
C18:2n6	5.49 ± 0.59	11.3 ± 1.54	13.8 ± 0.42
C18:3n3	<0.5	0.67 ± 0.50	<0.5
C20:0	<0.5	1.02 ± 0.44	0.74 ± 0.21
C20:1n9	<0.5	0.75 ± 0.43	<0.5
C20:3n6	1.98 ± 0.20	<0.5	<0.5
C20:4n6	2.87 ± 0.29	1.03 ± 0.15	3.21 ± 0.24
C22:0	<0.5	0.71 ± 0.31	<0.5
C22:4n6	1.48 ± 0.08	<0.5	9.00 ± 0.69
C22:5n6	36.5 ± 1.18	16.0 ± 2.37	10.3 ± 0.83
C22:5n3	<0.5	0.95 ± 0.29	0.80 ± 0.09
C22:6n3	<0.5	<0.5	0.75 ± 0.28
Total saturates	46.4 ± 2.24	46.1 ± 4.11	41.8 ± 1.22
Total monounsaturates	4.5 ± 0.57	23.1 ± 2.15	19.1 ± 0.53
Total PUFAs	49.1 ± 2.21	30.6 ± 2.59	39.0 ± 1.59
Total n-3	0.73 ± 0.34	1.75 ± 0.76	1.93 ± 0.45
Total n-6	48.3 ± 1.96	28.9 ± 2.48	36.8 ± 1.51
S/P	0.96 ± 0.08	1.57 ± 0.26	1.09 ± 0.07

nd = not detected; PUFAs = polyunsaturates; S/P = saturates/polyunsaturates ratio
* only fatty acids accounting for more than 0.5% are reported

The total amount of tocopherol in 1 ml of semen was 46.9 ng. Alpha-tocopherol was the most important form identified and quantified in 31.7 ng/ml; δ-tocopherol was also identified, in agreement with previous results, and the content was 15.2 ng/ml. The larger proportion of both tocopherols was present in spermatozoa (Figure 1) followed by progressive lower proportions in granules and plasma. The preferential distribution of vitamin E in spermatozoa confirms its major antioxidant function to prevent sperm lipid peroxidation in the rabbit, as already shown in other domestic animal species.

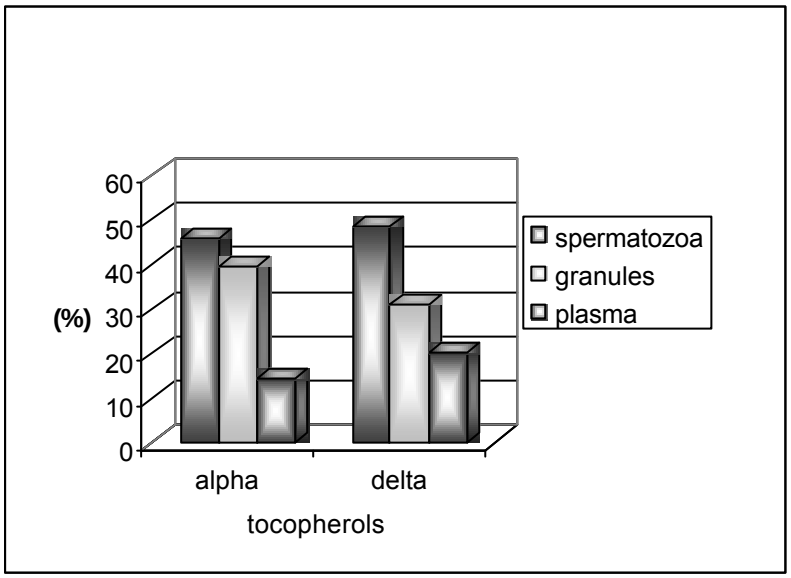

Figure 1. Proportional distribution of tocopherols, alpha and delta, in rabbit semen components.

CONCLUSION

The characteristic fatty acid composition of rabbit spermatozoa has been confirmed: polyunsaturates accounted for the major proportion of fatty acids and were mainly represented by C22:5n-6 (36%); n-3 polyunsaturates were present in very low proportion, and also the proportion of monounsaturates was low. The fatty acid composition of phospholipid in seminal plasma and granules showed a lower level of unsaturation and a greater proportion of oleic acid in comparison with the gamete composition. Of interest was the presence of consistent proportion of different n-6 polyunsaturates, from C18:2 to C22:5, in seminal plasma. Two forms of tocopherols, α and δ, have been found in all components of rabbit semen, and spermatozoa were the preferential site of accumulation.

REFERENCES

BIERI J.G. AND PRIVAL E.L. 1965. Lipid composition of testes from various species. *Comp. Biochem. Physiol*. **15**:275-279.

CEROLINI S., GLIOZZI T.M., PIZZI F., PARODI L., MALDJIAN A., NOBLE R. 2002. Importanza strutturale e funzionale delle componenti lipidiche negli spermatozoi degli animali domestici di interesse domestico. *Progress in Nutrition*. **4**:151-154.

CHRISTIE W.W., NOBLE R.C. AND MOORE J.H. 1970. Determination of lipid classes by gas chromatographic procedure. *Analyst*. **95**:940-944.

DVORSKA J.E., SURAI P.F. SPEAKE B.K. AND SPARKS N.H.C. 2002. Antioxidant systems of the developing quail embryo are compromised by mycotoxin aurofusarin. *Comp. Biochem. Physiol.* **131C**:197-205.

EVERSHED L.P. 1992. Gas chromatografy of lipids. Lipid analysis, a practical approach. Oxford University Press, New York, pp 113-151.

FARRELL P.B., FOOTE R.H. AND DOUGLAS-HAMILTON D.H. 1996. Effect of inert particles in rabbit/bull semen on sperm concentration/motility measured by CASA and variability due to chamber differences. *Mol. Androl*. **8**:213-224.

FOLCH J., LESS M. AND SLOANE-STANLEY G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem.* **226**:497-509.

GLIOZZI T.M., LUZI F.. AND CEROLINI S. 2003. Assessment of sperm viability in boar, rabbit and rooster: a modification of the fluorometric ethidium bromide exclusion procedure. *Theriogenology*. **60**:635-645.

HOLTZ W. AND FOOTE R.H. 1978. Composition of rabbit semen and the origin of several constituents. *Biol. Reprod.* **18**:286-292.

NISSEN H.P., AND KREYSEL H.W. 1983. Polyunsatura ted fatty acids in relation to sperm motility. *Andrologia*. **15**:264-269.

PARENTE R., MELOTTI C., ZACCHINI A., DI STASIO D., AND POLI A. 1994. Il laboratorio di andrologia. II. Indagini seminologiche per la valutazione del potenziale di fertilità. *Analysis*. **1**:22-23.

POULOS A., DARIN-BENNETT A., AND WHITE A.G. 1973. The phospholipid bound fatty acids and aldheydes of mammalian spermatozoa. *Comp. Biochem and Physiol.* **46B**:541-549.