

## **Dietary antioxidant supplementation did not affect declining sperm function with age in the mouse but did increase head abnormalities and reduced sperm production**

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**Summary** – The present study aims to ascertain whether dietary administration of a mixture of vitamins C and E may prevent the negative effects of paternal age on male fertility variables in the mouse. Experimental males were fed a standard diet supplemented with either a low or high dose of vitamins C and E. Oocytes enclosed in cumulus masses were inseminated using a  $2 \times 2$  factorial design established according to whether males were young (3–4 months of age) or aged (22–24 months of age) and whether they were fed a control or antioxidant diet. Aged males showed a significant decrease in number of spermatozoa/mg epididymis when compared to young males. Dietary supplementation with low doses of vitamins C and E did not have any effect on sperm quality, fertilization and conceptus development *in vitro*. However, high doses of dietary vitamins C and E decreased the number of spermatozoa/mg epididymis, and increased the percentage of spermatozoa with misshapen heads distended in the distal part. These findings suggest that dietary supplementation with pharmacological doses of vitamins C and E may disturb spermatogenesis. The mechanism involved may be associated with either the antioxidant properties of vitamins C and E or their inhibitory action on steroidogenesis by Leydig and/or Sertoli cells.

**dietary supplementation / *in vitro* fertilization / paternal age / sperm quality / vitamins C and E**

**Résumé** – Un régime supplémenté en antioxydant n'empêche pas la décroissance de la spermatogenèse en fonction de l'âge de la souris, mais induit des anomalies de la tête et même diminue la production du sperme. L'étude suivante a été entreprise pour savoir si l'administration d'un mélange de vitamines C et E sur la diète peut éviter les effets négatifs de l'âge paternel sur les

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paramètres de fertilité masculine sur la souris. Les mâles du groupe expérimental ont été alimentés avec un régime standard, augmenté d'une dose faible ou forte de vitamines C et E. Les femelles ont été inséminées en utilisant un schéma factoriel  $2 \times 2$ , établi en fonction de l'âge des mâles (jeunes : 3-4 mois, ou âgés : 22-24 mois) et ont été alimentés avec un régime témoin ou additionné d'antioxydant. Les mâles âgés ont montré une diminution significative du nombre de spermatozoïdes/mg d'épididyme par rapport aux jeunes mâles. La supplémentation du régime avec une dose faible de vitamines C et E n'a eu aucun effet sur la qualité du sperme, la fécondation, et le développement du conceptus *in vitro*. Pourtant, une dose élevée de vitamines C et E a diminué le nombre de spermatozoïdes/mg d'épididyme et augmenté le pourcentage de spermatozoïdes malformés à têtes distendues dans la partie distale. Ces résultats indiquent que des doses pharmacologiques de vitamines C et E peuvent altérer la spermatogenèse grâce à leurs propriétés antioxydantes potentielles ou à leur action inhibitrice sur la stéroïdogénèse par des cellules de Leydig ou de Sertoli.

#### **âge paternel / fécondation *in vitro* / qualité du sperme / supplément diététique / vitamines C et E**

## **INTRODUCTION**

There are many studies that show negative effects of paternal age on male fertility. Ageing in human testes is associated with vascular degeneration, and a reduction in number and function of Leydig cells, interstitial cells, myoid cells and Sertoli cells. These changes are accompanied by reduction in the capability to produce testosterone, and rising plasma levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and oestradiol (Johnson, 1986; see Cummins et al, 1994 for a review). Increases in LH and FSH plasma levels, together with reduction in the number of Sertoli cells, are correlated with a decrease in daily sperm production which, in turn, is associated with a major degeneration of germ cells (Johnson et al, 1984; Johnson, 1986, 1989). Furthermore, as testicular vascularity decreases, spermatogenic failure increases, which may result in a reduction in sperm output in rats (Auroux et al, 1985) and humans (Johnson, 1989). The study of age-related histologic changes in the adult mouse testis reveal the appearance of vacuoles in the seminiferous epithelium after 6 months of age, with atrophied tubules that initially appear in patches and then spread throughout the testis. The thickness of the basement membrane surrounding the atrophied tubules is positively

correlated with the degree of atrophy (Takano and Abe, 1987). With increasing paternal age, spermatozoa may show morphological abnormalities during their development. They may degenerate and be phagocytosed by Sertoli cells, which show age-associated lipid accumulations within their cytoplasm (Lynch and Scott, 1950; Paniagua et al, 1987).

The study of the reproductive performance of C57BL/6NNia male mice (Parkening et al, 1988), revealed that only 42% of aged males mated versus 100% in young males, with 65% siring young versus 96% in young males. Aged males that failed to mate had atrophied testes, fewer and less motile sperm, lower testosterone levels and a high percentage of degenerating epithelium lining seminiferous tubules. A longitudinal analysis of 2- to 30-month-old CBF1 male mice showed progressive losses in mating success and sperm production between 18 and 30 months, with decreased levels of serum LH and testosterone (Bronson and Desjardins, 1977). In another study, Parkening (1989) found that aged males that did not mate or mated without producing offspring, showed a reduced number of spermatozoa/mg epididymis, a decreased sperm motility, increased percentage of abnormal spermatozoa and decreased fertilization rate

and number of concepti reaching the blastocyst stage.

According to the 'free radical theory of ageing' (Harman, 1956), free radicals, generated metabolically or accidentally by ionizing radiation, are the main factors for explaining the degenerative ageing process (Harman, 1981). Miquel and co-workers established an important variation to this theory, assigning a key role in the senescent process to the damage exerted by oxygen radicals to mitochondria (Miquel and Fleming, 1986). In this way, reactive oxygen species (ROS) such as the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and the highly toxic hydroxyl free radical ( $OH\cdot$ ), are produced continuously in mitochondria because of the 'leakage' of high energy electrons along the electron transport chain. Mitochondrial proteins and lipids can be damaged, but mitochondrial DNA (mtDNA) is the main target for oxidative attack because of its location near the inner mitochondrial membrane sites where the oxidants are formed. Furthermore mtDNA lacks protective histones and has no DNA repair activity (for a review, see Shigenaga et al, 1994).

Age-associated oxidative stress may be prevented by antioxidants such as vitamins C and E. Fraga et al (1991), reported a protective effect of dietary intake of vitamin C against endogenous oxidative damage to human sperm DNA. Dawson et al (1987) reported an improvement in sperm quality in men with sperm agglutination in excess of 25% after supplementing their diets with vitamin C. An improvement in sperm quality of heavy smokers was also reported after ascorbic acid supplementation (Dawson et al, 1992). Furthermore, oral administration of vitamin E significantly improved sperm function as assessed by the zona binding test (Kessopoulou et al, 1995) and fertilization rate (Geva et al, 1996).

In the present study, we aim to ascertain whether dietary administration of a mixture

of vitamins C and E may prevent the negative effects of paternal age on sperm quality, fertilization, and conceptus development *in vitro* in the mouse.

## MATERIALS AND METHODS

### Animals, dietary supplements and oocyte retrieval

F1 hybrid male and female mice (C57BL/6Jlco female X CBA/Jlco male) were given a standard diet (containing 590 g carbohydrates, 30 g lipids and 160 g protein/kg diet) and tap water 'ad libitum'. In the experimental group, males were fed the same standard diet but supplemented with an antioxidant mixture of vitamin C and vitamin E. In a first set of experiments, diet was supplemented with 0.025 g vitamin C/kg and 0.06 g vitamin E/kg. These supplements resulted in an average daily intake of 0.1 mg vitamin C and 0.24 mg vitamin E/kg body weight. These doses are based on several studies of human beings at these concentrations (Fraga et al, 1991; Kessopoulou et al, 1995). Males were fed this diet from the second month of life until their death at 4 months of age (young males) or during the last 3 months before their death at 22–24 months of age (aged males). In a second set of experiments, males were fed an antioxidant mixture containing 10 g vitamin C/kg diet and 0.6 g vitamin E/kg diet. These supplements resulted in an average daily intake of 1 600 mg vitamin C and 96 mg vitamin E/kg body weight. This particular mixture of vitamins was chosen based on a previous study demonstrating a protective effect of this mixture against GSH oxidation in blood and mitochondria from livers and brains of aged rats (De-la-Asunción et al, 1996). Young males were fed this diet from the second month of life until their death at 4 months of age, and aged males during the 7 months before their death at 22–24 months of age. Females fed a control diet were superovulated at 2 months of age by an intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (PMSG) (Folligon; Intervet-International, Boxmeer, Holland) followed 48 h later by 10 IU of human chorionic gonadotrophin (hCG) (Chorulon; Intervet-International). They were killed by cervical dislocation 14 h after the hCG injection. Their oviducts were excised and placed into 2 mL medium M2 (Quinn et al, 1982) where they were supple-

mented with 4 mg bovine serum albumin (BSA; Fraction V, Sigma Chemical Co, St Louis, MO, USA)/mL. Oocytes enclosed in cumulus masses were released from the ampullae, washed and transferred directly to insemination droplets.

### **Fertilization and conceptus development in vitro**

In each experiment, four males (two young and two aged, fed a standard or antioxidant diet) were killed by cervical dislocation 2 h before insemination. Cauda epididymes were weighed, excised and placed into 0.5 mL medium T6 (Quinn et al, 1982) supplemented with 15 mg BSA/mL. Ten minutes later, the caudae were discarded and the sperm solution adjusted to a concentration of  $5 \times 10^6$  motil spermatozoa/mL. Spermatozoa were then incubated until insemination in 20  $\mu$ L droplets overlaid with mineral oil in an atmosphere of 5% CO<sub>2</sub> in air at 37 °C. Oocytes enclosed in cumulus masses from four different females were inseminated using a 2  $\times$  2 factorial design. At 8 h after insemination, oocytes of each group were washed, transferred, and incubated in groups of ten into 20  $\mu$ L droplets of medium M16 (Whittingham, 1971). At this time, oocytes were assessed for fertilization, by observing if they extruded a second polar body (PB) and had two pronuclei. Cleavage was monitored every 24 h for 5 days.

### **Sperm vitality**

To evaluate sperm vitality, sperm was stained in a 1:1 aqueous solution containing 7.23 mM yellow eosin (Sigma Chemical Co) and 154 mM NaCl. A minimum of 100 spermatozoa per group was observed using a  $\times 40$  objective. Eosin does not pass through intact membranes and so living spermatozoa remain colourless.

### **Sperm morphology**

Ten microlitres of sperm suspension were extended on microscope slides and were air dried. They were then fixed for 1 h in Carnoy (absolute ethanol/glacial acetic acid, 3:1, v/v) and stained for 5 min with an aqueous solution containing 5% yellow eosin. Slides were then washed

with running water and air dried. A minimum of 200 spermatozoa per group was observed (using  $\times 100$  immersion objective) to assess their morphology. Spermatozoa were considered abnormal if they contained head malformations corresponding to classification 1–5 as reported by Krzanowska (1981) or if they contained more than one flagellum or an unusually shaped flagellum. According to Krzanowska's sperm classification, class 1 corresponds to spermatozoa with almost normal heads but with a changed curvature in the distal part of their heads (1a) or with slightly bent acrosomes (1b). Class 2 corresponds to spermatozoa with heads of completely normal shape, except that they possess canals of low stainability, situated transversely, obliquely or longitudinally to the long axis of their heads (2a). The most typical form of this class has a narrow, condensed head and contains a longitudinal canal of very low stainability (2c); sometimes the canal is not visible (2b). Class 3 corresponds to misshapen and flat heads distended in the apical part (3a) or in the distal part (3b). Spermatozoa of class 4 have severely misshapen heads showing abnormalities in distribution of chromatin (4a); some of these heads possess vacuoles or short canals of less stainable material (4b). Finally, spermatozoa of class 5 have severely misshapen 'thread-like heads'.

### **Fluorescence staining and counting of nuclei**

In some experiments, concepti that had reached the 16-cell or early blastocyst stage at 81 h post-insemination were fixed and their nuclei labelled. After removing their zona pellucidae with Tyrode acid (pH 2.4), concepti were fixed and labelled overnight in absolute alcohol containing 94  $\mu$ M bisbenzimidazole (Hoechst 33258; Sigma Chemical Co) at 4 °C. They were then washed for 1 h in absolute alcohol and mounted on microscope slides in glycerol beneath a coverslip and carefully squashed using a pencil rubber. The numbers of nuclei in each conceptus were then counted. Nuclei showing metaphase chromosomes were counted as single cells. The mitotic index of each conceptus was defined as the percentage of the number of metaphases/total number of cells.

## Statistical analysis

Two-way analysis of variance (Anova) was applied for comparisons of means. Significance was defined as  $P \leq 0.05$ . The entire statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS).

## RESULTS

Tables I and II show the effect of paternal age and dietary supplementation with low doses of vitamins C and E on sperm quality, fertilization and conceptus development in vitro. The number of spermatozoa/mg epididymis ( $P \leq 0.05$ ) (table I), percentage of spermatozoa with misshapen heads distended in the apical part (3a) ( $P \leq 0.005$ ) (table I), and mitotic index ( $P \leq 0.05$ ) (table II) decreased significantly with paternal age. Dietary supplementation with low doses of vitamins C and E did not have any effect on seminal, fertilization or conceptus development parameters.

Tables III and IV show the effect of paternal age and dietary supplementation with high doses of vitamins C and E on sperm quality, fertilization and conceptus development in vitro. Paternal age was associated with decreased number of spermatozoa/mg epididymis ( $P \leq 0.05$ ) (table III). Dietary supplementation with high doses of antioxidants decreased the number of spermatozoa/mg epididymis ( $P \leq 0.05$ ) and increased the percentage of spermatozoa with misshapen heads distended in the distal part (3b) ( $P \leq 0.05$ ) (table III). A similar increase in number of spermatozoa with misshapen 'thread-like heads' (type 5) was obtained, but it was ignored because of uncertainty in the data due to the small number of misshapen spermatozoa recorded. No effect of diet on fertilization and conceptus development was observed (tables III and IV).

## DISCUSSION

The present study shows a decrease in number of spermatozoa/mg epididymis with paternal age. This result is in agreement with a previous study by Parkening (1989) which also demonstrated an age-associated decrease in number of spermatozoa/mg epididymis.

A reduction in number of spermatozoa/mg epididymis may be due to a decrease in number and function of Sertoli cells, since each of them can support only 28 germ cells (28:1) (Johnson, 1986, 1989). Gosden et al (1982) reported differences in structure and gametogenic potential of seminiferous tubules in ageing mice, which affected the spermatogenesis process. Auroux et al (1985), in their work on testicular ageing in the Wistar rat, found a decrease in testicular vascularity with age. This, in turn, may increase spermatogenic failure and reduce sperm output (Johnson, 1989). Paternal-age-associated oxidative stress may explain also, at least in part, the decreased number of spermatozoa/mg epididymis found in the present study.

The mitochondrial genome of post-mitotic somatic cells is a key target for free radicals and thus for the ageing process (Miquel and Fleming, 1986). Accumulation of oxygen radical damage to mitochondria may be especially patent in post-mitotic differentiated cells such as Sertoli cells and Leydig cells. There are many studies suggesting the presence of oxidative stress in these cells. Ageing of Leydig cells is associated with defective mitochondrial function and steroid metabolism (Kinoshita et al, 1985). Moreover, in humans and mice there is age-associated accumulation of lipofuscin pigment (insoluble end-product of ROS-induced lipid peroxidation) in Leydig and Sertoli cells. Elastin deposits may also appear in the lamina propria of the ductuli efferentes and epididymis as well as lipofuscin accumulations in the principal cells of

**Table I.** Effect of diet supplementation with low doses of vitamins C and E on sperm quality.

Paternal age	Diet	n	No of spermatozoa ( $\times 10^6$ )/mg epididymis	Sperm vitality (%)	Normal sperm morphology (%)	Abnormal sperm morphology									
						1a	1b	2a	2b	2c	3a	3b	4a	4b	5
Young	control	7	1.4 $\pm$ 0.2 <sup>a</sup>	88.0 $\pm$ 2.6	87.4 $\pm$ 1.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	13.4 $\pm$ 1.5	1.1 $\pm$ 0.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0
	vitamins C and E	7	1.7 $\pm$ 0.2	90.2 $\pm$ 1.5	84.9 $\pm$ 1.9	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.4 $\pm$ 0.2	0.0 $\pm$ 0.0	12.2 $\pm$ 1.8	2.3 $\pm$ 0.4	0.0 $\pm$ 0.0	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0
Aged	control	7	1.0 $\pm$ 0.1	90.4 $\pm$ 1.2	88.6 $\pm$ 1.7	0.0 $\pm$ 0.0	0.5 $\pm$ 0.3	0.1 $\pm$ 0.1	0.5 $\pm$ 0.2	0.1 $\pm$ 0.1	7.2 $\pm$ 1.6	1.0 $\pm$ 0.5	0.2 $\pm$ 0.2	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1
	vitamins C and E	7	1.2 $\pm$ 0.3	91.4 $\pm$ 1.0	88.7 $\pm$ 1.2	0.3 $\pm$ 0.3	0.2 $\pm$ 0.2	0.0 $\pm$ 0.0	0.5 $\pm$ 0.2	0.0 $\pm$ 0.0	8.8 $\pm$ 0.9	1.1 $\pm$ 0.3	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1
Two-way Anova															
Paternal age			$P \leq 0.05$	ns	ns	ns	ns	ns	ns	ns	$P \leq 0.0005$	ns	ns	ns	ns
Diet			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Age $\times$ diet			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

<sup>a</sup>Values are means  $\pm$  standard errors.

**Table II.** Effect of paternal age and diet supplementation with low doses of vitamins C and E on fertilization and mouse conceptus development in vitro.

Paternal age	Diet	n	No oocytes	Fertilized (%)	n	No cells at 81 h post-insemination	Mitotic index at 81 h post-insemination (%)	n	Day-5 blastocysts (%)	Day-5 hatching blastocysts (%)	
Young	control	7	27.7 ± 4.8 <sup>a</sup>	87.0 ± 3.2	3	21.4 ± 0.4	7.3 ± 0.5	4	54.6 ± 16.7	41.2 ± 15.8	
	vitamins C and E	7	31.7 ± 5.0	90.2 ± 3.8	3	21.8 ± 1.3	6.5 ± 1.2	4	79.8 ± 5.7	68.8 ± 9.1	
Aged	control	7	28.9 ± 6.2	92.7 ± 3.5	3	21.3 ± 0.6	3.4 ± 1.4	4	62.8 ± 18.1	47.6 ± 15.6	
	vitamins C and E	7	37.1 ± 5.9	88.4 ± 2.2	3	21.4 ± 2.2	5.1 ± 0.5	4	73.0 ± 3.2	66.8 ± 7.0	
Two-way Anova											
Paternal age			ns	ns		ns			ns	ns	
Diet			ns	ns		ns			ns	ns	
Age × diet			ns	ns		ns			ns	ns	
							<i>P</i> ≤ 0.05				

<sup>a</sup>Values are means ± standard errors.

**Table III.** Effect of diet supplementation with high doses of vitamins C and E on sperm quality.

Paternal age	Diet	n	No of spermatozoa ( $\times 10^6$ )/mg epididymis	Sperm vitality (%)	Normal sperm morphology (%)	Abnormal sperm morphology																
						1a	1b	2a	2b	2c	3a	3b	4a	4b	5							
Young	control	9	1.8 $\pm$ 0.2 <sup>a</sup>	89.7 $\pm$ 1.1	89.2 $\pm$ 1.1	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2	8.9 $\pm$ 0.9	1.1 $\pm$ 0.3	0.1 $\pm$ 0.1	0.4 $\pm$ 0.2	0.0 $\pm$ 0.0							
	vitamins C and E	9	1.4 $\pm$ 0.2	91.9 $\pm$ 1.4	88.3 $\pm$ 1.0	0.1 $\pm$ 0.1	0.3 $\pm$ 0.2	0.2 $\pm$ 0.2	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	9.0 $\pm$ 0.8	1.5 $\pm$ 0.3	0.1 $\pm$ 0.1	0.3 $\pm$ 0.2	0.1 $\pm$ 0.1							
Aged	control	9	1.3 $\pm$ 0.1	89.7 $\pm$ 0.7	87.2 $\pm$ 3.0	0.6 $\pm$ 0.3	1.1 $\pm$ 0.7	0.0 $\pm$ 0.0	0.9 $\pm$ 0.5	0.2 $\pm$ 0.1	8.6 $\pm$ 1.7	1.2 $\pm$ 0.5	0.0 $\pm$ 0.0	0.6 $\pm$ 0.2	0.0 $\pm$ 0.0							
	vitamins C and E	9	1.1 $\pm$ 0.1	86.6 $\pm$ 1.6	83.7 $\pm$ 1.8	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	11.2 $\pm$ 1.7	3.0 $\pm$ 0.6	0.1 $\pm$ 0.1	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1							
Two-way Anova																						
Paternal age			$P \leq 0.05$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
Diet			$P \leq 0.05$	ns	ns	ns	ns	ns	ns	ns	ns	$P \leq 0.05$	ns	ns	ns	ns	ns	ns	ns	$P \leq 0.05$		
Age $\times$ diet			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

<sup>a</sup>Values are means  $\pm$  standard errors.

**Table IV.** Effect of paternal age and diet supplementation with high doses of vitamins C and E on fertilization and mouse conceptus development *in vitro*.

Paternal age	Diet	n	No oocytes	Fertilized (%)	n	No cells at 81 h post-insemination	Mitotic index at 81 h post-insemination (%)	n	Day-5 blastocysts (%)	Day-5 hatching blastocysts (%)
Young	control	9	37.8 ± 4.5 <sup>a</sup>	89.5 ± 4.2	3	20.4 ± 1.7	3.6 ± 1.1	6	71.3 ± 11.0	61.2 ± 11.2
	vitamins C and E	9	39.1 ± 3.0	88.3 ± 1.5	3	19.7 ± 2.9	6.1 ± 2.4	6	77.6 ± 2.7	64.0 ± 4.3
Aged	control	9	38.6 ± 2.7	88.7 ± 2.5	3	17.7 ± 1.3	4.7 ± 1.9	6	66.2 ± 4.7	55.3 ± 5.0
	vitamins C and E	9	41.2 ± 3.6	83.8 ± 3.5	3	18.0 ± 2.5	6.2 ± 2.0	6	77.8 ± 4.3	66.2 ± 5.4
Two-way Anova										
Paternal age			ns	ns		ns	ns		ns	ns
Diet			ns	ns		ns	ns		ns	ns
Age × diet			ns	ns		ns	ns		ns	ns

<sup>a</sup>Values are means ± standard errors.

the epididymis (see Cummins et al, 1994 for a review).

The present study showed no effects on sperm quality, fertilization and conceptus development *in vitro* when the diet was supplemented with low doses of vitamins C and E. However, high doses of vitamins had negative effects on sperm quality. In fact, the number of spermatozoa/mg epididymis diminished and the percentage of spermatozoa with misshapen heads distended in the distal part increased in males fed a diet supplemented with pharmacological doses of vitamins C and E.

Vitamins C and E, are considered 'antioxidants' but in fact they are redox (reducing-oxidizing) agents, and, in some circumstances, they can act as pro-oxidants (Herbert, 1994, 1996; Schwartz, 1996). Normally, an antioxidant effect appears when cells are exposed to physiological concentrations found in food, whereas a pro-oxidant effect appears at higher concentrations (Herbert, 1994). Thus, the high doses of antioxidants used in this work may have produced a pro-oxidant effect on the Sertoli and/or Leydig cells and so have disturbed the process of spermatogenesis. However, it is necessary to bear in mind that this particular 'cocktail' of vitamins protects against GSH oxidation in blood and mitochondria from livers and brains of aged rats (De-la-Asunción et al, 1996). Furthermore, we have demonstrated that this mixture of vitamins C and E prevents age-associated decrease in ovulation rate after exogenous ovarian stimulation and precludes the concomitant increase in percentage of aneuploid and diploid oocytes in the mouse (Tarín JJ et al, unpublished). This body of evidence suggests, therefore, that dietary supplementation with pharmacological doses of vitamins C and E may have an antioxidant effect on sperm and/or Sertoli and/or Leydig cells. However, this antioxidant mixture may act by other mechanisms rather than keeping cells in a reduced state. In fact, the

presence of high concentrations of vitamin C in the testes may disturb steroidogenesis by the Leydig and Sertoli cells and so induce a drop in number of spermatozoa/mg epididymis as well as an increase in percentage of spermatozoa with misshapen heads distended in the distal part. This notion is supported by several studies reporting that high concentrations of ascorbic acid may inhibit ovarian and adrenal steroidogenesis (for a review, see Levine and Morita, 1985).

In humans, there exists a negative correlation between sperm morphology, fertilization rate (Zenzes et al, 1985; Kruger et al, 1988) and pre-implantation conceptus development (Ron-El et al, 1991; Parinaud et al, 1993; Janny and Menezo, 1994). However, in mice treated with high doses of vitamins C and E, we did not find any association between the presence of spermatozoa with misshapen heads distended in the distal part, fertilization and conceptus development. This may be due to the relatively high concentration of spermatozoa used at insemination together with the relatively low incidence of abnormal forms observed in the present study. This effect would be reinforced by the fact that misshapen spermatozoa are selected against at fertilization by the zona pellucida (Kot and Handel, 1987; Menkveld et al, 1991) and/or cumulus cells (Krzanowska and Lorenc, 1983).

The present study shows that paternal age does not affect the percentage of fertilized oocytes that reach the blastocyst stage *in vitro*. The only significant difference with respect to conceptus development *in vitro* between young and aged males is the negative effect of paternal age found in the mitotic index of concepti 81 h post-insemination in the first set of experiments with low doses of vitamins C and E. This effect, however, may be an artefact because it was not found in the experiments made with high doses of vitamins C and E.

In summary, the present study i) confirmed previous studies demonstrating that

paternal age decreased the number of spermatozoa/mg epididymis; and ii) showed that dietary supplementation with high doses of vitamins C and E decreased the number of spermatozoa/mg epididymis and increased the percentage of misshapen spermatozoa with distended heads in the distal part. It remains to elucidate whether the negative effects of high doses of vitamins C and E on spermatozoa are due to their potential antioxidant effect or their inhibitory action on steroidogenesis by Leydig and/or Sertoli cells.

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