Effects of dietary vitamin A intake on acrosin- and plasminogen-activator activity of ram spermatozoa

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Abstract

Acrosin and plasminogen activators are proteolytic enzymes of ram spermatozoa that play an essential role in the induction of the acrosome reaction, as well as the binding of spermatozoa to the oocyte and their penetration through the layers that surround the oocyte. Since vitamin A can alter gene expression in various tissues, testis included, this study was undertaken to evaluate the possible effect of vitamin A intake on acrosin- and plasminogen-activator activity. During a 20-week experiment, 15 rams of the Greek breed Karagouniki, divided to three groups, received different amounts of vitamin A *per os* in retinyl acetate capsules (group A, controls, 12 500 iu/animal per day; group B, 50 000 iu/animal per day; group C, 0 iu/animal per day up to the 13th week, then 150 000 iu/animal per day until the end of the experiment). Acrosin- and plasminogen-activator activity were determined by spectrophotometric methods. Vitamin A was determined in blood plasma by HPLC. No statistical differences were detected regarding the body weight of the rams or the qualitative and quantitative parameters of their ejaculate throughout the whole experiment. No statistically significant alterations of enzyme activity were detected in group B. In group C, both enzyme activities started declining in week 9. Compared with controls, maximum reduction for acrosin was 49% on week 11 and for plasminogen activators 51% in week 14. Activities returned to normal rates after vitamin A resupplementation. To date, the main result of vitamin A deficiency was known to be arrest of spermatogenesis and testicular degeneration. A new role for vitamin A may be suggested, since it can influence factors related to male reproductive ability before spermatogenesis is affected.

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Introduction

There is accumulated evidence that acrosomal enzymes play an essential role in the induction of the acrosome reaction, and the binding of sperm to and their penetration through the layers that surround the oocyte (Yanagimachi 1994). The most widely studied acrosomal enzyme is the endoproteinase acrosin (EC 3.4.21.10). The presence of this sperm acrosomal serine proteinase in the acrosome of ram spermatozoa was proved 30 years ago (Zaneveld *et al.* 1973, Srivastava *et al.* 1974). Today we know that acrosin is involved in the acrosome reaction (Tesařik *et al.* 1988, De Jonge *et al.* 1989), the recognition and binding of spermatozoa to the zona pellucida (Saling 1981, Jones 1991), the penetration of the zona pellucida by spermatozoa (Kennedy *et al.* 1983, Schill 1991), the fusion of the gamete membranes (Tesařik 1995) and

development of the block to polyspermy by zona hardening (Smith *et al.* 2000).

There are at least two types of plasminogen activator (PA) in mammals: the tissue-type PA (t-PA) and the urokinase-type PA (u-PA); both convert the abundant extracellular zymogen plasminogen into plasmin, which is the active proteinase that stimulates fibrinolysis and proteolysis. PAs, as well as PA inhibitors (PAIs) and plasmin inhibitors, have been found in ejaculated spermatozoa from various species, sheep included (Smokovitis *et al.* 1987). The PAs and their inhibitors are localized to the outer acrosomal membrane and plasma membrane of human and boar spermatozoa (Smokovitis *et al.* 1992). It has been proved that, during the acrosome reaction, PAs are released in human, boar, bull and ram spermatozoa (Taitzoglou *et al.* 1996), and that plasmin induces the acrosome reaction (Taitzoglou *et al.* 2003) and influences various motility

parameters (Taitzoglou *et al.* 2004) in bull spermatozoa. A number of studies show a positive correlation between PA activity (PAA) of human spermatozoa and their fertilizing ability (Lison *et al.* 1993, Liu *et al.* 1996, Huang *et al.* 1997).

Vitamin A is required for several critical life processes, including reproduction, metabolism, differentiation, hematopoiesis, bone development, and pattern formation during embryogenesis (Sporn *et al.* 1994). In the testis, vitamin A is required for the normal production of spermatozoa (Mason 1933). In the seminiferous epithelium of vitamin A-deficient mice, a depletion of germ cells occurs. Only Sertoli cells, type A spermatogonia, and occasionally a few degenerating spermatids are present (van Pelt & de Rooij 1990). Resupplementation of retinol (Morales & Griswold 1987) or retinoic acid (van Pelt & de Rooij 1991) results in the re-initiating of spermatogenesis in a synchronized manner. The mechanism by which retinoids elicit their effects ultimately resides in their ability to regulate gene expression at specific target sites (Mangelsdorf 1994). Several types of retinoid receptor have been identified; among them, the types retinoic acid receptor α_1 (RAR α_1), RAR γ (Kim & Wang 1993) and retinoid X receptor γ (RXR γ ; Huang *et al.* 1994) have been identified in the testis. These receptors are active during the whole of spermatogenesis and until the phase of elongating spermatids in spermiogenesis (Dufour & Kim 1999).

It is known that retinoids can affect various parameters of the fibrinolytic system. Indeed, experiments *in vitro* showed that retinoids increase t-PA synthesis by human endothelial cells (Kooistra *et al.* 1991), while they enhance t-PA levels in rat tissues *in vivo* (van Benneccum *et al.* 1993). Furthermore, retinoids, when supplemented *per os*, affect various parameters of the human fibrinolytic system (Declerck *et al.* 1993). On the contrary, to our knowledge the possible effect of vitamin A on acrosin activity has not been investigated to date. Therefore, the present study was undertaken to investigate the effect of excessive vitamin A intake or vitamin A deprivation on the activity of acrosin and PAs of ram spermatozoa, and their possible correlation.

Materials and Methods

The study was carried out between 16 January and 29 May 2002. 15 rams of the Greek breed Karagouniki were used. At the onset of the study all animals were 23 months old and had approximately the same body weight (78 ± 6 kg). The rams were weighed weekly throughout the experiment. During the experiment all rams were kept under natural daylight conditions; they were in good health and were not treated pharmaceutically. Starting on 1 December 2001, and until the end of the experiment, they were kept on a vitamin A-deficient diet consisting of barley (62.07%, w/w), wheat bran (19.99%, w/w), soya (12.80%, w/w), limestone (3.76%, w/w), Ca₃(PO₄)₂ (0.1%, w/w), NaCl (0.7%, w/w), NH₄Cl (0.40%, w/w), sufrum

(0.08%, w/w), vitamin and mineral supplement (0.1%, w/w), and barley straw *ad libitum*. On 16 January 2002 (week 1 of the experiment) the rams were divided into three groups with five animals each, and received different amounts of vitamin A *per os* as retinyl acetate (ROVIMIX[®] A 500; kindly offered by Roche Vitamins, Hellas, Greece), in capsules produced in our laboratory (group A, controls, 12 500 iu/animal per day; group B, 50 000 iu/animal per day; group C, 0 iu/animal per day up to 13th week, then 150 000 iu/animal per day until the end of the experiment). Except when mentioned otherwise, all chemicals were provided by Sigma-Aldrich (Steinheim, Germany). The plasminogen used was isolated from ovine blood plasma by affinity chromatography on lysine-Sepharose 4B as described by Deutsch and Mertz (1970).

Blood samples and semen samples were collected weekly. The ejaculated semen was collected using a small ruminant artificial vagina. Immediately after collection, sperm motility was evaluated subjectively by preparing a wet mount of the spermatozoa and estimating the percentage of motile spermatozoa in four fields. Additionally, sperm concentration was determined using a Neubauer hemocytometer. Acrosomal status, viability and percentage of abnormal forms were evaluated using the staining procedure eosin/nigrosin (Thompson & Cummins 1985). Spermatozoa were classified as (1) dead with intact acrosome, (2) dead with detached acrosome, (3) live with intact acrosome and (4) live with detached acrosome. Slides were examined with a bright-field microscope. For classification, microscope fields were selected at random and all spermatozoa in each field were evaluated until 200 spermatozoa per slide were examined.

Acrosin assay

Preparation of solutions was as follows. Ficoll: 11% (w/v) Ficoll was diluted in 0.12 M NaCl/0.025 M Hepes buffer, pH 7.4. Benzamidine solution was made by dissolving a peptidase inhibitor in water to the concentration of 0.5 M. N- α -benzoyl-DL-arginine para-nitroanilide-HCL (BAPNA) extender was made with 0.01% (v/v) Triton X-100 in 0.055 M Hepes/0.055 M NaCl at pH 8.0. BAPNA was diluted in DMSO to a concentration of 23 mM. Immediately before use it was further diluted nine times with BAPNA extender.

Acrosin activity was determined in ram spermatozoa by spectrophotometry (Kennedy *et al.* 1989). In brief, 5 μ l of each ejaculate were placed in 1000 μ l NaCl/Hepes, pH 7.4, vortexed, and a volume containing 2×10^6 spermatozoa was washed with Ficoll solution. After rediluting the washed spermatozoa, 0.5×10^6 sperm cells were diluted in NaCl/Hepes to a final volume of 100 μ l. 100 μ l benzamidine solution were added to control tubes and 1000 μ l BAPNA solution were added to all tubes after vortexing. After 90 min incubation at 37°C, 100 μ l benzamidine solution were added to test tubes and all tubes were centrifuged at 1000 g for 15 min. Supernatants were collected

and the absorbance was read at 410 nm. The difference between control and test tubes was adopted as net absorbance. Acrosin activity was expressed as $\mu\text{iu } 10^{-6}$ spermatozoa, as calculated by the formula suggested by Kennedy *et al.* (1989).

PAA assay

The following solutions were used: 0.73 mM chromogenic substrate solution (S-2251; Chromogenix Milano, Italy); 50% (v/v) acetic acid; and extraction solution prepared by mixing 4 vol 0.1 M Tris/HCl buffer and 1 vol 0.5 M potassium thiocyanate.

Spermatozoa were centrifuged twice with natural saline in order to wash away the seminal plasma. A sample volume containing 1×10^8 spermatozoa was vortexed in 1000 μl extraction solution and centrifuged at 3000 *g* for 10 min, and supernatants were collected and stored at -70°C until assayed.

PAA was determined by spectrophotometry as described in detail by Smokovitis *et al.* (1987). A total volume of 500 μl mixture consisting of 200 μl substrate solution, 100 μl plasminogen solution (final concentration, 0.5 casein units ml^{-1}), 100 μl Tris/HCl buffer and 100 μl of the acrosomal extract was incubated for 30 min at 37°C . The reaction was terminated by adding 1000 μl 50% (v/v) acetic acid. The absorbance was read at 405 nm. The difference between control and test tubes was adopted as net absorbance. PAA was expressed as iu t-PA 10^{-8} spermatozoa, referring to a standard curve designed using 1–50 iu t-PA (Biopool AB, Umeå Sweden).

Vitamin A determination in blood plasma

Three metabolites of vitamin A (retinoic acid, retinol, and retinyl palmitate) were determined in ram blood plasma using a HPLC method that was specially designed for this project (Moulas *et al.* 2003). Figure 1 shows typical chromatograms of a standard solution (Fig. 1a) and a ram blood plasma sample (Fig. 1b). In brief, samples of 200 μl were extracted with 2-propanol-dichloromethane, the extracts were centrifuged, and supernatants were collected, evaporated, reconstituted in mobile phase, and analyzed on a C_8 column using two consecutive isocratic elutions with methanol tetrahydrofuran-acetate buffer. Detection was performed at 350 and 325 nm using wavelength changes during the run. The method exhibits analytical characteristics well within the acceptable limits. Overall recoveries were 73.7% for retinoic acid, 90.2% for retinol, and 87.7% for retinyl palmitate. Precision values, expressed as the percentage relative standard deviation, were in the range of 1.16–6.18%, while limits of detection were 0.3 ng ml^{-1} for retinoic acid, 6.0 ng ml^{-1} for retinol, and 10.0 ng ml^{-1} for retinyl palmitate.

Statistical analysis

For the statistical analysis, the SPSS (version 10.0) was used. The level of significance was $P < 0.05$. When

Figure 1 Typical chromatogram of a standard solution (a) and a ram blood plasma sample (b) containing 198.1 ng ml^{-1} retinol and 16.2 ng ml^{-1} retinyl palmitate. Peak identification: 1, retinoic acid; 2, retinol; 3, retinyl palmitate.

distributions and variations permitted the use of parametric hypothesis tests, ANOVA was used to calculate differences among the three groups, followed by the Duncan test, while the calculation of median differences between two independent groups was performed by Student's *t* test. When parametric hypothesis tests could not be used, the Kruskal–Wallis test was performed in order to calculate differences among the three groups, followed by the Mann–Whitney U test to specify differences between two independent groups. The Pearson correlation coefficient between retinol concentration and acrosin or PAA was calculated with a simple linear-regression equation.

Results

The body weight of the animals did not change significantly throughout the experiment. The sperm concentrations did not show any statistically significant differences among the three groups, throughout the whole experiment, and neither did the spermatozoal motility or the percentage of morphologically normal forms (Table 1). Sperm viability and acrosomal status of control and treated samples showed no statistical differences.

Acrosin activity

Acrosin activity in group B showed no statistically significant differences compared with controls. On the contrary,

Table 1 Mean ($\pm$ s.d.) values of sperm concentration (10^6 ml^{-1}), mean percentage ($\pm$ s.d.) of motility, and mean percentage ($\pm$ s.d.) of normal forms in ram ejaculates. Group A (controls), 12 500 iu vitamin A/animal per day; group B: 50 000 iu vitamin A/animal per day; group C: 0 iu vitamin A/animal per day until the 13th week, the 15 000 iu vitamin A/animal per day until the end of the experiment.

Week	Group A			Group B			Group C		
	Concentration	Motility	Normal forms	Concentration	Motility	Normal forms	Concentration	Motility	Normal forms
1	3984 $\pm$ 48 035	74 $\pm$ 5.48	91.2 $\pm$ 5.80	3281 $\pm$ 62 581	70 $\pm$ 9.35	91.8 $\pm$ 3.96	3360 $\pm$ 51 056	74 $\pm$ 5.48	88 $\pm$ 5.56
2	3945 $\pm$ 46 384	73 $\pm$ 7.58	89.6 $\pm$ 4.33	4781 $\pm$ 89 573	72 $\pm$ 6.71	88.2 $\pm$ 4.49	3605 $\pm$ 71 223	73 $\pm$ 5.70	91.6 $\pm$ 4.72
3	3620 $\pm$ 69 169	75 $\pm$ 3.54	88 $\pm$ 6.89	4468 $\pm$ 84 515	72 $\pm$ 7.58	90.6 $\pm$ 3.97	4637 $\pm$ 75 427	75 $\pm$ 3.54	91.6 $\pm$ 4.21
4	4791 $\pm$ 109 812	76 $\pm$ 5.48	91 $\pm$ 4.35	4286 $\pm$ 79 666	71 $\pm$ 8.22	88.6 $\pm$ 5.27	3290 $\pm$ 9554	71 $\pm$ 5.48	88 $\pm$ 5.95
5	3870 $\pm$ 49 454	73 $\pm$ 5.70	89.2 $\pm$ 4.08	4470 $\pm$ 81 556	70 $\pm$ 6.12	90.2 $\pm$ 3.63	4470 $\pm$ 64 121	72 $\pm$ 7.58	88.4 $\pm$ 4.39
6	4295 $\pm$ 92 368	73 $\pm$ 5.70	90.6 $\pm$ 5.27	3052 $\pm$ 82 354	71 $\pm$ 7.42	87 $\pm$ 6.041	3480 $\pm$ 53 667	75 $\pm$ 0	88.2 $\pm$ 3.96
7	3270 $\pm$ 75 765	76 $\pm$ 2.24	89.6 $\pm$ 4.72	3350 $\pm$ 74 891	72 $\pm$ 8.37	89.2 $\pm$ 3.83	4365 $\pm$ 85 405	76 $\pm$ 6.52	87 $\pm$ 5.52
8	4115 $\pm$ 8276	74 $\pm$ 6.52	90.4 $\pm$ 3.84	3050 $\pm$ 61 522	74 $\pm$ 5.48	87.8 $\pm$ 3.96	3085 $\pm$ 60 733	75 $\pm$ 3.54	88.4 $\pm$ 3.71
9	2940 $\pm$ 71 565	74 $\pm$ 8.22	90 $\pm$ 4.30	2793 $\pm$ 78 556	71 $\pm$ 8.22	89.6 $\pm$ 4.50	4066 $\pm$ 98 377	71 $\pm$ 5.48	87.8 $\pm$ 3.89
10	4035 $\pm$ 52 064	73 $\pm$ 7.58	89 $\pm$ 3.08	3637 $\pm$ 71 603	77 $\pm$ 2.74	90.2 $\pm$ 4.38	4685 $\pm$ 77 414	74 $\pm$ 4.18	92.2 $\pm$ 4.43
11	3700 $\pm$ 67 265	72 $\pm$ 9.08	90.4 $\pm$ 6.22	4825 $\pm$ 11 255	74 $\pm$ 5.48	88.4 $\pm$ 4.92	4785 $\pm$ 88 341	72 $\pm$ 7.58	91 $\pm$ 5.29
12	3210 $\pm$ 82 598	71 $\pm$ 8.22	90.2 $\pm$ 5.31	3240 $\pm$ 74 988	73 $\pm$ 5.70	88.6 $\pm$ 3.04	3460 $\pm$ 86 751	74 $\pm$ 5.48	92.4 $\pm$ 5.31
13	4150 $\pm$ 53 265	72 $\pm$ 7.58	87 $\pm$ 6.89	4335 $\pm$ 72 856	72 $\pm$ 5.70	87.2 $\pm$ 4.96	4900 $\pm$ 68 525	75 $\pm$ 6.12	90 $\pm$ 3.74
14	4130 $\pm$ 52 978	72 $\pm$ 5.70	87 $\pm$ 6.32				3690 $\pm$ 55 474	74 $\pm$ 2.24	86.6 $\pm$ 4.27
15	4305 $\pm$ 54 314	74 $\pm$ 8.22	88.2 $\pm$ 6.37				4410 $\pm$ 61 79	74 $\pm$ 4.18	90.2 $\pm$ 5.01
16	4875 $\pm$ 55 132	72 $\pm$ 5.70	84.6 $\pm$ 5.45				4275 $\pm$ 58 422	71 $\pm$ 6.52	86 $\pm$ 4.24
17	4155 $\pm$ 50 602	71 $\pm$ 8.22	92 $\pm$ 3.53				3775 $\pm$ 59 672	73 $\pm$ 5.70	90.2 $\pm$ 4.26
18	4100 $\pm$ 49 987	70 $\pm$ 9.35	88 $\pm$ 5.70				3795 $\pm$ 52 356	74 $\pm$ 5.48	90.4 $\pm$ 6.02
19	4010 $\pm$ 6525	70 $\pm$ 7.07	88.6 $\pm$ 5.12				3200 $\pm$ 50 608	73 $\pm$ 4.47	91.6 $\pm$ 8.20
20	4456 $\pm$ 98 219	72.5 $\pm$ 6.45	89.2 $\pm$ 5.16				3190 $\pm$ 75 984	75 $\pm$ 8.66	89.8 $\pm$ 5.35

in group C there was a reduction in acrosin activity, starting in the 10th week and lasting up to the 18th week. In this group, the lowest activity was noticed in the 18th week, when a 54.5% reduction was observed (Fig. 2).

PAA

Rams of group B had no significant difference in their spermatozoal PAA, compared with controls. PAA in group C followed a pattern similar to that of acrosin, showing a reduction between weeks 10 and 18. Nevertheless, the strongest effect of vitamin A deprivation was shown earlier than in the acrosin case (a reduction of 51.5% was observed in week 12; Fig. 3).

Retinol concentration

Retinol concentration in ram blood plasma of the rams was always kept within the normal range ($20\text{--}50 \mu\text{g } 100 \text{ ml}^{-1}$). This happened in all three groups and throughout the experiment. It should be mentioned that during the last 4 weeks of the experiment, retinol concentration showed a short increase in the control group, exceeding $40 \mu\text{g } 100 \text{ ml}^{-1}$. Although group B received vitamin A in a dose 4-fold higher than group A (controls), no statistically significant difference in retinol concentration was observed between these two groups. In group C, vitamin A was kept low during vitamin A deprivation, and returned to normal range by vitamin A resupplementation (Fig. 4).

Retinoic acid concentration

In week 1, retinoic acid was not detectable in the blood plasma of any ram group. From week 2, retinoic acid

appeared in small concentrations in groups B and C, and was kept at these levels through the whole experiment. In weeks 2, 3, and 13 retinoic acid concentration in group B showed statistically significant differences compared with

Figure 2 Mean ($\pm$ s.d.) values of acrosin activity of ram spermatozoa in $\mu\text{iu } 10^{-6}$ spermatozoa. ◇ indicate statistical differences compared with controls ($P < 0.05$). Group A (controls), 12 500 iu vitamin A/animal per day; group B, 50 000 iu vitamin A/animal per day; group C, 0 iu vitamin A/animal per day until the 13th week, then 150 000 iu vitamin A/animal per day until the end of the experiment.

Figure 3 Mean ($\pm$ S.D.) values of PAA of ram spermatozoa in $\text{iu } 10^{-8}$ spermatozoa. $\diamond$ indicate statistical differences compared with controls ($P < 0.05$). Groups are as in the Fig. 2 legend.

controls, but it should be noted that this observation was not consistent, while the P value was very close to 0.05. The comparison of the respective means of these specific weeks showed that this metabolite can be either higher or lower compared with controls. In group C, retinoic acid was not detectable until week 12. From week 13 it was steadily detectable and always in concentrations higher than those of the control group (Fig. 5).

Retinyl ester concentration

In week 1, retinyl ester showed its lower concentration in group A, while it was not detectable in the other two groups. This metabolite increased through the following weeks in the control group. The same observation was made in group B. The only statistically significant differences between group B and the control group were

Figure 4 Mean ($\pm$ S.D.) values of retinol concentration in ram blood plasma (in $\mu\text{g } 100\text{ml}^{-1}$). Open symbols indicate statistical differences compared with controls ($P < 0.05$). $\blacksquare$, group A (controls); $\bullet$, group B; $\diamond$, group C. Groups are as in the Fig. 2 legend.

Figure 5 Mean ($\pm$ S.D.) values of retinoic acid concentration in ram blood plasma ($\mu\text{g } 100\text{ml}^{-1}$). Open symbols indicate statistical differences compared with controls ($P < 0.05$). $\blacksquare$, $\square$, group A (controls); $\bullet$, $\circ$, group B; $\diamond$, group C. Groups are as in the Fig. 2 legend.

observed in weeks 2, 4, 7, and 12 but this was an inconsistent observation and the P value was very close to 0.05 in these cases. In group C retinyl ester was not detectable until week 13. From week 14 it was steadily detectable, and after week 15 its concentration was significantly higher compared with controls (Fig. 6).

Correlation

There was a positive correlation between retinol concentration in blood plasma and the acrosin activity of group C ($r = 0.605$, $P < 0.05$), as well as between retinol concentration and PAA ($r = 0.744$, $P < 0.001$). This correlation appeared with a 5-week delay. It is noteworthy that the Pearson coefficient was statistically significant even when it was estimated with a delay of 6 weeks for acrosin activity, and a delay of 2, 3, or 4 weeks for PAA, but its highest value was recorded when calculated after of 5-week delay. No significant correlation was found between retinoic acid concentration or retinyl ester concentration and any enzyme activity.

Figure 6 Mean ($\pm$ S.D.) values of retinyl ester concentration in ram blood plasma ($\mu\text{g } 100\text{ml}^{-1}$). Open symbols indicate statistical differences compared with controls ($P < 0.05$). $\blacksquare$, $\square$, group A (controls); $\bullet$, $\circ$, group B; $\diamond$, group C. Groups are as in the Fig. 2 legend.

Discussion

To our knowledge this is the first time that the effect of vitamin A on acrosomal enzyme activity has been investigated, taking into consideration not only retinol, but two more of its metabolites as well; retinoic acid and retinyl palmitate. These three retinoids were selected for the following reasons. First, retinol, bound to retinol-binding protein (RBP), is the main metabolite of vitamin A transported from the liver to target tissues. Second, retinyl palmitate is practically the only retinoid carried from the small intestine to the liver, in order to be either used or stored. Finally, retinoic acid is the intracellular active form of vitamin A, affecting the expression of various genes. For this purpose, retinoic acid is produced in the cell from retinol, while only a small quantity can diffuse from the blood into the cell (Blaner & Olson 1994). Nevertheless, since the 75% of plasma retinoic acid is of tissue origin, this retinoid can also be an indicator of vitamin A status in the body (Wolf 2001).

Due to well-known homeostatic mechanisms, retinol concentration was maintained within the range of $20\text{--}50\text{ }\mu\text{g ml}^{-1}$ during the whole experiment, as suggested by the reviewed literature. The slight increase in retinol concentration during the last few weeks of the experiment should be expected, since rams tend to have such an increase at the onset of the summer (Asadian *et al.* 1995). On the contrary, retinyl ester and retinoic acid concentrations showed significant changes during the experiment, being non-detectable during vitamin A deprivation, but reaching considerably higher levels after vitamin A resupplementation.

The Sertoli cell is the main site of retinol uptake by the testis. In these cells, vitamin A can be either stored or oxidized to retinoic acid and, after binding to specific nuclear receptors, affect the expression of various genes. Spermatocytes and spermatids also need vitamin A, which is delivered to them by the Sertoli cells essentially as retinol bound to RBP (Davis & Ong 1992) or to cellular RBP (CRBP; Ong 1985), but also as retinyl ester (Kim & Wang 1993) or retinoic acid (Livera *et al.* 2002). Since spermatocytes and spermatids are protected by the blood–testis barrier, the Sertoli cells are their basic suppliers of vitamin A (Kim & Wang 1993). Nevertheless, during spermatogenesis, from spermatogonia A0 up to the stage of pachytene spermatocytes, germ cells can uptake retinol directly from the blood since they are not protected by the blood–testis barrier. Additionally, retinyl ester, when in high concentrations can diffuse into these germ cells from the blood (Blaner & Olson 1994), and so can retinoic acid (Wolf 2001). It is noteworthy that vitamin A deprivation results in a decrease of RAR α in rat testis (Haq *et al.* 1991), as well as in a transportation of these nuclear receptors to the cytoplasm, where they are inactive (Livera *et al.* 2002).

In group B, the supplementation of vitamin A at a dose four times higher than the daily needs of the rams, and for

13 weeks, did not affect either enzyme activity. It seems that the supplementation of excessive vitamin A cannot affect the synthesis of these proteolytic enzymes, at least once the needs of the target cells have been covered. On the contrary, acrosin activity and PAA in group C decreased 108 days after vitamin A deprivation, and were kept low for 9 weeks. When retinol concentration in plasma is low, there is also a decrease of CRBP in testis (Rajan *et al.* 1990). This probably reduces the delivery of retinol to germ cells (Ong 1985). Since retinol is esterified only when bound to CRBP, germ cells receive less retinyl esters as well (Kim & Wang 1993). Finally, CRBP is necessary for the oxidation of retinol to retinoic acid (Blaner & Olson 1994). Since CRBP is not present in spermatocytes or spermatids (Huggenvik & Griswold 1981), it seems that the only source of retinoic acid for these cells are the Sertoli cells (Cavazzini *et al.* 1996), which produce less retinoic acid in vitamin A deprivation. This results in a decrease of the retinoic acid delivered to the nucleus of the target cell, because delivery of retinoic acid occurs when this is bound to cellular retinoic acid-binding protein (CRABP), and this binding protein acts as a passive vehicle, which binds and releases its ligand according to concentration gradients (Noy 2000).

Retinoic acid, bound to CRABP, modulates nuclear gene expression by binding to specific receptors. These receptors bind as homo- or heterodimers to specific DNA sequences known as retinoic acid-response elements. To date, two such response elements have been identified, direct repeats DR-5 and DR-2, which are found in the promoters of several retinoic acid-responsive genes (Mangelsdorf 1994). At least in humans, DR-5 has been localized on the t-PA gene, and when retinoic acid binds to it the expression of the gene is enhanced (Bulens *et al.* 1995). On the contrary, no such response element was found on the mouse t-PA gene; this gene and its enhancer activator protein 1 are synergistically activated by cAMP and retinoic acid (Mira-y-Lopez *et al.* 1998).

During spermatogenesis, the biosynthesis of acrosin takes part in the meiotic phase or immediately after this (Kremling *et al.* 1991, Raab *et al.* 1994). A specific time point for the biosynthesis of PAs by the spermatogonia or the spermatids has not been determined. Nevertheless, pachytene spermatocytes and spermatids show an increased t-PA activity during stages IX–XII (Bardin *et al.* 1994). Therefore, the increased activity of t-PA, the type of PA to which the greatest percentage of PAA of ram spermatozoa is attributed (Smokovitis *et al.* 1987), coincides with the maximum concentration of CRABP and RAR α in germ cells as well.

Apart from direct regulation of gene expression, retinoic acid can also act indirectly, by regulating an intermediary, which in turn regulates the gene of interest, influencing mRNA stability, or activating nuclear receptor dimers other than the RAR–RXR complex (Balmer & Blomhoff 2002). These include other receptors of the steroid receptor superfamily, such as the receptors of vitamin D₃ and

thyroid hormone (Maden 2000). The two parts of the heterodimer have a synergistic effect on the activation of the target gene (Mangelsdorf 1994). Indeed, a response element on the human t-PA gene has been identified where the heterodimer of the RXR and the vitamin D₃ receptor binds and activates its transcription (Merchiers *et al.* 1999). In fact, it is known that vitamin D₃ regulates PA synthesis by normal and malignant osteoblasts (Hamilton *et al.* 1985), the production of u-PA and PAI-1 by monocytes (Gyetko *et al.* 1988), and the synthesis of PA by epithelial and fibroblastic cells (Koli & Keski-Oja 1996) or by rat heart microvascular cells (Puri *et al.* 2000).

Vitamin A deficiency is able to reduce plasma testosterone concentration in rats (Bartlett *et al.* 1989), and testosterone does affect acrosin activity (Theodosiadou 2000) and PAA (Rekkas *et al.* 1993). Nevertheless, in the Bartlett *et al.* (1989) study, the reduction of mean testosterone concentration was observed 112 days after the beginning of a vitamin A-deficient diet, while it is proved that 70–75 days after the supplementation of such a diet to rats spermatogenesis is arrested and the seminiferous epithelium degenerates irreversibly (Ismail *et al.* 1990). Since sperm counts, motility, and percentage of abnormal forms were not altered during our experiment, while our findings appeared a lot earlier in a species – the sheep – with a definitely greater capacity of storing vitamin A in the liver, we conclude that the possible effect of vitamin A deprivation on any enzyme activity via alterations of testosterone concentration should be excluded.

It is noteworthy that the effect on the activity of either enzyme was shown with a delay of 108 days for vitamin A deprivation and 42 days for vitamin A resupplementation. This is not surprising if we take into consideration that (1) sheep can cover their needs using vitamin A that they have already stored and (2) spermatogenesis in the ram lasts approximately 49 days, followed by the transportation of the spermatozoa in the epididymis for 16 days. This delay possibly supports the suggestion that the effect of vitamin A on acrosin and PAs of spermatozoa is exerted by regulating the biosynthesis of these enzymes during spermatogenesis, rather than the synthesis of PAs, PAIs, or acrosin inhibitors that are taken up by the spermatozoa after they are released into the seminiferous tubule.

In conclusion, excessive vitamin A intake did not affect acrosin or PAA, but vitamin A deprivation reduced the activity of both enzymes in a reverse manner. Recent knowledge about vitamin A metabolism and action in the cell allows us to suggest that it may have influenced acrosin and PA synthesis during spermatogenesis by regulating gene expression, although other, indirect mechanisms of action should not be excluded. To date, the main result of vitamin A deficiency was known to be the arrest of spermatogenesis and testicular degeneration. A new role for vitamin A in the male reproductive system may be suggested, since it can influence factors related to male reproductive ability such as acrosin and PAs, before spermatogenesis is affected.

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