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Effects of selenium deficiency on testicular morphology and function in rats

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For four generations rats were fed a low selenium diet (2–7 μg Se kg^{-1}) or the same diet with 250 or 300 μg Se kg^{-1} added as selenite. In male rats of the first generation that had been fed the diets from the age of 20 days onwards, selenium depletion led to slightly delayed testis growth during pubertal development that was compensated for in the later stages of maturation. In adult rats fed the low selenium diet for nearly a year no changes in testicular mass and morphology were observed. The serum concentration of testosterone of 6-month-old, selenium-depleted animals was, however, slightly lower than that of adequately supplied controls, and the stimulation of testosterone secretion by administration of GnRH or LH resulted in a significantly less marked rise in the serum concentration of testosterone. From the second generation onwards the testis mass, expressed as a percentage of the body mass, decreased and in the fourth generation was less than 50% of that of the controls. The male gonads of fourth generation animals showed a severe bilateral atrophy, in which the seminiferous tubules were considerably reduced in diameter and almost entirely lined by Sertoli cells and a few stem cells. Differentiated spermatozoa could not be detected. The alterations were reversible and spermatogenesis was restored by feeding the selenium-adequate diet. The findings indicate that testicular morphology and functions are affected by severe selenium deficiency and that the element is necessary for testosterone biosynthesis and the formation and normal development of spermatozoa.

Introduction

Selenium (Se) has been established as an essential trace element that is necessary for the maintenance of various physiological processes. In the form of glutathione peroxidases (Rotruck *et al.*, 1973; Ursini *et al.*, 1985) it complements the effects of vitamin E in the antioxidant defence system, and as part of the type I iodothyronine deiodinase it is needed in the production of tri-iodothyronine from thyroxine (Arthur *et al.*, 1990; Behne *et al.*, 1990).

Several investigations have shown that the testis is one of the main target organs for Se and that the element, independent of vitamin E, plays an important role in the male reproductive system. After administration of [^{75}Se]selenite to mice (Gunn *et al.*, 1967), rats (Brown and Burk, 1973), bulls (Smith *et al.*, 1979; Pond *et al.*, 1983) and rams (Pond *et al.*, 1983), a relatively large percentage of the tracer accumulated in the testis. Retention was especially high in Se-deficient animals, and compared with the main storage tissues of the element, muscle and liver, there is a preferential supply to the male gonads in periods of insufficient Se intake (Behne *et al.*, 1982; Behne *et al.*, 1988a). When related to the dry mass of the organ, the Se concentration in the testis is among the highest in the tissues of adult rats (Behne and Wolters, 1983). Selenium in the testis is regulated by gonadotrophic hormones (Behne *et al.*, 1982) and its concentration increases considerably during puberty with the onset of spermatogenesis (Behne *et al.*, 1986).

In the tracer experiments mentioned above, most of the labelled element was incorporated into the spermatozoa. Here, Se was found in the outer membrane of the sperm mitochondria in the form of a specific selenoprotein (Pallini and Bacci, 1979; Calvin *et al.*, 1981). Several studies have shown that Se is necessary for the normal development of spermatozoa. Rats and mice fed low Se diets over long periods produced spermatozoa with impaired motility and morphological anomalies that most frequently occurred in the midpiece region of the tail (McCoy and Weswig, 1969; Wu *et al.*, 1969, 1973, 1979; Wallace *et al.*, 1983, 1987). In humans, low concentrations of Se in spermatozoa were associated with abnormally low and high sperm counts (Behne *et al.*, 1988b), and the motility of spermatozoa of subfertile men could be improved with Se supplementation (MacPherson *et al.*, 1993).

There were also some indications that Se deficiency affects the testicular mass and morphology of rats and mice (Wu *et al.*, 1969; Sprinker *et al.*, 1971; Wallace *et al.*, 1987), but detailed information on the nature and severity of these lesions is scarce. It was therefore the aim of the present study to investigate the effects of an insufficient supply of Se on the morphology and function of the male gonads of rats, during the first stages of Se depletion and after long-term Se

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deficiency. As Se is transferred to the offspring during pregnancy and lactation, severe deficiency can be achieved only in animals born to already deficient dams. The experiments were therefore carried out on rats which were either depleted during pubertal maturation or were extremely Se-deficient after having been fed a low Se diet for several generations.

Some of the effects of selenium deficiency on the secretion of testosterone have been described in a preliminary report (Behne *et al.*, 1987).

Materials and Methods

Wistar rats were fed either a low Se diet or a Se-adequate diet. The basal diet (ICN Biochemicals, Cleveland, OH) consisted of 58.7% sucrose, 30% Torula yeast, 5% tocopherol-stripped lard, 5% salt mix (Hubbell *et al.*, 1937), 1% vitamin mix [Vitamin mix composition (g kg^{-1}): vitamin A concentrate (500 000 iu g^{-1}): 1.8; *p*-aminobenzoic acid: 5.0; vitamin B 12: 0.00135; biotin: 0.02; calcium pantothenate: 3.0; choline chloride: 75.0; vitamin D concentrate (850 000 iu g^{-1}): 0.125; dextrose: 900.46; folic acid: 0.09; inositol: 5.0; menadione: 2.25; niacin: 4.25; pyridoxine–HCl: 1.0; riboflavin: 1.0; thiamin–HCl: 1.0] and 0.3% D,L-methionine. 100 mg of D,L-α-tocopherol acetate kg^{-1} diet was added. The Se content in the different batches ranged from 2 to 7 μg kg^{-1} diet. The Se-adequate diet was the basal diet with 250 μg Se kg^{-1} or 300 μg Se kg^{-1} added in the form of sodium selenite. The animals were kept under standardized laboratory conditions and were given free access to the diet and distilled water. In the first generation weanling offspring of dams that had been fed a normal commercially available rat diet (Altromin, Lage) were given either the low or the adequate diet. As Se-depleted males become infertile from the second generation onwards (McCoy and Weswig, 1969; Wu *et al.*, 1969), the females were matched for mating with males that had been fed the normal rat diet. The study was carried out on Se-depleted and Se-adequate male rats of four generations obtained in this way. At the end of the experiments the animals were killed by injection of an overdose of sodium phenobarbital after blood had been taken by cardiac puncture in the anaesthetized state. The experiments were approved by the 'Senatsverwaltung für Gesundheit und Soziales', Berlin, Germany.

Experiment 1

Fifty-five 20-day-old Se-adequate male rats were used. Five animals were killed at the beginning of the experiment. The others were divided into two groups of twenty-five animals and were fed either the low Se diet or the diet supplemented with 250 μg Se kg^{-1}. On days 40, 45, 55, 125 and 256 of the experiment five animals from each of the two groups were killed and the testis mass and the seminiferous tubule diameter were determined.

Experiments 2 and 3

Twenty-four 20-day-old male rats were fed the low Se diet and twenty-four the diet with 250 μg Se kg^{-1} added. On day 170, the animals were divided into groups of six rats each, with two Se-depleted and two Se-adequate groups used in each of the experiments. In Expt 2, 1 μg of GnRH (Schering AG, Berlin) in 0.5 ml saline was injected s.c. into the animals from a Se-depleted and from a Se-adequate group. The rats in the remaining Se-depleted and Se-adequate groups were injected with 0.5 ml of the solvent. In Expt 3, in a similar experimental design, 15 iu LH in the form of hCG (Schering AG) in 0.5 ml saline or 0.5 ml of the solvent alone were injected i.p. into the animals from a Se-depleted and a Se-adequate group. Immediately before, and 2 h after the injections, blood samples (1 ml) were taken from the orbital venous plexus under appropriate ether anaesthesia for the determination of the serum concentrations of testosterone. The testosterone analysis was carried out by means of a solid phase radioimmunoassay (Hasan *et al.*, 1971).

Experiment 4

Rats were fed the low Se diet or the basal diet supplemented with 300 μg Se kg^{-1} for four generations. The effects of Se deficiency on testis mass were determined in animals of the 1st, 2nd and 4th generation. Morphological and histomorphometrical investigations were carried out on the testes of Se-depleted and Se-adequate animals of the 4th generation and on Se-depleted rats of the same generation which from the age of 3 months onwards had been fed the diet supplemented with 300 μg Se kg^{-1} for a period of 4 months.

Preparation of specimens for histological examination

In Expts 1 and 4, formalin-fixed, paraplast-embedded, 4 μm sections of the organs were stained with haematoxylin and eosin. The histomorphometrical analysis was performed on a semi-automatic image analyser (Leitz, Wetzlar).

Statistical analyses

For the statistical evaluation of the differences in testis mass, seminiferous tubule diameter and testosterone secretion between the Se-adequate and Se-deficient groups Student's *t* test was applied.

Results

Se depletion in maturing rats

The data obtained in Expt 1 on the effects of Se depletion on some testicular parameters during pubertal maturation are shown (Table 1; Fig. 1). As Se deficiency leads to growth retardation, the testis mass was expressed as a percentage of the body mass so that specific changes in this organ could be recognized. Significant differences between the two groups were found in the 45- and 55-day-old animals, but not in the adult 125-day-old rats (Fig. 1). No significant alterations in the relative testis masses between the Se-adequate group (0.84 ± 0.04%) and the Se-depleted group (0.89 ± 0.03%) were observed when the animals were fed the diets for a further 130 days. The values for the diameter of the seminiferous tubules (Table 1) show that in the first generation, Se depletion had relatively little effect on testicular morphology.

Table 1. Effects of selenium depletion on testicular development of maturing rats[a]

Age (days)	Body mass (g) Se +	Body mass (g) Se −	Testes mass (g) Se +	Testes mass (g) Se −	Diameter of seminiferous tubules (μm) Se +	Diameter of seminiferous tubules (μm) Se −
20	41.4 ± 7.9[b]		0.21 ± 0.03[b]		—	
40	99.0 ± 11.4	89.0 ± 11.6	1.11 ± 0.12	0.98 ± 0.06	—	
45	106.2 ± 8.4	114.4 ± 12.6	1.54 ± 0.24	1.23 ± 0.22	—	
55	129.2 ± 16.7	150.8 ± 17.8	2.03 ± 0.25	1.92 ± 0.26	209.4 ± 4.3	212.6 ± 5.6
125	307.8 ± 18.7	238.2 ± 11.8*	3.36 ± 0.22	2.77 ± 0.17*	234.4 ± 10.2	246.1 ± 11.8
256	417.8 ± 11.5	371.8 ± 15.4*	3.48 ± 0.10	3.30 ± 0.23	234.3 ± 11.8	236.2 ± 11.1

[a]20-day-old rats were fed either a low selenium diet (Se −) or the same diet with 250 μg kg^{-1} added as sodium selenite (Se +).
[b]Each value represents the mean ± SD of five animals.
*Indicates significant differences ($P < 0.01$) between the two groups.

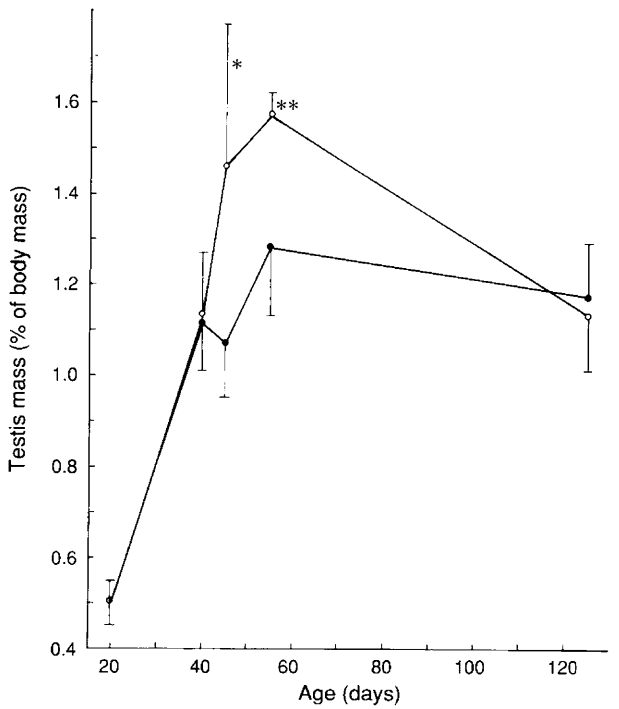

Fig. 1. Changes in the testis mass of selenium-depleted and selenium-adequate rats during pubertal maturation. Animals (20 days old) were fed either a low selenium diet (•) or the same diet with 250 μg Se kg^{-1} added as selenite ( ). Values are the means ± SD of five animals. Significant differences between the two groups (*$P < 0.05$ and **$P < 0.01$).

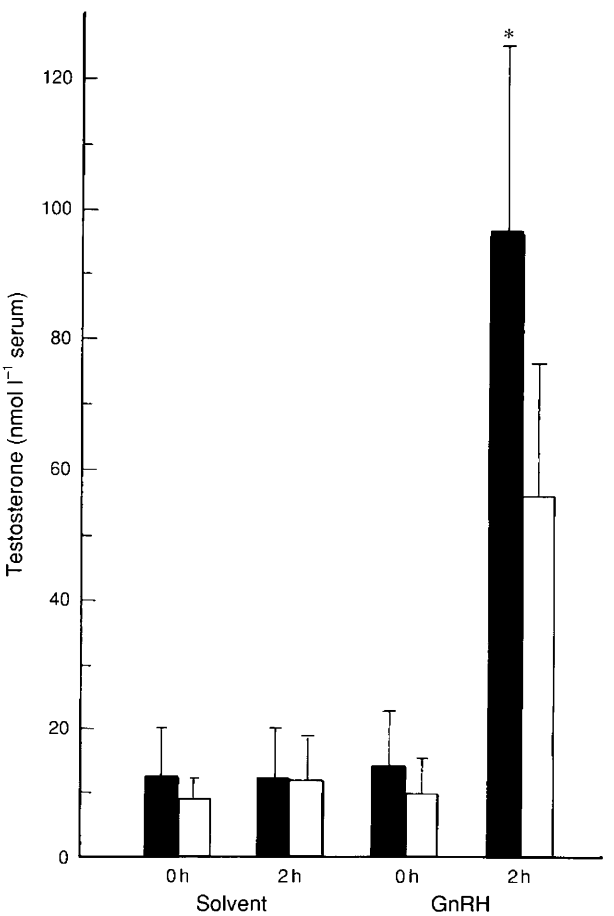

Fig. 2. Testosterone secretion in selenium-depleted and selenium-adequate rats after stimulation with GnRH. Animals (20 days old) were fed a low selenium diet (□) or the same diet with 250 μg Se kg^{-1} added as selenite (■) for 150 days. Serum concentrations of testosterone (mean ± SD of six animals) were measured before and 2 h after an injection of GnRH in saline or of saline only. *Significant difference between the two groups ($P < 0.02$)

Effects of Se depletion on testosterone secretion

Differences in testosterone secretion were observed in rats fed the two diets for 170 days. The serum testosterone concentration (mean ± SD of 24 animals) in the Se-depleted group (7.4 ± 4.3 nmol testosterone l^{-1} serum) was slightly lower ($P < 0.05$) than that in the Se-adequate group (11.2 ± 7.0 nmol testosterone l^{-1} serum). After the administration of GnRH, which regulates testosterone production in the Leydig cells via the hypophyseal secretion of LH, the increase in serum concentration of testosterone 2 h after the injection was significantly lower ($P < 0.02$) in the depleted animals than in the adequately supplied control group (Fig. 2). A similar, statistically significant decrease ($P < 0.001$) in testosterone secretion was found after direct stimulation of the

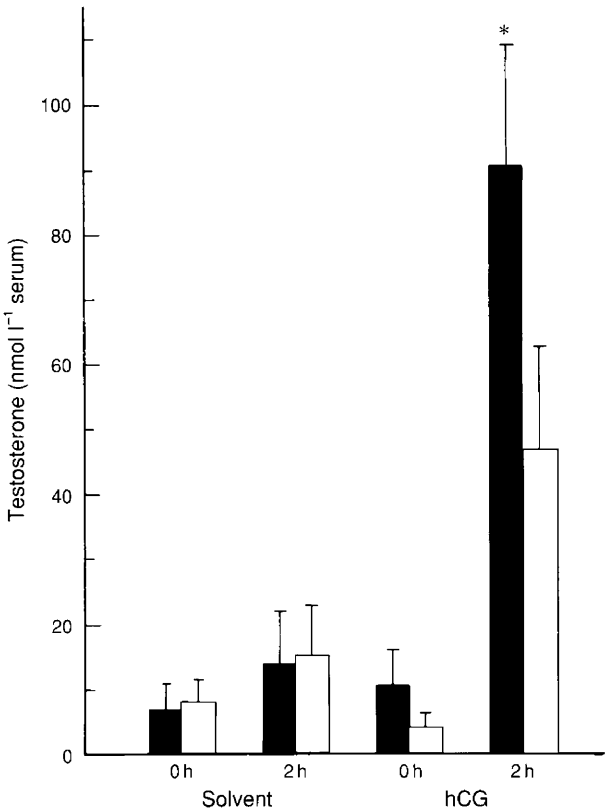

Fig. 3. Testosterone secretion in selenium-depleted and selenium-adequate rats after stimulation with LH. Animals (20 days old) were fed a low selenium diet (□) or the same diet with 250 μg Se kg^{-1} added as selenite (■) for 150 days. Serum testosterone concentrations (mean ± SD of six animals) were measured before and 2 h after an injection of LH (in the form of hCG in saline) or of saline only. *Significant difference between the two groups ($P < 0.001$).

Leydig cells by injection of LH in the form of hCG (Fig. 3). This result indicates that the difference between the two groups is not due to changes in the hypothalamic–hypophyseal axis of testosterone regulation but to an effect of Se deficiency on the Leydig cells.

Effects of severe, long-term Se deficiency

The results show that when the animals were fed the low Se diet or the Se-adequate diet for several generations, there was a decrease in the relative testis mass in the Se-deficient rats from the second generation onwards, and by the 4th generation the relative testis mass was less than 50% of that of the adequately supplied animals (Fig. 4). Correspondingly, the pathomorphological examination revealed a severe bilateral atrophy of the male gonads with a considerable decrease in the seminiferous tubule diameter. In the Se-adequate animals, the diameter was 258.2 ± 35.9 μm (mean ± SD of five animals), with 55.5% of the tubular diameters ranging from 250 to 300 μm and 31.5% from 200 to 350 μm. By comparison, the mean value of 123.5 ± 14.5 μm in five Se-deficient rats was significantly less ($P < 0.001$), with 52.5% of the diameters ranging from 100 to 125 μm and 39.2% from 125 to 150 μm.

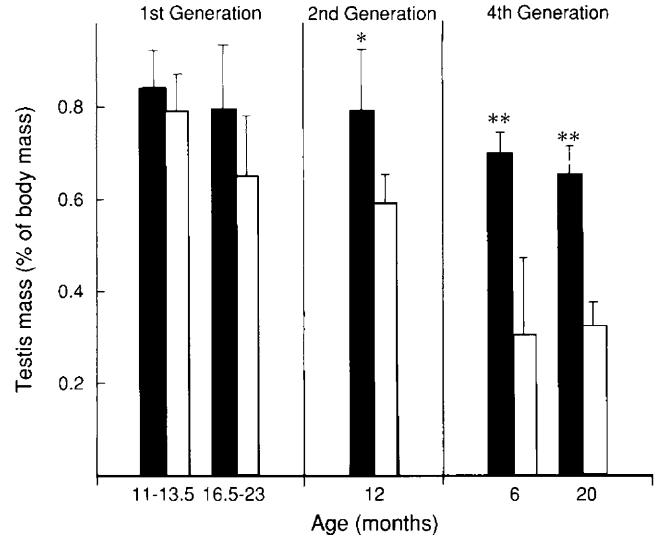

Fig. 4. Effects of progressive selenium deficiency on testis mass. Rats were fed either a low selenium diet (□) or the same diet with 300 μg Se kg^{-1} added as selenite (■). The values are the means ± SD of five animals. Significant differences between the two groups (*$P < 0.02$ and **$P < 0.001$).

The effects of feeding the diets for four generations on testicular morphology are shown (Fig. 5). Omission of Se from the diet led to severe pathological changes. The degenerative process involved the whole testis uniformly. The seminiferous tubules were almost entirely lined by Sertoli cells, or Sertoli cells and a few stem cells, or spermatogonia that did not show mitotic activity. The basement membranes were thickened and translucent. A few seminiferous tubules showed various degrees of mineralization or osseous metaplasia. Peritubular connective tissue was slightly increased and showed a marked oedema with a very few focal infiltrates of inflammatory cells. The number of Leydig cells appeared to be increased. The atrophic seminiferous tubules intermingled with a few tubules showing incomplete spermatogenic activity with differentiation proceeding only to the spermatocyte stage. Differentiated spermatozoa could not be detected either in the seminiferous tubules or in the epididymis.

Spermatogenesis was restored by feeding 3-month-old Se-deficient animals the Se-adequate diet for four months (data not shown). The seminiferous tubules then had a diameter of 247.7 ± 48.2 μm (mean ± SD of five animals) with 41.6% of the tubule diameters in the range from 250 to 300 μm and 27.9% in the range from 200 to 250 μm. Differentiated spermatozoa developed as would be expected in the case of undisturbed spermiogenesis and were present in the epididymis in numbers equivalent to normal values. Correspondingly, the number of Leydig cells appeared to have decreased compared with the Se-depleted rats.

Discussion

It has been found that during pubertal maturation there is a considerable increase in the concentration of Se in the testis and, thus, an increased Se requirement of the male gonads

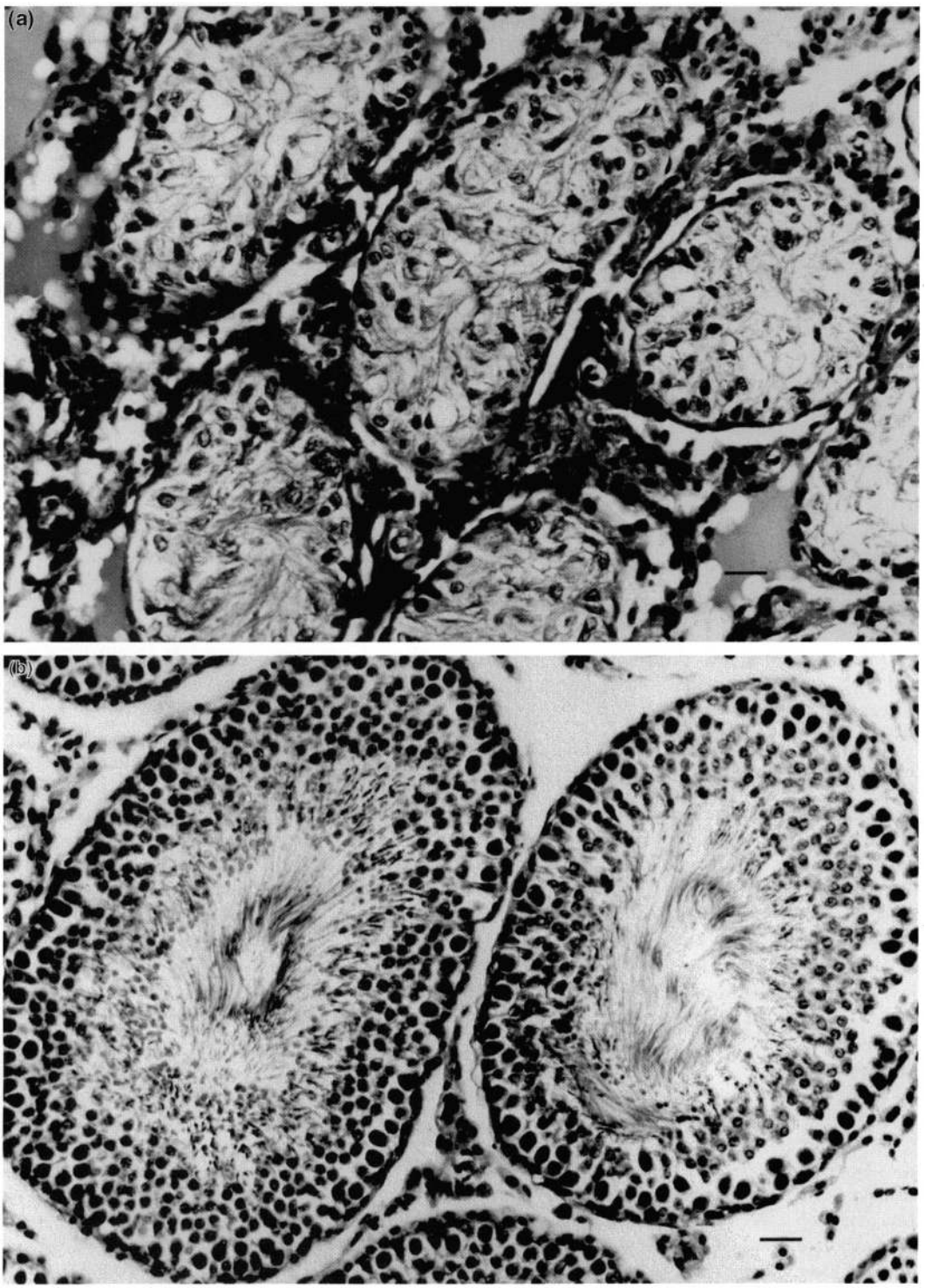

Fig. 5. Effects of selenium deficiency on testicular morphology. Rats were fed either (a) a low selenium diet or (b) a selenium-adequate diet for four generations. The seminiferous tubules of the selenium-deficient animals had considerably smaller diameters and were almost entirely lined by Sertoli cells and a few stem cells (staining with haematoxylin and eosin). Scale bars represent 25 μm.

(Behne *et al.*, 1986). However, the results of the present study show that in rats born to Se-adequate dams an insufficient supply of the element during puberty has only a minor effect on testicular development. Significant differences in the relative testis mass between the Se-adequate and Se-depleted group were found in the 45- and 55-day-old rats, but not in the adult

animals. This indicates that Se depletion results in a slightly delayed growth of the male gonads during puberty that can be compensated for in the later stages of maturation. Even feeding the deficient diet for more than a year did not significantly affect the relative testis mass. The similarity in the mean diameter of the seminiferous tubules in the two diet groups likewise shows that insufficient Se supply during sexual maturation has no severe effects on testicular development.

From the second generation onwards the testis mass of the deficient animals decreased continuously and changes in the testicular morphology became increasingly apparent. Histologically, the atrophy appeared as complete azoospermia and the seminiferous tubules were considerably reduced in diameter and almost entirely lined by Sertoli cells and a few stem cells. The changes were reversible and it was possible to restore spermatogenesis by feeding a Se-adequate diet.

The fact that changes in the testicular mass and morphology were not noticeable in the first generation of Se depletion was also noted in a study on mice, which did, however, show a sharply reduced sperm count and a decrease in the diameter of the seminiferous tubules in the second generation (Wallace *et al.*, 1983). The delay in the appearance of lesions in the testis may be due to homeostatic mechanisms which in periods of insufficient Se supply strive to maintain the Se concentration in the gonads and certain other tissues, such as the endocrine organs and the brain (Behne *et al.*, 1988a). Consequently, during Se depletion the concentration of the element declined much more slowly in the testis than in the main Se storage tissues, muscle and liver (Behne *et al.*, 1982, 1988a).

A slight decrease in serum testosterone concentrations in the rats fed the low Se diet for nearly 6 months suggests that Se deficiency affects testosterone secretion in animals of the first generation. This relationship was more distinct after stimulation of testosterone production. The secretion of testosterone in the Leydig cells is regulated by LH released from the pituitary gland, which itself is controlled by hypothalamic GnRH. An injection of GnRH or of LH, in the form of hCG, led to a less marked increase in the testosterone concentration in the serum of the Se-depleted rats compared with that in animals that had been supplied with adequate amounts of the element. This suggests a biological role for Se in the biosynthesis of testosterone and indicates that the Leydig cells are affected at a relatively early stage of Se depletion. The hyperplasia of the Leydig cells that appeared to be present in the testis of the extremely Se-deprived animals might, therefore, be the result of a compensatory process to counteract testosterone deficiency.

Sperm morphology and motility were likewise found to be susceptible to Se depletion. In mice maintained on a low Se diet in the first generation, the incidence of abnormal spermatozoa was greater, and the abnormalities were most frequent in the sperm head (Watanabe and Endo, 1991) and in the midpiece of the tail (Wallace *et al.*, 1983). In rats, sperm morphology and motility appeared to be normal after feeding the diet for 4 months, but after 11 months on the low Se diet, half of the animals produced spermatozoa with impaired motility and characteristic midpiece damage (Wu *et al.*, 1979).

As Se is concentrated in the outer membrane of sperm mitochondria, in the form of a specific selenoprotein (Calvin *et al.*, 1981), it is most likely that lesions found in the midpiece region are caused by a direct effect of insufficient Se supply on the formation of this compound.

The diminished testosterone secretion after stimulation with GnRH or LH might be due to an effect of Se deficiency on the LH receptors or on the biosynthesis of testosterone in the Leydig cells. It has been shown that the concentration of the seleno-enzyme glutathione peroxidase decreases faster with insufficient Se supply than that of most of the other selenoproteins (Behne *et al.*, 1988a). In the Leydig cells, glutathione peroxidase has been localized immunocytochemically in the cytoplasm in close relationship to the smooth endoplasmic reticulum (Murakoshi *et al.*, 1983), and it is possible that the metabolic pathway of testosterone biosynthesis requires protection against peroxidation and is thus affected by a decrease in the activity of this enzyme. In this respect, the 20 kDa phospholipid hydroperoxide glutathione peroxidase (Ursini *et al.*, 1985), which specifically catalyses the reduction of membrane-bound phospholipid peroxides, has also to be taken into consideration, as, in a study on the distribution of Se-containing proteins in testicular fractions, a compound with the same relative molecular mass was found suggesting the presence of this enzyme in the Leydig cells (C. Weiss-Nowak, A. Kyriakopoulos and D. Behne unpublished).

The mechanisms responsible for the appearance of lesions in the male gonads in the more advanced stages of Se depletion await further clarification. In rats, testosterone has been shown to control the initial differentiation of spermatogonia (Steinberger, 1971), and the administration of antiandrogens to rats (Neumann and Schenk, 1978) and dogs (Neumann and Schenk, 1980) results in a considerable testicular atrophy with azoospermia and pathomorphological changes similar to those in the Se-deficient animals and also reversible within a few weeks after normal conditions had been restored. This finding suggests that Se deficiency affects the testicular morphology only indirectly via the decrease in testosterone production. However, the testes are known to be susceptible to a variety of insults. Several other Se-containing proteins have been found in the male gonads in addition to the glutathione peroxidases (Calvin *et al.*, 1987; Behne *et al.*, 1988a), and it is therefore possible that the testicular lesions observed in severe Se-deficiency are due to the decrease in the biological activity of more than one Se compound.

The findings of the present study provide further evidence for the importance of Se in the male reproductive system. They show that, besides being involved in the formation of spermatozoa, Se is also needed for normal testosterone metabolism and the maintenance of testicular morphology. It will therefore be of interest to investigate in more detail the relationship between Se metabolism and male reproductive processes, and to clarify the role of the different selenoproteins in the testis and spermatozoa.

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