BIOLOGY OF REPRODUCTION 46, 1114–1118 (1992)

Impaired Detoxification of Reactive Oxygen and Consequent Oxidative Stress in Experimentally Cryptorchid Rat Testis[1]

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ABSTRACT

The effect of experimental cryptorchidism on the level of oxidative stress and antioxidant functions in rat testis was studied. Adult male Sprague-Dawley rats were rendered unilaterally cryptorchid (by suturing one testis to the abdominal wall) and killed 1, 3, or 7 days after the operation. As an indicator of oxidative stress, lipid peroxidation was measured by the diene conjugation method in testis homogenates. The activities of the antioxidant enzymes were determined either in the 10 000 × *g* supernatant fraction (glutathione [GSH] peroxidase, GSH transferase, hexose monophosphate shunt) or in crude testis homogenates (superoxide dismutase, catalase). An expected reduction (48%) in weight of the abdominal testes was evident by postoperative Day 7. The catalytic activities per testis of superoxide dismutase (Cu/Zn form) and catalase were found to decrease in cryptorchidism. The effect was seen on the first postoperative day and was most profound on Day 7 after surgery. The principal antioxidant enzyme, superoxide dismutase, was most sensitive to cryptorchidism, the activity in the abdominal testes being 74% or 85% (per gram of tissue or per whole testis, respectively; $p < 0.01$). After impairment of the reactive oxygen detoxifying capacity, lipid peroxidation was increased in the abdominal testis by 46% ($p < 0.01$) on postoperative Day 7. Slight concomitant increases were detected in the activities of GSH-peroxidase ($p < 0.01$), GSH-transferase ($p < 0.001$), and the hexose monophosphate shunt ($p < 0.001$). This effect was seen only when calculated per gram of tissue, not per whole testis. When decapsulated control testes were incubated in HEPES-buffered Medium 199, 18% ($p < 0.05$) or 42% ($p < 0.01$) of superoxide dismutase activity was lost after 3 h at 37°C or 40°C, respectively, whereas the other enzyme activities were not affected. This result suggests that cryptorchidism particularly affects superoxide dismutase because of elevated abdominal temperature. In conclusion, the present investigation showed that superoxide dismutase activity of rat testis is sensitive to inactivation by elevated temperatures. In addition, the catalase activity is decreased, and thus the reactive oxygen detoxifying capacity is reduced, resulting in increased oxidative stress of the cryptorchid rat testis. These events may be implicated in the impaired spermatogenesis and increased tendency to malignant transformation of the cryptorchid testis.

INTRODUCTION

Cellular prooxidant states are defined as increased concentrations of active oxygen, organic peroxides, and radicals. A prooxidant state may be due either to overproduction of reactive substances or to a deficiency in the antioxidant defense system [1]. The role of prooxidant states in various physiological and pathological phenomena is currently under active investigation, and these states are thought to be implicated in processes as diverse as aging, carcinogenesis, drug action and drug toxicity, inflammation, defense against protozoa, and many others [1–4].

Recently accumulated evidence has revealed the vulnerability of human sperm function to oxidative stress. Infertile men whose spermatozoa cannot fuse with oocytes are characterized by extremely high basal levels of active oxygen production [5, 6]. Accordingly, a low level of the principal antioxidant enzyme, superoxide dismutase, is implicated in human sperm dysfunction [7]. These data are consistent with the proposed causative role for lipid peroxidation in the etiology of defective sperm function [6–8].

Experimental cryptorchidism is known to result in disruption of spermatogenesis [9, 10] and has been widely used as a model for elucidation of normal and pathologic testicular function. In the present study, we have investigated the effect of experimental cryptorchidism on the level of oxidative stress and the function of the enzymatic antioxidant defense in rat testes. The aim was to use this experimental model to obtain more information on testicular prooxidant states and factors controlling them under physiological and pathological conditions.

MATERIALS AND METHODS

Chemicals

Butylated hydroxyanisole, cumene hydroperoxide, epinephrine, glucose-6-phosphate, hydrogen peroxide, reduced glutathione (GSH), reduced nicotinamide dinucleotide diphosphate (NADPH), as well as the enzymes catalase (bovine liver), glutathione reductase (Bakers Yeast), and superoxide dismutase (Cu/Zn form, bovine erythrocytes) were all purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Male Sprague-Dawley rats (age 3–4 mo; 330–370 g) produced in our own vivarium were fed a standard laboratory diet (Hankkija OY, Finland) with free access to tap water and were kept under controlled L:D cycle. Rats under ether

Accepted January 30, 1992.

Received August 23, 1990.

[1]The study was financially supported by a research contract from The Finnish Life and Pension Insurance Companies and by grants from the Academy of Finland, The Finnish Cancer Foundation. The Sigrid Juselius Foundation, and the Juho Vainio Foundation, Finland.

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anesthesia were rendered unilaterally cryptorchid by attaching the testis with a suture to the abdominal wall. The contralateral testis served as control. The animals were killed with 100% CO_2 1, 3, or 7 days after the operation; after they reached unconsciousness they were exsanquinated by cutting the throat.

Tissue Preparation

The testicles were dissected out, rinsed in saline, and weighed. A 20% (w/v) homogenate was prepared in a 0.25 mol/L sucrose solution (0°C) with a Potter-Elvehjem glass-Teflon homogenizer driven by an electric drill at 500 rpm. A postmitochondrial supernatant was prepared by centrifugation (10 000 × *g* max for 10 min at 4°C).

In Vitro Incubations

Control testes were decapsulated and incubated for 3 h at various temperatures (34°C, 37°C, 40°C, and 42°C) in sealed 50-ml Falcon tubes (Falcon Plastics, Los Angeles, CA) in a large volume (1 g/100 ml) of HEPES-buffered Medium 199, pH 7.4. In addition, slices of rat liver were incubated as above for controls. After incubation, tissues were rinsed and tissue fractions were prepared as described above.

Lipid Peroxidation

The appearance of conjugated diene double bonds in polyunsatured fat was used to estimate the level of oxidative stress in testes. Lipids extracted from tissue homogenates by chloroform-methanol, dried under a nitrogen atmosphere, and then redissolved in cyclohexane were analyzed spectrophotometrically (at 232 nm) for quantitation of the diene conjugation [11].

Enzyme Assays

Care was taken to perform all enzyme activity measurements under optimal conditions with respect to incubation time and protein concentration so that the initial velocities were determined. Superoxide dismutase (Cu/Zn form) was assayed spectophotometrically by inhibition of epinephrine autoxidation [12]. Catalase activity was determined by measuring the rate of disappearance of 15 mmol/L hydrogen peroxide at 240 nm [13]. In the analysis of superoxide dismutase activity, 1 μg of the purified enzyme preparation (from bovine erythrocytes) corresponds to 3.6 U, and 1 μg of catalase (bovine liver catalase) corresponds to 2.5 U. The activities of GHS-peroxidase (with cumene hydroperoxide as the substrate) [14], glutathione-transferase (with 1-chloro-2, 4-dinitrobenzene as the substrate) [15], and hexose monophosphate shunt [16] were measured spectrophotometrically by the methods described previously [15, 16]. Because the total NADPH production was measured, the hexose monophosphate shunt activity represents the sum of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. Superoxide dismutase and catalase activities were determined in tissue homogenates, whereas the 10 000 × *g* supernatant fluid was used in the GHS-peroxidase, GHS-transferase, and hexose monophosphate shunt assays.

TABLE 1. Protein contents and weights of rat testis 1, 3, and 7 days after induction of unilateral cryptorchidism.*

Day	Protein contents (mg/g wet weight)		Weight (g)	
	Scrotal	Abdominal	Scrotal	Abdominal
1	152 ± 7	162 ± 11	1.82 ± 0.06	1.64 ± 0.05
3	149 ± 8	164 ± 18	1.61 ± 0.06	1.75 ± 0.16
7	152 ± 2	149 ± 3	1.70 ± 0.06	0.99 ± 0.05†

*Mean ± SEM; for Days 1 and 3, n = 4; for Day 7, n = 8.
†$p < 0.001$.

Retinol

For analysis of retinol, samples of testis homogenates were extracted with methanol and diethylether, and the amount of retinol was quantified with HPLC using retinol acetate as internal standard [17].

Protein

Protein content was measured by the biuret method [18] with BSA as the reference protein.

Statistics

Dunnet's test was used in the statistical evaluation of results. A p value less than 0.05 was selected as the limit of statistical significance.

RESULTS

As expected, the abdominal testes became atrophied; a reduction of weight was evident by postoperative Day 7 (Table 1). The protein contents of testicles (per gram tissue) remained unchanged through the 7-day follow-up period (Table 1). The earliest change noted in the biochemical parameters measured was a reduction in superoxide dismutase activity of abdominal testes, which appeared on postoperative Day 1 (Figs. 1 and 2). The decline in the activity of superoxide dismutase was the same whether calculated per gram of tissue (Fig. 1) or per whole testis (Fig. 2). In the case of catalase activity, the effect was significant only when given as the total activity in tissue. Superoxide dismutase activity continued to decrease and was lowest at the last time point (Fig. 1 and 2).

The level of oxidative stress, as measured by appearance of conjugated diene double bonds in polyunsaturated fat, was the same in both scrotal and abdominal testes when measured 1 or 3 days after the operation. However, substantially increased levels of diene conjugation (per gram of tissue) were found in abdominal testes on Day 7 (Fig. 1). This effect was seen only when the results were calculated per unit weight of tissue (Fig. 2). In parallel, GSH-

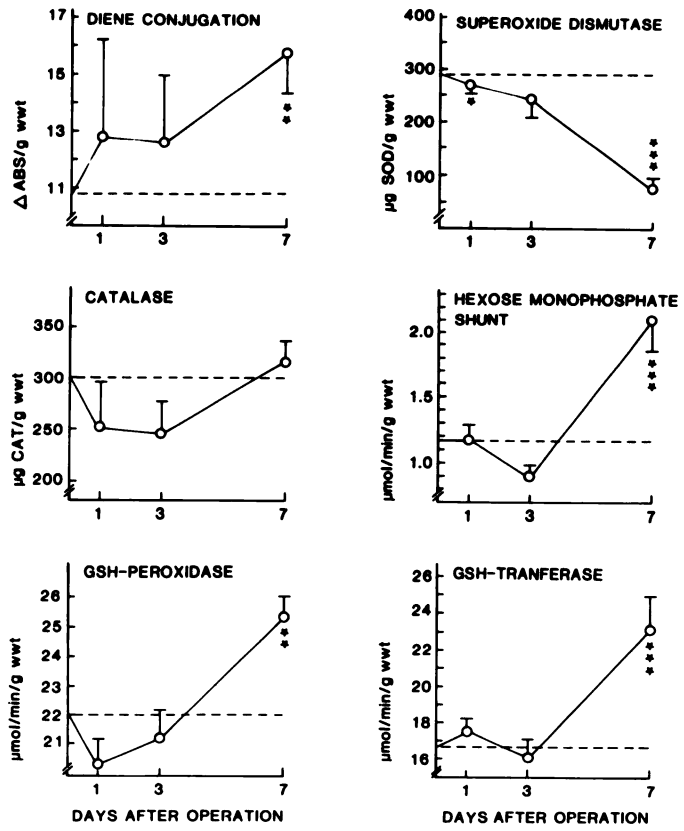

FIG. 1. Diene conjugation and antioxidant enzyme activities in rat testis 1, 3, and 7 days after induction of cryptorchidism. The results are expressed per gram of tissue (dotted lines = control activities). For the two first time points n = 4, for the last time point n = 8. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

peroxidase, GSH-transferase, and the hexose monophosphate shunt activities were found to be elevated within the 7-day cryptorchid rat testis, but only when the activities were expressed per gram of tissue (Figs. 1 and 2).

To determine the sensitivities of testicular and hepatic antioxidant enzymes to elevated temperatures, the activities of the enzymes were measured after incubation of decapsulated testicles and liver slices for 3 h at different temperatures. Superoxide dismutase activities of both tissues were inactivated by heat; the testicular enzyme was clearly more sensitive, losing 18% or 42% of the catalytic activity after 3 h at 37°C or 40°C, respectively (Fig. 3). In contrast, other enzyme activities were not affected or were slightly increased by the higher temperatures tested (data not shown).

In addition to the antioxidant enzyme activities, testicular retinol concentrations were determined but were not found to be affected by cryptorchidism (data not shown).

DISCUSSION

The cryptorchid testis is characterized by increased Leydig cell volume and hypertrophy of cellular organelles involved in steroidogenesis [19, 20], but the weights of the seminal vesicle and ventral prostrate are reduced [21–23], indicating an overall impairment of androgenic function. Several lines of evidence indicate gross impairment of the endocrinological functions of testes in cryptorchidism. There is a drastic decrease in the number of LH receptors [24, 25] and impairment of gonadotropin uptake in vivo [26] by the cryptorchid testis. Moreover, a decline in the activity of enzymes associated with androgen biosynthesis [27, 28], and decreased androgen-binding protein production [29, 30] have been noted in the cryptorchid testis. It has been suggested, however, that impaired Leydig cell function and shortage of testosterone would not be of decisive importance in the cryptorchidism-induced impairment of spermatogenesis [31].

In the present study, we investigated the effects of experimental cryptorchidism on the level of oxidative stress, implicated in human testicular dysfunction (see *Introduction*), and the function of the antioxidant enzymes in rat testes. We found that the activity of superoxide dismutase was drastically affected by cryptorchidism. A decrease in catalytic activity was seen on the first postoperative day, and the activity was even lower 7 days after induction of cryp-

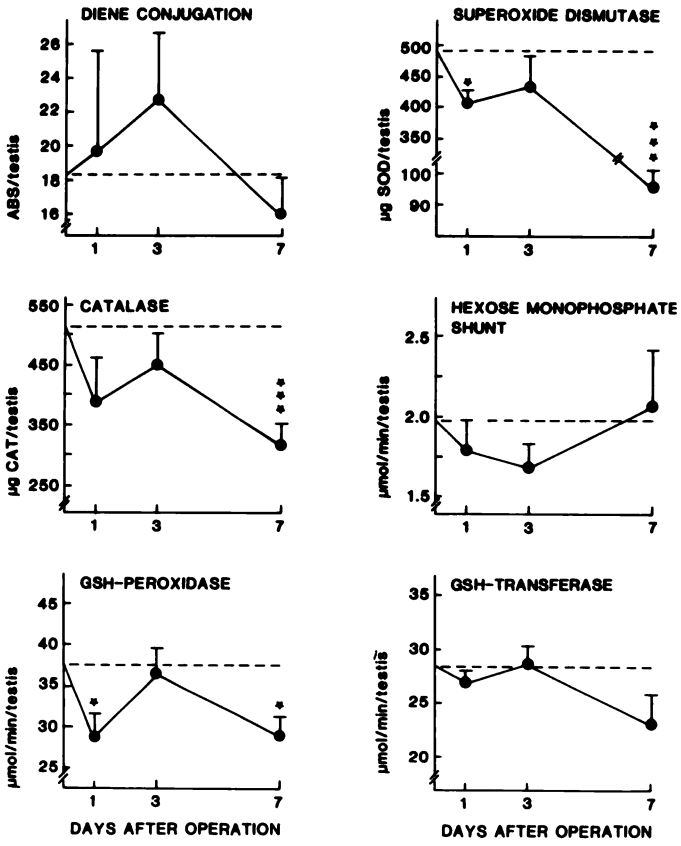

FIG. 2. Diene conjugation and antioxidant enzyme activities in rat testis 1, 3, and 7 days after induction of cryptorchidism. The results are expressed as total activities in whole testes (dotted lines = control activities). For the two first time points n = 4; for the last time point n = 8. * $p < 0.05$; *** $p < 0.001$.

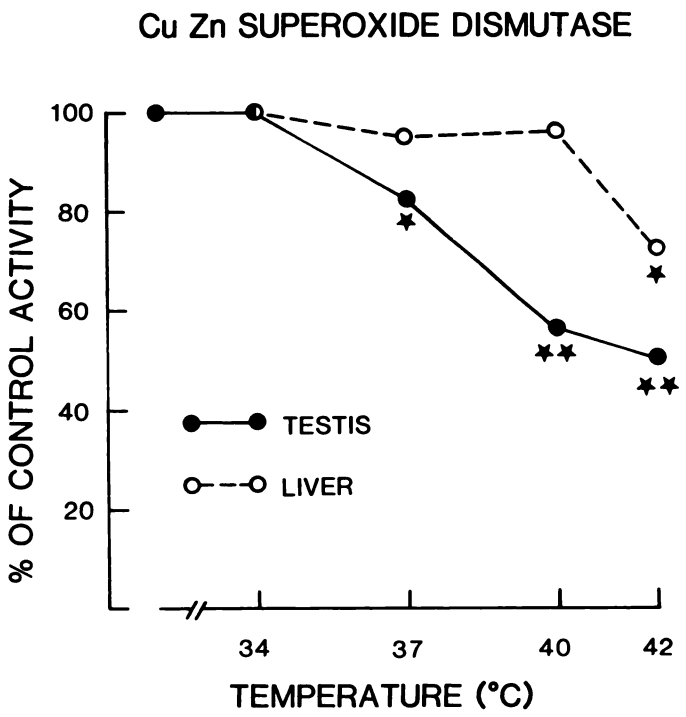

FIG. 3. Inactivation of testicular and hepatic superoxide dismutase activities by elevated temperatures in vitro. Decapsulated testes and liver slices were incubated for 3 h in HEPES-buffered Medium 199. Means from five separate assays are shown (variation between assays less than 10%). * $p < 0.05$; ** $p < 0.01$.

torchidism. Catalase activity was also decreased in the abdominal testis.

The results of biochemical analyses performed in the present study were given both per whole testis and per gram tissue. Presenting the results per whole testis gives an indication of the total content/detoxication capacity of the testicles, but may in this case also reveal apparent changes that in fact were due to alterations in relative amounts of the different testicular cell types. However, presenting the results per gram of tissue indicates the actual amounts of reactive species, and enzymes detoxifying them, present in testicular tissue and is thus more informative when the consequences for the functioning of testes are considered.

High levels of superoxide dismutase activity are found in round spermatids and pachytene spermatocytes, the cell types known to be reduced in number in cryptorchid testicles [32]. Thus, the possibility cannot be ruled out that the decreased superoxide dismutase activity of cryptorchid testicles reflects cell death. Yet the activity of superoxide dismutase was found to decrease very rapidly after induction of cryptorchidism, whereas earliest changes in the activities of various tubular and interstitial enzymes, due to alterations in relative amounts of different cell types, seem to take place 2–4 days after induction of cryptorchidism [28]. In the present study, superoxide dismutase activity was decreased 24 h after operation. In in vitro experiments, superoxide dismutase lost catalytic activity after 3 h at elevated temperatures. Moreover, catalase activity, which also was found to be decreased due to cryptorchidism, is absent in round spermatids and pachytene spermatocytes of testes [32]. Thus, a change in relative amounts of different cell types is hardly the sole explanation for the decreased activities of reactive oxygen-detoxifying enzymes in cryptorchid rat testicles.

The mechanisms for the cryptorchidism-induced decreases in the activities of the reactive oxygen-detoxifying enzymes remain to be explored. In case of superoxide dismutase, the sensitivity of the testicular enzyme to elevated temperatures was suggested by incubations with crude tissue preparations in vitro (Fig. 3). This would suggest that the cryptorchidism-caused effects on superoxide dismutase activity may be due to a direct effect of the elevated temperature in the abdominal testis.

Impaired detoxication functions may lead to elevated cellular concentrations of oxygen radicals and, consequently, to increased peroxidation of cellular lipids, as was seen in the present study in the cryptorchid testis. Diene conjugation, regarded as a specific indicator of lipid peroxidation [33], was significantly elevated, but not until the last time point, 7 days after induction of cryptorchidism. Thus, the elevation in the amount of diene conjugation took place after impairment of the enzymatic antioxidant functions.

Concomitant with the increased levels of oxidative stress, activities of the antioxidant enzymes that function in detoxication of reactive products formed in lipid peroxidation (i.e., GSH-peroxidase, GSH-transferase, and hexose monophosphate shunt) were found to be elevated by the seventh postoperative day. This may indicate principally that compensatory mechanisms similar to those described for the lung [34] are present in testis.

The findings of the present study, impaired detoxication of reactive oxygen species and concomitant oxidative stress, may be implicated in biochemical mechanisms responsible for testicular dysfunction in cryptorchidism. Retaining the integrity of the highly specialized structure should be of vital importance to sperm function. The high levels of readily peroxidizable polyunsaturated material expose spermatozoa to excessive oxidative stress. In support of this, a surprisingly high level of superoxide dismutase is present in the human testis [35], and the superoxide dismutase activity of sperm samples is a good predictor of their survival time [7]. Moreover, the antioxidant vitamins A and E, as well as zinc (a component of the superoxide dismutase molecule) are known to be essential for the initiation and maintenance of spermatogenesis [26, 36, 37]. The increased level of oxidative stress in the cryptorchid testis, as seen in the present study, may also help to explain why cancer (mainly seminoma) is about 30 times more common in cryptorchid than scrotal testes [38], since the cellular prooxidant states are known to be implicated in carcinogenic processes [4].

While the importance of undisturbed antioxidant defence for testicular function remains to be elucidated, the vulnerability of testes to prooxidative factors opens new insights into possible impairment of testicular functions by environmental factors. Previously it has been shown that antioxidant functions in rat liver and human skin are impaired by external factors, such as carcinogenic chemicals [39] and UVB-irradiation [40]. Our present data show that similar factors may also affect normal testicular function.

ACKNOWLEDGMENTS

The technical assistance of Mrs. Raija Söderholm and Mrs. Leena Söderholm is gratefully acknowledged.

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