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Protective Effects of Antioxidants on Testicular Functions of Varicocelized Rats

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Left varicocele detrimentally affects spermatogenesis and the epididymal sperm maturation process bilaterally. We analyzed the effect of antioxidants on sperm production and quality in rats which were varicocelized. Non-varicocelized rats served as controls (group A). Left varicocele was done in 5 groups (B, C , D , E and F) of rats of the same age. Antioxidants taurine, catalase or superoxide dismutase were administered intraabdominally twice a week in groups C, D or E, respectively. Group F received taurine, catalase and superoxide dismutase. Groups A and B received a vehicle dose of saline equally. Eight weeks after varicocele induction bilateral epididymal caudal sperm content, motility and fertilizing ability were analyzed. All the parameters were significantly lower in group B than in groups A, C, D, E and F, although the values in group A were higher than in the other groups. Bilateral testicular weight and testosterone responses to human chorionic gonadotropin stimulation were significantly higher in groups A, C, D, E and F than in the group B. There were no significant differences in serum testosterone basal profiles among groups A, B, C, D, E and F. These results indicated the effectiveness of antioxidants for the preservation of testicular function and the epididymal sperm maturation process in varicocelized subjects.

Key words: antioxidants; reactive oxygen species; sperm quantity and quality; varicocele

Reactive oxygen species (ROS) are cytotoxic and cause tissue injury in several pathophysiologies, such as cancer, glomerular nephritis, atherosclerosis and arthritis. In addition, hyperoxia, ischemia/reperfusion, chemotherapy and radiotherapy result in the production of ROS (Sie, 1991). The generation of ROS is linked to professional phagocytes which utilize these pernicious molecules during the destruction of invading cells and organisms. However, many non-phagocytic cell types also have the capacity to produce ROS. Thus, ROS produced by endothelial cells, mesangial cells, fibroblasts, thyroid cells, Leydig cells and oocytes can play a positive, functional role in the electron transfer reactions that regulate cell activity. Although the controlled generation of these highly reactive molecules may serve as a second messenger system in many different cell types, the uncontroled production of ROS is considered as an important factor in the tissue damage induced by several pathophysiologies.

Since human sperm plasma membranes have a high content of polyunsaturated fatty acids, the membranes are sensitive to damage by free radicals (Alvarez et al., 1987). As a result of the lipid peroxidation induced by free radicals, spermatozoa may not be able to undergo acrosomal reaction and may be impaired for motility (Rao et al., 1989; Aitken and West, 1990). Spermatozoa and infiltrating leucocytes are considered to be the major sources of ROS in semen samples (Aitken and West, 1990; Kessopoulou et al., 1992). Spermatozoa and seminal plasma have their own anti-oxidative mechanisms to protect ROS-induced cellular damage. Several studies have demonstrated the presence of enzymatic antioxidants such as

Abbreviations: hCG, human chorionic gonadotropin; IVF, in vitro fertilization; LTV, left testicular vein; ROS, reactive oxygen species; SOD, superoxide dismutase

catalase, superoxide dismutase (SOD), and glutathione peroxidase/reductase in human semen (Alvarez et al., 1987; Jeulin et al., 1989; Kobayashi et al., 1991). Besides these enzymatic systems, other compounds with antioxidant properties have been found in human semen. Albumin (Halliwell and Gutteridge, 1987), taurine and hypotaurine (Alvarez and Storey, 1983), pyruvate (de Lamirande and Gagnon, 1992), urate (Grootveldt and Halliwell, 1987), vitamins E and C (Chow, 1991; Thiele et al., 1995) and β-carotene (Burton and Ingold, 1984), play an important role in the protection of tissues against free radical attack.

Varicoceles are the main cause of primary and secondary male infertility. The large incidence of varicoceles in men with secondary infertility and the common observation that varicoceles generally develop at the time of puberty suggest that the presence of varicoceles can cause a progressive decline in fertility. Although varicocelectomy is considered as a highly effective treatment of infertility due to varicoceles a significant number of men remain infertile after varicocelectomy (Sofikitis and Miyagawa, 1992). Therefore there is an increasing interest to develop conservative modes of therapy of infertility due to varicoceles. Considering that oxygen radical scavengers provide significant restoration of testicular function after testicular vascular diseases (Henry and Turner, 1996), we studied the effects of the administration of antioxidants on testicular spermatogenic and steroidogenic functions in rats with varicoceles.

Materials and Methods

Grouping of rats, induction of varicoceles and administration of antioxidants

Four-week-old male Wistar rats (Shimizu Experimental Co., Kyoto, Japan) weighing 90 to 100 g were divided into 6 groups (A, B, C, D, E and F). Groups B, C, D, E and F were anesthetized with intraperitoneal nembutal (25 mg/kg) and left varicocele was induced as previously described (Saypol et al., 1981). In brief, partial left renal vein ligation was performed at a point medial to the junction of the adrenal and spermatic veins, so as to reduce the renal vein to an external diameter of 1 mm. Then the anastomotic branch between the left testicular vein (LTV) and the left common iliac vein was ligated, and all the small anastomotic branches of the LTV were carefully ligated (Saypol et al., 1981; Sofikitis and Miyagawa, 1992). Nine rats were similarly anesthesized and only underwent dissection of the LTV and the left renal vein medial to the sites of the entry of the adrenal and spermatic veins. These rats were consider as the controls (group A) . Groups B, C, D, E and F included 7, 9, 10, 9 and 8 rats, respectively. Each group received various medical treatments by intraabdominal injections from the first week after operation for 8 weeks. All drugs were administered via the intraabdominal route (Beaman et al., 1987), since this route is less time consuming and less invasive than that of intravenous injection. A vehicle dose of 0.5 mL of saline (0.85% w/v) was administered to groups A and B twice a week. Taurine (100 mg), catalase (10 μg) or SOD (150 IU) (Sigma Chemical Co. St Louis, MO) was diluted in 0.5 mL of saline and given to groups C, D or E, respectively, twice a week. A mixture of taurine (100 mg), catalase (10 μg) and SOD (150 IU) diluted in 0.5mL of saline was given to group F equally. We adopted the above doses of antioxidants for testicular dysfunction with experimentally induced varicoceles according to our preliminary studies.

Determination of testicular temperature

Testicular and intraabdominal temperatures were measured by percutaneous insertion of a 29-gauge needle probe attached to a digital thermometer (Unique Medical, PTC 201 model, Tokyo, Japan). The difference between the intraabdominal and intratesticular temperatures on each side was recorded.

Assessment of fertility

Eight weeks after operation (experimental period), each rat from each group was placed in the

same cage with three 10-week-old female Wistar rats for 5 days. Twenty-one to 27 days after the beginning of the mating experiment, the females were checked daily for parturition. The male rats were considered as fertile if its matings resulted in at least one pregnancy.

Determination of serum testosterone concentration

Serum testosterone concentration was evaluated twice; prior to intraabdominal injection of human chorionic gonadotropin (hCG; 1,500 IU/animal; Teikoku Zohki Co., Tokyo) and 3 h after injection. Serum was transferred to the Japanese Special Reference Laboratory and the testosterone concentration was determined by radioimmunoassay using kits from Nihon DPC Corporation (Tokyo) as previously described (Coyotupa et al., 1973). The intra- and interassay coefficients of variation were 5% and 9%, respectively. The sensitivity of the assay was 0.1 ng.

Determination of the weight of the testicles and seminal vesicles

The rats were sacrificed. The testicles and seminal vesicles were excised free of surrounding tissue and weighed on a Mettler Basbal scale (Delta Range, Tokyo).

Epididymal sperm content and quantitative sperm motility

Epididymides were separated carefully from the testicles under a 10-time magnification provided by a stereo zoom microscope (model TL2, Olympus Co., Tokyo). The epididymis was divided into 3 segments; head, body and tail. The epididymal tail was trimmed and minced in 5 mL of Toyoda and Chang medium (Toyoda and Chang, 1974). The minced epididymal tissue was separated from the liberated spermatozoa by filtration through a stainless steel wire mesh with a pore size of 60 μm (Tomoda, Matsue, Japan). Five drops of the filtrate were used for assessing the sperm count (number of spermatozoa/mL) which was determined using a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel). Subsequently, 10 droplets of the filtrate were counted to calculate the percentage of motile spermatozoa. The proportion of motile spermatozoa was determined by counting 100 cells in randomly selected fields. Samples for motility evaluation were kept at 35˚C.

Sperm processing for in vitro fertilization (IVF)

Samples containing 2×10^6 spermatozoa/mL were prepared from the sperm suspensions as described. These samples were incubated at 37˚C under 5% CO_2 for 12 h. Then, 0.1 mL of the sperm suspensions was introduced to 0.9 mL of the Toyoda and Chang medium containing the oocytes in a different dish by calibration with a Pasteur pipette as previously described (Toyoda and Chang, 1974).

Collection of oocytes and insemination

Immature female rats were injected subcutaneously with 30 IU pregnant mare's serum gonadotropin (Sigma Chemical Co.) 54 h prior to an intraabdominal injection of 30 IU hCG (Teikoku Zohki Co.). Rats were killed 19 h after hCG injection. The oviducts were removed and the ampullar portion was put into a plastic dish containing Toyoda and Chang medium. The eggs in cumulus masses were dissected out of the oviducts and introduced into the medium. Oocytes were inseminated with 2×10^5 spermatozoa per oocyte. After 24 h of insemination, the number of oocytes with 2 pronuclei plus a second polar body was assessed. Five male rats from each group were processed for the IVF analysis. Ten oocytes were inseminated by spermatozoa from the left epididymal cauda of each male rat. Ten oocytes were inseminated by spermatozoa from the right epididymal cauda of each male rat.

Statistical analysis

Statistical analysis was performed on all data using the paired *t*-test to compare quantitative

Table 1. Testicular weight and the differences between the intraabdominal and testicular temperatures

Group		Left testis		Right testis	
		Weight (mg)	Δ (˚C)	Weight (mg)	Δ (˚C)
A:Control	[9]	1328 ± 174*	4.3 ± 0.3*	1401 ± 157*	4.5 ± 0.3*
B:Saline	[7]	613 ± 108**	2.5 ± 0.4**	824 ± 82**	2.7 ± 0.4**
C:Taurine	[9]	801 ± 79***	2.6 ± 0.3**	1061 ± 64***	2.8 ± 0.3**
D:Catalase	[10]	824 ± 89***	2.9 ± 0.4**	1102 ± 75***	3.0 ± 0.3**
E:SOD	[9]	811 ± 85***	2.8 ± 0.4**	1098 ± 73***	3.0 ± 0.3**
F:Mix	[8]	846 ± 97***	2.8 ± 0.3**	1106 ± 82***	2.9 ± 0.4**

[ ], number of animals in each group; Δ, differences between intraabdominal and testicular temperatures; Mix, taurine + catalase + SOD; SOD, superoxide dismutase.
Within the same column, * versus **; ** versus ***; * versus ***: $P < 0.05$.

parameters referring to paired organs within a group and one-way analysis of variance ($P < 0.05$) followed by the Duncan's multiple range test ($P < 0.05$) to analyze intergroup differences of quantitative parameters. All values were expressed as the mean ± SD. Qualitative parameters were analyzed by chi-square test. A probability of < 0.05 was considered to be statistically significant.

Results

Testicular temperature

The differences between intraabdominal and intratesticular temperatures are summarized in Table 1. There were no significant differences between the left and right testes in all groups. However, group A showed significantly higher values to the varicocelized groups B, C, D, E and F.

Table 2. Weights of the seminal vesicles

Group		Weight†
A: Control	[9]	781 ± 82*
B: Saline	[7]	426 ± 77**
C: Taurine	[9]	579 ± 61***
D: Catalase	[10]	601 ± 58***
E: SOD	[9]	608 ± 53***
F: Mix	[8]	640 ± 61***

[ ], number of animals in each groups; Mix, taurine + catalase + SOD; SOD, superoxide dismutase.
† Weight (mg) of the left and right seminal vesicles. Within the column, * versus **; ** versus ***; * versus ***: $P < 0.05$.

Testicular weight

Left testicular weights were significantly lower than right testicular weight in groups B, C, D, E and F, except group A (Table 1). The left and right testicular weights in group B were significantly lower than in all other groups.

Weights of the seminal vesicles

The weights of seminal vesicles are summarized in Table 2. The weight was significantly lower in group B than in all other groups. The weights in groups C, D, E and F were right in between groups A and B.

Epididymal sperm content, motility and fertilization rate

The epididymal caudal sperm content, motility and fertilization rate are summarized in Table 3. The sperm contents on the left or right side in group A were significantly higher than in all other groups. Left and right epididymal sperm contents were significantly lower in group B than in all other groups. In group B sperm content was significantly lower on the left side than on the right side. The percentage of motile sperm on left and right sides in group B was significantly lower than in all other groups. The

Table 3. Epididymal sperm content, motility and IVF outcome

Group		Left epididymis			Right epididymis		
		Sperm content (millions of sperma-tozoa/mL)	Motile sperm (%)	Oocytes with 2-PN + 2nd PB (FO/IO)	Sperm content (millions of sperma-tozoa/mL)	Motile sperm (%)	Oocytes with 2-PN + 2nd PB (FO/IO)
A: Control	[9]	74 ± 8*	78 ± 8*	43/50*	71 ± 6*	75 ± 7*	44/50*
B: Saline	[7]	13 ± 8**	19 ± 7**	4/50**	33 ± 8**	26 ± 6**	8/50**
C: Taurine	[9]	40 ± 7***	41 ± 6***	21/50***	48 ± 6***	49 ± 7***	27/50***
D: Catalase	[10]	46 ± 5***	45 ± 6***	25/50***	53 ± 7***	55 ± 8***	27/50***
E: SOD	[9]	45 ± 6***	45 ± 5***	27/50***	51 ± 5***	56 ± 6***	28/50***
F: Mix	[8]	48 ± 6***	49 ± 8***	29/50***	55 ± 7***	58 ± 6***	29/50***

[ ], number of animals in each group.
FO, fertilized oocytes; IO, inseminated oocytes; IVF, in vitro fertilization; Mix, taurine + catalase + SOD; 2-PN + 2nd PB, 2 pronuclei plus a second polar body; SOD, superoxide dismutase.
Within the same column, * versus **; ** versus ***; * versus ***: $P < 0.05$.

percentages of motile spermatozoa on the left and right sides were significantly higher in group A than in all other groups. The proportion of fertilized oocytes to inseminated oocytes on both the left and right sides was significantly smaller in group B than in all other groups. The largest values were observed in group A.

hCG test and mating test

Basal testosterone serum profiles were not significantly different among all groups (Table 4). However testosterone responses were significantly lower in group B. The highest values were found in group A. The proportion of fertile rats in group B was significantly lower than the other groups. The frequency of fertile rats was increased in antioxidant treated groups C, D, E and F, although they were not extended to group A.

Discussion

The present study confirms previous studies in our laboratory (Sofikitis and Miyagawa, 1992) that the induction of left varicocele in Wistar rats results in a bilateral decrease in testicular versus intraabdominal temperature difference. The increase in testicular temperature may be the cause of the impaired Leydig cell secretory function (Table 4) in this study since it is known

Table 4. Serum concentrations of testosterone, testosterone responses to hCG and the results of the mating test

Group		hCG (ng/mL)		Fertile rats/tested rats
		Basal testosterone	Testosterone response	
A: Control	[9]	120 ± 45*	857 ± 51*	9/9****
B: Saline	[7]	119 ± 32*	501 ± 61**	1/7*****
C: Taurine	[9]	116 ± 31*	692 ± 85***	3/9
D: Catalase	[10]	111 ± 39*	701 ± 97***	4/10
E: SOD	[9]	120 ± 36*	708 ± 86***	3/9
F: Mix	[8]	123 ± 22*	728 ± 65***	4/8

[ ], number of animals in each group; hCG, human chorionic gonadotropin; Mix, taurine + catalase + SOD; SOD, superoxide dismutase.
Within the same column, * versus **; ** versus ***; * versus ***; **** versus *****: $P < 0.05$.

that an increase in testicular temperature results in Leydig cell secretory deficiency (Sofikitis and Miyagawa, 1992). The significantly lower testosterone responses in varicocelized rats indicated a defect in Leydig cell secretory function. The secretory defect detrimentally influenced the spermatogenesis and epididymal sperm maturation process in varicocelized rats in this study. This hypothesis is supported by the reduced epididymal caudal sperm content and motility profiles (Tables 3 and 4). It is known that optimal Leydig cell function is necessary for the activation and maintenance of spermatogenesis and epididymal sperm maturation process (Saypol et al., 1981; Hurt et al., 1986). The lighter seminal vesicular weight in varicocelized rats may be attributed to the impaired Leydig cell secretory function since a small alteration in androgen production can affect seminal vesicular weight in rats (Sofikitis and Miyagawa, 1992). The absence of significant difference in serum basal testosterone concentration between varicocelized rats and control rats does not indicate an absence of difference in Leydig cell secretory function between the 2 groups since delicate alterations in Leydig cell secretory function may not be accompanied by alterations in peripheral serum androgen levels (Saypol et al., 1981).

The present study also confirm previous studies (Hurt et al., 1986; Takihara et al., 1990) providing evidence of a reduction of spermatogenesis in varicocelized rats by cytometric DNA analysis or by calculation of sperm concentration directly in the epididymal fluid. We revealed a bilateral detrimental effect of left varicocele on sperm fertilizing capacity. This is supported by the significantly smaller IVF trial outcome bilaterally in varicocelized rats. The diminished sperm fertilizing ability may be due to defects in the epididymal sperm maturation process since it is known that failure of spermatozoa to complete the cascade of events that characterize the epididymal sperm maturation process resulted in impaired sperm fertilizing ability (Hurt et al., 1986). Development of spermatozoal ability to expose forward motility, undergo capacitation, and penetrate the zona pellucida of the oocyte are examples of the several important properties spermatozoa acquire during epididymal sperm passage.

ROS are regularly formed during the process of normal respiration. However, the production is kept at physiologically low levels by intracellular free radical scavengers (Southorn and Powis, 1988). While eukaryotic life is impossible without adequate oxygen, exposure to excess concentrations of oxygen results in progressive and lethal damage in several tissues. Several studies have shown that a large production of ROS in semen was detected in more than 25% of infertile men (Aitken and Clarkson, 1987b; Iwasaki and Gagnon, 1992). Aitken and West (1990) and Kessopoulou and co-workers (1992) have demonstrated that the major sources of ROS in semen were derived from the spermatozoa and infiltrating leucocytes. Kessopoulou and co-workers (1995) reported that oral administration of vitamin E significantly improved the in vitro function of human spermatozoa of men with a high ROS production. We showed a benefical effect of the administration of antioxidants on testicular spermatogenic and steroidogenic functions in varicocelized rats. Detrimental effects induced by the administration of drugs were not observed in all rats during this experimental period (data not shown). The significantly larger epididymal caudal sperm content and the enhanced testosterone responses were observed in varicocelized rats treated with antioxidants rather than in sham-treated varicocelized rats (Tables 3 and 4). Furthermore, treatment with antioxidants improved bilateral testicular functions and ameliorated the epididymal sperm maturation process as indicated by the significantly higher epididymal caudal sperm motility and fertilizing ability. The number of fertile rats was increased in varicocelized rats treated with antioxidants than in sham-treated varicocelized rats. The improvement in testicular functions in varicocelized rats treated with antioxidants could not be attributed to the testicular countercurrent heat exchange system since the alterations in testicular temperature was not observed. However, it should be emphasized that the values of quantitative and qualitative sperm parameters remained signif-

icantly lower than the control values, although the antioxidants improved testicular and epididymal parameters in varicocelized rats.

SOD specifically scavenges the superoxide radicals by catalyzing their dismutation to hydrogen peroxide and oxygen. Hydrogen peroxide has been proposed to be the most toxic ROS for human spermatozoa (Alvarez et al., 1987; Aitken et al., 1993). Catalase enhances the degradation of hydrogen peroxide to oxygen and water. Treatment with SOD and catalase reduced the degree of testicular damage in experimental acute torsion (Henry and Turner, 1996). Taurine is known to reduce the cellular production of ROS (Alvarez and Storey, 1983). Morsny and co-workers (1979) have reported that taurine stimulated hamster sperm motility. In our study each of the administered antioxidants had benefical effects on sperm parameters. Although the testicular function in group F was higher compared with that in groups C, D and E, the difference was not significant. The small number of animals in each group might have been responsible for the lack of significant difference. This implies that there was an increased intracellular testicular production of ROS that exceeds the capacity of the testicular tissue by removing oxidants and restoring oxidized components in varicocelized rats. The overall result may be disorganization of intracellular enzymes and membranes. The inflammatory response that generally follows tissue damage due to ROS (Aitken and Clarkson, 1987a; Southorn and Powis, 1988; Ahotupa and Huhtaniemi, 1992; Mackenna and Kessopoulou, 1993) may amplify the primary result. Administration of antioxidants may support the natural antioxidant mechanism of testicular tissue and partially protects the tissue damage due to the induction of varicoceles.

The present study demonstrated a protective effect of antioxidants on testicular spermatogenic and steroidogenic functions and raised the possibility of administering antioxidants to men with varicoceles who have failed to improve sperm qualitative and quantitative parameters after varicocelectomy.

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