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Factors Inducing Vascularization Improve the Spermatogenic and Steroidogenic Function of the Varicocelized Testis

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We evaluated whether alterations in testicular vascularization and fluid dynamics have a role in the development of testicular damage in varicocelized animals. Left varicocele was induced in 6 groups of rats (A, B, C, D, E and F; 7 rats in each group). Four times a week, rats of group A, B, C, D, E and F underwent intrascrotal injections of saline, insulin-like growth factor-I, fibroblast growth factor, tumor necrosis factor-α, vascular endothelial growth factor or all the previous factors together, respectively. Additionally 7 rats were sham-operated (group G). At 8 weeks post-operation, groups E, F and G showed significantly higher left epididymal sperm content and motility, testosterone responses to human chorionic gonadotropin stimulation and intraabdominal versus intratesticular temperature differences than group A. Histological sections showed that the number of vessels per seminiferous tubule was significantly larger in groups E and F than in group A. Factors inducing vascularization have a beneficial effect on the function of the varicocelized testis attributable to improvement of testicular tissue nutrition or to amelioration of the functionality of the countercurrent heat exchange system.

Key Words: growth factors; testis; spermatozoa; varicocele

In most Western and a few Eastern countries male infertility is both a private and social problem. Although several assisted reproduction techniques provide an attractive mode of management for oligospermia (in vitro fertilization [IVF] trials), severe oligospermia/asthenospermia (intracytoplasmic sperm injection trials), azoospermia with testicular spermatozoa (intracytoplasmic sperm injection trials), or even azoospermia without testicular spermatozoa (ooplasmic injections of round spermatid nuclei or secondary spermatocytes) (Palermo et al., 1992; Sofikitis et al., 1998a, 1998b), conservative and surgical therapy of male infertility should always be present in the physician's mind and should be applied prior to assisted reproduction techniques for the improvement of qualitative, quantitative and functional sperm parameters. Improvement of the quality of ejaculation prior to the introduction of the assisted reproduction techniques offers the possibility of natural conception, has a lower cost than assisted reproduction, provides an opportunity for a second child, obviates the need for ovarian stimulation known to include risks for the female partner, and finally increases the probabilities for a positive outcome of assisted reproduction technology that may be applied if natural conception has not been achieved by post-medical or surgical treatment. Therefore, IVF, intracytoplasmic sperm injection and ooplasmic injections of round spermatid nuclei or secondary spermatocytes should be applied only after having attempted to improve testicular function and semen quality with the most contemporary surgical or medical (conservative) treatments.

Abbreviations: FGF, fibroblast growth factor; hCG, human chorionic gonadotropin; IGF, insulin-like growth factor; IVF, in vitro fertilization; LΔT, difference between intraabdominal and left intratesticular temperature; PBS, phospate buffered saline; TC, Toyoda and Chang; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VIF, vascularization-inducing factor

Varicocele is one main cause of male infertility (Sofikitis and Miyagawa, 1993). Varicocelectomy is an efficient procedure in improving semen characteristics in the majority of men with varicocele (MacLeod, 1965). However, a significant number of men with varicocele remain infertile post-varicocelectomy. For the latter population of men with varicocele, additional surgical or conservative treatments are necessary prior to the introduction of assisted reproduction techniques. Ligation of the right testicular vein or hormonal administration have been recommended for men with varicocele who do not improve post-varicocelectomy (Mehan and Chahval, 1982; Ameler and Dubin, 1987; Sofikitis et al., 1992a, 1992b). However, a considerable number of subjects with varicocele remain infertile even after the introduction of several surgical and medical modes of treatment.

In the current study we evaluated the role of growth factors inducing vascularization in the spermatogenic and steroidogenic function of the varicocelized testis taking into consideration that testicular malnutrition has been implied as one of the mechanisms mediating the detrimental effect of left varicocele on ipsilateral testicular function (Harrison et al., 1983, 1986; Sofikitis and Miyagawa, 1993). The rat was used as an experimental animal because it is relatively inexpensive, it can be maintained easily, anesthesia can be readily performed, sperm and blood samples can be collected, and induction of left varicocele in the rat is accompanied by most of the consequences known to occur in the human varicocelized testis.

Table 1. Surgical and medical treatments in the various groups of rats

Group	Varicocele induction	Medical treatment
A	+	Saline
B	+	IGF-I
C	+	FGF
D	+	TNF-α
E	+	VEGF
F	+	All the above factors together
G	–	Saline

FGF, fibroblast growth factor; IGF, insulin-like growth factor; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor.

Materials and Methods

Treatment of animals

Left varicocele was introduced in 6 groups (A, B, C, D, E and F; 7 rats in each group) of 8-week-old Sprague-Dawley rats during anesthesia with intraperitoneal pentobarbital sodium (25 mg/kg). Partial left renal vein ligation was performed by the method of Sypol and coworkers (1981) at a point medial to the junction with the adrenal and spermatic veins, so as to reduce the renal vein to an external diameter of 1 mm. Then all the anastomostic branches of the left testicular vein were carefully ligated (Sofikitis et al., 1992a, 1992b). Additionally, seven 8-week-old rats were sham-operated (group G). The latter rats underwent dissection of the left testicular vein and the left renal vein medial to the drainage of the adrenal and spermatic veins. Four times a week, rats of groups A, B, C, D, E, F or G underwent intrascrotal injections of saline, insulin-growth factor-I (IGF-I; 250 ng) (Sigma Chemical Co., St. Louis, MO), fibroblast growth factor (FGF; 25 ng) (Sigma Chemical Co.), tumor necrosis factor-α (TNF-α; 25 ng) (Sigma Chemical Co.), vascular endothelial growth factor (VEGF; 25 ng)(Sigma Chemical Co.), all the previous factors (same doses) together or saline, respectively (Table 1).

At 8 weeks post-operation, testicular and intraabdominal temperatures were measured. Blood was aspirated from the right renal vein and processed for testosterone assay (basal testosterone). Human chorionic gonadotropin (hCG, Teikoku Zouki Company, Tokyo, Japan) was then injected (1,500 IU) intramuscularly. Three hours later blood was aspirated from the right renal vein and processed for testosterone assay (testosterone responses). At that time the left testicular vein was checked for dilation. Then, the left testis and left epididymal cauda were carefully dissected. Left testicular weight was measured and a piece of testicular tissue

was processed for immunohistochemistry using a monoclonal antibody against endothelial protein Factor VIII-related antigen to observe and count the testicular vessels. The number of vessels per seminiferous tubule was recorded. Spermatozoa were recovered from the left epididymal cauda. Epididymal sperm content and motility were appreciated. Then epididymal caudal spermatozoa were processed for IVF trials.

Determination of testicular temperature

Left testicular and intraabdominal temperatures were assessed by percutaneous insertion of a 29-gauge needle probe attached to a digital thermometer (Unique Medical, Model PTC 201, Tokyo, Japan). Intraabdominal temperature was monitored with a rectal probe and body temperature was maintained between 36.7 and 37.3˚C with radiant heat throughout the procedure. The difference between the intraabdominal and intratesticular temperature was recorded.

Determination of testicular weight

The left testis was excized, dissected free of surrounding tissue, and weighed on a Mettler Basal Scale (Delta Range, Tokyo).

Testosterone assay

Testosterone was determined by radioimmunoassay using kits from Nihon DPC Corporation (Tokyo) according to the method of Coyotupa and coworkers (1972). The intra- and interassay coefficients of variation were 5% and 9%, respectively. The sensitivity of the assay was 0.1 ng.

Epididymal caudal sperm content, quantitative and qualitative sperm motility

Each epididymis was separated carefully from its testicle under a magnification of 10 provided by a stereo zoom microscope (Model SZS, Olympus Corp., Tokyo). The epididymal tail was recovered and minced in 5 mL of Toyoda and Chang (TC) medium (Toyoda and Chang, 1974) titrated to pH 7.4 at 37˚C with sodium hydroxide. 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. Ten droplets of the filtrate were used for calculation of sperm count and motility. The average sperm count, quantitative motility and qualitative motility (motility grade) were recorded. The sperm count was determined with a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel). The chamber was placed on an ordinary microscope slide, and a 20-power objective and 10-power eyepiece were used for viewing. Quantitative motility refers to the percentage of motile spermatozoa. Motility grade was evaluated using an arbitrary grading system on a scale of 0 to 4, with 0 representing no movement, 1 meaning weak forward progression of some spermatozoa, 2 indicating steady forward progression of most motile spermatozoa, 3 meaning strong forward progression, and 4 representing very vigorous and rapid progression.

IVF trials

Sperm samples were centrifuged and resuspended in a volume of TC medium to produce samples of sperm at a concentration of 2×10^6/mL. Sperm samples were incubated for 8 h at 37˚C under 5% carbone dioxide to allow for sperm capacitation.

Oocytes were collected from immature rats as previously described (Yamamoto et al., 1998). Cumulus masses containing 10 oocytes were placed into drops of 0.9 mL of TC medium. The latter drops were mixed with 0.1 mL of the sperm suspensions. Thus, the sperm concentration was 200,000 spermatozoa/mL. Oocytes were cultured at 37˚C for 24 h and were then observed via an inverted microscope (IX-70, Olympus, Tokyo, Japan). The number of oocytes with 2 pronuclei plus a second polar body (fertilized oocytes) was recorded. IVF outcomes are defined as ratio of fertilized oocytes to inseminated oocytes.

Table 2. Effects of growth factors inducing vascularization on LΔT, left testicular weight, basal testotsterone profiles and testoterone responses to hCG

Group	LΔT (˚C)	Left testicular weight (mg)	Basal testosterone (ng/dL)	Testosterone response (ng/dL)
A	2.5 ± 0.3	803 ± 81	163 ± 56	619 ± 68
B	2.8 ± 0.3**	996 ± 92*,**	148 ± 50	803 ± 94*
C	2.9 ± 0.4**	1013 ± 101*,**	152 ± 53	782 ± 70*
D	2.8 ± 0.3**	1032 ± 108*,**	181 ± 67	878 ± 82*
E	3.1 ± 0.3*,**	1192 ± 94*,**	204 ± 72	851 ± 92*
F	3.2 ± 0.3*,**	1281 ± 99*,**	210 ± 66	854 ± 103*
G	4.1 ± 0.4*	1433 ± 77*	201 ± 63	864 ± 101*

hCG, human chorionic gonadotropin; LΔT, difference between intraabdominal and left intratesticular temperature.
Within each column, * indicates a significant difference ($P < 0.05$) compared with varicocele group A, whereas ** indicates a significant difference ($P < 0.05$) compared with control group G.

Immunohistochemistry

Testicular tissue was fixed with 20% formalin for 48 h and embedded in paraffin. Using the serial section technique (7 μm section), anti-Factor VIII stain was used for immunohistochemical analysis.

Endogenous peroxidase activity was quenched by incubation for 30 min with 3% H_2O_2, followed by washing in a phosphate buffered saline (PBS, pH 7.2). The sections were incubated with the antiserum for 18 h at 4˚C. PBS was used to replace the antiserum in the controls. Normal rabbit serum served as a blocking agent. Rabbit anti-mouse IgG was used to detect bound antibodies. 3,3-Diaminobenzidine tetrahydrochloride was used as the final chromogen. The sections were counterstained with hematoxylin.

Each section was viewed under a light microscope (× 200). The number of blood vessels per seminiferous tubule was counted in the 5 different areas from each testis.

Statistical analysis

Statistical analysis was performed using one-way analysis ($P < 0.05$) of variance followed by Duncan's multiple range tests ($P < 0.05$) to analyze intergroup differences. All values were expressed as mean ± SD. A probability $P < 0.05$ was considered to be statistically significant. All parameters were assayed in a blind fasion. IVF outcome was analyzed using the χ^2-test.

Results

Testicular temperature

The difference between intraabdominal and left intratesticular temperature (LΔT) was significantly larger in groups E, F and G than in group A (Table 2).

Testicular weight

Left testicular weight was significantly larger in groups B, C, D, E, F and G than in group A (Table 2).

Testosterone profiles

There were no significant differences in basal serum testosterone profiles among all groups. In contrast, serum testosterone responses to hCG stimulation were significantly larger in groups B, C, D, E, F and G than in group A (Table 2).

Epididymal caudal sperm qualitative, quantitative and functional parameters

Left epididymal caudal sperm content, quantitative and qualitative motility were signifi-

Table 3. Effects of factors inducing vascularization on epididymal caudal sperm content, quantitative sperm motility, motility grade and IVF outcome

Group	Epididymal caudal sperm content ($\times 10^6$/mL)	Quantitative sperm motility (% motile sperm)	Motility grade	IVF outcome (fertilized oocytes/ inseminated oocytes)
A	18 ± 8	24 ± 10	1.6 ± 0.2	19/70
B	27 ± 10**	36 ± 11**	2.4 ± 0.8**	44/70*,**
C	28 ± 10**	33 ± 9**	2.3 ± 0.6**	46/70*
D	30 ± 11*,**	47 ± 11*,**	2.7 ± 0.5*	47/70*
E	53 ± 8*,**	59 ± 11*	2.9 ± 0.4*	47/70*
F	62 ± 9*	59 ± 13*	3.2 ± 0.3*	47/70*
G	68 ± 10*	66 ± 10*	3.3 ± 0.4*	53/70*

IVF, in vitro fertilization.
Within each column, * indicates a significant difference ($P < 0.05$) compared with varicocele group A, whereas ** indicates a significant difference ($P < 0.05$) compared with control group G.

cantly smaller in group A than in groups D, E, F and G. IVF outcome (ratio of fertilized oocytes/ inseminated oocytes) was significantly smaller in group A than in groups B, C, D, E, F and G (Table 3).

Blood circulation in the varicocelized testis

Figure 1 shows typical vessels in interstitial testicular tissue (anti-Factor VIII stain, × 200). The number of vessels per seminiferous tubule was significantly larger in groups E and F than in groups A, B, C, D and G (Table 4).

Discussion

Left varicocele is one main cause of male infertility and is still an enigmatic disease, offering an exciting and attractive field for research. Thus, during the last 15 years several studies on

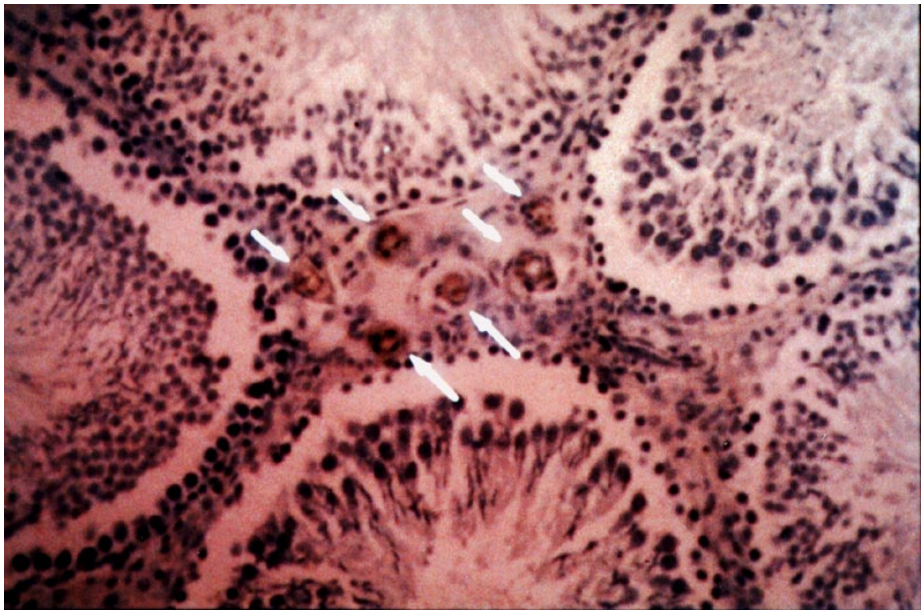

Fig. 1. Immunohistochemical staining for Factor VIII antigen. Arrows indicate vessels in interstitial testicular tissue (× 200).

experimental varicocele models attempted to illustrate varicocele pathophysiology. However, several questions regarding this enigmatic disease (Turner, 1983) still exist: Why is left varicocele rare prior to the age of 8? Why does obesity protect us from varicocele development? Why do some men with varicocele remain fertile? How does varicocele affect testicular function? Several theories have been proposed to explain the detrimental effect of left varicocele on bilateral testicular function: increase in testicular temperature (Saypol et al., 1981), increase in testicular hydrostatic pressure resulting in testicular tissue malnutrition (Harrison et al., 1983, 1986), retrograde blood flow of adrenal/renal metabolites via the left testicular vein (Javert and Clark, 1944), testicular hypoxia, testicular defects in glucose metabolism (Sofikitis and Miyagawa, 1992), alterations in the function/sensitivity of the hypothalamo-pituitary-testicular axis (Weiss and Rodriguez-Rigau, 1978), Sertoli cell anatomical defects (Snydle and Cameron, 1983) or secretory dysfunction (Sofikitis and Miyagawa, 1992) and Leydig cell secretory dysfunction (Rajfer et al., 1987; Sofikitis et al., 1996). Among these theories, elevation of testicular temperature due to dysfunction in the countercurrent heat exchange system and testicular tissue malnutrition are the only theories referring to fluid dynamics and the testicular vascular system. In the current study we attempteed to investigate the influence of alterations in testicular tissue vasculature in the development of testicular damage induced by varicocele.

The present investigation confirms previous studies showing that induction of left varicocele in the rat leads to an increase in testicular temperature resulting in Leydig secretory dysfunction. Rajfer and coworkers (1987) have demonstrated that increase in testicular temperature causing dysfunction of 17,20 desmolase impairs intratesticular testosterone biosynthesis. That study is consistent with the finding of significantly smaller testosterone responses in varicocelized rats than in control rats in the current study. It appears that during normal conditions the diminished intratesticular testosterone profiles are not reflected in the peripheral serum testosterone levels, whereas when Leydig cells are hyperstimulated, the impaired Leydig cell secretory function in varicocelized rats is manifested by smaller testosterone responses to hCG stimulation. These findings are also compatible with our previous observations of the rabbit varicocele model (Sofikitis and Miyagawa, 1992, 1994; Sofikitis et al., 1996). The Leydig cell secretory dysfunction in varicocelized rats may be responsible for the impaired spermatogenesis and epididymal maturation process manifested by smaller epididymal cauda sperm content and motility in the current study. It is known that optimal concentrations of intratesticular and intraepididymal testosterone are important for activating and maintaining the spermatogenesis and epididymal sperm maturation process. Considering that Leydig cell function influences Sertoli cell secretory function, which in turn affects spermatogenesis and the epididymal sperm maturation process, defects in Sertoli cell secretory function may be present in rats with varicocele further impairing spermatogenesis and the epididymal sperm maturation process. In fact, studies in varicocelized rabbits have revealed anatomic (Snydle and Cameron, 1983) and functional (Sofikitis and Miyagawa, 1992) defects in Sertoli cells.

The epididymal sperm maturation process is known to involve a cascade of events resulting in the development of the sperm's capacity to penetrate and fertilize oocytes. Alterations in spermatozoal lipid and carbohydrate composition and transportation of spermatozoal

Table 4. The number of vessels per seminiferous tubule

Group	Number of vessels
A	14 ± 6
B	20 ± 6
C	23 ± 7
D	21 ± 7
E	41 ± 11*
F	46 ± 13*
G	19 ± 5*

* indicates a significant difference ($P < 0.05$) compared with varicocele group A.

cytoskelton proteins occur during the sperm's passage through the epididymis (Turner, 1979). The subnormal/abnormal epididymal sperm maturation process detrimentally affects the overall sperm fertilizing capacity. Thus, the smaller IVF outcome demonstrated in the current study in varicocelized rats may be due to the inability of the epididymal lumen to induce those alterations that secure optimal sperm fertilizing potential.

Previous studies of monkeys with varicocele (Harrison et al., 1983, 1986) have shown an increase in intratesticular hydrostatic pressure. High intratesticular hydrostatic pressure is expected to impair the exit of oxygen and nutrients from the arterial ends of capillaries resulting in hypoxia and malnutrition. Hypoxia and malnutrition can explain the hypospermatogenesis and Leydig cell and Sertoli cell dysfunction in patients and animals with varicocele (see review Sofikitis and Miyagawa, 1993). We attempted to improve testicular spermatogenic and steroidogenic processes in the varicocelized rats by administering factors inducing vascularization. Angiogenic factors are expected to improve tissue nutrition and restore hypoxia. The primary stimulus for neovascularization in the vast majority of tissues is hypoxia. Hypoxia stimulates tissue cells to secrete several paracrine factors including VEGF that specifically induces proliferation of adjacent capillaries in the tissue (Hammes et al., 1996). In response, endothelial cells acquire the angiogenic phenotype with a subsequent rearrangement of gene expression that leads to upregulation of downstream effector mechanisms such as adhesion receptors (Bischoff, 1995) and pericellular proteolysis (Bacharach et al., 1992). Other angiogenic factors include basic FGF, IGF-I, TNF-α, angiotensin II and platelet derived growth factor.

Administration of each of the vascularization-inducing factors (VIFs) in varicocelized rats increased the number of vessels per seminiferous tubule, but a significantly larger number of vessels was found only in rats treated with VEGF or a combination of all the factors. Treatment with a combination of VIFs, TNF-α and VEGF significantly improved spermatogenesis and the epididymal sperm maturation process. The latter thesis is supported by the significant improvements in epididymal caudal sperm content, quantitative motility and qualitative motility. These improvements may be attributable to amelioration of Leydig cell secretory function as indicated by the significantly larger values of testosterone responses compared with varicocelized rats treated with saline. The large and significant improvement in Leydig cell secretory function in rats treated with a combination of VIFs, TNF-α and VEGF is attributable to the significant increases in LΔT values compared with varicocelized rats treated with saline since it is known that increase in testicular temperature detrimentally affects 17,20 desmolase in Leydig cells (Rajfer et al., 1987). The improvement in testicular temperature regulation in varicocelized rats treated with a combination of VIFs, TNF-α and VEGF is a finding of clinical importance and may be attributable to improvement in vascularization. If we adopt the hypothesis of Harrison and coworkers (1981, 1986), we are able to propose that improvement in vascularization and an increase in the number of testicular tissue capillaries decreases intratesticular hydrostatic pressure and ameliorates the tissue edema known to occur in varicocelized subjects (Harrison et al., 1981, 1986). Improvement in fluid dynamics in varicocelized rats may account for the improvement in the functionality of the vascular countercurrent heat exchange system in the varicocelized testis resulting in a decrease in testicular temperature.

Although improvements in epididymal caudal sperm content and motility were significant only in varicocelized rats treated with a combination of VIFs, TNF-α and VEGF, we believe that administration of FGF or IGF-I also improve (although in a smaller degree) the function of varicocelized testis and the epididymal sperm maturation process. This is supported by the significantly larger testicular weight and IVF outcome in the groups of rats that received FGF or IGF-I. Testicular weight is known to correlate significantly with testicular endocrine and exocrine function (Takihara et al., 1987) and in vitro sperm fertilizing capacity is largely dependent on the efficiency of the

epididymal lumen to induce the necessary alterations in spermatozoa in order to achieve fertilizing capacity.

The current study shows that intrascrotal administration of specific VIFs can induce neovascularization in the varicocelized testis, improve the efficiency of the vascular coutercurrent heat exchange system and subsequently decrease testicular temperature. A decrease in LΔT in varicocelized rats is accompanied by improvements in spermatogenesis, the epididymal sperm maturation process and testicular steroidogenic capacity. Improvement in testicular tissue nutrition may also contribute to improvements in testicular and epididymal function. In addition, the present study is the first report in the literature providing an attractive hypothesis to explain the presence of a subpopulation of fertile men with varicocele: men with varicocele and abundant testicular vascularization may be able to a) have adequate testicular nutrition, b) maintain a satisfactory function of the countercurrent heat exchange system avoiding larger increases in testicular temperature and c) alleviate the testicular edema proven to exist in men with varicocele. Therefore, the varicocelized fertile men may be able to alleviate testicular tissue fluid dynamics and compensate for the effects of the large testicular venous pressure on the testis.

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