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Effect of folic acid and zinc sulphate on endocrine parameters and seminal antioxidant level after varicocelectomy

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Summary

Varicocele is among the most common problems which may lead to male infertility. Spermatogenesis is impaired as a consequence of this vascular defect, through mechanisms that are not well described. This study aimed to evaluate serum hormonal level (inhibin B, FSH and testosterone) and seminal plasma antioxidant defence levels after folic acid and zinc sulphate administration in varicocelectomised patients. Participants were randomly allocated to four experimental groups. Our randomisation schedule was as follows: zinc sulphate/folic acid, folic acid, zinc sulphate and placebo. The patients underwent varicocelectomy, before which a blood and semen sample were obtained and also three and six months after varicocelectomy for evaluation of blood hormonal level (FSH, testosterone, inhibin B) and seminal oxidative stress status (nitric oxide, superoxide dismutase, total antioxidant capacity). Patients in different groups took orally one capsule per day after dinner following varicocelectomy for 6 months. A significant rise in peripheral blood inhibin B and seminal plasma activity was detected in the zinc sulphate/folic acid group after 6 months. The present clinical trial indicates a change in the hormonal status of varicocelectomised patients following long-term administration of zinc sulphate and folic acid.

Introduction

Varicocele is an abnormal degree of vasodilatation in the pampiniform plexus and the internal spermatic vein. The varicose alteration is favoured by the extended free passage of the testicular vein in the retroperitoneum, by the lack of supporting muscle pump, by congenitally weak vessel walls or maybe by an atonic cremaster muscle in part accompanying the spermatic vein (Naughton *et al.*, 2001).

The incidence of clinical varicocele in the general population is approximately 15%, but it has been implicated as a cause in 35–50% of patients with primary infertility and up to 81% of men with secondary infertility (Evers & Collins, 2003).

Recently, there has been great emphasis on the role of oxidative stress in the pathogenesis of infertility in patients with varicocele. Varicocele reduces the antioxidant defence of both seminal and blood plasma causing an oxidative stress status (Agarwal *et al.*, 2009; Abd-Elmoaty *et al.*, 2010). A meta-analysis of four studies performed before 2006 showed that patients with varicocele had significantly greater reactive oxygen species (ROS) and lower total antioxidant capacity (TAC) level in their seminal plasma. Moreover, decrease in superoxide dismutase (SOD), catalase and glutathione peroxidase in infertile patients with varicocele was also reported (Anderson & Sharpe, 2000).

Production of high levels of ROS in the reproductive tract affects not only the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus (Cocuzza *et al.*, 2008). Infertile men with varicocele were shown to have high levels of sperm DNA damage caused by elevated levels of oxidative stress (OS)

(Chen & Chow, 2006). Although many individual studies reported improvement after varicocele repair, there are still conflicting opinions as to whether a varicocelectomy improves antioxidant defences and fertility (Naughton *et al.*, 2001; Evers & Collins, 2003).

DNA synthesis plays an essential role in germ cell development, and evidently, zinc and folate are important nutrients for DNA synthesis as well as RNA transferring. Therefore, it could be concluded that these two elements play a role in reproduction system (Wong *et al.*, 2000; Favier, 1992).

It has also been indicated that folic acid, the synthetic form of folate and zinc sulphate, effectively scavenges oxidising free radicals, and thus can be regarded as antioxidants (Joshi *et al.*, 2001). Furthermore, in a study carried out by Wong *et al.* (2002), zinc was implicated as an important trace element involved in testicular development, spermatogenesis and sperm motility (Ebisch *et al.*, 2007).

Also based on the results of our under published double blind placebo-controlled study, beneficial effect of zinc sulphate and folic acid co-administration on sperm parameters and DNA integrity following varicocelectomy was observed. In another study, in which fertile and subfertile males were included, a 74% increase in normal sperm concentration in the subfertile males after folic acid and zinc sulphate intervention was demonstrated (Wong *et al.*, 2002).

However, the fundamental mechanisms involved in the proper effects of synthetic folic acid and zinc sulphate on spermatogenesis are not yet clarified (Fujisawa *et al.*, 2001). Therefore, our hypothesis is that folic acid and zinc sulphate may affect endocrine parameters, for instance, by stimulating the function of the Sertoli or Leydig cells to modify spermatogenesis microenvironment.

Sertoli cells are the main producers of inhibin B in the human body. The serum inhibin B concentration positively correlates with sperm concentration, testicular volume and the state of the spermatogenetic epithelium (Von Eckardstein *et al.*, 1999; Pierik *et al.*, 2003). Thus, the quality of the Sertoli cell function and spermatogenesis could be evaluated according to inhibin B concentrations, so it could be used as a sensitive marker of spermatogenesis in humans. The assessment of serum hormonal level (inhibin B, FSH and testosterone) and seminal plasma antioxidant defence levels post-folic acid and zinc sulphate intervention following varicocelectomy is the goal of this study.

Material and methods

Participants

The research was approved by ethics committee for research involving human subjects at Kerman Medical University, Kerman, Iran and all subjects participated in this study after giving informed consent. The study was also registered at Iranian Registry of Clinical Trials (IRCT registration number: IRCT138802261910N1). One hundred and sixty infertile subjects were enroled in this study who had already consulted with an urologist for varicocelectomy. The presence of varicocele (grade III) was the criterion to enter the study. It was assessed by clinical parameters and was confirmed by Doppler ultrasound scanning. Patients with the evidence of leukocytospermia, hypogonadism (testicular volume <15 ml), congenital urogenital abnormalities and urogenital infections were excluded from the study.

Study design

Participants were randomly allocated to four experimental groups. Our randomisation schedule was as follows: zinc sulphate/folic acid (ZF), folic acid (FA), zincs sulphate (ZS) and placebo (PL). The patients underwent varicocelectomy, before which a blood and semen sample were obtained for laboratory assessments. Patients in each group took orally one capsule per day after dinner following varicocelectomy for 6 month. The dosage of the zinc sulphate was 66 mg per capsule, and for folic acid, it was 5 mg per capsule. Patients in placebo group received the same capsules without the effective drug.

Semen collection and antioxidant evaluation

Before varicocelectomy, one sample was obtained from each participant and also three and 6 months after varicocelectomy the other two samples were collected. The semen samples were provided by the participants via masturbation after an abstinence period of 3–5 days and delivered to the laboratory within 30 min after production. After liquefaction, semen was centrifuged at 1100 g for 6 min to obtain the spermatozoa pellet and a supernatant. The latter was centrifuged at 3300 g for 10 min to obtain the seminal plasma. Markers of seminal plasma oxidative stress (SOD, NO, TAC) were evaluated by kits from Sigma company (Sigma-Aldrich, MO, USA).

Blood plasma hormonal determination

Venous blood samples were taken from individuals, usually in the morning, and serum was separated by centrifugation after clotting and was kept frozen until its analyses. The frozen serum was sent on dry ice to the Department of Biochemistry, in Kerman medical school for a centralised analysis of the reproductive hormones: FSH, inhibin B, total testosterone. FSH was measured by time-resolved immunofluorometric assay (Delfia, Wallac,

Turku, Finland), Testosterone by a time-resolved flouro-immunoassay (Delfia, Wallac, Turku, Finland) and Inhibin B by a specific two-sided enzyme immunometric assay (Serotec,UK). Intra- and inter-assay coefficients of variation (CV) for measurements of both FSH and LH were 3 and 4.5%, respectively and for testosterone was 8. The intra- and inter-assay CV for inhibin B was 15 and 18%, respectively.

Statistical analysis

Mixed model analysis was applied to investigate difference between treatment groups (between factor variable) and between times points (within factor variable) simultaneously. *P*-value <0.05 was considered as significance level. All analysis were carried out using SPSS software version 15 for Windows.

Results

Based on the results (Table 1), plasma testosterone levels were not improved significantly in the group of patients who received zinc sulphate and folic acid supplements 6 month post-varicocelectomy(4 ± 0.15 versus 3.8 ± 0.16). Similar results were observed for groups of patients who received folic acid, zinc and placebo(4.2 ± 0.22 versus 3.6 ± 0.21, 3.9 ± 0.36 versus 3.5 ± 0.15, 4.0 ± 0.31 versus 3.5 ± 0.17, respectively). In the group of patients with 6 month zinc sulphate and folic acid co-administration, a significant rise in peripheral blood inhibin B was detected (127.9 ± 56.2 versus 190.1 ± 45.1; $P \leq 0.05$). Comparable results were observed in the group of patients who were treated with zinc sulphate (98.3 ± 32.9 versus 172.8 ± 52.9; $P \leq 0.05$). A significant alteration in FSH values after 6 months of treatment was not indicated.

Biochemical evaluation of seminal plasma showed that NO concentrations decreased nonsignificantly in all groups of this study except placebo 3 and 6 months after operation (56.6 ± 5.6 versus 48.8 ± 7.5, 60.2 ± 6.4 versus 56.1 ± 8.7, and 56.6 ± 7.2 versus 54.7 ± 4.3, respectively). In addition, total antioxidant capacity of seminal plasma samples did not show a considerable raise as a consequence of varicocelectomy in all intervention groups. Table 2 demonstrates significant improvement in SOD activity in patients under zinc sulphate and folic acid co-administration (58.3 ± 3.3 versus 68.4 ± 2 $P \leq 0.05$) and the group of individuals who were treated with zinc sulphate (63.3 ± 1.9 versus 71.4 ± 1.7 $P \leq 0.05$). Also, no significant differences in blood hormonal concentration and semen antioxidant capability were observed in all four groups during different times of assessment.

Discussion

Varicocele is one of the most commonly identifiable causes of male infertility which results in defective spermatogenesis through mechanisms that are not well understood (Hauser *et al.*, 2001). Disregarding the different hypotheses for altered spermatogenesis, it is commonly accepted that semen quality improves in a majority of patients following varicocele repair. However, pinpointing the underlying mechanisms may open new horizons in the treatment of male infertility with varicocele (Hauser *et al.*, 2001).

Some studies have confirmed the relationship between infertility and the generation of ROS in infertile men with varicocele (Agarwal *et al.*, 2009).

Excess production of ROS or decreased antioxidant defences in the seminal plasma induces OS, which

Table 1 Inhibin B, FSH and testosterone blood plasma level: the mean ± SEM of Inhibin B, FSH and testosterone blood plasma level before varicocelectomy versus three and six month after varicocelectomy treatment following administration of zinc, folic acid or co–administration of them in the different randomized groups

	Group time	Before (mean ± SE)	3 Mount mean ± SE)	6 month (mean ± SE)
Zinc/Folic	Inhibin B (pg/ml)	127.9 ± 56.2	130.4 ± 76.6	190.1 ± 45.1[a]
	FSH (IU/l)	4.2 ± 0.64	3 ± 0.44	4.9 ± 1.08
	Testosterone (ng/ml)	4 ± 0.15	3.8 ± 0.13	3.8 ± 0.16
Folic acid	Inhibin B (pg/ml)	117.1 ± 80.6	123.3 ± 71.2	120.5 ± 57.2
	FSH (IU/l)	3.2 ± 6.2	2.9 ± 0.69	4.3 ± 1.01
	Testosterone (ng/ml)	4.2 ± 0.22	3.9 ± 0.2	3.6 ± 0.21
Zinc	Inhibin B (pg/ml)	98.3 ± 32.9	159.8 ± 73.9	172.8 ± 52.9[a]
	FSH (IU/l)	3.1 ± 0.43	3.9 ± 0.58	4.7 ± 0.52
	Testosterone(ng/ml)	3.9 ± 0.36	3.8 ± 0.16	3.5 ± 0.15
Placebo	Inhibin B (pg/ml)	114.5 ± 48.9	118 ± 70.9	126.3 ± 60.5
	FSH (IU/l)	3.6 ± 0.98	3.3 ± 0.95	5.3 ± 1.64
	Testosterone (ng/ml)	4.0 ± 0.31	4.3 ± 0.44	3.5 ± 0.17

Different superscripts show significant difference ($P < 0.05$) in each row.

Table 2 NO, SOD and TAC semen plasma level: the mean ± SEM of NO, SOD and TAC semen plasma level before varicocelectomy versus three and six month after varicocelectomy treatment following administration of zinc, folic acid or co–administration of them in the different randomized trial groups.

Groups	Sequence	Before	3 Months	6 Months
	NO μm	56.6 ± 5.6	57 ± 9.1	48.8 ± 7.5
Zinc/Folic	SOD% Inhibition	58.3 ± 3.3	70.1 ± 1.3[a]	68.4 ± 2[a]
	TAC μm	1.1 ± 0.29	1.2 ± 0.33	1.2 ± 0.27
	NO μm	60.2 ± 6.4	55.9 ± 6.5	56.1 ± 8.7
Folic acid	SOD% Inhibition	62.3 ± 3.2	68.6 ± 1.7	69 ± 1.7
	TAC μm	1.2 ± 0.31	1.1 ± 0.52	1.2 ± 0.47
	NO μm	56.6 ± 7.2	51 ± 4.9	54.7 ± 4.3
Zinc	SOD% Inhibition	63.3 ± 1.9	72.4 ± 1.5[a]	71.4 ± 1.7[a]
	TAC μm	1.2 ± 0.39	1.1 ± 0.64	1.2 ± 0.32
	NO μm	59.1 ± 9.5	58.9 ± 9.0	59.3 ± 5.7
Placebo	SOD% Inhibition	63.3 ± 2.6	71.9 ± 1.5	69.8 ± 1.2
	TAC μm	1.2 ± 0.33	1.2 ± 0.62	1.1.47

Different superscripts show significant difference ($P < 0.05$) in each rows.

impairs spermatogenesis by affecting sperm plasma membrane and the genomic and mitochondrial spermatozoa DNA, causing fragmentation and base degradation. This may lead to molecular or genetic changes that are responsible for several different pathologic features, including infertility (Köksal *et al.*, 2000; Chen *et al.*, 2004).

The changes in the testicular microenvironment and haemodynamics can increase the generation of ROS and/or decrease the local antioxidant capacity, resulting in OS and effects of increased OS in the serum, semen, and testicular tissues of varicocele patients have been discussed in numerous studies (French *et al.*, 2008).

Unlike these studies showing OS as an important pathophysiologic factor that causes impairment of sperm parameters in varicocele, the exact aetiology of OS elevation in association with varicoceles is unclear. This could be due to various compensatory mechanisms in these patients that help to maintain spermatogenesis (Benoff *et al.*, 2004; Nallella *et al.*, 2004; Cocuzza *et al.*, 2008).

Locally produced NO is involved in the regulation of testicular vasculature. NO is synthesised from L-arginine by the catalytic activity of three different isoforms of nitric oxide synthase: constitutive neuronal, endothelial and inducible NO synthase (NOS). Endothelial NOS forms are normally expressed in Leydig cells and testicular vasculature. In varicocele testes, the expression of inducible NOS is upregulated to maintain testicular arterial blood flow as a compensatory mechanism to increase blood flow resulting from the hypoxia induced by venous stasis, which may be detrimental to spermatogenesis (Türkyilmaz *et al.*, 2004).

In some adolescent patients with varicocele, increased malondialdehyde (MDA) levels occurred together with elevated NO levels, indicating excessive lipid peroxidation (Romeo *et al.*, 2003).

There may be a causal relationship between elevated intratesticular temperatures with increased apoptosis in varicocele due to increased NO levels (similar to that seen in cryptorchidism). Studies have also demonstrated a role for NO in heat-induced apoptosis (Agarwal *et al.*, 2009).

A more recent study showed a significant increase in the levels of oxidants (MDA and NO) and reduced levels of enzymatic (CAT, SOD, GPX) and nonenzymatic (ascorbic acid) antioxidants in the semen of infertile men with varicocele (Abd-Elmoaty *et al.*, 2010).

Similar results have been confirmed by Mostafa *et al.* (2009) who compared MDA, hydrogen peroxide and five antioxidants (CAT, SOD, GPX, and vitamins C and E) in fertile and infertile men with and without varicocele. On the basis of these results, it was concluded that varicocele is associated with OS even in fertile normozoospermic men (Mostafa *et al.*, 2009; Abd-Elmoaty *et al.*, 2010).

A meta-analysis of four studies published until 2006 showed that patients with varicocele had significantly greater ROS and lower total antioxidant capacity (TAC) levels (Agarwal *et al.*, 2009).

Despite the above-mentioned findings, the role of OS and semen antioxidants in the aetiology of varicocele associated infertility is not without controversy.

As some of the studies have reported similar OS levels in fertile men with varicoceles and infertile men with varicoceles and some others observed no significant difference in ROS levels between fertile men with and without varicocele and a lack of any correlation of ROS level with varicocele grade (Weese *et al.*, 1993; Hendin *et al.*, 1999). In view of these findings, it appears surgical treatment does not seem to modify absolute values of TAC (Mancini *et al.*, 2004).

Our study is in agreement with those indicating no significant alteration in NO, SOD and TAC post-varicocelectomy.

Inhibin B has been shown to be an important factor related to testicular hormonal function, and several reports have been made suggesting correlation between inhibin B and FSH or testosterone (Fujisawa *et al.*, 2001). The present findings are consistent with those of earlier studies performed on infertile patients with varicocele which observed no significant increase in serum mean concentration of inhibin B and other hormones after varicocelectomy (Fujisawa *et al.*, 2001).

There are evidences showing that folate is important for the synthesis of DNA, transfer of RNA and the amino acids cysteine and methionine. DNA synthesis plays an important role in germ cell development, and thus in reproduction. It has also been reported that folic acid, the synthetic form of folate, effectively scavenges oxidising free radicals and as such can be regarded as an antioxidant (Joshi *et al.*, 2001; Ebisch *et al.*, 2006).

Despite its water-soluble character, folic acid inhibits lipid peroxidation (LPO). Therefore, folic acid can protect bio-constituents such as cellular membranes or

DNA from free radical damage (Joshi *et al.*, 2001). Only limited knowledge is available on the impact of dietary folate and synthetic folic acid on subfertility.

Besides, zinc serves as a cofactor for more than 80 metalloenzymes involved in DNA transcription and protein synthesis. Because DNA transcription is a major part of germ cell development, zinc is also likely to be important for reproduction. Moreover, it has antiapoptotic and antioxidant properties and reacts with SOD as cofactor (Zago & Oteiza, 2001; Chimienti *et al.*, 2003).

In spite of zinc and folate administration benefits on male fertility, no considerable impact of zinc and folate intervention was reported on endocrine parameters in the study by Ebisch *et al.* (2006), whereas in our study, a major rise of inhibin B was observed following 6 months of treatment in 2 groups receiving zinc sulphate alone and zinc combined with folic acid, respectively. However, no significant result was found for the group treated with folic acid alone.

There were significant alterations in the SOD level in 2 groups of individuals treated with zinc sulphate alone and zinc plus folic acid. Significant alterations in SOD and inhibin B levels in the two groups receiving zinc alone and zinc plus folate, respectively, and also lack of any significant differences for these two factors in the group treated with only folate, implicate a role for zinc in these consequences.

Conclusion

While beneficial effects of zinc sulphate administration on endocrine parameters and seminal antioxidant condition after varicocelectomy were found, more studies on the effects of zinc sulphate and folic acid administration in male fertility are needed.

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