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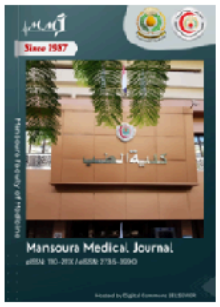

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ACCUMULATION OF CADMIUM AND IRON IN THE HYDROCELE FLUID OF INFERTILE MEN WITH PRIMARY VARICOCELE; A POSSIBLE ROLE IN MALE INFERTILITY

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ACCUMULATION OF CADMIUM AND IRON IN THE HYDROCELE FLUID OF INFERTILE MEN WITH PRIMARY VARICOCELE; A POSSIBLE ROLE IN MALE INFERTILITY

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ABSTRACT

Varicoceles are a common cause of male infertility, but despite data from human studies the pathophysiology remains unclear. Seminal plasma cadmium concentrations were found to be increased in infertile men. In addition, increases in blood plasma cadmium concentrations in infertile men have been associated with teratozoospermia. Cadmium contributes to infertility by induction of apoptosis in testicular tissue. Methods: An ejaculate and blood sample were collected immediately before surgery followed by aspiration of hydrocele fluid from the tunica vaginalis at the time of subinguinal varicocelectomy. In each specimen, cadmium and iron levels were determined by atomic absorption and the effect of hydrocele fluid on the sperm velocity was determined by examining aliquots of sperms suspended in hydrocele fluid compared to those suspended in seminal plasma. Results: The cadmium and iron levels were higher in the hydrocele fluid than the peripheral blood in 72% and 46% of patients with bilateral varicocele respectively. Hydrocele fluid added to the sperms, initially increased the motility for 10 to

15 minutes then the velocity diminished gradually and eventually all the sperms became immotile in 30 minutes.

Conclusions : 1- The hydrocele fluid in patients with varicocele has a deleterious effect on the sperm vitality. 2- The increased cadmium and iron concentrations in the hydrocele fluid are probably derived from the increased transvascular fluid exchange which occurs with varicocele. 3- These metal ions may diffuse from the hydrocele fluid to the sperms during their maturation in the epididymis and induce their acrosomal insufficiency effect. 4- Impregnation rate was higher after surgical correction of varicocele and removal of hydrocele fluid in those patients who presented with high preoperative levels of cadmium and iron

INTRODUCTION

The possible influence of the trace elements, especially iron, cadmium, copper and zinc, on male infertility is a matter of great interest. Increased levels of metal ions in human semen appear to be significantly and positively correlated with male infertility, suggesting that trace elements in human seminal plasma are important factors in the male reproductive function [1]. There was a significant positive correlation between sperm density and the concentration of zinc in seminal plasma in normospermic subjects [2]. Similarly, other authors also concluded that zinc concentration in semen decreased with decreasing numbers and motility of spermatozoa [3]. There are, however, reports in which no significant correlation between zinc concentration in seminal fluid and sperm density or motility was found [4,5]. Cadmium (Cd) is associated with a putative effect on human sperm, causing impairment of fertility [6]. When blood Cd is above 1.5 mg/L, there is significant reduction in sperm density, suggesting a possible threshold effect of Cd on spermatogenesis [2]. Mechanisms of the putative effect of Cd on sperm include inhibition of activities of certain key enzymes of sperm carbohydrate metabolism, androgen biosynthesis and metabolism and effects on T and B cells [7]. Mainly induction of apoptosis of T cells [8]. Seminal plasma Cd concentrations were found to be increased in infertile, compared with

fertile men [9]. In addition, increases in blood plasma Cd concentrations in infertile men have been associated with teratozoospermia and an inverse correlation between blood plasma Cd/seminal plasma Cd and sperm density and semen volume has been reported [2,10]. In contrast, in other studies, it has been reported that Cd exposures have not been associated with a significant reduction in semen quality [11]. or with a decreased probability of a live birth [12]. Metal-ion toxicity could result from the formation of hydroxyl free radicals from hydrogen peroxide and superoxide ion radicals via the Fenton and Habber-Weiss reactions as in the case of iron [13]. Varicocele-associated infertility is widespread as up to 40%of males from infertile couples present with varicocele [14]. The mechanisms underlying varicocele-associated infertility, however, are poorly characterized. The most widely accepted explanation for the pathophysiology of varicocele in male infertility is abnormally elevated testicular temperature due to impaired heat transfer by the scrotum and/or changes in testicular blood flow [15]. The fact that only 13% of men with varicocele are infertile [16] and although varicocele repair has been documented to reduce testicular temperature, only 1/3 of infertile men with varicocele will experience a return of fecundity following varicocele correction [14]. These findings suggest that varicocele may not be a primary cause of infertility and that it is the interaction of varicocele with other as yet unidentified factors that produces the infertile state. We suggest that one such factor is the concentration of cadmium and iron in the male reproductive tract. Considering that the studies conducted so far are mainly focused on the heavy metal content in the seminal plasma and its correlation with semen quality and considering that seminal plasma may not be the most appropriate fluid to be used as an elemental indicator of male infertility [17]. The present study proposes to measure the trace elements namely, cadmium and iron, in the blood plasma and hydrocele fluid in patients suffering from varicocele associated infertility as well as elucidating the effect of direct exposure of the sperms to hydrocele fluid.

MATERIALS AND METHODS

The study group consisted of 45

infertile males, selected from patients scheduled for varicocelectomy at Mansoura International and Mansoura Insurance Hospitals during the period between 2000 to 2004. The age of these patients ranged from 22 to 35 (mean 29 ± 2.4) and all of them had been married for more than 2 years. Men selected for the study were not occupationally exposed to heavy metals and had no genitourinary infections. Participants collected the semen samples by masturbation immediately preoperative after 2-5 days of abstinence into sterile glass vials. A questionnaire pertaining to age, occupation, marital status and reproductive history was prepared. Semen analysis was performed following WHO guidelines, 1999. Two aliquots of sperms were prepared from each specimen to be examined for sperm density and percentage motility with a Makler chamber.

OPERATIVE TECHNIQUE

All subjects subsequently chosen for study presented with grade II (audible by Doppler ultrasonography, palpable, not visible) or grade III varicocele (audible, palpable, visible). Subjects with grade I varicocele ('subclinical'; audible, not visible, not palpable) were excluded from the study population as there are significant questions remaining unanswered concerning the benefit of varicocele repair in this group. Two centimeters skin crease incision is made in the groin, 2 finger breadth above the pubic tubercle. The spermatic cord is picked up at its emergence of the external ring (Fig.1). The fascial covering of the cord is opened and the pampiniform plexus of veins as well as the cremasteric plexuses are identified and separated from the vasal plexus that covers the vas deferens. The upper pole of the testis is delivered into the groin incision by gentle traction on the spermatic cord assisted by scrotal upward pushing until the insertion of the cremasteric muscle into the tunica vaginalis is visualized. The hydrocele fluid in the tunica vaginalis is aspirated, and then the tunica is opened and everted behind the testis (Fig.2). The pampiniform and cremasteric veins are ligated and divided, carefully preserving the accompanying arteries. The testis is replaced into the scrotum and the wound is closed in a single continuous layer. The semen results were

averaged for each patient and a single value was computed for each parameter, 0.5 ml of the hydrocele fluid is mixed with 0.5 ml of sperm suspension on a glass slide and examined with a phase-contrast microscope every 5 minutes for a total of 30 minutes.

BIOCHEMICAL ANALYSIS

Approximately 2 ml of peripheral blood and 2ml of hydrocele fluid were collected from each patient. The sample was digested twice with 5ml of an acid mixture (HNO_3: HCLO4, 6:1) in a glass tube to almost dryness. The residue was finally dissolved in 1ml of 1% of HNO_3 and applied on the graphite tube atomizer for detection of cadmium and iron by atomic absorption spectrophotometer. The recovery of cadmium and iron in the samples was observed to be 96% and 97%, respectively. The instrument was calibrated using different standards for iron and cadmium. The precision was calculated to be 1.2 and 1.09 and the accuracy was 10% and 15% for iron and cadmium, respectively. A sample blank was also prepared with each set of samples in order to control for possible metal contamination by external source.

STATISTICAL ANALYSIS

Data were expressed as Mean ± SD. The statistical analyses were performed using linear regression and Student's t-test. A probability value of $P<0.05$ was considered to be significant.

RESULTS

As suggested in the WHO guidelines (18), the semen specimens were divided into four groups: normospermia, oligospermia, asthenospermia, and oligoasthenospermia. Table 1 shows the semen characteristics of all the examined groups. None of the patients was normospermic. In all cases, no significant differences in semen volume were observed. The sperm density in oligospermia and oligoasthenospermia was significantly lower than in asthenospermia ($P < 0.05$). The sperm motility was significantly higher in the oligaspermic than in the asthenoor oligoasthenospermic groups ($P<0.05$).

Table (2) shows the effect of addition of hydrocele fluid to the sperms.

There was a significant increase in the sperm velocity and linear velocity in the three groups which remained for 15 minutes in the oligospermic group, for 10 minutes in the athenospermic, and for 7 minutes in the oligoasthenospermic group. Afterwards, the sperm motility gradually diminished and all the sperms became immotile in 30 minutes. The most remarkable effect of hydrocele fluid on the sperm motility was observed in the oligoasthonospermia (almost 3 fold increase of motility) (Fig3).

The blood plasma and hydrocele fluid concentration of cadmium and iron are shown in Table 3. The concentration of cadmium is significantly higher in the hydrocele fluid of all groups than in the plasma ($P<0.01$). The concentration of iron in asthenospermia are significantly higher than in oligospermia ($P<0.05$). There was a significant correlation between the Cd concentration in the hydrocele fluid and the increased sperm velocity in the oligoasthenospermic group ($p<0.05$) No significant correlations were observed between the iron concentration in the hydrocele fluid and plasma.

Table (1): Semen characteristics of the study groups (Mean±SD)

Subjects	Volume (ml)	Sperm density ($x10^6$/ml)	Motility (%)	Normally formed sperms (%)
Oligospermia (n=15)	3.8±1.1	9.3±6.2	35.2±9.3	62±15
Asthenospermia (n=15)	3.3±1.0	72±31	27.2±7.7	73±11
Oligoasthenospermia (n=15)	3.4±1.2	11.2±7.3	32.0±5.2	57±12

Table (2): The effect of addition of hydrocele fluid (HF) on sperm velocity

Subjects	Before HF addition		After HF addition	
	Velocity*	Linear velocity**	Velocity	Linear velocity
Oligospermia	56.3±6.2	36.6±4.1	74.3±11.0	47.1±6.2
Asthenospermia	21.2±2.2	18.6±3.7	43.3±6.4	36.8±2.2
Oligoasthenospermia	24.4±6.5	17.2±5.3	66.3±3.0	40.6±6.1

Velocity (n>26μm / sec)

Linear velocity (n= 22 μm / sec)

Table (3): Concentrations of cadmium and iron in hydrocele fluid and plasma of infertile men (mean±SD)

Subjects	Plasma		Hydrocele Fluid	
	Cd	Fe	Cd	Fe
Oligospermia	0.52±0.6	170±42	1.23±0.4	164±22
Asthenospermia	0.63±0.2	185±36	1.2±0.7	193±24
Oligoasthenospermia	0.58±0.2	179.2±38	1.45±0.3	181.4±23

Cd = Cadmium μg / L

Fe = Iron μg / dl

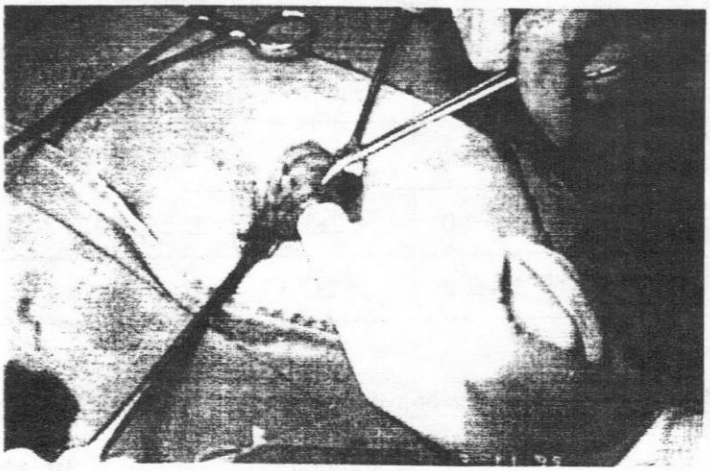

Fig 1. Delivery of the spermatic cord out of the incision .

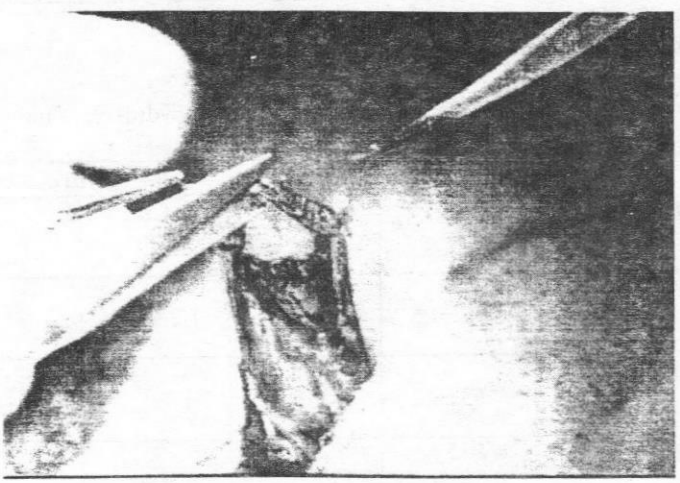

Fig 2. Isolation and division of the pampiniform plexus of veins.

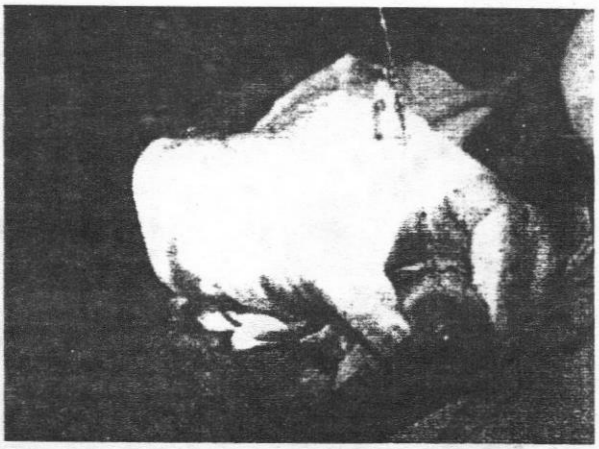

Fig (2-a) The hydrocele fluid in the tunica is aspirated

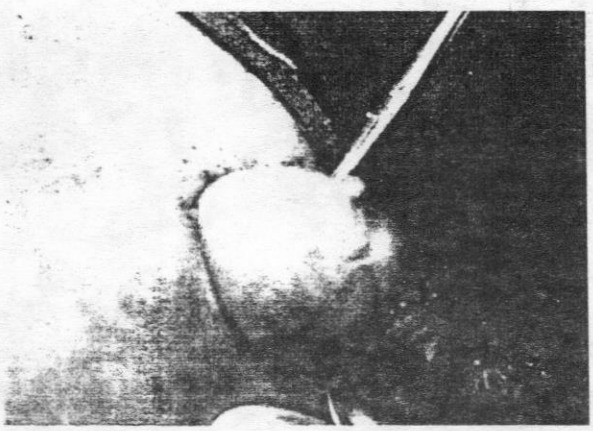

Fig (2-b) Tunica is opened and everted behind the testis.

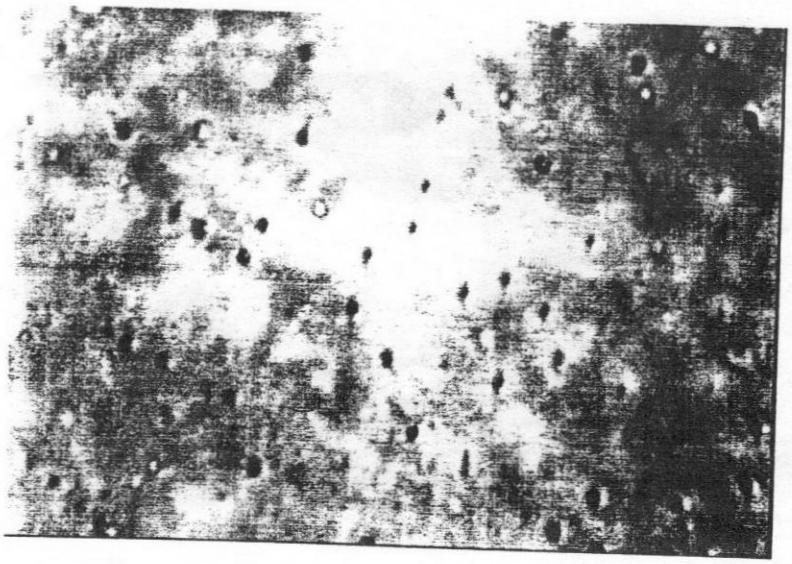

Fig (3-a) Sperm motility before thes addition of hydrocele fluid.

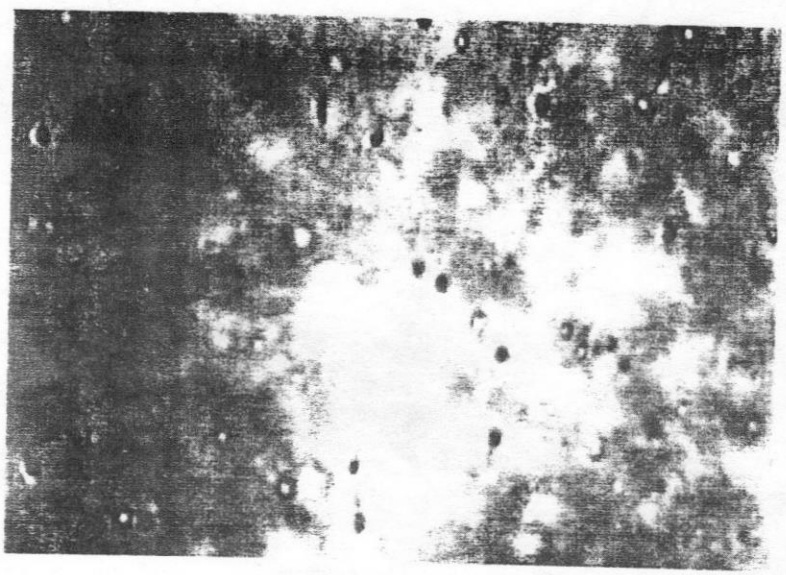

Fig (3-b) Decreased sperm motility after addition of hydrocele fluid.

DISCUSSION

The prevalence of infertility has increased from 8 to 15% over the past 2 decades in industrialized countries [19]. To evaluate the roles of trace elements on the male reproductive function, the most commonly used specimens are blood and seminal plasma. Measurement of metal concentrations in seminal plasma offers a better opportunity to understand the relationships between a given element and sperm quality [2, 5]. The concentration of Cd in seminal plasma from infertile men with varicocele is markedly increased compared with fertile men with varicocele or men with other forms of infertility. Cadmium concentrations are similarly increased in the testis of infertile men with varicocele. After varicocele repair, pregnancy by coitus was observed only in those men whose Cd concentrations were at the lower end of the range [6]. Cadmium has been suggested as a toxicant that leads to apoptosis [20]. Moreover, a significant correlation was found between Cd content and the number of apoptotic cells within the seminiferous tubules. As Cd accumulation may disrupt the actin cytoskeleton, which in fertile men contributes to shaping of the acrosome around the sperm nucleus [21]. Cadmium in sperm appears to act as an effector that mechanistically accounts simultaneously for the oligospermia that accompanies varicocele, the 'stress' sperm morphology seen in varicocele [22] and the acrosome reaction insufficiency typical among men with varicocele.

The observation that Cd concentration in the hydrocele fluid is higher than that in plasma raises the possibility that this may be the simple result of an increase in local venous blood pressure in the pampiniform plexus leading to progressive filtration of Cd out of the blood into the hydrocele fluid. Support for this comes from the fact that Cd itself alters the permeability of vascular endothelium [23] producing edema [24], rendering the blood-testis barrier more porous, and rendering the hydrocele fluid in the tunica able to accumulate Cd more rapidly. Benoff et al [6] have also reported that blood plasma Cd levels are in the normal range in infertile patients with varicocele irrespective of whether on not reproductive tract Cd concentration are elevated.

Therefore, the high local venous pressure in the testis affected by varicocele must be responsible for Cd accumulation in the tunica vaginalis of these patients. The presence of Cd-rich hydrocele fluid around the testis and epididymis may provide a route of entry for toxic metal to the seminiferous tubules and sperms. Histological studies of adolescent varicocele showed actin loss by cells near the basement membrane of seminiferous tubules, overcome of the blood-testis barrier [25] and damage of the seminiferous endothelium [26]. Once accumulated, the Cd itself can alter the properties of the cell membranes by altering calcium ion channels. The pore region of these channels results from alternate splicing and microdeletions in the pore region may lead to less selective ion channels which would permit Cd to pass more easily through cell membranes into the cytoplasm of germ cell lineage [27].

Examination of testis biopsies from infertile men with varicocele showed that actin immunoreactivity was decreased and apoptosis, which normally occurs at low concentrations during spermatogenesis, was increased compared with biopsies from men without varicocele [28]. Increased Cd concentrations in the reproductive tract are probably derived from the increased transvascular fluid exchange which occurs with varicocele [29].

One may argue that other studies have failed to find any significant difference in seminal plasma Cd concentrations of fertile and infertile men [30]. There are two possible explanations for this. Firstly, measurements of metal ion concentrations in seminal plasma may be of less significance than direct measurements of metal ion concentrations in spermatozoa themselves. It has been reported that sperm motility was inversely correlated with aluminium in spermatozoa but not in seminal plasma [31]. Secondly, some factors associated with varicocele and Cd, possibly iron or other metal ion, must interact to regulate apoptosis.

Oxidative stress induced by reactive oxygen species (ROS) has been proposed as one of the major causes of human male infertility [32]. The importance of seminal plasma antioxi-

dants in the protection of spermatozoa against oxidative stress is increasingly recognized. The outstanding role of ascorbic acid has received particular attention [33], as a reducing agent it is able to reduce catalytically active transion metal ions such as iron, thereby accelerating vitamin consumption and promoting metal-induced oxidative damage [34].

In extracellular and intracellular compartments, the binding of iron ions to specific proteins prevents any form of metal-dependent catalysis, including ascorbate consumption. However, in the presence of ROS, iron can be released from binding proteins, inducing reactive radical species and ascorbate oxidation [35]. Iron was suggested to induce lipid peroxidation and inhibit sperm motility as evidenced by significant inhibition of sperm motility associated with marked rise in malondialdhyde levels in the semen incubated with iron [36]. However pre-existing membrane lipid peroxides were not detected in spermatozoa. It is therefore suggested that lipid peroxidation takes place only after radical oxygen stress has exhausted the cellular defense system. Lipid peroxidation is not the initial, but one of the later, events leading to the death of spermatozoa [37].

Human spermatozoa are rich in polysaturated fatty acids, so they may be highly susceptible to lipid peroxidation. This phenomenon was found to be related to mid-piece sperm abnormality and motility. Although there are reports [38] that lipid peroxide concentration in seminal plasma showed no relationship with sperm concentration, sperm motility or the number of immotile spermatozoa. However other studies [39] are contradictory and reported inhibited motility of sperms by lipid peroxidation.

It seems that cord congestion as a result of varicocele leads to impaired venous drainage of the testicle with a consequent testicular congestion and accumulation of toxic metal ions as Cd and iron in the hydrocele fluid that could be responsible for inhibiting the spermatogenic function of the testicle by several possible mechanisms. The presence of high Cd concentration in the hydrocele fluid surrounding the epididymis in which maturation of the sperms takes place together with the

high temperature provided by the varicocele and the persistence of this elevated temperature by the insulating action of the hydrocele fluid. The combined effect of high temperature and high Cd concentration may change sperm protein expression (e.g. loss of head actin, reduced percentages of spermatozoa expressing surface mannose receptors) and function (e.g. acrosome reaction insufficiency). In-vitro experiments support our postulation, where fertile donor spermatozoa exposed to high temperature and high Cd concentration produced sperm function changes more than those produced by high Cd concentration alone [6], indicating that temperature and Cd act synergistically. The lipid peroxidation effect of iron on the sperms leads to decreased sperm motility. The observation of deceased sperm motility after addition of the hydrocele fluid to the sperms in the present study supports this possibility. Furthermore, the record of positive correlation between increased iron, decreased zinc and decreased sperm motility (39) is additional support. Dawra et al [40] observed complete inhibition of lipid peroxide formation in the spermatozoa by addition of seminal plasma to incubation mixture which could be the result counteraction of zinc in the seminal plasma.

Although varicocele surgery has been used in clinical practice for decades, these procedures have been the source of some controversy and discussion. A large body of literature suggests improved semen parameters and fertility following varicocelectomy [14] but some investigation have challenged the benefit of these procedures [41]. We had previously reported [42] that varicocelectomy together with removal of the hydrocele fluid and evertion of the tunica was associated with higher impregnation rate than varicocelectomy alone and assumed a possible role of toxic materials in the hydrocele fluid that could be responsible for persistence of infertility despite varicocelectomy.

CONCLUSION

In the present study we emphasize on the role of hydrocele fluid and the metal ions accumulated in it as a potential factor of male infertility. Therefore, we recommend that all varicocelectomy operations should be

completed by removal of the hydrocele fluid and evertion of the tunica.

If preoperative Cd and iron levels in the hydrocele fluid could be tested, there may be more appropriate patient selection for varicocelectomy. In fact, investigators might utilize these screening techniques for constructing prospective randomized trials to include patients that may truly benefit from this type of surgery and avoid procedures on those who have little or no chance of success. Assisted reproduction or IVF/ICSI may be utilized for those identifiable patients who would not benefit from surgery.

In the future, percutaneous aspiration of hydrocele fluid and identification of Cd and iron levels, may add to the selectivity for surgery. These procedures may be performed in an office setting with local anaesthesia. Where patients are selected for surgery on the basis of these markers, the results may present a new challenge to those reports that categorically state that varicocelectomy is of no benefit.

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