Beneficial effects of microsurgical varicocelectomy on sperm DNA fragmentation, distribution of nuclear sulfhydryl groups and sperm maturation: a prospective trial

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ABSTRACT

Background: There is evidence to show that varicocele repair can improve conventional sperm parameters and sperm DNA integrity in infertile men with a clinical varicocele.

Objective: To further examine the effect of varicocelectomy on sperm quality, specifically, sperm nuclear chromatin integrity, distribution of nuclear sulfhydryl groups and sperm maturation.

Design, Setting and Participants: We prospectively evaluated a consecutive series of infertile men (n=29) presenting to Ovo clinic with one year or more history of infertility, a clinically palpable varicocele and abnormal semen parameters. Six sperm donors with normal sperm parameters served as controls.

Surgical Procedure: Microsurgical sub-inguinal varicocelectomy.

Outcome Measures: (1) Conventional sperm parameters, (2) aniline blue staining (AB is specific to histone lysines), (3) iodoacetamide fluorescein (IAF targets free protamine sulfhydryl groups) and (4) sperm chromatin structure assay (SCSA) with the results expressed as % DNA fragmentation index (%DFI) and percent high DNA stainability (%HDS) before and 4 months after microsurgical varicocelectomy.

Results: The sperm %DFI, %HDS (a measure of chromatin compaction), % 5-IAF staining (diffuse head staining), % AB staining (dark blue) were all significantly lower in the control group compared to infertile men with varicocele (8 vs. 20%, 4.0 vs. 9.6%, 1.7 vs. 16.3%, and 2.5 vs. 13.5% respectively). The %5-IAF and %AB staining decreased significantly after surgery (from 16.3 to 5.4%, and from 13.5% to 5.4%, respectively). Similarly, the %HDS and %DFI also decreased significantly after surgery (from 10% to 6% and from 20% to 13%, respectively). The only notable relationships were between aniline blue staining and %HDS post varicocelectomy ($r= 0.57$, $P < 0.05$), and both %IAF staining and %DFI were inversely correlated with motility (r=-0.44 and -0.43, respectively).

Conclusion: The data show that varicocelectomy is associated with a consistent improvement in sperm DNA integrity and chromatin compaction using three different assays of sperm chromatin integrity (SCSA, IAF, Aniline Blue).

RÉSUMÉ

Contexte: Il y a la preuve(l'évidence) pour montrer que la réparation de varicocele peut améliorer des paramètres de sperme conventionnels et l'intégrité d'ADN de sperme dans des hommes infertiles avec varicocele clinique..

Objectif: Examiner l'effet de varicocelectomy sur la qualité de sperme, spécifiquement, le sperme l'intégrité chromatin nucléaire, la distribution de groupes sulfhydryl nucléaires et la maturation de sperme.

Schéma, environnement et participants : Nous avons éventuellement évalué une série consécutive d'hommes infertiles (n=29) présentant à la clinique Ovo avec un an ou plus d'histoire d'infertilité, varicocele cliniquement palpable et des paramètres de sperme anormaux. Six donneurs de sperme avec des paramètres de sperme normaux ont servi de contrôles.

Intervention chirurgicale: Microchirurgie sous-inguinale varicocelectomie.

Mesures des résultats: (1) Des paramètres de sperme conventionnels, (2) l'aniline bleu teintant(tachant) (d'AB est spécifique à histone lysines), (3) iodoacetamide fluorescein (libère protamine sulfhydryl des groupes) et (4) le sperme chromatin l'essai de structure (SCSA) avec les résultats (DFI) de fragmentation d'ADN de % et le pour cent haut ADN stainability (%HDS) auparavant et 4 mois après varicocelectomy microchirurgical

Résultats: Le pourcentage de spermatozoïdes avec le sperme de DFI%, HDS%, positif de 5-IAF coloration, une coloration positive AB (bleu foncé) étaient significativement inférieure dans le groupe témoin par rapport aux hommes

infertiles ayant une varicocèle (8 vs 20%, 4,0 vs 9,6%, 1,7 vs 16,3%, et 13,5 vs 2,5% respectivement). Le pourcentage de spermatozoïdes avec positifs 5-IAF coloration et AB nucléaire positif diminué de façon significative après la chirurgie (de 16,3 à 5,4%, et de 13,5% à 5,4%, respectivement). Les HDS% et DFI% également diminué de façon significative après la chirurgie (de 10% à 6% et de 20% à 13%, respectivement). Les seules relations entre les notables étaient coloration au bleu d'aniline et varicocélectomie après HDS ($r = 0,57$, $P < 0,05$), et les deux taches IAF et DFI% ont été inversement corrélée avec la motilité ($r = -0,44$ et de $-0,43$, respectivement).

Conclusion: Les données montrent que varicocélectomie est associée à une amélioration constante de l'intégrité d'ADN de sperme et compaction de la chromatine en utilisant trois différents dosages cytochimiques de l'intégrité chromatine des spermatozoïdes (SCSA, l'IAF, le bleu d'aniline).

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A-Introduction

Infertility is defined as the inability of a couple to achieve a natural pregnancy after one year or more of unprotected intercourse. Infertility is a very common condition where 10-20% of the population is considered infertile [1, 2]. This prevalence can be as high as 30% in some rural communities [3]. Infertility is classified into primary, those who never fathered a child, or secondary, those who fathered a child before but unable to conceive again despite unprotected sex. Male factor infertility can be found in 40-50% of couples that present for workup of infertility [4]. The causes of male factor infertility are many, and they can be classified generally into pre-testicular, like endocrinopathy; intra-testicular, like varicocele; and post-testicular, like ejaculatory duct obstruction.

Varicocele (from the Latin word Varix "dilated vein" and Greek kele "tumor") has been described a long time ago and is defined as dilatation of veins in pampiniform plexus with the presence of reflux. Despite the correct description of varicocele "dilated and twisted vein over the testicles" nearly 2000 year ago by Roman writer Celsius, an association between varicocele and male-factor infertility was not identified until the nineteenth century at which time the first report of improvement in semen parameters after correction of bilateral varicocele was noted [5]. It is the most commonly identified factor in men with male factor infertility and it is more prevalent on the left side. A varicocele is identified in approximately 40% of men with primary infertility, whereas the prevalence can be as high as 80% in men with secondary infertility [6]. Only one out of every five infertile varicocele patients seeks treatment for infertility [7]. The mechanisms of

varicocele-induced pathology and the pathophysiology of varicocele will be discussed in depth at later sections.

The human sperm chromatin is highly compact and consists of nuclear DNA and associated nucleoproteins. This condensed chromatin is crucial for protection of paternal genome from external environment during sperm transit through the male and female genital tracts. Indeed, sperm quality used to be defined by common parameters that are measured by any standard sperm analysis, namely sperm count, motility and morphology. However, alterations in human sperm genome are not normally detected by such standard WHO criteria. Hence, aberrant chromatin packing and sperm DNA damage maybe present in otherwise normal semen sample examined by traditional sperm testing. More important, abnormalities in human sperm genome have been shown to lead to post-fertilization failure [8, 9].

There is a good body of literature to show that spermatozoa of infertile men possess more chromatin defects than spermatozoa of fertile men, which has led some investigators to suggest that sperm DNA damage may be predictive of male fertility potential [10-12]. The etiology of sperm DNA damage is multi-factorial and the molecular factors believed to cause sperm DNA damage are oxidative stress, aberrant chromatin remodeling (compaction) and abortive apoptosis [13-16]. Of special interest, is the association between clinical (palpable) varicocele and the presence of sperm DNA damage. Several investigators have examined this relationship and showed a negative effect of

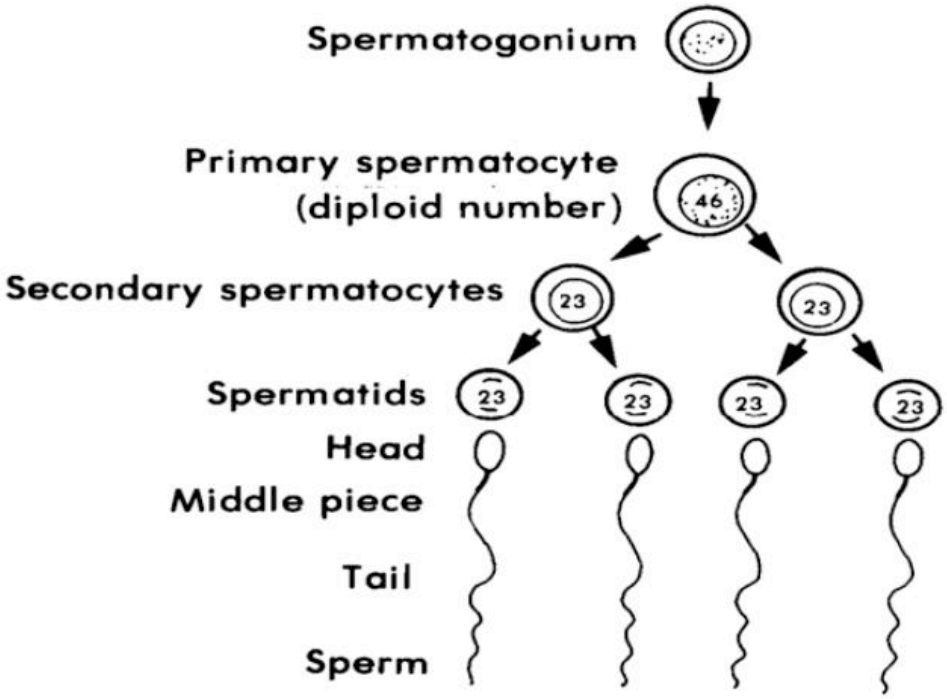
varicocele on sperm DNA integrity and fertility potential [17-21]. The relationship between varicocele, oxidative stress and sperm DNA damage will be explained in greater detail later in this thesis.

B- Review of literature

B1: Spermatogenesis

Spermatogenesis is the process of production and development of spermatozoa from male primordial germ cells. In humans, spermatogenesis starts with relatively undifferentiated diploid stem cells that undergo reductive divisions (meiosis) to produce highly specialized haploid sperm cells (Figure 1).

Figure B1: Stages of spermatogenesis



The main goal of spermatogenesis is the production of a genetically unique male gamete that is able to fertilize an ovum and generate offspring. While a complete cycle of sperm production is lengthy and can take up to 64 days, it is efficient and approximately 70 million spermatozoa are produced daily [22, 23]. It is crucial to have an intact hypothalamo-pituitary-gonadal axis in order to complete the process of spermatogenesis. This usually starts with the release of gonadotropin-releasing hormone from the hypothalamus, which in turn stimulates the secretion of follicular stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary gland. FSH interacts with Sertoli cells in the gonads which provides support and nutrition necessary for sperm development, while LH, on the other hand, stimulates Leydig cells to produce testosterone that is important for maintenance of spermatogenesis [24].

The last stage of sperm production is the called spermiogenesis. This stage is highly relevant to the hypothesis of this thesis project as human sperm chromatin undergoes progressive condensation mainly at this part of spermatogenesis. Chromatin condensation (compaction) is secondary to replacement of nuclear histones by transition proteins, and finally, by protamines. The protamine-DNA complex is very tightly compacted in part due to the numerous inter- and intra-protamine disulfide bonds [24]. Spermiogenesis takes place at post-meiotic phase and results in a mature gamete with highly compacted chromatin to protect paternal genome during transit of spermatozoa through male and female reproductive tracts. Varicocele repair is believed, in part, to improve

sperm quality through spermiogenesis to produce mature and chromatin compacted spermatozoa.

B 2- Causes of male-factor infertility

There are many factors that can influence male fertility potential. These causes are either divided according to sperm analysis, i.e. Oligospermia, teratospermia and azoospermia, or according to the location of insult in relation to the testicles, i.e. pre-testicular vs. post-testicular. The following is list of the main causes of male-factor infertility stratified by location in relation to the testicle:

Pre testicular:

- Hormonal abnormalities, hypogonadotropic hypogonadism
- Advanced age
- Poor nutrition
- Medications (especially, hormonal agents)
- Bad social habits like smoking
- Obesity

Testicular (sperm production):

- Genetic defects (e.g. chromosome abnormality)
- Varicocele
- Trauma
- Testicular tumor (e.g. Seminoma)
- Gonadotoxic drugs, e.g. Chemotherapy
- Cryptorchidism
- Radiation therapy
- Mumps orchitis
- Medications
- Testicular hyperthermia (e.g. occupational)
- Idiopathic

Post testicular (obstruction)

- Ejaculatory duct obstruction
- Congenital bilateral absence of vas deference
- Retrograde ejaculation
- Infections (e.g. epididymitis)
- Urethral strictures

Potential causes of male factor infertility are many and require a thorough evaluation to identify possible confounding factors. The key to diagnosis and

management is complete medical history taking and physical examination. In general, some factors can be modified, for example obesity, smoking, alcohol consumption, some medications. On the other hand, significant portion of infertile men have irreversible causes like genetic abnormalities. The main objective of identifying reversible cause is to try to modify them and increase the male fertility potential, in contrast, the objective of recognizing genetic causes is to council the couple about risk of transmission of certain genetic anomalies to the child, either naturally or through the use of assisted reproduction techniques. Initial evaluation will direct the physician towards the required laboratory and/or imaging investigations, however, at least 2 semen analyses are required at the beginning of evaluation for potential male factor infertility.

The prognosis of a couple presenting for infertility workup depends largely on the identified causes, if any. In addition, After identifying abnormal semen parameters, the care of a sub fertile male varies greatly, depending on the desires of the affected couple, available resources, local referral patterns, and treatment style of the involved physicians [25]. Discussing each cause of male infertility and the proposed treatment is outside the scope of this project. We will focus mainly on the diagnosis, pathophysiology and treatment outcomes of the most common and reversible condition associated with infertility, varicocele.

B3-Etiology of varicocele

Etiology of varicocele is only poorly understood. Clinically, 75-95% of varicoceles are left sided. This predisposition to the left side can be explained anatomically by the fact that left gonadal vein is longer than the right and the left gonadal vein enters the left renal vein perpendicularly (at right angle). These factors increase the hydrostatic pressure when the patient is in the upright position which will overcome the unidirectional vein valve mechanism, and, eventually cause backflow and dilatation of pampiniform plexus of veins [26]. The left internal spermatic vein drains into the narrower and higher pressure left renal vein compared to the right internal spermatic vein which drains into the wide and lower pressure inferior vena cava [23].

Increases in the left gonadal hydrostatic pressure might also result from back pressure caused by the compression of left renal vein between the aorta and superior mesenteric arteries “nutcracker phenomenon” [27]. In addition, it has been shown from cadaveric studies that men with varicoceles had absent valves in the internal spermatic vein, mainly on the left side, thus, incompetent or absent valves play a role in the etiology of varicocele [28]. Moreover, demonstration of regurgitation (reversal) of the blood flow during scrotal duplex ultrasound exam is a landmark and perquisite for diagnosis of varicocele.

B4- Mechanisms of varicocele-induced sperm dysfunction

The mechanism (s) by which varicocele induces sperm dysfunction is (are) still unknown, but are believed to be multifactorial. This has led to the generation of multiple theories to explain the relationship between varicocele and male-factor dysfunction. The most widely accepted and studied mechanism of varicocele-induced sperm dysfunction is scrotal hyperthermia (with negative impact on spermatogenesis). Other possible mechanisms that will be discussed in the following sections are reflux of renal/adrenal products, hormonal imbalance and changes in testicular microvasculature.

B4-1 Varicocele and scrotal hyperthermia

Scrotal temperature is maintained physiologically at lower values than body temperature, which is necessary for normal spermatogenesis. Moreover, intact blood circulation helps scrotal cooling. Hence, the presence of varicocele with the resultant regurgitation and stagnation of venous blood may render the mechanism of testicular cooling ineffective [29]. Several reports exist in the literature to support the notion that varicocele causes increase in scrotal temperature either through comparison between men with and without varicocele, or the observed decline in scrotal temperature following varicocele repair. Zorgniotti et al (1973) reported significant bilateral elevations of scrotal temperature in adolescents with palpable grade II-III varicocele compared to

control subjects [30]. Furthermore, this work was supported by the demonstration of scrotal cooling concomitant with improvement in testicular volume following successful varicocele repair [31]. Agger et al also reported a decrease of 0.5 C in scrotal temperature after varicocelectomy in those patients who experienced a significant improvement in sperm count [32].

B4-2 Reflux of adrenal/renal toxins

Tributaries to the left renal vein include the left internal spermatic and adrenal veins. Theoretically, the reverse blood flow (reflux) in the internal spermatic vein might cause renal and/or adrenal metabolites (such as catecholamines, cortisol) to reach the testicles and cause injury. This hypothesis, has led many to believe that adrenal catecholamines can be exchanged from venous system to the testicular artery, through the pampiniform plexus, causing arterial vasoconstriction and subsequent hypoxic injury to the testicles [29]. In animal studies, Camoglio et al have induced left varicocele through rings introduced through the left renal vein to cause renospermatic reflux and followed the rat models with hormonal profile and testicular biopsies. Varicocele was associated with testicular atrophy, histological changes, high follicular stimulating hormone (FSH) and low testosterone. The authors concluded that renal and adrenal metabolites may enhance varicocele-induced testicular damage [33]. In addition, it was shown that catechoamines concentration was 3-folds higher in a refluxing left gonadal vein compared to peripheral blood [34].

On the other hand, microspheres injected into the left renal vein did not appear in either testis in experimentally induced animal models with varicocele, a finding against the hypothesis of metabolites reflux into the testes from the kidney and adrenal gland [29]. In addition, ablation of left adrenal gland did not reduce the ongoing insult on rat testes 12-weeks following induced varicocele, i.e. reduced fertility, increased intra-testicular temperatures [35]. Steeno et al (1976) reported no difference in other adrenal products, like cortisol, between spermatic and peripheral blood samples in infertile men with varicocele [36].

B 4-3 Hormonal imbalance (dysfunction)

As mentioned earlier, intact hypothalamo-pituitary-gonadal axis is important for the initiation and maintenance of spermatogenesis. Varicocele was hypothesized to negatively alter such hormonal axis and hence, could be one mechanism by which varicocele induces sperm dysfunction [23]. Significant differences were found in levels of follicular stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) between infertile men with varicocele and without varicocele [37]. Furthermore, a world health organization (WHO 1992) multicenter study have shown the mean testosterone concentration was significantly lower in men >30 years old with varicocele compared to younger men without varicocele [38]. Inference of this study would be that varicocele exerts negative effects on testosterone production through Leydig cells, not only on Sertoli cell and spermatogenesis. Although testosterone was shown to

be suppressed in men with varicocele, it is not clear whether such decline has effects on spermatogenesis, especially in patients with unilateral varicocele. FSH, mostly considered as indicator of spermatogenesis, was reported to be significantly higher in infertile men with varicocele (average 7.8 IU/L) than infertile men without varicocele (average 3.5 IU/L) [39]. Furthermore, higher levels of estradiol and sex-hormone binding globulin associated with a decrease in free testosterone were found in oligospermic patients with varicocele [40].

Since varicocele was associated with hormonal dysfunction and alterations of the hypothalamo-pituitary-gonadal axis (HPG axis), varicocele repair could improve such hormonal imbalance. Gonadotropin-releasing hormone (GnRH) stimulation test was used to examine the integrity of HPG axis after varicocelectomy. Baseline and GnRH-stimulated FSH levels were higher in varicocele men with abnormal pre-operative semen parameters when compared to adolescents with varicocele and normal sperm parameters. This has led the authors to conclude that FSH and GnRH stimulation tests can be used to select adolescents for varicocele repair [41]. On the other hand, normal response of LH to GnRH stimulation was shown to predict good fertility potential and, possibly, pregnancy rates following varicoceletomy [42]. Varicocele can cause changes in HPG axis and selection of infertile patients with varicocele and low testosterone could improve outcomes of varicocele repair semen parameters.

B5 - PATHOPHYSIOLOGY OF VARICOCELE

The true effect of varicocele on male fertility potential is not known. Numerous studies have demonstrated an association between varicocele and reduced male fertility potential (e.g. poor semen parameters, infertility). However, most varicocele studies involve highly selected populations (e.g. infertile men) and rarely examine unselected men, representing an important reason for the difficulty in relating varicoceles with male fertility. Moreover, the lack of reliable end-points for measuring fertility represents another challenge in relating varicoceles with male infertility. Conventional sperm parameters (sperm concentration, motility and morphology) are generally monitored in varicocele studies but these parameters exhibit a high degree of biological variability and are of modest value in predicting male fertility potential [43] Pregnancy is also of limited value in assessing the influence of varicocele on male fertility potential because this outcome is heavily influenced by female factors.[44] Overall, studies of non-infertility populations provide conflicting results on the relationship between varicocele and fertility. As such, a cause and effect relationship between varicocele and male infertility has not been established.

B 5-1 Testicular atrophy

An adverse effect of varicocele on male fertility is suggested by the testicular atrophy that is generally associated with this condition.[38, 45-50] Indeed, it has been objectively demonstrated that in men with a left varicocele, mean left testicular volume is less than right testicular volume. [47, 50] However, the relationship between varicocele grade and the degree of testicular atrophy is less clear. Zini et al.[51] found that in men with unilateral left varicocele, the loss

of left testicular volume relative to the right (i.e. right minus left) increased with increasing varicocele grade, whereas Alukal et al.[52] found no such correlation between varicocele grade and volume differential.

The impact of testicular atrophy on male fertility remains to be established although most studies indicate that atrophy is associated with reduced sperm parameters. Sigman and Jarow[49] have reported that in men with left varicocele, those with testicular atrophy have poorer sperm parameters than do men without atrophy. Similarly, in a study of adolescents, Diamond et al.,[53] have shown that a volume differential greater than 10% between the normal and affected testis correlates with a significantly decreased sperm concentration and total motile sperm count. However, loss of testicular volume is not clearly associated with loss of fertility[48].

B 5-2 Testicular histology

A varicocele is associated with bilateral spermatogenic abnormalities and Leydig cell dysfunction.[54-57] The testicular histology in infertile men with varicocele is variable, but most studies report reduced spermatogenesis (hypospermatogenesis).[32, 58] More recently, Santoro and Romeo[59] described abnormalities in the ultrastructure of testicular tissue of men with varicocele. They noted that histologic changes were less pronounced in adolescents than in adults, implying that uncorrected adolescent varicoceles will be associated with greater testicular injury later in life. The observed increase in germ cell apoptosis associated with varicocele is thought to occur as a result of hyperthermia and low testosterone levels in the testis.[60] Testosterone concentration (testosterone is secreted by Leydig cells) is lower in older (>30 years) compared to younger men

with varicocele, a trend not seen in men without varicocele, suggesting a progressive, adverse effect of varicocele on Leydig cell function[38].

B 5-3 Sperm quality and fertility

The influence of varicocele on sperm parameters has not been established conclusively. In studies of infertile men, varicoceles have been associated with abnormal sperm parameters. MacLeod[61] and other investigators[38] observed that the majority of semen samples from infertile men with varicocele have poorer sperm parameters (lower sperm counts, increased numbers of spermatozoa with abnormal forms and decreased sperm motility) than fertile men. However, the "stress pattern" described by MacLeod (i.e. increased proportions of sperm with tapered heads and immature forms) is not a specific marker for varicocele and, therefore, is not diagnostic of this condition.[62] In studies of unselected men (i.e. non-infertile), the relationship between varicocele and sperm parameters is less clear. Johnson[63] showed that in a cohort of asymptomatic military recruits, nearly 70% of men with a palpable varicocele had an abnormality on semen analysis. In contrast, Zargooshi[64] observed that most young military recruits with significant (grade 2 and 3) varicoceles have normal semen parameters.

Although studies on the prevalence of varicocele in men with primary and secondary infertility suggest that the presence of a varicocele may cause a progressive decline in fertility this has not been confirmed by prospective studies. Chehval and Purcell[65] conducted a prospective, uncontrolled study of untreated varicocele and observed a significant deterioration in both sperm density and motility at 9-96 months follow-up. In contrast, Lund et al.,[66] conducted a

prospective, controlled trial of untreated men with and without varicocele and found no decline in semen parameters in either group after 8 years of follow-up.

B6: Treatment of varicocele

Varicocele is the most common correctable factor in men presenting with male infertility. Treatment of varicocele is commonly through an interventional procedures, i.e. there is no medical treatment. These interventions can range from simple radiological embolization to more invasive surgical varicocelectomy. We will discuss the different interventions used for the treatment of varicocele.

B6-1: Percutaneous venous (occlusion) embolization

Angiographic embolization of varicocele is an outpatient office-based procedure done under local anesthesia. The first report of successful trans venous sclerotherapy for occlusion of internal spermatic vein was on 1978 [67] It involves radiological-guidance catheterization of internal spermatic vein with subsequent occlusion using sclerosant agents, detachable balloons, coils or combination of different modalities [23]. Access is achieved via either internal jugular vein, usually for bilateral or right varicocele, or via common femoral vein.

Percutaneous venous occlusion has several advantages and disadvantages. Advantages include the minimally invasive nature, outpatient procedure, local anesthesia and the reasonable success rates. Disadvantages include exposure to radiation and the fact that it can be difficult and time consuming. Success rates as low as 69% have been reported with 27% of procedures deemed unsuccessful

during the procedure it self [23, 68]. In addition, there are concerns of coil migration and the need for a second intervention due to higher recurrence rate than surgery. Cayan et al (2009) reported 12.7% recurrence rate associated with radiological embolization, compared to 1.05% when microsurgical varicocelectomy is done [69].

B6-2 Sclerotherapy for varicocele

Sclerotherapy is considered an alternative therapy for varicocele. It was first described in 1988 by Tauber [23]. Antegrade scrotal scleratherapy is a simple and quick technique with a low complication rate. The success rate varies from 87% to 95% [70]. An incision is made through upper scrotum and the spermatic cord is identified and isolated. Venography is performed through a spermatic vein and a sclerosing agent is injected in an antegrade fashion. A meta-analysis of literature showed the reflux persistence rate to be 5% and 13%, with a mean 8% depending on the severity of reflux. While success rate as high as 100% was reported in grade 1 reflux, success rate decreased to 85% in severe (grade 3) reflux [70].

B6-3 Surgical approach

Surgery is considered the gold standard approach for treatment of varicocele and any new approach should be compared to this treatment. The basic principles of any varicocele surgery is the ligation of all internal spermatic veins with preservation of testicular artery, vas deference and cord lymphatic channels

as inadvertent injury to the artery may result in testicular atrophy and ligation of lymphatic channels can lead to subsequent formation of a hydrocele [23]. Several surgical approaches are available and generally can be divided into open or laparoscopic approaches, open techniques are further divided into retroperitoneal, inguinal, subinguinal and scrotal approaches.

Laparoscopic varicoceletomy (LV) involves division of internal spermatic vein using laparoscopic clips and preservation of spermatic artery. This approach is simple, safe, effective and does not need sophisticated laparoscopic skills to be performed [71]. Although preservation of the testicular artery (TA) during this approach would logically add to the overall improvement in sperm parameters, scarifying the TA tends to decrease the rate of varicocele recurrence because all of the vessels (including veins) are ligated. LV was proposed as the technique of choice in cases with previous inguinal surgery as laparoscopy in this situation would help to avoid operating through a scared area [71]. The improved vision and magnification during LV makes it ideal for identification and avoidance of injury to TA and lymphatic channels. Complications for LV are related either to access, like major vascular or bowel injuries, or due to pneumoperitoneum. In addition, trials to preserve the TA might result in missing small internal spermatic vein tributaries that usually run on the wall of artery and considered potential source of varicocele recurrence [72]. In a meta-analysis by Cayan et al comparing varicocelectomy techniques, laparoscopic varicocelectomy was associated with 30% pregnancy rate, 4.3% recurrence rate, and 2.8% hydrocele formation [69].

Scrotal approach for varicocelectomy was abandoned because of the risk of injury to testicular artery and potential subsequent testicular atrophy. In addition, this approach renders surgery complex due to the several small venous tributaries of the pampiniform plexus at this level. Therefore, this technique is no longer being used for high failure and complication rates [23].

The retroperitoneal (modified Palomo) approach involves high ligation of internal spermatic vein after it exits the internal inguinal ring. The main advantage of this approach is the simplicity of the procedure because fewer vein are encountered at this level and fewer veins need to be ligated, therefore, reducing the risk of varicocele recurrence [23]. Another potential advantage of retroperitoneal access is avoidance of inguinal canal and the avoidance of operating in a scarred operative field in case of previous inguinal surgery. However, this approach is associated with a recurrence rate of 6.8% to 11% [73]. Etriby et al reported that a major disadvantage of the modified Palomo approach is the lack of identification and ligation of external spermatic vein, a potential cause of varicocele recurrence [74].

Inguinal varicocelectomy has gained popularity before the description of microsurgical subinguinal varicocelectomy. This is mainly due to familiarity of the inguinal canal to urologists and the fact that internal spermatic veins are relatively large and few in numbers at this level. Due to the complexity of the microanatomy of the spermatic cord at subinguinal compared to inguinal varicocelectomy, some surgeons have recommended the latter approach for

surgeons who do not have extensive experience with these procedures, especially when using the operating microscope [75]. However, inguinal varicocelectomy requires opening the inguinal canal, making this surgery more painful than a subinguinal approach (which avoids opening the inguinal canal). In addition, inguinal varicocelectomy carries the risk of subsequent inguinal hernia [76]. Gontero et al compared inguinal to subinguinal varicocelectomy and his results showed a trend towards fewer spermatic veins at the inguinal canal, less injury to the testicular artery and reduced incidence of persistent venous reflux after surgery [76]. Microsurgical inguinal varicocelecomy was associated with more than 50% increase in total motile sperm count in 46.61% infertile men, 42.8% pregnancy rate, 2.1% recurrence rate and only 0.6% hydrocele formation [77].

Finally, subinguinal varicocelectomy involves a smaller incision at the external inguinal ring, without the need to open inguinal canal. Obviously, this approach is associated with less pain and discomfort. On the other hand, the spermatic vein has many branches at this level which makes the surgery demanding. The use of surgical microscope with this approach has yielded excellent results in terms of improved success rates, reduced recurrence and hydrocele formation rates as well as a significant decrease in the incidence of testicular artery injury [78]. Similar to the inguinal approach, subinguinal varicocelectomy allows easy delivery of the testes and ablation of all possible venous channels to prevent future varicocele recurrence. In addition, external spermatic veins, which could cause recurrence, can be readily identified using this approach and ligated [78, 79].

We will describe the technique of microsurgical subinguinal varicocelectomy because this is the procedure was done for all infertile men included in this cohort. A one-inch oblique incision is made over the external inguinal ring over the pubic bone. The spermatic cord is mobilized immediately at the level it exits the external inguinal ring, the external oblique fascia is not incised. The testis is delivered and all associated gabernacular and external spermatic veins are divided. The Vas deference with its adventitia and vascular bundle are separated on a Penrose drain and the cord with its contents are separated on a second Penrose drain. The operating microscope is used at this stage to identify and preserve lymphatic channels and testicular artery (arteries). All internal spermatic veins are ligated. Complications from this approach are not common and lower than other surgical approaches for varicocele [80]. In a recent review of literature of 5000 men in 33 studies who investigated the effect of varicocelectomy, microsurgical subinguinal/inguinal varicocelectomy offer the best outcomes, in terms of pregnancy, complication and recurrence rates. Microsurgical subinguinal varicocelectomy showed the best pregnancy rates and the lowest recurrence rate of varicocele, in contrast, microsurgical inguinal approach provided the lowest rate of hydrocele formation while laparoscopic approach had the highest complication rates [81].

B 7-Treatment outcomes of varicocele repair

Varicocele is repaired mainly to correct semen parameters in infertile men. However, other indications include scrotal pain, hypogonadism and cosmetic reasons. The principle outcome measures for varicocele repair are improvement in sperm parameters (sperm count, motility, morphology) and pregnancy rates. The utilization of objective outcome endpoints is of importance especially when subjective measures are used, like scrotal pain. The following section will mainly focus on effect of varicocele repair on pregnancy rate and sperm parameters.

B 7.1 Sperm parameters

The terms "oligo", astheno" and "terato" are frequently used in the literature to describe abnormalities in sperm parameters. Oligospermia refers to a low concentration of sperm in semen, asthenospermia refers to poor motility and teratospermia refers to abnormal shape of spermatozoa. Patients without varicocele whose sperm parameters are abnormal might not benefit much of treatment compared to men with varicocele and semen abnormalities as varicocele repair was associated with 60-70 % improvement in semen quality [82, 83]. Despite paucity in the literature of randomized controlled studies examining the effect of varicocelectomy on sperm parameters, there is evidence from uncontrolled and/or non-randomized studies that varicocele repair results in improvement in sperm quality. Interestingly, the Cochrane review of multiple randomized studies concluded the lack of good evidence to support that varicocele repair improves sperm parameters [84, 85]. We will discuss in the following

sections the available randomized and non-randomized trials on the effect of varicocele repair on sperm count, motility and morphology.

Madgar et al (1995) reported the effect of high spermatic vein ligation for varicocele in infertile men. In this series, sperm concentration had increased from 15 to 32 millions/ml ($p < 0.05$), sperm motility improved from 30% to 55% (<0.001) in addition to improved percentage of sperm normal shape from 27% to 40% (<0.005) [86]. Another randomized study (Yamamoto et al, 1996) reported improvement in sperm parameters in a cohort of subclinical varicocele. Sperm concentration increased from 15 to 20.9 millions/ml, however, sperm motility and morphology did not change significantly [87]. On the other hand, several other randomized trials did not observe a statistically significant improvement in sperm parameters after varicocelectomy [88-90]. There are many reasons for such variability in study outcomes but the heterogeneity of the studied populations, different surgical techniques used and the disparity of varicocele grade could form the basis of such differences. Most of the available literature on the positive effects of varicocele repair on sperm parameters originate from uncontrolled studies [23]. Kibar et al (2002) performed subinguinal varicocelectomy on 90 uncontrolled varicocele patients and observed significant improvement in sperm density (22.1 to 38.3 m) sperm motility (23.2% to 45.1%) and sperm morphology (2.6 % to 10.2%) [91]. Furthermore, Hsieh et al (2006) studied the effect of varicocele repair on semen parameters in 254 patients and reported significant improvement in sperm density (24.2 to 41 m), motility (30% to 47%) [92]. The lack of control group in these studies threatens the credibility of the positive effects of varicocele repair on sperm quality as these changes can be easily

explained by the biologic variability of sperm parameters.

B 7.2 Pregnancy rate

The definition of infertility is the inability of couples to conceive for at least one year of unprotected intercourse. Hence, most of infertile men who present to the clinic want to maximize their odds for spontaneous (unassisted) pregnancy. Unfortunately, up to date there is insufficient evidence to support the notion that varicocele repair improves the rate of couple's natural pregnancy [84, 93]. This is due to the fact that previous publications on the effect of varicocele repair on spontaneous pregnancy either lack a control arm, randomization, a clinically palpable varicocele or included patients with normal sperm parameters [93]. Furthermore, several meta-analysis were published on the effects of varicocelectomy on fertility potential but the results were weakened by the heterogeneity of the included RCTs [94]. The dilemma arises partly from the fact that the mechanism by which varicocele induces male infertility is poorly understood, hence, the mechanism by which varicocele repair improves sperm parameters and pregnancy rates is still controversial.

Marmar et al (2007) conducted a meta-analysis of 16 studies reporting on the effects of varicocelectomy on sperm parameters and pregnancy rates. The odds rates of spontaneous pregnancy after varicocele repair compared to no treatment or medical therapy was 2.63 (95% CI, 1.60–4.33], P=0.00001) when a fixed-

effects model was used [95]. This translated into a number needed to treat (NNT) of 5.7 (95% CI, 4.1–9.5). A wide range of pregnancy rates was reported after varicocele repair. Madgar et al reported a pregnancy rate as high as 60% in a randomized study, in addition to significant improvement in sperm parameters [86]. Cayan et al (2009) published a meta-analysis on the best technique for treatment of palpable varicocele in infertile men. Out of 4473 men, 39.07% initiated spontaneous pregnancy after repair of varicocele with different techniques. Pregnancy rates were 25-55.2% with retroperitoneal Palomo technique, 33-50.9% with microscopic subinguinal, 30-43 with inguinal, 16-40% with laparoscopy, 20-40% with radiological embolization and 34-39 with microscopic inguinal [69]. On the other hand, a new meta-analysis published in European Urology concluded that definitive evidence is still lacking as to the positive effect of varicocele repair on spontaneous pregnancy [93].

International guidelines have reported conflicting statements regarding recommendations for repair of varicocele in infertile men with varicocele. The American Society of Reproductive Medicine has recommended varicocelectomy in the presence of clinically palpable varicocele and abnormal sperm parameters provided the female partner has no (or potentially treatable) condition that prevents conception. The European Association of Urology guidelines has stated that repair of varicocele to improve spontaneous pregnancy is still controversial. Interestingly, other committees have recommended against varicocele repair [93].

B 8-Sperm DNA integrity

The sperm genomic material is located at the head and intact sperm DNA integrity is of great importance for a weighted transmission of paternal genetic information to off springs. During spermatogenesis, the diploid spermatocytes divide in meiosis to produce haploid secondary spermatocytes that contain a single copy of each chromosome [96]. A mature sperm has to travel, first, through the entire length male genital tract starting from the testicle through epididymis then the vas deference and finally exits through urethra, then, has to pass the entire length of female genital tract in order to fertilize the ovum and form a zygote with stable genetic material. Hence, the sperm genome has to be protected from external insult during such transit. This is especially relevant due to the fact that spermatozoa have no known DNA repair mechanisms [97]. Such protection mechanism consists mainly of dense sperm chromatin compaction (see later sections).

B 8.1 Sperm chromatin compaction

Human sperm chromatin is highly condensed and compacted. In contrast to somatic cells, This tight compaction is unique to mammals and vital for the transfer of mature sperm through male and female genital tracts in a genetically stable condition [98]. Sperm chromatin compaction takes place primarily during spermiogenesis, the last stage of spermatogenesis, where the elongated spermatids form mature spermatozoa [97]. The human sperm DNA is tightly condensed by protamine molecules (basic nuclear proteins) that render the sperm genome

inactive until it is reactivated post-fertilization [99]. Disulfide bonds begin to form at the spermatid stage of spermatogenesis and further linkage occurs during sperm-epididymis transit where each neighbor pair of protamine molecules become linked to each other [99]. During this stage, majority of the nuclear histones are replaced by protamines, for further compaction and organization [97]. Sperm chromatin compaction has some other functions, other than protection of sperm DNA, that include making the DNA more compact for more active motility during sperm transit through genital tracts, silencing most of the genome and minimize cross species fertilization [97]. Defects in sperm chromatin compaction, and hence in DNA integrity, can cause low sperm fertilizing capacity, as will be explained later. There are now several available tests (both cytochemical and flow cytometry-based) that can measure human sperm chromatin and DNA integrity.

B 8.2 Sperm Chromatin Structure Assay (SCSA)

Among several available sperm DNA tests, the Sperm Chromatin Structure Assay-SCSA is one of the most widely utilized and perhaps best studied. The SCSA was described more than 30 years ago and it measures the percent sperm DNA fragmentation index (%DFI) and chromatin compaction. The SCSA has been studied extensively (in animals and humans alike) and the results validated by studying fertile populations [98]. The clinical utility of the SCSA stems from the fact that a well-defined threshold has been established for this test (a DFI>30% is considered significant based on studies of fertile populations), such that in men

with a test result above the set threshold, changes in life style and/or medical interventions may be indicated [98].

SCSA utilizes metachromatic features of acridine orange and principles of flow cytometry. SCSA results are expressed as sperm %DFI (an index of sperm DNA damage) and sperm %HDS (high DNA stainability, a measure of nuclear chromatin compaction) Figure 7.2.1. The original description by Evenson et al in his pioneering study showing green (intact DNA) and red (damaged DNA) [100], as in figure.7.2.2. Clinical studies have indicated that with high sperm %DFI levels (%DFI >30), the probability of natural conception is reduced and couples may consider avoiding intra-uterine insemination (IUI) and move directly to intra-cytoplasmic sperm injection (ICSI) [98]. Some investigators have further suggested that couples with very high %DFI (>50%) and failed ICSI might consider use of testicular sperm for subsequent ICSI but strong evidence behind this is still lacking [98].

Figure 8.2.1: SCSA with good DNA integrity

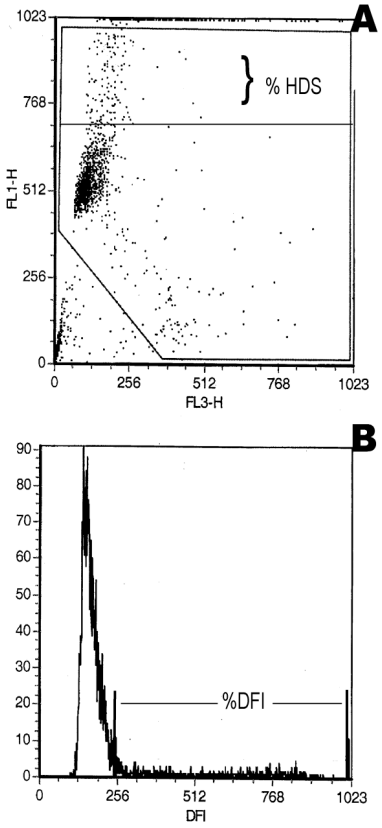
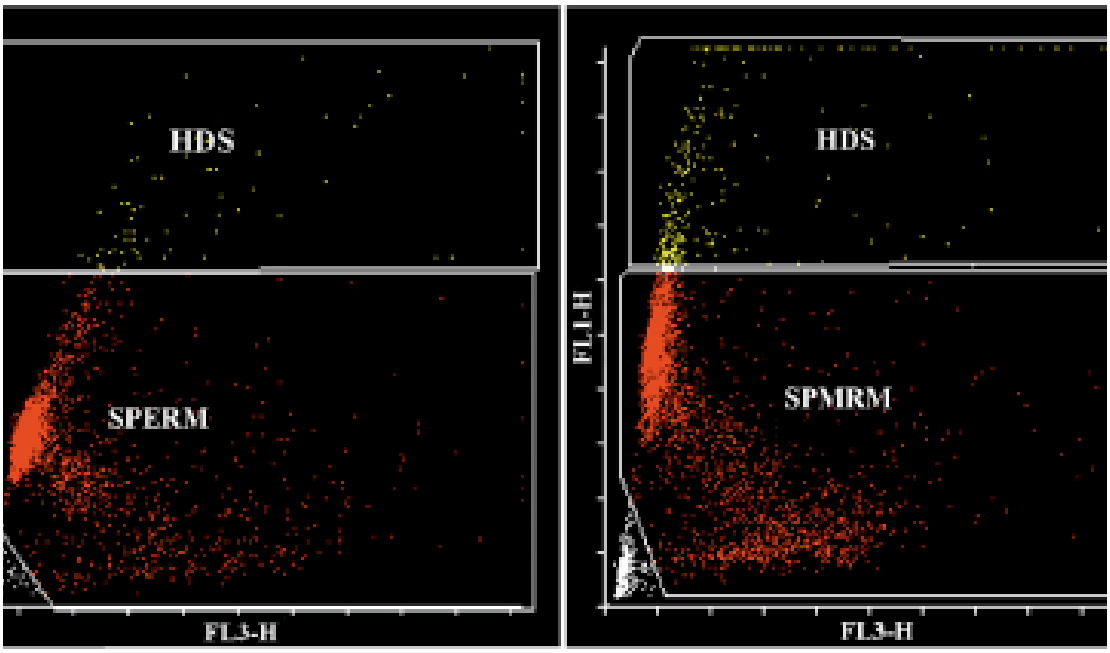


Figure 8.2.2: SCSA with green (intact DNA) and red (damaged DNA)



B 9- Varicocele and oxidative stress

Oxidative stress (OS) occurs when there is imbalance between the rate of production of reactive oxygen species (ROS) and scavenging by anti-oxidant capacity. These ROS include (superoxide anions, hydrogen peroxide, hydroxyl radical, hydroperoxyl radical and nitric oxide) [101]. A controlled low threshold of ROS production if considered physiological and important for sperm function [102-104], however, excess ROS can cause sperm dysfunction. Spermatozoa are vulnerable to oxidative injury due to the fact that sperm plasma membrane is abundant in polyunsaturated fatty acids, hence, lipid peroxidation results in sperm dysfunction and loss of viability [105]. Furthermore, it was reported that approximately 25% of the seminal fluid of infertile men posses more ROS, and defective anti-oxidant capacity, compared to fertile men [104, 106].

The seminal plasma provides anti-oxidant capacity against oxidative stress [107]. Anti-oxidants can be enzymatic or non-enzymatic and include superoxide dismutase, glutathione peroxidase, catalase, uric acid, vitamins C&E and albumin [107]. The protective role of seminal antioxidants against oxidative stress has been reported by several researchers [108-110]. In addition, reduced seminal anti-oxidant capacity has been associated with sperm dysfunction and constitutes a potential cause of male factor infertility [106, 111, 112].

There is evidence to support the proposed mechanism for varicocele-induced sperm dysfunction through generation of ROS making the testicles unable to handle oxidative stress [23]. However, it is not yet clear whether elevated levels

of ROS in infertile men with varicocele are due to the pathophysiology of varicocele or due to infertility [23]. Nonetheless, several studies have shown a higher ROS production, supported by elevated OS markers, in infertile men with varicocele compared to fertile men and infertile men without varicocele [101, 113-115]. Furthermore, varicocele repair was associated with reduced oxidative stress and/or increase in seminal antioxidant capacity [101, 116, 117]. This provides an additional mechanism to the beneficial effect of varicocele repair on sperm parameters and pregnancy rate.

B 10- Varicocele and sperm DNA integrity

High sperm DNA fragmentation is negatively associated with spontaneous pregnancy and outcomes of assisted reproduction [118, 119]. The etiology of sperm DNA damage is multifactorial and many studies have proposed oxidative stress, defective (aberrant) chromatin compaction and abortive apoptosis as the main factors [13-16].

The true pathophysiology of varicocele is unknown. In addition, the relationship between varicocele repair and improvement in sperm parameters and pregnancy rate is one of the most controversial issues in Andrology. This is mainly due to the fact that most studies have evaluated highly selected patient populations (e.g. infertile men) and have examined outcome measures that have poor reproducibility [120]. Sperm parameters are not highly reliable parameters due to the high degree of biologic variability. Hence, there is a need for a reproducible end-point variables that can better diagnose and predict fertility potential.

Several investigators studied the relationship between varicocele and sperm DNA damage. It has been shown that infertile men with varicocele posses a higher degree of sperm DNA fragmentation [17-21]. The mechanism for varicocele-induced sperm DNA damage is not completely understood but the increased levels of ROS in the semen of infertile men with varicocele can cause DNA damage resulting in sperm dysfunction and poor fertilizing capacity [120, 121]. In addition, to further augment the evidence that varicocele-mediated oxidative stress is associated with sperm DNA damage, varicocele repair was

associated with reduction in sperm DNA damage [120, 122, 123]. See discussion of the manuscript below for more details.

***B 11: Sperm DNA damage and reproductive outcomes (natural and assisted reproduction pregnancy rate*s)**

There is mounting evidence to suggest that intact genomic material in the nucleus of male gametes is important for fertility potential of spermatozoa. Several studies have shown that sperms with abnormal chromatin organization are more frequent in subfertile and infertile men [12]. Furthermore, couples in which the male partner has significantly high levels of high sperm DNA damage have low natural pregnancy rates and, if pregnancy occurs, it is usually after waiting for extended time interval [12, 124]. Poor sperm DNA integrity was also associated with recurrent miscarriage [12].

Assisted reproductive techniques (ART) have revolutionized the management of infertile couple and being increasingly utilized. In vitro fertilization (IVF) and intra cytoplasmic injection (ICSI) have become the standard of care for male factor infertility. During natural pregnancy, there is a physiological selection of spermatozoa and only healthy sperm with intact DNA integrity can fertilize the ovum. However, when ICSI is utilized (in which the sperm, regardless of its DNA integrity, is injected directly into the cytoplasm of the oocyte), it bypasses all the

natural barriers that are made to block (prevent) fertilization of a sperm with high DNA damage [125]. Hence, there is increasing concern on the genomic stability and later embryonic development when pregnancy occurs after ICSI utilizing a sperm with high DNA damage. Pregnancy rates after ARTs (IUI and IVF, specifically) are inversely related to sperm DNA integrity [125, 126]. The adverse effect of sperm DNA damage on reproductive outcomes after ARTs may be due, in part, to the effects of sperm DNA damage on embryo development [127].

B 12: Discussion

Varicocele is the most reversible cause of male factor infertility. Varicocele repair in infertile men with clinical varicocele has been shown in several occasions to improve sperm parameters by 60-80%. The accurate pathophysiological mechanisms by which varicocele induces sperm dysfunction is only poorly understood. Sperm DNA integrity has emerged as adjunct to predict/diagnose male infertility and might play a major role in the selection of method of assisted reproduction technique in infertile couple seeking pregnancy. However, despite the mounting evidence on the association between varicocele and sperm dysfunction, it is still of controvercy.

Whether the relationship between varicocele and sperm DNA damage is one of cause-and-effect is unproven. Zini et al recently published a systematic review on all studies that have examined the relationship between varicocele and sperm DNA integrity [120]. Out of 16 enrolled studies, five studies showed that the level of sperm DNA damage in infertile men with varicocele was similar to that of infertile men

without varicocele [18, 128-131]. In four other studies, the level of sperm DNA damage in infertile men with varicocele was higher than that of infertile men without varicocele [19, 132-134]. On the other hand, an evaluation of studies of non-infertility populations demonstrates a strong association between varicocele and sperm DNA damage [120]. Furthermore, several investigators have examined the effect of varicocele repair on sperm DNA damage. A total of twelve studies identified on the effect of varicocelectomy on sperm DNA damage, and these studies have all shown that varicocele repair is associated with reduced sperm DNA damage [116, 122, 123, 135-141]. To our best of knowledge, this is the first study to show improvement in sperm DNA integrity after varicocelectomy using 3 different assays simultaneously on the same cohort of patients. In contrast, previous studies have used single sperm DNA assay (SCSA, TUNEL, COMET) to assess the effect of varicocele repair on human sperm DNA integrity.

C-Aim of Thesis

1- To prospectively examine the effect of varicocele repair on sperm DNA fragmentation, distribution of nuclear sulfhydryl groups, sperm maturation.

2- To study correlations between sperm DNA (chromatin) assays and different sperm parameters.

3- Report the long-term followup pregnancy rates in relation to improvements in sperm DFI%.

D- "MANUSCRIPT"

BENEFICIAL EFFECTS OF MICROSURGICAL VARICOCELECTOMY ON SPERM DNA FRAGMENTATION, DISTRIBUTION OF NUCLEAR SULFHYDRYL GROUPS AND SPERM MATURATION: A PROSPECTIVE TRIAL

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Key words: Sperm DNA / Iodoacetamide fluorescein / Aniline Blue / male infertility / varicocele

D.1: ABSTRACT

Background: There is evidence to show that varicocele repair can improve conventional sperm parameters and sperm DNA integrity in infertile men with a clinical varicocele.

Objective: To further examine the effect of varicocelectomy on sperm quality, specifically, sperm nuclear chromatin integrity, distribution of nuclear sulfhydryl groups and sperm maturation.

Design, Setting and Participants: We prospectively evaluated a consecutive series of infertile men (n=36) presenting to Ovo clinic with one year or more history of infertility, a clinically palpable varicocele and abnormal semen parameters. Six sperm donors with normal sperm parameters served as controls.

Surgical Procedure: Microsurgical sub-inguinal varicocelectomy.

Outcome Measures: (1) Conventional sperm parameters, (2) aniline blue staining (AB is specific to histone lysines), (3) iodoacetamide fluorescein (IAF targets free protamine sulfhydryl groups) and (4) sperm chromatin structure assay (SCSA) with the results expressed as % DNA fragmentation index (%DFI) and percent high DNA stainability (%HDS) before and 4 months after microsurgical varicocelectomy.

Results: The sperm %DFI, %HDS (a measure of chromatin compaction), % 5-IAF staining (diffuse head staining), % AB staining (dark blue) were all significantly lower in the control group compared to infertile men with varicocele (8 vs. 20%, 4.0 vs. 9.6%, 1.7 vs. 16.3%, and 2.5 vs. 13.5% respectively). The %5-IAF and %AB staining decreased significantly after surgery (from 16.3 to 5.4%, and from 13.5% to 5.4%, respectively). Similarly, the %HDS and %DFI also decreased significantly after surgery (from 10% to 6% and from 20% to 13%, respectively). The only notable

relationships were between aniline blue staining and %HDS post varicocelectomy (r= 0.57, P <0.05), and both %IAF staining and %DFI were inversely correlated with motility (*r*=-0.44 and -0.43, respectively).

Conclusion: The data show that varicocelectomy is associated with a consistent improvement in sperm DNA integrity and chromatin compaction using three different assays of sperm chromatin integrity (SCSA, IAF, Aniline Blue).

D.2: Introduction:

Varicocele is a patho-biological condition associated with dilatation of veins of Pampiniform plexus within spermatic cord. Varicocele is found in approximately 15% of the general population but the prevalence of clinical varicocele is approximately 40% in men with history of infertility [142, 143]. Although controversial, it is postulated that varicocele induces sperm dysfunction through increased scrotal temperature, reflux of blood from the spermatic vein, and impaired microcirculation [144]. In general, it is reported that varicocele repair results in improved semen quality in 60-80% of infertile men [145]. However, the true effect of adult varicocelectomy on male fertility remains controversial largely because of the paucity of randomized and controlled trials [138]. Recently, there has been mounting evidence to show that increased levels of semen

reactive oxygen species (ROS) and sperm apoptotic markers are associated with varicocele [146].

During spermatogenesis, spermatid nuclear re-modeling and compaction is associated with displacement of nuclear histones by transition proteins and then by protamines [147]. Disrupted spermatogenesis may result in the generation of spermatozoa with impaired protamination, poor chromatin compaction and an increased susceptibility to DNA damage [148, 149]. There is evidence to suggest that spermatozoa of infertile men possess substantially more chromatin defects and DNA damage than spermatozoa of infertile men [10, 12, 150]. The etiology of sperm DNA damage is multi-factorial and most investigators have proposed that ultimately, oxidative stress, aberrant chromatin remodeling (compaction) and abortive apoptosis can result in sperm DNA damage [13-16].

Conventional sperm parameters (sperm concentration, motility, and morphology) are generally evaluated in varicocele studies. However, the use of conventional sperm parameters as outcome measures is weakened by virtue of the high degree of biological variability of these parameters and their modest value in predicting male fertility potential [43, 120]. On the other hand, pregnancy is not a good parameter to assess outcomes of varicocele repair as it is highly influenced by female factors. Furthermore, there is no consistent relationship between varicocele repair and sperm parameters in non-infertility populations [120, 151, 152]. As such, an improvement in sperm DNA integrity would provide more credibility as to the therapeutic effect of varicocelectomy because compared with standard semen parameters, measures of sperm DNA damage

exhibit a lower degree of biologic variability and may be better predictors of male fertility potential.

A number of investigators have recently examined the association between varicocele and sperm DNA damage. All of these studies have shown that varicocele repair is associated with reduced DNA damage. However, the majority of these studies lack randomization, control arm, and rarely used more than one sperm DNA assay for assessment of the effect of varicocele repair. As such, the purpose of this study was to prospectively examine the effect of varicocele repair on several sperm DNA assays (sperm DNA fragmentation index, distribution of nuclear sulfhydryl groups, sperm maturation) and to study the correlations between these assays and sperm parameters.

D.3: Materials and Methods:

Materials:

Acridine orange (AO) was purchased from PolySciences (Warrington, PA, USA). IAF (5-iodoacetamide-fluorescein) was purchased from Invitrogen (Burlington, ON, Canada). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA) and were at least of reagent grade.

Patient population:

We conducted a prospective study of couples presenting for infertility evaluation at the OVO fertility clinic in Montreal, Canada over one year period. This cohort includes patients evaluated in a prior varicocelectomy study [137]. Men presenting to our clinic with one year or more of infertility, a clinically palpable varicocele and abnormal semen parameters (reduced sperm concentration, motility, or morphology on two or more semen samples) were deemed to be candidates for varicocele repair. Baseline testicular volumes (estimated with an orchidometer) and serum FSH, LH and testosterone levels were obtained. Men with azoospermia, severe oligozoospermia (<5 million sperm/mL), complete asthenozoospermia or evidence of genital tract infection were excluded. Men were not selected based on the results of sperm DNA damage. Couples in whom the wife had tubal obstruction or ovulatory failure were not included. All of the operations (microsurgical varicocelectomy) were performed by the same surgeon (AZ), as previously described [153]. The study was approved by the ethics review board at McGill University and all men signed an informed consent prior to participating. Patient information for this study remained confidential and within the institution.

We recruited and evaluated 29 consecutive men who satisfied the inclusion and exclusion criteria. The recruited men were asked to submit three semen samples (one at 1–2 months before surgery and another two at 4 and at 6 months after varicocelectomy) for evaluation of standard sperm parameters and sperm DNA and chromatin integrity (assessed by SCSA – sperm chromatin structure assay). All 29 men underwent microsurgical varicocelectomy over the study period and these men were contacted (over the phone) to maximize compliance with the study protocol.

Semen handling:

Samples were obtained by masturbation after 3–5 days of sexual abstinence. After liquefaction of semen, standard semen parameters (volume, concentration, motility) were obtained using a computer-assisted semen analyzer – CASA. All of the semen samples had motile sperm and none had significant numbers of round cells or leukocytospermia as per WHO guidelines (<1 million round cells/mL).

Following liquefaction, two 25–100 μL aliquots of semen (containing approximately 2 million spermatozoa) were collected from the original sample and frozen at −70 °C for later evaluation of sperm chromatin structure assay (SCSA) parameters (%DNA fragmentation index-%DFI, %high DNA stainability-%HDS) and cytochemical chromatin tests (%IAF fluorescence and %AB staining).

Sperm DFI and HDS:

Sperm DNA damage was assessed by the sperm chromatin structure assay (SCSA) and the results were expressed as sperm percentage DFI (an index of DNA damage) and sperm percentage HDS (a measure of nuclear chromatin compaction) as previously described [10, 11]. Stored semen samples were thawed on ice and treated for 30 sec with 400 µL of a solution of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.2. After 30 sec, 1.2 mL of staining buffer (6 µg/mL AO, 37 mm citric acid, 126 mM Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0) was admixed to the test tube. The sample was placed into the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the sample flowing to establish excellent sheath/sample flow, and then measurements were taken at exactly 3 min after AO staining. A minimum of 5000 cells from two aliquots of each sample were analyzed by FACS scan interfaced with a data handler (cellquest 3.1; Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA, USA). WinList (Verity Softwarehouse Inc., Topsham, ME, USA) was used to generate the cytogram (red vs. green fluorescence) and histogram (total cells vs. DFI) plots, as well as, percentage DFI and percentage HDS readings. A mean of the two sperm percentage DFI and percentage HDS values was reported. The variability of the replicate SCSA measures (percentage DFI and percentage HDS) was <5%. Testing of paired samples (pre- and post-surgery) was always carried out on the same run.

We have shown that testing fresh and frozen-thawed samples gives comparable results (<5% variability) and that the inter-assay variability of sperm percentage DFI is low (<5%) by repeat assessments of reference semen samples [11, 150]. Over 300 aliquots

of the same semen sample ('reference sample') have been stored at −70 °C for ongoing assessment of inter-assay variability. We have previously validated our assay by assessing sperm DNA fragmentation (by terminal nucleotidyl transferase dUTP nick end labeling – TUNEL assay) in parallel with sperm percentage DFI and have shown a strong association ($r = 0.71$) between these two measures of DNA damage [11, 150].

Cytochemical tests of sperm chromatin: aniline blue and iodoacetamide fluorescein

Thawed semen samples were fixed with 70% ethanol and kept at −20 °C before further processing. Smears were prepared from the fixed semen samples, left to air-dry at 20°C for 30 min and immediately stained. For aniline blue (AB) staining (15), smears are incubated with the dye (5% AB in 4% acetic acid) for 5 minutes, then 3 times with dH2O and mounted with glycerol. For iodoacetamide-fluorescein (IAF) fluorescence (IAF, for free sulfhydryl group)(15-16) the smears were incubated with 0.1 M Tris (pH 6.8) for 5 min and then with 0.1 mM IAF for 15 min. The IAF-stained smears were rinsed briefly with dH2O, washed with Tris and then mounted with DABCO.

To overcome the subjective variation we counted at least 200 sperms per slide. We followed the same grading systems adapted by de Lamirande et al. (16) and divided the counted sperm into three categories: Dark blue (dark blue over the whole head), pale (whole head pale staining), or medium (post acrosomal region intensely stained) for AB staining. For IAF, the fluorescence is graded as pale (whole head pale), medium (post-acrosomal region bright) or intense (whole head bright).

Data analysis:

Results are expressed as means ± one SD. Differences between the pre- and 4-month post-varicocelectomy parameters were estimated by Wilcoxon signed-ranks test. Differences between the pre- and 4-month and 6-month post-varicocelectomy parameters were estimated by ANOVA (Student–Newman–Keuls method). The calculations of correlation coefficients between parameters (variables) were performed using a nonparametric procedure, the Spearman rank-order correlation. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. Analyses were conducted using the sigma stat program (SPSS, Chicago, IL, USA).

D.4: Results:

We recruited 29 infertile men with clinical varicocele in this study. Mean (±SD) left and right testicular volumes were 14 ± 2 and 15 ± 4 mL, respectively. The mean (±SD) serum FSH, LH and total testosterone levels were 6 ± 4, 4 ± 1 IU/L and 13 ± 5 nmol/L respectively. At baseline, the 29 men had a mean sperm concentration of 42 million/mL (range: 6–78 million/mL), progressive motility of 25% (range 10–40%) and a percentage DFI of 20% (range 10-30%). Each of the 29 men had a reduced sperm concentration (<20 million/mL) or reduced progressive motility (<50%) or both at baseline. On the other hand, positive 5-IAF staining (diffuse and intense head staining) (figure D.4.2), and positive AB staining (dark blue) (figure D.4.2) were both significantly higher in infertile men with varicocele compared to control group (16.3% vs. 1.7%, and 13.5 vs. 2.5% respectively) [see table 1).

Sperm DNA integrity improved significantly at 4 months after surgery ($n = 29$; percentage DFI decreased from 20 ± 10% (range 4–38%) before surgery to 12 ± 6% (range 3–23%) at 4 months after surgery (p=0.001). Similarly, sperm chromatin compaction also improved significantly at 4 months after surgery ($n = 29$; percentage HDS decreased from 10 ± 6% before surgery to 6 ± 5% at 4 months after surgery (Table 2).

On the other hand, cytochemical tests have also demonstrated significant differences in semen samples before and after surgery. The percentage of sperms with dark aniline blue stain has significantly decreased at 4-month after varicocele repair (from pre-op 13.5%±7 to post-op 5.4%±3.4, p=0.00003) Table3. Similarly, The percentage of spermatozoa with intense IAF staining showed a statistically significant

reduction after surgery (from pre-op 16.3%±6 to post-op 5.4%±2.7, p=0.0004) Table 4. Sperm concentration and progressive motility also improved significantly at 4 months after surgery.

We found no significant relationships between AB staining and %DFI, motility or concentration. The only notable relationship was between aniline blue staining and %HDS post-varicocelectomy (r= 0.57, P <0.05). In addition, there were no significant relationships between %IAF staining and %DFI, %HDS or sperm concentration. The only notable relationship was between %IAF staining and sperm motility (r= - 0.44, P 0.01).

Finally, a follow-up of patients by either phone or clinic appointment was done to report pregnancy rates and new DFI% results. Table 3 summarizes the results. We were able to gather information on 18 patients. There couples achieved natural pregnancy during follow-up. Nine patients considered ARTs (IUI or IVF) and, overall, 4 patients achieved pregnancy.

D.5: Discussion:

In the present study, we have observed that varicocelectomy is associated with a significant decrease in the proportion of cells with DNA damage (decreased sperm %DFI). Varicocelectomy was also associated with a significant decrease in the percentage of cells with HDS (%HDS by SCSA). In addition, the mean sperm concentration and percent progressive sperm motility have improved significantly after varicocele repair. The improvements in sperm DNA integrity and chromatin compaction were observed as early as 4-month after surgery, and further sustained at 6-month post-varicocelectomy.

Aniline blue (AB) binds to lysine-rich nuclear proteins and mostly histones in spermatozoa [154]. In infertile men, spermatozoa with dark staining are often obviously abnormal and considered immature [155, 156]. In the present study, we observed that varicocele repair was associated with a significant reduction in sperm cells with dark AB staining (from 13.5%±7 to 5.4%±3.4). The higher AB staining may either reflect a change in the level of histones and/or a change in histone orientation making it more accessible to AB, both of which are associated with lower sperm chromatin compaction [156]. In addition, infertile men with varicocele demonstrated a higher percentage of dark stained spermatozoa compared with the fertile men without varicocele. These findings are similar to previously published reports by Sadek et al [139] and Foresta et al [157]. In contrast, it was reported that dark AB staining spermatozoa was not significantly different between infertile men with varicocele compared to men with idiopathic infertility [128]. This could lead to the possibility that sperm DNA damage

might be due to infertility itself and not due to varicocele per se. on the other hand, the only notable relationship was between aniline blue staining and %HDS post-varicocelectomy. This can be explained by the fact that both assays measure the same outcome, chromatin compaction

One of the strength of the current study is the addition of iodoacetamide-fluorescein (IAF) stain to validate and confirm the previous findings. IAF is an excellent sulfhydryl-targeted reactive with proven usefulness to label sperm proteins (head and flagellum) [158]. In the current study, sperm heads with intense IAF fluorescence stain were significantly higher in the varicocele group, before surgery, compared to controls. Furthermore, the percentage of spermatozoa with intense IAF fluorescence decreased significantly after varicocele repair (16.3%±6 to 5.4±2.7). Mechanisms such as increase in sulfhydryl content, as well as, lower degree of disulfide bond formation can explain the high IAF fluorescence observed in the sperm heads of infertile men with varicocele [159].

A lower degree of disulfide bond formation can lead to lower chromatin compaction and explain the observed increases in AB staining in sperm heads before varicocele repair [160]. In addition, the high levels of DNA stainability (%HDS) is indicative of increased accessibility of acridine orange stain to the DNA suggesting that the chromatin is less compact (more porous) [10]. Tenaka et al have shown that targeted disruption of histone to protamine exchange in mouse spermatids results in increased sperm DNA stainability, reduced chromatin stability and increased sperm head morphologic abnormalities [161]. We have previously observed a strong relationship between percentage of HDS and sperm nuclear histone H2B staining, suggesting that

percentage HDS is associated with an incomplete histone to protamine exchange during spermiogenesis [162, 163].

Although several studies have reported improved semen parameters and pregnancy rates after varicocele repair [145], the true effect of adult varicocelectomy on male fertility remains controversial [137]. A number of investigators have shown that varicocele is associated with an increased level of seminal oxidative stress and that varicocele repair may lower the levels of oxidative stress [18, 116, 123]. More convincing, number of reports have shown that the presence of varicocele was specifically associated with oxidative sperm DNA damage (e.g., high levels of 8-hydroxy-2-deoxyguanosine), suggesting that varicocele impairs spermatogenesis and induces sperm DNA damage as a result of increased oxidative stress [133, 164]. A decrease in sperm DNA damage using 3 different assays (SCSA, AB, IAF), as observed in this study, is a more credible outcome measure that conventional sperm parameters owing to the lower degree of biologic variability of sperm DNA damage [11, 165].

Sperm DNA damage has been associated with reduced potential for natural and IUI-assisted pregnancy [10] . Sperm DNA damage has also been associated with modest reduction in in vitro fertilization (IVF) pregnancy rate and, more importantly, with a significant increase in the risk of pregnancy loss after IVF and intracytoplasmic sperm injection (ICSI) [162]. Of importance is the fact that we did not have a control group of couples who had ICSI before surgery in order to compare them to pregnancy rates of ICSI after varicocelectomy.

E: Conclusion and Summary

In summary, in this prospective study of infertile men with varicocele, we have shown that varicocelectomy is associated with a durable improvement in sperm chromatin compaction and DNA integrity, using 3 different assays. The beneficial effect of varicocelectomy on sperm DNA damage further supports the premise that varicocele may impair sperm DNA integrity, and provides an additional mechanism for the reported improvement in pregnancy rates after varicocele repair. We recognize the limited sample size of the current study, hence, larger and well-designed studies are needed to better define the relationship between varicocele and sperm DNA damage.

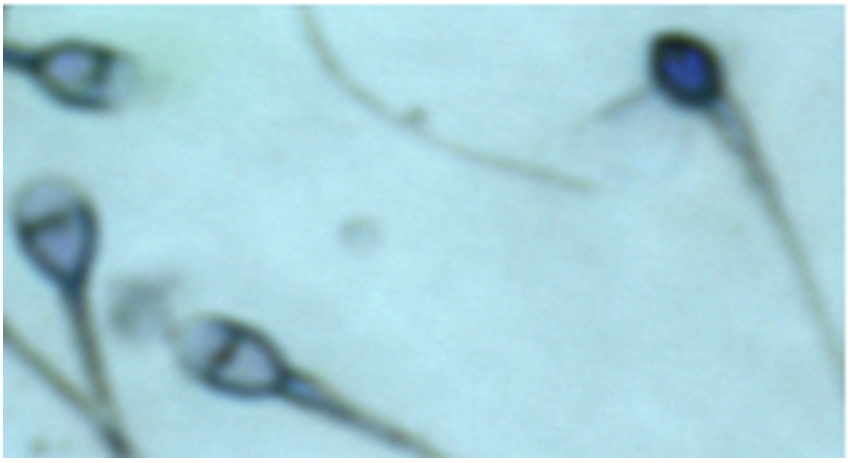

Figure D.4.1: AB Cytochemistry showing a positive AB stain with dark blue and abnormal shape sperm head (upper right) compared to light stain normal looking sperm head (left side of the picture)

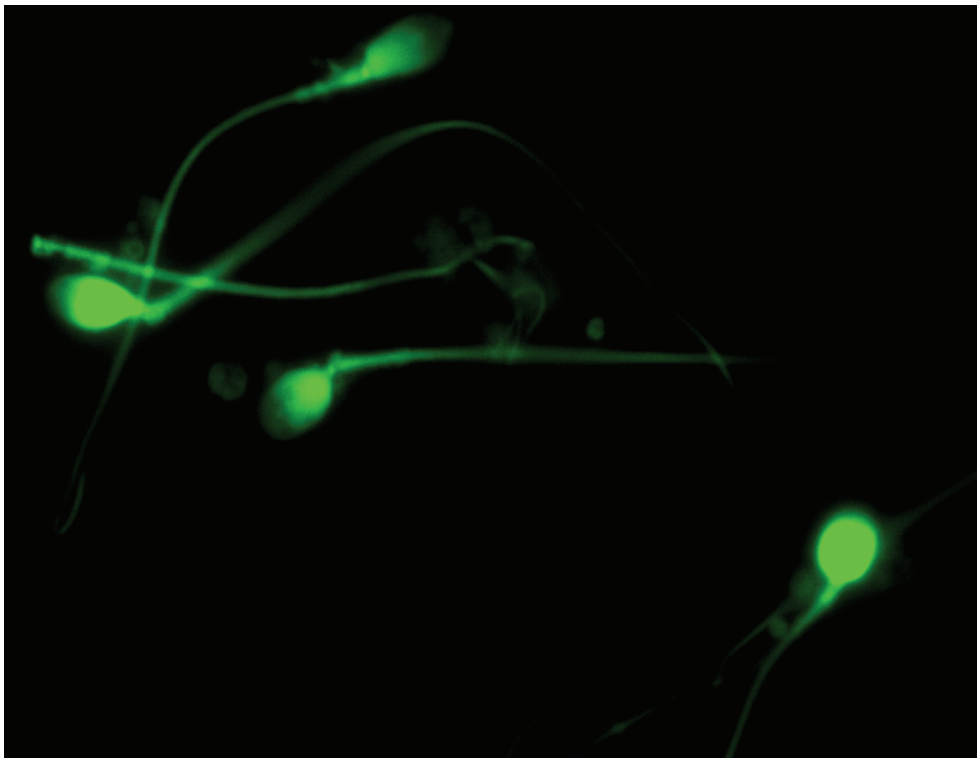

Figure D.4.2: 5-IAF cytochmistry showing intense IAF stain of abnormal looking sperm head (right lower) compared intermediate and light IAF stain (left upper part of the picture).

Table 1. Comparison of sperm DNA fragmentation index (% DFI), high DNA stainability (%HDS), %IAF fluorescence (diffuse head fluorescence) and positive aniline blue stain (dark staining) between infertile patients with varicocele (before surgery) and sperm donors (controls).

Parameter	Varicocele	Controls	P-value
% AB stain	13.5 ± 7	2.5 ± 1	0.0009[a]
%Positive 5-IAF	16.3 ± 6	1.7 ± 1	0.0001[a]
Sperm % DFI	20 ± 10.6	7.4 ± 5	0.011[a]
Sperm % HDS	10.4 ± 6.1	3.6 ± 3.6	0.018

Values are means ± SD;

[a]Wilcoxon signed-ranks test

Table 2. Conventional sperm parameters, %IAF fluorescence (diffuse head fluorescence), positive aniline blue stain (dark staining), sperm DNA fragmentation index (% DFI) and high DNA stainability (%HDS) before, and, 4 months after microsurgical varicocelectomy (n=29).

Parameter	Pre-Op	Post-Op (4-months)	P-value
Sperm concentration ($x10^6$/mL)	42 ± 36	103 ± 123	0.018[a]
Progressive motility (%)	25 ± 15	36 ± 24	0.04
%Positive aniline blue stain	13.5 ± 7	5.4 ± 3.4	0.00003
Positive 5-IAF	16.3 ± 6	5.4 ± 2.7	0.0004[a]
Sperm % DFI (%)	20 ± 10.6	12 ± 5.7	0.001[a]
Sperm % HDS (%)	10.4 ± 6.1	6.4 ± 4.6	0.0009

- Values are means ± SD;
- [a] Wilcoxon signed-ranks test;

Table 3: Pregnancy rates and DFI% change following varicocelectomy

Number	Natural pregnancy	IUI trials/ Pregnancy?	IVF trials/ Pregnancy?	DFI before	DFI after	Comments
1	n	0	0	6.3	8.5	
2	N	1/n	2/n			
3	N	0	0	15.1	8.2	
4	N	1/n	0	5.6	4.4	
5	N	0	0	24.1	21.4	
6	N	3 trials/twin preg (delivered)	0	23	12.4	
7	N	0	0	34.5	22.9	
8	Y	0	0	8.1	14.5	
9	Y	0	0			Pregnant 3 months
10	Y	1, n	0			No more FU
11	N	0	2, y			ICSI twice, first pregnancy lost, second successfull
12	N	0	0	35.8	15.4	
13	N	2, n	1, y			Pregnant at time of telephone call
14	N	0	2, y			Has two kids by icsi
15	N	0	0	13.5	12.6	
16	N	0	0	5.5	11.9	
17	N	1,n	0	38.2	14.9	
18	N	2,n	1,n			

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