

## Original Research Article

# Improved semen parameters and sperm deoxyribonucleic acid fragmentation following microscopic varicocelectomy: short term results of a prospective study

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## ABSTRACT

**Background:** Assessment of sperm deoxyribonucleic acid (DNA) damage post varicocelectomy, has brought about new prospects in treating men with infertility. Improvements in spermatogenesis with decrease in sperm DNA damage has been demonstrated which has led to significant enhancement in fertility rates. Therefore, we evaluated the efficacy of microscopic varicocelectomy on the reduction in sperm DNA damage in concurrence to the conventional improvement of semen parameters.

**Methods:** All patients with a diagnosis of varicocele leading to infertility and planned for microscopic varicocelectomy from January 2013 through September 2019, were included in the study. Sperm chromatin structure assay (SCSA) was done preoperatively and at 3 and 6 months postoperatively for sperm DNA integrity assessment and the results expressed as sperm percentage DFI were compared. A p value <0.05 was considered statistically significant.

**Results:** 105 infertile men underwent microscopic varicocelectomy. Mean age and infertility period from the date of unprotected sex were 26.5±5.6 years and 30 months respectively. Baseline mean sperm concentration was 29 million/ml, mean progressive motility 24% and a percentage DFI ranged from 5–36% with a mean of 16%. Sperm DNA integrity improved significantly with a DFI decrease from 25±11% to 18±6%, 3 months post-surgery which was consistent at 6 months (13±5%). Sperm concentration and progressive motility increased with a mean sperm count improvement by 21.9 million/ml and mean motility by 20.3%.

**Conclusions:** Microscopic varicocelectomy provides durable improvement in DNA integrity and semen parameters. Sperm DNA integrity assessment using SCSA is a useful tool to demonstrate change in the semen quality post treatment.

**Keywords:** Sperm DNA fragmentation, Varicocelectomy, Semen analysis

## INTRODUCTION

Male can be the sole factor responsible for infertility with a frequency almost as high 20-25% of the time.<sup>1</sup> Although various factors are responsible for male infertility, abnormal semen analysis is pivotal to its diagnosis. A routine semen analysis may not provide adequate information and fails to meet the current demands of artificial conception techniques. This calls for the use of

advanced and reliable indicators in diagnosing male factor infertility. Evaluation of sperm deoxyribonucleic acid (DNA) damage is one such indicator which may aid in specific case scenarios.

Sperm DNA fragmentation (SDF) is noted to be significantly higher in patients with infertility leading to poorer outcomes of natural conception.<sup>2</sup> Sperm DNA integrity is considered vital to normal embryogenesis, and

can be damaged by various intrinsic or extrinsic factors.<sup>3</sup> Intrinsic factors include apoptosis during sperm maturation, oxidative stress during transit or following ejaculation and protamination failure. Lifestyle factors such as smoking/obesity, varicocele, infections, radiation exposure and exposure to toxins constitute the predominant extrinsic factors.<sup>4</sup>

Varicocele, an extrinsic factor, leads to oxidative stress contributed by testicular hyperthermia causing a duration-dependent decline in testicular function. The generation of excessive reactive oxygen species (ROS) induced by oxidative stress damages the membrane function and the sperm DNA integrity.<sup>5</sup> Although varicocelectomy is said to improve semen quality by 60–80% in infertile men, contrasting results from various randomized controlled trials has generated controversy questioning its credibility as a therapeutic procedure.<sup>6</sup> Improvisation of conventional semen parameters as an outcome measure after varicocelectomy is essential to maintain its veracity. Testing for sperm DNA integrity and demonstrating improvement is useful in this regard; however, data surrounding this assessment is scarce.

Therefore, we evaluated the efficacy of varicocelectomy, prospectively, with reference to the sperm DNA integrity by assessing the reduction of sperm DNA damage post-surgery in concurrence to the conventional improvement of semen parameters post-surgery.

## METHODS

A prospective study was conducted at department of urology, M.S. Ramaiah Medical College, Bengaluru from January 2013 through September 2019, after obtaining institutional ethical committee clearance. All consecutive patients attending our outpatient clinic with a diagnosis of varicocele causing infertility and planned for microscopic varicocelectomy as per the treatment guidelines, after detailed discussion with the primary consultant regarding the pros and cons, were included in the study.

### *Inclusion criteria*

Married overall healthy men <40 years with infertility with >1 year of unprotected intercourse, clinically palpable varicoceles (grades 1–3), impaired semen analysis (at least one of the following semen characteristics: sperm concentration <20 million/ml or progressively motile sperm <50%) and increased DNA fragmentation index (DFI) were included. All of the spouses <35 years old, had undergone evaluation by gynaecologists and were reported to be normal.

### *Exclusion criteria*

Unilateral or bilateral subclinical varicoceles, recurrent varicoceles, normal semen parameters, azoospermia, additional causes of infertility present concurrently, significant comorbidities, occupational heat exposure and associated female-factor infertility were excluded.

Initial evaluation in the outpatient clinic included a detailed clinical history followed by a thorough clinical examination; ultrasonography (USG) of the scrotum with colour doppler, complete hemogram, random blood sugar, serum creatinine, urine analysis±urine c/s (when indicated), thyroid profile, lipid profile, semen analysis, hormonal assessment inclusive of serum follicle stimulating hormone (FSH), serum luteinizing hormone (LH), and serum total testosterone.

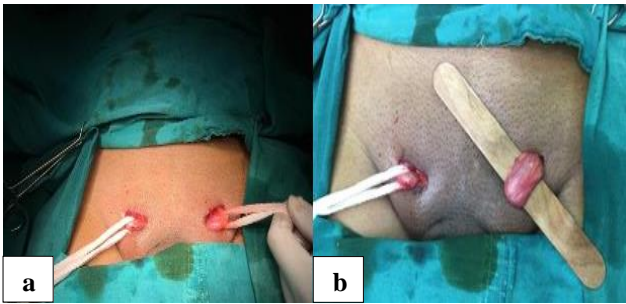
A pre-anaesthetic evaluation was done in all patients prior to the admission/procedure. Informed written consent for the proposed procedure was taken prior to the procedure. An appropriate antibiotic was administered intravenously just before induction and continued until post-operative day one. All of the operations (microsurgical varicocelectomy) were performed by a single surgeon under general anaesthesia in supine position.

### *Surgical technique*

A 3-4 cm subinguinal incision was made below the external ring and deepened through Camper's and Scarpa's fascia (Figure 1). The spermatic cord is grasped using a Babcock clamp, delivered and placed over a wooden stick (Figure 2a and b). The operating microscope (Leica MS1) was then brought into the field (Figure 3). Under 5-6 power magnification, the external and internal spermatic fasciae were opened and the magnification of microscope increased to 10X. Subtle pulsations were utilized as a guide to locate the underlying internal spermatic artery (or arteries). In case identification was not possible, an intraoperative USG probe was used to identify the same. Once identified, the artery was dissected free of all surrounding veins and lymphatics. Internal spermatic veins within the cord, and cremasteric veins with the exception of vasal veins, were ligated by using two 5-0 silk ligatures beneath the vein (Figure 4a and b). At the end of the procedure, only the testicular arteries, cremasteric arteries, cremaster muscle fibers, nerves, lymphatic vessels, and vas deferens with its vessels were preserved. Spermatic cord was then returned to its bed following adequate haemostasis and skin closed using a running 4-0 monocryl subcuticular suture reinforced with steri-strips.



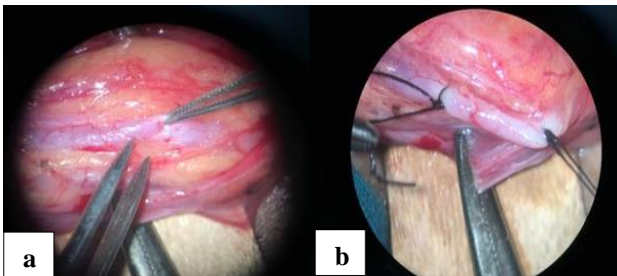
**Figure 1: Initial step: a 3-4 cm subinguinal incision made below the external ring.**



**Figure 2: (a) Incision deepened through Camper's and Scarpa's fascia, and (b) the spermatic cord grasped using a Babcock clamp, delivered and placed over a wooden stick.**



**Figure 3: The operating microscope (Leica MS1) being brought into the field after delivering the spermatic cord.**



**Figure 4: (a) Identification and dissection of internal spermatic veins within the cord, and (b) followed by ligation using two 5-0 silk ligatures.**

#### *Semen sample collection*

Semen samples were collected after 3–5 days of sexual abstinence, preoperatively at least a week apart (with the second taken into account) and postoperatively at the end of 3 and 6 months. Post liquefaction, standard semen parameters (volume, concentration, motility) were obtained using a computer-assisted semen analyzer (CASA). All of the semen samples demonstrated a motile sperm and none had significant numbers of round cells or leucocytospermia as per World Health Organization (WHO) guidelines (<1 million round cells/ml).

Further, a sample from the original sample were frozen at 70-degree C for later evaluation of sperm DNA damage

integrity by the sperm chromatin structure assay (SCSA) and the results were expressed as sperm percentage DFI (an index of DNA damage).

Descriptive analysis was carried out by mean and standard deviation for quantitative variables, frequency and proportion for categorical variables and results expressed as mean±one SD. Differences between the pre and post treatment parameters were estimated by Wilcoxon signed-ranks test. Analysis of variance (ANOVA), used wherever appropriate. The calculations of correlation coefficients between parameters (variables) were performed using a nonparametric procedure, the Spearman rank-order correlation. A p value <0.05 was considered statistically significant. IBM statistical package for the social sciences (SPSS) version 22 was used for statistical analysis.

## **RESULTS**

A total of 105 infertile men were included in the final analysis. The average age of the 105 infertile men recruited for this study was 26.5±5.6 years (21-40) and the infertility period from the date of unprotected sex was 30 months (15-96). Grade of the varicoceles of the 105 men varied as depicted in Table 1 with unilateral varicocele in 23 and bilateral in 82 men. Mean left and right testicular volumes were 13.2±4.2 and 13.9±3.2 ml, respectively. FSH, LH and total testosterone levels were well within normal limits (Table 1). Baseline mean sperm concentration was 29 million/ml, mean progressive motility 24% and a percentage DFI ranged from 15–36%.

**Table 1: Demographic characteristics of the study population (n=105).**

Parameters (n=105)	Value
Age (years)	26.5±5.6 (21-40)
Age, female partner (years)	25.7±6.9 (19-35)
Infertility interval in months	30 (15-96)
<b>Grade of Varicocele</b>	
1	20 (19.04)
2	74 (70.4)
3	11 (10.4)
<b>Unilateral</b>	23 (21.9)
<b>Bilateral</b>	82 (78.09)
<b>Volume of left testis (ml)</b>	13.2±4.2
<b>Volume of right testis (ml)</b>	13.9±3.2
<b>Sperm counts (sperms/ml)</b>	29.0±20.6
<b>Progressive motility (%)</b>	25±10.8
<b>FSH (IU/ml)</b>	5.4±2.8
<b>Testosterone (nmol/l)</b>	4.2±1.1
<b>LH (IU/ml)</b>	4±1 IU/l

Table 2 demonstrates the distribution of semen parameters below threshold levels in our study population. Sperm DNA integrity improved significantly at 3 months after surgery (% DFI decreased from 25±11% before surgery to 18±6% at 3 months after surgery). Sperm concentration and progressive motility showed improvement in 92 and

84 patients (87.6% and 80%) respectively at 3 months post-surgery. Sperm count improved by 21.9 million/ml, and mean motility by 20.3%. Both sperm concentration and progressively motile sperms improved for 82 patients (78.09%). 96 patients of the 105 infertile men recruited for this study submitted a second post-operative semen sample at 6 months after surgery. Sperm DNA integrity showed persistent improvement at 6 months after surgery (n=96; 13±5%, at 6 months after surgery). Although the differences did not significantly improve when compared with the third month values, sperm concentration and progressive motility improved with reference to the before surgery values.

**Table 2: Preoperative semen parameters in the study cohort (n=105).**

Parameters below threshold (n=105)	No. of pts (%)
<b>Sperm count &lt;15 million sperms/ml</b>	25 (23.8)
<b>Progressive motility &lt;32%</b>	23 (21.9)
<b>Morphology &lt;4% of normal forms</b>	4 (3.8)
<b>Count + motility</b>	30 (28.57)
<b>Count + morphology</b>	8 (7.6)
<b>Motility + morphology</b>	7 (6.6)
<b>3 parameters</b>	8 (7.6)

**DISCUSSION**

Although assessment of semen analysis is the key step in the management strategy, the advent of assessing the sperm DNA damage has opened up new prospects in men with infertility. DNA breaks are a physiological phenomenon occurring throughout life, needed for transient relief from torsional stress. These temporary breaks, when left unrepaired lead to genetic mutations or DNA fragmentation in the ejaculate.<sup>7,8</sup> De Iuliis et al hypothesised that an increase in histone to protamine ratio following oxidative stress leads to defective chromatin compaction.<sup>9</sup> ROS play an important physiological role of modulation gene and protein activities vital for sperm proliferation. When in excess, ROS is pathological to spermatozoa leading to DNA fragmentation.<sup>4</sup>

Sperm DNA damage is known to be associated with diminished potential for natural and IUI-assisted pregnancy decreased in in-vitro fertilization (IVF) pregnancy rate and, a significant rise in pregnancy loss risk following IVF and intracytoplasmic sperm injection (ICSI).<sup>10-13</sup> The impact of sperm DNA damage on late reproductive (foetal and post-natal) outcomes in humans still remain unknown. However, experimentally, sperm DNA damage has negative effect on ICSI embryo development, pregnancy rates and offspring health and genomic stability.<sup>14</sup>

Currently, sperm DNA fragmentation test is indicated in male infertility in couples with unexplained infertility,

history of multiple failed IVF/ICSI treatment, history of recurrent miscarriage in partner, varicocele, poor semen parameters and advanced age.<sup>2,15</sup> Tests for sperm DNA damage can be either direct or indirect and are labelled by single or double stranded DNA breaks. Direct methods include Comet – single cell gel electrophoresis, Tunel – terminal deoxy nucleotide transferase mediated UTP nick end-labelling and dye tests. Sperm chromatin dispersion and sperm chromatin structure assay (SCSA) are indirect techniques that require denaturation of DNA.<sup>16</sup>

Our study used SCSA, an indirect flow cytometry test which is at present the only test with clear and clinically useful cut off levels. The concept of this test based upon the fact that DNA in a sperm with abnormal chromatin structure is more prone to acid or heat denaturation. SCSA estimates the susceptibility of sperm DNA by quantifying the metachromatic shift of acridine orange (AO) from green to red following acid treatment. Using flow cytometry, the extent of DNA denaturation is determined unlike the visual counting of red and green cells in AO test. Its advantages are high reproducibility, rapid examination of many cells, examination of both fresh and frozen samples with well-established clinical thresholds. On the contrary, it detects only single strand DNA breaks, is expensive and not available as commercial kits.<sup>17</sup>

Our study cohorts included male infertility associated with varicocele and we looked into post treatment improvement in DNA sperm integrity using SCSA. Previous studies have shown that varicocelectomy is associated with a decrease in sperm DNA fragmentation (by SCSA or TUNEL assay), but majority were of retrospective design unlike our current prospective study.<sup>18</sup> Our observations following microscopic varicocelectomy revealed significant decrease in the sperm DNA damage (decreased sperm DFI) with improvements in sperm DNA integrity and overall seminal parameters) post intervention. An increase in the mean sperm count and mean motility was also associated. The observed increase in sperm DNA integrity i.e. reduction in percentage DFI is in concurrence with the reported reduced effect of sperm DNA damage post-intervention. The reduction in DFI percentage at 3 months post-surgery is sustained at 6 months, is strong evidence in support of a favourable effect of varicocelectomy on sperm DNA integrity The observed improvements in sperm DNA integrity and overall semen parameters following varicocele repair support the hypothesis that varicocelectomy improves spermatogenesis and lower the levels of oxidative stress.<sup>19</sup> A decrease in sperm DNA damage (as observed in our study) has more credibility than semen analysis parameters owing to the lower degree of biologic variability of sperm DNA damage.<sup>20</sup> The mean baseline percentage DFI was not high (i.e. >30%) in our population, which further validates the results. Reduction in sperm percentage DFI (from 25 to 13%) is potentially clinically significant based on the reported influence of sperm DNA fragmentation on reproductive outcomes.<sup>14-16</sup>

However, our study lacks a long term follow-up of the patients and lack of measurement of the ultimate end result of pregnancy outcome either spontaneous pregnancy or the efficacy of ART methods post intervention which is crucial to defining success outcomes. A prospective study with a larger sample size with multi-institutional involvement with assessing the final outcome of pregnancy rates either spontaneously or by ART methods is warranted to ascertain the fact of improvement of the sperm DNA integrity post varicocelectomy. Furthermore, SCSA for different scenarios in infertility and treatments other than varicocelectomy needs to be validated. Whether sperm DNA integrity would replace the more conventional easily available semen analysis is yet to be seen.

## CONCLUSION

For male infertility associated with varicocele, microscopic varicocelectomy provides durable improvement in DNA integrity and semen parameters. Sperm DNA integrity assessment is a useful tool to demonstrate change in the semen quality post treatment and should be used routinely.

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