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A Randomized Clinical Trial Study of the Effects of Varicocelectomy on Sperm Clinical Analysis and DNA Fragmentation: A Preliminary Data

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ABSTRACT

Varicocele is a disease characterized by abnormal dilatation of the testicular veins within the pampiniform plexus due to difficult blood return in this region. The prevalence of varicocele is 30 to 40% in infertile men, who frequently show seminal changes and a high rate of sperm DNA Fragmentation Index (DFI), one of the conditions responsible for failure of spermatogenesis. Several studies have demonstrated that varicocelectomy may improve the seminal parameters and even the quality of genetic material. However, published papers are predominantly retrospective rather than prospective. In this study, we show some effects of varicocelectomy on the seminal quality and DFI rate of men who were submitted compared to men who did not undergo the procedure, before and after nine months of the intervention. This preliminary data was a prospective, randomized and controlled clinical trial in which 15 men with varicocele and five men without varicocele (control group) were included. In the varicocele group, five patients were submitted to varicocelectomy and 10 were not. The DFI rate was analyzed using the Halosperm G2® kit and the seminal parameters were assessed according to the criteria established by the World Health Organization (WHO), 2010. A difference in DFI rate was detected between the varicocele (52% ± 0.28) and the control group (27% ± 0.07). In the varicocele group, no differences in DFI rate were observed between patients submitted to varicocelectomy and patients who were not submitted to the procedure, however, we could observe a sharp increase in the sperm concentration of patients who underwent surgery (28.33 x 10^6/ml ± 26.91) compared with not submitted varicocelectomy (3.03 x 10^6/ml ± 14.74). These preliminary results emphasize that men with varicocele have a higher DFI rate.

KEYWORDS: Varicocele; Varicocelectomy; DNA fragmentation; Spermatozoa; Seminal parameters.

ABBREVIATIONS: DFI: DNA Fragmentation Index; ROS: Reactive Oxygen Species; SCD: Sperm Chromatin Dispersion.

INTRODUCTION

Varicocele is a disease characterized by abnormal dilatation of the testicular veins within the pampiniform plexus due to difficult blood return in this region. The disease is detected in approximately 20% of adults and adolescents and in 19 to 41% of men who seek treatment of infertility.[1] Among infertile patients, 30 to 35% have primary infertility and 69 to 81% have secondary infertility.[2]

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This anatomical anomaly is one of the major and most recurrent causes of low and poor quality sperm production. In 1965, MacLeod[3] was the first to report that the spermatozoa in most of the semen samples from patients with varicocele showed a low concentration, reduced motility and, more frequently, morphological abnormalities compared to the gametes of fertile men. Similar results were recently reported by Xue, et al.[4] and Kadioglu, et al.[5]

The mechanisms leading to failure of spermatogenesis in patients with varicocele are not fully known. Increased scrotal temperature is pointed out as one of the major causes of sperm alterations since the debilitated circulation caused by varicocele in the pampiniform plexus induces an increase in temperature in the region, which may lead to failure of spermatogenesis and damage to sperm DNA.[6,7]

Damage to sperm DNA such as DNA Fragmentation Index (DFI) is associated with male infertility, with unsuccessful pregnancy and genetic changes in the offspring.[8] Several factors can lead to DNA damage, such as mutations by radiation, errors introduced during DNA replication, environmental insults, epigenetics alterations,[9] apoptosis and increased production of Reactive Oxygen Species (ROS).[10] A global estimate reported by Wang[11] indicated that patients with varicocele have significantly greater sperm DNA damage than fertile controls, with a mean difference of 9.84%. Enciso[12] reported that the frequency of spermatozoa with fragmented DNA was 32.4% in patients with varicocele, a value 2.6 times higher than that observed in fertile individuals.

The DFI present in patients with varicocele can be reversible. Zini et al.[13] reported that infertile men showed improved sperm DNA integrity six months after surgical repair of varicocele. Similarly, Kadioglu, et al.[5] observed a significant improvement of all seminal parameters and of DFI compared to preoperative values.

Several studies have demonstrated improved seminal parameters after surgery, although the studies were predominantly retrospective rather than prospective and were not randomized.[7] Another weak point of previous studies is that the patients served as their own controls in the assessment of the effect of varicocelectomy. We still do not know whether the simple counseling received by the patients during the months on the waiting list could have been a treatment comparable to surgical treatment of varicocele, and therefore a group of randomly selected patients should be considered for the observation of this effect.[14]

The true effect of varicocelectomy on male fertility continues to be a matter of controversy and many studies have been conducted to elucidate the effectiveness of surgical remediation in sperm concentration, morphology and motility. On this basis, the objective of the present study was to determine the effect of surgical correction of varicocele on seminal quality and DFI compared to subjects who did not undergo the procedure, before and nine months after the intervention.

MATERIALS AND METHODS

Patients

This was a preliminary, controlled, open and randomized study with random subject allocation to parallel groups, approved by the Research Ethics Committee of the University Hospital, Ribeirao Preto Medical School of University of Sao Paulo (UH-RPMS/USP) and by the National Ethics and Research Committee (CONEP).

The varicocele group consisted of men aged 18 to 45 years invited to participate, who had grade II or III varicocele and who accepted surgical intervention (varicocelectomy). The patients were recruited at the Infertility Outpatient Clinic of the Sector of Human Reproduction, Department of Gynecology and Obstetrics, UH-RPMS/USP, from February 2013 to March 2015. The control group consisted of men without varicocele, with normal seminal parameters aged 18 to 45 years. All subjects were included in the study after giving informed consent to participate.

The selected patients with varicocele were divided randomly into two groups: group A consisted of five patients submitted to microsurgery for the correction of varicocele and group B consisted of 10 patients who were not submitted to surgery. Sealed envelopes were used for random allocation of the participants, with equal quantities for each group. Each envelope contained a single number corresponding to intervention or not as determined by using the *Random Allocation Software* (Isfahan University of Medical Sciences, Isfahan, Iran). A control group consisted of five men without varicocele with normal seminal parameters was also included in this study.

Exclusion criteria were: a history of smoking, excessive alcohol use or drug consumption, previous surgery for the correction of varicocele, suspected urogenital infections, cancer or endocrinopathies and their treatment that might influence testicular function.

Varicocelectomy was performed by microsurgery with a microscope and subinguinal incision. The procedure consisted of dissection and isolation of the spermatic cord at the level of the external inguinal ring followed by opening of the cremaster muscle and ligation of the dilated veins with the aid of a microscope for better identification of the veins and preservation of the artery.

Sperm Clinical Analysis

For the execution of the sperm clinical analysis (spermogram) the patients were refrain from sexual activity for 3 to 5 days the semen were collected by masturbation and ejaculation

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into sterile glass cups. The samples were evaluated according to the protocols of the Laboratory of Andrology, Sector of Human Reproduction, UH-RPMS/USP. The semen was analyzed for sperm concentration, percentage motility, and morphology according to WHO criteria (WHO).[15]

Macroscopic analysis of the sperm was performed, with observation of liquefaction time, color, volume, viscosity and pH. For microscopic analysis, the *Makler* counting chamber (Sefi-Medical Instruments Ltd. Haifa, Israel) was used for the sperm concentration count and the determination of sample motility. Vitality was measured using *Eosin Y* 0.5% *dye* (Eosin Gelblich – Merck-CI 45380, Darmstadt, Germany). Sperm morphology was determined according to Krüger criteria using *Nigrosin 8%* staining technique (Nigrosin, Water Soluble – Sigma-CI 50420, Darmstadt, Germany).[16]

Analysis of Sperm DNA Fragmentation

To determine DFI was used the Sperm Chromatin Dispersion (SCD) technique with the *Halosperm G2® kit* (Halotec DNA, Madrid, Spain). The sperm sample was diluted in Phosphate Buffer Saline (PBS) to $20x10^6$ spermatozoa/ml. Next, a 50 µL sample of diluted sperm was added to 100 µL agarose previously melted in a water bath at 95-100 ºC for 5 minutes. Of the semen-agarose mix, 20 µL were pipetted onto slides precoated with agarose provided in the kit, and covered with coverslip. The slide was then placed in a refrigerator (4 ºC) for 5 minutes for agarose solidification. The coverslips were gently removed. Approximately 32 µL of denaturing acid solution belonging to the kit was placed over the sperm sample, and incubated for 7 minutes. The same quantity of lysis solution, also belonging to the kit, was added and incubated for 20 minutes. After washing 5 minutes in a tray with abundant distilled water, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 2 minutes each and then air-dried. Dye 1 and 2 (belonging to the kit) were added and left in contact with the samples for 20 minutes each one. The slides were allowed to dry at room temperature. Readings were taken with a phase contrast microscope equipped with a 40X objective and 100 spermatozoa of each patient were counted.[17]

RESULTS

Most patients presented the varicocele on the left side and only two had in both sides (bilateral). The degree of the disease observed was six with grade II (40%) and nine grade III (60%).

The descriptive variables of the seminal parameters and DFI of men with and without varicocele is represented in Table 1. The participants showed similar mean age, pH, morphology and volume. With regard to seminal parameters evaluated between two groups, we observed that men with varicocele presented lower mean of sperm count, motility and vitality and higher rate of immotile sperm compared to controls. The mean DFI seems to be higher in those patients with varicocele (52% ± 0.28) than controls (27% ± 0.07).

Variables	Varicocele (N=15) Mean ± SD	Control (N=5) Mean ± SD
Age (years)	29.33 ± 5.72	27.80 ± 3.56
DFI (%)	52.00 ± 0.28	27.00 ± 0.07
Sperm count ($x10^6$/mL)	28.19 ± 28.63	67.80 ± 53.08
Motility PR (%)	23.00 ± 0.17	36.00 ± 0.09
Motility NP (%)	40.00 ± 0.14	42.00 ± 0.11
Immotile sperm (%)	37.00 ± 0.23	21.00 ± 0.08
Vitality (%)	80.00 ± 0.20	85.00 ± 0.06
Morphology (%)	3.00 ± 0.02	3.00 ± 0.01
pH	8.63 ± 0.35	8.80 ± 0.27
Volume (mL)	2.79 ± 1.21	2.54 ± 0.75

N: number of patients in each group; DFI: DNA fragmentation index; PR: progressive; NP: non-progressive; SD: standard deviation.
Table 1: Descriptive data of the age, seminal parameters and DNA fragmentation index (DFI) of the patients with and without varicocele.

Figure 1 illustrates a light microscope field visualized after the technique of chromatin dispersion in human sperm. Spermatozoa with intact DNA exhibit a halo and spermatozoa with no halo have fragmented DNA.

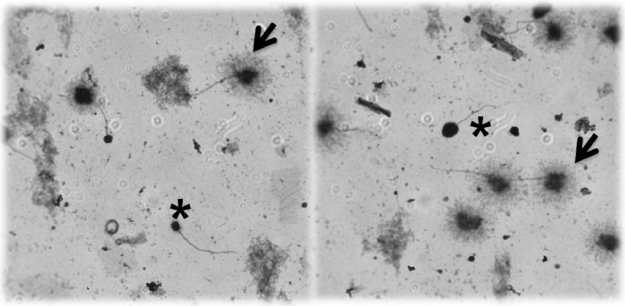

Figure 1: Microscopic field visualized after staining with Sperm Chromatin Dispersion (SCD) technique. Those sperm cells with fragmented DNA are indicated by an asterisk, and sperm with big halo of DNA dispersion are indicated by an arrow. (100x)

The mean of the difference scores (delta) evaluated before and after nine months in the groups who underwent or not the varicocelectomy are presented in Table 2. The analyzed variables age, DFI, motility, vitality, morphology, pH and volume seems not chance with surgery. However, there was a marked increase in sperm count in the group that was submitted to surgery.

DISCUSSION

Our preliminary findings show that the mean DFI was higher in those patients with varicocele than controls. The DFI and seminal parameters between patients submitted or not to varicocelectomy appears to be similar. However, we observed an increase in sperm count in the group submitted to surgery.

Despite contradictory studies, varicocele is considered to be one of the major causes of male subfertility. The etiology

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Variables	Non-varicocelectomy (N=10) Mean ± SD	Varicocelectomy (N=5) Mean ± SD
Age (years)	29.00 ± 6.45	30.00 ± 4.47
DFI (%)	-0.12 ± 0.26	-0.26 ± 0.32
Sperm count (x10^6/mL)	3.03 ± 14.74	28.33 ± 26.91
Motility PR (%)	-0.01 ± 0.06	0.00 ± 0.06
Motility NP (%)	0.02 ± 0.09	-0.01 ± 0.09
Immotile sperm (%)	-0.02 ± 0.11	0.01 ± 0.07
Vitality (%)	0.02 ± 0.12	-0.04 ± 0.08
Morphology (%)	-0.02 ± 0.02	-0.01 ± 0.03
pH	0.08 ± 0.39	0.30 ± 0.27
Volume (mL)	-0.08 ± 1.35	0.30 ± 1.86

N: number of patients in each group; DFI: DNA fragmentation index; PR: progressive; NP: non-progressive; SD: standard deviation.

Table 2: Mean of the difference scores (delta) evaluated before and after nine months in the groups who underwent or not the varicocelectomy

of sperm DNA damage is multifactorial and the condition may occur in a spontaneous or accidental manner.[5] A failure spermatogenesis process may result in the generation of sperms with high levels of DNA damage.[18] This damage may lead to apoptosis during spermatogenesis or may generate spermatozoa which started to undergo this process but for some reason escaped programmed cell death.[19] Apoptotic cells show a sequence of characteristic morphological events including chromatin condensation and margination, changes that are associated with genomic DNA cleavage into multiple 180 base of pairs (bp) fragments.[20]

We observe that men with varicocele presented higher DFI than controls. Sperm DNA fragmentation is a multifactorial disease and environmental conditions may improve the DFI, in which oxidative stress and apoptosis are main causes.[21] In accordance with our findings, Wang, et al. showed an increasing in DNA damage in sperms from patients with varicocele[11] and DNA fragmentation may be an expression of increased ROS. ROS may cause defective sperm function as a result of lipid peroxidation of the polyunsaturated fatty acids in the plasma membrane, alter sperm morphology, lead to decreased motility and ineffective spermatozoon-oocyte fusion.[22] In addition, spermatozoa from patients with varicocele exhibit increased levels of 8-hydroxy-2 deoxyguanosine associated with a deficiency of the pro-oxidant defense system and may cause oxidative DNA damage, base modification, DNA chain breaks and chromatin crosslinking since the sperms have limited defense mechanisms against oxidative attack to their DNA.[23]

It has been reported that the surgical treatment of varicocele improves semen quality of infertile men with a clinically palpable varicocele.[24] The DFI values between the groups submitted or not to varicocelectomy seems not be different between the groups. In addition, we observed an increase in sperm concentration in the group submitted to surgery. A significant increase in the sperm concentration rate after varicocelectomy has been reported previously, together with an increased sperm motility and morphology.[5,25]

Venous hypertension, which is the hydrostatic column that generates pressure on diseased gonadal venous valves in patients with varicocele, together with reflux of toxic renal metabolites in the testicles, may cause chronic vasoconstriction of testicular arterioles.[10] Changes in the gametes may be related to reflux of the metabolites, reduction of blood flow volume, and anoxia,[26] in addition to an increase in temperature in the region.[7] Varicocelectomy, which involves ligation of all internal spermatic veins, prevents a retrograde blood flow[7] and normalizes the system of countercurrent heat exchange that involves the pampiniform plexus, thus improving the venous circulation at this site and reducing failure of spermatogenesis.

The limitation of the present study was that we did not perform the statistical analysis because the sample size is small and it would not be possible to complete the normality test, since neither the shape of the error distribution nor the presence of outliers can be judged with reasonable confidence. Thus, increasing the sample size is necessary to confirm these findings.

Based on these preliminary data, we observed that varicocele may be related to a higher percentage of spermatic DNA damage. A larger patient series is needed to assess the effect of surgical correction. The varicocelectomy could be a possible treatment for the reduction of damage to male gametes and improvement of seminal parameters.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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DISCLOSURES

This study was approved by the Research Ethics Committee of the University Hospital, Ribeirao Preto Medical School of University of Sao Paulo (UH-RPMS/USP) (CEP) and by the National Ethics and Research Committee (CONEP) and all subjects were included in the study after giving informed consent to participate (TCLE).

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