Original Article

Can Altered Expression of HSPA2 in Varicocele Patients Lead to Abnormal Spermatogenesis?

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Abstract

Background: Heat shock protein A2 (HSPA2) is correlated with sperm maturity and function. Therefore, dysfunctional expression of this gene results in abnormal spermatogenesis. On the other hand, DNA damage in spermatozoa is considered to be an important cause of male infertility, and the presence of sperm with DNA fragmentation and chromatin abnormalities in human ejaculates is well documented, in particular in men with poor semen quality. Therefore, the aim of this study is to evaluate HSPA2 expression and its relation with DNA fragmentation, protamine deficiency involved in DNA packaging and semen parameters in varicocele patients in comparison to fertile men before and after varicocelectomy.

Materials and Methods: This study included 52 fertile individuals as the control group and 70 infertile individuals with varicocele as the experimental group. Sperm DNA fragmentation, protamine deficiency and relative HSPA2 expression were evaluated by the sperm chromatin dispersion test, chromomycin A3 staining and RT-PCR, respectively.

Results: The mean values of abnormal morphology, protamine deficiency and DNA fragmentation were significantly lower in varicocele individuals following varicocelectomy when compared to fertile individuals. The correlation between these parameters were studied and discussed in the text.

Conclusion: There is a decrease in relative HSPA2 expression which is possibly due to chronic induced hyperthermia in varicocele individuals. Removal of this stress increases HSPA2 expression and results in the proper folding of proteins involved in spermatogenesis; therefore resulting in improved DNA packaging, as well as better sperm morphology and motility which may indirectly reduce sperm DNA fragmentation.

Keywords: Varicocele, HSPA2, Protamines, DNA Fragmentation, Surgery

Introduction

Abnormal tortuosity and enlargement of the pampiniform plexus vein within the spermatic cord is called varicocele. This abnormality is associated with male infertility and may result in distress for couples hoping to conceive (1-4). The incidence of varicocele ranges from 9-23% in the fertile population and its frequency increases to 40% in infertile patients. The negative impact of varicocele and its related mechanism on male infertility, although well debated in the literature, remains to be elucidated (5-7). Despite several advocated theories describing the mechanisms through which varicocele may lead to infertility; gonadal hyperthermia is the most accepted (8). This condition can make germ cells more likely to undergo apoptosis during specific stages of spermatogenesis (9), possibly by reducing DNA, RNA and protein synthesis or make them susceptible to damage (10, 11).

Hyperthermia, being involved in DNA damage, has been advocated as one of the underlying causes of varicocele. Three major mechanisms have been suggested for sperm DNA damage in varicocele, which are: 1. high produc-

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tion of reactive oxygen species (12), 2. reduced antioxidant capacity (9) and 3. protamine deficiency (13).
For adaptation and overcoming exogenous stress, such as exposure to high temperatures, prokaryotic and eukaryotic organisms have implemented sophisticated approaches such as the production of conserved cellular proteins named heat-shock proteins (HSPs) (11, 14). Some members of the HSPs are tissue specific and have a bioregulatory function, especially in protein biosynthesis, folding, transport and clearance. HSPA2, a member of the 70-kDa family, is testis tissue specific and has been shown to function as a chaperone protein which aids in protein folding (11, 15). Furthermore, deficiency of this gene in knockout mice or a mutation in this gene results in the arrest of primary spermatocytes at stage I of meiosis; thus leading to azoospermia or infertility in mice and thereby indicating an important role for HSPA2 in spermatogenesis (16).
Protamines are one of the major proteins involved in DNA packaging and protecting sperm from DNA damage. Therefore, considering the role of HSPA2 in protein folding and previous reports showing that expression of protamines is relatively lower in varicocele individuals; thus the aim of this study is to investigate and compare the relative expression of HSPA2 in varicocele patients to fertile individuals. In addition, this study also aims to observe if there is a relation between relative expression of HSPA2, protamine deficiency, and sperm chromatin integrity and to investigate the role of varicocelectomy on these parameters.

Materials and Methods

Semen sample preparation

Semen samples were obtained from 70 individuals with male factor infertility who presented with grades II and III varicocele and from 52 fertile individuals without varicocele, as the control group. Individuals included in this study did not have a history of systemic illnesses, cryptorchidism, orchitis, epididymitis, urethritis or testicular atrophy. Female infertility was ruled out in the wives of these 70 individuals and wives of the 52 individuals were pregnant at the time of semen collection. The study was approved by the Institutional Review Board and informed consents were signed by individuals who provided semen for this study.
Semen density and motility were analyzed according to World Health Organization guidelines (17) and sperm morphology was assessed according to strict criteria (18) by a single observer using CASA Sperm Analysis (Computer Aided Sperm Analysis). Semen samples were washed twice using Ham's F-10 solution. The resulting sperm pellet was used for RNA extraction, assessment of DNA fragmentation, protamine deficiency and sperm morphology.

Total RNA preparation and reverse transcription polymerase chain reaction (RT-PCR) analysis

In this study, RT-PCR was mainly based on Lima et al. (11). RNA extraction was carried out using RNXplus reagent (Cinnagen, Iran). To eliminate any DNA contamination, extracted RNA was treated with DNase I. According to the manufacturer's protocol, 2 μg of treated RNA was used for complementary DNA (cDNA) synthesis with RevertAid H Minus M-MuLV reverse transcriptase (Fermentase Corporation, Lithuania) and oligo (dT) 18. By the use of specific primers for β-actin and HSPA2 (Table 1), polymerase chain reaction (PCR) was carried out using 2 μL of cDNA as follows: DNA denaturation for 10 minutes at 94°C, followed by 36 cycles (1 minute at 94°C, 1 minute at 57°C and 1 minute at 72°C) and a final extension for 10 minutes at 72°C. Each PCR was performed under linear conditions. cDNA amplification was assessed on 1.5% agarose gels that were stained with ethidium bromide (Cinnagen, Iran). The gels were then analyzed with scanner system gel documentation (Gene Flash, UK). The HSPA2/β-actin ratio was assessed in patients with varicocele and fertile individuals by Syngene Tools from Syngene software (version 3.06) (11).

Table 1: Oligonucleotide sequences used for RT-PCR analysis

Transcript	(Direction 5′-3′) Sequence
HSPA2	F: TTG TTG GAA GTC TTT GGT ATA R: CAT TTG CAT TTA TGC ATT TGT
β-actin	F: CGT GAC ATT AAG GAG AAG CTG TGC R: CTC AGG AGG AGC AAT GAT CTT GAT

Sperm chromatin dispersion test (SCD test) and chromomycin A3 (CMA3) staining

There were 5 - 10 million/ml washed and diluted semen samples used for the SCD test, which was carried out according to a procedure by Fernandez et al. Slides were stained with Wright's stain (Merck 1.01383.0500) for light field microscopy viewing. Sperm nuclei without fragmented DNA exhibited large or medium-sized halos, whereas sperm that DNA were fragmented appeared with small halos, no halos and solidly stained cores, or no halos and irregular or faintly stained cores (degraded). A minimum of 200 sperm per sample were scored under the x100 objective of the microscope. CMA3 staining was carried out according to Nasr-Esfahani et al. (19, 20).

Statistical analysis

The Kolmogorov-Smirnov Z test was used to assess normal data distribution. Coefficients

of correlation and the student's t-test were carried out using the Statistical Package for the Social Studies (SPSS 11.5; Chicago, IL) software. When comparison was carried out between the fertile and varicocele group the independent t test was used; while for comparison between varicocele before and after surgery, the paired t test was used.

Results

There were 70 varicocele individuals included in this study of which all underwent varicocele surgery. At six months post-surgery each individual was asked to provide a second semen sample, out of which only 30 agreed. Only 39 out of 70 varicocele individuals provided information on the status of fertility post-surgery. Of these, the partners of 15 individuals were pregnant which resulted in a pregnancy rate of 38.5%. From these 15 individuals, only 8 provided a second semen sample six months post-surgery.

RT-PCR analysis of HSPA2 and β-actin gene expression in sperm from four varicocele (lanes 1-4) and four fertile individuals (lanes 5-8) are shown in figure 1A, thus revealing a uniform pattern of β-actin gene expression and a variable expression of HSPA2 in different individuals. RT-PCR analysis of the expression of HSPA2 and β-actin genes in an individual with varicocele before, three and six months post-surgery, show a gradual increased expression of HSPA2 post-surgery (Fig 1B). The mean values of HSPA2/β-actin in varicocele patients and fertile individuals were 0.414 ± 0.04 and 0.667 ± 0.08, respectively, which was significantly different ($p<0.01$). Six months post-surgery the relative expression of HSPA2 increased to 0.66 ± 0.08 which was significantly higher than prior to varicocelectomy ($p<0.01$) and not significantly different from fertile individuals (Fig 1C).

In figure 2, the images of CMA3 staining in a varicocele individual before and six months post-varicocelectomy are shown. The mean CMA3 value in fertile individuals was 29.62 ± 2.07 which was significantly lower than in varicocele individuals before surgery (42.15 ± 1.75). In varicocele individuals, the CMA3 value reduced to 31.94 ± 1.74 six months following surgery ($p<0.01$) and was not significantly different from fertile individuals (Fig 2C). A significant correlation between CMA3 positivity and HSPA2 expression (Fig 2D) in the fertile or control group was observed ($r=0.437$, $p=0.03$) whereas the correlation was insignificant in the varicocele group both before ($r = 0.209$, $p=0.11$) and post-surgery ($r = -0.279$, $p=0.13$).

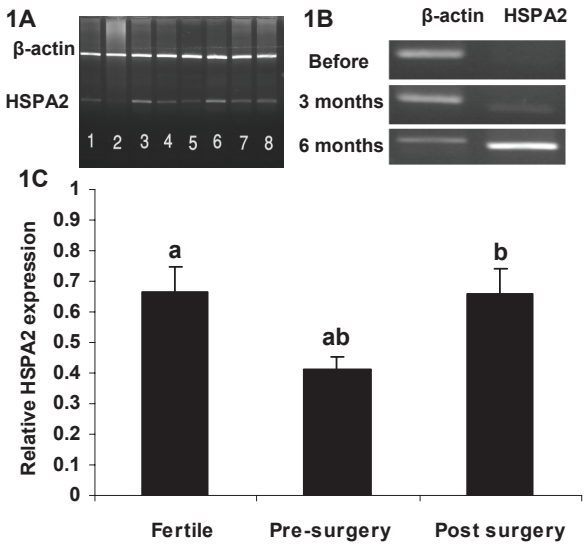

Fig 1: A. RT-PCR analysis of HSPA2 and β-actin in varicocele (lanes 1-4) and fertile (lanes 5-8) individuals. B. RT-PCR analysis of HSPA2 and β-actin genes in an individual with varicocele before, three and six months after varicocelectomy. C. The mean values of relative expression of HSPA2 β-actin in fertile and varicocele individuals before and after surgery. Common letters are significantly different for $p<0.05$.

An image of the SCD test, representing DNA fragmentation, before and post- surgery has been shown in figures 3A and 3B, respectively. A comparison of the mean sperm DNA fragmentation between the fertile and varicocele groups before and six months following varicocelectomy (Fig 3C) has shown a significantly lower value in the fertile group (36.73 ± 2.57) versus the varicocele group (45.69 ± 2.43, $p=0.01$). Of note, the mean sperm DNA fragmentation value in the varicocele group reduced to 35.9 ± 2.4 ($p= 0.01$) by six months following surgery which was not significantly different from the control group ($p=0.83$). The correlation between sperm DNA fragmentation as assessed by the SCD test with relative expression of HSPA2 in fertile and varicocele individuals before and six months post-surgery showed only a significant negative correlation between relative HSPA2 expression with percentages of DNA fragmentation in fertile individuals ($r = -0.585$, $p<0.01$, Fig 3D).

Images of sperm morphology before and post-surgery are shown figures 4A and B, respectively. The mean percentages of abnormal sperm morphology in the control group, and in varicocele individuals before and six months post-surgery were 65.78 ± 1.38, 74.93 ± 1.34 and 67.23 ± 2.33, respectively (Fig 4C). The results were significantly different between

fertile and varicocele individuals before surgery and were insignificant with six months post-surgery ($p<0.05$). An analysis of the correlation between relative HSPA2 expression and the percentage of abnormal morphology (Fig 4D) in fertile and varicocele individuals, before and six months post-surgery, showed no significant correlation in fertile ($r = 0.296$, $p= 0.19$) and varicocele individuals before surgery ($r=0.185$, $p=0.21$); however a significant negative correlation was observed between these two parameters in varicocele individuals six months post-surgery ($r = - 0.576$, $p<0.01$). The percentages of sperm motility were 57.30 ± 1.71, 48.23 ± 1.81 and 54.35 ± 3.03, in fertile and varicocele individuals, before and six months post-surgery, respectively and were significantly different between fertile individuals when compared with varicocele individuals prior to surgery ($p<0.01$).

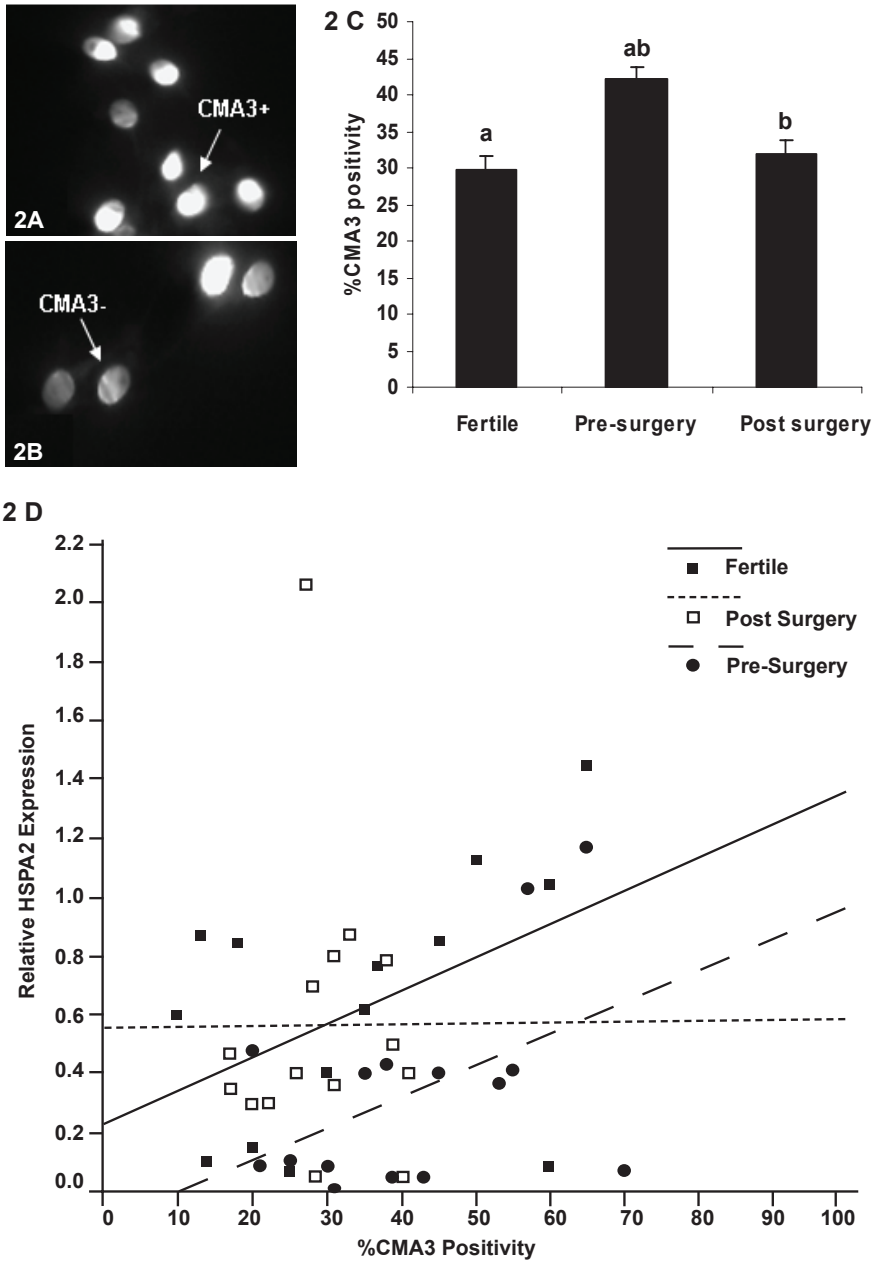

Fig 2: Images of CMA3 staining in varicocele individuals before (A) and six months after varicocelectomy (B). The mean percentages of CMA3 positivity in fertile, varicocele pre-surgery and six months after surgery (C). The correlation between CMA3 positivity with relative expression of HSPA2 in fertile and varicocele individuals before and six months after surgery (D). Common letters are significantly different for $p<0.05$.

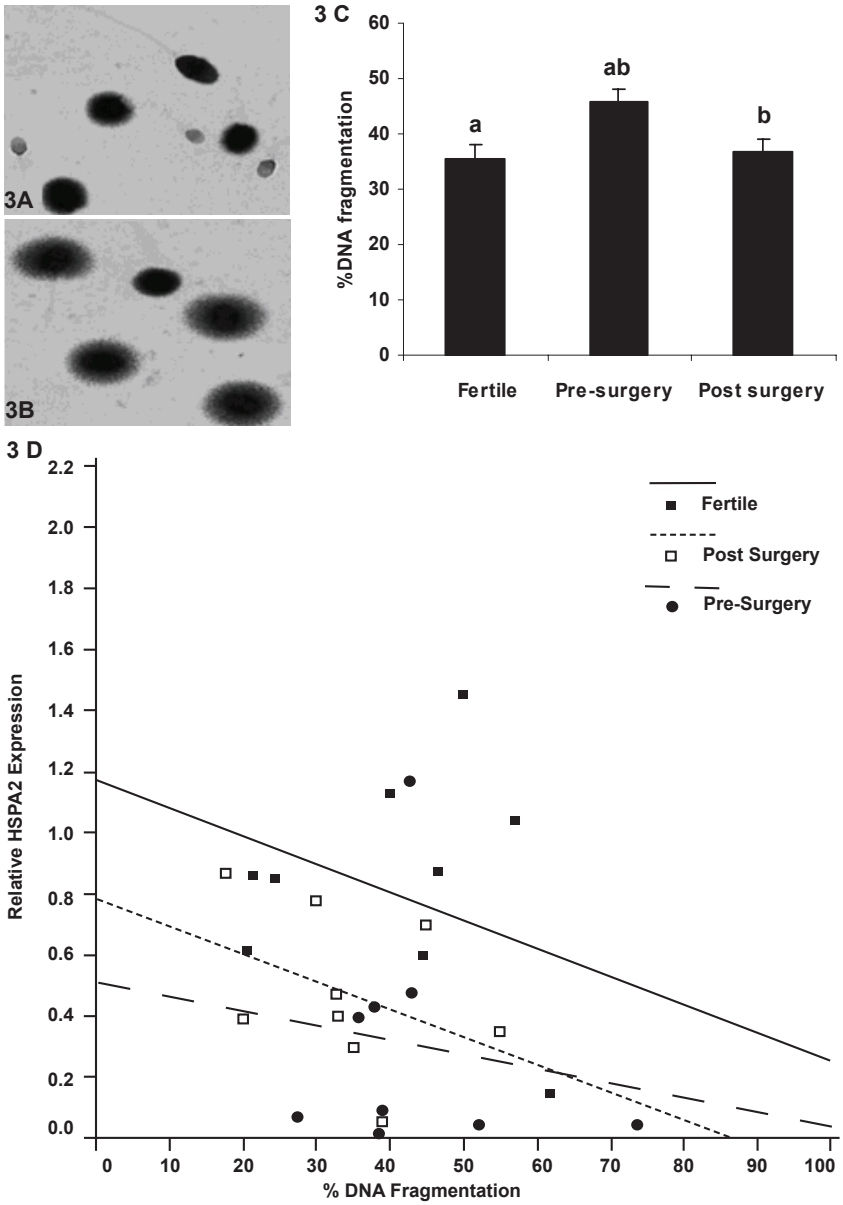

Fig 3: Images of sperm DNA fragmentation in varicocele individuals before (A) and six months after varicocelectomy (B).The mean percentages of sperm DNA fragmentation in fertile, varicocele before surgery and six months after surgery (C). Correlation between sperm DNA fragmentation with relative expression of HSPA2 in fertile, and varicocele individuals before and six months after surgery (D).Common letters are significantly different for p< 0.05.

Sperm density in fertile and varicocele individuals before and six months after varicocelectomy were 57.08 ± 3.75, 47.89 ± 3.78 and 70.76 ± 3.56, respectively. This value was not significantly different between fertile individuals and varicocele individuals before surgery (p=0.1), while sperm density was significantly different between varicocele individuals before and post-surgery (p<0.01). No significant correlation was observed between HSPA2 and sperm density in the fertile and varicocele individuals before and post-surgery. Correlation analysis between HSPA2 and motility revealed only a significant association between these two parameters six months post-surgery (r = 0.392, p=0.039). An assessment of the correlation between CMA3 and sperm DNA fragmentation revealed no significant relationship between these two parameters in the three groups, however, a significant negative correlation (r = -0.422, p=0.02) was observed between CMA3 and motility six months post-surgery.

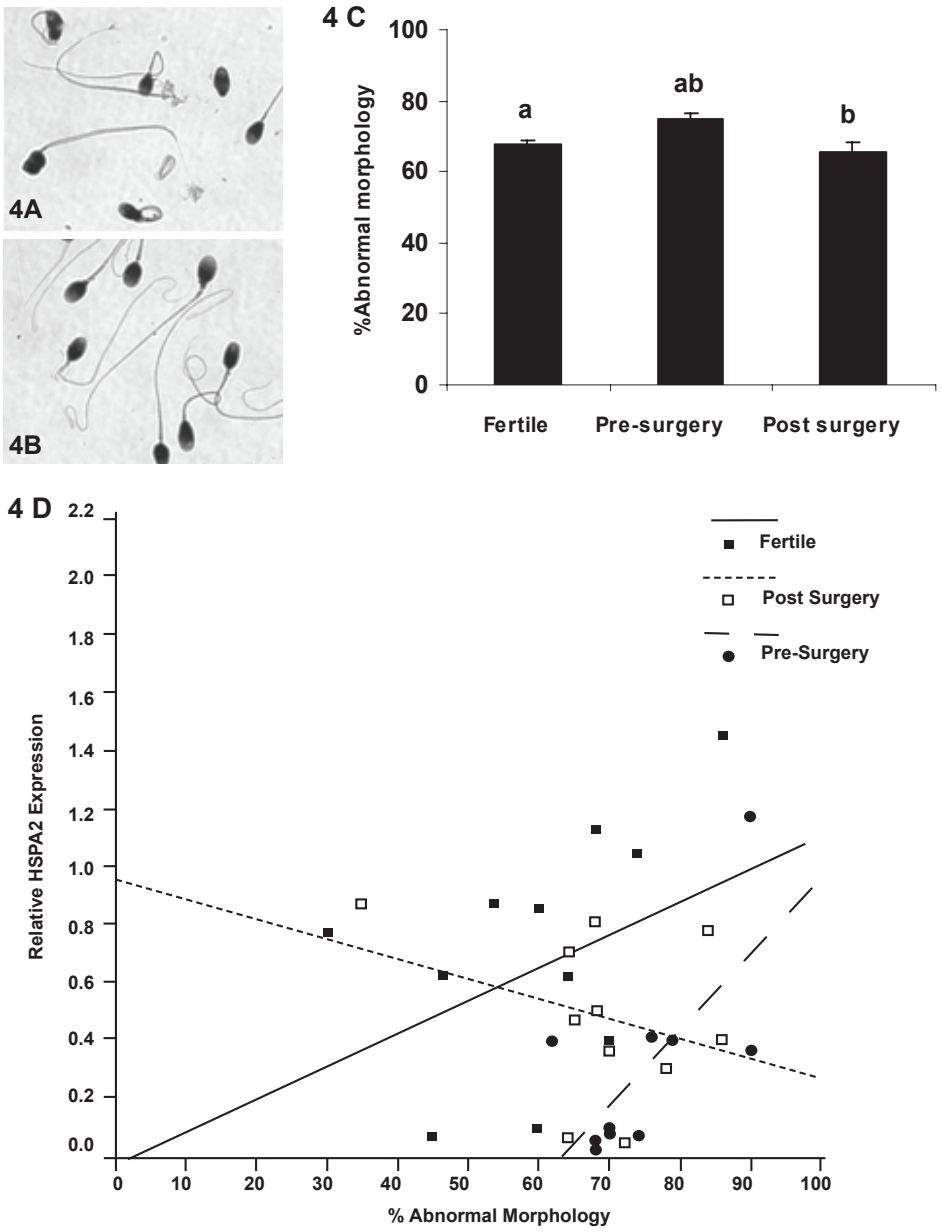

Fig 4: Images of abnormal sperm morphology in varicocele individuals before (A) and six months after varicocelectomy (B). The mean percentages of abnormal sperm morphology in fertile, and varicocele individuals before and six months after surgery (C). The correlation between abnormal sperm morphology with relative expression of HSPA2 in fertile and varicocele individuals before and six months after surgery (D). Common letters are significantly different for $p<0.05$.

Discussion

Considering the abundant literature studies, it is well accepted that varicocele induces hyperthermia, impairs spermatogenesis and can result in infertility (21). However, the precise mechanism remains to be elucidated. One of the physiological defense mechanisms to acute heat induced stress is over-expression of chaperone proteins such as HSPA2 (22-24). However, in accordance with previous studies, the results of this study has shown that unlike acute conditions, chronic hyperthermia induced by varicocele results in not only impaired spermatogenesis but also reduces HSPA2 expression when compared to the fertile population (11, 25-27). In addition, the results of this study reveal that surgical correction of this pathophysiological condition not only improves semen parameters (28) but also results in normal expression of HSPA2 (Fig 1). Considering HSPA2's biphasic expression, therefore the main roles envisaged

for this protein include: DNA recombination and repair, folding, transport, assembly of proteins necessary for progression and completion of first meiosis as well as the final stages of sperm maturation in the process of cytoplasmic extrusion, plasma membrane remodeling and displacement of histones with protamine for chromatin condensation (15, 23, 29, 30). Indeed, the importance of HSPA2 expression has been well validated by its absence of expression in Sertoli cell syndrome and the low incidence of genetic variation for this protein (23). Therefore, the question which remains to be answered is the relationship between HSPA2 expression with protamine deficiency involved in nuclear condensation, DNA fragmentation and sperm morphology as well as their impact on fertility.

One of the most important steps in spermiogenesis is DNA packaging which coincides with the second phase of HSPA2 expression in spermatogenesis (23, 29). During this step somatic histones are initially substituted by testis specific histones, then by transitional proteins and finally by proteins named protamines (31). Protamine deficiency is associated with low sperm counts, decreasing sperm motility and morphology, diminishing fertilization ability and increasing sperm DNA damage (32-34). Similar to the previous study, the results of this study show that the percentage of CMA3 positive sperm which reflects protamine deficiency is higher in varicocele patients and reduces to normal levels by six months post-surgery (35). Thus, this suggests an association between protamine deficiency and varicocele individuals. In this study a significant positive correlation between relative HASP2 expression and CMA3 positive sperm has been observed in fertile individuals. Thus, this reveals that in individuals with high degrees of protamine deficiency the expression of HSPA2, a chaperone protein involved in protein folding, is possibly up-regulated in order to overcome protamine deficiency. A similar, but insignificant correlation was observed in varicocele individuals before surgery however their overall expression of HSPA2 was initially significantly lower and increased following surgery. Therefore, according to the literature, varicocelectomy not only eliminated chronic heat stress, in addition, it significantly increased the overall expression of this chaperon protein which might have lead to normal protamine expression and therefore, an overall reduction in CMA3 positive sperm. As shown in Figure 2, the number of individuals with high CMA3 positivity decreased following surgery and a more homogenous population in terms of HSPA2 expression and CMA3 positive sperm was observed. This population was located between the fertile and varicocele populations prior to surgery. In accordance with these findings, Govin et al. reported that HSPA2 was a chaperone for transitional proteins involved in histone/protamine exchange (36).

A comparison of DNA fragmentation in fertile and varicocele individuals before and post-surgery revealed that varicocele individuals had higher levels of DNA fragmentation which, as expected, improved upon surgery (28). This possibly suggested that chronic hyperthermia induced by varicocele might result in DNA fragmentation which was ameliorated by surgery.

Assessment of the relationship between HSPA2 and DNA fragmentation revealed a significant negative correlation between these two parameters which suggested that fertile individuals with high levels of HSPA2 expression were less prone to DNA damage. Although insignificant, a similar pattern of correlation was observed between these two parameters in varicocele individuals. The overall expression of HSPA2 was lower in varicocele individuals which suggested that this may have predisposed them to higher levels of DNA fragmentation. Although correction of this condition improved HSPA2 expression, however the correlation observed in fertile patients was not attained. This might suggest that increased DNA fragmentation may not be the primary cause of infertility in varicocele individuals and may be the consequence of another disorder such as distorted DNA condensation. If one considers the latter to be the cause for DNA fragmentation in varicocele individuals, therefore, a significant positive relation between DNA fragmentation and the percentage of CMA3 positivity should have been obtained. Our results, however, have shown an insignificant correlation between these two parameters. Thus, it has been suggested that two other mechanisms may have resulted in higher DNA fragmentation in these individuals. Indeed, increased apoptosis and ROS levels have been reported to be significantly higher in varicocele individuals (37).

HSPA2 expression is biphasic and the first phase regulates progression through first meiosis while the second phase regulates late spermiogenic events such as DNA condensation (23, 29). Therefore, improved expression of HSPA2 may partly account for increased sperm concentration at six months post-surgery. Indeed it has been shown that continuous expression of HSPA2 is of paramount importance for preventing apoptosis and completion of spermatogenesis (23). In this study no significant correlation was observed between

sperm density and HSPA2. This may be explained by the fact that HSPA2 was not assessable in semen samples at low concentrations due to limitations of the number of sperm required for RT-PCR and assessment of other parameters. In addition, severe alteration in the expression of HSPA2 in the first phase may lead to azoospermia (16).

Among semen parameters, sperm morphology has been considered to have a higher impact on fertilization and thereby fertility (38, 39). Different researchers have shown a close correlation between nuclear normalcy with fertilization and further embryonic development (40-42). The results of this study have shown that the mean numbers of sperm with abnormal morphology were higher in varicocele individuals and decreased to fertile levels six months post-surgery. In addition, the results showed an insignificant positive correlation between the percentages of abnormal morphology with HSPA2 expression in both fertile and varicocele individuals. However six months post-surgery, a negative significant correlation existed between these two parameters. This was likely due to the fact that, by removal of heat stress in individuals whose percentages of abnormal morphology decreased post-surgery, an increase in the relative HSPA2 expression was seen. However, in those who had persistently high percentages of abnormal sperm morphology following varicocelectomy, when the heat stress was removed and resulted in a decrease in HSPA2 expression, other reasons than hyperthermia may have accounted for the high degree of sperm abnormalities.

From correlation analysis between relative HSPA2 expression with DNA fragmentation, the percentages of CMA3 positive sperm and abnormal morphology, it appears that, the relationships between HSPA2,with morphology and CMA3, after surgery, are completely different when compared to fertile and varicocele individuals before surgery. However, the relationship of HSPA2 with DNA fragmentation after surgery shows a similar trend to the fertile individuals and prior to surgery. This is possibly due to the role of HSPA2 in proper folding of protamine and other related proteins that are involved in sperm maturation during spermiogenesis, while the occurrence of DNA fragmentation could be secondary to this phenomenon or induced through a different pathway which could occur during spermatogenesis and even after ejaculation. The pregnancy rate in this study was 38.5%. Therefore, differential analysis of varicocele individuals whose partners became pregnant in comparison with those who did not would have been of great value for this study. However, due to the lack of cooperation and low number of participants' partners who became pregnant post- surgery, this analysis was not carried out.

As with sperm morphology, similar results were also obtained for sperm motility. No relation between sperm motility and HSPA2 prior to surgery in the varicocele individuals and in the fertile individuals was seen, while the trend was reversed six months post-surgery. Therefore, in individuals with increased HSPA2 expression, an increase in sperm motility also occurred which indicated that proper folding of proteins during spermatogenesis has an important consequence for sperm motility. This observed effect could be related to the increased DNA packaging which leads to a more hydrodynamic structure, resulting in better motility. Indeed the negative significant relation obtained between motility and the percentages of CMA3 positive sperm may further verify this postulation.

Conclusion

Since infertility induced by varicocele and its analysis at the pregnancy level is multi-factorial; therefore, it is difficult to determine the causative factors that are involved. However, from the results of this study it can be concluded that at the molecular level there is a decrease in relative HSPA2 expression which is possibly due to chronic hyperthermia induced in varicocele individuals. Removal of this stress increases HSPA2 expression which results in the proper folding of proteins involved in spermatogenesis, thus causing an improvement in DNA packaging, in addition to better sperm morphology and motility which may indirectly reduce sperm DNA fragmentation. It is of interest to note that a recent study has suggested altered expression of specific HSPs or their related factors which are specific to chronic varicocele and a study of these proteins may reveal more information on the pathogenesis of varicocele (22).

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