Heat-Shock Protein Expression and Oxidative Stress in Male Infertility

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ABSTRACT

In infertile men, it has been demonstrated that heat shock protein (HspA2) is *expressed in spermatogonia parallel the loss of spermatogenic function. Low level of HspA2 expression in spermatogonia might lead to an altered level of protection, which in turn could be involved in low spermatogenic efficiency.* ***Aim:*** *The present study aimed to investigate the relationship between expression of heat shock protein (HspA2) in ejaculated human sperm and oxidative stress in male infertility.* ***Patients & Methods:*** *This study included 96 men attending the Andrology Outpatient Clinic, Mansoura University Hospital. The semen samples obtained from men were grouped according to WHO criteria into: Normozoospermia (N) was used as control group (n=24), Asthenozoospermia (A) (n= 21), Astheno-Teratozoospermia (AT) (n=23) and Oligo-Astheno-Teratozoospermia (OAT) (n=28). Computer assisted semen analysis (Autosperm), hypo-osmotic swelling (HOS) test and acrosin activity of spermatozoa by gelatinolysis test were performed. Also, malondialdehyde (MDA)/spermatozoa and total antioxidant capacity (TAC) were assessed in seminal plasma. Expression level of HspA2 mRNA of spermatozoa was determined by RT-PCR and DNA fragmentation was detected by agarose gel electrophoresis.* ***Result:*** *The current study showed that, percentage of DNA fragmentation was significantly increased in OAT group compared to control group (N). Also, the present study showed significantly negative correlation between MDA/spermatozoa with sperm concentration, grade A motility grade A+B motility, velocity, linear velocity linearity index, normal morphology, acrosin activity index, HOS test TAC and HspA2 expression. HspA2 expression and TAC level showed significantly positive correlation with sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, linearity index, normal morphology, acrosin activity index, HOS test and HspA2 expression.* ***Conclusion:*** *From results of the current study, it could be concluded that HspA2 gene expression in ejaculated sperm from infertile might be associated with spermatogenic and/or spermiogenic dysfunction involved in the pathogenesis of some cases of male infertility.*

INTRODUCTION

It is estimated that about 15% of couples present with reduced fertility, and male infertility is responsible for half of these cases. The reduction in sperm count is generally accompanied with high rate of sperm with abnormal

morphology and/or low progressive motility, which leads to lower fertility[1].

Of the many causes of male infertility, oxidative stress (OS) has been identified as one factor that affects fertility status and thus, has been extensively studied. Spermatozoa, like any other aerobic cell, are constantly facing the "oxygen-paradox"[2]. The presence of seminal oxidative stress in infertile men suggests its role in the pathophysiology via several mechanisms acting in synergism which can impair sperm characteristics and functional capacity[3].

Much remains to be learned about the regulation of gene expression during spermatogenesis process. Some developmentally regulated genes are expressed exclusively in spermatogenic germ cells, whereas others are expressed in both germ cells and somatic cells. Some of these genes expressed only in germ cells are homologues of genes transcribed in somatic cells and are members of specific gene families[4], such as the heat-shock proteins (Hsps) families, which are highly conserved cellular stress proteins, present in every organism from bacteria to man [5].

The HspA2, a member of the 70-kDa Hsp family, is a molecular chaperone that assists in the folding, transport and assembly of proteins in the cytoplasm, mitochondria and endoplasmic reticulum[6]. Hsps have a protective action on the cellular auto-regulation in response to an array of insults, including oxidative stress[5] and on the mechanism of homeostasis, providing a balance between protein synthesis and degradation[7]. The protective action of HspA2 could be mediated by anti-apoptotic, anti-inflammatory and anti-DNA damage effects [8].

During spermatogenesis, three distinct phases can be differentiated: mitotic proliferation of spermatogonia, meiotic development of spermatocytes, and postmeiotic development of spermatids and maturation of the spermatozoon[9]. Since all these developmental stages represent situations where dramatic transformations and cellular differentiation take place, it is not astonishing that spermatogenesis is accompanied by expression of different Hsps [10].

HspA2 has an essential role for normal spermatogenesis. Dysfunctional expression of regulated genes may result in abnormal spermatogenesis [11]. In maturation arrest, testes HspA2 protein levels were shown to be lower than those in normal men and are completely absent in testes with Sertoli cell-only syndrome. The authors also demonstrated that HspA2 is expressed during the spermatocyte and spermatid stages of spermatogenesis[12]. These results agree with those of **Son et al.** [13], who demonstrated repression of HspA2 mRNA in testicular biopsy material from men with abnormal spermatogenesis.

In the current study, the relationship between the expression of heat shock protein HspA2 in ejaculated human sperm and oxidative stress in male infertility was investigated.

MATERIALS & METHODS

Study included 96 semen samples obtained from patients attending the Andrology Outpatient's Clinic, Mansoura University Hospital, Egypt. The semen samples were grouped according to criteria of WHO into, Normozoospermia (n=24), Asthenozoospermia (n= 21), Astheno-Teratozoospermia (n=23) and Oligo-Astheno-Teratozoospermia (n=28). All semen samples were investigated for semen analyses and acrosin activity as described by **Henkel *et al.*[14].** Determination of malondialdehyde (MDA)/spermatozoa was assessed by the method of **Draper *et al.*[15].** Total antioxidant capacity (TAC) of seminal plasma was measured by colorimetric method using commercially available kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the method of **Rice-Evans and Miller[16]**. The results were expressed as mM Trolox equivalent.

Hypo-osmotic Swelling Test: 1 ml aliquot of swelling solution (0.735 g sodium citrate dihydrate and 1.351 g fructose dissolved in 100 ml distilled water) was added to 0.1ml of liquefied semen and mixed well. Then the mixture was incubated at 37 ºC for 30 minutes[1] and sperms were then examined under phase–contrast microscope.

***RT-PCR* analyses:** Total RNA was extracted from sperms using AxyPrep Multisource Total RNA Miniprep kit (Axygen Biosciences, Union City, CA 94587, USA). cDNA synthesis from total RNA and PCR was performed by RT/PCR Master Mix. Gold Beads Kits (BIORON The ENZYME company, Germany) using Open Reading Frame sequence-specific primers. β-actin: sense, 5′-CGT GAC ATT AAG GAG AAG CTG TGC-3′; antisense, 5′-CTC AGG AGG AGC AAT GAT CTT GAT-3′, and HspA2: sense, 5′-TTG TTG GAA GTC TTT GGT ATA-3′ and antisense, 5′-CAT TTG CAT TTA TGC ATT TGT-3′[13]. PCR was performed by heating for 10 min. at 94°C for DNA denaturation followed by 36 cycles (1 min. at 94°C, 1 min. at 57°C, 1 min. at 72°C) and a final extension for 10 min. at 72°C. The cDNA amplification was assessed on 2% agarose gel by electrophoresis [17]. The gel was analyzed with image analysis as it was possible to estimate comparatively the amount of cDNA (semiquantified) referring to the genes under study. The cDNA amplifications of each sample were assessed for the β-actin and HspA2 genes. The samples were first analyzed for β-actin, which characterizes the presence of total RNA in the material under study, and then for HspA2. The HspA2/β-actin ratio was assessed in all groups (Figure 1).

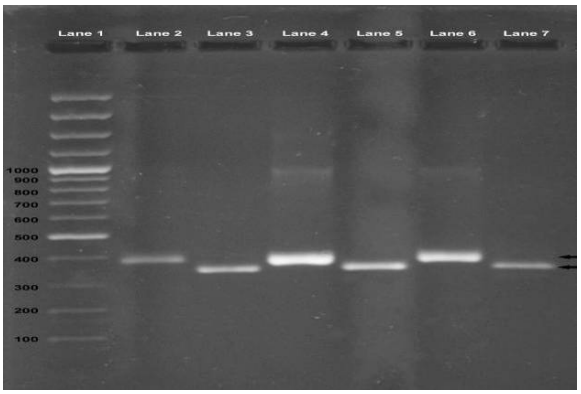

Fig. (1): RT-PCR detection of HspA2 and β-actin mRNA expression in RNA isolated from ejaculated spermatozoa. Lane 1 shows molecular weight marker (in base pairs), lane 2, 4 and 6 show HspA2 mRNA expression and lanes 3, 5 and 7 show β-actin mRNA expression.

DNA Fragmentation Analysis by Agarose Gel Electrophoresis[18]:

Spermatozoa were collected after centrifugation and DNA fragmentation was assessed by Enhanced Apoptotic DNA Ladder Detection kit (BioVision Research Products 980 linda ista Avenue, Mountain View, CA 94043 .USA). Sperm pellet 5-10 x 10^6 cells in a 1.5 ml microcentrifuge tube was washed with PBS and the pellet was centrifuged for 5 min at 500 x g. Supernatant was removed and the cells were lysed with 35 µl. TE lysis buffer. 5µl of enzyme A solution were added and mixed by gentle vortex and incubated at 37°C for 10 min. 5µl of enzyme B solution were added into each sample and incubated at 50°C for 30 min. Also, 5 µl of 9843ammonium acetate solution and 50 µl of isopropanol were added and mixed well. DNA pellet was washed with 0.5 ml 70% ethanol, and air dried. The DNA pellet was dissolved in 20µl DNA suspension buffer. The sample was loaded onto a 1.8% agarose gel. The gel was run at 5 V/cm for 1 hour. The gel was stained by staining buffer (provided by the kit) with gentle shaking for 30 minutes. DNA ladder was visualized by illumination of short UV wavelength (254nm) and photographed with camera equipped with 520 nm filter (Figure 2).

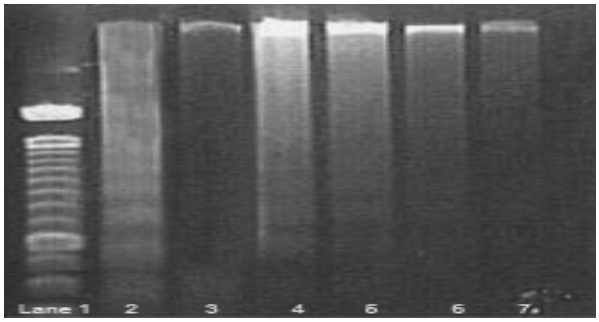

Figure (2): Analysis of genomic DNA fragmentation in human spermatozoa (sperm pellet 10 x 10^6 cells) from different groups. Total DNA from spermatozoa was prepared, and fragmentation was assessed by agarose gel electrophoresis. Lane 1: 100-bp ladder (GIBCO-BRL). Lanes 2, 4, 5: show DNA fragmentation (DNA ladder), lanes 3, 6, 7: show no DNA fragments (no DNA ladder).

Statistical analysis

Statistical analysis was performed using the MedCalc program (MedCalc Software, Mariakerke, Belgium)[19]. The significance of differences between groups was assessed using Wilcoxon's test. Correlations were calculated using Spearman's rank order coefficient. P value of <0.05 was considered statistically significant.

RESULTS

The sperm characteristics exhibited by all groups are given in Table 1.

Table (1) Semen parameters in all studied groups (Median, Range):

Parameters	N n=24	A n=21	AT n=23	OAT n=28
Volume (ml)	3.0 (2.0-7.0)	3.5 (1.0-7.5)	3.0 (1.0-8.2)	3.7 (1.5-8.4)
Concentration (million /ml)	67.2 (44.8-96.0)	49.5 (24.9-67.2)	29.5 (20.8-53.3)	4.7 (0.4-18.6)
Grade A motility (%)	54.0 (41.0-60.0)	33.0 (14.0-42.0)	14.0 (0.0-28.0)	2.5 (0.0-20.0)
Grade A+B motility (%)	60.0 (51.0-67.0)	39.0 (35.0-57.0)	28.0 (2.0-44.0)	10.0 (0.0-41.0)
Velocity (µm/sec)	80.0 (63.4-95.4)	74.4 (32.3-109.7)	36.4 (17.6-69.9)	22.1 (0.0-74.5)
Linear velocity (µm/Sec)	61 (42.9-70.7)	50.8 (18.1-84.1)	23 (11.1-49.4)	11.8 (0.0-52.6)
Linearity index (%)	76.9 (67.5-86.7)	65.8 (43.3-77.6)	60.8 (48.7-86.5)	63.5 (0.0-97.4)
Normal morphology (%)	64.0 (58.0-66.0)	44.0 (34.0-64.0)	12.0 (2.0-22.0)	2.0 (0.0-10.0)
WBCs (million/ml)	0.5 (0.4-0.6)	0.6 (0.4-3.6)	0.8 (0.4-4.4)	0.7 (0.3-3.6)

N= Normozoospermia, **A=** Asthenozoospermia, **AT=** Astheno-Teratozoospermia, **OAT=** Oligo-Astheno-Teratozoospermia and **n=** number of cases

Table (2): Acrosin activity index, HOST (%), MDA/spermatozoa, and TAC in all studied groups (median, range)

Parameter	N n=24	A n=21	AT n=23	OAT n=28	p-value
Acrosin activity index	12.6 (8.9-19.4)	10.7 (5.5-13.9)	5.0 (0.9-11.0)	2.2 (0.6-7.5)	P1 < 0.0001 P2 < 0.0001 P3 < 0.0001
HOST (%)	84.0 (72.0-94.0)	62.0 (48.0-89.0)	54.0 (32.0-67.0)	33.5 (6.0-65.0)	P1 < 0.0001 P2 < 0.0001 P3 < 0.0001
MDA/spermatozoa	3.00 (1.40-6.58)	5.48 (2.01-14.36)	7.57 (2.17-16.65)	50.84 (9.47-521.73)	P1 = 0.0005 P2 < 0.0001 P3 < 0.0001
TAC (mM Trolox equivalent)	1.8 (1.2-2.6)	1.4 (0.9-1.8)	1.2 (0.6-1.7)	0.9 (0.7-1.4)	P1 = 0.0001 P2 < 0.0001 P3 < 0.0001

P_1: significance between N and A, **P_2**: significance between N and AT and **P_3**: significance between N and OAT

Table (2) shows the acrosin activity index, HOS test (%), malondialdehyde (MDA) (nmol/10^8 sperm) and TAC (mM Trolox equivalent) in all studied groups. Acrosin activity index, HOS test and total antioxidant capacity were significantly higher in control (N) group compared with A, AT and OAT groups. However, seminal plasma concentration of malondialdehyde (MDA) per 10^8 sperm was significantly increased in A, AT and OAT groups than in the control (N) group.

Table (3): DNA fragmentation of spermatozoa and HspA2 expression among different groups.

Parameter	N n=24	A n=21	AT n=23	OAT n=28	p-value
DNA fragmentation (%)	3/24 (12.5%)	5/21 (23.8%)	9/23 (39.1%)	14/28 (50%)	P1=0.5496 P2=0.0790 P3=0.0100
HspA2 expression	2.50 (1.40-3.57)	1.29 (0.92-1.54)	0.92 (0.74-1.19)	0.69 (0.41-1.08)	P1 < 0.0001 P2 < 0.0001 P3 < 0.0001

Table (3) shows DNA fragmentation% and HspA2 expression in all studied groups. The DNA fragmentation % was significantly increased in OAT group compared to control. HspA2 expression was significantly lower in A, AT & OAT compared to control group (N).

Table (4): Correlation of MDA/spermatozoa, TAC and HspA2 expression with sperm parameters (n=96).

Parameter	MDA/ spermatozoa	TAC (mM Trolox equivalent)	HspA2 Expression
Concentration (million /ml)			
-r	-0.913	0.728	0.779
-P	P<0.0001	P<0.0001	P<0.0001
Grade A motility (%)			
-r	-0.860	0.709	0.771
-P	P<0.0001	P<0.0001	P<0.0001
Grade A+B motility (%)			
-r	-0.809	0.713	0.757
-P	P<0.0001	P<0.0001	P<0.0001
Velocity (µm/sec)			
-r	-0.783	0.664	0.667
-P	P<0.0001	P<0.0001	P<0.0001
Linear velocity (µm/Sec)			
-r	-0.814	0.655	0.700
-P	P<0.0001	P<0.0001	P<0.0001
Linearity index (%)			
-r	-0.417	0.335	0.386
-P	P<0.0001	P=0.0009	P=0.0001
Normal morphology (%)			
-r	-0.838	0.741	0.811
-P	P<0.0001	P<0.0001	P<0.0001
WBCs (million/ml)			
-r	0.346	-0.293	-0.407
-P	P=0.0006	P=0.0037	P<0.0001
Acrosin activity index			
-r	-0.767	0.679	0.763
-P	P<0.0001	P<0.0001	P<0.0001
HOST (%)			
-r	-0.764	0.724	0.734
-P	P<0.0001	P<0.0001	P<0.0001
MDA/spermatozoa			
-r	----------	-0.678	-0.707
-P		P<0.0001	P<0.0001
TAC			
-r	-0.678	----------	0.718
-P	P<0.0001		P<0.0001
HspA2 Expression			
-r	-0.707	0.718	----------
-P	P<0.0001	P<0.0001	

Table (4) shows the correlations between seminal MDA/spermatozoa, TAC (mM Trolox equivalent), HspA2 expression, and semen characters. This table shows significantly negative correlation between MDA/spermatozoa with sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, linearity index, normal morphology, acrosin activity index, HOS test, TAC and HspA2 expression. Significant positive correlation was shown between MDA/spermatozoa and WBCs.
HspA2 expression and TAC level showed significantly positive correlation with sperm concentration, grade A motility, grade A+B motility, velocity, linear velocity, linearity index, normal morphology, acrosin activity index, HOS test and HspA2 expression.

Table (5): acrosin activity index, HOST (%), MDA/spermatozoa, TAC and HspA2 expression in samples without and with DNA fragmentation. (median, range)

Parameter	Samples without DNA fragmentation (n=55)	Samples with DNA fragmentation (n=25)	Significant comparison
Acrosin activity index (%)	8.98 (0.62-19.49)	3.26 (0.72-17.25)	P = 0.0052
MDA(nmol/l)/10^8 sperm)	5.48 (1.40-521.73)	10.50 (1.47-290.00)	P = 0.0212
HOST (%)	62.00 (9.00-94.00)	52.00 (6.00-86.00)	P = 0.0548
TAC(mM Trolox equivalent)	1.38 (0.69-2.64)	1.13 (0.78-2.26)	P = 0.0327
HspA2 expression	1.19 (0.50-3.57)	0.77 (0.41-1.95)	P < 0.0001

Table (5) shows acrosin activity index, HOS test, TAC, and HspA2 expression decreased in samples with DNA fragmentation compared with samples without DNA fragmentation. However, MDA/spermatozoa was significantly increased in samples with DNA fragmentation than those samples without DNA fragmentation.

DISCUSSION

Heat shock proteins play a cyto-protective role in preventing irreversible damage to cellular proteins by binding to unfolded or partially malfolded peptides to retard thermal denaturation and aggregation of cellular proteins [(20)]. It is well demonstrated that some Hsp are expressed in normal conditions in the testis [(21)], some are developmentally regulated and some are only induced in response to an array of insults, including hyperthermia, oxidative stress, heavy metals, ethanol, amino acid analogues, inflammation and infection [(5)].

In the present study, semiquantitative RT–PCR analysis demonstrated a positive correlation between HspA2 expression and semen parameters in accordance with **Son *et al.*** [13], who demonstrated repression of HspA2 mRNA in testicular biopsy material from men with abnormal spermatogenesis. **Huszar *et al.*** [22] have demonstrated that HspA2 is present in ejaculated human sperm. In addition, a lower expression of HspA2 was correlated with a higher percentage of cells with residual cytoplasm, determined by creatine kinase (CK) activity [23]. This suggests that HspA2 is essential for normal spermatogenesis, which is confirmed by the fact that lower HspA2/CK ratios correlate with lower fertility rates in *in vitro* fertilization programs[24]. **Naaby-Hansen and Herr** [25] have demonstrated that the constituency of HSP chaperones on the human sperm surface is far more diverse, abundant and immunogenically crossreactive than previously recognized, and suggested that plasma membrane-associated chaperones serve multiple functions in human sperm, some of which appear to be critical for sperm–ZP interaction and fertilization.

Feng *et al.* [12] have reported that, HspA2 was expressed in spermatocytes and spermatids in normal testes and in cases with maturation arrest but that expression was decreased in the latter. These authors concluded that lower HspA2 expression might be associated with the pathogenesis of male infertility. **Di Domenico *et al.*** [26] showed that the production of Hsp70 is quantitatively correlated with the degree of stress. The level of synthesis is controlled both transcriptionally and post-transcriptionally through repression of Hsp70 mRNA synthesis and destabilization of Hsp70 transcripts.

Of the many causes of male infertility, is oxidative stress (OS) which result from imbalance between the production of ROS and the body's antioxidant defense mechanisms[27]. Although it has been shown that small amounts of ROS are essential for regulation of normal sperm functions like sperm capacitation, acrosome reaction and oocyte fusion [28], but at high levels they have potential toxic effects on sperm quality and function.

The present data showed that MDA/spermatozoa in seminal fluid were significantly higher in patients with DNA fragmentation than those in patients without fragmentation. Meanwhile, in patients with DNA fragmentation, TAC and HspA2 expression were significantly decreased. These data were in accordance with previous studies that showed high production of ROS and low antioxidant capacity in infertile men with varicocele [29, 30]. This study showed a high frequency of DNA fragmentation in patients with abnormal spermatogenesis that could be explained by the pathologic action of oxidative stress demonstrated by MDA per spermatozoa and the decreased level of protection demonstrated by HspA2 expression and total antioxidant Capacity. Because of the known association between sperm immaturity and increased rate of lipid peroxidation and fragmentation of DNA in men[31,32], it could be hypothesized that both lipid peroxidation and DNA

fragmentation are related to apoptosis in immature sperm.

In conclusion, the present study demonstrated that HspA2 gene expression was significantly down-regulated in ejaculated sperm from infertile men sustain oxidative stress. Anomalies in the expression of that gene are associated with spermatogenic and/or spermiogenic dysfunction involved in the pathogenesis of some cases of male infertility, and sperm mRNA analyses may thus be a useful tool in evaluating the infertile man.

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تعبير بروتين الصدمة الحرارية و الاجهاد التأكسدى فى عقم الرجال

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جامعة المنصورة

هذه الدراسة تهدف لتحرى العلاقة بين تعبير بروتينِ الصدمة الحرارية (HspA2) في الحيوانات المنوية والإجهادِ التأكسدى في عقم الرجال.وتتضمن الدراسة ٩٦ عينةً مني تم الحصول عليها من المرضى المترددين على العيادة الخارجية لأمراض الذكورة في مستشفى جامعة المنصورة حيث تم تقسيم العينات تبعا لتوصيات منظمة الصحة العالمية إلي:عينات بها حيوانات منوية طبيعية (٢٤ عينة). وعينات بها حيوانات منوية قليلة الحركة (٢١ عينة) وعينات بها حيوانات منوية قليلة الحركة وضعيفة الاشكال الطبيعية (٢٣ عينة) وعينات بها حيوانات منوية قليلة العدد و الحركة وضعيفة الاشكال الطبيعية (٢٨ عينة) و قد تم تحليل عينات السائل المنوي طبقا لتوصيات منظمة الصحة العالمية باستخدام جهاز يعمل بالكمبيوتر. و تم تقييم نشاطِ الاكروزين باستخدام الشرائح الدقيقة المغطاة بالجيلاتين والتحلل الجيلاتينى. و تحديد مستوى المالون ثنائى الألدهيد فى البلازما كعلامة للإجهادِ التأكسدى. و الكشف عن ترابط غشاء الحيوان المنوى عن طريق اختبار الانتفاخ الناتج عن انخفاض الضغط الأسموزى تبعا لتوصيات منظمة الصحة العالمية. والكشف عن تعبير بروتين الصدمة الحرارية باستخراج الحمض النووى الريبوزى من الحيوانات المنوية المترسبة بالطرد المركزى وتحلل باستخدام تقنية النسخ العكسى وتفاعل البلمرة المتسلسل. والكشف عن تلف الحمض النووى للحيوانات المنوية باستخدام سلم الحمض النووى الريبوزى.

وقد أوضحت نتائج الدراسة وجود علاقة ارتباط طردية ذات دلالة احصائية بين تعبير بروتين الصدمة الحرارية (HspA2) ومعايير المنى، مما قد يشير الى الدور الذى يلعبه هذا البروتين فى حماية الحيوانات المنوية. كما اوضحت الدراسة ايضا وجود علاقة ارتباط عكسية ذات دلالة احصائية بين تعبير البروتين سالف الذكر وكلا من المالون داى الدهيد و عد خلايا الدم البيضاء فى المنى. وتبين زيادة فرصة تلف الحامض النووى الريبوزى مع انخفاض تعبير البروتين محل الدراسة.

و قد خلصت الدراسة أن تحديد أنواع معينة من الجزيئات البيولوجية (كبروتين الصدمة الحرارية HspA2 على سبيل المثال) فى السائل المنوى ربما يؤدى إلى معلومات مفيدة عن وظيفة الحيوان المنوى و ترابط غشاؤه الخلوى و أيضا إلى إيضاح الأسباب المحتملة للعقم عند الرجال و هذا يفيد فى تطوير خطط علاجية موجهة لأسباب و طرق حدوث العقم