

ROLE OF APOPTOSIS IN THE EVALUATION OF SPERM QUALITY IN INFERTILE OLIGOZOOSPERMIC MEN

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Objective To assess the role of apoptosis in the evaluation of the quality of spermatozoa in infertile oligozoospermic patients.

Patients and Methods Sperm samples from infertile oligozoospermic patients (n=32) and proven fertile men (n=20) were tested by conventional semen analysis, hypo-osmotic swelling (HOS) test and TUNEL (terminal deoxynucleotidyl transferase mediated dUTP [deoxy-uridine triphosphate] nick-end labeling) assay, which measures the occurrence of DNA strand breaks.

Results In the fertile men (control group), none of the sperms was positive for DNA strand breaks, while in the oligozoospermic patients $0.9 \pm 0.7\%$ of the sperms were positive for DNA strand breaks. This result was statistically highly significant when compared to the control group ($p < 0.001$). A significant inverse correlation was seen between the percentage of TUNEL-positive

cells and the concentration of spermatozoa ($r = -0.86$, $p < 0.05$). Also a significant inverse correlation was seen between the percentage of apoptotic cells and forward motility ($r = -0.6$, $p < 0.05$). While a positive correlation was found between abnormal sperm cells and the percentage of TUNEL-positive cells ($r = 0.49$, $p < 0.05$), a significant inverse correlation between the percentage of apoptotic cells and the percentage of HOS-positive cells ($r = -0.9$, $p < 0.05$) was found.

Conclusion Detection of DNA strand breaks is an important parameter in improving the evaluation of sperm quality and might play an important role as a predictor for fertilization success and in advanced fertilization treatments.

Keywords apoptosis, DNS strand breaks, oligozoospermia, HOS test, TUNEL assay

INTRODUCTION

Sperm quality plays an important role in human reproduction, and various tests and many different parameters for evaluation have been suggested¹⁻³. Although conventional semen analysis provides considerable information, its limitations in predicting the ability to achieve pregnancy are well acknowledged; other, more reliable technologies are still needed⁴.

In the past few years, attention has been paid to the significance of physiologic cell death, which occurs during normal spermatogenesis^{5,6}. Apoptosis is a result of DNA strand breaks. It is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alteration, leading to suicide⁷⁻⁹. Unlike numerous studies carried out in intratesticular cells, data

on apoptosis in human spermatozoa are scarce^{10,11}. In our study, the presence of apoptosis in human spermatozoa in proven fertile men and those with oligozoospermia has been correlated to sperm cell concentration, morphology, motility and to the hypo-osmotic swelling test (HOS test). This correlation was done to assess the role of apoptosis in evaluating the quality of spermatozoa in infertile oligozoospermic patients.

PATIENTS AND METHODS

During the year 2000/2001 more than one hundred infertile men were evaluated at the outpatient clinics of Al-Zahraa Hospital, Al-Azhar University, Cairo, Egypt. Only 32 oligozoospermic patients aged between 30 and 45 years fulfilled the criteria of our study.

Table 1: Hormonal Profile of the Infertile Oligozoospermic Patients

	Mean Value \pm SD
FSH (mIU/ml)	6.8 \pm 2.5
LH (mIU/ml)	4.4 \pm 1.4
Testosterone (ng/ml)	4.5 \pm 1.2
Estradiol (pg/ml)	28.1 \pm 8.7

All the 32 oligozoospermic patients had been suffering from primary infertility for at least 2 years. They were all non smokers, not medically treated and none of them had had previous scrotal surgery in the last 6 months prior to the study. All had a normal physical examination. Clinical scrotal and ultrasound examination showed no testicular abnormalities or varicocele. All cases included in the study had no pus in their semen. Serum levels of FSH, LH, testosterone and estradiol were measured (using the electrochemiluminescence technique on Elecsys 1010) and are summarized in Table 1. Peripheral blood culture and G banding for chromosomal analysis were performed to exclude chromosomal abnormalities¹². As controls, 20 normal fertile volunteers aged between 30 and 40 years were included in the study. Semen samples from patients and controls were collected by masturbation after 5 days of abstinence from intercourse. Routine screening, hypo-osmotic swelling (HOS) test and a test for the presence of DNA strand breaks (TUNEL assay) in the spermatozoa were performed.

Immunohistochemistry for the detection of apoptotic cells (TUNEL assay)

The seminal fluid was washed twice in phosphate buffered saline (PBS) and centrifuged for 5 minutes at 200g on polylysine-coated slides.

DNA fragmentation associated with apoptosis was detected by terminal deoxytransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining utilizing the ApopTag in-situ apoptosis detection peroxidase kit (Oncor, USA). In principle, the end result of apoptosis is DNA fragmentation. The labeling target of the ApopTag kit is the multitude of new 3'-OH DNA ends generated by intranucleosomal

cleavage in apoptotic bodies. Non-isotopic digoxigenin-nucleotide, dUTP is catalytically added to the DNA by terminal deoxynucleotidyl transferase (TdT) enzyme. Anti-digoxigenin peroxidase conjugate binds to the reaction site catalytically generates an intense signal from chromogen substrate.

Post-fixation was carried out in ethanol: acetic acid 2:1 for 5 min. at -20°C , then washed again in phosphate buffered saline (PBS). Endogenous peroxidase was quenched using perox block (Zymate) for 45 sec. The sections were rinsed in PBS, blotted around it and immediately 2 drops of equilibration buffer were added. They were then incubated for 10 min. in a humid chamber at RT. After tapping off and blotting, 20ul of working strength TdT enzyme (1 drop of TdT enzyme + 2 drops of reaction buffer) were added. The sections were covered and incubated for 1 hour at 37°C in a humid chamber. Negative slides received PBS instead of the working strength TdT enzyme. The slides were then immersed in a coplin jar containing pre-warmed stop wash buffer and incubated for 30 min. at 37°C . Two drops of antidigoxigenin-peroxidase were added and the slides were reincubated for 30 min. at room temperature. After 4 washes AEC chromogen (1 drop of substrate buffer + 1 drop of AEC chromogen + 1 drop of 0.6% H₂O₂ + 1ml D.W.) was applied and the slides were incubated for 5-15 min. at RT. Following wash in tap water and DDW, the slides were dried, mounted with aquamount (Sigma) and covered with glass coverslips for photomicroscopy according to the manufacturer's instructions. A positive control slide of human tonsils was included in each test. Focal in situ staining inside intact apoptotic nuclei and apoptotic bodies was visualized using the high power (400X) of an ordinary light microscope (Zeiss). Apoptosis was scored by counting the proportion of apoptotic cells to normal ones in at least 10 randomly selected fields that contain approximately 10000 cells¹³. The visualization of a small percentage of stained apoptotic cells yields biologically significant data.

Hypo-Osmotic Swelling Test (HOS test)

The HOS test was done on all semen samples as described by Jeyendran et al.¹⁴ and as reported elsewhere^{4,15}. The HOS test is based on the semipermeability of the intact cell membrane, which causes spermatozoa to swell under hypo-osmotic conditions. It gives

Table 2: Results of Conventional Semen Analysis, HOS Test and TUNEL Assay in Normal Fertile Volunteers and Oligozoospermic Patients

Sperm function	Normal Fertile Volunteers (n=20) Mean \pm SD	Oligozoospermic Patients (n=32) Mean \pm SD	P Value
Concentration $\times 10^6$ /ml	79 \pm 15	10 \pm 4	p< 0.001
Motility %	64 \pm 14	14 \pm 5	p< 0.001
Atypical forms %	20 \pm 4	26 \pm 5	p< 0.01
HOS-positive sperms %	71 \pm 5	28 \pm 10	p< 0.001
TUNEL-positive sperms %	-	0.9 \pm 0.7	p< 0.001

information about the compliance of the cell membrane of the sperm tail.

A total of 0.5 ml of hypo-osmotic solution (0.735 g sodium citrate dihydrate, 1.351 g fructose, and 100 ml H₂O) and 0.050 ml semen were gently mixed and incubated for 60-120 minutes at 37°C. At least 100 random spermatozoa were then examined at a magnification of $\times 400$. Spermatozoa were considered HOS positive when they showed signs of swelling, identified as changes in the shape of the tail, as described by Jeyendran et al.¹⁴. The percentage of sperm swelling was then calculated.

A statistical study was done using the SPSS statistical software package, version 9.02, Echo. Soft Corp., USA, 1998. Student's t-test was used for comparison between the two groups and the Spearman correlation test was used to study the association between each two parameters or variables among each group. Probability of error (p) was considered significant at p<0.05, while p>0.05 was considered non-significant.

RESULTS

Our results are shown in Tables 1 and 2. The investigated semen samples of the oligozoospermic patients had a sperm concentration of $10 \pm 4 \times 10^6$ /ml, 14 \pm 5% of the spermatozoa appeared to be motile, 26 \pm 5% of the spermatozoa showed atypical forms. The semen samples from the normal fertile volunteers had a sperm concentration of $79 \pm 25 \times 10^6$ ml, 64 \pm 14% of the spermatozoa

appeared to be motile, 20 \pm 4% of the spermatozoa showed atypical forms.

HOS Test

It is considered normal for a semen sample, if more than 60% of the spermatozoa undergo tail swelling. If less than 50% of the spermatozoa show tail swelling, the semen specimen is considered to be abnormal³.

The HOS test was positive in 28 \pm 10% of spermatozoa in samples of the oligozoospermic patients, while in the normal fertile volunteers it was positive in 71 \pm 5% of spermatozoa.

When the results were compared between patients and controls there was a highly significant decrease in sperm count (p<0.001) and in forward motility (p<0.001) and a highly significant increase in atypical forms (p<0.01). The percentage of HOS-positive cells showed a highly significant decrease in our patients when compared to the controls (p<0.001). In the oligozoospermic patients there was a positive significant correlation between the percentage of HOS-positive cells and sperm count (r= 0.84, p<0.05) and between the percentage of HOS-positive cells and forward motility (r=0.5, p<0.05).

Apoptosis as measured by DNA strand breaks (TUNEL assay):

In the investigated semen samples of fertile men, none of the sperms was positive for DNA strand breaks (Fig. 1) using TUNEL technique while in the oligozoospermic patients 0.9 \pm 0.7% of the sperms were positive for DNA

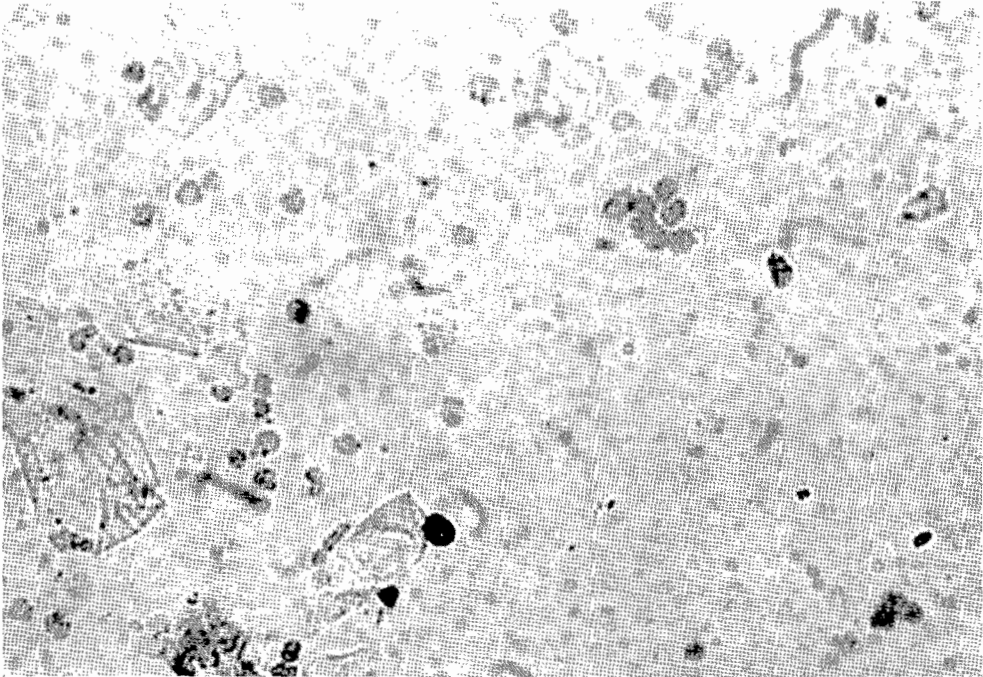


Fig. 1: TUNEL-positive cells in an oligozoospermic patient (weak-positive) (x400)

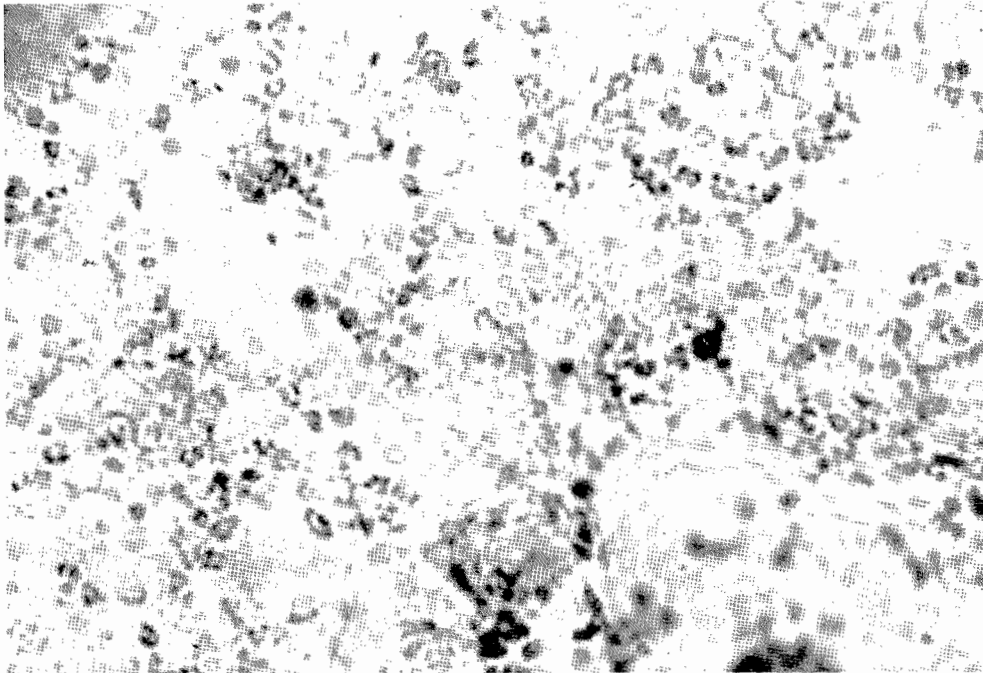


Fig. 2: TUNEL-positive cells in an oligozoospermic patient (strong-positive) (x400)

strand breaks (Fig. 2). This result was statistically highly significant when compared to the control ($p < 0.001$). A significant inverse correlation was seen between the percentage of TUNEL-positive cells and the concentration of spermatozoa ($r = -0.86$, $p < 0.05$). An inverse significant correlation was seen between the percentage of apoptotic cells and forward motility ($r = -0.6$, $p < 0.05$). A positive correlation was found between abnormal sperm cells and the percentage of TUNEL-positive cells ($r = 0.49$, $p < 0.05$), while a significant inverse correlation between the percentage of apoptotic cells and the percentage of HOS-positive cells ($r = -0.9$, $p < 0.05$) was found.

DISCUSSION

Programmed cellular death is an extremely interesting biological phenomenon and is involved in the regulation of the growth, differentiation and homeostasis of many multicellular organisms¹⁶.

Spermatogenesis is a dynamic and rather complex process, which includes proliferation and differentiation of germ cells. As observed in the majority of proliferating and differentiating tissues and also during spermatogenesis, a considerable number of germ cells, especially during the first spermatogenic wave, die by apoptosis throughout their development¹⁷⁻²⁰.

In response to various stimuli, cells are able to trigger or to hinder the expression of genes responsible for cell suicide⁷.

The most relevant biochemical characteristic of apoptotic death is the activation of endogenous endonucleases. These enzymes induce numerous breaks in the double strand following degradation of DNA and chromatin condensation. This breakage can be detected using terminal deoxynucleotidyl transferase (TdT) which is a specific test, that allows distinguishing DNA strand breakage in apoptotic cells from that of necrotic cells²¹.

Apoptosis has received a great deal of attention in the last few years. Høst et al.²² reported that by conventional methods of sperm preparation either separating sperm cells due to morphology or swim-up capacity, spermatozoa with DNA strand breaks could not be avoided. These spermatozoa that have a normal morphological appearance and are

motile might be capable of fertilizing human oocyte either *in vivo* or *in vitro* although they have activated enzymes for DNA strand breaks. Such activated spermatozoa may start the apoptotic process in the oocyte or simply prohibit final activation of the oocyte^{22,23}. This might explain why Gabrielsen et al.²⁴ did not find any beneficial effect of ICSI in patients previously seen with failed fertilization and normal conventional sperm cell parameters. Høst et al.²³ have demonstrated a possible relation between DNA strand breaks in the spermatozoa and a low fertilization rate. Gandini et al.²⁵ confirmed that a high DNA fragmentation was one of the characteristics of spermatogenetic failure.

Høst et al.²² have found a significantly higher incidence of spermatozoa with DNA strand breaks in men with oligozoospermia compared to men with a normal spermiogram. In our study using the TUNEL technique none of the semen samples of fertile men was positive for DNA strand breaks while in oligozoospermic patients, the sperms were positive for DNA strand breaks. This result was statistically highly significant when compared to the control.

Data published by Gandini et al.²⁵ revealed that most of the cells with apoptosis had atypical head forms. Also Høst et al.²² found a correlation between an increasing percentage of DNA strand breaks in the head of the spermatozoa and the number of abnormal sperm cells. Our work has confirmed these findings; a positive significant correlation between abnormal sperm cells and the percentage of apoptotic cells was noted.

Gandini et al.²⁵ reported an increased DNA fragmentation in line with the decrease of sperm concentration and motility. In our study, a significant inverse correlation was seen between the percentage of TUNEL-positive cells and the concentration of spermatozoa. Also a significant inverse correlation was seen between the percentage of apoptotic cells and forward motility.

The HOS test done in our study has shown that in oligozoospermic patients, the sperm count was significantly correlated with the percentage of HOS-positive cells.

However, there was a significant inverse correlation between the percentage of apoptotic cells and the percentage of HOS-positive

cells. This is in accordance with the work done by Oosterhuis et al.⁴ which revealed an inverse correlation between the percentage of TUNEL-positive cells and the percentage of HOS-positive cells, showing a trend towards significance.

In conclusion, we believe that detection of DNA strand breaks can improve the evaluation of the quality of sperms and, therefore, might play an important role as a predictor for fertilization success and in advanced fertilization treatments.

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RESUME

Rôle de l'Apoptose dans l'Evaluation de la Qualité du Sperme chez des Hommes Infertiles Oligospermes.

Objectif: Déterminer le rôle de l'apoptose dans l'évaluation de la qualité du spermatozoïde chez des patients infertiles présentant une oligozoospermie. **Patients et Méthodes :** Des échantillons de sperme de sujets oligospermes (n=32) et de sujets fertiles (n=20) ont été testés par méthodes d'analyse conventionnelle de sperme, test de turgescence hypo-osmotiques (HOS) et TUNEL essai, qui mesurent la survenue de cassures de chaînes d'ADN. **Résultats :** Dans le groupe de sujets fertiles, aucun sperme n'était positif pour les cassures de bandes de DNA, tandis que chez les patients oligospermes, $0,9 \pm 0,7$ % des spermatozoïdes étaient positifs pour les cassures de chaînes d'ADN. Ce résultat était hautement significatif comparé au contrôle ($p < 0.001$). Une corrélation inverse significative a été retrouvée entre la proportion de cellules TUNEL-positives et la concentration de spermatozoïdes ($r = -0.86$, $p < 0.05$). De même, une corrélation inverse significative a été retrouvée entre la proportion de cellules apoptotiques et la motilité rectiligne ($r = -0.6$, $p < 0.05$). Une corrélation positive a été retrouvée entre le taux de formes anormales et la proportion de cellules TUNEL-positives ($r = 0.49$, $p < 0.05$). Une corrélation inverse significative a été trouvée entre la proportion de cellules apoptotiques et le pourcentage de cellules HOS-positives ($r = -0.9$, $p < 0.05$). **Conclusion :** La détection des cassures de chaînes d'ADN est un important paramètre dans l'amélioration de l'évaluation de la qualité du sperme et pourrait être un facteur prédictif de succès de fertilisation et dans les traitements avancés de fertilisation.

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