Differences in Poly(ADP-ribose) Polymerase1- (PARP1-) and Proliferative Cell Nuclear Antigen (PCNA) Immunoreactivity in Patients Who Experienced Successful and Unsuccessful Microdissection Testicular Sperm Extraction Procedures

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Purpose: The aim of this study is to evaluate expression of deoxyribonucleic acid (DNA) synthesis and repair markers in testicular tissues of azoospermic men in whom sperm retrieval could and could not be achieved as a result of microdissection testicular sperm extraction (micro-TESE) procedure.

Materials and Methods: In this prospective cohort study, testicular tissues were retrieved from 60 Non-obstructive Azoospermia (NOA) patients who underwent micro-TESE procedure. These patients were divided into two groups: micro-TESE positive group, which included 30 NOA patients from whom sperm could be extracted via micro-TESE procedure; and micro-TESE negative group, which included 30 NOA patients from whom sperm retrieval could not be achieved via micro-TESE procedure. Expression and distribution patterns of poly(ADP-ribose) polymerase-1 (PARP-1) and proliferative cell nuclear antigen (PCNA) in extracted tissues were assessed by immunohistochemical staining to reveal any differences in DNA synthesis and repair between the two groups.

Results: Micro-TESE positive group exhibited significantly stronger immunoreactivity for both PCNA and PARP-1 ($P = .001$ and $P = .001$ respectively). The results of this study reveal that both DNA synthesis and repair markers were expressed strongly in patients who experienced successful micro-TESE procedure.

Conclusion: Although further studies are needed to support these findings, PARP-1 and PCNA expression in testicular tissues of NOA patients could be promising predictive factors for micro-TESE procedure success.

Key words: Azoospermia, Micro-Tese, PCNA, PARP-1

INTRODUCTION

Male factor is an important cause of infertility, contributing to 40-50% of all cases. Varicocele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, erectile dysfunction, trauma, and tumors are very well known etiologies of male infertility as well as defective spermatozoa.[1]
Non-obstructive azoospermia (NOA), which is defined as the absence of spermatozoa in semen due to impaired spermatogenesis, is the contributing factor for approximately 10% of cases of male factor infertility.[2] Sexual chromosomal abnormalities, Y chromosome translocations and microdeletions, trauma, oxidative stress, cryptorchidism, and radiation are possible etiologies for NOA.[3] Retrieval of spermatozoa is achieved by testicular sperm extraction (TESE) in 50% of NOA cases, which was described for the first time in 1994.[4,5] Over time, micro-TESE, which allows direct visualization of tubules containing more germ cells with active spermatogenesis via an operating microscope, became the method of choice for spermatozoa retrieval (SR).[6]
Poly ADP-ribosylation is a post-translational modification of proteins that plays a key role in the maintenance of genomic integrity and DNA repair.[7] Poly (ADP-ribose) polymerase-1 (PARP-1) is involved in many molecular and cellular processes such as DNA damage detection and repair, cell differentiation, apoptosis, and chromatin structure modulation.[8]
Sufficient spermatogenesis depends on the proliferative activity of spermatogonia and the loss of germ cells during meiosis and spermiogenesis.[9] Proliferative cell nuclear antigen (PCNA) is a nuclear matrix protein involved in DNA synthesis and repair. PCNA is also a useful tool in the diagnosis of germinal arrest, which is a result of DNA synthesis deterioration.[10]
This study aimed to explore the differences in DNA synthesis and repair in testicular tissues of NOA patients from whom sperm retrieval could and could not be achieved, by way of evaluating the expression and distribution patterns of PARP-1 and PCNA immunohistochemically.

PATIENTS AND METHODS

Study Population
The study population included 60 primary infertile azoospermic men with no endocrinopathy, known chromo-

Table 1. Comparisons of PARP-1 and PCNA immunoreactivity in TESE negative and positive groups.

Group[a]		n	Mean ±SD	Min-Max	p
Parp_1	TESE-negative	30	73,30 ± 20,31	45-117	,001
	TESE-positive	30	203,97 ± 18,23	176-240	
PCNA	TESE-negative	30	28,23 ± 4,71	19-37	,001
	TESE-positive	30	61,90 ± 7,71	50-75	

[a]Data is presented as mean ± SD or number (percent)

some disorder, retrograde ejaculation, history of urogenital surgery or erectile dysfunction, aged between 31-39 years, who applied to Izmir University IVF Clinic, between September 2015 and April 2016. Patients with numerical, structural chromosome abnormalities and with partial translocation were not included in the study. The participants were subjected to micro-TESE procedure by the same urologist and divided into two matched groups, those with sperm in tissue specimens, and those without In 30 men, SR could not be achieved (micro-TESE negative group), while in the remaining 30 men, SR could be achieved (micro-TESE positive group). The local ethics committee approved the study protocol. After obtaining informed written consent from each of the participants, general health data were collected.

Procedure

For each individual, chromosome number and structures were studied by examining 20 metaphase numbers using lymphocyte cell culture from peripheral blood and GTG banding. All of the participants had normal karyotypes and hormonal status including FSH. Azoospermia was confirmed in at least two different semen analyses of 60 primary infertile men, according to World Health Organization guidelines.[11] To evaluate testicular size and texture, all participants underwent testicular ultrasound and Doppler sonographic examination.

Testicular tissue samples were extracted via microdissection-TESE method as defined by Schlegel.[12] The extracted tissues were fixed in neutral formalin for 24 hours. After washing the tissues in tap water for a night long, they were dehydrated through ascending grades of alcohol and cleared in xylene. 5 micron thick sections were taken to poly-lysine coated glass slides and parted for immunohistochemistry.

Evaluation

Immunohistochemistry

The sections were incubated at 60 °C for overnight, deparaffinized in xylene, and dehydrated through descending grades of alcohol. For antigen retrieval, the sections were boiled in citrate buffer (10 mM, pH 6.0) for 15 minutes in a microwave processor (Thermo, CA, USA). To prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide (Thermo, CA, USA) for 15 minutes. The sections were then blocked with blocking serum (Ultra V Block, Thermo, CA, USA) for 10 minutes. Subsequently, the sections were incubated with primary antibodies PARP-1 (Scbt, CA, USA) and PCNA (Thermo, CA, USA) for 60 minutes at room temperature and humid air. Then, antigen-antibody complex was fixed with biotinylated secondary antibody and streptavidin-peroxidase complex (20 minutes). AEC (Thermo, CA, USA) was used for labeling. The sections were then counterstained with Mayer's hematoxylin and mounted for microscopic evaluation.

The images were recorded via an Olympus microscope with an attached camera (CX31 Germany).

Ten fields on each slide were chosen randomly under X400 magnification, and the H score was established according to density and percentage of involvement. The density of involvement was scored as 0 (no involvement), 1(+, weak immunoreactivity), 2(++, moderate immunoreactivity), or 3(+++, strong immunoreactivity). The percentage of involvement/immunoreactivity was calculated by division of the number of immunoreactive cells to total cells and scored as 1 (0-10%, focal), 2 (11-50%, regional), and 3 (51-100%, diffuse). For each field, density and amount scores were calculated with the formula: " HScore= $\Sigma Pi.(i+1)$". The sum of 10 fields' scores in each slide made up the individual slide score.[13] Image analysis software (Leica Q Win V3 Plus Image, Leica, Germany) was used for each field's score calculation.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences version 20.0 (SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). For discrete and continuous variables, descriptive statistics (mean, standard deviation, median, minimum value, maximum value, and percentile) are given. In addition, the homogeneity of the variances, which is one of the prerequisites of parametric tests, was checked through Levene's test. The assumption of normality was tested via the Shapiro–Wilk test. A total of 60 individuals participated in the study. All the individuals were independent of each other. For this reason, two independent group comparisons were used for the study. To compare the differences between the two groups, the Student's *t*-test was used when the parametric test prerequisites were fulfilled, and the Mann–Whitney U test was used when such prerequisites were not fulfilled.

RESULTS

Table 1 summarizes the statistical differences between groups.

In the micro-TESE negative group, fewer spermatogenic cells showed PARP-1 expression, and immunoreactivity was weaker as well. In the micro-TESE positive group, spermatogenic cells that showed PARP-1 expression were more frequent, and immunoreactivity, especially in primary spermatocytes, was stronger (**Figure 1**).

Regarding PCNA staining, the micro-TESE negative group included few immunoreactive cells, while in the micro-TESE positive group, the number of immunoreactive cells was greater (**Figure 2**).

DISCUSSION

The main purpose of this study was to determine whether there is a difference in PARP-1 and PCNA immunoreactivity in two groups of infertile men suffering from
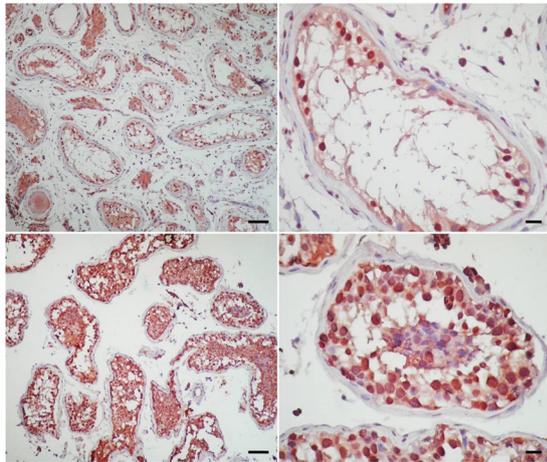
Figure 1. Immunohistochemical expression of PARP-1 in testicular tissues of NOA patients. A1 and A2 belong to TESE negative group; B1 and B2 belong to TESE positive group. □: Immun positive spermotogenic cells. A1 and B1 X100 = 50 μm, X400 Bar = 10 μm. Mayer's hematoxylin background staining.
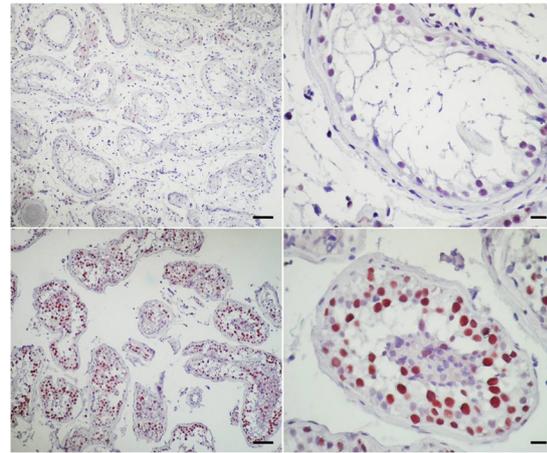
Figure 2. Immunohistochemical expression of PCNA in testicular tissues of NOA patients. A1 and A2 belong to TESE negative group; B1 and B2 belong to TESE positive group. □: Immun positive spermotogenic cells. A1 and B1 X100 = 50 μm, X400 Bar = 10 μm. Mayer's hematoxylin background staining.

non-obstructive azoospermia. The first group of patients consisted of men in whom SR could be achieved as a result of micro-TESE procedure, while the other group included azoospermic men in whom SR could not be achieved. Our data revealed that the micro-TESE positive group showed stronger PARP-1 and PCNA immunoreactivity, which indicates better DNA synthesis and repair.

Apart from genetic disorders, the etiologic factors for NOA, such as alkylating agents, radiation, and toxins, are suggested to contribute NOA by accumulating reactive oxygen species (ROS). Accumulation of ROS causes DNA damage and impairs DNA integrity.[14] As a result of DNA damage and loss of integrity, spermatogenesis and sperm-oocyte interaction is destructed.[15] Furthermore, DNA damage in sperm cells is related to fertilization failure, lower IVF success, poor placentation, and pregnancy complications.[16]

ROS-related DNA damage causes a substantial amount of single and double strand DNA breaks that lead to activation of PARP enzymes.[17] PARP enzymes act in many cellular processes including DNA repair, cellular proliferation, differentiation, apoptosis, and necrosis. PARP-1 is the enzyme responsible for more than 90% of PARP activity in human testes.[18] PARP-1 achieves DNA repair by DNA base excision, homologous recombination, and non-homologous end-joining processes. There is a large amount of evidence in the literature that poly ADP-ribosylation plays an important role in safeguarding DNA integrity in spermatogenesis.[19] However, there is evidence regarding a possible role of PARP overactivation due to excessive amount of ROS that can lead to male reproductive disorders via necroptotic cell death. It is considered to be related with the type and amplitude of the factor that brings out ROS.[20,21]

Most of the studies regarding PARP expression in testicular tissue have been implemented in rats. Moreover, in the context of male fertility disorders, only a few studies exist. Two different studies investigated PARP expression in testicular tissue of varicocele patients and reported stronger immunoreactivity in the varicocele

group, indicating increased DNA repair.[22,23] Two other studies investigated the effect of aging on PARP expression in testicular tissues, and both studies reported stronger immunoreactivity in elder patients, similar to varicocele patients, indicating more DNA repair due to increased amount of DNA strand breaks.[24,25] The present study is the first to investigate PARP expression in testicular tissues of NOA patients undergoing micro-TESE procedure. The results of this study revealed stronger immunoreactivity in micro-TESE positive group regarding PARP-1 staining, possibly indicating a better response to stress stimulus via increased DNA repair.

Proliferative cell nuclear antigen (PCNA) is a well-known marker of DNA synthesis that has a key role in DNA replication. It also has a role in cell cycle regulation and DNA repair.[10] The PCNA index, which was developed to assess aggressiveness of tumors, has also been shown to be a useful marker to assess germ cell kinetics.[26,27] Our data revealed stronger immunoreactivity for PCNA in the micro-TESE positive group, indicating increased DNA synthesis and proliferation activity in this group.

The testicular cell number in the seminiferous tubules is maintained by a dynamic balance between cell proliferation and apoptotic cell death[28] Fragmentation of DNA in the nucleus is one of the morphological changes in the apoptotic process and can be detected in histological sections using the TUNEL (In situ terminal deoxynucleotidlytransferase-mediated deoxy-UTP nick end labeling) method.[29] This assay identifies single and double-stranded DNA breaks, labeling free 3□OH termini with modified nucleotides in a template-independent manner[30] In this experimental study, we did not perform the apoptosis pathway. However, we are planning to investigate apoptotic signal relations in testicular tissue using the micro-TESE procedure as a continuation of the present study.

CONCLUSIONS

In summary, the results of the present study revealed in-

creased PARP-1 and PCNA immunoreactivity reflecting greater DNA repair and synthesis activity in testicular tissues of NOA patients in whom SR could be achieved by micro-TESE procedure. In other words, increased PARP-1 and PCNA immunoreactivity was evident in successful TESE procedures. Based on these findings, it is possible to suggest that stronger PARP-1 and PCNA immunoreactivity in testicular tissue of NOA patients can be a promising predictor of successful SR following TESE procedures. However, we should emphasize that it would be inappropriate to make such a decision solely on the basis of the immunohistochemical findings of a single study. Further studies with additional DNA repair, synthesis, and apoptosis markers will be useful to enlighten the issue.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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