Objective: To explore the relationship between men’s age and DNA damage repair proteins related to apoptosis in human testicular germ cells.

Design: Retrospective case–control study.

Setting: Academic institutions.

Patient(s): Testicular specimens were obtained from 22 fertile volunteers aged 20–82 years.

Intervention(s): Deoxyribonucleic acid repair markers were assessed using immunohistochemical staining for the cell proliferation marker [proliferating cell nuclear antigen (PCNA)]; DNA repair markers [poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1), poly(adenosine diphosphate-ribose) (PAR), X-ray repair cross-complementing1 (XRCC1), and apurinic/apyrimidinic endonuclease 1 (APE1)]; and apoptosis-associated markers (caspase 9, active caspase 3, and cleaved PARP-1).

Main Outcome Measure(s): The prevalence and cellular localization of the above markers in testicular tissues of young, middle aged, and old men.

Result(s): Statistically significant differences in DNA damage repair–associated proteins (PARP-1, PAR, XRCC1, and APE1), and apoptosis markers (caspase 9, active caspase 3, and cleaved PARP-1) were observed in testicular samples from older men. These differences were most marked in spermatocytes.

Conclusion(s): The study demonstrates that there is an age-related increase in human testicular germ cell DNA break repair and apoptosis with age. (Fertil Steril 2009;91:2221–9. ©2009 by American Society for Reproductive Medicine.)

Key Words: Aging, DNA repair, apoptosis, germ cells, PARP-1, caspase.

There are several lines of defense against the induction and persistence of DNA damage in cells. First, there are agents that prevent the formation of DNA damage, such as detoxifying peptides and proteins, and oxyradical scavengers, such as vitamins E and C. Second, DNA damage already present in the genome may be sensed by DNA damage response pathways, and the damage may be removed by an array of DNA repair pathways to reduce the possibility of inducing mutations. Third, the damaged cell itself may be eliminated by spontaneous death or apoptosis (1).

Advancing paternal age has been reported to be implicated in a broad range of abnormal reproductive and genetic consequences (2, 3), including diminished semen quality (4), reduced fertility (5), increased nuclear DNA fragmentation (6), and increased frequencies of spontaneous abortions (7, 8).

The accumulation of macromolecular damage, especially damage to genomic DNA, is believed to underlie the aging process. Poly(adenosine diphosphate [ADP]-ribosyl)ation has been classically shown to be a pivotal pathway in DNA repair, apoptosis, and necrosis (9). Upon DNA damage, poly(ADP-ribose) polymerase 1 (PARP-1) is rapidly auto-modified and catalyzes the poly(ADP-ribosylation) of nuclear acceptor proteins, such as histone H1 and H2B, which leads to the relaxation of chromatin structure (10). El Khamisy et al. (11) have also shown that PARP-1 is required for the assembly or stability of X-ray repair cross-complementing group 1 (XRCC1) nuclear foci after oxidative DNA damage, and the formation of these foci is mediated through interaction with poly(ADP-ribose). They proposed a model in which the rapid activation of PARP-1 at sites of DNA strand breaks facilitated DNA repair by recruiting the molecular scaffold protein, XRCC1.
Given their roles in cellular responses to various types of genomic insults, PARP-1 and other PARPs have been implicated in aging and longevity (12, 13). Germ cell loss associated with aging occurs by apoptosis, probably because of a combination of a primary testicular defect and secondary hypothalamic pituitary dysfunction. Reproductive aging in the rat is characterized by decreased Leydig cell steroidogenesis associated with seminiferous tubule dysfunction. Accelerated germ cell apoptosis involving spermatogonia, spermatocytes, and spermatids is greater in the testes of aging rats than in the testes of younger animals (14).

The aim of this study was to evaluate human testicular germ cell proliferation, DNA break repair, and apoptotic markers in testicular biopsy specimens of fertile men of different age groups. Characterization of some of these markers would improve our understanding of the balance between mechanisms of proliferation, DNA repair, and apoptosis in germ cells that lead to production of sperm with good DNA integrity.

**MATERIALS AND METHODS**

**Testicular Specimens**

The study included 22 fertile married volunteers who had completed their families. They were patients of different age groups attending the Andrology Clinic and General Surgery Department at Al-Minya University Hospital (Egypt) for inguinoscrotal procedures, such as inguinal hernia repair and orchietomy for prostate cancer treatment, who agreed to donate a testicular specimen after providing written consent. Prostate cancer was in early stages, localized to the prostate with no metastasis to other organs, and the patients were in good general condition. The study was approved by the Ethics Committee of Postgraduate Studies and Research of Al-Minya University and the Human Investigations Committee of the Yale University School of Medicine. The subjects were divided into three groups: the young age group consisted of 10 patients aged 20–30 years, the middle age group consisted of 6 patients aged 30–40 years, and the old age group consisted of 6 patients aged 58–82 years.

All groups had proven fertility and had completed their families, without seeking any medical advice or treatment for infertility. For the young and middle age groups, no abnormalities were detected in the semen analysis (according to World Health Organization semen analysis standards) or their hormonal status (FSH, LH, and T). Duplex ultrasonography was also performed for all groups to exclude the presence of varicocele. Specimens were fixed in Bouin’s solution after surgical retrieval and were embedded in paraffin blocks. Hematoxylin and eosin staining and immunohistochemistry for cell proliferation marker (proliferating cell nuclear antigen or PCNA), DNA base excision repair (BER) markers [PARP-1, poly(ADP-ribose) (PAR), XRCC-1, and apurinic/apyrimidinic endonuclease 1 (APE1)], and apoptosis markers (caspase 9, active caspase 3, and cleaved PARP-1) were performed for all testicular specimens. Immunohistochemical studies were performed in the Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine.

**Immunohistochemistry**

Immunohistochemistry was performed using a VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Paraffin sections (5 μm) were deparaffinized in xylene and then rehydrated in graded alcohol. Antigen retrieval was performed by boiling the sections in 0.1 mol/L sodium citrate for 20 minutes, followed by 0.2% Triton X-100 in phosphate-buffered saline for 10 minutes. The slides were then treated with 3% hydrogen peroxide in deionized water for 10 minutes to quench endogenous peroxidase activity. The sections were blocked with normal serum from the strain used for raising secondary antibody for 30 minutes at room temperature. Each slide had two adjacent sections, with one serving as a negative control. Negative control sections were treated in the same way as above, using the strain-specific blocking serum while omitting the primary antibody labeling.

The specimens were labeled with first antibody overnight at 4°C [PCNA (MBL, Woburn, MA; 1:100), PARP-1 (R&D Systems, Minneapolis, MN; 1:100), PAR (Trevigen, Gaithersburg, MD; 1:200), XRCC1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), APE1 (Santa Cruz Biotechnology; 1:500), caspase 9 (Stressgen Bioreagents, Victoria, BC, Canada; 1:100), active caspase 3 (Promega, Madison, WI; 1:50), and cleaved PARP-1 (Epitomics, Burlingame, CA; 1:50)] and then labeled with biotinylated secondary antibody for 30 minutes at 37°C, followed by incubation with ABC reagent for 30 minutes at 37°C. Intervening washes were performed after each incubation. The sections were developed using a DAB Substrate Kit (Vector Laboratories) for 5–10 minutes and then were counterstained with VECTOR Hematoxylin QS (Vector Laboratories) and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

Specimens were examined with objective magnification of ×10, ×40, ×60, and ×100 using a Zeiss light microscope (Carl Zeiss, Jena, Germany). The different stages of germ cells were assessed for staining. Photographs were taken by a Zeiss digital camera attached to the microscope. Specimens were evaluated for positive cells that showed marker expression. In each specimen at least 10 seminiferous tubules were randomly evaluated, and the percentages of the positive cells were recorded. Positive cells were stained brown, and negative cells remained blue. In the young age group 10 patients were assessed and more than 100 tubules evaluated; in the middle age group 6 patients were assessed and more than 60 tubules evaluated; and in the old age group 6 patients were assessed and more than 60 tubules evaluated. To compare the percentage expression levels the data were then scored into six groups: 0 = 0, 1%–20% = 1, 21%–40% = 2, 41%–60% = 3, 61%–80% = 4, and >80% = 5.

Statistical analysis was performed by comparing means using analysis of variance and the Kruskal-Wallis and
Mann-Whitney U tests. All statistical analyses were performed using the SPSS 12.0 statistical package for Windows (SPSS, Chicago, IL).

RESULTS
Comparison of Changes of Marker Expression in Different Age Groups in Different Testicular Cell Types

Sertoli cells All the poly(ADP-ribosyl)ation-related DNA BER markers (PARP-1, PAR, and XRCC1) were expressed in Sertoli cells (Table 1). In particular, PAR showed a significant trend to increased expression in older patients. APE1 was not expressed in Sertoli cells. The cell proliferation marker PCNA was expressed in all Sertoli cells in the different age groups. As for the apoptotic markers, all Sertoli cells in all age groups expressed caspase 9 and active caspase 3, but they did not express cleaved PARP-1 in any age group (Table 2).

Spermatogonia All the DNA BER markers (PARP-1, PAR, XRCC1, and APE1) were also expressed in spermatogonia (Table 1). In particular, APE1 showed a significant trend to increased expression in older patients. The cell proliferation marker PCNA was expressed in all spermatogonia in the different age groups. As for the apoptotic markers, spermatogonia in all age groups expressed active caspase 3 and cleaved PARP-1, whereas there was a small but significant increase in expression of caspase 9 in the old age group. Both active caspase 3 and cleaved PARP-1 expression were significantly higher in the older patient’s spermatogonia (Table 2).

Spermatocytes The DNA BER markers PARP-1, PAR, and XRCC1 showed the greatest variability in expression in spermatocytes, with the old specimens showing significantly higher expression for PARP-1 and XRCC1 (Table 1). No spermatocytes were stained for APE1 in any age group. The cell proliferation marker PCNA was observed in all spermatocytes in the different age groups. As for the apoptotic markers, spermatocytes in all age groups expressed variable levels of active caspase 3 and cleaved PARP-1, but they did not express caspase 9 in any age group. (Table 2).

Round spermatids All round spermatids showed the presence of the DNA BER markers (PARP-1, PAR, and XRCC1); however, none showed the presence of APE1 (Table 1). Expression of the cell proliferation marker PCNA in round spermatids in different age groups showed a slight but significant increase in the old age group. For the apoptotic markers, all round spermatids in the three age groups expressed active caspase 3 and cleaved PARP-1, whereas there was a small but significant increase in expression of caspase 9 in round spermatids in the old age group (Table 2).

Sperm Sperm did not express any proliferation markers or DNA BER markers. In the young age group 96% of them expressed active caspase 3, and 100% of the sperm expressed the same marker in the middle and older age groups. All sperm from the young and middle age groups expressed cleaved PARP-1 in the cytoplasm, whereas 98% were

<table>
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<th>TABLE 1</th>
<th>Comparison of expression of DNA base excision repair markers in Sertoli cells, spermatogonia, spermatocytes, and round spermatids and sperm within young (n = 10 patients), middle (n = 6 patients), and older (n = 6 patients) age groups.</th>
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positive in the older age group. No sperm expressed caspase 9 in any of the groups. There was no significant difference in the expression levels of the markers in the different age groups (Table 2).

**Post-testicular spermatozoa** Further examination of cleaved PARP-1 showed that some epididymal spermatozoa displayed staining in the cytoplasm (Fig. 1A); however, ejaculated spermatozoa failed to show cleaved PARP-1 staining (Fig. 1B).

**Marker Expression in Different Age Groups**

**Young age group** Proliferating cell nuclear antigen and DNA BER proteins (PARP-1, PAR, and XRCC1) were expressed in the nuclei of all cell types in the seminiferous tubules except spermatoozoa. For both PARP-1 (Fig. 2A and B) and XRCC1 the expressions were lowest in spermatocytes (Table 1). APE1 was only expressed in spermatogonia nuclei (Fig. 2C and D). The apoptotic marker caspase 9 was only expressed in Sertoli cells, whereas the active form of caspase 3 was expressed in the cytoplasm of all cell types but less in spermatogonia and spermatocytes (Fig. 2E and F). Cleaved PARP-1 was expressed in the cytoplasm of all germ cells lining the seminiferous tubules except Sertoli cells (Fig. 2G and H).

**Middle age group** Proliferating cell nuclear antigen and DNA BER proteins (PARP-1, PAR, and XRCC1) were expressed in all cell types in the seminiferous tubules except sperm. For both PARP-1 and XRCC1 the expression was the lowest in spermatocytes (Table 1). APE1 was expressed only in spermatogonia nuclei (Fig. 2C and D). The apoptotic marker caspase 9 was only expressed in Sertoli cells, whereas the active form of caspase 3 was expressed in all cell types but to a lower extent in the spermatogonia. Cleaved PARP-1 was expressed in the cytoplasm of all germ cells lining the seminiferous tubules except Sertoli cells (Table 1).

**Old age group** Proliferating cell nuclear antigen and DNA BER proteins (PARP-1, PAR, and XRCC1) were detected in all germ cells lining the seminiferous tubules except sperm. APE1 was only detected in spermatogonia nuclei (Fig. 2A and B). The apoptotic marker caspase 9 was detected only in Sertoli cells and rarely in round spermatids. Active caspase 3 was detected in all cell types in the seminiferous tubules (Fig. 2E and F). Cleaved PARP-1 was detected in all cells in the seminiferous tubules except Sertoli cells (Fig. 2G and H).

**DISCUSSION**

Spermatogenesis is a complex process of proliferation and differentiation transforming spermatogonia into mature spermatozoa. This unique process involves a series of mitoses and a meiotic division followed by marked changes in cell structure, in addition to proliferation and differentiation. It is clear that spermatozoa can arise in the ejaculate possessing various nuclear anomalies related to chromatin damage (15). These arise owing to the possible failure of a number of processes that can either cause and/or fail to detect or eliminate the abnormal spermatozoa. Two processes that may fail in their task...
During spermatogenesis, leading to abnormal sperm in the ejaculate, are DNA repair and apoptosis.

In the present study we have examined one of the key DNA repair pathways involved in BER. The poly(ADP-ribosyl)ation pathway plays a multipurpose role in a wide range of biologic processes, including DNA repair and maintenance of genomic stability, transcriptional regulation, centromere function and mitotic spindle formation, centrosomal function, structure and function of vault particles, telomere dynamics, trafficking of endosomal vesicles, apoptosis, and necrosis (16). The PARP-1 enzymes are believed to be activated by DNA strand breaks. During meiosis, endogenous DNA double-strand breaks occur during the first meiotic prophase, which are repaired to instigate genetic diversity and chromosome segregation (17, 18). A second round of DNA breakage, which is presumably linked to chromatin compaction, occurs in differentiating spermatids as the chromatin is tightly compacted (19–23). We have shown the presence of key members of this pathway in normal testicular specimens and that changes occur in their presence during aging.

The pathways for single-strand break repair in mammalian cells involve a number of coordinated, sequential reactions responsible for damage detection: end processing, gap filling, and ligation. In the case of the short-patch pathway of BER, for example, a damaged base is recognized and removed by a damage-specific glycosylase, thus creating an abasic site whose phosphodiester bond is cut by APE1. Subsequently, DNA-polymerase β acts at the cleaved abasic site and excises the base-free sugar phosphate residue. Finally, this ligatable nick is sealed by DNA ligase IIIa. Substantial evidence indicates an important role for XRCC1 in single-strand break repair and BER. Apparently devoid of any enzymatic activity, this protein is thought to act as a scaffolding protein for other repair factors. XRCC1 has been shown to physically interact with several enzymes known to be involved in the repair of single-strand breaks, including DNA ligase IIIa, DNA-polymerase β, APE1, PARP-1, and PARP-2 (24).

Only a limited number of reports have investigated the role of poly(ADP-ribosyl)ation during mammalian spermatogenesis, and most of these have been limited to the rodent species. Interestingly, a recent study by Dantzer et al. (25) has shown that PARP-2-deficient mice exhibit severely impaired spermatogenesis, with a defect in prophase of meiosis I characterized by massive apoptosis at pachytene and metaphase I stages. A number of previous biochemical investigations that described the localization of PARP-1 activity in rat (26–28) and rooster testes (29) reported high PARP-1 activity localized in spermatogonia and pachytene nuclei of spermatocytes. Interestingly a study by Meyer-Ficca et al. (30) noted that PAR expression was localized in the spermatids and was related to chromatin remodeling occurring during spermiogenesis. Human spermatogenesis, however, proceeds very differently from that in rodents. First, in rodents spermatogenesis occurs in waves in the seminiferous tubules so they express one stage of spermatogenic cells, whereas in the human different stages of spermatogenic cells are expressed at the same time. Second, human spermatogenesis is known to produce a heterogeneous population of mature spermatozoa when compared with rodents, in particular when one examines DNA quality (31).

Recently Maymon et al. (32) investigated the role of PAR and PARP-1 during human spermatogenesis in azoospermic men who underwent testicular biopsies for sperm recovery. Expression of both markers was localized in germ cells nuclei in seminiferous tubules showing full spermatogenesis; PAR expression was exhibited in round spermatids and sperm in a subpopulation of primary spermatocytes. In seminiferous tubules showing maturation arrest at the spermatocytes level, strong PAR expression was identified in spermatocytes. In contrast to our results, Sertoli cells lacked immunoreactivity for both markers.

In the present study PARP-1 was expressed in all spermatogonia in the testes from young men. In spermatocytes the expression levels decreased dramatically, whereas in...
Representative images of immunohistochemistry of testicular sections from young (A, C, E, G) and old men (B, D, F, H). Expression of PARP-1 in spermatocyte nuclei of the young (A) age group is less frequent compared with that in the old (B) age group spermatocytes. Expression of APE1 in spermatogonia nuclei of the young age group (C) is less compared with that in the old age group (D). Expression of active caspase 3 is decreased in spermatogonia and in the perinuclear area of spermatocytes of the young age group (E) compared with those of the old age group (F). Cleaved PARP-1 expression was decreased in the young age spermatogonia and in the perinuclear area of spermatocytes (G) compared with in the old age group (H). Sperm show the apoptotic-associated markers: active caspase 3 (E, F) and cleaved PARP-1 (G, H) around the tail before complete removal of the cytoplasm. Original magnification, ×60 in A and B, ×100 in C–H.
round spermatid expression was again increased and seen in all spermatids. Interestingly, XRCC1 expression mirrored that of PARP-1 indicating the joint association between these proteins in presumed response to the presence of DNA strand breaks. PAR expression, however, was also observed in all spermatocytes, indicating that poly(ADP-ribosyl)ated proteins are present throughout the early stages of spermatogenesis until at least the round spermatid stage. In relation to a second marker of DNA BER, APE1 showed the most specific staining in that it was only found in spermatagonia. This indicates that poly(ADP-ribosylation) may indeed be involved in other aspects of cell function distinct from DNA repair. The presence, in particular, of PARP-1 in all the germ cell stages shows that poly(ADP-ribosylation) is involved in a broad range of cellular processes. The presence of PARP-1 in the spermatid stage has already been linked to a role in facilitating DNA strand break management during the chromatin remodeling steps of spermiogenesis, where controlled DNA double-strand breaks are introduced into round spermatids to support the exchange of protamines and histones (30). The presence of PAR did not identically mirror that of PARP-1 in that it was also observed in all spermatocytes. Recently, PAR has also been shown to be not only involved in DNA damage and apoptosis but to have important regulatory functions in the normal physiology of the cell. An example of this is during mitosis, when NuMa, a protein involved in mitotic spindle function, is poly(ADP-ribosyl)ated (33–35).

The following scenario could therefore be perceived to occur during normal spermatogenesis in relation to poly(ADP-ribosylation). Spermatogonia display an innate ability to undergo DNA BER. This is shown by the presence of APE1 as a marker and also the presence of PARP-1, XRCC1, and PAR. Interestingly, APE1 was expressed in fewer cells than PARP-1, indicating that either PARP-1 is a preferred mode of DNA repair or that PARP-1 is also functioning by another mechanism. The second is more likely because PARP-1 is also being increasingly implicated in more complex events, such as a previously uncharacterized nucleosome binding property that promotes the formation of compact, transcriptionally repressed chromatin structures (36). In the spermatocytes PARP-1 expression was less abundant, and here it is more likely involved with the onset of meiosis, whereby DNA strand breaks are introduced during the first meiotic prophase; these DNA strand breaks would recruit PARP-1 and XRCC1. The poly(ADP-ribosyl)ation system is believed to regulate protein association dynamics at the site of DNA damage in vivo. This is supported by studies that demonstrated rapid recruitment of XRCC1 to sites of poly(ADP-ribose) formation in DNA-damaged living cells (11, 37). Finally, in the spermatid stage PARP-1 clearly seems implicated in the intricate nuclear restructuring that occurs when the sperm nuclei is compacted with the introduction of protamines (38, 39).

The most well-characterized role of poly(ADP-ribosyl)ation is its link to activate apoptosis in the event of dramati-
Advancing male age has multiple genomic defects in human sperm (DNA fragmentation index, chromatin integrity, gene mutations, and numeric chromosomal abnormalities). Wyrobek et al. (6) proposed that there are multiple spermato- genetic targets for genomically defective sperm, with substantially variable susceptibilities to age. They concluded that as healthy men age, they would have decreased pregnancy success and increased risk for producing offspring with acro- droplasia mutations, but no increased risk for fathering aneuploid offspring (Down, Klinefelter, Turner, triple X, and XYY syndromes). One theory is that testicular apoptosis may be enhanced in the aging man owing to the decline in the T level that occurs in advancing age (51). This germ cell apoptosis resulting from reduced intratesticular T concentration is caspase 3 dependant (52).

In aged testes, significant differences were observed in DNA repair and apoptotic marker expression in both spermatogonia and spermatocytes. In Sertoli cells there was a difference observed in the expression of PAR (P<0.001), being higher in older men. Although spermatogonia did not show any significant difference in the poly(ADP-ribosyl)ation-related markers PARP-1, PAR, and XRCC1 between young and old subjects, APE1 expression (P<0.001) was significantly higher in the old age samples. PARP-1 (P<0.001) and XRCC1 (P<0.001) expression were significantly higher in spermatocytes in the old age group testes specimens compared with the young. Apoptotic markers active caspase 3 (P<0.001) and cleaved PARP-1 (P<0.001) expression were significantly higher in the spermatogonia and spermatocytes in the old testes compared with the young.

Hence, it seems that aging is associated with increased DNA repair due to increased DNA breaks. When, repair mechanisms such as poly(ADP-ribosyl)ation are over-activated, it may lead to apoptosis (53). This could explain the increased expression of active caspase 3 and cleaved PARP-1 in spermatocytes after PARP-1 over-activation and the initiation of apoptosis in them.

In this study we have used immunohistochemistry as a tool to investigate expression of various apoptotic and DNA repair markers. Although it is difficult to accurately quantify expression using immunohistochemistry, the results provided clearly indicate both the presence of key poly(ADP-ribosyl)ation pathway members and changes during age. Limitations in the antibodies available and their own intrinsic differences can lead to a mis- or over-interpretation of results when using immunohistochemistry. The results at hand do, however, clearly indicate that differences exist in the DNA repair and apoptosis markers with age in the testes; however, whether this is due to a global aging in all tissues needs to be verified. With this information we can confidently pursue more elaborate techniques of examination of testicular tissues at both the RNA and protein levels, and this will provide additional insights into the importance of these pathways.

In conclusion, the classic duties of the poly(ADP-ribosyl)ation pathway–related DNA repair and apoptosis pathways are largely carried out in the early stages of spermatogenesis. In the final stages of spermiogenesis, apoptosis is used not only as a mechanism of programmed cell death but as a means to facilitate the cytoplasmic remodeling that occurs during the final stages of sperm maturation. With age, there is an increased expression of DNA repair and apoptosis-associated markers in the testicular germ cells, supporting the idea that changes occur in sperm DNA quality with age (6).

REFERENCES


