

Apoptosis, spermatogenesis and male infertility

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Germ Cell Apoptosis
4. Sperm Apoptosis
5. Apoptosis in cryptorchid testis
6. Oxidative stress and apoptosis
7. Apoptosis and male infertility
8. Apoptosis and male reproductive ageing
9. Apoptosis as a marker of semen quality
10. Conclusion and future directions
11. Acknowledgement
12. References

1. ABSTRACT

Apoptosis is an essential physiological process demonstrated to play important roles in diverse physiological processes. As true for several other organs, apoptosis occurs at a high rate in the primary male reproductive organ, testis. Apoptosis is also exhibited by spermatozoa in the human ejaculate. Caspase activation, externalization of phosphatidylserine, alteration of mitochondrial membrane potential and DNA fragmentation are markers of apoptosis found in ejaculated human spermatozoa. These markers appear in excess in sub-fertile men and functionally incompetent spermatozoa. The importance of apoptotic pathway in spermatogenesis and sperm maturation is also indicated by the expression of several markers of this pathway in the testis and epididymis, respectively. This process of regulated cell death serves several important functions in the testis, a few of which include maintaining appropriate germ cell to Sertoli cells ratio, removing defective germ cells and maintenance of overall quality control in sperm production. This review presents an update on the role of apoptosis in male reproduction and fertility, and implications of altered apoptosis in male infertility.

2. INTRODUCTION

Unwilling childlessness in the couples in spite of having regular unprotected intercourse within a certain period (12 months or more) of time is defined as infertility (1, 2). Approximately 15 % of sexually active couples, not using any contraceptive are unable to conceive (3). In about half of them, male factor is the sole cause or contributes to the infertility problem. Research on a variety of possible causes has helped understanding the basic process of spermatogenesis and male fertility. The main causes of infertility in men are diverse such as genetic, physiopathologic and anatomopathologic abnormalities, intense and prolonged physical exercise, aging, drugs, and even excessive time of sexual abstinence (4).

Spermatogenesis is a complex biological process which depends on a precisely controlled cascade of developmental genes orchestrating spermatogonial cell proliferation, chromosomal reduction divisions to produce a haploid genome in each daughter cell and finally, morphological differentiation of these cells into mature sperm. The expression of a large number of genes is developmentally regulated during spermatogenesis (5).

Both transcriptional and translational control mechanisms are responsible for temporal and stage specific expression pattern (6). The transition through different steps of spermatogenesis is tightly regulated and a defect at any of these steps may impair spermatogenesis. Among several processes important at this stage, quality control is one of the most important aspects. Apoptosis is the best known quality control mechanism in testis.

Apoptosis is an evolutionary-conserved regulatory physiological mechanism that allows eukaryotes to eliminate unneeded, senescent, or aberrant cells (7). A cell will undergo apoptosis as a result of information received from its environment interpreted in the context of internal information, such as cell type, state of maturity and developmental history. Pathways to activate apoptosis are different in various cell types; however, the mechanism of death itself may be the same, that is, a final common pathway (8). A cell first encounters a signal to activate the relevant genetic machinery before it enters into the apoptotic pathway (9). A stimulus, which originates from outside the cell exists which can advance or delay apoptotic cell death. Agents that can penetrate the cell directly, and modulate the apoptotic cascade in the absence of specific cell surface receptors can trigger apoptotic stimulation. These agents include: heat shock, stress factors, free radicals, ultraviolet radiation, numerous drugs and synthetic peptides, toxins and potent lymphocyte enzymes (10). Two distinct pathways exist for the initiation of apoptosis: extrinsic or receptor-linked apoptosis and intrinsic or mitochondria-mediated apoptosis (11).

Mitochondrion is an important intracellular organelle participating in the process of apoptosis. Mitochondria are multitasking organelles involved in ATP synthesis, reactive oxygen species (ROS) production, calcium signalling and apoptosis. Mitochondrial defects are known to cause physiological dysfunction, including infertility (12). The interrelated mitochondrial systems are assembled from roughly 1000 genes distributed between the two very different genetic systems of the mammalian cell: the nuclear genome and the mitochondrial genome (13). Hence, the complexities of mitochondrial diseases reflect the intricacies of both the physiology and the genetics of mitochondria. Apoptosis begins with a series of cellular, morphological and biochemical alterations which lead to cell shrinkage, membrane blebbing and DNA fragmentation (14). In somatic cells, mitochondria are the prime regulator of apoptosis (programmed cell death), by initiating the activation of the mitochondrial permeability transition pore (mtPTP). Caspases, a family of cysteine aspartic acid proteases, are important in regulating apoptosis (15).

Investigation of apoptosis in spermatogonia, spermatocytes and spermatids in the testis has been done extensively and many apoptotic factors have been identified (16). Testicular apoptosis has been reported in human specimens, but its correlation with serum gonadotropins and testosterone levels is not clear (17). It is assumed that somatic cells may die in the apoptotic, the autophagic, or the necrotic way but the mechanisms

involved in the sperm death are obscure and the biological significance of apoptosis in ejaculated sperm is yet to be elucidated (18). Externalization of phosphatidylserine to the sperm outer membrane leaflet is considered to mark terminal apoptosis. Activated caspases-3, loss of the mitochondrial membrane potential and DNA fragmentation are other markers of terminal apoptosis expressed by varying proportion of ejaculated sperm (19, 20). Apoptosis plays an important role in regulating spermatogenesis of various mammalian species including human (18). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (21). The portions of apoptotic sperm are reported to be higher in ejaculated semen samples from infertile men compared with fertile men (22). This is also evident from the fact that sperm caspases become more activated in patients with infertility than in healthy men (22, 23). There is an established consensus on the implication of apoptosis in male infertility; however, the exact mechanism of its involvement remains to be elucidated. The present review is an effort to update the role of apoptosis in normal spermatogenic process, sperm quality control management and male infertility.

3. GERM CELL APOPTOSIS

Spermatogenesis, the dynamic process of cell differentiation is a precisely controlled and cyclical timed process comprising of proliferation of spermatogonia, meiotic divisions of spermatocytes, and the differentiation of spermatids into spermatozoa (24, 25). Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact. Sertoli cells, spanning the thickness of the seminiferous epithelium, supervise spermatogenesis by providing structural and nutritional support to the germ cells (23). The proliferation and differentiation of germ cells depends on the release of two hormones from the anterior pituitary gland, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (26). The removal of these hormones induces germ cell apoptosis through indirect effects (27). Spermatogenesis is accompanied by germ cell apoptosis in the seminiferous epithelium and apoptosis here could be triggered by a variety of stimuli (25, 28). Kim et al., 2002 proposed four possible functional roles for the presence of apoptosis during spermatogenesis (24). He postulated that germ cell apoptosis is necessary to maintain an optimal germ cell to Sertoli cell ratio, to eliminate any abnormal germ cells and that the formation of the blood-testis barrier requires the elimination of excessive germ cells and an apoptotic surge of germ cell apoptosis occurs prior to puberty regulating the ratio of germ cells to Sertoli cells. Approximately 75% of the spermatogonia die in the process of programmed cell death before reaching maturity (27, 28).

It is generally accepted that Sertoli cells control the germ cell population through one of the best-known apoptotic pathways, the Fas/FasL paracrine signal transduction system, in which the Fas ligand (FasL) expressed by Sertoli cells induces apoptosis when it binds with its receptor, Fas, expressed by the germ cells (29). In addition to the physiological germ cell apoptosis that occurs

continuously throughout life (30), increased germ cell apoptosis results from external disturbances such as irradiation or exposure to toxicants (31). Over proliferation of early germ cells is tempered by selective apoptosis of their progeny (18). Large numbers of spermatocytes undergo apoptosis in the testis of a 4-week-old rat, and spermatogonia become the main cell type undergoing apoptosis in the adult rat (18). Testicular germ cell apoptosis occurs normally and continuously throughout life (32). In addition, massive testicular germ cell loss is known to result from toxicant exposure (33) depletion of growth factors (34), alterations of hormonal support (testosterone or pituitary hormones including FSH and LH) (35, 36), heat exposure (37), radiation (38), or treatment with chemotherapeutic compounds (39). In many of these situations, germ cells are known to undergo apoptosis, indicating that a specific pathway is activated when the testicular environment cannot support spermatogenesis. However, the elements that control this process have not been identified

4. SPERM APOPTOSIS

Mature sperm cells have been reported to express distinct markers of apoptosis-related cell damage although they lack transcriptional activity and have a very small amount of cytoplasm. Externalization of phosphatidylserine (PS) to the sperm outer membrane leaflet is considered to mark terminal apoptosis. Activated caspase-3, loss of the integrity of the mitochondrial membrane potential (MMP) and DNA fragmentation are other markers of terminal apoptosis expressed by a varying proportion of ejaculated sperm (19, 20). Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency (21). The proportions of apoptotic sperm are reported to be higher in ejaculated semen samples from infertile men compared with healthy men. Moreover, sperm caspases become more activated in patients with infertility than in healthy fertile men (22, 23). Sperm apoptosis remains a controversial issue as uncertainty exists if apoptotic sperm are sperm with poor functional activity or in fact indicative of sperm that have failed to complete maturation during spermiogenesis. Several investigations demonstrated the occurrence of possible apoptosis in ejaculated human sperm (40) and in bull sperm (41). However, a study conducted by Lachaud et al., 2004 examined the ability of human spermatozoa to initiate apoptosis *in vitro* and their findings suggested that these cells lack the capacity to initiate the apoptotic pathway of cell death (42). Therefore, it would be interesting to find out who actually triggers apoptosis in sperm. It must start when the sperm are inside the body and may continue later on or we may just observe the remnants of this process in the ejaculate.

Apoptosis in sperm may also be initiated by ROS-independent pathways involving the cell surface protein Fas. Fas is a type I membrane protein that belongs to the tumour necrosis factor-nerve growth factor receptor family and mediates apoptosis. When Fas ligand or agonistic anti-Fas antibody binds to Fas, apoptosis occurs. On the other hand, bcl-2, the inhibitor gene of apoptosis, protects the

cell, most likely by mechanisms that reduce ROS production. Although the Fas protein often leads to apoptosis, some of the Fas-labelled cells may escape apoptosis through abortive apoptosis. This result in a failure to clear all of the spermatozoa destined for elimination and thus, leads to a large population of abnormal spermatozoa in the semen. This failure to clear Fas- positive spermatozoa may be due to a dysfunction at one or more levels. First, the production of spermatozoa may not be enough to trigger apoptosis in men with hypospermatogenesis. In this case, Fas-positive spermatogonia may escape the signal to undergo apoptosis. Second, Fas-positive spermatozoa also may exist because of problems in activating Fas-mediated apoptosis. In this scenario, apoptosis is aborted and fails to clear spermatozoa that are earmarked for elimination by apoptosis. In men with abnormal sperm parameters (oligozoospermia, azoospermia), the percentage of Fas-positive spermatozoa can be as high as 50 per cent. Samples with low sperm concentrations are more likely to have a high proportion of Fas-positive spermatozoa.

5. APOPTOSIS IN CRYPTORCHID TESTIS

Cryptorchidism is the most common congenital disorder in newborn boys. The incidence of cryptorchidism has increased during the last few decades (39) from about 1% to 1.5%. It is a serious risk factor for testicular cancer (43) and an important cause of infertility (43). The pathogenesis of the reduced fertility seen in cryptorchidism has not been fully clarified; however, it is known that heat stress in cryptorchidism reduces sperm count and hence compromise fertility. Several studies have detailed the morphometric features of cryptorchid testes during the early years of life (44, 45). The germ cell counts of cryptorchid testes are within normal limits during the 1st year of postnatal life. They fall below the normal range between 1 and 2 years of age, reaching the lowest level of germ cells per tubule at approximately 2 years of age (45, 46). The reduced fertility has been linked to the reduced number of germ cells, because the cryptorchid patients with the lowest total germ cell counts have the poorest spermiograms in adulthood (47). The unfavorable temperature affecting the undescended testis may also be an important factor in the occurrence of germ cell loss and infertility. The temperature in the scrotum, which is a few degrees lower than the body temperature, is believed to maintain an optimal environment for testicular function. Surgical induction of cryptorchidism in experimental animals causes disruption of spermatogenesis, which leads to infertility. It has now been shown that the expression of apoptotic markers increases in the cryptorchid testis which compromises fertility (48). Absalan et al., 2009 demonstrated strong decrease in the expression of surviving 140 and 40 variants and an increase in the expression of p53 and Bax (48). Observations on infertile men with oligozoospermia and azoospermia indicate that some of the patients may have higher scrotal temperature due to an intrinsic defect in thermoregulation, varicocele or occupational exposure to higher temperatures (49, 50).

6. OXIDATIVE STRESS AND APOPTOSIS

Amongst numerous causes of male infertility, oxidative stress is one important contributor. Reactive oxygen species (ROS) production and its effects on semen quality have been extensively studied in recent years. Sperm, like any other aerobic cell, are constantly facing the “oxygen-paradox”. Oxygen is essential to sustain life and physiological levels of ROS are necessary to maintain normal cell functions. Conversely, breakdown products of oxygen such as ROS can be detrimental to cell function and survival (51). Oxidative stress is a consequence of an imbalance between ROS production and body’s antioxidant defense capacity (52, 53). ROS are detrimental to sperm survival and function due to its adverse effects on sperm membrane and genetic material. High frequency of single- and double-stranded DNA breaks due to oxidative stress, activates apoptosis by inducing cytochrome c and caspases 9 and 3 (18). Disruption of inner and outer mitochondrial membranes results in release of cytochrome ‘C’ protein which activates caspases and induces apoptosis. Studies in infertile men showed that high levels of cytochrome ‘C’ in seminal plasma indicate significant mitochondrial damage by ROS (54).

Considerable evidence exists showing disruption of mitochondrial functions (e.g., loss of transmembrane potential, permeability transition, and release of cytochrome ‘C’ leading to impaired electron transport) are important events in many apoptotic cell deaths (55). Mitochondrial exposure to ROS results in the release of apoptosis inducing factor (AIF), which directly interacts with the DNA and leads to DNA fragmentation (18). A positive correlation was demonstrated between increased sperm damage by ROS and higher levels of cytochrome C and caspase 9 and 3, which indicate positive apoptosis in patients with male factor infertility (54). Activation of caspases 8, 9, 1, and 3 in ejaculated human spermatozoa has been studied to examine the main pathways of apoptosis (55). Potential functional impact of this phenomenon and possible activation mechanisms were examined by subjecting cells to freezing and thawing, and testing the dependence of caspase activity on membrane integrity (56, 57). Therefore, in the context of male infertility, seminal oxidative stress, sperm DNA damage and apoptosis are interlinked, and constitute a unified pathogenic molecular mechanism. Therefore, apoptosis in semen could be a useful indicator of semen quality.

7. APOPTOSIS AND MALE INFERTILITY

A dramatic increase in germ cell apoptosis occurs in some pathological conditions, which include idiopathic infertility in males (26). Spontaneous apoptosis has been observed frequently in spermatocytes, less frequently in spermatogonia and seldom in spermatids (58). The presence of apoptosis in human germ cells in the testes, specifically in spermatocytes has been reported by Fujisawa *et al.* (59). The authors found that the rate of apoptosis decreased in the testis of infertile men. A study undertaken by Martinčić *et al.*, 2001 determined the presence and frequency of germ cell apoptosis in the human testis from infertile patients while the Sertoli cells were not apoptotic (28). The exact incidence of male adult

germ cell apoptosis remains unclear, since not all degenerating germ cells display the classical morphology of apoptosis (27). It has been postulated that androgen withdrawal alters the expression of caspase activity in different cell types of human seminiferous epithelium (60). The Fas-system has been shown to participate in the regulation of the spontaneous germ cell apoptosis. It has been hypothesized that in the normal state, Sertoli cells express Fas ligand and this triggers apoptosis of a few Fas-positive germ cells revealing a paracrine control between the Sertoli and germ cells (61, 62, 63). The authors concluded that this death-delivering process ensures testicular homeostasis by the elimination of ill-supported germ cells. Fas signaling pathway between Sertoli cells and germ cells is crucial not only in normal apoptosis but also in toxicant induced apoptosis (64). The development of mature sperm is the product of a precisely regulated sequence of events in which germ cell proliferation, differentiation, self-renewal and apoptosis is controlled.

Aziz *et al.*, 2007 compared the apoptotic and non-apoptotic fractions of ejaculated human spermatozoa (65). The authors separated apoptotic and non-apoptotic fractions of human sperm for evaluation of morphology and sperm deformity index. Evaluation of sperm morphology and other quality parameters revealed a superior quality of the non-apoptotic fraction in comparison to the apoptotic fraction. The morphology markers used by the authors did not show any change post-ejaculation. Unlike other animals, human sperm epididymal passage is not associated with any morphological remodeling detectable under the light microscope (66). Even the cytoplasmic extrusion is completed before the commencement of epididymal transport (67). Therefore, these markers are likely to present these morphological changes which started developing within the seminiferous tubules. The presence of such cells in the ejaculate represents the remnants of the process of quality control by apoptosis. This is also supported by the fact that the healthy spermatozoa after ejaculation do not become apoptotic over time if incubated under standard conditions (42, 68). These cells ultimately die by necrosis and not by apoptosis (42).

FAS interact with its natural ligand FASLG to initiate the extrinsic apoptotic pathway (69). In testis, the expression of FAS and FASLG is predominant and confined mainly to germ cells and Sertoli cells, respectively (62), which have been proposed to be the key regulators of the process of germ cell apoptosis (63). The downstream function of FAS is mediated by various caspases including caspase-8. This makes the genes encoding these proteins very important candidate genes for screening in the infertility cases. Ji *et al.*, 2009 analyzed 620 infertile individuals for identification of polymorphisms in FAS, FASLG and CASP9 genes in the infertile population (70). The authors observed correlation between FAS-670A/G (rs1800682:A.G) and CASP8-6526N ins/del (rs3834129:–/CTTACT) polymorphisms and semen quality. Individuals with FAS-670GG showed low apoptosis rate and decreased sperm concentration, compared with the FAS-670AA genotype. Similarly, in comparison with the CASP8-6526N ins/ins genotype, the CASP8-6526N (ins/del + del/del)

genotypes also showed significantly decreased sperm apoptosis rate and poor sperm motility. The study showed for the first time that functional variants in apoptosis related genes could affect semen quality and fertility (70). In a recent study we compared the level of apoptosis between fertile control individuals and infertile individuals characterized by normozoospermia, oligozoospermia and asthenozoospermia (71). Statistically significant difference between cases and controls was observed and apoptosis was increased in all groups of infertile individuals. All the above taken into account indicates increased level of apoptosis in male infertility.

8. APOPTOSIS AND MALE REPRODUCTIVE AGEING

In the testes, apoptosis increases with age, producing an accelerated germ cell loss (72, 73). This might be related to the fall in androgen levels (74) and/or an increase in oxidative stress in the tissue (75). These changes also occur in the epididymis and other reproductive organs, so it is not unreasonable to think of an increase in apoptotic indices in the ageing male tract. In addition, in the epididymal epithelium, some striking segment-specific changes occur at the histological and biochemical levels prior to the major loss of spermatogenesis. These changes include some ultrastructural features which are characteristic of ageing, such as accumulation of lipofuscin granules, a dramatic increase in the thickness of the basement membrane and the number of halo cells (76), and changes in the junctional complexes between epithelial cells (77). In addition, changes in the expression of genes related to oxidative stress in the epididymis due to age have also been described (78). Changes in gene expression related to ageing include the decrease of several mRNAs, such as the 5 alpha-reductase isozymes and proenkephalin, that occurs in the epididymis (79). In fact, this decrease is the earliest marker for ageing in the male reproductive tract (80). These changes are strongly correlated with the process of apoptosis. Therefore, it appears that apoptosis plays a very significant role in reproductive ageing.

9. APOPTOSIS AS A MARKER OF SEMEN QUALITY

Sperm DNA damage and sperm apoptosis have been considered as potentially useful indices of male fertility. Cellular apoptosis is a normal event that occurs both during and after embryonic development. Germ cell loss (80, 81), now recognized as occurring via apoptosis, is a dominant process during spermatogenesis and is regulated by p53, p21, caspases, bcl-2, and Fas expression levels, with many alternate pathways (82). It has been found that the number of sperm with Fas expression was low in subjects with normal sperm parameters but high in men with abnormal sperm parameters (83). The presence of immature sperm may contribute to increased levels of apoptotic markers in ejaculated sperm (84). Although apoptosis is considered a mechanism to ensure selection of sperm cells with undamaged DNA, sperm with DNA

damage that are not eliminated by apoptosis may fertilize an ovum (83).

Many studies have reported increased levels of apoptosis in poor quality semen samples (18). There are a number of indications that apoptosis occurs during spermatogenesis in humans (85). Apoptosis has been reported even in ejaculated sperm (86). The observation that mature ejaculated spermatozoa are positive for the TUNEL assay has led to the theory that apoptosis is occurring (18, 87). Whether apoptosis accounts for a significant proportion of DNA damage seen in the spermatozoa of infertile men is still an open question. Studies have shown DNA fragmentation in ejaculated spermatozoa (83). DNA damage has been further linked to poor pregnancy outcome (1). There are three hypotheses that have been put forward to explain the source of DNA damage in sperm. First, it is believed that DNA damage is caused by improper packaging and ligation during sperm maturation (88). Secondly, oxidative stress causes DNA damage (74) and the increased levels of specific forms of oxidative damage such as 8-hydroxydeoxyguanosine in sperm DNA supports such a theory (89). Thirdly, observed DNA fragmentation is caused by apoptosis (90, 91).

We have measured the level of apoptosis in a recent study in the individuals undergoing infertility treatment. Increased level of apoptosis was measured in all groups of infertile individuals before treatment (71). The use of herbal supplements resulted in improvement in overall semen quality. This was accompanied by a significant decrease in the level of apoptosis post-treatment. All the above taken into account indicates that measurement of apoptosis could be an indicator of semen quality. Measuring apoptosis level could also be used as a marker for follow up of male infertility treatment to restore natural fertility or in assisted reproduction. However, studies need to determine the level of useful apoptosis in the semen samples against the level that indicates compromised semen quality.

10. CONCLUSION AND FUTURE DIRECTIONS

From the above studies, the role of mitochondria mediated apoptotic pathway and extrinsic apoptotic pathway appears to be critical in reproductive physiology and has been established beyond doubt. It has been reported to affect several physiological processes pertaining to male fertility, most importantly the semen quality by removing defective cells. Apoptosis in addition to maintaining an appropriate ratio between Sertoli and germ cells is one of the well known mechanisms of quality control in the testis. The role of sperm production in propagation of species and the enormous number of cell divisions in the testis justifies the critical role of apoptosis in this process. Though certain level of apoptosis is always required to effectively deliver health sperm, increased level of apoptosis could indicate adverse effects on sperm production, ultimately compromising male fertility. Further research in this field could explore the following aspects:

The presence of apoptotic markers in the sperm needs further investigation regarding its origin, requirement and male infertility. There are several members to function at each step of the pathway and different members may work in tissue / organ specific manner. Activation of apoptotic pathway in testicular dysfunction and normal tissues may use another protein for the same function. Therefore, studies of apoptotic gene expression in normal and infertile testis could provide further insights into the process of spermatogenesis and male infertility. Defect in a proteins participating in this pathway may result in reduced activity, which could hamper normal apoptotic process. Abortive apoptosis leads to DNA damage and lowering of the semen quality. Therefore, individuals with abnormal apoptosis and DNA damage are candidates for mutation analysis on genes participating in apoptotic pathways. Role of ions in maintenance of normal cellular environment is well known. Ion channels also play an important role in the apoptotic signaling. By understanding the molecular mechanism of these ion channels during defective and normal apoptotic process may provide further insights into the idiopathic male infertility. Effect of environmental factors including drugs such as chemotherapy on apoptosis in testis could help us explain their effect on fertility and reproduction.

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