Chapter 12
Apoptosis in Ejaculated Spermatozoa and in the Normal and Pathological Testes: Abortive Apoptosis and Sperm Chromatin Damage

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12.1 Introduction

The term "programmed cell death" was originally used to describe the coordinated series of events leading to cell demise during development. The term "apoptosis" refers to a morphologically distinct form of cell death that plays a major role during the normal development and homeostasis of multicellular organisms. This mode of cell death is a tightly regulated series of energy-dependent molecular and biochemical events orchestrated by a genetic program [1].

Apoptosis is either developmentally regulated (launched in response to specific stimuli, such as deprivation of survival factors, exposure to ionizing radiation and chemotherapeutic drugs, or activation by various death factors and their ligands) or induced in response to cell injury or stress. It is now widely accepted that apoptosis serves as a prominent force in sculpting body parts, deleting unneeded structures, maintaining tissue homeostasis, and as a defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes, virus-infected cells and tumor cells. Apoptosis has also been recognized in the pathogenesis of many diverse human diseases including cancer, acquired immune deficiency syndrome, neurodegenerative disorders, atherosclerosis, and cardiomyopathy. Maintaining the homeostatic relationship between apoptosis and cell proliferation is important for tissue development and degeneration. Decreased apoptosis may lead to neoplasia, whereas increased apoptosis may lead to a dystrophic condition [1].

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Table 12.1 Key events occurring during spermatogenesis when comparing apoptosis and necrosis

	APOPTOSIS		NECROSIS
	ACTION	ROLE IN SPERMATOGENESIS	
STIMULATED BY	Tissue remodeling Maintenance of cell pool size Genomic Damage Metabolic derangement hypoxia Imbalance in signaling pathways	Cytoplasmic remodelling Maintains spermatogonia numbers Important in Spermatocytes	Metabolic stresses Absence of nutrients Changes in pH, temperature Hypoxia, anoxia
MORPHOLOGICAL CHANGES			
Affected cells	Individual Cells	Can impact overall sperm morphology	Groups of cells
Cell volume	Decreased		Increased
Chromatin	Condensed		Fragmented
Lysosomes	Unaffected		Abnormal
Mitochondria	Initially remain normal		Morphologically Aberrant
Inflammatory response	None		
Cell fate	Apoptotic bodies consumed by neighboring cells	Sertoli Cells can act as macrophages	Lysis
MOLECULAR CHANGES			
Gene activity	Required for program	Replacement of Histones by Protamine shuts down gene activity and also changes the ability for uniform DNA fragments to occur	Not needed
Chromosomal DNA	Cleaved at Specific sites leading to uniform sized DNA fragments		Random Cleavage

The impact of apoptosis on aspects of spermatogenesis is highlighted in the Role in Spermatogenesis column

12.1.1 Cellular Characteristics of Apoptosis Versus Necrosis

The process of apoptosis is associated with well-defined morphological and biochemical changes, including a reduction in cell volume, blebbing of the cell membrane, chromatin condensation and margination, and formation of apoptotic bodies (Table 12.1). In contrast to physiological cell death or apoptosis, necrosis is a passive process that does not require energy expenditure by the cell and occurs in response to a wide variety of noxious agents. Necrosis (Table 12.1) does not occur in a developmental context, usually affects a group of contiguous cells, and is characterized by swelling of the cell and its organelles (as a result of ion pump failure) and results ultimately in membrane rupture and cell lysis [1].

A unique biochemical event in apoptosis is the activation of calcium–magnesium-dependent endonuclease activity, which specifically cleaves cellular DNA between regularly spaced nucleosomal units. Such fragments are a characteristic DNA pattern, which is considered the hallmark of apoptosis. In necrosis, as opposed to apoptosis, the genomic DNA is degraded randomly by a host of cytosolic and lysosomal endonucleases, producing a continuous spectrum of sizes [2, 3].

Another important distinguishing feature of apoptosis is the rapid clearance of dead cells by "professional" phagocytes (such as macrophages) before they can lyse, spill their noxious contents, and cause an inflammatory reaction. This clearance mechanism is efficient and rapid. In contrast, during the pathological or accidental cell death that results from overwhelming cellular injury, cells swell and lyse, releasing noxious contents that often trigger an inflammatory response. An additional change associated with cells during the early phases of apoptosis is the alteration of plasma membrane phosphatidylserine asymmetry. In normal cells, the phosphatidylserine is located on the cytoplasmic side or on the inner leaflet of the plasma membrane. Early in apoptosis, phosphatidylserine is translocated from the inner to the outer surface of the plasma membrane and, consequently, is exposed to the external cellular environment. Surface exposure of phosphatidylserine occurs along with chromatin condensation that precedes the increase in membrane permeability and constitutes one of the principal targets of phagocyte recognition [4].

A disruption in the mitochondrial transmembrane potential occurring before nuclear changes has been observed in many cells undergoing apoptosis. This permeability transition involves the opening of a large channel in the inner membrane of the mitochondrion that leads to the release from mitochondria to the cytosol of apoptosis-inducing factors (AIF). In addition, permeability transition causes the mitochondrial generation of reactive oxygen species (ROS) and rapid expression of phosphatidylserine residues in the outer plasma membrane leaflet [5].

Moreover, during apoptosis, mitochondrial inner membrane proteins, such as cytochrome c, leak out into the cytosol. At least two other cytosolic proteins, apoptotic protease activating factors Apaf-1 and Apaf-3, have been identified that collaborate with cytochrome c (also known as Apaf-2) to induce proteolytic processing and CASPASE activation and, in turn, kill cells by apoptosis [3, 6].

These key differences between classic apoptosis and necrosis are confounded by the intricate changes occurring during spermatogenesis to the nuclear and cytoplasmic architecture (Table 12.1). For example, the replacement of histones by protamines: (i) creates a shutdown of gene activity during spermiogenesis hence inhibiting any active orchestrated contribution of apoptosis to this process and (ii) alters chromatin architecture so that the classic ordered fragmentation of nucleosomes seen in most cells cannot occur in sperm. For these reasons, we believe that although aspects of apoptosis are used to control spermatogenesis, it cannot be viewed as true apoptosis; hence some of the signals we associate with apoptosis become more complicated to understand.

12.1.1.1 Programmed Cell Death Cascade

Broadly, the programmed cell death cascade can be divided into at least three to four phases: signal activation, control, execution, and structural alterations. Multiple signaling pathways lead from death-triggering extrinsic signals to a central control and execution stage [1].

Three major pathways are involved in the process of caspase activation and apoptosis in mammalian cells. The intrinsic pathway for apoptosis involves the release of cytochrome c into the cytosol where it binds to Apaf1. Once activated by the cytochrome c, Apaf-1 then binds to procaspase 9 resulting in the activation of the initiator caspase 9 and the subsequent proteolytic activation of the executioner caspases 3, 6, and 7. The active executioners are then involved in the cleavage of a set of proteins, such as poly ADP ribose polymerase (PARP), and causes morphological changes to the cell and nucleus typical of apoptosis. A major player in the process is the B-cell lymphoma/leukemia 2 (BCL2, Bcl2) family of proteins [7] which act to regulate apoptosis through the interplay of the pro- and anti-apoptotic BCL family members. Members of the Bcl2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as Bax functioning as inducers and proteins such as Bcl2 as suppressors of cell death [3].

The extrinsic pathway for apoptosis involves ligation of a death receptor (e.g., Fas) to its ligand (e.g., Fas ligand (FasL). For the Fas pathway, binding of FasL to Fas activates Fas receptors, which recruit the Fas-associated death domain, which in turn binds to the initiator caspase 8 or 10 [8].

A third subcellular compartment, the endoplasmic reticulum has also shown to be involved in apoptotic execution. Crosstalk between these pathways does occur at numerous levels. In certain cells, caspase 8 through cleavage of Bid, a pro-apoptotic Bcl2 family member, can induce cytochrome c release from mitochondria in Fas-mediated death signaling. All these pathways converge on caspase 3 and other executioner caspases and nucleases that drive the terminal events of programmed cell death [8].

In this chapter, we will discuss apoptosis in relation to how and if it occurs in mature spermatozoa and how apoptosis functions in testes of men with normal spermatogenesis and different pathologies.

12.1.2 Apoptosis in Mature Spermatozoa

Numerous studies have now reported the presence of apoptotic protein markers on sperm membranes, including Fas [9], Bcl family proteins [10], and annexin V [11, 12]. As stated above the question of whether spermatozoa undergo apoptosis has perplexed a number of researchers. This question was raised in our initial study [9] of the presence of apoptotic proteins in ejaculated sperm, and we even coined the phrase abortive apoptosis [9] to convey a distinction from normal apoptosis. In addition, the finding that human spermatozoa can exhibit high levels of DNA fragmentation [13–20] has further pointed to apoptosis being a key mechanism in the control of spermatogenesis. Unfortunately, it has not been helped by the use of various DNA assessment techniques that have been confused with the diagnosis of apoptosis. The distinct mechanisms described above and in Table 12.1, including morphological and biochemical changes, reduction in cell volume, blebbing of the cell membrane, chromatin condensation, controlled DNA fragmentation and

margination, and formation of apoptotic bodies, are not always evident in such a specialized cell like a mature spermatozoon.

The discovery of the internucleosomal fragmentation of genomic DNA to regular repeating oligonucleosomal fragments generated by Ca/Mg-dependent endonuclease is accepted as one of the best characterized biochemical markers of apoptosis (programmed cell death). In 1970, Williamson [21] described that cytoplasmic DNA isolated from mouse liver cells after culture was characterized by DNA fragments with a molecular weight consisting of multiples of 135 kDa. This finding was consistent with the hypothesis that these DNA fragments were a specific degradation product of nuclear DNA. In 1978, Zakharyan and Pogosyan presented a paper revealing that glucocorticoid-induced DNA degradation in rat lymphoid tissue, thymus, and spleen occurred in a specific pattern producing fragments of DNA that were electrophoretically similar to those observed after treatment of chromatin with micrococcal nuclease, which indicated that an internucleosomal cleavage pattern of DNA degradation occurred during apoptosis [22–24].

This classic ordered DNA fragmentation seen in apoptotic cells is not evident in human spermatozoa because of the differences in chromatin packaging imparted by protamines [13] (Table 12.1). There are however some hallmarks of apoptosis. For example, it has been shown that human sperm contains the proteins necessary for the autophagy process. Proteins related to the autophagy/mitophagy process (LC3, Atg5, Atg16, Beclin 1, p62, m-TOR, AMPKα 1/2, and PINK1) were all found present in human spermatozoa. Aparicio et al. [25] showed that autophagy-related proteins and upstream regulators were present and functional in human spermatozoa.

Overall, mature spermatozoa display several features of apoptotic cells; however, they also appear to be able to escape programmed cell death once transcription is shut down. Improving our understanding of this enigma is one area of research that requires further attention.

12.1.3 Testicular Germ Cell Apoptosis in Normal Spermatogenesis

In contrast to mature ejaculated spermatozoa, the role of apoptosis is quite clear in the testes. The testes of normal men produce more than 100 million spermatozoa daily; however up to 75% of the spermatogonia die in the process of programmed cell death before reaching maturity. Spermatogenesis is therefore a dynamic process, and both germ cell proliferation and differentiation need to be tightly regulated. This output depends on proliferative activity in the basal compartment of the seminiferous epithelium where the spermatogonial cells are found and differentiate toward the lumen where meiosis and spermatogenesis occur. During regular spermatogenesis, testicular germ cells therefore degenerate by an apoptotic process. In mammals, germ cell death is conspicuous during spermatogenesis and occurs spontaneously at various phases of germ cell development such that seminiferous epithelium yields fewer spermatozoa than might be anticipated from spermatogonial proliferations [26].

In normal newborns, apoptotic cells in the seminiferous cords were identified as being mostly spermatogonia, even though Sertoli cells were also detected. The extent of testicular cell proliferation during fetal and neonatal development determines the final adult testis size and potential for sperm output in the human with subsequent stabilization during the first years of prepuberty. Even though gonadotropins start to increase during the first month of life, it is remarkable that the peak of the activation of the hypothalamic–pituitary–gonadal (HPG) axis that takes place during the second and third months of life was not associated with a lower rate of apoptosis or with increase in testis weight. Hormonal or growth factors present in the feto-placental unit might influence testicular cell growth for a few weeks after birth. The newborn period is characterized by increased cell mass in the two compartments of the testis. This cell growth seems to be mainly mediated by decreased apoptosis. The main mechanism for modulation of cell number in the prepubertal testis is the regulation of apoptotic cell death relative to cell proliferation [27].

Similarly, apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis in adult humans. Human testes exhibit a spontaneous occurrence of germ cell apoptosis involving all three classes of germ cells, including spermatogonia, spermatocytes, and spermatids. The incidence of spontaneous germ cell apoptosis in humans varies with ethnic background. For example, the incidence of spermatogonial and spermatid apoptosis was higher in Chinese men than in Caucasian men. The triggering factors for spontaneous germ cell apoptosis during normal spermatogenesis are not known, and it is uncertain why there are ethnic differences in the inherent susceptibility of germ cells to programmed cell death. However, it should be noted that, in testes, as in many other tissues, the contribution of spontaneous germ cell apoptosis has been grossly underestimated due to the rapid and efficient clearance of apoptotic cells by professional phagocytes (Sertoli cells) [1]. The Sertoli cells, lining the seminiferous epithelium, supervise spermatogenesis by providing structural and nutritional support to germ cells.

The survival of conjoined spermatogonial cell progenies depends in part on maintaining structural and functional relationships with both neighboring Sertoli cells and with the basal lamina of the seminiferous tubular wall. Spermatocytes are less dependent on the basal lamina relationship and more dependent on Sertoli cell support. When apoptosis signaling is activated, the CASPASEs initiate a cell disassembling procedure, generating apoptotic bodies leading to the final demise of entire spermatogonial and spermatocyte progenies [28].

During spermatogenesis, spermatogonia and round spermatids almost certainly die by apoptosis [29]. Peak germ cell loss has been observed during the stages of mitosis of type A spermatogonia, meiotic division of spermatocytes, and during spermiogenesis [30]. Apoptotic germ cells are either sloughed into the tubule lumen or phagocytosed by Sertoli cells. Spermatozoa also demonstrate changes consistent with apoptosis. The percentage of germ cells undergoing apoptosis in normal subjects is significantly lower than that seen in men with oligoasthenoteratozoospermia, Hodgkin's disease, and testicular cancer [31].

Five possible functional roles have been proposed in the literature for the presence of apoptosis during normal spermatogenesis:

- Maintenance of an optimal germ cell/Sertoli cell ratio. It has been established that each Sertoli cell can support only a finite number of germ cells throughout their development into spermatozoa. Therefore, supraoptimal numbers of spermatogonia may undergo apoptosis to maintain an optimal ratio [32].
- Elimination of abnormal germ cells. There may be a selective process in which abnormal germ cells, especially chromosomally abnormal germ cells, are eliminated from the population by apoptosis [26].
- The formation of the blood-testis barrier by tight junctions between Sertoli cells requires the elimination of excessive germ cells. Suppression of germ cell apoptosis by means of inactivating Bax, an apoptosis-inducing gene, prevents the formation of these tight junctions [33].
- Creation of a prepubertal apoptotic wave facilitates the eventual functional development of mature spermatogenesis. A massive wave of germ cell apoptosis normally takes place as mammalian species approach puberty. This wave serves as a regulator of the ratio between germinal cells in various stages and Sertoli cells. There is evidence that preventing this wave of apoptosis by expression of apoptosis inhibitory proteins, such as BclxL or Bcl2, results in highly abnormal adult spermatogenesis accompanied by sterility [34].
- Selective removal of unneeded portions of sperm cytoplasm. Apoptosis contributes during spermatogenesis in the process of removing abnormal sperm. For example, spermatids display many of the histological and molecular fingerprints of apoptosis. Maturing spermatids form darkly staining basophilic bodies and express multiple CASPASEs within these “residual bodies.” In addition, these bodies contain proteins linked to the regulation of cell death such as Fas and p53. The cytoplasm of maturing spermatids is collected and removed by residual bodies. This is probably done by neighboring Sertoli cells, which recognize and phagocytose them as they are shed. All of this has led to the idea that developing spermatozoa use the apoptotic machinery to selectively dissipate unneeded portions of their cytoplasm. In this view, apoptotic factors are somehow segregated to the cytoplasm—away from the nucleus—and this segregation permits the emerging sperm to utilize the apoptotic machinery without dying [35].

12.1.4 Regulators of Testicular Apoptosis

Apoptotic cell death seems to be strictly regulated by extrinsic and intrinsic factors and can be triggered by a wide variety of stimuli. Examples of extrinsic stimuli potentially important in testicular apoptosis are irradiation, trauma, viral infection, toxin exposure, and the withdrawal of hormonal support. It has been widely assumed that certain hormones, growth factors, or cytokines are necessary for cell survival and cell cycle progression and that their absence leads to apoptosis of their target

cells. Moreover, genetic control plays a prominent role in apoptosis through molecular regulatory factors, which act as intrinsic mediators [36].

12.2 Intrinsic Regulators

12.2.1 Genes Regulating Germ Cell Apoptosis

Disruption of a number of genes results in infertility through accelerated germ cell apoptosis in mice. These findings give a first glimpse of the mechanisms involved in the regulation of germ cell apoptosis and may help in defining important genetic principles that may apply to genes important for human fertility. Male mice deficient in Bax were infertile and displayed accumulation of premeiotic germ cells with complete loss of advanced spermatids. In addition, mice misexpressing Bcl2 in spermatogonia displayed an accumulation of spermatogonia before puberty but, during adulthood, exhibited loss of germ cells in the majority of the tubules [37].

12.2.1.1 Fas-FasL

The cell surface receptor, Fas, is a transmembrane glycoprotein that belongs to the tumor necrosis factor/nerve growth factor family. The Fas-FasL interaction triggers the death of cells expressing Fas. Expression of Fas and FasL is not only detected on the Sertoli cells but also in germ cells and Leydig cells [38].

In testis, the Fas system has been implicated in maintaining immune privilege. According to this hypothesis, FasL-expressing Sertoli cells eliminate Fas-positive activated T cells, providing general protection against rejection in the testicular environment. Moreover, if Sertoli cells are injured, they increase the expression of FasL to eliminate Fas-positive germ cells, which cannot be supported adequately. These findings and the response of FasL and Fas, expressed by Sertoli cells and germ cells, respectively, to environmental conditions by initiating germ cell death implicate the Sertoli cell in the paracrine control of germ cell output during spermatogenesis by a Fas-mediated pathway [39].

Although Fas may contribute to germ cell homeostasis, it is not essential. Mice with complete lack of Fas are fertile without any overt defects in germ cell apoptosis [40]. It may still play a key role in coordinating the number of sperm in human. Recently, Wang et al. [41] investigated whether single nucleotide polymorphisms (SNP) in the promoter regions of two Fas pathway genes can influence their transcriptional activities and result in abnormal cell apoptosis, thus leading to impairment of spermatogenesis. They showed that frequencies of FASLG -844CC, CT, and TT genotypes among infertile men were significantly different from those among controls ($P = 0.024$). Men with FASLG -844TT genotype had an increased risk of idiopathic azoospermia or severe oligozoospermia compared with those with

CC and CT genotype (odds ratio 2.72, 95% confidence interval 1.25–5.93). The results suggest that FASLG -844C/T SNP may be a genetic predisposing factor of idiopathic azoospermia or severe oligozoospermia.

12.2.1.2 Bcl2 Family

Bcl2 is the first identified member of a growing family of genes that regulates cell death in either a positive or a negative fashion. The Bcl2 family of proteins, which contains both pro-apoptotic (Bax, Bak, Bclxs, Bad) and anti-apoptotic (Bcl2, Bcl-xL, Mcl, A1) proteins, constitutes a critical, intracellular checkpoint within a common cell death pathway that determines the susceptibility of a cell to apoptosis. It is generally believed that the ratio of pro-apoptotic to anti-apoptotic Bcl2 family proteins is the critical determinant of cell fate, with an excess of Bcl2 resulting in cell survival but an excess of Bax resulting in cell death. Although these molecules compete, it has not been established firmly yet whether anti-apoptotic or pro-apoptotic members are dominant in determining the key survival-promoting decision point. Paradoxically, a given family member may perform either function, depending on the cell systems used [8].

Bcl2 protects cells from apoptosis by its capacity to reduce production of ROS. Other members of the Bcl2 family, including Bax, Bak, and Bad, can block the ability of Bcl2 to inhibit apoptosis and subsequently to promote cell death. Bax, for example, functions to increase the sensitivity of cells to apoptotic stimuli [42]. Disruption of Bax, an apoptosis-inducing gene, prevented the process of apoptosis in the testis and resulted in an accumulation of immature germ cells (mainly spermatocytes) in the tubules [33].

The impact of the Bcl pathway may differ in varying male infertility phenotypes as Stronati et al. [43] have shown that when exposed to environmental pollutants, certain chemicals might alter sperm DNA integrity and BclxL levels in European adult males. Finally, it is also known that normal testicular function is dependent upon hormones acting through endocrine and paracrine pathways both in vivo and in vitro. Sertoli cells provide factors and it has been shown that their removal induces germ cell apoptosis. One classic example is the proteins of the Bcl-2 family. These key apoptotic proteins in particular provide one signaling pathway which appears to be essential for male germ cell homeostasis controlled hormonally [44].

12.2.1.3 p53

The p53 family of transcription factors, including p53, p63, and p73, are critical for many physiological processes, including female fertility, but little is known about their functions in spermatogenesis. p53 suppresses oncogenic transformation by promoting apoptosis. p53 is found in high concentration in the testis and plays a significant role in temperature-induced germ cell apoptosis. This cell cycle

regulator also seems to be required for radiation-induced apoptosis of spermatogonia, as evidenced by de novo induction of p53 expression in spermatogonia and degenerating giant cells in the testis following irradiation [36].

p53-induced testicular apoptosis involves:

1. Activation of redox-related genes also known as p53-induced genes
2. Generation of ROS
3. Oxidative degradation of mitochondrial components permitting the release of apoptosis-inducing factors, including AIF, cytochrome c, Apaf1, and Apaf3, into the cytosol to activate the CASPASEs [45]

In mouse models, it has also been reported that deficiency of the TAp73 isoform, but not p53 or DeltaNp73, results in male infertility because of severe impairment of spermatogenesis [46]. These results indicate that abnormal regulation of p53 family members could impact human male infertility.

12.2.1.4 CASPASEs

CASPASEs are cysteine proteases that promote apoptosis in mammals. Evidence for the role of CASPASEs in cell death is based on findings that their inhibition can prevent apoptosis, whereas their overexpression and activation cause apoptosis. CASPASEs mediate apoptosis by cleaving selected intracellular proteins, including poly (ADP-ribose)polymerase (PARP), lamin, and actin, and cause morphological changes to the cell and nuclei [47, 48].

In vitro, apoptosis of human male germ cells can be prevented by CASPASE inhibition [49]. On the other hand, CASPASE activity could not be detected in human adult germ cells obtained from men with normal spermatogenesis and cultured in vitro under conditions that led to massive DNA fragmentation, suggesting the implication of an alternative, CASPASE-independent mechanism [50, 51]. In contrast, Kim et al. [52] have shown that the expression of FasL is upregulated in the testes of patients with SCO and MA, which suggests that it may be associated with apoptotic elimination or altered maturation of Fas-expressing germ cells through the activation of caspase 3.

12.2.1.5 c-Myc

c-Myc is a nuclear phosphoprotein, encoded by a proto-oncogene, c-Myc. It plays a key role in the control of cell proliferation by acting as a transcription factor. Overexpression of the c-Myc gene in transgenic rats induces germ cell apoptosis at the meiotic prophase of primary spermatocytes. Depletion of sperm and seminiferous tubule atrophy causing sterility have been observed in the male transgenic rats [53].

12.2.1.6 Cyclic Adenosine Monophosphate Responsive Element Modulator (CREM)

The transcriptional activator, cyclic adenosine monophosphate (cAMP)-responsive element modulator (CREM), which is highly expressed in postmeiotic cells, may be responsible for the activation of haploid germ cell-specific genes involved in the structuring of the spermatozoa. CREM is responsive to the cAMP signal pathway and is required for expression of postmeiotic germ cell-specific genes. Mice that are CREM-deficient are phenotypically normal but have a maturation arrest at the early spermatid stage associated with a marked increase in apoptosis [54].

CREM is expressed in nuclei of round spermatids but not in elongated spermatids. CREM may be important for spermatid development and as a stage-specific regulator of human spermatogenesis. Absence of CREM may play a causative role in testicular failure associated with various types of human male infertility [55].

12.2.1.7 c-kit

c-kit has been identified as a germ cell apoptosis-preventing gene. Blockade or loss of the c-kit receptor results in the inability of mature spermatozoa to undergo the acrosome reaction. Decreased expression of the c-kit receptor and its ligand, stem cell factor, may alter the balance between cell proliferation/differentiation and cell death, resulting in increased apoptosis in the testes [56].

In mice, c-kit is involved in the migration of primordial germ cells and is expressed early in spermatogenesis. It is expressed in type A, intermediate, and type B spermatogonia, and its ligand is expressed in Sertoli cells [57].

12.2.2 Genetic Regulators of DNA Repair

DNA damage is one of the most potent triggers of apoptosis. DNA damage (e.g., chromosomal abnormalities, failure of DNA repair or genetic recombination, ionizing radiation, chemotherapy) leads to the elimination of damaged cells scattered within the epithelium via apoptosis [58].

PARP is a chromatin-associated enzyme with a presumptive role in DNA repair during replication and recovery from strand breaks caused by genotoxic agents. It is particularly active in the testis, where its expression varies according to the stage of germ cell differentiation. The degradation of PARP is also one of the classic indicators of apoptosis [59].

12.3 Extrinsic Regulation (Hormonal Regulation)

Withdrawal of gonadotropins or testosterone can markedly accelerate germ cell apoptosis. In rodents, spermatogenesis and apoptosis have been shown to be hormonally dependent. As in other hormonally sensitive reproductive organs, such as the prostate, endometrium, and ovary, the withdrawal of hormonal stimulation results in the selective degeneration of specific cell types [36].

Assessing the relationship between hormonal deprivation and the induction of germ cell apoptosis in adult rats following the withdrawal of testosterone demonstrated a significant rise in testicular cells with a low DNA content in combination with a decrease in haploid cells after testosterone deprivation [60].

Glucocorticoids act at the level of the pituitary and testis to suppress testosterone secretion and as a result may generate testicular apoptosis [61, 62]. Also, administration of exogenous glucocorticoid resulted in testicular germ cell apoptosis in rats [61, 62]. Severe stress may provoke the release of endogenous glucocorticoids in men, resulting in decreased serum testosterone and possibly triggering apoptosis [63].

There is an increase in DNA fragmentation in seminiferous tubules after hypophysectomy [64], further supporting the concept that androgen deprivation increases programmed cell death in the seminiferous epithelium. GnRH antagonist-induced germ cell apoptosis is most prominent among meiotic spermatocytes. Administration of a GnRH antagonist resulted in morphologic signs of germ cell degeneration in spermatocytes and spermatids [1].

Gonadotropin-dependent germ cell apoptosis seems to be age-related. A marked increase in apoptotic DNA fragmentation was seen in aging rats treated with a potent GnRH antagonist to suppress circulating levels of FSH, LH, and testosterone. Testicular apoptosis may, therefore, be enhanced in the aging male, given the decline in free testosterone levels that occur with advancing age [65].

12.3.1 Testicular Germ Cell Apoptosis During Testicular Dysfunction Conditions

12.3.1.1 Aging

With aging, both potential daily sperm production and Leydig cell function decline. As for spermatogenesis, histopathological examination reveals that there is a significant decline in the number of Sertoli cells per seminiferous tubule and the number of spermatids and primary spermatocytes per Sertoli cell [66].

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 [67].

We have previously explored the relationship between men's age and DNA damage repair proteins related to apoptosis in human testicular germ cells [68]. Statistically significant differences in DNA damage repair-associated proteins (PARP1, PAR, XRCC1, and APE1) and apoptosis markers (caspase 9, active caspase 3, and cleaved PARP1) were observed in testicular samples from older men. These differences were most marked in spermatocytes. It is clearly apparent that there is an age-related increase in human testicular germ cell DNA break repair and apoptosis with age.

Diminished spermatogonial proliferation was also found concomitant with low spermatogonial apoptosis. The decline of spermatogonial apoptosis might reflect a compensatory role of apoptosis in spermatogonia for the diminished proliferation that occurred during aging. Accelerated apoptosis of primary spermatocytes was detected in the testis of elderly men. It was speculated that apoptosis of primary spermatocytes might be the most relevant cause of impaired spermatogenesis in the aged testis. Sertoli cells might already have digested many apoptotic spermatids at the time of the detection of DNA fragmentation, because those cells are phagocytosed in the early phase of the apoptotic process in the rat testis [69].

The aspect of declining sperm quality in aged men has further implications with a number of studies now showing that there is a paternal age-related decline in fertility, a higher rate of certain neurodegenerative pathologies in offspring fathered by aged men, and possible transgenerational effects related to the paternal lineage [70–75].

12.3.1.2 Varicocele

Several varicocele-associated factors, including heat stress, androgen deprivation, and exposure to toxic elements, may induce pathways, which result in apoptosis [76]. Our own studies have shown that that there is an increase in human testicular germ cell DNA repair and apoptosis in infertile varicocele patients and that their profile resembles that of premature aging [77].

Apoptosis in the Ejaculate of Men with Varicocele

Varicocele induces apoptosis, which is initiated in the testicular tissue and is then expressed in the semen. Up to 10% of sperm cells in the ejaculate of men with a varicocele were apoptotic, as compared with 0.1% in fertile controls [78]. Saleh et al. [79] showed that infertile men with varicoceles had significantly greater DNA damage in spermatozoa than had normal men. Bertolla et al. [80] also evaluated DNA fragmentation in adolescents with clinically diagnosed varicoceles and determined that these boys had a higher percentage of cells with DNA fragmentation than did adolescents with no varicocele.

The expression of Fas protein was upregulated in semen samples obtained from patients with varicocele when compared to a control group, whereas little or no changes in FasL expression were detected in both groups. The relationship between varicoceles and apoptosis was explored by monitoring the concentrations of the soluble form of Fas (s-Fas) in seminal plasma, to characterize the Fas-signaling system with regard to hypospermatogenesis as a result of varicocele. By screening the seminal plasma of oligospermic men with varicoceles, oligospermic men with no varicocele, and normal controls, for the levels of s-Fas and the s-Fas ligand, s-Fas ligand was not detected in any of the cases, whereas s-Fas levels were specifically lower only in cases of varicocele [81, 82].

These reduced s-Fas levels were reversed by varicocelectomy. However, although higher temperatures may inhibit s-Fas production in patients with varicocele, the reason for this decrease in s-Fas levels remains unknown [81, 82].

In contrast, Chen et al. [83] identified no relationship between semen quality and apoptosis in fresh semen samples obtained from 30 patients with varicocele and 15 fertile controls. Although the varicocele patients had a significantly higher apoptotic index (AI) than fertile controls, semen quality and sperm motion characteristics were not significantly different between the two groups.

Seminal ROS may result in sperm DNA damage in patients with varicoceles. At the molecular level, ROS affect DNA directly and alter the levels of intracellular Ca+2, which is known to be one of the most effective means of inducing apoptosis. Morphological alterations in testicular tissues have been reported as "stress patterns" in patients with varicoceles. This stress pattern is reminiscent of, although not identical to, the cytomorphological changes in apoptosis [76].

High levels of seminal ROS and reduced total antioxidant capacity were detected in both fertile and infertile men with a clinical diagnosis of varicocele. Therefore, it was hypothesized that spermatozoal dysfunction in association with varicoceles may be related, at least in part, to elevated levels of sperm DNA damage induced by the high levels of ROS which are common in such patients [84].

Interestingly in a recent study, Agarwal and colleagues [85] identified and analyzed proteins of interest in infertile men with unilateral varicocele by searching for differentially expressed proteins (DEP) compared to fertile men. They identified 29 proteins of interest involved in spermatogenesis and other fundamental reproductive events such as sperm maturation, acquisition of sperm motility, hyperactivation, capacitation, acrosome reaction, and fertilization. Proteins expressed uniquely in the unilateral varicocele group were cysteine-rich secretory protein 2 precursor (CRISP2) and arginase-2 (ARG2). They concluded that expressions of these proteins of interest are altered and possibly functionally compromised in infertile men with unilateral varicocele.

Apoptosis in the Testicular Tissue in Men with Varicocele

Simsek et al. [86] evaluated the presence of apoptosis in testicular tissue, using the TUNEL assay. Apoptosis was very rare in the testicular tissues of the control group compared to the varicocele group. The mean percentage of apoptotic cells per total

germ cell was 2% in the control and 14.7% in the varicocele group. Hassan et al. [87] showed that testicular apoptosis is increased in varicocele-associated men either fertile or infertile. They found that the occurrence of apoptotic changes comprised all types of germ cells but did not affect Sertoli cells. Mean tubular apoptotic indices of fertile or infertile men with varicocele were significantly higher than controls (mean of 4.55 and 6.29% versus 2.71; $P < 0.05$). Mean Leydig cells apoptotic indices of infertile men with varicocele were also significantly higher than those of fertile men without varicocele as well as controls.

Benoff et al. [88] also reported that there were far more apoptotic nuclei, as assessed by TUNEL labeling in testis biopsy sections, in the seminiferous tubules of men with varicocele than in normal controls and that the percentage of apoptotic nuclei was noticeably higher in some men with varicoceles.

Although Bcl2 was not expressed in the germ cells in infertile patients with varicocele, these cells expressed low levels of Bax, with no significant differences to the specimens from fertile men. In the testes from infertile patients with varicoceles stained for Caspase 3, significantly fewer germ cells were detected than those in the testes of normal controls. It was suggested that apoptosis might be suppressed as the result of reduced expression of caspase 3 and that the mitochondrial pathway involving Bcl2 and Bax may not be involved in apoptotic regulation in germ cells [89].

12.3.1.3 Failure of Spermiogenesis

The causes of complete spermiogenesis failure are not completely known. These include the withdrawal of some developmentally important ligands, such as testosterone [90] or vitamin A [91]; mutations of the receptors with which these ligands and their metabolites can act, such as the retinoic acid receptor A [92] or the retinoid X receptor B [93]; alterations of molecules involved in signal transduction pathways, downstream of receptors, such as CREM protein [54]; or mutations of components of cell DNA repair enzyme systems [94]. Such conditions are often associated with germ cell apoptosis [95].

Reduced expression of CREM was also detected in patients with predominant round spermatid maturation arrest in comparison with men with normal spermatogenesis or with mixed testicular atrophy [55], and increased apoptosis of testicular cells has been demonstrated in patients with abnormal spermatogenesis [96]. It can thus be postulated that the low efficacy of round spermatid sperm injection in cases of complete spermiogenesis failure is due to the activation of apoptosis-promoting mechanisms similar to those operating in the experimental models of spermiogenesis arrest [97].

Apoptosis is involved in the removal of arrested germ cells from the testis of patients with spermatogenic disorders. The degree of spermatocyte and spermatid DNA fragmentation in the group of patients with incomplete spermiogenesis failure appears higher as compared to men with normal sperm production [1].

In addition to DNA fragmentation, apoptotic cells also undergo a rearrangement of plasma membrane lipids, leading to translocation of phosphatidylserine from

the inner side of the plasma membrane to the outer layer, probably as a result of disintegration of plasma membrane cytoskeleton that, in healthy cells, stabilizes membrane structure by connecting plasma membrane components to the cellular interior. It was suggested that this plasma membrane modification may serve to mark apoptotic cells for subsequent recognition and removal by the phagocytotic machinery [98].

Tesarik et al. [99], using double labeling with TUNEL and annexin V, concluded that patients with complete spermiogenesis failure (round spermatids is the latest stage detected histologically in the testicular biopsy in azoospermic patients) had significantly higher frequencies of primary spermatocytes and round spermatids carrying the apoptosis-specific DNA damage in comparison with patients with incomplete spermiogenesis failure (elongated spermatids is the latest stage detected histologically in the testicular biopsy in azoospermic patients). Apoptosis-related phosphatidylserine externalization occurs rarely until the advanced stages of spermiogenesis. Since externalized phosphatidylserine is expected to be involved in the recognition of apoptotic cells by phagocytes, apoptotic spermatocytes and round spermatids may not be removed easily by phagocytosis. The high frequency of DNA damage in round spermatids from patients with complete spermiogenesis failure explains the low success rates of spermatid conception in these cases. They also recommended that the evaluation of apoptosis could help to predict success rates of spermatid conception.

CASPASE activation and DNA fragmentation are frequent phenomena in germ cells from men with non-obstructive azoospermia, especially in cases of meiotic and postmeiotic maturation arrest. The incidence of CASPASE activation and DNA fragmentation is somewhat lower in samples from patients with hypospermatogenesis, in which some germ cells achieve the late elongated spermatid stage [50].

12.3.1.4 Obstructive Azoospermia

The mechanism inducing apoptosis after obstruction remains unknown. Since the obstruction of the vas deferens would also induce an increase of pressure in the seminal tract, it may cause apoptosis. Increased pressure occurring prior to testicular development might have a more adverse effect than that occurring in adulthood. The difference in apoptotic change between prepubertal and adult cases might thus relate to the susceptibility to pressure. However, these pressure increases also seem to be reduced by epididymal development [100].

Flickinger et al. [101] reported that obstruction of the seminal tract in immature rats caused epididymal granulomas, which might in turn have caused fairly high pressure to the seminal tract. In case of prepubertal obstruction when epididymis is not well developed, the increased pressure may directly affect the testis to cause increased germ cell apoptosis.

Patients with congenital absence of the vas deferens who generally have good spermatogenesis are somewhat different from acquired obstructions. They have life-long history of seminal tract obstruction; however, the increase or the fluctuation of

the pressure may not occur. This could be supported by the report that the vasectomized men showed significantly greater seminiferous tubular wall thickness than the patients who had congenital absence of the vas deferens [102].

12.4 Conclusion

The importance of understanding how apoptosis functions in both the normal and abnormal testes is paramount. It is becoming clear that subtle abnormalities in a sperm can become a significant factor in defining the progress of not only pregnancy but also of fetal and childhood development [72, 74, 103]. Improving our understanding of apoptosis and the factors that control it in the testes may allow us to better define male infertility and also treat it in a way that can limit any adverse paternal effects from spermatozoa that escape apoptosis.

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