

# Role of caspases in male infertility

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**Apoptosis is characterized by a variety of changes resulting in the recognition and phagocytosis of apoptotic cells. Caspases (cysteiny aspartate-specific proteinases) play a central role in the regulation of apoptosis in the human seminiferous epithelium. They are expressed as inactive proenzymes and participate in a cascade triggered in response to pro-apoptotic signals. To date, 14 caspases have been implicated in the human apoptotic pathway cascade. Among these, caspase-3 is considered to be a major executioner protease. Since apoptosis is a universal suicide system in almost all cells, a close control via molecular, endocrine and physical factors establishes homeostasis of cell growth and death. The proper regulation of the caspase cascade plays an important role in sperm differentiation and testicular maturity. However, caspases have been implicated in the pathogenesis of multiple andrological pathologies such as impaired spermatogenesis, decreased sperm motility and increased levels of sperm DNA fragmentation, testicular torsion, varicocele and immunological infertility. Future research may provide a better understanding of the regulation of caspases, which may help us to manipulate the apoptotic machinery for therapeutic benefits. In this review, we summarize the consequences of caspase activation, aiming to clarify their role in the pathogenesis of male infertility.**

*Key words:* apoptosis/caspases/human/male infertility/sperm

## Introduction

Programmed cell death (PCD), also known as apoptosis, is required for normal spermatogenesis in mammals and is believed to ensure cellular homeostasis and maintain the fine balance between germ cells and Sertoli cells. A central component of apoptotic machinery involves members identified as a family of aspartic acid-directed cysteine proteases called caspases (CP, cysteinyl aspartate-specific proteinases). Together they play a central role in the regulation of apoptosis in the human seminiferous epithelium. They are expressed as inactive proenzymes (pCP), which are further processed into active (cleaved, aCP) forms in cells undergoing apoptosis and thus play a crucial role in the transduction of apoptotic signals in cells that are destined to die. The caspases are under the influence of diverse regulators including activators and inhibitors (Reed, 2000).

Caspase activation occurs by self-proteolysis and/or results from the actions of other caspases or regulator proteins. Ligation of Fas ligand to Fas in the cellular membrane triggers activation of CP8. Once activated, CP8 transduces a signal to effector caspases, including CP3, CP6 and CP7. This leads to degradation of the cellular substrates of these caspases, including cytoplasmic structural proteins such as actin and/or nuclear proteins such as poly-ADP-ribose polymerase (PARP).

To date, 14 caspases have been implicated in the human apoptotic pathway cascade. Among these, CP3 is considered to be a major executioner protease. This article provides an overview on the activation of the caspase family, its regulation and the series of events that plays a major role in the pathogenesis of male infertility. Specifically, disorders such as impaired spermatogenesis, sperm DNA damage, asthenozoospermia, varicocele and immunological infertility will be discussed.

## *Caspases: definition*

Apoptosis is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of endoplasmic reticulum, a decrease in cell volume and alterations in plasma membrane; these changes result in the recognition and phagocytosis of apoptotic cells (Arends and Wyllie, 1991). Early studies on apoptosis concentrated on the role of nucleases. More recently, a role has been proposed for a family of different cysteine proteases. The name proposed for all family members is caspase, the 'c' denoting a cysteine protease and the 'aspase' referring to the ability of the enzymes to cleave after an aspartic acid residue. Individual family members are then referred to in order of their publication; therefore, interleukin-1 $\beta$ -converting enzyme (ICE), the first family member, acquired the name CP1 (Cohen, 1997).

**Table I.** Function, subtype and used adapter of important caspases

Function	Caspase subtype	Prodomain and adapter element
Initiator caspases	Caspase 2, 8, 9, 10	Long: DED (8, 10) or CARD (2, 9)
Effector caspases	Caspase 3, 6, 7	Short: without DED or CARD
Cytokine processor	Caspase 1, 4, 5, 11–14	Long: CARD

Caspases have been implicated in apoptosis with the discovery that cell death abnormal-3 (CED-3), the product of a gene required for cell death in the nematode *Caenorhabditis elegans*, is related to mammalian interleukin-1 $\beta$ -converting enzyme (ICE or CP-1). Although CP1 has no obvious role in cell death, it was the first identified member of a large family of proteases whose members have distinct roles in inflammation and apoptosis. Caspases play a dual role; they function in cell disassembly (effectors) and in initiating this disassembly in response to proapoptotic signals (initiators). Three subfamilies of caspases exist: an ICE subfamily, comprising CP1, CP4 and CP5, a CED-3/CP3 subfamily, comprising CP3 and CP6–10, and a CP2 (ICH-/Nedd2) subfamily (Thornberry, 1998). Caspases can be differentiated according to the length of prodomain as long versions (CP1, 2, 4, 5, 8, 9, 10 and 12) and short versions (CP3, 6, 7 and 11) (Table I) (Cryns and Yuan, 1998; Kumar, 1999).

Most proteases are synthesized as precursors that have insignificant catalytic activity. The precursor is usually converted to the active enzyme by proteolytic processing either by another protease or by autocatalysis and triggered by the binding of cofactors or removal of inhibitors. Caspases share similarities in amino acid sequence, structure and substrate specificity. They are all expressed as proenzymes (30–50 kDa) that contain three domains: an NH<sub>2</sub>-terminal domain, a large subunit (~20 kDa) and a small subunit (~10 kDa). During the caspase activation, proteolytic processing occurs between domains, followed by association of the large and small subunits to form a heterodimer (Thornberry and Lazebnik, 1998). Although the majority of caspases are situated within the cytoplasm, some members can be found at the Golgi apparatus (CP12) or in association with the mitochondria (CP2, CP3 and CP9) (Cohen, 1997; Nicholson, 1999). However, the actual presence of caspases within the intermembrane space of mitochondria could not be confirmed by three apoptosis models (van Loo *et al.*, 2002). The most important initiator caspases are CP8 for type I apoptosis, CP9 for type II apoptosis, and CP12 for type III apoptosis. To date, eight different CP8 isoforms have been identified at the mRNA level (Scaffidi *et al.*, 1997). CP3 is the most important effector caspase. Its activation hallmarks the point of no return in PCD signalling (Earnshaw *et al.*, 1999). CP12 segregates with the inflammatory CP1, CP4, CP5 and CP11 (Kalai *et al.*, 2003). Studies in CP12-deficient mice suggested that the enzyme plays a major role in ER stress-induced apoptosis (Nakagawa *et al.*, 2000).

Although caspases share an absolute requirement for cleavage after aspartic acid, they are highly specific in their substrate preferences. During the execution phase of apoptosis, several proteins (e.g. PARP, lamin B and histone H1) are cleaved. The number of proteins identified as being cleaved during apoptosis is increasing rapidly. Some caspases show overlapping specificities for certain substrates (CP3 and CP7 can both cleave PARP),

whereas other caspases may have a unique substrate specificity (to date, CP6 is the only caspase known to cleave lamins) (Kaufmann, 1989). The biological significance of these proteolytic cleavages and their relationship with the ensuing apoptotic morphology is largely unknown.

Caspases were probably one of the first obvious therapeutic targets for modulating apoptosis and they remain the most viable approach to blocking apoptotic cell death. In fact their therapeutic effect was considered even before the discovery that they play a central role in apoptosis (Alnemri *et al.*, 1996). Findings from animal models have highlighted the protective/therapeutic role of caspase inhibitors in many systemic diseases such as cardiac arrest, neurological, and rheumatoid diseases or in cases undergoing organ transplantation (Nicholson, 2000). The possibility of applying the same concept in the field of male infertility remains to be explored.

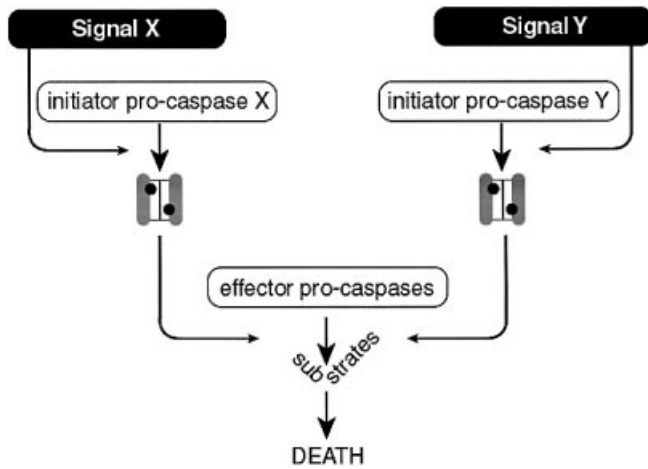
#### Target sites for caspases

Cleavage and activation of proapoptotic substrates provide signals, which can link the two different cascade programmes or allow for positive feedback of apoptotic signals, thus enhancing the apoptotic process. The kinases MEK kinase 1, Mst1 and p21-activated kinase2/hPak65 are all cleaved and activated by caspases, resulting in potential feedback activation through c-Jun kinase/stress-activated protein kinase-regulated portions of the apoptotic pathway (Nunez *et al.*, 1998). CP8 cleavage of another proapoptotic substrate, the Bcl-2 family member Bid, triggers cytochrome *c* release from the mitochondria, conceivably linking the death receptor and the apoptosis protease-activating factor 1 (Apaf-1)/CP9 pathways (Li *et al.*, 1998). Similar cross-talk between the two pathways may also result from CP8 cleavage of the effector CP3, which can in turn cleave and activate CP9. On the other hand, caspase cleavage and inhibition of proteins that relay survival and proliferation signals are also common in apoptosis. Such proteins include the phosphatidylinositol-3 kinase/Akt, Raf-1 and focal adhesion kinase (FAK) (Nunez *et al.*, 1998).

Cleavage of a number of structural or housekeeping proteins by caspases is thought to facilitate the disassembly of a cell undergoing apoptosis. As an example, nuclear lamins, the major cytoskeletal structural component of the nucleus, are cleaved by CP6 during apoptosis (Rao *et al.*, 1996). The cytoskeletal protein actin as well as a number of actin-regulatory proteins including a-fodrin, aII-spectrin, fIII-spectrin and gelsolin are also cleaved by caspases during apoptosis (Nunez *et al.*, 1998).

#### Signalling pathways

A diversity of mechanisms exists for activating caspases (Hengartner, 2000); effector caspases are usually activated proteolytically by an upstream caspase, whereas initiator caspases



**Figure 1.** Caspase cascade in apoptotic cells. Effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals. Reprinted with permission from Thornberry and Lazebnik (1998), © 1998 AAAS.

are activated through regulated protein–protein interactions (Figure 1).

#### Activation by membrane receptors

This pathway involves a group of receptors characterized by the presence of an intracellular region, called the death domain. These receptors are required for the transmission of the cytotoxic signal. Death domain receptors induce apoptosis by similar signalling pathways (Schulze-Osthoff *et al.*, 1998).

Upon engagement of members of the tumour necrosis factor (TNF) receptor superfamily with their extracellular ligands [such as TNF, Fas ligand (FasL)/CD95L, and TNF-related apoptosis-inducing ligand (TRAIL)], oligomerization and/or a conformational change in the receptor occur. Seconds after receptor engagement, an intracellular death-inducing signalling complex (DISC) is formed through recruitment of adapter molecules, such as FADD (Fas-associated death domain protein), that also contain death domain motifs. FADD also contains a DED and recruits the DED-containing caspase, CP8, into the DISC through homotypic interactions between the death effector domain (DED) motifs present in both proteins (Muzio *et al.*, 1996).

The cytokine TNF- $\alpha$  is secreted by testicular germ cells. The expression of the TNF receptor protein in the human seminiferous epithelium is found predominantly in the Sertoli cells, which means that the antiapoptotic effect of TNF- $\alpha$  is probably mediated via these somatic cells. Interestingly, expression of the Fas ligand, a known inducer of testicular apoptosis, is down-regulated by TNF- $\alpha$ . Thus, in the seminiferous tubules, germ cell-derived TNF- $\alpha$  may regulate the level of the Fas ligand and thereby control physiological germ cell apoptosis (Pentikainen *et al.*, 2001).

Fas ligand (FasL) is a type II transmembrane protein. Upon engagement of FasL to Fas, an intrinsic program of apoptotic death is stimulated in a target cell leading to the activation of CP8 (Nagata, 1999). Among receptor-mediated PCD, CP8 plays the most important role in transduction of death signals. Recently, we demonstrated that the signal that transmits CP8 is also present in ejaculated sperm and during spermatogenesis (Paasch *et al.*, 2001).

#### Mechanism of mitochondrial apoptosome-driven pathway

A variety of key events in apoptosis focuses on mitochondria, including the release of caspase activators such as cytochrome *c*, a haem-containing protein. Cytochrome *c* resides in the space between the outer and inner membranes of mitochondria and transports electrons from complex III to complex IV in the respiratory chain. Mammalian testes express two types of cytochrome *c*: somatic cytochrome *c* (Cyt *c*<sub>S</sub>) and testis-specific cytochrome *c* (Cyt *c*<sub>T</sub>). Cytochrome *c* plays an important role during apoptosis. Activated mitochondria release cytochrome *c* from the intermembrane space into the cytoplasm, and the released cytochrome *c* binds to Apaf-1.

Other key events include changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation–reduction and participation of pro- and antiapoptotic Bcl-2 family proteins. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death (Green and Reed, 1998).

#### Cross-talk among activation pathways

Upon activation of CP8 within the DISC, the death signal is propagated by two alternative mechanisms, depending on the cell type (Scaffidi *et al.*, 1998). In certain cell types (type I cells), stimulation of death receptors results in a robust activation of CP8, followed by direct activation of effector caspases downstream. In contrast, other cell types (type II cells) fail to activate sufficient CP8 within the DISC (for reasons that are currently unknown) to permit direct activation of the caspase cascade. Instead, death receptor-mediated activation of CP8 in type II cells results in the proteolysis of Bid, a BH3-only member of the Bcl-2 protein family. Truncated Bid (tBid) translocates to mitochondria where it promotes the Bax/Bak-dependent release of cytochrome *c*, thereby triggering apoptosome assembly (Lee *et al.*, 1999). Therefore, a level of cross-talk exists between the membrane receptor and mitochondrial pathways of apoptosis (Creagh *et al.*, 2003).

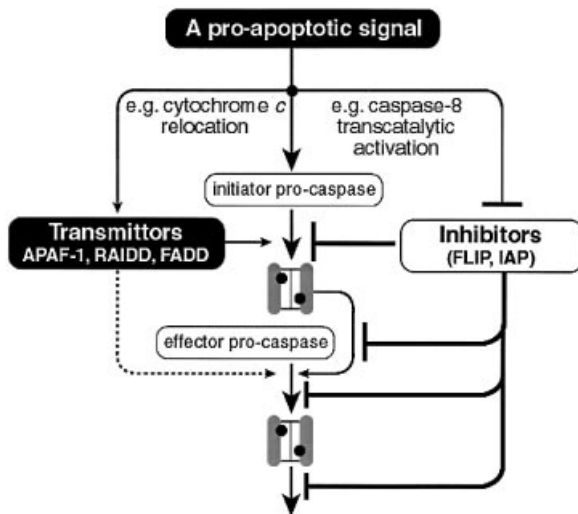
#### Endoplasmic reticulum-mediated pathway

A novel caspase-activation pathway in which CP12 functions as the initiator caspase in the context of ER stress has recently been proposed (Morishima *et al.*, 2002).

Thymocytes from CP12-null mice, which normally die in response to cytotoxic drugs, were shown to be slightly resistant (~20%) to agents such as brefeldin A, tunicamycin or thapsigargin that are considered to produce ER stress (Nakagawa *et al.*, 2000). This finding suggests that CP12 may be involved in ER stress-mediated apoptosis to some degree. However, owing to the incomplete inhibition of cell death seen in CP12-null animals, a certain amount of redundancy between ER stress-associated caspase-activation pathways must pertain. Upon ER-associated activation, CP12 has been proposed to activate CP9 downstream in an Apaf-1-independent manner (Morishima *et al.*, 2002). However, the mechanism of ER-mediated CP12 activation remains unknown.

#### Alternative pathways

A caspase-independent pathway is triggered first by lipids (e.g. ceramides), chemotherapeutics and neurotoxins and does not rely



**Figure 2.** Regulation of caspases by opposing effects of activators and inhibitors. A signal apparently initiates three pathways involving transmitters, initiator caspases, and inhibitors. Transmitters: *APAF-1* = apoptotic protease activating factor; *RAIDD* = RIP-associated Ich-1/CED3 homologous protein with death domain; *FADD* = Fas-associated protein with death domain; inhibitors: *FLIP* = FADD like ICE inhibitory proteins; *IAP* = inhibitor of apoptosis. The dashed line from co-factors to effector caspases reflects the possibility that effector caspases may be activated by an autocatalytic mechanism. Reprinted with permission from Thornberry and Lazebnik (1998), © 1998 AAAS.

on receptors with death domains (Bidere and Senik, 2001). In addition, reactive oxygen species (ROS) may induce functional defects by inducing necrotic PCD without necessarily activating caspases (Leist and Jaattela, 2001). Fatty acids and fatty acid oxidation products, however, do affect signal transduction pathways, particularly those involved in apoptosis (Tang *et al.*, 2002). A freezing–thawing cycle of sperm markedly influences the lipid composition with the appearance of lysophosphatidylcholines and ceramide (Glander *et al.*, 2002).

Granzyme B, a serine protease, is released in response to viral infect and tumour cells. The first substrate identified for granzyme B was found to be CP3, which immediately suggested a molecular basis for CTL/NK-mediated killing (Martin *et al.*, 1996). Granzyme B is also capable of directly processing several other caspases *in vitro*, suggesting that this granule component may induce apoptosis by triggering the simultaneous activation of multiple caspases. However, only CP3 and CP8 have been demonstrated to be direct substrates for granzyme B in intact cells, suggesting that these may be the most proximally activated caspases (Creagh *et al.*, 2003).

CP2 has long been suspected to act as an initiator caspase, but evidence for this role has been slow to emerge. CP2 contains an N-terminal caspase recruitment domain (CARD) within its prodomain, consistent with a possible role for this protease as an initiator caspase (Kumar *et al.*, 1994). Recently, several studies have proposed that CP2 can act as an apical caspase in pathways that were considered to be Apaf-1/CP9-initiated. These studies claim that CP2 acts upstream of mitochondrial apoptosome formation to promote mitochondrial cytochrome *c* release and apoptosome-dependent CP9 activation downstream (Lassus *et al.*, 2002).

### Terminal execution by CP3, CP6 and CP7

Cleavage of regulator proteins such as members of the Bcl-2 family or activation of kinases such as protein kinase  $\delta$  and  $\theta$ , PAK2/hPAK65 as well as MEKK-1 results in a coordinated disassembly of the affected cell (Adams and Cory, 1998). This causes loss of function, an increase in enzyme capacity, which in turn lead to conversion of downstream elements (e.g. lamins, actin and cytokeratines) and disassembly of the core structure and regulators of the cytoskeleton (FAK) (Thornberry and Lazebnik, 1998).

### Control of caspase cascade

Since PCD is a universal suicide system in almost all cells of the human, close control is necessary to establish homeostasis of cell growth and death.

### Molecular control

PCD can be initiated by receptors in mitochondria or by other external factors. Once activated, the process may be stopped or modulated until it reaches the beginning of the terminal phase. Regulators of the signalling cascade and proteins, which directly interfere with the caspases, establish this molecular control (Figure 2).

### Regulators of the BCL2 subfamily

The most tightly regulated part of PCD reflects the creation of the apoptosome by cytochrome *c* (CyC) and Apaf-1 ‘apoptotic protease activating factor’ in the presence of pCP9 (Li *et al.*, 1997). Bcl-2 proteins control the segregation of Apaf-1 and CyC at the outer membrane of mitochondria and the activation of pCP9. More than 15 members of that family have been identified. Interestingly, despite shared homologies, they represent different functions. According to their sequences, three subfamilies can be identified (Table II).

The protein Bcl-2 is found on the cytoplasmic site of the mitochondrial membrane, at the endoplasmic reticulum and at the core membrane (de Rooij and Grootegeod, 1998). Some of these proteins may form dimers, allowing them to change between pro- and antiapoptotic functions (Vander Heiden *et al.*, 1997).

### NF- $\kappa$ B

The transcription activator nuclear factor NF- $\kappa$ B is a transcription factor expressed in the testis (Ghosh *et al.*, 1998). There are five known members of the NF- $\kappa$ B family in mammals that include p50/p105, p65/RelA, c-Rel, RelB and p52/p100. NF- $\kappa$ B can be activated within minutes by a variety of stimuli such as inflammatory cytokines (e.g. TNF- $\alpha$ , growth factors and stress inducers). When activated, NF- $\kappa$ B suppresses apoptosis through the transcriptional activation of genes whose products block apoptosis. However, NF- $\kappa$ B has been found to be associated with antiapoptotic as well as proapoptotic mechanisms (Barkett and Gilmore, 1999). Thus, NF- $\kappa$ B has been suggested to be a stress response factor that controls whether a cell lives or dies.

### IAP

‘Inhibitors of apoptosis’ (IAP) represent one of three viral protein families (cow pox protein, CrmA, baculovirus gene product p35),

**Table II.** Bcl-2 subfamilies, their function, localization and sequence homologies

Antiapoptotic integral membrane proteins of mitochondria, endoplasmic reticulum and core		Proapoptotic cytosolic proteins or associated with the cytoskeleton, translocated during PCD to mitochondrial membrane>	
Bcl-2 subfamily	Sequence homologies	Bax subfamily	Sequence homologies
Bcl-2, Bcl-x <sub>L</sub> , Bcl-w Mcl-1, A1/BFL-1 BOO/DIVA	BH1-4	Bax, Bak, BOK/MTD BH3 subfamily BIK/NBK, BIM/Bod, Bid, Bad, BLK, Hrk Others Bcl-x <sub>S</sub>	BH1-3 Sequence homologies BH3 Sequence homologies BH1 and BH4

which prevent apoptosis in infected cells (Cryns and Yuan, 1998). The precise caspase targets of the IAP remain elusive. Potent, selective inhibition of CP3 and CP7 was observed *in vitro* with X-linked IAP suggesting that IAP inhibit apoptosis through inhibition of effector caspases. The story is not so simple, however, because they also prevent the activation of these enzymes upon over-expression, suggesting that effector caspase proenzymes or other proteins in the activation complex are the real targets in cells (Deveraux *et al.*, 1997). Alternatively, if effector caspases amplify the apoptotic signal by activating initiator caspases, IAP may function as negative regulators of this feedback. IAP and other caspase inhibitors are involved in the regulation of apoptosis by establishing thresholds that determine the concentration of active effector caspases required to initiate cell disassembly, thus also preventing the consequences of accidental or spontaneous proenzyme activation. Inhibitors may also be used to confine the activity of these enzymes to specific cellular locations (Thornberry and Lazebnik, 1998).

#### FLIP

FLIP ('FADD-like ICE inhibitory proteins), are endogenous CP inhibitors that are analogue-to-viral inhibitors. There are two splice variants: FLIP<sub>S</sub> and FLIP<sub>L</sub>. FLIP<sub>L</sub> resembles structures such as CP8 and CP10 but does not have catalytic activity. The short form, FLIP<sub>S</sub>, contains two death effector domains and is structurally related to the viral FLIP inhibitors of apoptosis, whereas the long form, FLIP<sub>L</sub>, contains in addition a caspase-like domain in which the active-centre cysteine residue is substituted by a tyrosine residue. FLIP<sub>S</sub> and FLIP<sub>L</sub> interact with the adapter protein FADD and the protease FLICE, and potentially inhibit apoptosis induced by all known human death receptors. Thus FLIP may be implicated in tissue homeostasis as an important regulator of apoptosis (Irmiler *et al.*, 1997). These proteins probably compete with pCP8 for binding to its cofactor, FADD, thus preventing caspase activation (Thornberry and Lazebnik, 1998).

#### ProT and PHAP proteins

ProT is an oncoprotein required for cell proliferation. One of the biochemical functions of ProT is to prevent apoptosome formation. Such a biochemical activity is consistent with its oncogenic function, because other previously known negative regulators of apoptosis such as Bcl-2, and IAP have also been shown to have oncogenic activities (Jiang *et al.*, 2003).

PHAP proteins are tumour suppressor proteins that promote apoptosis by accelerating CP9 activation, thus suggesting that it may inhibit cell growth by promoting apoptosis. Further, certain PHAP proteins are preferentially expressed in mouse cerebellum during its most active developmental period characterized by massive apoptosis (Jiang *et al.*, 2003).

#### Control of nuclear degradation

To disassemble DNA, it is necessary to overcome the strictly regulated cell cycle. There are three main controllers of the cell cycle: the tumour suppressor protein p53, the oncoprotein MDM2 and PARP.

#### p53

In normal growing cells, p53 is activated if DNA is damaged (e.g.  $\gamma$ -radiation, drugs). If the DNA has been irreversibly damaged, the cell p53 may initiate the elimination by PCD and may stop the cell cycle from starting DNA repair. In addition, direct transcription of CP1 by p53 activates pCP3 (Gupta *et al.*, 2001). The importance of the p53 system is reflected by the molecular background of neoplasia. In 50–60% of all tumours, including testicular cancer, mutations of p53 are detectable (Gao *et al.*, 2001). Oncogenic transformation is suppressed by p53 via promotion of apoptosis. p53 is found in high concentration in the testis and plays a significant role in temperature-induced germ cell apoptosis (Socher *et al.*, 1997). It is intriguing also that a nuclear oncoprotein, such as the p53, and p21, which is considered an apoptosis regulatory protein, both increase concomitantly with CP1 (Blanco-Rodriguez and Martinez-Garcia, 1999).

#### MDM2

The protein MDM2 (mouse double minute 2) is overexpressed in testicular cancer. It inhibits p53, which initiates its degradation. Activated CP3 in turn deactivates MDM2. Therefore, MDM2 plays a role in disrupting cell cycle control (Guillou *et al.*, 1996).

#### PARP

Another target of caspases is PARP. This highly conserved enzyme specifically repairs DNA strand breaks. PARP is deactivated by cleavage of aCP3 into a catalytic fragment of 89 kDa and a DNA binding unit of 24 kDa. Cleaved PARP is detectable only in ongoing PCD. Therefore, it can be used to differentiate between PCD and necrosis (D'Amours *et al.*, 1999).

### Endocrine factors

Selective PCD can be induced by a wide variety of cofactors, e.g. heat, irradiation, ischaemia, toxicants and withdrawal, in addition to supraphysiological levels of different hormones (Erkkila *et al.*, 1997; Turner *et al.*, 1997; Sinha Hikim and Swerdloff, 1999; Rockett *et al.*, 2001).

#### Prolactin

In general, unregulated prolactin metabolism can severely damage human male reproduction. It has an antigonadal effect and is known to stimulate proliferation of human prostate cancer cell lines (Janssen *et al.*, 1996). Testicular fragments treated with prolactin have higher levels of apoptosis than those untreated when assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) assay. Thus, spermatogonial apoptosis is induced by elevated titres of plasma prolactin. When testicular fragments were exposed to prolactin and the pesticide cycloheximide, significantly high levels of caspase activity were detected (Yazawa *et al.*, 2000). In support of the role of caspases, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk), which is a pan-caspase inhibitor, decreased the prolactin-induced germ cell apoptosis (Yazawa *et al.*, 2001).

#### Estrogen

Spermatogenic cells express estrogen receptors, and estrogen-like chemicals present in the environment adversely affect male reproductive health. Such chemicals can affect gene expression and cellular function by binding to the hormone receptors and have been implicated in the declining trend of male fertility, an increase in testicular cancer, cryptorchidism and the so-called testicular dysgenesis syndrome (Sharpe and Skakkebaek, 1993; Akingbemi and Hardy, 2001). It is well established that primarily haploid spermatogenic cells undergo apoptosis in response to estrogens in contrast to what is found in other toxin-induced cell death models, where the diploid population of spermatocytes are most affected (Hikim *et al.*, 1995).

Estrogen exposure increases the expression of spermatogenic cell Fas-FasL leading to the activation of components downstream of Fas. Most important is the observation that the number of apoptotic cells is highest in seminiferous tubule stages where both Fas and FasL are expressed in large amounts (Nair and Shaha, 2003).

#### Testosterone/FSH

Artificial separation of Sertoli cells from the basement membrane induces apoptosis, but may be prevented by a mixture of testosterone and FSH (Dirami *et al.*, 1995). Thus, it appears that Sertoli cell apoptosis following testosterone/FSH withdrawal is an isolated finding detected only under *in vitro* conditions (Bockers *et al.*, 1994). If seminiferous tubules are incubated with testosterone/FSH, no significant differences in the proportion of cells containing active caspases or fragmented DNA are detected in any cell type examined over the 48 h *in vitro* incubation period. However, a significant ( $P < 0.01$ ) increase in the proportion of cells with fragmented DNA is detected in the primary spermatocyte and elongating/elongated spermatid populations as early as 24 h after the beginning of incubation, and this increase continues between 24 and 48 h. These changes are not accompanied by any

detectable increase in the proportion of germ cells containing active caspases (Tesarik *et al.*, 2002). These findings support reports of animal models that used LH-suppressive testosterone and estradiol, a regimen known to rapidly reduce testosterone production by the testes and to produce azoospermia within 8 weeks. Examination of the testicular tissue revealed that germ cell apoptosis resulting from a reduced intratesticular testosterone concentration is CP3 dependent and that the translocation of active CP3 and caspase-activated deoxyribonuclease to the nucleus may be involved in the induction of germ cell apoptosis (Kim *et al.*, 2001).

#### Antiandrogens

In general, the apoptotic germ cell death process could be related to an increased expression and activation of effector CP3 and CP6. Exposure to the antiandrogen flutamide increases CP3 and CP6 mRNA (in RT-PCR approaches) as well as pro-CP3 and pro-CP6 protein (in western blot analyses) levels in the adult rat testis. Active cleaved CP3 and CP6 protein appeared following the exposure to the antiandrogen in a dose-dependent manner, whereas they disappeared at cessation of exposure to flutamide (Omezzine *et al.*, 2003).

#### Reactive oxygen species

Reactive oxygen species are known to impair basic sperm functions, especially if neutrophil granulocytes are the source of ROS during chronic inflammation. Stimulated neutrophils release the haem enzyme myeloperoxidase that produces the powerful oxidant hypochlorous acid (HOCl) from  $H_2O_2$  and  $Cl^-$ . HOCl may trigger other signalling cascades related to apoptosis via oxidation of amino acid residues and other cell constituents (oxidative tagging). The percentage of cells with bound annexin V increased continuously upon incubation with HOCl up to 97.4% at  $5 \times 10^{-4}$  mol/l HOCl using annexin V, which cannot pass the intact plasma membrane (C. Gey, V. Paasch, J. Arnold and H. J. Glander, unpublished data).

Apoptosis was significantly correlated with ROS within infertile patients in the whole ejaculate [ $r$  (95% CI) = 0.53 (0.19, 0.86)]. Furthermore, in patients diagnosed with idiopathic male infertility, a positive relationship exists between sperm damage by ROS and higher levels of cytochrome *c* and CP9 and CP3, which indicates the presence of apoptosis in patients with male factor infertility (Wang *et al.*, 2003).

#### Drugs

##### Ethanol

Ethanol was found to have apoptogenic effects on different tissues and cell lines (Deaciuc *et al.*, 2001). Chronic alcohol intake in experimental animals has been associated with marked degenerative changes such as germ cell damage and loss of giant cell formation resulting in testicular atrophy (Martinez *et al.*, 2000). Moreover, spermatogenic arrest, hypogonadism and infertility have been reported in humans with a history of chronic alcohol abuse. Ethanol-induced testicular damage was reported to be mediated mainly by DNA damage, oxidative stress and androgen suppression (Zhu *et al.*, 2000). Sertoli cells have been considered to be the primary cellular target of ethanol toxicity, with marked Sertoli cell vacuolization being a morphological marker of

ethanol-induced testicular injury. Ethanol exposure enhances testicular germ cell apoptosis as increased immunoreactivity of CP3, CP8 and CP9 is detected in germ cells of ethanol treated rats. The Fas death receptor pathway through active forms of CP8 and CP3 most probably mediates increased CP3 reactivity in germ cells. Therefore, the apoptogenic effect of ethanol may be mediated via up-regulation of the Fas system and activation of CP3, CP8 and CP9, and may be involved in infertility associated with chronic alcohol intake (Eid *et al.*, 2002).

#### Chemotherapy

Cisplatin is a chemotherapeutic agent that may play a role in the treatment of *in vivo* carcinoma. It inhibits tumour growth by enhancing the expression of CP8 and Apaf-1 genes, which are associated with apoptosis (Mizutani *et al.*, 2002). A testicular germ cell cancer model was used to support the role of CP8 pathway in cisplatin-induced apoptosis. In CP9-blocked cells, initiation of apoptosis occurred in a CP9-independent manner accompanied by activation of CP2 and CP3 (Mueller *et al.*, 2003).

#### Physical agents

##### Hyperthermia

Exposure of the rat testis to mild heat results within 6 h in stage- and cell-specific activation of germ cell apoptosis. Initiation of apoptosis was preceded by a redistribution of Bax from a cytoplasmic to paranuclear localization in heat-susceptible germ cells (Yamamoto *et al.*, 2000). During the course of heat-induced apoptosis, Bax relocates in the ER, which constitutes the site for localization of CP12. The relocation of Bax is accompanied by cytosolic translocation of cytochrome *c* and is associated with activation of the initiator CP9 and the executioner CP3, CP6 and CP7 and cleavage of PARP (Hikim *et al.*, 2003). In other studies using the FasL-defective mice, the heat-induced germ cell apoptosis was not blocked, thus providing evidence that the Fas signalling system may be dispensable for heat-induced germ cell apoptosis in the testis (Nagata and Golstein, 1995). Taken together, these results demonstrate that the mitochondria- and possibly also endoplasmic reticulum-dependent pathways are the key apoptotic pathways for heat-induced germ cell death in the testis.

##### UV irradiation

UV radiation induces apoptosis via the generation of ROS and via a large number of unrelated pathways such as enhanced Fas transcription and/or mRNA stability. Most noteworthy, activation of CP3 and cleavage of PARP are characteristics of UV irradiation-induced apoptosis (Untergasser *et al.*, 2001).

### Consequences of caspase activation

#### Apoptosis

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations leading the cell to suicide (Vaux and Korsmeyer, 1999).

In mammalian testes, germ cells expand clonally through many rounds of mitoses before undergoing the differentiation steps that result in mature sperm. This clonal expansion is excessive and thus

requires a mechanism such as apoptosis to match the number of germ cells with the supportive capacity of Sertoli cells. Therefore, in the context of male reproduction, apoptosis controls the overproduction of male gametes and restricts the normal proliferation levels during conditions unsuitable for sperm development. Animal studies have shown that apoptosis is a key regulator of spermatogenesis in normal and pathological conditions (Furuchi *et al.*, 1996).

Although there are  $\geq 14$  caspases in humans, only a subset of these enzymes is proteolytically activated by various distinct death stimuli in different cell types (Porter and Janicke, 1999). However, pro-CP3 is usually present and processed by autoproteolytic cleavage that leads to assembly of the active heterotetrameric enzyme. The frequent activation of CP3 in different cell types raises the fundamental question of whether this protease is required for the death of the cell and for the many morphological changes associated with apoptosis. Unfortunately, there is one other known CP3-like protease in vertebrates (CP7) with an analogous *in vitro* substrate preference to CP3 (Nicholson and Thornberry, 1997). Moreover, the existence of other CP3-like proteases cannot be ruled out. Therefore, the activation of CP3 is not by itself proof that it is required for apoptotic events and cell death.

#### Loss of membrane structure—exposure of phosphatidylserine

Reduced integrity of sperm membrane is more frequent in sperm from infertile men, and this reduced integrity contributes to childlessness despite normal spermogram parameters (Glander and Schaller, 1999; Duru *et al.*, 2001). The deterioration of membrane integrity is also associated with an activation of caspases in somatic cells (Vermes *et al.*, 1995).

In those cells and in sperm with an intact plasma membrane, the phospholipid phosphatidylserine (PS) is located on the inner leaflet of the plasma membrane only. PS has a high and selective affinity for annexin V, a 35–36 kDa phospholipid binding protein (van Heerde *et al.*, 1995). Annexin V cannot pass through an intact plasma membrane; therefore, annexin V binding to sperm characterizes a disturbed integrity of the membrane. The binding can be the result of translocation of PS from the inner to the outer leaflet of plasma membrane resulting in an expose of PS (EPS) on the external surface (Vermes *et al.*, 2000). This translocation of PS is one of the earliest detectable features of cells undergoing the terminal steps of apoptosis (Vermes *et al.*, 1995; van Engeland *et al.*, 1998).

#### Impaired spermatogenesis

Ectopic expression and inactivation of apoptosis-related genes have been shown to cause abnormalities in spermatogenesis. For example, the testis of the Bax knockout (KO) mouse showed accumulation of atypical premeiotic germ cells and an absence of elongated spermatids, suggesting that the mitochondria-mediated cascade of apoptosis is important in spermatogenesis (Knudson *et al.*, 1995). During spermatogenesis—the process of germ cell proliferation and maturation from diploid spermatogonia to mature haploid sperm—a number of the developing germ cells die by apoptosis before reaching maturity, even under normal conditions (Billig *et al.*, 1995). In addition to the physiological germ cell apoptosis that occurs continuously throughout life, increased germ

cell apoptosis results from such external disturbances as irradiation or exposure to toxicants (Pentikainen *et al.*, 1999).

Reports indicate that when the testicular environment can no longer support spermatogenesis, the FasL system is activated leading to germ cell apoptosis, a process in which the Sertoli cells play a major role (Lee *et al.*, 1999). The downstream effectors of Fas include various caspases, the most upstream of them being caspase 8. Activation of CP8 is followed by activation of CP3, which then cleaves substrates involved in genome function, such as PARP. In support, the universal caspase inhibitor, Z-VAD-fmk, was also able to inhibit testicular germ cell apoptosis (Pentikainen *et al.*, 1999). It must be pointed out that there is a contradictory report that refutes the role of Fas/FasL system in male germ cell apoptosis (Ohta *et al.*, 1996; D'Alessio *et al.*, 2001).

### **Sperm DNA damage**

While some apoptotic events are caspase-independent, caspase activation is believed to be a well-defined point of no return for apoptosis progression, and a number of apoptotic events downstream of caspase activation have been characterized among which DNA fragmentation stands as a relatively late apoptotic event (Thornberry and Lazebnik, 1998). Evidence suggests that within the cellular component of the testicular tissue, caspases play a central role in the apoptotic process that leads to DNA fragmentation of Sertoli cells (Tesarik *et al.*, 2002). From a functional point of view, caspases involved in apoptosis act either as initiators (CP8, CP9 and CP10) or as effectors (CP3, CP6 and CP7) of apoptosis. Among the effector caspases, activated CP3 appears to induce activation of caspase-activated deoxyribonuclease (CAD; also called DNA fragmentation factor-40 or caspase-activated nuclease), which is integrally involved in degrading DNA. Therefore, CP3 appears to be the most important among them as it executes the final disassembly of the cell by generating DNA strand breaks (Kim *et al.*, 2001; Paasch *et al.*, 2003). Sperm DNA fragmentation was prevalent in fractions of sperm with positive immunostaining for active CP3, suggesting a relationship between them (Weng *et al.*, 2002). Further, a significant positive correlation was seen between *in situ* aCP3 in the sperm midpiece and DNA fragmentation in low motility semen specimens, suggesting that caspase-dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria and function in the nucleus (Wang *et al.*, 2003).

### **Effect on sperm motility**

Sperm fractions with low motility exhibit more active caspase-positive cells than the high-motility fractions in donors and patients. In support, higher levels of aCP3 (17 kDa) and the inactive pCP3 (32 kDa) were present in the low-motility fraction compared with the high-motility fractions from donors and patients (Weng *et al.*, 2002). Furthermore, there are lower and more variable levels of pCP3 in high-motility fractions of patients and donors and a virtual absence of aCP3. However, it is of interest to note that preliminary studies suggest that the addition of a pan caspase inhibitor did not lead to any improvement in sperm post-thaw motility (Peter and Linde-Forsberg, 2003).

The activation of caspases in low-motility sperm samples may be attributed to the role played by cytochrome *c*-Apaf-1 complex, which can activate CP9 and is followed by activation of

downstream death effectors such as CP3, CP6 and CP7 (Thornberry and Lazebnik, 1998).

### **Sperm differentiation**

Although apoptosis is a morphologically distinct form of cell death that usually serves to remove unwanted and potentially dangerous cells, there are some examples where apoptosis-like events do not lead to death but rather the terminal differentiation of certain cell types. For example, lens epithelial cells and mammalian red blood cells lose their nucleus and other subcellular organelles during terminal differentiation but continue to be metabolically active (Jacobson *et al.*, 1997).

Terminal differentiation of sperm shares many morphological and biochemical features with apoptosis. However, rather than causing the death of the entire cell, caspases are used to specifically eliminate cytoplasmic components, thereby producing a highly specialized living cell. In mammals, the cytoplasm collections in the residual bodies (RB) display several features of apoptosis (Blanco-Rodriguez and Martinez-Garcia, 1999). Although the role of caspases in the removal of bulk cytoplasm during mammalian spermatogenesis remains to be established, there are preliminary data showing that aCP3 is present in the RB in the testes of mice (Arama *et al.*, 2003). This is of academic interest as various types of caspase inhibitors are being considered as drugs for therapeutic purposes and the effects on human fertility have not been studied yet.

Recently, the role of caspases during capacitation of human sperm was investigated. Media containing bicarbonate was used to induce protein kinase-mediated alterations in the phospholipid bilayer (PL), which correlate with capacitation markers. Caspase inhibitors failed to block the capacitation-related PL, and CP3 was not detected in the mature sperm. Therefore, it appears that there is no current evidence implicating CP3 in mammalian capacitation (de Vries *et al.*, 2003).

## **Role of caspases in male reproduction**

### **Puberty**

Homeostatic control of cell number is the result of the dynamic balance between cell proliferation and cell death. In the testis, during fetal and newborn periods, it determines the final adult size and fertility potential.

The newborn period is characterized by a lower rate of apoptosis in germ cells, Sertoli cells and interstitial cells compared with that of the rest of early prepuberty. When comparing the difference in the rate of apoptosis using immunohistochemical staining for CP3, newborns have significantly lower values than older prepubertal infants (Table III). Moreover, the rate of proliferation of germ cells is higher in the newborn period than in later periods. This regulation of apoptotic cell death contributes markedly to the modulation of cell number in the prepubertal testis (Berensztein *et al.*, 2002).

### **Varicocele**

Germ cell apoptosis during normal spermatogenesis has been estimated to result in the loss of 25–75% of potential mature sperm cells in the adult testis. However, apoptosis is decreased in the testes of men affected by varicocele (Fujisawa *et al.*, 1999). Levels



**Table III.** Immunohistochemical expression of active caspase-3 in the cytoplasm of Sertoli, germ and interstitial cells (Berensztein *et al.*, 2002)

Group	Age	No.	Sertoli cells (%)	Germ cells (%)	Interstitial cells (%)
1	1 to 21 days	10	6.59 <sup>a</sup> ± 3.81	10.3 <sup>a</sup> ± 5.42	7.86 <sup>a</sup> ± 3.61
2	1 to 6 months	9	12.64 ± 6.66	23.0 ± 12.0	11.4 ± 3.69
3	1 to <6 years	13	12.62 ± 6.97	21.3 ± 11.6	13.1 ± 4.85

Values are percentage of positive cells per cell type (mean ± SD).

<sup>a</sup>Significantly different from group 2 or group 3.

of ICE and CP3 are both significantly lower in the testes of patients with varicocele than in specimens from healthy men. These results suggest that there is a connection between ICE and CP3 in the testes of infertile men with varicocele (Tanaka *et al.*, 2002).

### **Testicular torsion**

Testicular torsion is a urological emergency referred to as 'acute scrotum'. Early diagnosis and surgical intervention determines the prognosis of spermatogenesis. Until recently, the ischaemic injury of the ipsilateral twisted testis was called 'necrotic' and was followed by the loss of its endocrine and exocrine function. One line of evidence shows the activation of the caspase pathway during apoptosis in the testis after ischaemia; and that this ischaemia induces proteolysis of CP3 and PARP. A broad-spectrum caspase inhibitor, z-VAD-fmk, but not a CP3-specific inhibitor, DEVD-fmk, reduced the number of TUNEL-positive nuclei, DNA fragmentation and proteolysis of PARP. These data suggest that some hitherto unidentified caspase other than CP3 is involved in the evolution of apoptosis during ischaemia. Thus, it appears that the central component of the apoptotic machinery (caspases) plays a role in the pathogenesis of testicular torsion (Shiraishi *et al.*, 2000).

### **Male infertility**

To date, it is unclear whether apoptosis in ejaculated sperm occurs in a manner similar to that in somatic cells or if sperm, which are thought to have an almost transcriptionally inactive nucleus, undergo abortive forms of this process (Gandini *et al.*, 2000; Sakkas *et al.*, 2002).

#### *Caspases in spermatogenesis of infertile men*

CP3 has been detected not only within the cytosol but also in a perinuclear distribution of germ cells (Paasch *et al.*, 2001). Selective antibodies to aCP clearly show a cytoplasmic distribution of the caspases (C. Sorger, U. Paasch, S. Grunewald, J. Schaller and H. J. Glander, unpublished data). CP1, CP8 and CP3 were detected in Leydig and Sertoli cells as well as in spermatogonia and spermatocytes but not in spermatids and mature sperm (Paasch *et al.*, 2002a).

Experiments with immunofluorescent inhibitors of all active caspases types (pan-caspase, aCP-Pan) and by western blot analysis have clearly demonstrated the postacrosomal presence and functional competence of caspase in ejaculated human sperm (Paasch *et al.*, 2001, 2002b). It has been shown that sperm with intact membranes also contain to a lesser extent these enzymes. In contrast, samples from infertile patients were characterized by high numbers of cells with aCP, especially in cytoplasmic

residues, with a strong correlation to EPS (Paasch *et al.*, 2001). The presence of precursors and activated forms of initiator caspases 8 and 9 in conjunction with their shared effector CP3 in human sperm has also been confirmed (Paasch *et al.*, 2002b). This was also true for CP1 because it mediates alternative pathways of apoptosis via p53 and inflammatory signals (Solary *et al.*, 1998; Gupta *et al.*, 2001).

Consistent with the special structure of sperm, the active caspases were observed predominantly in the postacrosomal region (aCP8, aCP1 and aCP3) of live sperm. In addition, mitochondria-associated aCP9 was particularly localized in the midpiece.

In ejaculated sperm and in testicular tissue, the presence and functional competence of p53, MDM2 and PARP have been detected. In ejaculated sperm, PARP was always found while cleaved PARP was detected in 43% of semen samples. In addition, MDM2 and its cleaved residues were found in up to 74% of the semen samples. In 75–100% of the investigated samples, p53 was evident by western blot analysis (Paasch *et al.*, 2001, 2002b, 2003).

#### *Annexin V assays*

Superparamagnetic microbeads (~50 nm diameter) conjugated with annexin V (ANMB) were used to eliminate membrane damaged subpopulations of hamster sperm by magnetic cell sorting (MACS) (von Schonfeldt *et al.*, 1999). The procedure delivers two sperm fractions: ANMB+ and ANMB– sperm.

Flow cytometric analyses using anti-annexin V antibodies confirmed the separation effect of MACS in the sperm of donors. The specificity and sensitivity of the system were determined to be 94.8 and 72.6% respectively. The fraction of ANMB– sperm in the patients and donors showed significantly reduced amounts of aCP-Pan compared with unseparated sperm and with the ANMB+ fraction ( $P < 0.01$ ). In donors, the aCP-Pan decreased from  $21.8 \pm 2.6$  to  $9.2 \pm 1.4\%$  ( $P < 0.01$ ). In patients, it decreased from  $43.2 \pm 2.1$  to  $18.2 \pm 2.6\%$  in the membrane-intact fraction. Therefore, sperm with aCP-Pan were found mainly within the ANMB+ fractions ( $P < 0.01$ ). A significant correlation between the overall percentage of sperm with aCP-Pan and those binding annexin V microbeads was found in the donors and patients ( $r = 0.97$  and  $r = 0.72$  respectively;  $P < 0.01$ ) (Paasch *et al.*, 2003).

The MACS separation according to EPS resulted in a significant depletion of sperm having activated CP8, CP9, CP1 or CP3 within the EPS– fraction and a simultaneous enrichment of sperm bearing active CP8, CP9, CP1 or CP3 into the EPS+ fraction. The relationship between caspase activation and externalization of phosphatidylserine was found to be present by calculating the

log<sub>2</sub>OR. The closest association of caspase activation and membrane damage was detected for CP9. This effect could be attributed to the specialized structure of sperm in which the mitochondria are located in the midpiece near the outer membrane. On the other hand, the presence of active CP3 in sperm is accompanied by less loss of membrane asymmetry (EPS+ sperm) compared with the other CP. Because the protease is the shared effector enzyme of multiple apoptosis pathways, an activation triggered independently from membrane integrity is very likely (U. Paasch, S. Grunewald and H. J. Glander, unpublished data).

#### *Role of sperm preparation techniques*

Modern methods of sperm processing comprise essentially swim-up and density gradient centrifugation, which aim to separate high-quality sperm (Sterzik *et al.*, 1998). A significant cell-to-cell variation in ROS production in subsets of sperm characterized by different stages of maturation has been further elucidated (Gil-Guzman *et al.*, 2001). The oxidative damage of mature sperm by ROS-producing immature sperm could also activate PCD signalling cascade in addition to direct membrane and DNA damage (Ollero *et al.*, 2001).

Recently, we conducted a large study to investigate type I and II PCD pathways in mature and immature sperm as obtained by density gradient centrifugation. Both fractions significantly differed in the amount of aCP but not in the number of cells with disrupted MMP or TUNEL positivity. It has been shown that both fractions can be further subdivided by annexin V MACS. The separation effect of the annexin MACS was demonstrable within the mature and immature fraction for aCP8, aCP9 and aCP3 as well as for TUNEL positivity ( $P < 0.05$ ) (U. Paasch, A. Agarwal and A. K. Gupta, unpublished data). The results of DNA fragmentation from our study are similar to other studies that showed that the pellet fraction resulting from gradient centrifugation (90% layer, Percoll) of infertility patients is comprised of nuclei with decreased rates of DNA fragmentation (Zini *et al.*, 2000).

#### *Effect of cryopreservation*

Cryopreservation has been shown to activate caspases of different apoptotic pathways in human sperm. Density gradient centrifugation (DGC) applied after cryopreservation or in neat cells decreases the levels of active CP8, CP9 and CP3 in sperm from the 90% layer and from the 47–90% interface (unpublished data). For a complete understanding of the underlying subcellular effects, the levels of aCP8, aCP9 and aCP3 were measured in sperm from the 90% fraction and subjected to cryopreservation after DGC. It is of interest that sperm were characterized by low levels of aCP8, aCP9 and aCP3. After cryopreservation and thawing, CP8 and CP3 were re-activated in this sperm subset, but not CP9 (S. Grunewald, A. K. Grunewald, A. K. Gupta, N. Kattal, U. Paasch, R. K. Sharma, A. Agarwal and H. J. Glander, unpublished data).

Annexin V binding to EPS can measure the quantity of membrane damage during cryopreservation. Both the cryoprotective medium and cooling speed are equally important, (Glander and Schaller, 1999; Schuffner *et al.*, 2001). We were able to demonstrate that shock freezing compared with slow cooling induces significant EPS and activation of pan-caspase (Paasch *et al.*, 2002c). However, the sole presence of an active caspase is not the crucial lethal factor since overall survival time of a single

spermatozoon could not be prolonged by inhibitors of caspases (Weil *et al.*, 1998).

In a western blot study using 7 and 14% (v/v) of glycerol as cryoprotectant, CP8, CP9, CP1 and CP3 had decreasing amounts of pCP with a consecutive increase of active parts (Paasch *et al.*, 2002c). The use of the Test Yolk buffer and glycerol in comparison with the use of glycerol only resulted in a decrease in the EPS-positive cells (Duru *et al.*, 2001).

#### *Immunological infertility*

Antisperm antibodies (ASA) are the main cause of immunological infertility as they are capable of impairing sperm function by binding to the sperm membrane. Antibodies directed at sperm antigens can be detected in seminal fluid, where they may be bound to the sperm surface or solubilized in the seminal plasma, cervical mucus, oviductal fluid or follicular fluid of women. They also occur in the blood serum of men, but these appear to be iso-antisperm antibodies that are not important for fertilization. In the literature, the incidence of ASA in infertile couples (both men and women) ranges from 9 to 36% (Collins *et al.*, 1993).

The characterization and identification of human sperm antigens are important for understanding the mechanism by which ASA may impair sperm fertilization capacity. Using two-dimensional gel electrophoresis and immunoblot analysis, CP3 was identified as an antigen for ASA in men with immunological infertility (Bohring *et al.*, 2001).

## **Conclusion**

The importance of understanding the mechanisms of germ cell death has become evident during recent years as there is still a need for superior treatment modalities for male infertility. Caspase-dependent apoptosis is a well-characterized mechanism for removing senescent, defective or unneeded cells. The identification of caspases as critical components of the death machinery represents a major advance in our understanding of many molecular aspects of male infertility.

Once activated, caspases transduce a signal to effector caspases leading to degradation of cellular substrate. The main message is that caspases can exert multiple effects, which are critical for cell death to occur. These effects include cell shrinkage, blebbing, chromatin condensation and DNA fragmentation. CP3 appears to be the main executioner within the apoptotic cascade. In general, caspases play a major role in the pathogenesis of a multiplicity of andrological disorders such as impaired spermatogenesis, decreased sperm motility and increased levels of sperm DNA fragmentation. Although caspases have also been linked to testicular torsion, varicocele and immunological infertility, their regulation plays a beneficial role in sperm differentiation and testicular maturity. Future research should be directed towards gaining a better understanding of the regulation of these cysteine proteases. This may facilitate efforts to rationally manipulate the apoptotic machinery for therapeutic benefits.

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