

APOPTOSIS REGULATION IN THE MALE GERMINAL EPITHELIUM

REGULAÇÃO DA APOPTOSE NO EPITÉLIO GERMINAL MASCULINO

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inspirado na força e coragem
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Almeida C, Correia S, Rocha E, Alves A, Ferraz L, Silva J, Sousa M, Barros A (2010) Caspase signalling pathways in human testicular disorders. *Hum Reprod*, submitted.

Em cumprimento do disposto no referido Decreto-Lei declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a activa colaboração dos outros autores.

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ABSTRACT

In all human tissues, a balance between cell proliferation and cell death is essential to normal development. Deregulation of this balance was shown to be on the origin of proliferative and degenerative diseases. Apoptosis is a form of programmed cell death that involves genetically determined and regulated elimination of cells in response to specific stimuli, either internal or external. Caspases are the most important family of proteins involved, responsible for initiation and execution of the apoptotic cascade.

During normal human spermatogenesis, spontaneous apoptosis was demonstrated to be present and to be essential for the adjustment of the number of germ cells to the support capacity of Sertoli cells and for the removal of damaged and abnormal ones. In this mechanism, the Fas/FasL system was shown to be involved. Several spermatogenic disorders have been described including the presence of spermiogram abnormalities and absence of sperm production. Increased apoptosis have already been detected in cases of abnormal semen parameters. The significance of apoptosis in male infertility and the apoptotic mechanisms involved is an issue of debate and contradictory findings have been described in the literature.

In order to search for the significance of apoptosis in sperm from men with abnormal semen parameters (concentration, morphology and rapid progressive motility), active caspase-3 was analysed in ejaculated spermatozoa, both in the neat semen and after gradient centrifugation and swim-up techniques (swim-up fraction). Analysis was performed in ejaculated samples from 67 males undergoing spermiogram evaluation. Six men were normozoospermic and 61 had abnormal spermiogram parameters. A correlation was found between the presence of sperm with active caspase-3 and asthenozoospermia, in the neat semen. Active caspase-3 was confined to the midpiece suggesting that caspase-3 activity might correspond to the activation of the cell apoptotic machinery in response to mitochondrial lesions. Additionally, a correlation was shown with teratozoospermia in the swim-up fraction. However, the low rates of sperm with active caspase-3 observed in men with decreased sperm normal morphology suggest a low risk of selecting apoptotic spermatozoa during clinical treatments (Paper I).

It still remains to be understood if apoptosis in testicular spermatozoa from azoospermic men with complete spermatogenesis is related to the pathology. Testicular samples were obtained from 18 men undergoing treatment testicular biopsy: 5 oligozoospermic, 9 with obstructive azoospermia (4 with congenital bilateral absence of the vas deferens - CBAVD, and 5 with secondary obstructive azoospermia) and 4 with

hypospermatogenesis. Active caspase-3 was observed in different testicular sperm compartments: midpiece, equatorial region, acrosomal vesicle region, nucleus and cytoplasm. Hypospermatogenesis showed active caspase-3 mainly in the midpiece. In CBAVD, secondary obstructive azoospermia and oligozoospermia, active caspase-3 was mainly present in the nucleus and was 1.89-fold higher in secondary obstruction than in CBAVD. Results suggest that tubular obstruction may induce apoptotic cascade activation due to nuclear lesions. A stronger negative impact in testicular spermatozoa development was shown for duct obstruction for secondary causes. In hypospermatogenesis, disrupted sperm production might be a consequence of increased apoptosis activated through mitochondrial lesions. No relation between decreased sperm production and apoptosis in testicular spermatozoa was found for oligozoospermia (Paper IV).

After caspase-3 activation, phosphatidylserine externalization is observed. Although correlations were described between abnormal semen parameters and increased sperm with phosphatidylserine translocation, this apoptotic feature has also been related to sperm capacitation. In order to define the significance of phosphatidylserine translocation in ejaculated spermatozoa from men with abnormal semen parameters, 37 ejaculated samples were analyzed: 9 were normozoospermic and 28 had abnormal semen parameters. Both the neat semen and swim-up fraction were evaluated, and distinction among live, early and late apoptotic and necrotic sperm was performed. Although apoptosis seemed to be related to all individual parameters, total and late apoptosis rates were significantly increased only when the overall semen quality was decreased, i.e., in oligoasthenoteratozoospermic (OAT) samples. Even after sample purification, OAT men maintained increased rates of total and late apoptotic spermatozoa (Paper III).

Testicular spermatozoa analysis was performed in 19 patients, 8 with obstructive azoospermia (4 CBAVD and 4 with secondary obstructive azoospermia), 6 with hypospermatogenesis and 5 without azoospermia (3 with anejaculation, and 2 with oligozoospermia). Results showed significantly increased rates of sperm total and late apoptosis in hypospermatogenesis and obstructive azoospermia when compared with anejaculation and oligozoospermia. Increased rates of late apoptosis in CBAVD in relation to secondary obstruction and of early apoptosis in anejaculation in relation to oligozoospermia suggest a negative impact of long-term obstruction and sperm release absence on testicular sperm quality. Comparisons between semen and testis showed that oligozoospermic men presented significantly higher rates of sperm apoptosis in semen than in testis suggesting the presence of a post-testicular apoptotic induction factor contributing for the presence of apoptotic sperm in the ejaculate, and the potential beneficial use of testicular spermatozoa in clinical treatments (Papers II, III).

After describing the apoptotic rates and defending possible apoptotic mechanisms involved in ejaculated and testicular spermatozoa from men with abnormal spermatogenesis, the question about apoptosis being a stage-specific phenomenon during spermatogenesis arose. In order to answer this question Sertoli cells, spermatogonia, primary and secondary spermatocytes and round spermatids were analyzed for the presence of active caspases-3, -8 and -9 in testicular samples from 27 men: 5 oligozoospermic, 4 CBAVD, 5 with secondary obstructive azoospermia, 5 with hypospermatogenesis, 3 with maturation arrest and 5 with Sertoli-cell-only syndrome (SCOS). No significance for apoptosis was found in cases with maturation arrest. Sertoli-cell-only syndrome was suggested to be related to increased active caspase-3. In cases of oligozoospermia, CBAVD, secondary obstruction and hypospermatogenesis, apoptosis seemed to be present in meiotic germ cells from all pathologies. Apoptosis in secondary obstruction was suggested to depend on a cross-talk between the extrinsic and intrinsic apoptotic pathways, whereas in CBAVD only the intrinsic apoptotic pathway with mitochondrial lesions seemed to be present, mainly at the primary spermatocyte stage. Low numbers of germ cells observed in hypospermatogenic cases were suggested to result from Sertoli cell death by apoptosis and mitochondrial lesions at the primary spermatocyte stage. Finally, in oligozoospermic patients stem cell death by mitochondrial damage and meiosis malfunctioning during spermatogenesis might be on the origin of the decreased sperm output. In addition, the post-testicular apoptotic induction factor theory would be additionally responsible for the extreme low sperm numbers observed in these men (Paper V).

In conclusion, the present thesis allowed the establishment of a correlation between apoptosis and abnormal semen parameters. In addition, several apoptotic activation mechanisms involved in complete and non-complete spermatogenesis were suggested.

RESUMO

O desenvolvimento normal de todos os tecidos humanos depende de um equilíbrio entre a proliferação e a morte celular. Alterações deste equilíbrio estão na origem de doenças proliferativas e degenerativas. A apoptose é uma forma de morte celular geneticamente programada que, perante estímulos internos ou externos, conduz à eliminação de células de uma maneira controlada. Neste mecanismo, as caspases são as principais proteínas envolvidas, responsáveis tanto pela activação da apoptose como pela sua execução.

Durante a espermatogénese humana, a apoptose é responsável pelo ajuste do número de células germinais à capacidade de suporte das Células de Sertoli assim como pela eliminação de células anormais, ocorrendo este mecanismo numa maneira dependente do sistema Fas/FasL. Alterações no normal funcionamento da espermatogénese podem conduzir à presença de parâmetros seminais anormais ou mesmo à ausência de produção de espermatozóides. Em homens com parâmetros seminais anormais foi já descrita a presença de taxas de apoptose aumentadas. No entanto, têm surgido resultados contraditórios sobre a importância da apoptose na infertilidade masculina.

De modo a decifrar a relação entre espermatozóides em apoptose e parâmetros seminais anormais (concentração, morfologia e mobilidade progressiva rápida), determinaram-se as taxas de espermatozóides do ejaculado com caspase-3 activa após liquefacção (fracção sémen) e após centrifugação por gradientes e swim-up (fracção swim-up). Foram analisadas amostras seminais de 67 homens: 6 homens normozoospermicos e 61 homens com parâmetros seminais anormais. O estudo demonstrou uma relação entre espermatozóides com caspase-3 activa e a astenozoospermia, na fracção sémen. Adicionalmente, a presença de caspase-3 activa apenas na peça intermédia sugere que a activação da cascata apoptótica pode ter ocorrido devido a lesões mitocondriais. Na fracção swim-up foi encontrada uma relação com a teratozoospermia. No entanto, as reduzidas taxas de espermatozóides com caspase-3 activa em homens com teratozoospermia sugere um baixo risco de selecção de espermatozóides apoptóticos durante os tratamentos clínicos (Artigo I).

Outra questão por resolver é a relação entre a apoptose e a azoospermia. De modo a verificar a existência ou não de uma relação entre a presença de espermatozóides testiculares em apoptose e a azoospermia foram analisados espermatozóides de biopsias testiculares de 18 homens: 5 oligozoospermicos, 9 com azoospermia obstrutiva (4 com ausência congénita bilateral dos vasos deferentes - CBAVD e 5 com azoospermia obstrutiva secundária) e 4 com hipoespermatoogénese. A caspase-3 activa foi detectada em

vários compartimentos dos espermatozóides: peça intermédia, região equatorial, região da vesícula acrossómica, núcleo e citoplasma. Em pacientes com hipoespermatoogénese, a caspase-3 activa foi detectada essencialmente na peça intermédia enquanto nos pacientes com CBAVD, azoospermia obstrutiva secundária e oligozoospermia foi detectada essencialmente no núcleo. Os pacientes com azoospermia obstrutiva secundária apresentaram 1.89 vezes mais caspase-3 activa no núcleo que os CBAVD. Os resultados sugerem que a obstrução tubular, nomeadamente a obstrução secundária, pode induzir a activação da cascata apoptótica devido a lesões nucleares. Nos pacientes com hipoespermatoogénese observou-se um aumento das taxas de apoptose em consequência de lesões mitocondriais. Nos oligozoospermicos, a apoptose nos espermatozóides testiculares não parece estar na origem da patologia (Artigo IV).

Após activação da caspase-3, ocorrem alterações ao nível da membrana citoplasmática: translocação da fosfatidilserina para o folheto externo da membrana. Embora já tenham sido descritas algumas correlações com a presença de valores anormais do espermograma, a translocação da fosfatidilserina foi também relacionada com a capacitação do espermatozóide. De modo a definir a relação entre a translocação da fosfatidilserina nos espermatozóides do ejaculado e os parâmetros seminais anormais, analisaram-se 37 amostras seminais: 9 com normozoospermia e 28 com anomalias nos parâmetros seminais. Este estudo foi igualmente realizado na fracção de sémen e de swim-up, com distinção entre espermatozóides vivos, em apoptose inicial ou tardia ou necróticos. Embora tenha sido encontrada uma relação entre a apoptose e os 3 parâmetros seminais, as taxas de apoptose total (inicial + tardia) e tardia apresentaram-se significativamente aumentadas apenas em amostras de qualidade total diminuída (oligoastenoteratozoospermia - OAT). Mesmo na fracção swim-up, a percentagem de espermatozóides em apoptose total ou tardia permaneceu elevada nestes pacientes. Estes resultados sugerem que a translocação da fosfatidilserina nos espermatozóides está relacionada com as anomalias do espermograma (Artigo III).

Nos espermatozóides testiculares a análise foi realizada em 19 pacientes, 8 com azoospermia obstrutiva (4 CBAVD e 4 com obstrução secundária), 6 com hipoespermatoogénese e 5 sem azoospermia (3 com anejaculação e 2 com oligozoospermia). Os resultados demonstraram a presença de taxas de apoptose total e tardia aumentadas nos pacientes com hipoespermatoogénese e com azoospermia obstrutiva, em relação aos pacientes com anejaculação e oligozoospermia. Adicionalmente, foram observadas taxas de apoptose tardia aumentadas nos CBAVD em relação aos pacientes com obstrução secundária, e de apoptose inicial nos pacientes com anejaculação em relação aos oligozoospermicos. Os resultados sugerem um impacto negativo da

obstrução e ausência de ejaculação na qualidade dos espermatozóides testiculares. Comparações entre as taxas de apoptose nos espermatozóides do sémen e do testículo dos pacientes com oligozoospermia demonstraram taxas de apoptose aumentadas no sémen sugerindo a presença de um mecanismo pós-testicular de activação da apoptose e o possível benefício da utilização de espermatozóides testiculares durante os tratamentos clínicos destes pacientes (Artigos II, III).

Foram, portanto, descritas as taxas de apoptose e definidos possíveis mecanismos de activação envolvidos, nos espermatozóides do sémen e do testículo de homens com espermatogénese anormal. Colocou-se posteriormente a questão da importância da apoptose na linha germinal masculina patológica. De modo a investigar a hipótese de a apoptose ser activada em determinados estádios da espermatogénese e de modo a decifrar possíveis mecanismos de activação, a presença das formas activas das caspases-3, -8 e -9 foi analisada nas Células de Sertoli, espermatogónias, espermatócitos primários, secundários e espermatídeos redondos, de 27 homens: 5 oligozoospermicos, 4 com CBAVD, 5 com azoospermia obstrutiva secundária, 5 com hipoespermatogénese, 3 com paragem de maturação na segunda fase da meiose e 5 com Síndrome de Células de Sertoli (SCOS). Os resultados sugerem a ausência de uma relação entre a apoptose e a paragem de maturação. Nos casos de SCOS os resultados sugerem uma relação entre patologia e a presença de caspase-3 activa. Em todas as patologias com espermatogénese completa (oligozoospermia, CBAVD, obstrução secundária e hipoespermatogénese) observou-se apoptose aumentada nas células germinais meióticas (espermatócitos primários). Na azoospermia obstrutiva secundária, os resultados sugerem que a morte das células germinais por apoptose depende da interligação entre o mecanismo apoptótico externo e o interno, enquanto nos CBAVD apenas o mecanismo apoptótico interno activado por lesões mitocondriais, nomeadamente ao nível dos espermatócitos primários, parece estar presente. Nos pacientes com hipoespermatogénese verificou-se a morte das Células de Sertoli por apoptose e a presença de lesões mitocondriais ao nível dos espermatócitos primários. Nos pacientes com oligozoospermia, a presença de reduzidos números de espermatozóides no ejaculado parece resultar da morte de células estaminais por apoptose e de alterações na meiose. Adicionalmente, um mecanismo apoptótico pós-testicular seria responsável pela reduzida concentração de espermatozóides observada nestes pacientes (Artigo V).

Em conclusão, a presente tese permitiu estabelecer uma correlação entre a apoptose e a presença de parâmetros seminais anormais assim como possíveis mecanismos de activação da apoptose na linha germinal masculina de pacientes com espermatogénese completa e não completa.

ACRONYMS AND ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial transmembrane potential
A1	Bcl-2-related gene A1
AIF	Apoptosis-Inducing Factor
ANMBs	superparamagnetic annexin V-conjugated microbeads
ANT	Adenosine Nucleotide Translocator
Apaf-1	Apoptotic protease activating factor-1
Asp	Aspartate
AZF	Azoospermia Factor
BAD	BCL-2 antagonist of cell death
BAK	Bcl-2 antagonist/killer 1
BAX	Bcl-2 associated X protein
BCL-2	B-cell lymphoma protein 2
Bcl-w	Bcl-2 like 2 protein
Bcl-xL	Bcl-2-related protein, long isoform
Bcl-xS	Bcl-2-related protein, short isoform
BH	BCL-2 Homology
BID	BCL-2-interacting domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2-interacting mediator of cell death
BIR	Baculovirus IAP Repeat domain
Bruce	BIR repeat-containing ubiquitin conjugating enzyme system
BTB	Blood-Testis Barrier
CAD	Caspase-activated DNase
CARD	Caspase Recruitment Domain
Caspases	Cysteine aspartate-specific proteinases
CBAVD	Congenital Bilateral Absence of the Vas Deferens
c-FLIP	FLICE-inhibitory protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
c-IAP1	cellular IAP
c-IAP2	cellular IAP2
CUAVD	Congenital Unilateral Absence of the Vas Deferens
Cys	Cysteine
DD	Death Domain
DED	Death Effector Domain

DFF40	DNA Fragmentation Factor 40
DFF45	DNA Fragmentation Factor 45
DISC	Death-Inducing Signalling Complex
Endo G	Endonuclease G
FAAD	Fas-associated death domain
FasL	Fatty acid synthetase ligand
FasR	Fatty acid synthetase receptor
FLICE	FADD-like ICE
FSH	Follicle-Stimulating Hormone
GAAD	Gzma-activated DNase
Glu	Glutamic acid
GnRH	Gonadotrophin-Releasing Hormone
His	Histidine
HtrA2/Omi	High temperature requirement protein A2/Omi
IAP	Inhibitor of Apoptosis Protein
IBM	IAP binding motif
ICE	Interleukin-1 β Converting Enzyme
ICSI	Intracytoplasmic Sperm Injection
Ile	Isoleucine
ILP-2	IAP-like protein-2
IMM	Inner Mitochondrial Membrane
IVF	In Vitro Fertilization
lepST1	Leptotene Primary Spermatocyte
LH	Luteinising Hormone
MACS	Magnetic-activated cell sorting
MCL-1	Mieloid cell leukemia sequence 1
ML-IAP/Livin	Melanoma IAP/Livin
MOMP	Mitochondrial Outer Membrane Permeabilization
NAIP	Neuronal Apoptosis-Inhibitory Protein
NM	Normal Morphology
OMM	Outer Mitochondrial Membrane
PM	Progressive Motility
PS	Phosphatidylserine
pST1	pachytene Primary Spermatocyte
PTP	Permeability Transition Pore
PUMA	p53-Upregulated Modulator of Apoptosis
RING	Really Interesting New Gene

ROS	reactive oxygen species
rSd	round Spermatid
SCOS	Sertoli-cell-only syndrome
Sd.Sz	Elongated Spermatids
SG	Spermatogonia
Smac/DIABLO	Second mitochondria-derived activator of caspase/Direct IAP binding protein with a Low pI
ST1	Primary Spermatocyte
ST2	Secondary Spermatocyte
tBID	truncated Bid
TNF	Tumor Necrosis Factor gene superfamily
UBA	Ubiquitin-associated domain
VDAC	Voltage-Dependent Anion Channel
XIAP	X-linked IAP

INTRODUCTION

1 Spermatogenesis

1.1 Testicular organization

Testes are the male gonads and are responsible for male sexual hormone production. Human testes are divided into two compartments with distinct functions: tubular compartment and the interstitial compartment, both encased in the tunica albuginea. Spermatogenesis, the process involved in the production of male gametes, takes place in the tubular compartment, representing about 60-80% of the total testicular volume. This compartment consists of seminiferous tubules which originate and terminate at the rete testis, and present two types of somatic cells, peritubular and Sertoli cells. The former compose the myoid peritubular tissue that provides structural support and contains contractile elements capable of generating peristaltic waves that transport the immotile testicular spermatozoa along the tubule to the efferent ducts and the epididymis where final sperm cell maturation occurs. Sertoli cells constitute the main structural element of the seminiferous epithelium, and germ cells maintain an intimate contact with them at all stages of their development. The interstitial compartment represents about 12-15% of the total testicular volume and is composed by the Leydig cells that produce and secrete the most important male sexual hormone, testosterone. The process of male steroid hormones production is named steroidogenesis. The interstitium also contains macrophages, lymphatic channels, blood vessels and dendritic cells. From epididymis, mature spermatozoa are released into the deferent ducts, go through the ejaculator duct and, finally, the urethra (Weinbauer *et al.*, 2010).

Normal integrity and function of both compartments is essential to the normal production of sperm (quantitatively and qualitatively) and occurs in a regulated cyclical manner involving both endocrine and local (autocrine and paracrine) control mechanisms (Shalet, 2009).

1.2 Hormonal regulation of the testicular function

Normal spermatogenesis depends on the normal function of the hypothalamic-pituitary-testicular axis (Figure 1). This reproductive hormonal axis is formed by the hypothalamus, the pituitary gland and the testis, and functions in a strongly regulated manner in order to produce optimal concentrations of circulating male steroid hormones required for normal male sexual development, function and fertility (Weinbauer *et al.*, 2010). The hypothalamus secretes the **gonadotrophin-releasing hormone** (GnRH) that is delivered to the pituitary gland. In turn, the pituitary gland is stimulated to synthesize and secrete the gonadotropins, **luteinising hormone** (LH) and **follicle-stimulating hormone** (FSH) that, when released in circulation, activate receptors on Leydig cells and Sertoli cells, respectively. Upon LH receptor activation, Leydig cells produce testosterone that acts at the level of the Sertoli cells inducing and maintaining spermatogenesis. Contrarily, FSH acts directly on Sertoli cells promoting an increase on their proliferation rate (Shalet, 2009).

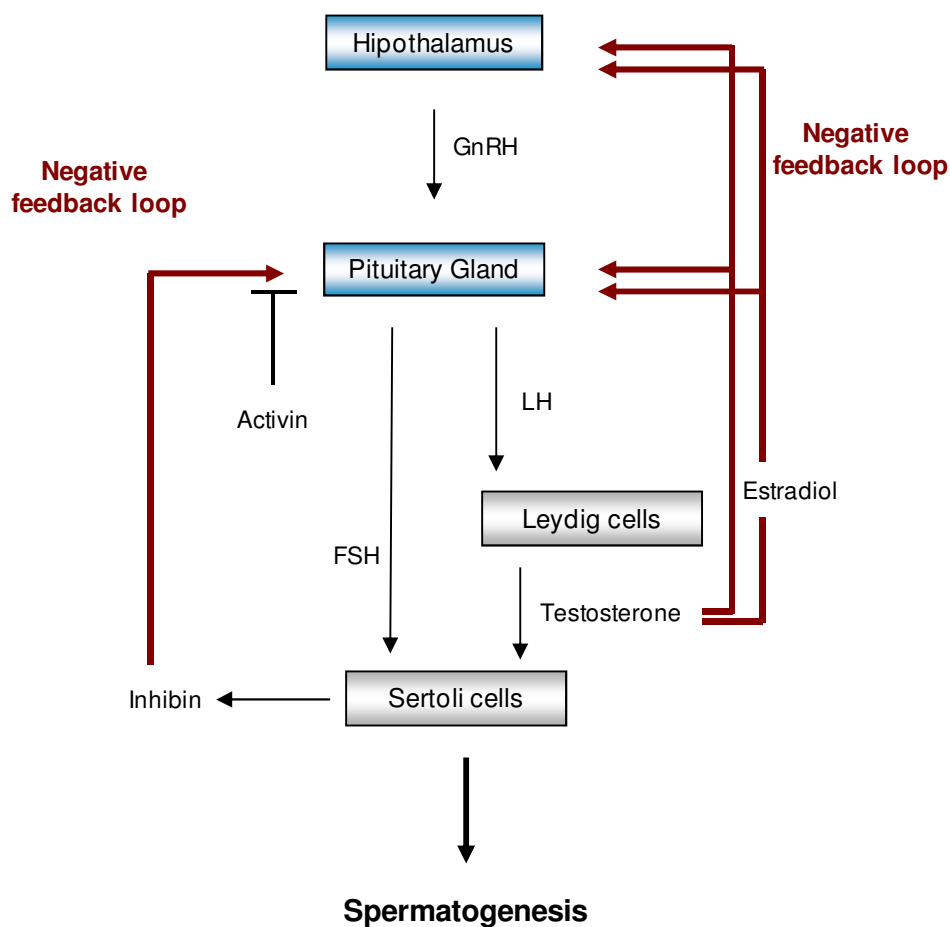


Figure 1. Hypothalamic-pituitary-testicular axis.

The hypothalamic-pituitary-testicular axis functioning is regulated by a negative feedback loop (Figure 1). Testosterone, produced by Leydig cells, regulates LH secretion by interaction with hypothalamus suppressing GnRH release and, consequently, LH release. In the pituitary gland the inhibitory effect, although present, is almost absent. FSH secretion is down regulated by testicular inhibin-B, a protein complex produced by Sertoli cells that acts directly at the pituitary gland (Tilbrook *et al.*, 2001). Inhibin-B mechanism of action may involve receptor-binding competition with activin, a FSH biosynthesis enhancement protein, or binding to specific inhibin receptors that will antagonize activin action (Plant *et al.*, 2001).

Beyond the spermatogenic control by gonadotropins and androgens, estrogens have also been suggested to influence testicular function at the level of Leydig and Sertoli cells and also germ cells. In men, estrogen is derived from aromatase activity, an enzyme that irreversibly transforms androgens in estrogens, in the endoplasmic reticulum of numerous tissues. Estrogen is involved in the negative feedback effect of testosterone on the hypothalamus being essential to a normal balance of the hypothalamic-pituitary-testicular axis (O'Donnell *et al.*, 2001; Carreua *et al.*, 2007).

Beside this endocrine control of spermatogenesis, local interactions between neighboring cells (paracrine regulation) or within the same cell (autocrine regulation) responsible for testicular function regulation have been described. Local factors like growth and immunologic factors, glycoproteins and others seem to modulate hormone activity and intra/intercellular communication between interstitial and tubular compartment, between Sertoli cells and germ cells, and between germ cells (Weinbauer *et al.*, 2010).

1.3 Sertoli Cells and the blood-testis barrier

During development of a functional testis, Sertoli cells play two different roles separated in time and function: testis formation and spermatogenesis. During fetal life, immature and proliferating Sertoli cells enable seminiferous cord formation and prevent germ cell meiosis and differentiation. After puberty, Sertoli cells complete maturation that involves loss of proliferative ability and tight junctions formation between adjacent Sertoli cells. At this time, spermatogenesis begins and the final number of mature spermatozoa is directly related to the number of mature Sertoli cells (Sharpe *et al.*, 2003).

Sertoli cells are located near the basal membrane and extend to the lumen of the seminiferous tubules, giving support to all morphological and physiological modifications that

occur during germ cells differentiation into mature spermatozoa. Sertoli cells are columnar in shape, have long and thin mitochondria and their nuclei exhibit a variety of shapes. Besides giving structural support, Sertoli cells are responsible to nurture developing germ cells, to phagocyte degenerating ones and also residual bodies, and are involved in the release of spermatids during spermiation. So, Sertoli cells are crucial to normal germ cell development (Johnson *et al.*, 2008).

These somatic cells create a unique environment for germ cell development. Between adjacent Sertoli cells exist cytoplasmic extensions connected by junctions that constitute a barrier, the **blood-testis barrier** (BTB). This barrier physically separates the germinal epithelium in two compartments, the basal and the adluminal compartment, and is capable of restricting the passage of larger hydrophilic molecules, particularly proteins, from the systemic circulation into the interstitium (Figure 2).

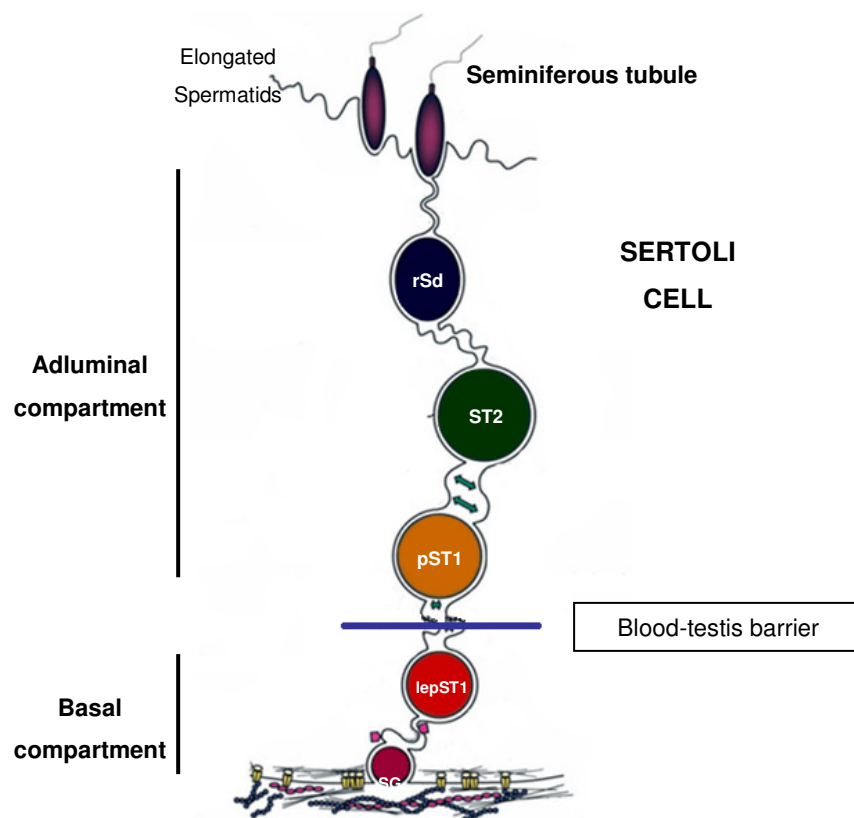


Figure 2. Blood-testis barrier and germinal epithelium compartments. SG - spermatogonia; lepST1 - leptotene primary spermatocytes; pST1 - pachytene primary spermatocytes; ST2 - secondary spermatocytes; rSd - round spermatocytes. Adapted from Mruk *et al.*, 2004.

This limited access, together with the secretory activity of Sertoli cells, ensures a significantly different composition of the tubular fluid from that of the interstitial fluid surrounding the seminiferous epithelium, and creates a unique environment for the developing germ cells (Fijak *et al.*, 2006). Spermatogonia renewal takes place in the basal compartment, additionally with meiotic leptotene and zygotene spermatocytes whereas meiotic pachytene and secondary spermatocytes, haploid spermatids and spermatozoa are located in the adluminal compartment (Figure 2) (Fijak *et al.*, 2006).

One of the main functions of the BTB is to protect the developing germ cells from the immune system. During spermatogonia development into spermatocytes many new surface and intracellular proteins are expressed representing new antigens to be tolerated by the immune system. The BTB isolate meiotic and postmeiotic germ cells from circulating antibodies and leukocytes, preventing an immunologic response (Fijak *et al.*, 2006).

The capability of Sertoli cells to regulate spermatogenesis depends on Sertoli cell number and on their support capacity. During germ cell development and until spermatids are released in the seminiferous tubule lumen, germ cells remain attached to Sertoli cells and extensive interactions occur between them. Once spermatids are released and directed into the epididymis, the BTB immune protection no longer exists. However, tight junctions between the principal cells of the epididymis epithelium form the blood-epididymis barrier creating an immunoprotection in the epididymal lumen, essential for final sperm cell maturation (Cyr *et al.*, 2007; Cornwall, 2009).

1.4 Germ cell development

In cross sections of seminiferous tubules, Sertoli and germ cells are intimately associated. Germ cells are organized in concentric layers with stem cells near the basal membrane and advanced stages of germ cells progressively close to the tubular lumen (Figure 3). Overall, the cycle of the seminiferous epithelium requires 74 days, including the spermatogenic cycle that last 16 days (spermatogonial renewal). During spermatogenesis 4 sequential processes in germ cell development can be observed: mitotic proliferation and differentiation of spermatogonia; meiotic division of spermatocytes; morphological and biochemical modifications of elongated spermatids (spermiogenesis); release of spermatozoa into the tubular lumen (spermiation) (Amann, 2008; Hermo *et al.*, 2010a; Weinbauer *et al.*, 2010).

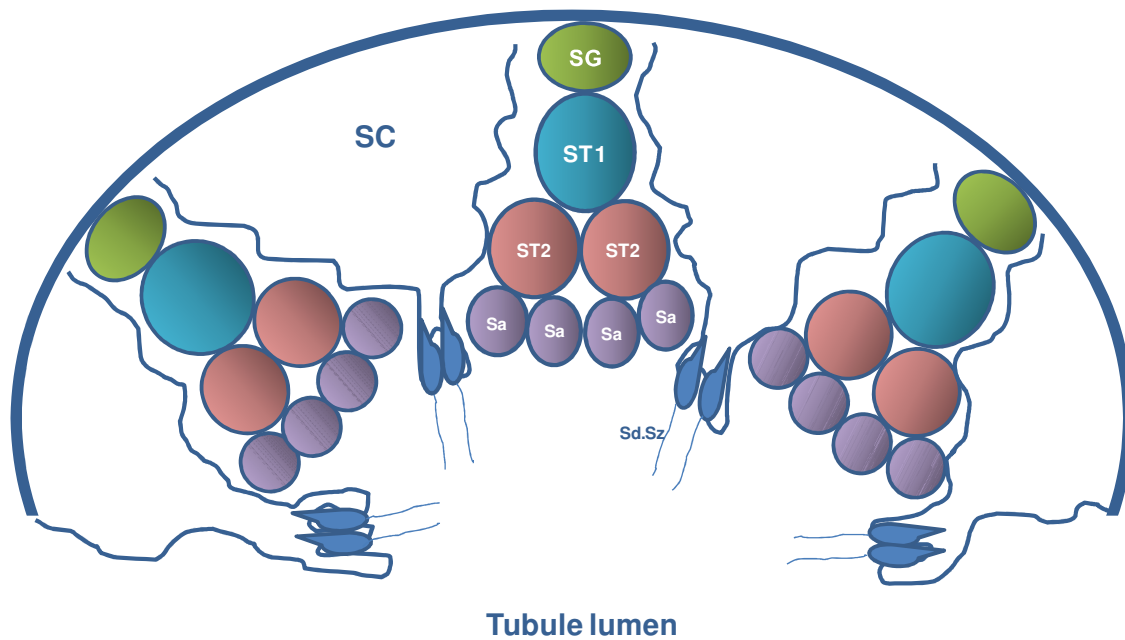


Figure 3. Germinal epithelium. SC - Sertoli cell; SG - spermatogonia; ST1 - primary spermatocyte; ST2 - secondary spermatocyte; Sa - round spermatid; Sd.Sz - elongated spermatids.

1.4.1 Mitotic proliferation and differentiation of spermatogonia

Spermatogonia are localized near the basal membrane of seminiferous epithelium and are classified in type A and type B spermatogonia. According to cell cytology and physiology, there are two types of spermatogonia A: Ad (dark) and Ap (pale). The Ad spermatogonia rarely present proliferation capacity and are considered the testicular stem cells. Only when the overall Ap spermatogonial population shows a drastically reduction in number, Ad spermatogonia divide by mitosis to produce more Ap spermatogonia. On the contrary, Ap spermatogonia show proliferating activity and divide to self renewal or differentiate into two type B spermatogonia. In turn, these divide into two primary spermatocytes beginning the meiotic phase of spermatogenesis.

1.4.2 Meiotic division of spermatocytes

Two sequential meiotic divisions are observed during spermatogenesis. The first division involves DNA replication (leptotene phase), homologue chromosomes synapsis (zygoten phase) followed by crossing-over (pachytene phase). Cell division occurs with DNA content reduction and secondary spermatocytes production. The second meiotic division is faster, without DNA replication, producing the haploid germ cells - round spermatid.

1.4.3 Spermiogenesis

This phase is characterized by structural and functional changes occurring during round spermatids development into elongated spermatids. Major transformations are observed on the cells and nucleus shape, on nuclear chromatin organization and on standard organelles. The Golgi apparatus of early spermatids forms the acrosomal vesicle which is a membrane-bound lysosomal structure formed over the nucleus apical surface and reaches its final shape at the end of spermiogenesis. This structure will be involved in the acrosome reaction at the time of fertilization (Hermo *et al.*, 2010b). During the early stages of spermiogenesis, the chromatin organization of round spermatids in nucleosomes (rich in histones) is disassembled and several modifications occur. During late spermiogenesis the incorporation of protamins allow an increase in the level of chromatin compaction and nucleus condensation (Oliva, 2006). Spermatids become elongated and the flagellum is formed.

1.4.4 Spermiation

This is the final step of spermatogenesis and involves the release of mature elongated spermatids from the Sertoli cells into the tubular lumen. During this phase, the excess of spermatids' cytoplasm (residual body), is extruded and phagocytosed by Sertoli cells. With residual body extrusion, the cytoplasmic droplet is formed. This is localized in the middle piece of the tail, contains vesicular elements from the Golgi apparatus and endoplasmic reticulum and seems to be involved in sperm cells maturation as they move through the epididymal duct. The final morphological and biochemical changes observed during the

maturation process take place within the epididymis and give rise to motile and fertile spermatozoa (Hermo *et al.*, 2010c).

1.5 The human mature spermatozoon

After spermiation, spermatozoa enter the epididymis as non-functioning gametes. The final sperm maturation process occurs during epididymal transit where spermatozoa acquire progressive motility and fertilization capacity (Cornwall, 2009). As mentioned before, during spermiogenesis sperm chromatin is reorganized into a nucleoprotamin complex. In the epididymis, the formation of disulfide bounds in protamins stabilizes this complex and increases the degree of compaction allowing an efficient protection of the paternal genome against external insults (Oliva, 2006; Miller *et al.*, 2010). The final sperm chromatin organization retains 15% of the nucleosome structure and 85% consist of nucleoprotamins. The presence of this histone-bound sperm chromatin have raised the question of whether sperm histones are associated with specific sequences of sperm chromatin or positioned randomly within sperm chromatin, and whether they are transmitted to the developing embryo. Data currently support a model of histone-associated chromatin in specific functional genes for both spermiogenesis and early fertilization (Hammoud *et al.*, 2009; Ward, 2010).

Although epididymal spermatozoa possess a cytoplasmic droplet, it is not present in the normal and mature spermatozoa. Mature spermatozoa are divided in head, mid-piece and tail (Figure 4). The head contains the nucleus with the paternal genome and is covered by the flattened acrosomal vesicle. It is attached to the mid piece where mitochondria are located and produce energy essential to flagellum movement and sperm motility (Cooper *et al.*, 2010).

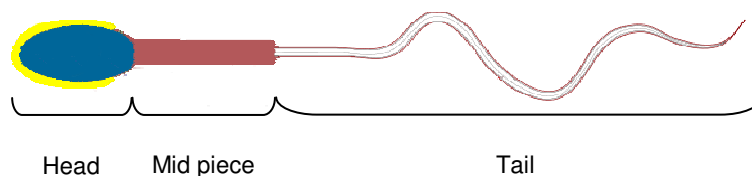


Figure 4. The human mature spermatozoon. Adapted from <http://www.search.com/reference/Spermiogenesis>.

2 Male Infertility

2.1 Evaluation of the infertile men

Infertility is usually defined as the incapability of a couple to conceive after one year of unprotected intercourse. Male factor is contributory in about 40% of the cases, the same as female factor and in 20% both male and female factors are present (Ferraz, 2000b; Gnoth *et al.*, 2005).

The diagnosis of male infertility firstly begins by collection of the male clinical history, physical examination and some complementary laboratory tests should be performed. One of the first tests requested is the spermiogram. Semen analysis, although not a real measure of fertility, can suggest a higher or lower probability of a couple achieving a conception naturally (Ferraz, 2000a; Shefi *et al.*, 2006). Due to the large biological variation, semen analysis should be performed on two or three different samples (Castilla *et al.*, 2006). On table 1 semen parameters routinely evaluated and their normal values are described. When all semen parameters are within normal values, men are classified as normozoospermic (WHO, 2010).

Table 1: Lower reference limits for semen characteristics.

Semen Parameters	Lower reference limit
Semen Volume	1.5 ml
Total sperm number	39 millions per ejaculate
Sperm concentration	15 millions per ml
Total motility (PR + NP)	40%
Progressive motility (PR)	32%
Sperm morphology (normal forms)	4% or 15% (Kruger criteria)
Vitality (live spermatozoa)	58%
<i>Other consensus threshold values</i>	
pH	≥7.2
Peroxidase-positive leukocytes	<1.0 million per ml
MAR test (motile spermatozoa with bound particles)	<50%
Immunobead test (motile spermatozoa with bound beads)	<50%
Seminal zinc	2.4 µmol/ejaculate
Seminal fructose	13 µmol/ejaculate
Seminal neutral glucosidase	20 mU/ejaculate

PR: progressive motility; NP: non-progressive motility. *Adapted from WHO 2010.*

When one or more semen parameters are outside normal values, different terminologies are used according to the pathology (Table 2).

Table 2: Nomenclature related to abnormal semen quality.

Nomenclature	Definition
Oligozoospermia	Total number (or concentration) of spermatozoa below the lower reference limit
Teratozoospermia	Percentage of sperm NM below the lower reference limit
Asthenozoospermia	Percentage of PR below the lower reference limit
Oligoteratozoospermia	Total number (or concentration) of spermatozoa and percentage of sperm NM below the lower reference limits
Oligoasthenozoospermia	Total number (or concentration) of spermatozoa and percentage of PR below the lower reference limits
Asthenoteratozoospermia	Percentages of both PR and sperm NM below the lower reference limits
Oligoasthenoteratozoospermia	Total number (or concentration) of spermatozoa and percentages of both PM and sperm NM below the lower reference limits
Criptoospermia	Spermatozoa absent from fresh preparations but observed in a centrifuged pellet
Necrozoospermia	Low percentages of live and high percentages of immotile spermatozoa in the ejaculate
Azoospermia	Absence of spermatozoa in the ejaculate
Leukospermia	Presence of leukocytes in the ejaculate
Haemospermia	Presence of erythrocytes in the ejaculate
Hipospermia	≤1.5ml of ejaculate volume
Aspermia	Absence of ejaculate

NM: normal morphology, PR: progressive motility. Adapted from WHO 2010.

Additional studies are needed in the presence of an abnormal spermiogram because it suggests a lower probability of achieving a conception. According to the clinical history, physical examination and spermiogram abnormality, the following tests should be performed (Shefi *et al.*, 2006; Costa, 2010):

- Hormonal evaluation: FSH, LH and testosterone levels measurement;
- Genetic Studies (Barros, 2010):
 - Karyotype - infertility in general;
 - Y chromosome microdeletions - deletions on the **azoospermia factor (AZF)** region, either AZFa, AZFb or AZFc [including deletions on the **deleted in azoospermia (DAZ)** gene family], are associated with secretory azoospermia and severe oligozoospermia (<1 million/mL) (Fernandes *et al.*, 2002; Ferrás *et al.*, 2002);

- Sperm chromosome aneuploidies studied by Fluorescence in Situ Hybridization - structural chromosomal abnormality carriers, idiopathic infertility (>3 years), ≥ 2 in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) failures, idiopathic recurrent miscarriages (Carrell, 2008);
 - Sperm DNA fragmentation evaluation - varicocele, prior therapy with chemo/radiotherapy, idiopathic infertility (>3 years), ≥ 2 IVF or ICSI failures, idiopathic recurrent miscarriages (Almeida, 2010; Sakkas *et al.*, 2010);
 - Molecular study of cystic fibrosis transmembrane conductance regulator (*CFTR*) gene: obstructive azoospermia due to congenital bilateral or unilateral absence of the vas deferens (CBAVD or CUAVD, respectively) (Grangeia *et al.*, 2008).
- Sonography: in cases of cryptorchidism and azoospermia (scrotal sonography), obstructive azoospermia suspicion (prostate and seminal vesicles) and unilateral renal vassal agenesis or presence of CBAVD without *CFTR* gene mutations (renal sonography) (Kartik *et al.*, 2010).

2.2 Causes of male infertility

After complete evaluation of the infertile men, the cause of infertility should be defined. However, in about 30-50% of the cases no cause for infertility can be found (idiopathic infertility) (Poongothai *et al.*, 2009; Lopes *et al.*, 2010). In 1% of men in the general population and about 10-15% of infertile men (American Society for Reproductive Medicine, 2008) no spermatozoa are found in the ejaculate - azoospermia. In cases of azoospermia with a non-obstructive cause, namely severe testicular disorders where no spermatozoa are found, histological analysis by biopsy sample is usually performed. However, one or more samples may be not representative of the whole testis and residual spermatogenesis may exist only in some seminiferous tubules. So, additional methods, hormone evaluation and sonography as previously referred, should be used in order to a correct diagnose (Tesarik *et al.*, 1999; Kartik *et al.*, 2010). Genetic disorders like chromosome abnormalities, Y chromosome microdeletions and cystic fibrosis gene mutations are associated with some testicular disorders (Huynh *et al.*, 2002).

According to localization and nature of the cause, azoospermia can be classified in three different categories: pre-testicular, testicular and post-testicular (Ferraz, 2000c; Kartik *et al.*, 2010; Lopes *et al.*, 2010). Pre-testicular causes, also named endocrine causes, are secondary to endocrine abnormalities that adversely affect spermatogenesis (secondary

testicular failure), resulting from hypothalamus/pituitary axis malfunctioning. Testicular causes or non-obstructive azoospermia can be congenital or acquired and are intrinsic to testicular function/spermatogenesis (primary testicular failure). Post-testicular causes are usually due to ducts obstruction (obstructive azoospermia) or ejaculatory dysfunction.

According to the male population studied in the present thesis, only testicular and post-testicular causes of male infertility will be discussed.

2.2.1 Testicular causes

2.2.1.1 Sertoli-Cell-Only Syndrome

Sertoli-cell-only syndrome (SCOS), also called germ cell aplasia, is present in about 30% of infertile men with azoospermia and is the most frequent cause of non-obstructive azoospermia. In this testicular disorder, there are no germ cells in seminiferous epithelium. Only Sertoli cells are observed after testicular biopsy. SCOS males are phenotypically normal, with testes from slightly reduced to normal size. LH and testosterone levels are normal but FSH levels can be increased (but not always) correlating positively with the degree of germ cell aplasia (Ferraz, 2000c; Sousa *et al.*, 2000b; Sousa *et al.*, 2002; Nieschlag *et al.*, 2010).

There are two distinct histological patterns of SCOS: pure or idiopathic and secondary or acquired. Both differ substantially in tubular wall histology, in morphology and function of Sertoli cells and in appearance of the interstitial tissue (Anniballo *et al.*, 2000; Lopes *et al.*, 2010).

In idiopathic SCOS, the total absence of germ cells results from embryonic disturbances where there is no primordial germ cell migration from the yolk sac to the seminiferous cords. It is a congenital disorder where the final destination of the germ cells is not achieved. However, basal membrane is intact, tubules show normal diameter with no histological alteration besides germ cell absence and the presence of numerous Sertoli cells with a normal morphology shape. The interstitial tissue also appears to be normal in cell number and shape (Anniballo *et al.*, 2000).

Secondary SCOS results from a postnatal pathogenic condition that induces progressive germ cells loss. Endogenous and exogenous damage, such as cryptorchidia, orchitis, chemo- and radiotherapy are associated with this condition. However, some tubules

survive these injuries and a focus of germ cells can be found, with the presence of mature elongated spermatids or even spermatozoa. So, when SCOS is diagnosed, more sections of the testicular biopsy should be processed to reduce the risk of sperm retrieval failure and to offer a correct diagnosis. In this SCOS pattern, histological alterations are observed. Basal membrane is altered, usually thickened or oedematous and all tubules show different diameters, though all smaller than normal. Contrarily to the congenital pattern, Sertoli cells are morphologically damaged and functionally compromised (Anniballo *et al.*, 2000).

Genetic abnormalities have been identified in azoospermic men. In about 5-15% of men with spermatogenic failure, Yq chromosome microdeletions were detected being the AZF region described as the genetic Y factor essential for male germ cell development (Fernandes *et al.*, 2006; Poongothai *et al.*, 2009). Deletions on the AZFa region are extremely rare (about 5% of all AZF deletions) and were associated to SCOS and to pure SCOS histological pattern. In secondary SCOS, where germ cells can be found in some seminiferous tubules, deletions on the AZFc region (the most frequent, about 65-70%) were identified suggesting a less severe phenotype (Vogt *et al.*, 1996; Kamp *et al.*, 2001; Ferrás *et al.*, 2004a; Ferrás *et al.*, 2004b). However, more recently, a study in a Chinese population revealed the presence of AZFb/b+c deletions in SCOS patients, suggesting that this massive deletion is also an important genetic cause of SCOS (Yang *et al.*, 2008).

2.2.1.2 Spermatogenic Arrest

Spermatogenic arrest is characterized by spermatogenesis interruption that can occur at the level of spermatogonia, primary and secondary spermatocytes or round spermatids. This testicular disorder is congenital and is present in about 4-30% of infertile men that perform a testicular biopsy. Physical evaluation is normal, including testicular volume and a complete normal diameter of seminiferous tubules is observed. FSH, LH, testosterone and inhibin B levels are normal, however, FSH and inhibin B may be also elevated or decreased, respectively (Sousa *et al.*, 2000b; Sousa *et al.*, 2002; Nieschlag *et al.*, 2010). It has also been described some partial arrest characterized by a decline in germ cell progression across meiosis but still with few elongated spermatids, a condition referred by some as an hypospermatogenesis variant (McLachlan *et al.*, 2007). In these cases of partial arrest, varied conditions of oligoasthenoteratozoospermia can also be observed, but the sperm production can be so low that spermatozoa is absent from the ejaculate and sperm retrieval may be only achieved by testicular biopsy (Nieschlag *et al.*, 2010).

Genetic causes of spermatogenic arrest are associated to Yq chromosome microdeletions on the AZFb region. However, secondary factors like radio and chemotherapy, heat or general diseases may also be on the origin of this testicular disorder (Ferrás *et al.*, 2004b; Costa *et al.*, 2008; Poongothai *et al.*, 2009; Nieschlag *et al.*, 2010).

2.2.1.3 Hypospermatogenesis

All stages of spermatogenesis are present but reduced in number. Tubules with incomplete cellular composition can also be found. There is often thickening of tunica propria, interstitial fibrosis and germ cells disorganization (McLachlan *et al.*, 2007; Nistal *et al.*, 2008). Two hypospermatogenesis variants quantitatively different can be found: pure hypospermatogenesis and hypospermatogenesis associated with degenerating spermatocytes. In pure hypospermatogenesis the number of primary spermatocytes is equal or higher than the number of spermatogonia; the number of round spermatids is higher than primary spermatocytes and the number of elongated spermatids is similar to spermatogonia. The second hypospermatogenesis variant is characterized by low number of spermatogonia and primary spermatocytes (but primary spermatocytes are more numerous than spermatogonia) and by many degenerating primary spermatocytes. The remaining ones give rise to the few spermatids observed in the seminiferous tubules from this disorder (Nistal *et al.*, 2008).

Genetically, hypospermatogenesis is associated to Yq chromosome microdeletions on the AZFc region (Fernandes *et al.*, 2002; Fernandes *et al.*, 2004; Vogt, 2004).

2.2.2 Post-testicular causes

2.2.2.1 Ducts obstruction (or absence)

Obstructive azoospermia is characterized by the total absence of spermatozoa and spermatogenic cells in the semen due to bilateral obstruction of the seminal ducts. It is a condition that occurs in about 15-20% of men with azoospermia. Men with obstructive azoospermia present normal testes size, normal FSH and conserved spermatogenesis. Obstruction can occur at the epididymal, vas deferens or ejaculatory duct levels and can be

congenital (primary obstruction) or acquired (secondary obstruction) (Dohle *et al.*, 2010b; Lopes *et al.*, 2010).

Epididymal obstruction is the most common cause of obstructive azoospermia (30-67%). In the congenital form, the distal part of the epididymis is absent and is usually accompanied by seminal vesicles agenesis. Acquired forms may occur after infections (epididymitis) or after epididymal surgery (cysts removal) (Dohle *et al.*, 2010b).

The most common form of congenital **obstruction at the vas deferens level** is the **congenital bilateral absence of the vas deferens (CBAVD)** and represents 6% of men with obstructive azoospermia (Sousa *et al.*, 2000a). These men frequently have epididymal malformations, vas deferens absence and absent or hypotrophic seminal vesicles. Renal anomalies can also be observed. Apart from being azoospermic, semen pH is acid and present abnormal volume values. CBAVD is often accompanied by cystic fibrosis (Quallich, 2006; Lopes *et al.*, 2010). Cystic fibrosis is an autosomal recessive disease common in Caucasian populations. The gene responsible for cystic fibrosis is **cystic fibrosis transmembrane conductance regulator (CFTR)** and is located on the short arm of chromosome 7. Cystic fibrosis phenotype is due to the presence of two severe *CFTR* gene mutations and more than 95% of men with cystic fibrosis have obstructive azoospermia due to CBAVD (Grangeia *et al.*, 2008). However, *CFTR* gene mutations are also the main cause of CBAVD in infertile men without any other cystic fibrosis phenotype. The most common genotype of men with CBAVD is heterozygosity for a severe and a mild mutation. However, 10-20% of CBAVD cases are not due to *CFTR* gene mutations. This subset of CBAVD patients with no *CFTR* gene mutation is associated with renal abnormalities (Sousa *et al.*, 2000a; Grangeia *et al.*, 2008; Behre *et al.*, 2010). Men with **congenital unilateral absence of the vas deferens (CUAVD)** are usually fertile. These men present normal seminal ducts and no *CFTR* gene mutations. However, they exhibit unilateral renal agenesis. Other CAUVD patients have *CFTR* gene mutations similar to CBAVD, no renal abnormalities, but exhibit contralateral obstruction of the seminal ducts making them azoospermic (Behre *et al.*, 2010; Dohle *et al.*, 2010b, 2010a).

Acquired vas deferens obstruction is secondary to vasectomy for sterilization or to surgery. The main difference for CBAVD is the vas deferens presence during physical examination (Dohle *et al.*, 2010b).

Ejaculatory duct obstruction is a congenital or acquired pathological condition found in about 1-3% of obstructive azoospermia that can affect one or both ejaculatory ducts (Dohle *et al.*, 2010b). Men with suspected ejaculatory duct obstruction have normal physical examination, including normal testes and palpable vas deferents, and normal hormone

profiles. In the presence of bilateral obstruction, only prostatic fluids contribute to the final ejaculate that presents decreased volume ($< 1.5\text{ml}$), increased acidity, absent seminal fructose, azoospermia and the seminal vesicles are usually, but not always, dilated. In partial obstructions, symptoms are less pronounced. Semen analysis can reveal decreased fructose levels, oligozoospermia and asthenozoospermia and, in some cases, semen analysis can approach normal parameters, maintaining only reduced sperm motility. However, partial obstructions can progress to complete obstructions. Congenital obstructions include Mullerian and Wolffian ducts cysts or atresia. Acquired causes may be secondary to trauma, infections or inflammations (Fisch *et al.*, 2006).

2.2.2.2 Ejaculatory disorders

Ejaculatory dysfunctions are uncommon and usually related to absence of ejaculation or semen volume lower than 1ml (Lopes *et al.*, 2010). Ejaculatory disorders are divided into 4 categories: anejaculation, retrograde ejaculation, delayed ejaculation and premature ejaculation. These dysfunctions can have anatomical or functional causes, can be congenital or result from psychological, neurological and endocrinological factors or can even be drug-related (Wolters *et al.*, 2006).

Anejaculation is characterized by the total absence of ejaculate and is caused by failure of semen emission from the seminal vesicles, the prostate and the ejaculatory ducts into the urethra. Anejaculation may be caused by acquired or congenital dysfunctions of the central or peripheral nervous system, like spinal cord injury and multiple sclerosis, respectively, or can be drug related. **Retrograde ejaculation** is the most common cause of ejaculatory dysfunction. Consist in the semen propulsion from the posterior urethra into the bladder. Retrograde ejaculation can be complete, with no antegrade ejaculation, or incomplete, with minimal antegrade emission. The causes can be surgical, neurogenic, urethral, bladder neck incompetence or even drug-related (Kamischke *et al.*, 1999; Dohle *et al.*, 2010c). **Delayed ejaculation** can result from psychological problems, organic lesions or drug related. **Premature ejaculation**, the inability to control time of ejaculation, is also associated to psychological or organic (e.g. prostatitis, hyperthyroidism) factors, but does not impair fertility (Dohle *et al.*, 2010c).

3 Apoptosis

3.1 Morphological and biochemical features of apoptosis

Apoptosis or programmed cell death is a mechanism of cell death that involves genetically determined and regulated elimination of cells in response to specific stimuli, either internal (e.g. DNA and organelle lesion) or external (e.g. trauma, radiation, growth factor withdrawal). A balance between apoptosis and cell proliferation is essential for tissue homeostasis and development. Dysregulation of this balance leads to proliferative (e.g. cancer) or degenerative (e.g. Alzheimer) diseases. Apoptotic cells are characterized by a distinct pattern of morphological features, first described in 1972 (Kerr *et al.*, 1972).

Morphological changes include cell shrinkage, chromatin condensation (pyknosis) and margination, plasma membrane blebbing followed by nucleus fragmentation (karyorrhexis) and formation of apoptotic bodies by a process named “budding” (Figure 5). These membrane surrounded structures consist of cellular fragments with organelles and nuclear fragments, which are quickly recognized due to plasma membrane structure changes, and phagocytosed. This process of recognition and digestion of the apoptotic bodies prevents the release of their cellular constitution into the surrounding tissue avoiding an inflammatory response (Saraste *et al.*, 2000; Elmore, 2007; Al-Rubeai *et al.*, 2009).

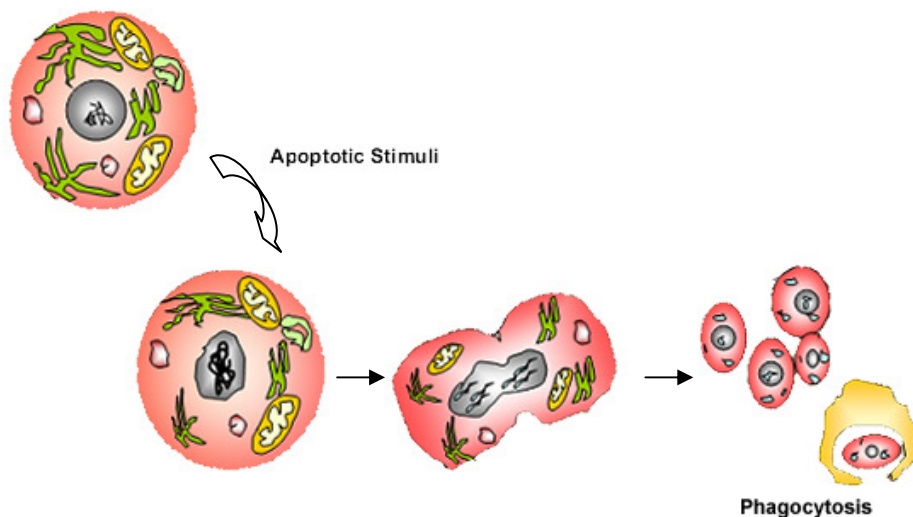


Figure 5. Morphological modifications in an apoptotic cell. Adapted from Rastogi *et al.*, 2009.

Changes in nuclear morphology coincide with the activation of endogenous DNases, like caspase-activated DNase (CAD) (Enari *et al.*, 1998), that is responsible for internucleosomal DNA fragmentation into double-stranded DNA fragments of 200 bp. This biochemical feature of apoptosis, detectable as a DNA ladder in electrophoresis, represents one of the hallmarks of apoptosis. Other apoptosis biochemical feature is the activation of proteases named caspases, which are going to initiate and execute the final cell disassembly (Saraste *et al.*, 2000).

3.1.1 Phosphatidylserine (PS) exposure

Viable cells present an asymmetric lipid distribution between the inner and the outer leaflets of the plasma membrane. The extracellular leaflet contains essentially choline-containing lipids, phosphatidylcholine and sphingomyelin, and the cytoplasmic leaflet contains amine-containing glycerophospholipids, as phosphatidylethanolamine, phosphatidylserine (PS) and phosphatidylinositol. However, during apoptosis plasma membrane asymmetry is lost due to PS translocation to the external leaflet. This exposure is involved in apoptotic cell recognition by macrophages and consequent engulfment without any inflammatory reaction (Daleke, 2003; van Meer *et al.*, 2008). This plasma membrane structure change occurs in an Mg^{2+} /ATP dependent manner, and under specific stimuli. In normal cells, plasma membrane asymmetry is maintained by the aminophospholipid translocase or flippase. However, in the presence of Ca^{2+} the translocase activity is inhibited and a non-specific bidirectional protein named scramblase is activated promoting the rapid PS translocation (Figure 6). So, in response to apoptotic stimuli, scramblase plays an important role in plasma membrane reorganization and apoptotic cells clearance by macrophages (Schlegel *et al.*, 2001; Daleke, 2003).

Apoptotic PS exposure can be detected using the annexin V-affinity assay due to the PS binding properties of annexin V in the presence of Ca^{2+} . As plasma membrane in normal and vital cells is impermeable and annexin V cannot penetrate the phospholipid bilayer, only upon PS exposure annexin V binding occur (van Engeland *et al.*, 1997). Loss of membrane asymmetry is an event of the apoptotic process initiated early during the execution pathway after effector caspases activation, until the final stage of apoptotic bodies formation (Naito *et al.*, 1997).

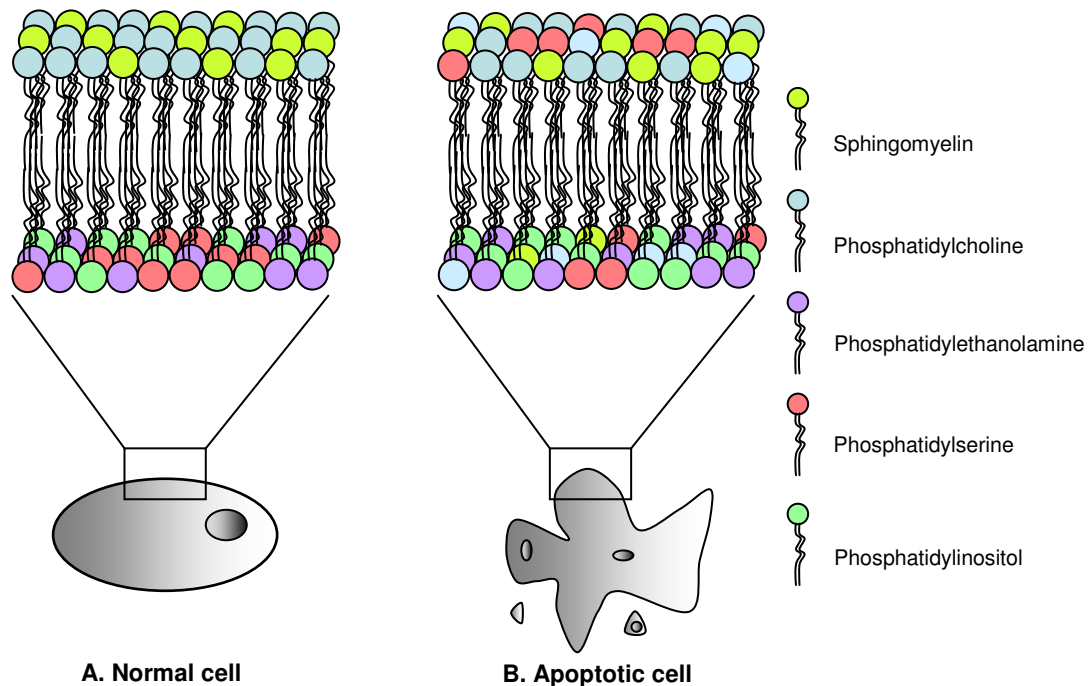


Figure 6. Plasma membrane asymmetry loss. A: Normal cell with plasma membrane asymmetry; B: Loss of plasma membrane asymmetry due to phosphatidylserine translocation to the extracellular leaflet. Adapted from <https://www.roche-applied-science.com>.

3.1.2 DNA fragmentation

The biochemical hallmark of apoptosis is the DNA fragmentation in oligonucleosomal fragments of about 200 bp. Two steps are involved in this event: firstly, there is an initial cleavage of DNA in long fragments of 50-300 kb and secondly the oligonucleosomal fragments of 200 bp are formed. Both cleavages are catalysed by the apoptotic nuclease **DNA fragmentation factor 40** (DFF40) (40kDa subunit from the DFF protein), also named **caspase-activated DNase** (CAD) in mouse models (Liu *et al.*, 1998; Widlak *et al.*, 2009). In normal healthy cells this magnesium-dependent nuclease is inactive in the nucleus due to association with its inhibitor protein, the 45kDa subunit (DFF45). Upon an apoptotic stimulus, apoptotic mechanisms are activated with consequent DFF45 cleavage and activation of DFF40 through its own homo-oligomerization. In the active form, DFF40 is able of binding DNA. Association with chromosomal proteins, like Histone H1 and topoisomerase II, allows the efficient cleavage in oligonucleosomes (Liu *et al.*, 1999). Further apoptotic DNA degradation seems to be performed by other apoptotic nucleases, like **endonuclease G** (Endo G) that is released from mitochondria and translocated to the nucleus. All these events

leading to DNA fragmentation are under apoptosis-inducing factor (AIF) action, a mitochondrial protein that upon apoptotic stimuli is released from mitochondria and translocates to the nucleus where cooperates with Endo G in a DNA degradation complex. Additionally, DNase II from phagocytes, a lysosomal acid DNase, seems to be involved in the final DNA degradation after apoptotic cells engulfment. DNA fragmentation is essential to complete apoptotic cell disassembly and clearance. Fragmentation failure can induce immune responses and development of autoimmune disorders (Parrish *et al.*, 2006).

3.2 Caspases

3.2.1 Structure and classification

The apoptotic cascade is a synchronized event that involves complex biochemical interactions between distinct protein families. The most important one, responsible for initiation and execution of apoptosis, is the protein family of caspases (cysteine-specific proteases). These are cysteine proteases that cleave specifically after aspartate (Asp) residues. Fourteen caspases were identified in mammals, 11 in humans. From these at least 7 have a known apoptotic role and the other 4 belong to the ICE (interleukin-1 β converting enzyme) subfamily (Nicholson, 1999; Riedl *et al.*, 2004). Functionally, human caspases can be divided in initiators (caspases-2, -8, -9 and -10), effectors (caspases-3, -6 and -7) and inflammatory mediated (caspases-1, -4, -5 and -13). The initiator caspases are responsible for starting the biochemical apoptotic cascade upon either intrinsic (intrinsic pathway) or extrinsic (extrinsic pathway) apoptotic factors. Effector caspases cleave proteins involved in DNA repair processes or cytoplasmic and nuclear proteins whose degradation will lead to the final cell disassembly. Inflammatory caspases mediate inflammatory reactions and show no apoptotic role (Riedl *et al.*, 2004).

Caspases are synthesized as inactive proenzymes, also named zymogens, and must undergo proteolytic activation in order to become functionally active. These procaspases are organized in an N-terminal prodomain followed by a large (~20 kDa) and a small (~10 kDa) subunits (Figure 7). The prodomain size is associated with caspase function. Effector caspases have short prodomains (20-30 aminoacid residues) and are not involved in protein interactions. Long prodomains (>90 aminoacid residues) are characteristic of initiator caspases and are involved in protein interactions, including caspase activation. Procaspases-8 and -10 show two death effector domains (DED) and procaspases-2 and -9 one caspase recruitment domain (CARD) (Earnshaw *et al.*, 1999; Fuentes-Prior *et al.*, 2004).

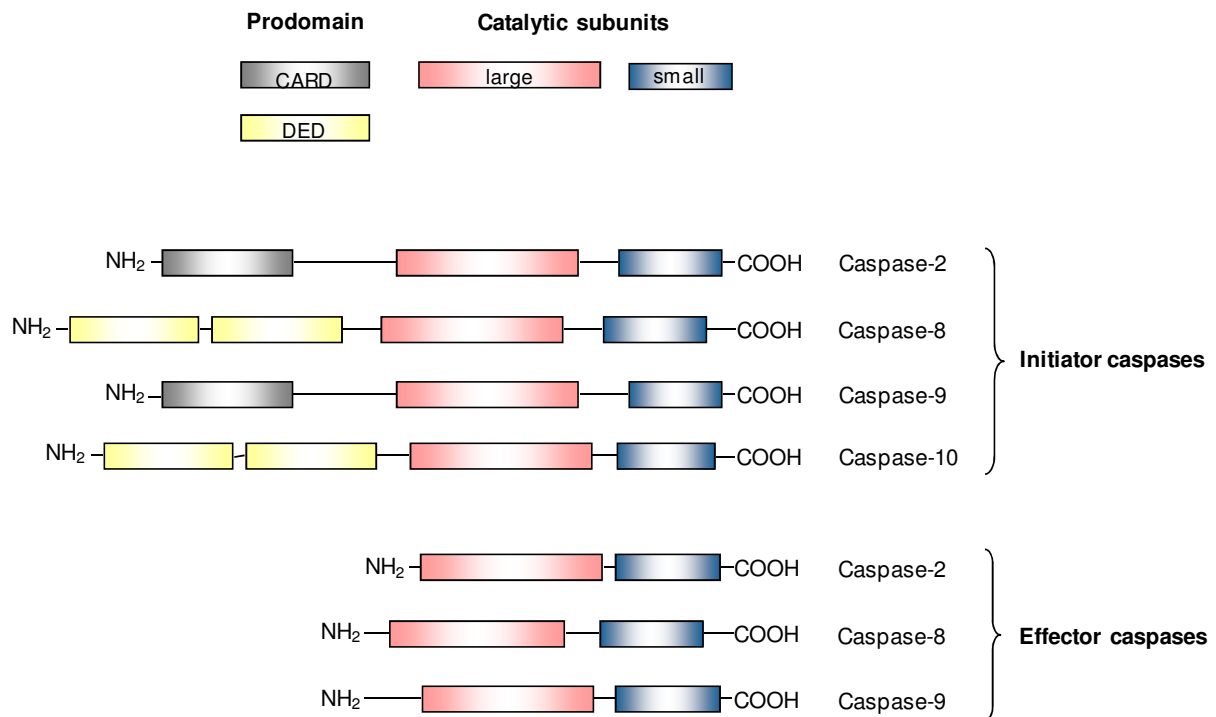


Figure 7. Caspases involved in apoptotic signaling pathways: classification based on structure and function. CARD - caspase recruitment domain; DED - death effector domain. Adapted from Grunewald *et al.*, 2009c.

3.2.2 Caspase activation and substrate recognition

Caspase activation usually requires two cleavages at Asp residues, first between the large and small subunits and a second cleavage between the prodomain and the large subunit. An active caspase is formed by the association of two caspase monomers (two small and two large subunits) with a heterodimer formation (Figure 8). The final enzyme presents a tetrameric structure with two active sites at opposite ends (Rastogi *et al.*, 2009).

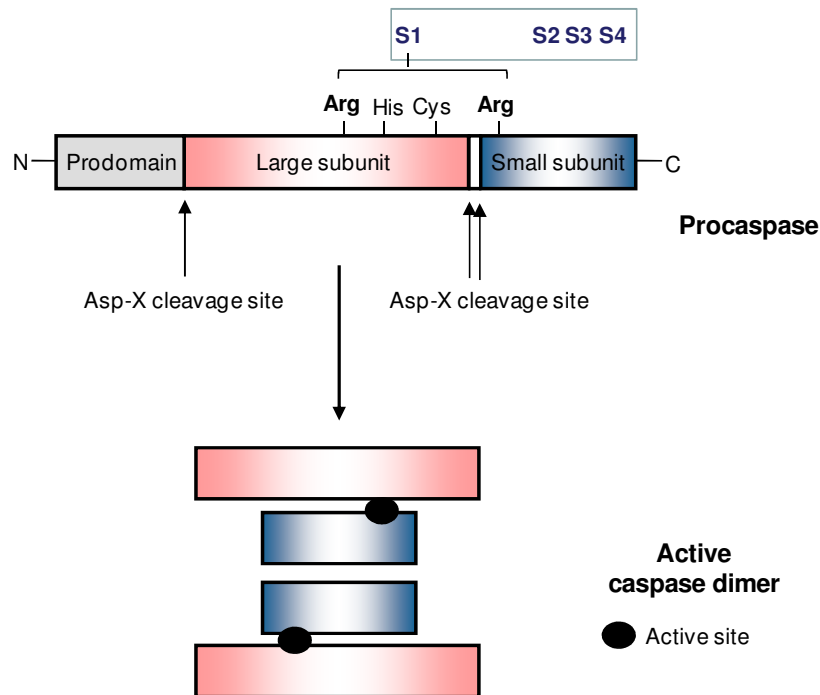


Figure 8. Proteolytic caspase activation. Adapted from Grütter, 2000.

Two ways of caspase activation have been proposed: proteolysis by other caspases or autoproteolysis. Effector procaspases exist within the cytoplasm as inactive dimers and activation is performed by initiator caspases. This way of activation, where caspases activate other caspases originates a protease cascade and leads to the apoptotic signal pathway amplification (Fuentes-Prior *et al.*, 2004; Rastogi *et al.*, 2009). Initiator caspases activation occurs through an autoproteolytic mechanism that follows the proximity-induced dimerization model (Shi, 2004). This model states that initiator caspases precursors exist in the cell as monomers, at low concentrations and in a conformation that prevents autocatalysis. They present some low catalytic activity and high concentration of monomers favors activation. So, when brought into close proximity of each other, initiator procaspases undergo dimerization and autoprocess themselves. In this model the presence of cofactors is essential. These cofactors are adaptor proteins that besides allowing procaspases conformational change, act also promoting dimerization by increasing the local concentration of caspases precursors. So, activation of initiator procaspases can be described as a cofactor-induced dimerization of monomeric zymogens (Bao *et al.*, 2007).

The active caspase presents four residues (S4-S3-S2-S1) that recognize a tetrapeptide sequence motif (P4-P3-P2-P1) in their substrates (Figures 8 and 9). The aminoacids

cysteine (Cys) and **histidine** (His), components of the active site, are harboured within the large subunit, the S1 subsite with two **arginine** (Arg) residues is located at the interface between the large and the small subunits, whereas the S2, S3 and S4 subsites are only from the small subunit. So, both subunits determine the active site (Nicholson, 1999; Riedl *et al.*, 2004; Rastogi *et al.*, 2009). Caspases cleave after the C-terminal residue (P1) requiring specifically an Asp in this position but are tolerant to aminoacid substitutions at the P2 and P3 subsites, although prefer His or **isoleucine** (Ile) in P2 and **glutamic acid** (Glu) in P3. However, the S4 subsite composition is critical and will determine the caspase substrate specificity (Nicholson, 1999; Fuentes-Prior *et al.*, 2004).

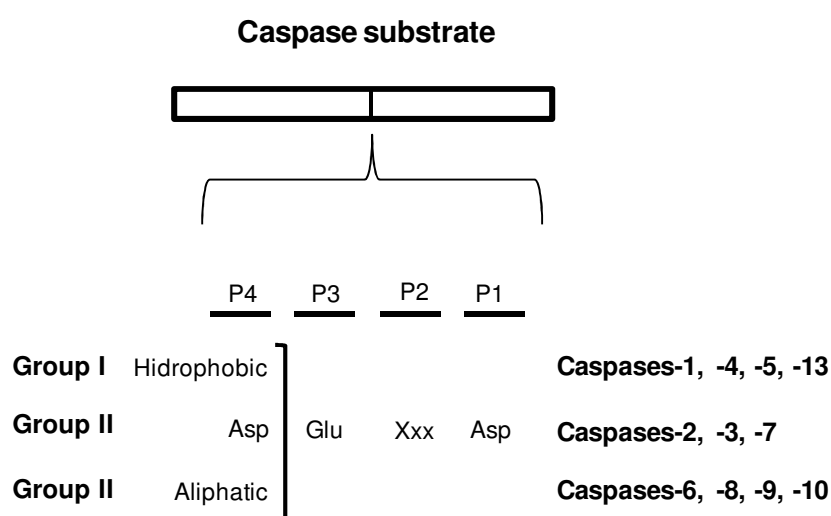


Figure 9. Caspase proteolytic specificity. Adapted from Nicholson, 1999.

According to substrate specificity, caspases can be subdivided into three groups (Figure 9). Group I (caspases-1, -4, -5 and -13) is tolerant for P4 substitutions. Group II (caspases-2, -3 and -7) is more stringent in S4 and require an Asp residue at P4. This aminoacid motif is found in many proteins cleaved during apoptosis, being consistent with the functions of effectors caspases. The other group of caspases, group III (caspases-6, -8, -9 and -10), prefer branched chain aliphatic aminoacids in P4, characteristic of the mature site of the initiator caspases. An exception in this catalytic structure is found for caspase-2 that requires also a S5 subsite for efficient substrate cleavage (Nicholson, 1999; Fuentes-Prior *et al.*, 2004).

3.3 Apoptosis Regulation

3.3.1 The BCL-2 protein family

Regulation of the apoptotic cascade is mediated by protein members of the **B-cell lymphoma protein 2 (BCL-2)** family. This family of proteins can be functionally divided in pro-apoptotic as anti-apoptotic (pro-survival) members. The relative expression of both is going to dictate cell fate. An increased expression of pro-apoptotic proteins will induce cell death by apoptosis. However, and on the contrary, an increased expression of anti-apoptotic proteins will induce cell proliferation, a situation observed in cases of neoplasias (Chipuk *et al.*, 2010).

Bcl-2 family protein members present conserved regions named **BCL-2 homology (BH)** domains. Structurally, proteins of this family can be divided according to the number of BH domains (Figure 10, van Delft *et al.*, 2006).

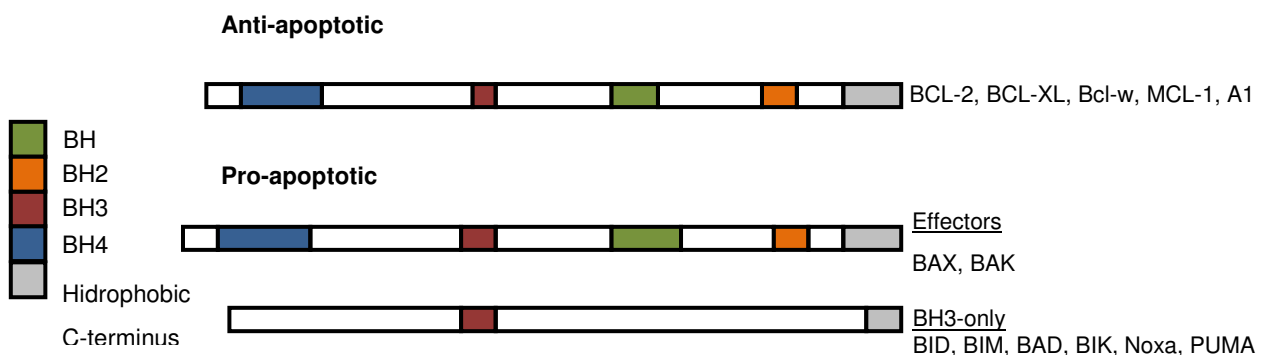


Figure 10. Bcl-2 family protein members. Adapted from van Delft *et al.*, 2006.

Anti-apoptotic members share four BH domains (BH1-4) and include BCL-2, BCL-2 like 2 protein (Bcl-w), BCL-2-related gene - long isoform (BCL-XL), BCL-2-related gene **A1** (A1) and **myeloid cell leukemia 1** (MCL-1). Pro-apoptotic members can be divided into two subfamilies: effector proteins and BH3-only proteins (Figure 10). Effector proteins (**BCL-2 associated X** protein - BAX and **BCL-2 antagonist/killer 1** - BAK) present also four BH domains and are required downstream to BH3-only proteins to induce apoptosis in response to BH3-only proteins stimulation. BH3-only proteins present only a BH3 domain and are divided according to their ability to bind only anti-apoptotic proteins (sensitizers/de-repressors BH3-only proteins) or either anti-apoptotic and effector BCL-2 family members (direct activators). Direct activators are capable of directly induce effectors oligomerization

and function and include **BCL-2-interacting mediator of cell death (BIM)** and **BCL-2-interacting domain death agonist (BID)**. Sensitizers/de-repressors BH3-only proteins promote indirect BAX/BAK activation through anti-apoptotic activity inhibition of the anti-apoptotic members. **BCL-2 antagonist of cell death (BAD)**, **BCL-2 interacting killer (BIK)**, **p53-upregulated modulator of apoptosis (PUMA)** and **PMA-induced protein 1 (Noxa)** are examples of members from this family (Chipuk *et al.*, 2010; Ghiotto *et al.*, 2010).

Interactions between the anti-apoptotic members and direct or indirect effector pro-apoptotic proteins activation, determine mitochondrial outer membrane permeabilization with consequent apoptotic intrinsic signalling cascade (Chipuk *et al.*, 2008).

3.3.2 IAP family members

The caspase cascade can be negative regulated by a family of proteins named inhibitors of apoptosis proteins (IAPs). There are eight mammalian IAPs: **X-linked IAP (XIAP)**, **cellular IAP1 (c-IAP1)**, **cellular IAP2 (c-IAP2)**, **melanoma IAP/Livin (ML-IAP/Livin)**, **IAP-like protein 2 (ILP-2)**, **neuronal apoptosis-inhibitory protein (NAIP)**, **BIR repeat-containing ubiquitin conjugating enzyme system (BRUCE)** and **Survivin (Wei *et al.*, 2008)**. All members of IAP family proteins contain **baculovirus IAP repeat (BIR)** domains at the N-terminus, and some also contain an **ubiquitin-associated domain (UBA)** following the last BIR domain, a **really interesting new gene (RING)** finger domain and a **CARD** domain near the C-terminus (Figure 11). The BIR domain is essential for caspase inhibition. The mechanism of inhibition relies on a tetrapeptid IAP-binding motif of the BIR domain, complementary to the tetrapeptid motif sequence of caspases active site. So, BIR domain binding to the caspase active site induces caspase inhibition, either by promoting active site degradation or by sequestering caspases away from their substrates (Tenev *et al.*, 2005; Mace *et al.*, 2010).

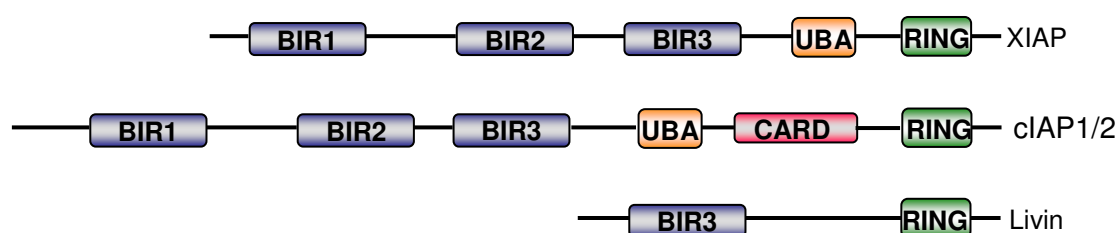


Figure 11. Members of the IAP protein family. Adapted from Mace *et al.*, 2010.

Only effector caspases-3 and -7 and initiator caspase-2 and -9 are affected by IAPs. XIAP, c-IAP1 and c-IAP2 contain three BIR domains (BIR1-BIR2-BIR3), each with a distinct function. The third BIR domain (BIR3) of XIAP interacts with processed caspase-9 (Shiozaki *et al.*, 2003) whereas the linker region between BIR1 and BIR2 is responsible for caspases-3 and -7 inhibition (Huang *et al.*, 2001). C-IAP1 and c-IAP2 can also bind caspases-3, -7 and -9, but with lower affinity than XIAP. NAIP, besides its inhibitory activity against caspases-3 and -7, like XIAP, inhibits caspase-9 activity through interaction with the BIR3 domain. ML-IAP/Livin, ILP2 and Bruce contain only one BIR domain and are weaker caspases inhibitors. In case of survivin, caspase inhibitory function was not shown to exist in vitro (Wei *et al.*, 2008).

IAPs anti-apoptotic function can be also regulated. **Second mitochondria-derived activator of caspase/direct IAP binding protein with a low pI** (Smac/DIABLO) and **high-temperature-regulated A2/Omi** (HtrA2/Omi) are pro-apoptotic proteins with a **IAP binding motif** (IBM) that interact with IAPs releasing caspases and promoting the apoptotic signalling cascade. Smac/DIABLO, however, is activated as a homodimer and presents two IBM. These IAP antagonists bind to XIAP BIR3 and BIR2 domains, competing with caspase-9 and caspase-3 binding, respectively (Mace *et al.*, 2010).

Although IAP family proteins are incapable of interacting with the initiator caspase-8, cellular **FADD-like ICE** (FLICE)-inhibitory proteins (c-FLIPs) binds to caspase-8 through DED interactions, and blocks caspase-8 recruitment to the activation platform. FLIP has a longer (FLIP-L) and a shorter (FLIP-S) isoforms. FLIP-S only shows caspase inhibition activity but FLIP-L can function as either a caspase inhibitor or activator. This dual function, in apoptosis or proliferation, depends on FLIP cellular levels. So, in order to the apoptotic function prevail, high levels of FLIP-L are needed to be present (Hyer *et al.*, 2006).

3.4 Apoptotic pathways

The apoptotic signaling cascade can be triggered through specific stimuli directed either to the cell surface where transmembrane receptors initiate the apoptotic cascade or to internal organelles, as mitochondria, where specific damage initiates biochemical transformations that will conduct the apoptotic events. In both pathways (extrinsic and intrinsic pathways, respectively) the cleavage of vital proteins by effector caspases execute the cell death (execution pathway). However, it is also important to refer that the apoptotic intrinsic pathway can also occur in a caspase-independent manner (Elmore, 2007).

3.4.1 Intrinsic pathway - Mitochondrial damage

Mitochondria play a central role in apoptosis. Several pro-apoptotic signals originated in other organelles (e.g. nucleus, ER, lysosomes) induce mitochondria alterations with consequent apoptotic cascade induction (Kroemer *et al.*, 2007).

In normal cells, the proton gradient formed across the inner mitochondrial membrane (IMM) is the basis of the mitochondrial transmembrane potential ($\Delta\Psi_m$) essential to oxidative phosphorylation with ATP production. During apoptosis $\Delta\Psi_m$ is lost, IMM integrity is disrupted and mitochondrial outer membrane permeabilization (MOMP) is observed (Grimm *et al.*, 2007). Consequently, the pro-apoptotic proteins cytochrome c, Smac/DIABLO and the serine protease HtrA2/Omi involved in the caspase-dependent pathway, AIF and Endo G involved in the caspase-independent pathway, are released from the mitochondrial intermembrane space to the cytosol (Figure 12) (Elmore, 2007). The MOMP was first reported to be associated to the opening of the voltage-dependent anion channel (VDAC), the most abundant protein of the outer mitochondrial membrane (OMM) that allows permeability to small metabolites and solutes up to ~5kDa (Peixoto *et al.*, 2010). Together with the adenosine nucleotide translocator (ANT) located on the IMM, VDAC is responsible for ATP and ADP transport through mitochondria. However, it was recently discovered that VDAC is a subunit of a supramolecular pore, the permeability transition pore (PTP) that also comprises the ANT in the IMM (Peixoto *et al.*, 2010; Pradelli *et al.*, 2010). So, PTP covers both IMM and OMM. Activation of PTP is associated to oxidative stress and increased Ca^{2+} levels and leads to IMM permeability increase, matrix swelling and OMM permeabilization and disruption, with release of the mitochondrial intermembrane space proapoptotic proteins to the cytosol (Grimm *et al.*, 2007).

All these events leading to $\Delta\Psi_m$ loss are modulated by proteins of the BCL-2 family (Jourdain *et al.*, 2009; Pradelli *et al.*, 2010). The BH3-domain of the pro-apoptotic proteins BAX and BAK target them to the membrane of different organelles, like endoplasmic reticulum, mitochondria and nucleus. BAX exist in the soluble form in the cytoplasm whereas BAK, in mitochondria, is bounded to the OMM. Upon pro-apoptotic stimuli, BH3-only proteins (e.g. BID, BAD and BIM) bind to BAX and BAK, direct BAX to the OMM where BAX and BAK oligomerize exerting a permeabilization effect. BH3-only proteins can also exert an indirect effect, binding to anti-apoptotic members such as BCL-XL and BCL-2 neutralizing them. In this case, BAX and BAK become free from their anti-apoptotic counterparts in order to oligomerize and induce $\Delta\Psi_m$ loss, either by direct pore formation on the OMM or simply by lipid bilayer destabilization (Jourdain *et al.*, 2009). However, interaction of BAX and BAK with

proteins from the PTP such as VDAC and ANT can also occur. Depending on cell type and apoptotic signal, these two mechanisms of $\Delta\Psi_m$ loss, either with IMM involvement or not, can occur simultaneously (Kroemer *et al.*, 2007).

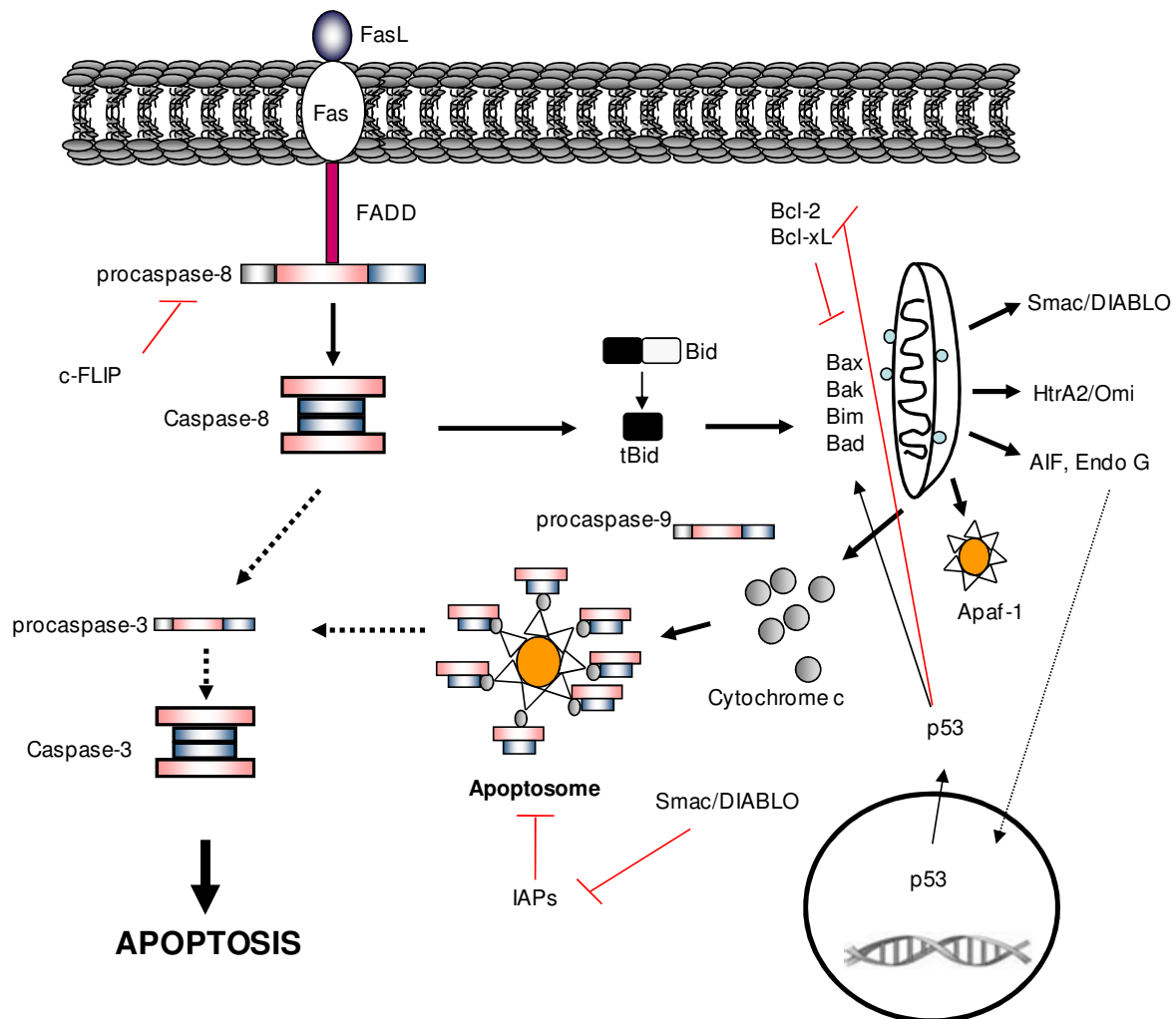


Figure 12. Apoptotic signalling cascade. Adapted from Shaha *et al.*, 2010.

The activation of the intrinsic apoptotic pathway by $\Delta\Psi_m$ loss can be also induced by nuclear DNA damage (Meulmeester *et al.*, 2008). The p53 tumor suppressor protein is present in the nucleus as an unstable protein at low levels. Upon DNA damage p53 levels increase both in the nucleus and cytoplasm. P53 is stimulated, binds to transcriptional regulators and functions as a transcription factor. Several p53 target genes encode proteins from the BCL-2 family. Cytoplasmatic p53 can induce MOMP through direct interaction with pro-apoptotic members (BAX and BAK) or indirectly inhibiting anti-apoptotic BCL-2 proteins

(BCL-2 and BCL-X). Nuclear p53 activates the BH3-only domain members, PUMA and Noxa, which inhibit anti-apoptotic proteins releasing BAX and BAK, triggering MOMP and the release of pro-apoptotic factors to the cytoplasm (Meulmeester *et al.*, 2008; Green *et al.*, 2009).

After translocation to the cytoplasm, cytochrome c binds to the adaptor protein **apoptotic protease activating factor-1** (Apaf-1) triggering the formation of a caspase-activating complex named apoptosome (Figure 12). Apaf-1 recruits procaspase-9 to the complex through CARD-CARD prodomain interaction (Kroemer *et al.*, 2007). The apoptosome is formed by seven Apaf-1, seven procaspase-9, seven cytochrome c, and also seven dATP molecules. This complex activates caspase-9 that, in turn, activates an effector caspase. Active caspase-9 seems to contain only one active site and so, recruitment of seven procaspase-9 molecules to the apoptosome complex seems to be related to caspase-9 high catalytic activity observed when bound to the apoptosome (Acehan *et al.*, 2002).

Beyond cytochrome c, Smac/DIABLO and HtrA2/Omi are also released from mitochondria after $\Delta\Psi_m$ loss. Both proteins are encoded by the nuclear genome, with a NH_2 -terminal mitochondrial localization sequence. Smac/DIABLO maturation involves NH_2 -terminus removal during translocation to mitochondria, whereas HtrA2/Omi maturation occurs in mitochondria with NH_2 -terminal sequence preservation. Both proteins present an **IAP binding motif** (IBM). So, when released in the cytoplasm, Smac/DIABLO and HtrA2/Omi bind IAPs, antagonize their inhibitor activity favouring caspase activation (Kroemer *et al.*, 2007).

The intrinsic caspase-independent pathway is activated through AIF and Endo G release from mitochondria. AIF is synthesized in the cytosol with also a NH_2 -terminal mitochondrial localization sequence but maturation occurs only after mitochondrial import into the intermembrane space. Upon $\Delta\Psi_m$ loss, AIF is released in the cytosol and translocated to the nucleus promoting chromatin condensation and large-scale DNA fragmentation. Similarly to AIF, Endo G is translocated to the nucleus and cleaves DNA in oligonucleosomal fragments (Elmore, 2007; Kroemer *et al.*, 2007).

3.4.2 Extrinsic pathway - Death receptor/ligand interaction

External apoptotic signal recognition involves transmembrane receptor/ligand interactions. These death receptors/ligands are members of the tumor necrosis factor (TNF) gene superfamily and all share a **death domain** (DD) essential for signal transmission from the cell surface. Fas receptor (Fas/CD95) is the major member of the death receptor family, a subgroup of the TNF superfamily, and the interaction with its ligand FasL (CD95L) is the best model to describe the apoptotic events observed during the extrinsic pathway (Guicciardi *et al.*, 2009).

Two extrinsic pathways of apoptosis activation were described, according to cell type. In both pathways, activation requires Fas oligomerization upon FasL binding through DD interaction. The cytoplasmic adaptor protein **Fas-associated death domain** (FADD) is recruited and through **death effector domain** (DED) interactions, FADD recruits procaspase-8 or procaspase-10 molecules to the complex (Barnhart *et al.*, 2003). Initiator caspases are then activated in this complex named **death-inducing signalling complex** (DISC). Upon initiator caspases activation, two distinct mechanisms can occur (Figure 12). In type I cells, large amounts of active initiator caspases are released from the DISC into the cytoplasm and cleave effector caspases triggering the proteolytic cascade. However, in type II cells DISC assembly is not so efficient. Small amounts of active initiator caspases are released in order that the signal is not strong enough to induce the apoptotic cascade. In these cells, the apoptotic signalling cascade is amplified through mitochondrial damage. Initiator caspases released from DISC, cleave the proapoptotic BCL-2 family member BID that once truncated (tBID) translocates to the mitochondria inducing oligomerization of other proapoptotic BCL-2 family members, BAX and BAK, which mediate the mitochondrial apoptotic pathway. Fas-mediated apoptotic pathway in type I cells can also induce mitochondrial damage but only to amplify the signal, whereas in type II cells mitochondrial apoptotic signalling is essential to the execution of apoptosis (Guicciardi *et al.*, 2009). The higher efficiency of the apoptotic signal transmission in type I cells was showed to be related to the co-localization of Fas and DISC assembly in lipid rafts and the more efficient internalization of the complex (Eramo *et al.*, 2004). Additionally, upon DNA damage with consequent p53 activation, apoptosis is induced through transcriptional activation of genes encoding Fas (Meulmeester *et al.*, 2008).

Activation of the initiator caspases by the extrinsic apoptotic pathway can be inhibited by a family of proteins known as **FLICE-inhibitor proteins** (FLIPs). FLIP has two DED and was shown to compete with procaspase-8 recruitment to the DISC, by DED interactions with FADD (Barnhart *et al.*, 2003; Guicciardi *et al.*, 2009).

3.4.3 Perforin/Granzyme Pathway

Cytotoxic T lymphocytes mediate apoptotic cell death mainly by the Fas/FasL signalling pathway. However, a different model of cytotoxic killing has also shown to exist. This model involves the protein pore formation perforin that, through pore formation, allows the release of exocytosed granules by cytotoxic T cells into the target cells cytoplasm. This release occurs in a calcium-dependent manner and the most important components of these granules are the serine proteases Granzyme A and Granzyme B (Barry *et al.*, 2002). Granzyme B cleaves after aspartate residues so, once released into the cytoplasm can induce apoptosis through 3 different mechanisms, all involving caspase activation. One mechanism involves procaspase-10 activation with cleavage of apoptotic factors like inhibitor of **CAD** (ICAD) and consequent DNA fragmentation and cell disassembly. Alternatively, Granzyme B can also amplify the apoptotic cascade by activating the mitochondrial pathway through BID cleavage. Finally, direct activation of caspase-3 with direct activation of the execution pathway bypassing the upstream apoptotic events was also shown to occur (Goping *et al.*, 2003). Granzyme A cleaves after lysine or arginine residues and, so, induces apoptosis in a caspase-independent manner. Granzyme A induced apoptosis occurs in the absence of some apoptotic features of apoptosis. No DNA fragmentation in oligonucleosomal fragments is observed. A specific DNase, **Gzma-activated DNase** (GAAD) is activated but only creates single-stranded DNA nicks. So, apoptotic cell induced by Granzyme A, although marked by some apoptotic features like loss of membrane integrity with blebbing, chromatin condensation and nuclear fragmentation, occurs with the absence of one of the hallmarks of apoptosis (Fan *et al.*, 2003).

3.4.4 Execution pathway

After effector caspases activation (caspases-3, -6 and -7), the final phase of apoptosis begins (Figure 12). Effector caspases, or also called execution caspases, cleave specific cellular substrates that will cause the morphologic and biochemical changes characteristic of apoptosis, leading to the final cell disassembly. Once activated, effector caspases mark the point of no return in apoptosis. The most important effector caspase is caspase-3 that specifically cleaves key substrates in this degradation phase. In normal cells, CAD is coupled with its inhibitor, ICAD. In apoptotic cells, caspase-3 cleaves ICAD releasing and activating CAD that cleaves DNA into oligonucleosomal fragments. Caspase-3 also cleaves

cytoskeletal proteins inducing cytoskeleton disruption with disintegration into apoptotic bodies that are phagocytised avoiding an inflammatory response (Slee *et al.*, 2001; Elmore, 2007).

4 Apoptosis during Spermatogenesis

4.1 Apoptosis as a regulator of normal spermatogenesis

Normal spermatogenesis depends on a balance between germ cell proliferation and death. In humans, spermatogenesis is usually referred as an inefficient process since daily sperm production is significantly lower in comparison to other species. Longer duration of spermatogenesis and lower density of germ cells in the testis, additionally with a 30-40% of germ cell loss occurring during meiotic divisions, are factors associated with such inefficiency (Johnson *et al.*, 2000).

Deciphering the apoptotic pathways in normal human spermatogenesis is a challenge. Scarce human testicular samples are available so mammalian germ cell apoptosis studies have been mostly relied on mouse and rat models. However, some studies in normal human testis have been published describing some of the apoptotic mechanisms involved in normal human spermatogenesis.

In mice, an early and massive wave of germ cell apoptosis, namely spermatogonia and spermatocytes, was shown to be present during testis development and to be essential for a functional spermatogenesis. The presence of a high levels of BAX expression, a pro-apoptotic member of the BCL-2 family proteins, was demonstrated to be associated with this apoptotic wave (Rodriguez *et al.*, 1997). However, in humans, this spontaneous apoptosis was shown to be absent in fetal and neonatal periods. Instead, during this period, increased rates of germ cell proliferation essential for testis growth and sperm output were observed. Only at puberty, under hormonal control, germ cell apoptosis occurs (Erkkila *et al.*, 1997; Francavilla *et al.*, 2000; Berensztein *et al.*, 2002). Contrarily to animals, spontaneous apoptosis in humans was shown to be present in all cell stages (spermatogonia, spermatocytes and spermatids) and also to be ethnic related, with higher rates in Chinese than Caucasian men (Hikim *et al.*, 1998).

The presence of spontaneous apoptosis during normal human spermatogenesis was shown to be essential for the adjustment of the number of germ cells to the support capacity of Sertoli cells and for the removal of damaged and abnormal ones (Francavilla *et al.*, 2000). In this mechanism, the Fas/Fas Ligand (Fas/FasL) system was demonstrated to be involved (Pentikainen *et al.*, 1999). FasL (Ligand) is expressed by Sertoli cells that recognize and bind to Fas (Receptor) expressed by germ cells, the apoptotic cascade is triggered and the externalization of phosphatidylserine leads to germ cell phagocytosis by Sertoli cells with no inflammatory reaction (Nakanishi *et al.*, 2004). However, since Fas/FasL is absent in the fetal

testis, human Fas/FasL system expression seems to be developmentally regulated and gonadotropin dependent (Francavilla *et al.*, 2000). Consistent with spontaneous apoptosis mediated through the Fas/FasL system is the absence of p53 expression in male germ cells from normal human testis (Oldereid *et al.*, 2001). Contrarily to testicular germ cells, apoptosis in normal human ejaculated spermatozoa seems to be independent from the Fas/FasL system (Sakkas *et al.*, 1999). However, additional information is needed to decipher the triggering pathways of apoptosis during normal human testicular development.

During the effector stage of apoptosis, the role of pro- and anti-apoptotic members from the BCL-2 family has already been described (Oldereid *et al.*, 2001). BCL-2 and BAK were shown to be present mainly close to the tubule lumen, in the cytoplasm of spermatocytes and spermatids. This co-localization of an anti- and a pro-apoptotic member, respectively, suggests an important function in human testicular apoptosis regulation. The pro-apoptotic member BAX was shown to be preferentially present in round spermatid nuclei suggesting a role in apoptotic induction due to DNA damage. However, since p53 was shown to be absent, BAX could be involved, instead, in spermatid maturation and differentiation. Additionally, the apoptotic promoter BAD, that was detected only in spermatid acrosomal region, was suggested to play a role in maturation and differentiation of the spermatid acrosomal region (Oldereid *et al.*, 2001). Finally, the anti-apoptotic BCL-XS and BCL-XL proteins were shown to be present in Leydig/peritubular cells and spermatogonia/spermatocytes, respectively, suggesting a role in germ cell density regulation (Beumer *et al.*, 2000).

Additional evidence of spermatogenesis modulation by apoptosis was demonstrated by the presence of apoptotic markers in ejaculated spermatozoa from normal fertile donors. Phosphatidylserine externalization, active caspase-3, Apoptosis-Induction Factor and DNA fragmentation were shown to be present in normal human sperm (Host *et al.*, 1999; Weng *et al.*, 2002; Taylor *et al.*, 2004). However, the presence of apoptotic markers in ejaculated spermatozoa from fertile men is lower than in infertile men indicating that increased levels of apoptosis may be associated with male infertility.

4.2 The presence of apoptotic markers in ejaculated spermatozoa from infertile men

As previously mentioned, modulation of spermatogenesis, during development, involves germ cell death by apoptosis. Elimination of damaged germ cells in men with normal spermatogenesis is mediated by the Fas/FasL system (Pentikainen *et al.*, 1999). So, in the presence of an efficient apoptotic mechanism, abnormal germ cells are eliminated and spermatozoa from the ejaculate should be normal. However, in semen samples from infertile men with abnormal sperm concentration, morphology and motility values, increased rates of Fas have already been shown to be present (Sakkas *et al.*, 1999). The presence of Fas-labeled spermatozoa was suggested not to be associated with death by apoptosis but to be a result of a mechanism named “abortive apoptosis”. This mechanism states that for somehow, a malfunction in the apoptotic mechanism led to the presence in the ejaculate of damaged spermatozoa that have failed to complete apoptosis (Sakkas *et al.*, 1999). Later, a second study also related the presence of apoptotic ejaculated sperm to this mechanism (Sakkas *et al.*, 2002). DNA damage, Fas, BCL-X and p53 were shown to be present in spermatozoa from all semen samples of men with normal and abnormal semen parameters. However, sperm DNA damaged and apoptotic markers were not always present in unison. So, the nature of DNA damage was suggested not to be associated to apoptosis but to an inefficient apoptotic elimination of damaged spermatozoa (Sakkas *et al.*, 1999). Additionally to the abortive theory, a second possibility for the origin of damaged spermatozoa in the ejaculate was suggested to be the result of incomplete DNA remodeling during sperm chromatin protamination (Sakkas *et al.*, 2002). Elimination of cytoplasmic components observed during terminal sperm differentiation share biochemical features with apoptosis (Blanco-Rodríguez *et al.*, 1999). Fas, p53 and BCL-X expression would represent cytoplasmic retention during spermiogenesis (Sakkas *et al.*, 2002).

On the contrary, it has been assumed by other authors that sperm cell death by apoptosis is one of the reasons of male infertility and that is associated to the presence of abnormal semen parameters. The possible relationship between the presence of apoptotic markers in ejaculated sperm and the presence of abnormal semen parameters is an issue addressed in many studies. In an infertile population mainly with poor sperm morphology, sperm apoptotic alterations were analyzed in the neat semen through the detection of phosphatidylserine exposure with distinction among live, early and late apoptotic/necrotic spermatozoa (Annexin V/Propidium Iodide assay), and for the presence of DNA fragmentation (Shen *et al.*, 2002). Sperm DNA fragmentation was positively correlated with late apoptosis but not with early apoptosis confirming DNA fragmentation as a late event in

the apoptotic mechanism. DNA fragmentation and late apoptosis inversely correlated with sperm motility and vitality and positively with abnormal sperm morphology including sperm head, midpiece and tail defects, demonstrating an association between apoptosis and abnormal sperm parameters. However, a positive correlation was found to sperm concentration or total sperm counts. As it is believed that mature sperm lacks the ability to undergo apoptosis (Weil *et al.*, 1998), this positive correlation may be explained by the abortive apoptosis theory. So, although the presence of apoptosis in ejaculated sperm does not affect sperm concentration and total sperm counts, sperm quality and function are affected and thus may contribute to infertility (Shen *et al.*, 2002).

Other studies were performed in order to search for the significance of apoptosis in ejaculated sperm from infertile men and different results were obtained. Negative correlations were found between dysfunctional mitochondria and sperm progressive motility (Donnelly *et al.*, 2000), DNA fragmentation and sperm motility and morphology (Gandini *et al.*, 2000), DNA fragmentation and only sperm concentration, phosphatidylserine externalization and sperm concentration and motility (Oosterhuis *et al.*, 2000a), DNA fragmentation and phosphatidylserine externalization with sperm motility (Weng *et al.*, 2002). Sperm DNA fragmentation together with Fas and p53 were negatively correlated to sperm concentration and normal morphology (Sakkas *et al.*, 2002) and active caspases-3 and -9 levels to sperm concentration, motility and morphology (Wang *et al.*, 2003). Additionally, sperm DNA damage associated to oxidative stress (Barroso *et al.*, 2000) and to the presence of active caspase-3 (Weng *et al.*, 2002) were found to be increased in the low motility fractions of semen samples from infertile men. Only one study failed to show a correlation between sperm apoptosis and semen quality (Ricci *et al.*, 2002). The wide variety of results observed relies not only on different apoptotic markers analyzed but also on male population characteristics and semen samples analyzed. Spermogram abnormalities were different among studies and in some cases selected semen fractions were analyzed instead of the neat semen. So, although a correlation between the presence of apoptotic markers in ejaculated sperm and abnormal sperm parameters was shown to exist in almost all studies, results remain inconclusive.

Sperm quality and functional viability can be determined by measurement of apoptosis in spermatozoa. Determination of a reliable method for sperm quality evaluation and *in vitro* fertilization (IVF) success prediction had already been investigated. Sperm quality was accessed by mitochondrial transmembrane potential ($\Delta\Psi_m$) measurements, reactive oxygen species (ROS) generation and DNA fragmentation in the neat semen and in prepared semen samples for IVF from patients with normal and abnormal semen parameters. A positive correlation was observed between sperm samples with high $\Delta\Psi_m$ and sperm concentration and motility. Prepared semen samples showed higher $\Delta\Psi_m$, lower levels of ROS production

and lower rates of sperm DNA fragmentation and were positively correlated with the fertilization rates after IVF. Loss of $\Delta\Psi_m$ is an early event of apoptosis and is associated with loss of sperm motility functions. Since changes in $\Delta\Psi_m$ occur during early apoptosis, this study suggest the measurement of $\Delta\Psi_m$ as the most sensitive test to evaluate sperm quality and predict successful IVF (Marchetti *et al.*, 2002).

Other techniques can be used to deplete apoptotic sperm in order to select the most viable ones for IFV. **M**agnetic-**a**ctivated **c**ell **s**orting (MACS) can be used to separate spermatozoa with deteriorated plasma membrane using superparamagnetic **a**nnexin V-conjugated **m**icro**b**eads (ANMBs). Determination of activated caspases was performed before and after sperm separation by MACS. Sperm from infertile men showed increased rates of annexin V-binding and activated caspases (8, 9, 1, 3) than sperm from donors. Additionally, the subpopulation positive for ANMBs binding showed higher rates of activated caspases than the neat semen, both in donors and infertile patients. So, ANMBs-MACS was shown to efficiently remove sperm with deteriorated plasma membrane and activated caspases and to produce a sperm fraction with increased sperm quality (Paasch *et al.*, 2003).

Determining the significance of the presence of apoptotic spermatozoa in ejaculated samples from infertile men with abnormal semen parameters is the first step towards sperm quality improvement when using assisted reproduction techniques.

4.3 Testicular germ cell apoptosis in conditions of abnormal spermatogenesis

Apoptosis in male infertility can be associated not only to increased levels of apoptotic markers in ejaculated sperm but also to increased levels of apoptotic testicular germ cells.

Evaluation of apoptosis was performed by DNA fragmentation rates determination in testicular tissue from men with Sertoli cell-only syndrome (SCOS), maturation arrest at the spermatocyte and spermatid level, hypospermatogenesis and obstructed spermatogenesis. Increased rates of apoptosis were observed in cases of maturation arrest and hypospermatogenesis in comparison to SCOS and normal spermatogenesis. Although present, apoptosis in normal testicular tissue was shown to be rare (Lin *et al.*, 1997a; Lin *et al.*, 1997b; Lin *et al.*, 1999b). In the same way, increased rates of DNA fragmentation were detected in testicular tissue from azoospermic men in comparison to men with obstructed spermatogenesis (Martincic *et al.*, 2001). Spermatogonia accelerated apoptosis, rather than

proliferative dysfunction, was, indeed, demonstrated to be on the origin of the decreased germ cells numbers associated to the hypospermatogenesis condition (Takagi *et al.*, 2001). However, the apoptotic related genes BAX, BCL-2 and p53 were shown to be absent irrespectively of the testicular histology (Lin *et al.*, 1999a).

On the contrary, the Fas/FasL system was shown to be expressed in testicular biopsies from men with maturation arrest. FasL was expressed by Leydig cells and Sertoli cells and Fas by germ cells. Increased numbers of Fas-positive primary spermatocytes were observed in maturation arrest, namely post-meiotic arrest, in comparison to normal testicular spermatogenesis (Francavilla *et al.*, 2000; Eguchi *et al.*, 2002). Additionally, ultrastructural studies showed a positive correlation between degenerating germ cells presenting apoptotic features, mainly primary spermatocytes and few round or elongated spermatids, and Fas-labeled germ cells (Francavilla *et al.*, 2002). DNA fragmentation was also detected in nuclei from primary spermatocytes (Eguchi *et al.*, 2002). In SCOS, however, Fas expression was never detected (Francavilla *et al.*, 2000). Apoptosis in maturation arrest seems to be mediated by the Fas/FasL system and to be related to the elimination of abnormal cells contributing to the low efficiency of spermatogenesis in these cases.

In azoospermic patients, intracytoplasmic sperm injection (ICSI) after testicular sperm extraction (TESE) enables the injection of a single spermatozoon into the oocyte (Palermo *et al.*, 1992). This technique has become an essential procedure in achieving a successful pregnancy in cases of azoospermia. However, in patients with spermatogenic arrest, infertility treatment includes elongated spermatid (ESI) or round spermatid (ROSI) injection instead (Tesarik *et al.*, 1996; Sousa *et al.*, 2000b; Sousa *et al.*, 2002). Apoptotic markers can be evaluated as an indicator of spermatids quality allowing the prediction of the fertilization rates success. DNA fragmentation and phosphatidylserine externalization were evaluated in men with complete (round spermatids as the most advanced cell stage) and incomplete (late elongated spermatids present) spermiogenesis failure (Tesarik *et al.*, 1998). Increased rates of DNA fragmentation were found in spermatocytes and round spermatids from cases with complete spermatogenic failure. In contrast, phosphatidylserine exposure was only detected in more advanced stages of spermiogenesis (elongated spermatids) in cases of incomplete spermiogenesis failure, suggesting that damaged spermatocytes and round spermatids may not be easily removed by phagocytosis (Tesarik *et al.*, 1998). Additionally, testicular samples from men with non-obstructive azoospermia showed high rates of DNA fragmentation in round spermatids (Jurisicova *et al.*, 1999) and altered chromatin condensation in mature elongated spermatids (Francavilla *et al.*, 2001). Increased rates of defective chromatin maturation and oocyte activating factors deficiency may be on the origin of the low success rates of round spermatid conceptions (Sousa *et al.*, 1999; Sousa *et al.*, 2002).

In order to improve sperm quality recovered from men with non-obstructive azoospermia, in-vitro culture procedures have been performed. Testicular tissue incubation with FSH was shown to decrease rates of spermatozoa with DNA fragmentation and phosphatidylserine externalization, with a post-culture enrichment of living and non-apoptotic spermatozoa. This quality improvement was suggested to be due to apoptotic spermatozoa disintegration and non-apoptotic round spermatid development into spermatozoa (Tesarik *et al.*, 2001).

Increasing evidence of the involvement of higher rates of germ cell apoptosis in testicular failure seems to exist. However, the molecular apoptotic mechanisms involved in the different testicular pathologies still need to be clarified.

AIMS

Several studies suggested that the increased levels of apoptotic markers in spermatozoa from infertile men are related to abnormal semen parameters. However, so far no conclusive results were achieved, as many studies presented confusing and contradictory data. In addition, no active apoptotic mechanism during abnormal spermatogenesis was identified.

In order to search for the significance of apoptosis in abnormal spermatogenesis conditions, the following questions were raised:

1. Is there any association between the rates of apoptotic ejaculated spermatozoa and the presence of abnormal spermiogram parameters, namely sperm concentration, percentage of sperm normal morphology and percentage of sperm progressive motility?
2. Is there any relation between the rates of testicular sperm apoptosis and obstructive and non-obstructive azoospermia syndromes?
3. Is germ cell apoptosis a stage-specific phenomenon? If so, what apoptotic mechanism could be involved in conserved and non-conserved spermatogenesis?

To answer these questions, the following methodologies were used:

➤ Active caspase-3 was quantified in ejaculated spermatozoa from 67 males undergoing spermiogram evaluation and in testicular samples from 18 men (5 oligozoospermic, 9 with obstructive azoospermia and 4 with non-obstructive azoospermia).

➤ Phosphatidylserine translocation can be detected by Annexin-V, a protein with high affinity to phosphatidylserine in the presence of calcium. Coupled with propidium iodide (PI) live, early apoptotic, late apoptotic and necrotic spermatozoa can be distinguished. Sperm samples from 56 patients undergoing an assisted reproduction attempt were analyzed for the presence of live, early and late apoptotic and necrotic spermatozoa. Ejaculated samples were obtained from 37 men and Annexin-V/PI was analyzed in the neat and prepared fractions. Testicular samples were obtained from 19 men (8 with obstructive azoospermia, 6 with non-obstructive azoospermia - secretory azoospermia, and 5 without azoospermia).

➤ Active caspase-3, active caspase-8 and active caspase-9 was quantified in Sertoli cells, spermatogonia, primary and secondary spermatocytes and round spermatids from 27 men with complete (5 oligozoospermic, 9 with obstructive azoospermia and 5 with hypospermatogenesis) and non-complete spermatogenesis (3 with maturation arrest and 5 with Sertoli-cell-only syndrome). Active caspases-3, -8 and -9 were detected using primary antibodies against the caspase active form and analyzed in paraffin sections by immunohistochemistry. Using stereological analysis, results were evaluated at the seminiferous tubule and the entire testicular volume level.

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Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality

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BACKGROUND: There are no studies relating the apoptotic marker caspase-3 in human sperm to different degrees of abnormal sperm concentration, morphology and rapid progressive motility. **METHODS:** Semen from 67 males with abnormal semen analyses ($n = 61$) and normozoospermia ($n = 6$) were used. In each case, sperm from the neat semen (semen fraction) and after gradient centrifugation and swim-up (swim-up fraction) were incubated with a caspase-3 profluorogenic substrate. Caspase-3 activity was quantified in 119 850 sperm, 67 488 from semen and 52 362 from swim-up fractions. Logistic regression was used to estimate odds ratio (with 99% confidence intervals) for the presence of caspase-3-positive sperm. **RESULTS:** In semen fractions, no relationship was found between abnormal semen analysis subgroups and sperm caspase-3 activity. On the contrary, a significantly increased number of sperm with caspase-3 activity was found in the swim-up fractions from samples with poor sperm morphology. When analysis was restricted to single semen analysis defects, a significant increase of caspase-3-positive sperm was found in the semen fractions of cases with asthenozoospermia, and in the swim-up fractions of cases with teratozoospermia. **CONCLUSIONS:** Sperm caspase-3 activity seems to be associated with teratozoospermia and asthenozoospermia, thus suggesting that nuclear, mitochondrial and cytoskeletal abnormalities induce caspase-3 activation during spermiogenesis or sperm maturation.

Key words: apoptosis/asthenoteratozoospermia/caspase-3 activity/male infertility/semen analysis parameters

Introduction

Programmed cell death (apoptosis) is a predetermined event in the cell developmental programme, being involved in senescence as well as in the cell response to injuries. Apoptosis is under the control of caspases, cysteine proteinases that cleave key cellular proteins after aspartate residues. Caspases are resident in cells as inactive proenzymes and become activated by multiple mechanisms. Initiator caspases can be activated either by external signals (procaspases-2, -8 and -10) or by internal signals (procaspase-9) derived from mitochondria (cytochrome c). Both pathways trigger membrane phosphatidylserine translocation from the cytosolic to the external leaflet of the plasma membrane, thus conveying cells to phagocytosis. Crosstalk events also occur between these two main pathways, with caspase-8 being able to activate caspase-9 either directly or through mitochondrial and nuclear modulators. Both mechanisms are also capable of transducing p53 (proapoptotic) nuclear stress signalling, and are modulated by Bcl2 (antiapoptotic) and Bax (proapoptotic). Effector caspases (procaspases-3, 6, 7) are then activated by initiator caspases, being responsible for cell proteolysis and

DNA degradation through activation of an endonuclease that cleaves DNA between regularly spaced nucleosomal units (Scaffidi *et al.*, 1998; Slee *et al.*, 1999; Wolf *et al.*, 1999; Boatright *et al.*, 2003; Said *et al.*, 2004).

Apoptosis regulates normal spermatogenesis. In the post-natal period, it is responsible for the death and phagocytosis of premeiotic germ cells in order to adjust their number to the number of Sertoli cells, and for the removal of damaged and abnormal germ cells during active spermatogenesis in the post-pubertal period (Print and Loveland, 2000; Sinha Hikim *et al.*, 2003). Germ cell apoptosis has been shown to increase after testicular injury, such as exposure to toxics, varicocele, testicular torsion, hormonal deprivation and genetic abnormalities (Francavilla *et al.*, 2000; Kim *et al.*, 2001; Said *et al.*, 2004), as well as a response to freeze–thawing (Glander and Schaller, 1999; Schuffner *et al.*, 2001; Paasch *et al.*, 2004).

In the present investigation we quantitatively analysed caspase-3 activity in sperm retrieved from the semen and swim-up fractions of males with normal and abnormal semen parameters.

Materials and methods

Semen samples

This study was initiated after Institutional approval and patient informed consent. A total of 67 consecutive males undergoing spermogram evaluation during infertility diagnosis were included. Criteria for inclusion were: (i) absence of known pathologies and intake of medicines; (ii) normal physical examination, hormonal profiles and karyotypes; (iii) spermogram analysis showing presence of sperm, normal pH, absence of agglutination and negative microorganism culture. The mean age was 34.9 ± 4.88 years SD (range 27–52), with 88.1% aged <39 years (age groups: 25–29 years, $n = 6$; 30–34 years, $n = 30$; 35–39 years, $n = 23$; 40–44 years, $n = 5$; 45–49 years, $n = 2$; 50–54 years, $n = 1$). Each patient contributed a single semen sample. Each semen sample was left to liquefy at 37°C for 30 min; a small fraction was taken for caspase-3 activity determination (semen fraction) and another aliquot was used for spermogram analysis (World Health Organization, 1999). The rest of the semen was submitted to gradient centrifugation at 437 g for 20 min at room temperature (Ixaprep; Medicult, Denmark), to select sperm with normal morphology and motility at the bottom of the tubes. The pellet was washed (194 g for 2×10 min at room temperature) in sperm preparation medium (SPM, with HEPES buffer; Medicult), layered with 50–100 μ l of SPM, and incubated (1 h, 37°C, 5% CO₂ in humidified air) to collect the swim-up fraction containing motile normal sperm that actively migrated from the pellet to the upper aqueous phase. This fraction was then taken for determination of caspase-3 activity in the swim-up. Seven cases were submitted to the swim-up technique only: three with oligoteratozoospermia (0.16 – 0.6×10^6 /ml; 25–33.3% rapid progressive motility; 0% of normal morphology but with insufficient sperm for rigorous quantitative normal morphology assessment) and four with oligoasthenoteratozoospermia (0.05 – 1.8×10^6 /ml; 3.3–13.5% rapid progressive motility; 0% normal morphology but with insufficient sperm for morphology assessment in three cases).

Caspase-3 activity detection

Fractions used for caspase-3 activity determination were centrifuged at 1300 g for 2 min at room temperature. The pellet was resuspended and incubated for 30 min (37°C, 5% CO₂ in humidified air) in previously warmed 50 μ l of medium containing (1:1) SPM and PhiPhiLux D2G2 (membrane permeant profluorogenic caspase-3 substrate; OncoImmunin Inc., USA; stock solution: 10 μ mol/l in Roswell Park Memorial Institute 1640 medium with 25 mmol/l HEPES, kept in aliquots at -20°C). Semen and swim-up sperm fractions were then washed (1300 g for 2×1 min at room temperature) with 500 μ l of phosphate-buffered saline (Sigma, Barcelona, Spain), and the pellet spread onto poly-L-lysine (Sigma)-coated glass slides. After air-drying, slides were mounted with 10 μ l Vectashield antifade medium (Vector Laboratories, USA), containing 1.5 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) to stain the DNA (blue), and sperm counted in an epifluorescence microscope (Eclipse, E-400; Nikon, Japan) fitted with a CCD camera (Sony, Japan) and an automated karyotyper software (Cytovision Ultra; Applied Imaging International, UK). In the presence of activated caspase-3, the specific substrate PhiPhiLux G2D2 is cleaved and emits red light. In all cases, sperm positive for caspase-3 activity showed an intense fluorescent red colour in the mitochondrial midpiece (Figure 1).

About 1000 sperm were counted (double-blind procedure) per semen and per swim-up fractions of each individual. The rate of caspase-3-positive sperm was expressed as the number of caspase-3-positive sperm per 100 sperm. In 25 cases, <1000 sperm could be counted (mean: 377.14 sperm per fraction, range: 22–986). Of

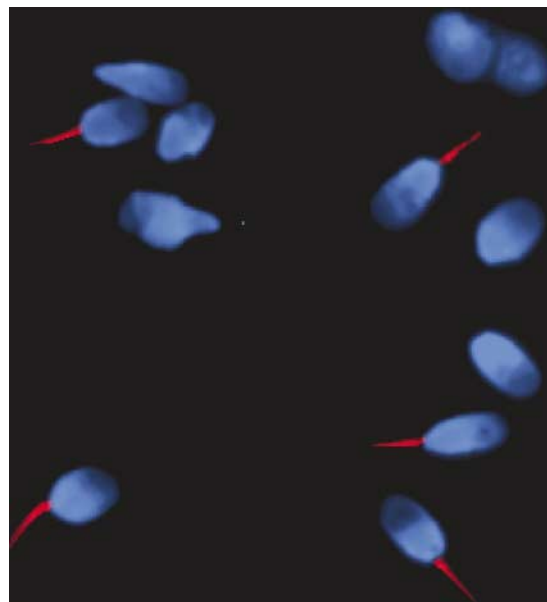


Figure 1. Epifluorescence image of sperm from a semen fraction of a patient with oligoasthenoteratozoospermia. Caspase-3 activity (red) appears restricted to the sperm mitochondrial midpiece. DNA is stained in blue with DAPI.

these, three cases had insufficient sperm in both semen and swim-up fractions, corresponding to two patients with oligoasthenoteratozoospermia (0.05 – 0.26×10^6 /ml; 3.3–5.4% rapid progressive motility; 0% normal morphology but with insufficient sperm for rigorous quantitative normal morphology assessment) and one patient with oligoteratozoospermia (0.16×10^6 /ml; 24.8% rapid progressive motility; 0% normal morphology but with insufficient sperm for morphology assessment). In the other 22 cases, there was insufficient sperm in the swim-up fraction: two patients with teratozoospermia (40 – 170×10^6 /ml; 44.4–46.5% rapid progressive motility; 6–11% normal morphology), two with asthenoteratozoospermia (20 – 75×10^6 /ml; 0.4–1.8% rapid progressive motility; 0–5% normal morphology), seven with oligoteratozoospermia (0.36 – 14×10^6 /ml; 25.6–59.3% rapid progressive motility; 0–5% normal morphology but with insufficient sperm for morphology assessment in one case), and 11 with oligoasthenoteratozoospermia (0.17 – 18.5×10^6 /ml; 1–20.4% rapid progressive motility; 0–11% normal morphology but with insufficient sperm for morphology assessment in one case).

Statistical analysis

Descriptive statistics were used for the initial characterization of the population, considering each male as an individual and a two-sided significance level of 5%. In this approach, Wilcoxon and Kruskal–Wallis tests were used to compare the percentages of caspase-3-positive sperm between semen and swim-up fractions, as well as between the defined levels of each parameter in each fraction. This approach is considered the traditional way for analysing this kind of outcome, where a number of cells are observed for each subject and the result is represented as a percentage of positive cells. A limitation of this traditional approach is that the total number of cells counted is not considered, and the characteristics observed for each cell is addressed only to the subject to which it belongs. Thus, data were then analysed at the cellular level, by which each spermatozoon scored was considered independently from other cells (Zhao *et al.*, 2001). In this approach, the cells are considered as

the individuals, and for each one the outcome, caspase-3-positive or caspase-3 negative sperm, corresponds to a binary variable. When data were analysed at the cellular level, the size of the sample studied and the power of the hypothesis tested were greatly increased. To control the possibility of committing a type I error, hypotheses were tested at a probability of 0.01 rather than 0.05, and thus a stringent significance level of 1% was chosen for statistical significance. The presence or absence of positive caspase-3 sperm was studied with logistic regression models and the odds ratio (OR) for risk of presence and their associated 99% confidence interval (CI) are presented. Firstly, a comparative analysis between semen and swim-up fractions was performed. To find any possible association between the presence of caspase-3 and the different spermogram parameters, semen and swim-up fractions were then analysed separately. All analyses were performed with the SPSS statistical package (version 11.0) for Windows.

Results

Two types of control groups were used: relative controls, specific for each spermogram parameter ($\geq 20 \times 10^6/\text{ml}$ sperm concentration; or $\geq 15\%$ normal morphology; or $\geq 25\%$ rapid progressive motility), and absolute controls, derived from samples showing all spermogram

parameters simultaneously normal (normozoospermia: $\geq 20 \times 10^6/\text{ml}$ sperm concentration + $\geq 15\%$ normal morphology + $\geq 25\%$ rapid progressive motility). Three study groups were used, based on the three main spermogram parameters. Each group was further divided into different subgroups of severity (Table I). A different study group classification was also made with dichotomous variables; an individual was classified based on single spermogram defects into oligo-, terato- and asthenozoospermia. In this case, only one semen parameter was affected in each individual. In the present study, there were no patients with pure oligozoospermia. When patients showed more than one affected semen parameter, they were classified into other groups, oligoasthenozoospermia, oligoterato-, asthenoterato- or oligoasthenoteratozoospermia (Table I). The number of cases and sperm analysed, as well as the means $\pm$ SD of caspase-3-positive sperm in all control and study groups, either in the semen and swim-up fractions, are shown in Table I.

The comparison of the presence of caspase-3-positive sperm between semen and swim-up fractions, considering both the males and the cells as individuals, revealed significant differences ($P < 0.001$), with semen fractions showing

Table I. Caspase-3-positive sperm in semen fractions and swim-up fractions per subgroup analysed

Groups/subgroups	Cases (n)	Semen fraction			Swim-up fraction		
		Sperm (n)	Caspase-3 ⁺ sperm		Sperm (n)	Caspase-3 ⁺ sperm	
			(n)	(%) ^a		(n)	(%) ^a
Concentration ($\times 10^6/\text{ml}$)							
≤ 1	6	4237	605	14.3 ± 6.6	1501	40	2.7 ± 4.9
$> 1 - \leq 5$	6	6129	775	12.6 ± 4.7	4349	96	2.2 ± 0.7
$> 5 - < 10$	11	11 215	1742	15.5 ± 6.3	6576	189	2.9 ± 2.0
$\geq 10 - < 20$	10	10 550	1175	11.1 ± 8.3	6859	199	2.9 ± 2.8
< 20	33	32 131	4297	13.4 ± 7.1	19285	524	2.7 ± 2.5
≥ 20	34	35 357	6560	18.6 ± 9.2	33 077	1748	5.3 ± 3.5
% normal morphology							
≤ 5	28	28 817	3773	13.1 ± 6.2	18 580	606	3.3 ± 2.4
$> 5 - < 10$	13	13 621	2600	19.1 ± 5.5	12 778	636	5.0 ± 4.0
$\geq 10 - < 15$	11	11 509	1895	16.5 ± 8.3	10 261	649	6.3 ± 3.3
< 15	52	53 947	8268	15.3 ± 7.0	41 619	1891	4.5 ± 3.5
≥ 15	9	9304	1984	21.3 ± 14.2	9242	341	3.7 ± 2.3
% rapid progressive motility							
≤ 5	7	6359	863	13.6 ± 7.6	3540	173	4.9 ± 2.5
$> 5 - < 10$	5	5066	818	16.1 ± 6.7	2532	188	7.4 ± 2.3
$\geq 10 - < 25$	22	21 910	3302	15.1 ± 8.1	17 685	606	3.4 ± 2.9
< 25	34	33 335	4983	15.0 ± 7.8	23 757	967	4.1 ± 3.1
≥ 25	33	34 153	5874	17.2 ± 9.2	28 605	1305	4.6 ± 3.6
Absolute control	6	6222	1272	20.4 ± 15.5	6137	234	3.8 ± 2.7
Asthenozoospermia	2	2054	524	25.5 ± 12.8	2088	79	3.8 ± 1.6
relative control	65	65 434	10 333	15.8 ± 8.3	50 274	2193	4.4 ± 3.4
Teratozoospermia	17	17 775	3064	17.2 ± 6.2	16 511	941	5.7 ± 3.7
relative control	50	49 713	7793	15.7 ± 9.3	35 851	1331	3.7 ± 3.0
Oligoasthenozoospermia	1	1028	188	18.3	1017	28	2.8
relative control	62	64 252	10 429	16.2 ± 8.7	50 967	2206	4.8 ± 3.4
Oligoteratozoospermia	8	8127	1173	14.4 ± 8.2	4834	128	2.6 ± 2.3
relative control	58	57 193	9309	16.3 ± 8.8	46 293	2137	4.6 ± 3.4
Asthenoteratozoospermia	9	9306	1700	18.3 ± 5.9	8341	494	5.9 ± 3.3
relative control	58	56 053	8932	15.9 ± 9.0	43 670	1743	4.0 ± 3.2
Oligoasthenoteratozoospermia	18	18 739	2331	12.4 ± 6.7	11 933	328	2.7 ± 2.2
relative control	45	46 541	8286	17.8 ± 8.9	40 051	1906	4.8 ± 3.5
Total	67	67 488	10 857	16.1 ± 8.6	52 362	2272	4.7 ± 3.9

^aMean $\pm$ SD.

Absolute control: $\geq 20 \times 10^6/\text{ml}$ concentration + $\geq 15\%$ normal morphology + $\geq 25\%$ rapid progressive motility.

Relative control: all other individuals of the population regarding each considered subgroup.

4.2-fold more caspase-3 activity than swim-up fractions (OR = 4.227, 99% CI = 3.975–4.495). In order to obtain a better knowledge of these differences and to investigate the possible association between the presence of caspase-3 activity and sperm concentration, normal morphology and rapid progressive motility, the two sperm fractions were analysed separately.

Semen fractions

Considering each male as an individual, the percentage values of caspase-3-positive sperm appeared much dispersed in the different groups of spermiogram parameters, thus suggesting that no relationship exists (Figure 2); nor were there significant differences with respect to subgroups ($P \geq 0.070$) for sperm concentration, normal morphology or rapid progressive motility.

Data were then treated at the cellular level, in which the individual was each spermatozoon scored. The presence or absence of caspase-3-positive sperm was analysed using logistic regression and the odds ratio calculated for each variable, with the reference category being the one referred to the normal value (relative control) of the parameter under analysis (Table II). All main spermiogram parameters were significantly associated with the presence of caspase-3-positive sperm ($P < 0.001$). The probability of the spermatozoon

being caspase-3 positive was significantly lower ($P < 0.001$) than the reference category (relative control) in all subgroups, except for the $> 5 - < 10\%$ rapid progressive motility subgroup, where no significant differences were found ($P = 0.063$). To further consolidate these results, a similar analysis was performed with a more strict reference group (absolute control), where all the spermiogram parameters were normal (Table II). Again, all main spermiogram parameters were significantly associated with the presence of caspase-3-positive sperm ($P < 0.001$), with higher risk in the reference category (absolute control), except for the $> 5 - < 10\%$ normal morphology subgroup where no significant differences were found ($P = 0.025$). To uncover the individual contribution of each abnormal spermiogram parameter to these results, dichotomous study groups were then analysed (Table III). Taking all other individuals of the population as control, there were significant differences in all cases with the exception of the oligoasthenozoospermic group ($P = 0.077$), with a higher risk for the presence of caspase-3-positive sperm being demonstrated in the astheno-, terato- and asthenoteratozoospermic subgroups ($OR \geq 1, P < 0.001$). When groups were compared to the absolute control, the oligoasthenozoospermic group again showed no significant differences ($P = 0.111$) and only the asthenozoospermic group showed significantly increased numbers of caspase-3-positive sperm ($OR = 1.333, P < 0.001$).

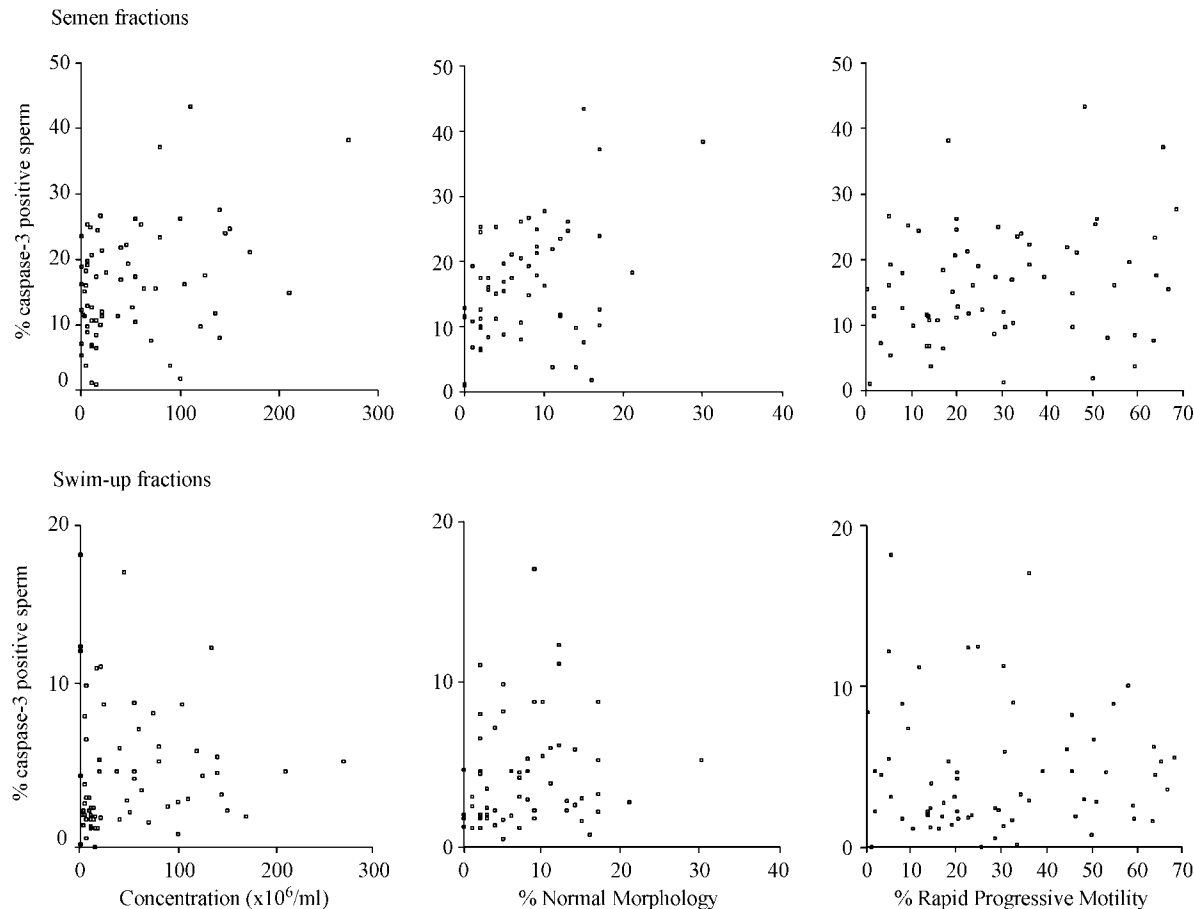


Figure 2. Scatters of the percentage of caspase-3-positive sperm in the semen fractions and in the swim-up fractions, among the different spermiogram parameters.

Table II. Odds ratio for risk of presence of caspase-3-positive sperm in semen and swim-up fractions per subgroup analysed

Groups/ subgroups	Semen fraction				Swim-up fraction			
	I		II		I		II	
	OR (99% CI)	P	OR (99% CI)	P	OR (99% CI)	P	OR (99% CI)	P
Concentration ($\times 10^6/\text{ml}$)		< 0.001		< 0.001		< 0.001		< 0.001
≤ 1	0.731 (0.650–0.823)	< 0.001	0.648 (0.564–0.745)	< 0.001	0.491 (0.323–0.745)	< 0.001	0.691 (0.442–1.080)	0.033
> 1– ≤ 5	0.635 (0.572–0.706)	< 0.001	0.563 (0.496–0.640)	< 0.001	0.405 (0.308–0.532)	< 0.001	0.570 (0.415–0.782)	< 0.001
> 5– < 10	0.807 (0.748–0.871)	< 0.001	0.716 (0.644–0.795)	< 0.001	0.530 (0.434–0.648)	< 0.001	0.747 (0.578–0.965)	0.003
≥ 10– < 20	0.550 (0.504–0.600)	< 0.001	0.488 (0.435–0.546)	< 0.001	0.534 (0.439–0.650)	< 0.001	0.752 (0.584–0.968)	0.004
≥ 20	0.678 (0.641–0.716)	< 0.001	0.601 (0.548–0.658)	< 0.001	0.500 (0.439–0.570)	< 0.001	0.704 (0.573–0.865)	< 0.001
% normal morphology		< 0.001		< 0.001		< 0.001		< 0.001
≤ 5	0.556 (0.514–0.602)	< 0.001	0.586 (0.534–0.643)	< 0.001	0.879 (0.736–1.050)	0.062	0.850 (0.694–1.040)	0.038
> 5– < 10	0.870 (0.799–0.949)	< 0.001	0.918 (0.832–1.013)	0.025	1.367 (1.146–1.631)	< 0.001	1.321 (1.081–1.616)	< 0.001
≥ 10– < 25	0.727 (0.663–0.797)	< 0.001	0.767 (0.692–0.851)	< 0.001	1.762 (1.477–2.102)	< 0.001	1.703 (1.393–2.082)	< 0.001
≥ 15	0.668 (0.621–0.718)	< 0.001	0.704 (0.646–0.768)	< 0.001	1.242 (1.064–1.450)	< 0.001	1.200 (1.001–1.440)	0.010
% rapid progressive motility		< 0.001		< 0.001		< 0.001		< 0.001
≤ 5	0.756 (0.683–0.837)	< 0.001	0.611 (0.540–0.692)	< 0.001	1.072 (0.865–1.327)	0.404	1.292 (1.092–1.684)	0.012
> 5– < 10	0.927 (0.835–1.030)	0.063	0.749 (0.660–0.851)	< 0.001	1.678 (1.362–2.067)	< 0.001	2.023 (1.560–2.624)	< 0.001
≥ 10– < 25	0.854 (0.804–0.908)	< 0.001	0.691 (0.628–0.759)	< 0.001	0.742 (0.652–0.845)	< 0.001	0.895 (0.731–1.096)	0.158
≥ 25	0.846 (0.802–0.893)	< 0.001	0.684 (0.625–0.748)	< 0.001	0.887 (0.793–0.992)	0.006	1.070 (0.883–1.295)	0.364

OR (99% CI) = odds ratio (99% confidence interval). $P < 0.01$ is considered significant.

Reference categories: (I) relative control groups for each spermiogram parameter; (II) absolute control group.

Relative control groups: $\geq 20 \times 10^6/\text{ml}$ concentration; $\geq 15\%$ normal morphology; $\geq 25\%$ rapid progressive motility.

Absolute control group: $\geq 20 \times 10^6/\text{ml}$ concentration + $\geq 15\%$ normal morphology + $\geq 25\%$ rapid progressive motility.

Table III. Odds ratio for risk of presence of caspase-3-positive sperm in semen fractions and swim-up fractions per subgroup analysed

Subgroups	Semen fraction				Swim-up fraction			
	I		II		I		II	
	OR (99% CI)	P	OR (99% CI)	P	OR (99% CI)	P	OR (99% CI)	P
A	1.826 (1.598–2.087)	< 0.001	1.333 (1.143–1.554)	< 0.001	0.862 (0.638–1.165)	0.205	0.992 (0.705–1.396)	0.952
T	1.120 (1.055–1.190)	< 0.001	0.811 (0.737–0.892)	< 0.001	1.568 (1.401–1.755)	< 0.001	1.525 (1.285–1.848)	< 0.001
OA	1.155 (0.936–1.424)	0.077	0.871 (0.697–1.089)	0.111	0.626 (0.381–1.029)	0.015	0.714 (0.424–1.205)	0.097
OT	0.868 (0.796–0.946)	< 0.001	0.656 (0.585–0.736)	< 0.001	0.562 (0.443–0.713)	< 0.001	0.686 (0.515–0.915)	0.001
AT	1.179 (1.093–1.271)	< 0.001	0.870 (0.782–0.968)	0.001	1.515 (1.323–1.734)	< 0.001	1.588 (1.288–1.958)	< 0.001
OAT	0.656 (0.615–0.700)	< 0.001	0.553 (0.501–0.610)	< 0.001	0.565 (0.483–0.661)	< 0.001	0.712 (0.569–0.891)	< 0.001

OR (99% CI): odds ratio (99% confidence interval). $P < 0.01$ is considered significant.

Reference categories: (I) all other individuals of the population or (II) absolute control group ($\geq 20 \times 10^6/\text{ml}$ concentration + $\geq 15\%$ normal morphology + $\geq 25\%$ rapid progressive motility).

O = oligozoospermia; A = asthenozoospermia; T = teratozoospermia.

Swim-up fractions

As observed for the semen fractions, when considering each male as an individual the percentage values of caspase-3-positive sperm appeared quite dispersed in the different groups of spermiogram parameters (Figure 2). With respect to subgroups, no significant differences were found ($P \geq 0.066$) for sperm concentration, rapid progressive motility or normal morphology.

When data were treated at the cellular level using comparisons to the relative controls, all main spermiogram parameters (concentration, normal morphology, rapid progressive motility) appeared significantly associated ($P < 0.001$) with the presence of caspase-3-positive sperm (Table II). No significant differences from the relative controls were found in the

$\leq 5\%$ subgroups of normal morphology ($P = 0.062$) and rapid progressive motility ($P = 0.404$). The probability of the spermatozoon being caspase-3-positive was significantly lower than the reference category in all other subgroups, except for a significant increase in the proportion of caspase-3-positive sperm relative to controls in the subgroups $> 5- < 10\%$, $\geq 10- < 15\%$ and $< 15\%$ of normal morphology, and $> 5- < 10\%$ of rapid progressive motility (OR ≥ 1 , $P < 0.001$). To further consolidate the present results, a similar analysis was performed using comparisons to the absolute control group (Table II). In this case, significant differences ($P < 0.001$) were found for the presence of caspase-3-positive sperm regarding all main spermiogram parameters. No significant differences from the absolute control

were found in the $\leq 1\%$ subgroup of sperm concentration ($P = 0.033$), in the $\leq 5\%$ ($P = 0.038$) and $< 15\%$ ($P = 0.010$) subgroups of normal morphology, and in the $\leq 5\%$ ($P = 0.012$), $\geq 10 - < 25\%$ ($P = 0.158$) and $< 25\%$ ($P = 0.364$) subgroups of rapid progressive motility. With the exception of a significant increase in the proportion of caspase-3-positive sperm relative to the control in the subgroups $> 5 - < 10\%$ and $\geq 10 - < 15\%$ of normal morphology, and $> 5 - < 10\%$ of rapid progressive motility ($OR \geq 1$, $P < 0.001$), the probability of the spermatozoon being caspase-3 positive was significantly lower than the reference category in all other subgroups. In the analysis of dichotomous study groups (Table III), comparisons with the relative controls or the absolute control showed significant differences in all subgroups, with the exception of the astheno- and oligoasthenozoospermic subgroups ($P > 0.01$). This also demonstrated a higher risk for the presence of caspase-3-positive sperm in the terato- and asthenoteratozoospermic subgroups ($OR \geq 1$, $P < 0.001$).

Discussion

Normal human spermatogenesis is modulated by apoptotic events that adjust the number of germ cells to the number of Sertoli cells and eliminate the abnormal gametes. Genetic and exogenous injuries influencing spermatogenesis potentiate the endogenous mechanism of germ cell apoptosis, and may lead to insufficient or absent sperm production. We show here that the presence of the major effector caspase-3 is related to asthenozoospermia and teratozoospermia.

Although it remains to be established whether sperm defects are a cause or a consequence of increased rates of apoptosis, several studies have shown an increase of DNA fragmentation in human sperm with low motility (Barroso *et al.*, 2000; Weng *et al.*, 2002), low sperm counts (Oosterhuis *et al.*, 2000) or decreased normal sperm concentration, morphology and motility (Gandini *et al.*, 2000; Shen *et al.*, 2002). This is of clinical relevance, as semen samples showing increased rates of sperm DNA fragmentation have been associated with decreased rates of fertilization (Marchetti *et al.*, 2002) and pregnancy (Tomlinson *et al.*, 2001). Other studies have also shown that mitochondrial integrity is decreased in samples with low sperm motility (Donnelly *et al.*, 2000; Weng *et al.*, 2002), and that phosphatidylserine exposure is increased in samples exhibiting low sperm motility (Weng *et al.*, 2002), decreased normal sperm motility and morphology (Shen *et al.*, 2002), or decreased normal sperm concentration and motility (Oosterhuis *et al.*, 2000).

Here we present a strict statistical analysis of 67 semen samples, in which 119 850 sperm were quantified for the presence of caspase-3 activity, 67 488 from semen fractions and 52 362 from swim-up fractions. The large number of cases studied enabled the analysis of different subgroups of severity of sperm defects within major groups of spermogram abnormalities. In the first approach, cases were divided according to decreased sperm normal concentration,

morphology and rapid progressive motility, and each group was then subdivided into different levels of severity of each sperm defect. In a second approach, and to uncover the possible contribution of simultaneous sperm defects, cases were analysed for single abnormalities (oligo-, astheno- and teratozoospermia). In the semen fractions, no relationship was found between the presence of caspase-3-positive sperm and any of the abnormal spermogram values. In accordance with previous results (Ricci *et al.*, 2002), the proportion of caspase-3-positive sperm was shown to be unrelated to sperm quality. However, we first show that this is due to the presence of simultaneous sperm defects, as the analysis of single sperm abnormalities revealed a strong relationship to asthenozoospermia. On the contrary, results obtained from the swim-up fractions showed a significant increase of caspase-3-positive sperm with decreased normal morphology and rapid progressive motility values, which after analysis of single sperm abnormalities was only confirmed for teratozoospermia. In accordance with previous reports that have used several other markers for detecting sperm apoptosis (Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Marchetti *et al.*, 2002), our current results confirm that gradient centrifugation and swim-up significantly deplete caspase-3-positive sperm. Taking into account that the presence of caspase-3-positive sperm was 4.2-fold higher in semen than in the swim-up fractions ($OR = 4.227$, 99% $CI = 3.975 - 4.495$), that the swim-up fractions were associated only with teratozoospermia ($OR \geq 1$ and $P < 0.001$), and that samples with decreased normal sperm morphology ($< 15\%$) have $4.5 \pm 3.5\%$ (mean $\pm$ SD) of caspase-3-positive sperm in the swim-up fractions, the data suggest a low risk of selecting apoptotic sperm during clinical treatments.

Caspase activation is a well-defined point of no return for apoptotic progression in somatic cells (Wolf *et al.*, 1999). Although procaspases are present in human mature sperm and might be activated by different stimuli such as cryopreservation (Paasch *et al.*, 2004), the role of caspases and apoptosis in ejaculated sperm is still an open question. Caspase activity in mature sperm may correspond to the activation of the apoptotic machinery leading to cell death as induced by different factors during spermiogenesis. In this mechanism, spermatids marked for elimination via apoptosis could somehow escape the removal mechanism and thus contribute to the poor sperm quality found in the ejaculate (Sakkas *et al.*, 2002; Said *et al.*, 2004). However, as active caspase-1 was demonstrated to be involved in the degradation of surplus spermatid cytoplasmic components (residual bodies) during spermiogenesis, caspase activity in ejaculated sperm may, alternatively, correspond to enzyme diffusion from residual bodies (Blanco-Rodriguez and Martinez-Garcia, 1999). Caspase-3 is the major effector enzyme causing cell disruption during apoptosis. Caspase-3 activity has been previously detected in the midpiece of ejaculated human sperm (Weng *et al.*, 2002) and shown to be significantly associated with low sperm motility (Weng *et al.*, 2002) or with decreased normal sperm concentration, motility and morphology (Wang *et al.*, 2003). Our present results confirm that sperm caspase-3 activity is restricted to the mitochondrial sheath, and show

no statistical association between the presence of caspase-3-positive sperm and decreased normal sperm concentration, motility or morphology in the neat semen. On the contrary, a significant increase of caspase-3-positive sperm is shown in the swim-up fractions from samples with decreased normal sperm morphology. These data also show that when the analysis is corrected for single sperm defects, caspase-3-positive sperm appear significantly associated with asthenozoospermia in the semen fractions and with teratozoospermia in the swim-up fractions. These data thus strongly favour the hypothesis that caspase activity is not the result of active enzyme diffusion from residual bodies and may correspond to the activation of the cell apoptotic machinery for discarding abnormal spermatids/sperm due to nuclear, mitochondrial and cytoskeleton structural defects.

In conclusion, no relationship was found between caspase-3-positive sperm and abnormal spermogram subgroups in the semen fractions, whereas a significant relationship was found between caspase-3-positive sperm and decreased normal sperm morphology and rapid progressive motility in the swim-up fractions. When the analysis was restricted to single spermogram defects, a significant increase in caspase-3-positive sperm was found in relation to asthenozoospermia in the semen and to teratozoospermia in the swim-up fraction.

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PAPER II

Almeida C, Sousa M, Viana P, Gonçalves A, Silva J, Ferras L, Barros A (2007)
Increased rates of phosphatidylserine translocation on spermatid and spermatozoa
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Aumento das taxas de translocação da fosfatidilserina nos espermátides e espermatozóides de homens com azoospermia

Increased rates of phosphatidylserine translocation on spermatid and spermatozoa from men with azoospermia

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Resumo

a) Objectivos: *Determinação das taxas de apoptose dos espermátides e espermatozóides de homens com azoospermia.* b) Desenho do estudo: *Avaliação quantitativa da translocação da fosfatidilserina com distinção entre células vivas, em apoptose e em necrose.* c) Âmbito onde se desenrolou o estudo: *Universidade do Porto, Portugal.* d) Características da população estudada: *Sob consentimento informado, estudaram-se 19 homens azoospermicos (5 com anejaculação, 7 com azoospermia obstrutiva, 7 com azoospermia secretora) e, como controlo, 6 homens normozoospermicos. Todos apresentam cariótipos normais e ausência de microdelecções do cromossoma Y.* e) Principais intervenções realizadas: *Foi realizada uma biopsia testicular de tratamento de modo a recuperar espermátides e espermatozóides testiculares. Nas amostras seminais o estudo da translocação da fosfatidilserina foi efectuado na fracção do sêmen e, após centrifugação por gradientes e swim-up, na fracção de swim-up. Após incubação das amostras com anexina V e iodeto de propídio, quantificou-se, ao microscópio de epifluorescência, os diferentes tipos de células.* f) Principais variáveis valorizadas: *Determinou-se a percentagem de espermátides e espermatozóides vivos, em apoptose inicial, apoptose tardia e em necrose nos diferentes grupos. As diferenças entre grupos foram determinadas através do teste (2 a um nível de significância de 5%).* g) Resultados: *O testículo apresenta percentagens superiores de espermátides e espermatozóides em apoptose inicial e em necrose, e o sêmen percentagens superiores de espermatozóide vivos e em apoptose tardia. Comparações entre as síndromes testiculares, revelaram proporções superiores de células vivas na azoospermia secretora seguida da obstrutiva, e per-*

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Apoios
FCT (60709/04, 60555/04, 59997/04, UMIB).

centagens significativamente superiores de células em apoptose inicial na anejaculação seguida da azoospermia obstrutiva. h) Conclusões: A presença de taxas de apoptose superiores no testículo, nomeadamente na anejaculação e na azoospermia obstrutiva, sugere um elevado impacto negativo da ausência de ejaculação e da obstrução dos canais excretores, nos espermatídeos e espermatozóides testiculares. Os valores aumentados de apoptose tardia na fracção de sémen sugerem a presença de um mecanismo pós-testicular de activação da cascata apoptótica.

Palavras-chave: Espermatogénese humana. Translocação da fosfatidilserina. Anexina V. Testículo. Sémen.

Summary:

a) Objectives: *Determination of the rates of apoptosis on spermatid and spermatozoa from men with azoospermia.* b) Study design: *Quantitative evaluation of phosphatidylserine translocation with distinction between live, apoptotic and necrotic cells.* c) Study localization: *University of Porto, Portugal.* d) Characteristics of the study population: *Under informed consent, 19 azoospermic men were analysed (5 with anejaculation, 7 with obstructive azoospermia, 7 with secretory azoospermia) and 6 normozoospermic men as controls. All have normal karyotypes and absence of Y chromosome microdeletions.* e) Main interventions: *A testicular biopsy was undertaken in order to recover testicular spermatids and spermatozoa. On semen samples, translocation of phosphatidylserine was studied on the semen fraction and, after gradient centrifugation and swim-up, on the swim-up fraction. After incubation with annexin V and propidium iodide, the different cells types were quantified on an epifluorescence microscope.* f) Main outcomes: *The percentage of live, early apoptotic, late apoptotic and necrotic spermatids and spermatozoa, was evaluated on the different groups. Differences between groups were analysed by (2 test with 5% significance level.* g) Results: *Testis shows higher percentages of early apoptotic and necrotic spermatids and spermatozoa, and semen shows higher percentages of live and late apoptotic spermatozoa. Comparisons between testicular syndromes revealed significant higher proportions of live cells in secretory azoospermia followed by obstructive, and significant higher percentages of early apoptotic cells in anejaculation followed by obstructive azoospermia.* h) Conclusions: *The higher percentages of apoptotic rates on testis, especially on anejaculation and obstructive azoospermia, suggest a strong negative impact of the absence of ejaculation and of excretory duct obstruction on testicular spermatids and spermatozoa. The increased rates of late apoptosis on the semen fraction, suggest a post-testicular event that activates the apoptotic cascade.*

Key words: Human Spermatogenesis. Phosphatidylserine translocation. Annexin V. Testis. Semen.

INTRODUÇÃO

A espermatogénese compreende uma série de eventos, desde a proliferação e diferenciação das espermatogónias, a divisão meiótica dos espermatócitos e a diferenciação e maturação dos espermatozóides (1). Durante este processo, mais de metade das células morre por apoptose antes de se diferenciarem em espermatozóides (2-5)

A apoptose ou morte celular programada é um mecanismo essencial na manutenção de uma homeostasia celular. O seu papel na espermatogénese tem sido largamente estudado (6) tendo sido já descrito, em animais, a presença de um mecanismo apoptótico activo em cada estadio da espermatogénese (7). Sabe-se

que é responsável pelo ajuste do número de células germinais à capacidade de suporte das células de Sertoli, assim como pela eliminação de células anormais ou danificadas (8).

São várias as alterações que ocorrem numa célula apoptótica, desde a redução do volume celular, a formação de corpos apoptóticos, a fragmentação do invólucro nuclear e a condensação e marginalização periférica da cromatina seguida de digestão internucleosómica do DNA (9). No entanto, uma das alterações morfológicas mais importantes e precoces na apoptose é a translocação do fosfolípido fosfatidilserina do folheto interno para o folheto externo da membrana citoplasmática (10-12). Esta alteração precoce ocorre após a activação das caspases (13), principal família de proteínas envolvida no processo

apoptótico, e é responsável pelo reconhecimento e fagocitose das células apoptóticas pelos macrófagos de modo a evitar um processo inflamatório (12, 14). Na espermatogénese, o reconhecimento e fagocitose das células germinais apoptóticas pelas células de Sertoli é efectuado de uma maneira dependente da fosfatidilserina, sendo este processo de fagocitose essencial para uma eficiente produção de espermatozóides (15).

Foram recentemente descritas, pelo nosso grupo de trabalho, as percentagens de apoptose no sêmen e na fracção de tratamento swim-up (16). Através do estudo da actividade da caspase-3, demonstramos a presença de caspase-3 activa unicamente na peça intermédia e em percentagens superiores em situações de astenozoospermia e de teratozoospermia. Estes resultados sugerem, então, que a maquinaria apoptótica se encontra activa no sêmen de modo a eliminar as células anormais devido a defeitos mitocondriais e do citoesqueleto não correspondendo, portanto, nem ao sequestro da enzima nos corpos residuais (17, 18) nem a um mecanismo de apoptose abortiva (19, 20). Permanece, no entanto, por descrever, as percentagens de apoptose nos espermatídeos alongados e espermatozóides nas situações de azoospermia.

Este estudo teve como objectivo determinar as percentagens de apoptose nas diferentes patologias testiculares (anejaculação, azoospermia obstructiva e azoospermia secretora). Para tal, recorreu-se ao estudo da translocação da fosfatidilserina, alteração que determina a eliminação das células apoptóticas por fagocitose. A expressão deste marcador apoptótico foi, adicionalmente, determinada a nível seminal.

MATERIAL E MÉTODOS

Seleção dos Pacientes

Foram estudados, sob consentimento informado, 19 homens azoospermicos em tratamento por infertilidade: 5 com anejaculação, 7 com azoospermia obstructiva - 4 CBAVD, 3 obstrução secundária, e 7 com azoospermia secretora - hipoespermatogénese (idade média 32.37 ± 3.06 ; range 28-40). Todos apresentam cariótipos normais (21-22) e ausência de microdelecções no cromossoma Y (23-24). De modo a recuperar espermatídeos ou espermatozóides para os tratamentos clínicos, foram submetidos a uma biopsia testicular de tratamento (25).

O grupo controlo consistiu em 6 homens (idade média 31.50 ± 1.05 ; range 30-33) com parâmetros seminais normais (concentração $\geq 20 \times 10^6/\text{ml}$; morfologia normal $\geq 15\%$; mobilidade progressiva rápida $\geq 25\%$), cariótipos normais e ausência de microde-

lecções no cromossoma Y. A determinação dos parâmetros seminais foi efectuada segundo as indicações da Organização Mundial de Saúde (26) e os critérios estritos de Kruger para a morfologia normal dos espermatozóides.

Preparação do tecido testicular

Após biopsia testicular de tratamento, o tecido foi colocado em Sperm Preparation Medium (SPM, tampão Hepes; Medicult) procedendo-se à sua dissociação mecânica, com o auxílio de bisturis cirúrgicos, de modo a libertar os espermatídeos e espermatozóides testiculares (27). O fluido resultante foi diluído em SPM e lavado duas vezes por centrifugação a 1200 rpm durante 5min. Foi removida uma média de 65 μl (range: 20-155 μl) de suspensão celular para estudo, no próprio dia da biopsia, da translocação da fosfatidilserina.

Preparação das amostras seminais

Cada paciente contribuiu apenas com uma amostra. Após liquefacção (37°C, 30min) e determinação dos parâmetros do espermograma, cada amostra foi dividida em duas fracções. Uma pequena fracção foi lavada com SPM (1500 rpm, 20min, temperatura ambiente) de modo a remover o líquido seminal. Removeu-se uma média de 251 μl (range: 180-265 μl) para determinação da fosfatidilserina translocada na fracção de sêmen. O restante foi sujeito à técnica de centrifugação por gradientes e swim-up. A amostra foi centrifugada (1500 rpm, 20min, temperatura ambiente) em gradientes Ixaprep (Medicult, Copenhagen, Dinamarca) de modo a remover o líquido seminal e as células imaturas. O centrifugado foi lavado duas vezes em SPM (1500 rpm, 10min, temperatura ambiente), recoberto com 50-100 μl SPM e incubado (1h, 37°C, 5% CO₂ em ar humedecido) para recolher na fase aquosa superior os espermatozóides móveis (swim-up). Foi removido uma média de 191 μl (range: 55-265 μl) de modo a determinar a translocação da fosfatidilserina na fracção de swim-up. Todo o procedimento foi efectuado no próprio dia da colheita.

Deteccção da translocação da fosfatidilserina

O estudo da translocação da fosfatidilserina do folheto interno para o folheto externo da membrana citoplasmática foi efectuado através do uso da proteína anexina V (An) que, na presença de cálcio, apresenta elevada afinidade para a fosfatidilserina (28). Quando conjugada com um fluorocromo, permite a

detecção da translocação da fosfatidilserina para o folheto externo da membrana citoplasmática, característica de uma célula num estado inicial de apoptose. Foi utilizado o Annexin V-FITC Apoptosis Detection Kit (Calbiochem, San Diego, USA) onde, a anexina V conjugada com o FITC (An-FITC), serve de marcador fluorescente para as células apoptóticas (emissão de cor verde). As células foram adicionalmente incubadas com o iodeto de propídio (IP), que é um corante vital para o qual a membrana citoplasmática não é permeável. Deste modo, apenas nas fases de perda de permeabilidade membranar (apoptose tardia e necrose), o IP é incorporado pela célula ocorrendo a emissão de fluorescência vermelha.

Quando necessário, foi adicionado ao volume inicial da amostra PBS (Phosphate Buffered Saline Solution, Sigma, St. Louis, USA) de modo a perfazer um volume final de 250µl. A amostra foi incubada (15min, temperatura ambiente) com 10µl de Media Binding Reagent (meio que favorece a ligação entre a anexina V e a fosfatidilserina) e AnV-FITC numa concentração final de 0.96µg/ml. Após centrifugação da amostra (900 rpm, 10 min, temperatura ambiente), ressuspendeu-se o pellet lentamente em 100µl de tampão previamente mantido no gelo. Finalmente adicionou-se o IP numa concentração final de 0.075µg/ml.

A observação dos resultados foi efectuada num microscópio de epifluorescência (Eclipse E-400; Nikon, Tóquio, Japão) com câmara CCD (Sony, Tóqui, Japão) e software apropriado para análise de imagem (Cytovision Ultra, Applied Imaging International, Sunderland, UK), tendo sido contabilizados cerca de 100 espermátides/espermatozóides por amostra testicular e 100 espermatozóides por amostra seminal.

A utilização simultânea da anexina V e do iodeto de propídio, permitiu distinguir quatro tipos de células: células vivas (An- IP-), células em apoptose inicial (An+ IP-), células em apoptose tardia (An+ IP+) e células necróticas (An- IP+) (Figura 1).

Análise estatística

Os resultados foram analisados ao nível celular onde o indivíduo de estudo é cada espermatozóide contabilizado. A determinação, entre grupos, da significância das diferenças entre a presença de células vivas, necróticas e apoptóticas, foi efectuada através do teste (2 a um nível de significância de 5%.

RESULTADOS

Testículo

Foi contabilizado um total de 1908 espermátides/espermatozóides, 512 na anejaculação, 724 na azoospermia obstrutiva (Azo) e 672 na azoospermia secretora (Azs) (tabela 1).

O estudo comparativo entre as três patologias testiculares, revelou a presença de percentagens significativamente superiores de células vivas na Azs ($P < 0.012$) seguida da Azo ($P < 0.0001$), relativamente à anejaculação, e de células em apoptose inicial na anejaculação ($P < 0.0001$), relativamente às Azo e Azs (figura 2). O estudo comparativo entre a azoospermia secretora e a azoospermia obstrutiva revelou valores significativamente superiores de células vivas na Azs ($P = 0.012$) e de células em apoptose inicial na Azo

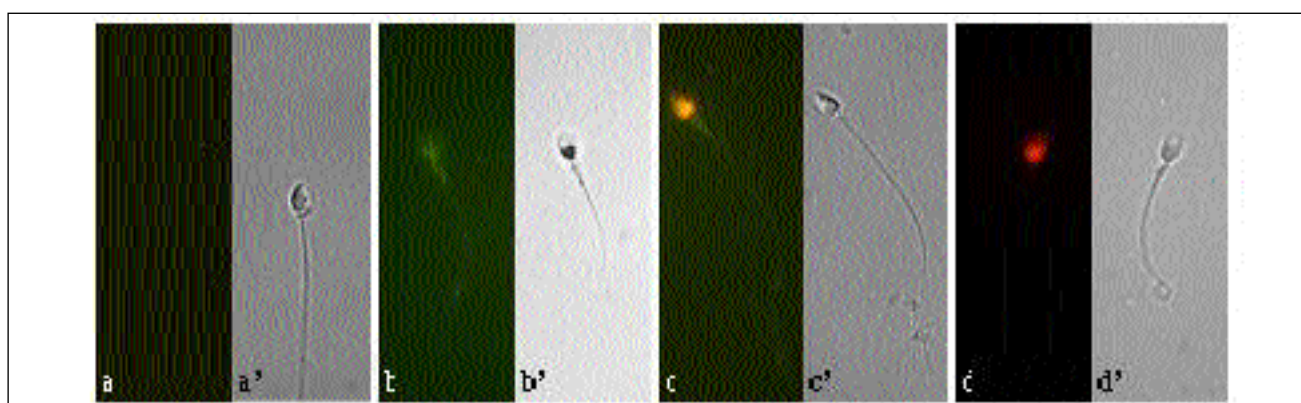


Figura 1

Imagens de fluorescência (a-d) e contraste de fase (a'-d') de espermátides e espermatozóides vivos (a, a'), em apoptose inicial (b, b'), apoptose tardia (c, c') e em necrose (d, d').

Tabela 1

Espermatídeos e espermatozóides vivos, em apoptose e em necrose nos grupos de patologia testicular.

	Anejaculação			Azo			Azs			Total		
	(n)	(%) ^a	P*	(n)	(%) ^a	P*	(n)	(%) ^a	P*	(n)	(%) ^a	P*
AnV-IP-	107	20.9 ± 21.8	<0.0001	215	29.7 ± 9.9	<0.0001	24	236 ± 23.6	<0.0001	564	29.6 ± 18.8	<0.0001
AnV+IP-	268	52.3 ± 22.6	<0.0001	298	41.2 ± 10.5	<0.0001	228	33.9 ± 16.8	<0.0001	794	41.6 ± 17.1	<0.0001
AnV-IP+	121	23.6 ± 12.2	<0.0001	190	26.2 ± 6.9	<0.0001	187	27.8 ± 13.2	0.006	498	26.1 ± 10.5	<0.0001
AnV+IP+	16	3.1 ± 3.8	0.041	21	2.9 ± 4.3	NS	15	2.2 ± 2.0	NS	52	2.7 ± 3.3	0.040
^a Média±DP												
*Versus Sémen												

(P = 0.006). Não se observaram diferenças significativas, entre os grupos, para a presença de células em apoptose tardia e em necrose (figura 2).

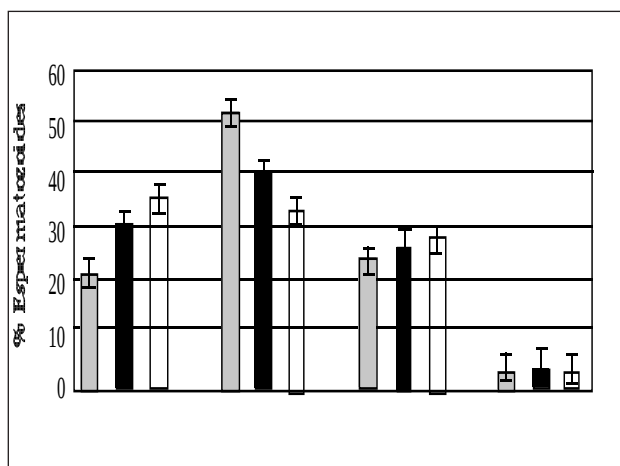


Figura 2

Diferenças para a presença de espermatídeos e espermatozóides vivos (AnV- IP-), em apoptose inicial (AnV- IP-), tardia (AnV+ IP+) e em necrose (AnV- IP+) entre os grupos de patologia testicular. *P<0.0001 (vs anejaculação); # P<0.012 (Azo vs Azs)

Sémen

O grupo controlo consistiu em 6 pacientes com parâmetros seminais normais, tendo sido realizada a técnica de centrifugação por gradientes e swim-up para cada amostra seminal.

Foi contabilizado um total de 674 espermatozóides na fracção de sémen e de 644 espermatozóides na fracção de swim-up (tabela 2). O estudo comparativo entre as duas fracções revelou um aumento significativo (P < 0.0001) da percentagem de células vivas após a realização da técnica de centrifugação por gradientes e swim-up, assim como uma redução signifi-

cativa da percentagem de células em apoptose inicial (P<0.0001) e em necrose (P=0.021) (tabela 1). Não se verificaram diferenças para a percentagem de células em apoptose tardia.

Testículo vs Sémen

Tabela 2

Espermatozóides vivos, em apoptose e em necrose nas fracções de sémen e de swim-up

	Fracção Sémen		Fracção Swim-Up		P*
	(n)	(%) ^a	(n)	(%) ^a	
AnV-IP-	314	46.6 ± 9.9	393	61 ± 12.4	<0.0001
AnV+IP-	116	17.2 ± 7.4	34	5.3 ± 2.3	<0.0001
AnV-IP+	235	34.9 ± 13.1	216	33.5 ± 10.9	NS
AnV+IP+	9	1.3 ± 1.8	1	0.2 ± 0.4	0.021
^a Média±DP					
*Sémen vs Swim-Up					

Os resultados obtidos ao nível testicular para a presença de células vivas, em apoptose inicial, tardia e em necrose, foram comparados com os resultados obtidos na fracção de sémen e swim-up. Este estudo revelou a presença de percentagens significativamente superiores de espermatozóides vivos (P<0.0001) no sémen e de espermatozóides em apoptose (P<0.0001) e em necrose (P=0.040) no testículo (tabela 1). No entanto, verifica-se que o aumento de células em apoptose no testículo, se deve apenas à presença de espermatozóides em apoptose inicial, apresentando o sémen percentagens significativamente superiores de espermatozóides em apoptose tardia (P<0.0001) (Figura 3). Os mesmos resultados se observaram quando comparando com a fracção de swim-up. Ao comparar especificamente cada patologia testicular com a fracção de sémen, observaram-se resultados semelhantes, excepto a ausência de diferenças significativas para a presença de células necróticas na Azo (P=0.063) e na Azs (P=0.225) relativamente ao sémen (tabela 1).

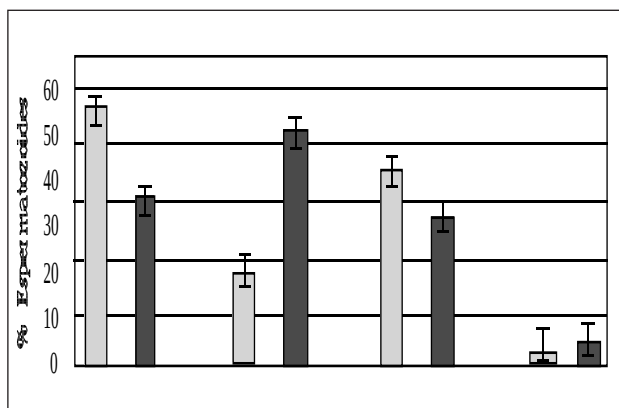


Figura 3

Diferenças para a presença de espermatozoides vivos (AnV- IP-), em apoptose inicial (AnV- IP-), tardia (AnV+ IP+) e em necrose (AnV- IP+) entre a fracção de sémén e o testículo.

* $P < 0.0001$, # $P = 0.040$

DISCUSSÃO E CONCLUSÕES

A apoptose é um mecanismo essencial no decorrer da espermatogénese. A exposição da fosfatidilserina no folheto externo da membrana citoplasmática é um marcador essencial no reconhecimento e eliminação de células germinais anormais pelas células de Sertoli de modo a permitir o desenvolvimento e a produção normal de espermatozoides.

Pensa-se que um dos factores envolvidos na alteração do funcionamento normal da espermatogénese, com consequente aparecimento de diferentes patologias (alteração dos parâmetros seminais e azoospermia), é a desregulação do mecanismo apoptótico (29). Estudos anteriores demonstraram a presença de percentagens aumentadas de apoptose em diferentes patologias testiculares, através da observação de percentagens elevadas de fragmentação do DNA (30 - 33) HT4, HT28, HT21, HT42) e do aumento da expressão das proteínas Fas/FasL (33, 34) (HT42, HT17).

A presença de fosfatidilserina translocada foi observada em todos os estadios da espermatogénese de indivíduos azoospermicos (35, 36), HT5/34 tendo-se verificado que a sua exposição raramente ocorre nos estadios de ST1, Sa, Sb e Sc (3.9%, range: 0-16%) comparativamente com o observado na fase de espermátidio alongado (49.3%, range: 20-54%) (35). No entanto, nem sempre houve uma clara definição do grupo de estudo, sendo avaliado um reduzido número de células.

Deste modo, e através do uso de uma proteína de elevada afinidade para a fosfatidilserina, a anexina V,

descrevemos, pela primeira vez, as percentagens de espermátides alongados e espermatozoides com fosfatidilserina translocada, em pacientes azoospermicos, assim como em pacientes com parâmetros seminais normais.

No presente trabalho, demonstramos a presença de percentagens significativamente superiores de espermatozoides vivos no sémén (46.6% vs 29.6%) e de espermátides e espermatozoides em apoptose inicial (41.6% vs 17.2%) e em necrose (2.7% vs 1.3%) no testículo, facto que sugere o envolvimento da apoptose nas diferentes patologias testiculares. No entanto, verificaram-se percentagens significativamente superiores de espermatozoides em apoptose tardia no sémén (34.9%) relativamente ao testículo (26.1%). Deste modo, e tal como Greco et al sugeriu através da demonstração de percentagens de fragmentação do DNA superiores no sémén (23.6%) relativamente ao testículo (4.8%) (37), confirma-se a presença de um factor pós-testicular de indução da activação da cascata apoptótica, que resulta na presença de espermatozoides numa fase avançada de degradação no ejaculado. Foi sugerido, recentemente, um eficiente tratamento de infertilidade com a administração oral de vitamina C e E (38) ou com diclofenac (39), nos casos de elevados níveis de apoptose no sémén.

A comparação entre os diferentes grupos de patologia testicular demonstrou a presença de percentagens significativamente superiores de espermátides e espermatozoides vivos (36%) nos casos de patologia testicular severa (azoospermia secretora) em comparação com os casos de espermatogénese conservada (29.7%, azoospermia obstrutiva e 20.9%, anejaculação). Por outro lado, a azoospermia obstrutiva e a anejaculação, apresentam níveis superiores de apoptose inicial (41.2% e 52.3%, respectivamente, vs 33.9%). Adicionalmente, não foram encontradas diferenças para a presença de células em apoptose tardia e em necrose. Estes resultados sugerem que, embora o mecanismo apoptótico se encontre activo no sentido de eliminar as células numa fase avançada de degradação, a acumulação de espermátides e espermatozoides por obstrução tubular (azoospermia obstrutiva) ou por incapacidade de ejaculação (anejaculação), tem um elevado impacto negativo sobre essas mesmas células conduzindo, então, à activação da cascata apoptótica.

Tal como demonstrado anteriormente (40), a técnica de centrifugação por gradientes e swim-up mostrou-se eficiente na redução da percentagem de espermatozoides em apoptose e em necrose e, consequentemente, no enriquecimento das amostras relativamente à percentagem de espermatozoides vi-

vos. Estes resultados vêm confirmar, novamente, o reduzido risco de selecção de espermatozóides apoptóticos durante os tratamentos clínicos (16).

Em conclusão, demonstramos a presença de percentagens superiores de espermátides alongados e espermatozóides em apoptose inicial e em necrose no testículo. No entanto, e nos casos de espermatogénese conservada, a apoptose parece surgir como consequência e não causa da patologia. No sémen, embora se verifique a presença de percentagens superiores de espermatozóides vivos, as elevadas percentagens de espermatozóides numa fase tardia da apoptose, mesmo na fracção do swim-up, demonstram a importância de aferir o melhor método de selecção de espermatozóides durante os tratamentos clínicos (40) e/ou de optimização da qualidade e funcionamento dos mesmos (41).

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Article

Phosphatidylserine translocation in human spermatozoa from impaired spermatogenesis



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Abstract

The aim of the present study was to evaluate phosphatidylserine translocation in specific patient groups and compare the rates of apoptosis between ejaculated and testicular spermatozoa. Fifty-six patients undergoing infertility treatments were included in the present study. Semen samples ($n = 37$) were obtained from cases with normozoospermia ($n = 9$) and abnormal semen parameters ($n = 28$). Testicular biopsy was performed in 19 patients, eight with obstructive and six with non-obstructive (hypospermatogenesis) azoospermia, and in five patients without azoospermia (anejaculation and oligozoospermia). Phosphatidylserine externalization was assessed using annexin-V binding and fluorescence microscopy, and propidium iodide exclusion tests were used to distinguish live from dead cells. In semen, oligoasthenoteratozoospermia showed significantly increased rates of sperm apoptosis (60.3 ± 12.9) than normozoospermia (47.5 ± 10.2). In testis, hypospermatogenesis (63.3 ± 10.3) and obstructive azoospermia (63.6 ± 15.1) showed significantly increased rates of sperm apoptosis than non-azoospermic patients (49.6 ± 25.5). Comparisons between semen and testis showed that oligozoospermia had significantly higher rates of sperm apoptosis in semen (57.9 ± 11.9) than in testis (29.4 ± 1.1). The results suggest the presence of a post-testicular apoptotic induction factor and the potential beneficial use of testicular spermatozoa in clinical treatments.

Keywords: *anejaculation, annexin-V, azoospermia, human sperm apoptosis, oligozoospermia*

Introduction

Apoptosis is a mechanism essential for normal development and homeostasis. Loss of plasma membrane asymmetry due to phosphatidylserine externalization is an early event of the execution phase of apoptosis that occurs after activation of caspases, the principal family of proteins involved in the apoptotic process, and before loss of plasma membrane integrity. This alteration is responsible for the recognition and phagocytosis of apoptotic cells by macrophages, in order to avoid an inflammatory response (Martin *et al.*, 1995; Naito *et al.*, 1997; Van den Eijnde *et al.*, 1997). Apoptosis also regulates human spermatogenesis, being essential to an efficient production of sperm through the adjustment

of the number of germ cells to the number of Sertoli cells and removal of the abnormal ones (Rodriguez *et al.*, 1997; Print and Loveland, 2000; Kierszenbaum, 2001). In this process, the Fas ligand secreted by Sertoli cells binds the Fas receptor in apoptotic germ cells, leading to activation of the caspase cascade (Pentikainen *et al.*, 1999), with translocation of phosphatidylserine from the inner to the outer membrane layer triggering the recognition and phagocytosis of apoptotic germ cells by Sertoli cells (Shiratsuchi *et al.*, 1997; Nakanishi and Shiratsuchi, 2004).

Previous studies demonstrated the presence of increased levels of oxidative stress, DNA fragmentation and caspase activity in human ejaculated spermatozoa from men with

abnormal semen parameters (Barroso *et al.*, 2000; Shen *et al.*, 2002; Weng *et al.*, 2002; Wang *et al.*, 2003; Marchetti *et al.*, 2004; Oosterhuis *et al.*, 2000; Taylor *et al.*, 2004; Almeida *et al.*, 2005; Varum *et al.*, 2007). The presence of translocated phosphatidylserine in human ejaculated spermatozoa has also been studied. In semen, increased rates of externalized phosphatidylserine were associated with decreased sperm motility (Oosterhuis *et al.*, 2000; Shen *et al.*, 2002; Weng *et al.*, 2002; Barroso *et al.*, 2006; Varum *et al.*, 2007), normal morphology (Shen *et al.*, 2002) or concentration (Ricci *et al.*, 2002), whereas no association to abnormal semen parameters was found by others (Barroso *et al.*, 2000; Ricci *et al.*, 2002; Lachaud *et al.*, 2004; Martin *et al.*, 2005). These contradictory results mostly derived from the criteria used for group classification, which were based on individual semen parameters without taking into consideration all of them together.

Conflicting results have also resulted from the study of apoptosis in human testicular germ cells from men with different testicular pathologies. In these studies, where the purpose was to analyse the whole spermatogenic cycle, the seminiferous germ cells were labelled and counted, without any particular quantification of spermatozoa. The other major problem of these studies relates to the fact that they have not used all subtypes of azoospermia and the same type of control group. Overall, and depending on the type of intergroup comparisons, all phenotypes gave an increased rate of DNA fragmentation, activated caspase activity, Fas and FasL expression, in anejaculation, obstructive azoospermia, hypospermatogenesis, post-meiotic arrest at the early and late spermatid stage, meiotic arrest and Sertoli cell-only syndrome (Tesarik *et al.*, 1998; Francavilla *et al.*, 2000, 2002; Takagi *et al.*, 2001; Eguchi *et al.*, 2002; Ramos *et al.*, 2002; Kim *et al.*, 2007; Tesarik *et al.*, 2004; Lin *et al.*, 1997). The presence of translocated phosphatidylserine in human testicular germ cells has also been studied, although yielding contradictory results for the same motives. In one study, increased concentrations of translocated phosphatidylserine were found in germ cells from cases with meiotic arrest and post-meiotic arrest at the late spermatid stage in comparison with Sertoli cell-only syndrome and hypospermatogenesis (Tesarik *et al.*, 2004), whereas another study found no increased rates of translocated phosphatidylserine in germ cells from cases with post-meiotic arrest at the early and late spermatid stage (Tesarik *et al.*, 1998).

This study quantifies the rates of phosphatidylserine translocation in spermatozoa from semen samples from normal subjects and with different semen parameter abnormalities. Additionally, analysis was applied to testicular spermatozoa from azoospermic patients with different spermatogenesis phenotypes, and to non-azoospermic patients.

Materials and methods

Patients

After institutional approval and patient informed consent, sperm samples were collected from a total of 56 patients undergoing infertility treatments. Ejaculated spermatozoa

were obtained from 37 cases, nine with normal semen parameters (controls) [$111.2 \pm 62.3 \times 10^6$ spermatozoa/ml, $17.5 \pm 2.7\%$ sperm normal morphology (NM), $47.7 \pm 14.0\%$ sperm rapid progressive motility (RPM)], and 28 with abnormal semen parameters, 12 with teratozoospermia ($100.3 \pm 43.0 \times 10^6$ spermatozoa/ml, $7.5 \pm 3.3\%$ NM, $41.7 \pm 10.9\%$ RPM), one with oligozoospermia (17.6×10^6 spermatozoa/ml, 17% NM, 60.6% RPM), one with oligoteratozoospermia (8×10^6 spermatozoa/ml, 7% NM, 40.5% RPM), eight with asthenoteratozoospermia ($160.4 \pm 237.6 \times 10^6$ spermatozoa/ml, $5.7 \pm 3.3\%$ NM, $14.0 \pm 5.2\%$ RPM) and six with oligoasthenoteratozoospermia ($7.8 \pm 5.8 \times 10^6$ spermatozoa/ml, $5.2 \pm 4.0\%$ NM, $15.2 \pm 7.2\%$ RPM). No patients had cryptorchidism, past evidence of genital infections, or abnormalities of hormone serum concentrations or testicular volume. They were studied only at the ejaculate level, as they had no indication for testicular sperm retrieval. Testicular spermatozoa were obtained from 19 patients, eight with obstructive azoospermia [four with primary obstructive azoospermia (OAZ) due to congenital bilateral absence of the vas deferens (CBAVD) four with secondary OAZ due to inflammatory obstruction of the epididymis], six with non-obstructive azoospermia (hypospermatogenesis) and five without azoospermia [three with anejaculation due to spinal cord injury; two with oligozoospermia, which after ICSI failures in previous treatment attempts with ejaculated spermatozoa underwent testicular sperm extraction (TESE)]. In these two cases of oligozoospermia, only explorative data on testicular samples exist. In all cases, males had normal karyotypes (Pinho *et al.*, 2005) and absence of Y chromosome microdeletions (Ferrás *et al.*, 2004).

Sperm collection and preparation

Testicular sperm retrieval or TESE by open biopsy were used, depending on the pathology (Sousa *et al.*, 2002). Testicular fragments were gently squeezed avoiding spermatozoa damage, and the resultant fluid was washed twice with sperm preparation medium (SPM; Medicult, Copenhagen, Denmark). A small part of the samples was taken for study. After liquefaction, semen samples were used for spermogram evaluation (World Health Organization, 1999) by WHO and Kruger criteria, and a part was taken for the present study. Study samples were divided into a total fraction (neat semen) and a swim-up fraction. For the neat fraction, the ejaculate was washed with SPM. For the swim-up fraction, the ejaculate was submitted to gradient centrifugation (Ixaprep; Medicult), the pellet washed twice with SPM and incubated for 1 h at 37°C , 5% CO_2 in humidified air.

Evaluation of phosphatidylserine translocation

Phosphatidylserine externalization was studied after sample collection and preparation. A pre-evaluation with trypan blue (0.4% trypan blue solution; Sigma, Barcelona, Spain) was made to control the integrity of the samples (**Table 1**). Phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane was analysed with the

Table 1. Vitality of samples measured by trypan blue exclusion test.

<i>Semen sample</i>	<i>Control</i>	<i>T</i>	<i>A</i>	<i>O</i>	<i>PT</i>	<i>AT</i>	<i>OAT</i>
Neat fraction	90.5 ± 10.3	79.5 ± 9.7	78.9 ± 9.4	75.4 ± 9.8	80.2 ± 10.8	82.7 ± 6.3	73.7 ± 10.9
Swim-up fraction	97.9 ± 2.2	94.9 ± 5.1	93.8 ± 5.5	96.7 ± 3.1	95.6 ± 4.7	92.3 ± 6.5	95.9 ± 3.2
<i>Testicular sample</i>	<i>Non-azoospermia</i>		<i>OAZ</i>		<i>SAZ</i>		
	<i>Total</i>	<i>AN</i>	<i>O</i>	<i>Total</i>	<i>CBAVD</i>	<i>IEO</i>	<i>HP</i>
	95.6 ± 4.2	98.2 ± 2.6	91.8 ± 2.5	93.9 ± 5.6	95.3 ± 2.9	92.3 ± 6.5	90.8 ± 9.6

Values are mean % ± SD.

Teratozoospermia (T), asthenozoospermia (A), oligozoospermia (O), cases with teratozoospermia and normal values of sperm concentration and sperm rapid progressive motility (PT), asthenoteratozoospermia (AT) and oligoasthenoteratozoospermia (OAT).

Obstructive azoospermia (OAZ), anejaculation (AN), oligozoospermia (O), congenital bilateral absence of the vas deferens (CBAVD), secondary OAZ due to epididymal inflammation (IEO), secretory azoospermia (SAZ) with hypospermatogenesis (HP).

Annexin V-FITC Apoptosis Detection Kit (Calbiochem, San Diego, CA, USA), where annexin-V (An), a protein with high affinity to phosphatidylserine in the presence of calcium (Van Engeland *et al.*, 1998), is conjugated with the fluorochrome FITC. In the presence of externalized phosphatidylserine, annexin-V conjugates with phosphatidylserine and the emission of a green fluorescence allows the distinction between apoptotic and non-apoptotic cells. Samples were simultaneously incubated with propidium iodide (PI) (Calbiochem), a non-permeable vital dye. Thus, when plasma membrane loses its integrity, propidium iodide is incorporated by the cell with emission of red fluorescence.

A volume of 250 µl of sample was incubated (15 min, room temperature) with 10 µl of media binding reagent and 1.25 µl of annexin-V (0.96 µg/ml final concentration). When necessary, PBS (phosphate-buffered saline solution; Sigma, St. Louis, USA) was added to the initial sample in order to have the final volume of 250 µl. After centrifugation (10 min, 86 g, room temperature), a volume of 100 µl of ice-cold buffer was added to the pellet. Finally, 0.25 µl of propidium iodide (0.075 µg/ml final concentra-

tions) was added to the previous mixture. Four distinct types of cell staining were distinguished: intact cells ($An^{-}PI^{-}$), early apoptotic cells ($An^{+}PI^{-}$), late apoptotic cells ($An^{+}PI^{+}$) and necrotic cells ($An^{-}PI^{+}$) (**Figure 1**). As in some testicular samples, only a low number of spermatozoa could be observed, and in order to have similar samples to compare, the analysis of about 100 cells was set for all samples (testicular and semen samples). Quantification and image analysis was performed with an epifluorescence microscope (Eclipse E-400; Nikon, Tokyo, Japan) fitted with a charge-coupled device camera (Sony, Tokyo, Japan) and an automated image software (Cytovision Ultra; Applied Imaging International, Sunderland, UK).

Statistical analysis

Results were analysed at the cellular level, where each spermatozoon was considered the individual of study. Data are presented as mean percentage values ± SD. Differences between groups of cells and groups of patients were considered significant at $P < 0.05$ (Fisher's exact test, two-sided).

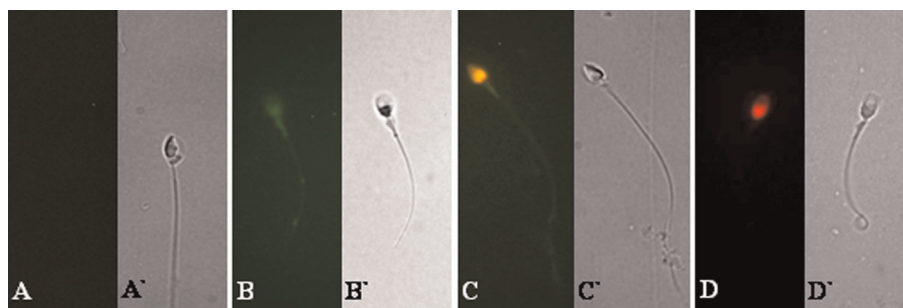


Figure 1. Examples of intact (A and A'), early apoptotic (B and B'), late apoptotic (C and C') and necrotic (D and D') spermatozoa. Images in fluorescence microscopy (A–D) and phase contrast (A'–D').

All analyses were performed using the Statistical Package for Social Sciences (SPSS version 14.0, SPSS, Inc., USA) for Windows.

Results

Neat fraction

Data showed a significant decrease of intact cells in teratozoospermia (T), asthenozoospermia (A) and oligozoospermia (O) in relation to controls ($P < 0.005$), and in O in relation to T and A ($P < 0.023$) (**Table 2**). Similarly, a significant increase of necrotic cells was observed in T, A and O in relation to controls ($P < 0.003$). Analysis of translocated phosphatidylserine showed that the rates of total apoptosis were significantly increased in T, A and O in relation to controls ($P < 0.022$), and in O in relation to T and A ($P < 0.025$). This was due to late apoptosis, with significant increases observed in T, A and O in relation to controls ($P < 0.001$), and in O in relation to T and A ($P < 0.001$). In contrast, decreased levels of early apoptosis were observed in all abnormal samples in relation to controls ($P < 0.001$), and in O in relation to T and A ($P < 0.042$).

In oligoasthenoteratozoospermia (OAT), data showed a significant decrease of intact cells in relation to pure teratozoospermia (PT), asthenoteratozoospermia (AT) and controls ($P < 0.001$), whereas significantly higher rates of necrotic cells were found in PT, AT and OAT in relation to controls ($P < 0.036$, **Table 2**). The rates of total apoptosis were also significantly increased in OAT in relation to PT, AT and controls ($P < 0.001$). This was due to late apoptosis, with significant increases observed in PT and OAT in relation to controls ($P < 0.001$), and in OAT in relation to PT and AT ($P < 0.01$). In contrast, decreased levels of early apoptosis were observed in PT, AT and OAT in relation to controls ($P < 0.011$), and in OAT in relation to AT ($P = 0.009$).

Swim-up fraction

Data showed a significant decrease of intact cells in A and O in relation to controls ($P < 0.001$), in O in relation to T and A ($P < 0.011$), and in A in relation to T ($P = 0.013$) (**Table 2**). A significant increase of necrotic cells was also observed in T, A and O in relation to controls ($P < 0.327$). Similarly, the rates of total apoptosis were significantly increased in A and O in relation to controls ($P < 0.003$), in O in relation to T and A ($P < 0.009$), and in A in relation to T ($P = 0.004$). This was exclusively due to late apoptosis, with significant increases observed in A and O in relation to controls ($P < 0.008$), in O in relation to T and A ($P < 0.042$), and in A in relation to T ($P = 0.036$).

OAT showed a significant decrease in the rates of intact cells in relation to PT, AT and controls ($P < 0.001$), and a significant increase in the rates of necrotic cells in relation to controls ($P = 0.007$, **Table 2**). The rates of total apoptosis were significantly decreased in PT ($P = 0.037$) and increased in OAT ($P < 0.001$) in relation to controls, and

in OAT in relation to PT and AT ($P < 0.001$). This increase was due to late apoptosis, with significant increases observed in OAT in relation to PT, AT and controls ($P < 0.001$). In contrast, early apoptosis was significantly increased in OAT in relation to PT ($P = 0.027$).

In comparison with the neat fraction, spermatozoa from the control group purified by gradient centrifugation followed by swim-up showed a notably significant increase in the rates of intact cells ($P < 0.001$). This was associated with a decreased in the rates of necrotic ($P = 0.006$) and total apoptotic cells ($P < 0.001$), and also in early apoptosis ($P < 0.001$). Comparisons between abnormal semen samples showed similar results, albeit with considerable decreases in both early and late apoptotic except for oligozoospermia, which showed no decreases in early apoptosis.

Testicular samples

Analysis of testicular retrieved spermatozoa (**Table 3**) showed a significant decrease of intact cells in OAZ and hypospermatogenesis (HP) in relation to non-azoospermic samples ($P < 0.001$). Similarly, the rates of total apoptosis were significantly increased in OAZ and HP in relation to non-azoospermic samples ($P < 0.001$). This was exclusively due to late apoptosis, with significant increases observed in OAZ and HP ($P < 0.002$). The same findings were observed when non-azoospermic samples were compared with primary OAZ due to congenital bilateral absence of the vas deferens (CBAVD) and secondary OAZ due to inflammatory obstruction of the epididymis (IEO). CBAVD also showed significant increased rates of necrotic cells and late apoptosis in relation to IEO ($P < 0.039$), and of late apoptosis in relation to HP ($P = 0.031$).

Anejaculation showed a significantly decreased rate of intact cells and increased rates of total apoptosis and early apoptosis in relation to OZ ($P < 0.001$), as well as an increased rate of early apoptosis and a decreased rate of late apoptosis in relation to OAZ ($P < 0.003$), CBAVD ($P < 0.001$), IEO ($P < 0.048$) and HP ($P < 0.021$). In contrast, and in comparison with OZ, decreased rates of intact cells ($P < 0.001$) and increased rates of total, early and late apoptosis were observed in OAZ ($P < 0.001$), CBAVD ($P < 0.001$), IEO ($P < 0.026$) and HP ($P < 0.016$).

Testicular spermatozoa were then compared with control samples from the neat fraction of ejaculated spermatozoa. Testicular spermatozoa from non-azoospermic cases showed similar rates of intact and total apoptosis cells, but significantly increased rates of early apoptosis and decreased rates of late apoptosis ($P < 0.001$) and also significantly increased rates of necrotic cells ($P = 0.004$). Necrotic cells were similarly increased in AN, OAZ and CBAVD ($P < 0.014$). In contrast, intact cells were significantly decreased and total apoptosis was significantly increased in AN, OAZ, CBAVD, IEO and HP. This was due to significant increases of early apoptotic cells, as late apoptosis was significantly decreased ($P < 0.002$). Testicular spermatozoa from oligozoospermic cases represented an exception. They exhibited increased rates of intact cells in relation to

Table 2. Rates of phosphatidylserine translocation in human spermatozoa from the neat semen and after gradient and swim-up purification.

Semen sample	Control		Seminal parameters					
			T	A	O	PT	AT	OAT
No. of patients	9	27	2791	1465	857	1217	820	645
<i>Neat fraction</i>								
No. of spermatozoa	989	2791	45.9 ± 10.9	43.9 ± 12.3	38.9 ± 12.6	48.2 ± 9.3	49.6 ± 7.9	36.3 ± 13.7
Intact	51.4 ± 10.4	51.9 ± 10.2	53 ± 11.9	9.3 ± 7.4	6.6 ± 3.7	8.9 ± 5.4	11.1 ± 8.9	6.9 ± 4.3
Total apoptotic	47.5 ± 10.2	14.9 ± 6.9	32.6 ± 11.1	43.7 ± 13.0	51.3 ± 11.5	40.5 ± 8.6	36.3 ± 7.4	53.4 ± 12.7
Early apoptotic	1.2 ± 1.6	2.8 ± 2.6	3.1 ± 2.8	3.1 ± 1.8	2.4 ± 2.6	2.9 ± 3.4	3.4 ± 2.0	
Late apoptotic								
Necrotic								
<i>Swim-up fraction</i>								
No. of spermatozoa	970	2785	1431	819	1252	822	609	
Intact	63.9 ± 10.9	60.9 ± 14.8	57.1 ± 15.4	51.3 ± 15.4	65.8 ± 13.9	64.1 ± 11.2	47.8 ± 16.1	
Total apoptotic	36 ± 11	38.4 ± 14.6	42 ± 15.1	47.9 ± 14.9	33.7 ± 13.7	35.3 ± 11.1	51.1 ± 15.7	
Early apoptotic	5.1 ± 2.3	5.6 ± 5.9	6.0 ± 7.1	7.4 ± 6.5	4.0 ± 1.7	5.8 ± 8.5	6.4 ± 5.4	
Late apoptotic	30.9 ± 9.6	32.8 ± 13.2	35.9 ± 13.6	40.4 ± 15.7	29.7 ± 12.8	29.4 ± 6.9	44.7 ± 15.9	
Necrotic	0.1 ± 0.3	0.7 ± 1.0	0.9 ± 1.2	0.9 ± 1.1	0.5 ± 0.7	0.7 ± 1.3	1.2 ± 1.2	

Values are mean % ± SD.

Teratozoospermia (T), asthenozoospermia (A), oligozoospermia (O), cases with teratozoospermia and normal values of sperm concentration and sperm rapid progressive motility (PT), asthenoteratozoospermia (AT) and oligoasthenoteratozoospermia (OAT).

AnV = annexin-V, PI = propidium iodide.

Intact = AnV⁻PI⁻, total apoptotic = AnV⁺, early apoptotic = AnV⁺PI⁻, late apoptotic = AnV⁺PI⁺, necrotic = AnV⁻PI⁺.

Table 3. Rates of phosphatidylserine translocation in human testicular spermatozoa.

	Non-azoospermia			OAZ			SAZ
	Total	AN	O	Total	CBAVD	IEO	HP
No. of patients	5	3	2	8	4	4	6
No. of spermatozoa	511	303	208	830	410	420	564
Intact	47.0 ± 23.7	33.2 ± 20.2	67.8 ± 1.1	33.6 ± 14.2	31.6 ± 10.0	35.6 ± 18.9	34.4 ± 9.9
Total apoptotic	49.6 ± 25.5	63.1 ± 24.8	29.4 ± 1.1	63.6 ± 15.1	64.3 ± 12.9	62.9 ± 19.1	63.3 ± 10.3
Early apoptotic	34.3 ± 28.2	47.5 ± 30.5	14.4 ± 2.5	37.8 ± 13.3	35.3 ± 5.5	40.2 ± 19.2	40.6 ± 11.5
Late apoptotic	15.3 ± 5.9	15.6 ± 7.9	14.9 ± 3.6	25.9 ± 6.5	28.9 ± 7.5	22.8 ± 4.3	22.7 ± 4.7
Necrotic	3.3 ± 3.4	3.7 ± 4.7	2.9 ± 0.7	2.8 ± 3.9	4.1 ± 5.7	1.5 ± 0.5	2.3 ± 2.1

Values are mean % ± SD.

Anejaculation (AN), oligozoospermia (O), obstructive azoospermia (OAZ), congenital bilateral absence of the vas deferens (CBAVD), secondary OAZ due to epididymal inflammation (IEO), secretory azoospermia (SAZ) with hypospermatogenesis (HP).

AnV = annexin-V, PI = propidium iodide.

Intact = AnV⁻PI⁻, total apoptotic = AnV⁺, early apoptotic = AnV⁺PI⁻, late apoptotic = AnV⁺PI⁺, necrotic = AnV⁻PI⁺.

controls due to a significant decrease in total apoptotic cells ($P < 0.001$). Testicular spermatozoa from oligozoospermic cases also demonstrated increased rates of intact cells in association with decreased rates of total apoptotic cells in relation to ejaculated spermatozoa from oligozoospermic patients and OAT ($P < 0.001$), while showing increased rates of early apoptosis ($P < 0.003$) and decreased rates of late apoptosis ($P < 0.001$).

Discussion

Phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane is an essential apoptotic feature in the prevention of an inflammatory response. The calcium-dependent glycoprotein annexin-V, which specifically binds to phosphatidylserine, has been used to detect this early apoptotic event (Van Engeland *et al.*, 1998). Previous studies have demonstrated a relationship between increased rates of externalized phosphatidylserine and abnormal seminal parameters in the neat semen, including decreased sperm motility and concentration in regard to total apoptosis (Oosterhuis *et al.*, 2000), as well as decreased sperm motility and normal morphology (Shen *et al.*, 2002) and asthenozoospermia (Varum *et al.*, 2007) only in regard to late apoptosis. Additionally, phosphatidylserine externalization was expressed with a higher frequency in the purified fractions of spermatozoa with low motility (Barroso *et al.*, 2000, 2006; Weng *et al.*, 2002). However, others did not find any correlation between sperm apoptosis and semen quality (Ricci *et al.*, 2002; Lachaud *et al.*, 2004; Martin *et al.*, 2005).

This study evaluated the relationship between semen and testis pathologies and apoptosis through evaluation not only of the total rates of apoptosis but also the rates of apoptosis in an early and a late stage of development.

Data showed significantly higher rates of total and late apoptosis in oligozoospermia, asthenozoospermia and teratozoospermia in spermatozoa from the neat semen in relation to normozoospermic subjects, thus showing a

relationship to all individual parameters. However, and also in relation to normozoospermic subjects, only oligoasthenoteratozoospermia showed significantly higher rates of total and late apoptosis, as pure teratozoospermia only showed significantly higher rates of late apoptosis with no differences observed for asthenoteratozoospermia. This indicates that although apoptosis seemed to be related to all individual parameters, the total rates are significantly increased only when the overall quality of the semen is poor. As samples showing abnormalities only in sperm normal concentration or motility are rarer, while samples with oligozoospermia are most frequently associated with decreased sperm normal motility and morphology, this could also explain the previous findings. Nevertheless, the present data also showed a significant increase of late apoptosis in samples from the neat semen with pure teratozoospermia in relation to normozoospermic subjects, which might suggest an additional relationship.

As the increased rates of total apoptosis were only associated with late apoptosis, data further suggest the presence of an active mechanism responsible for the removal of cells in an advanced degradation stage in cases of normal semen parameters. In counterpart, such mechanism seems to be deficient in men with abnormal semen parameters, especially in those with oligoasthenoteratozoospermia and pure teratozoospermia, where the apoptotic process seems to develop to later stages thus enriching the pool with dying and incapable spermatozoa. On the other hand, purification of spermatozoa by gradient centrifugation and swim-up was shown significantly to decrease the rates of apoptotic cells. Nevertheless, semen samples with asthenozoospermia and, especially, with oligoasthenoteratozoospermia, maintained an increased risk for the presence of apoptotic cells (total and late apoptosis), and only samples with pure teratozoospermia showed a significant loss of risk. This could be explained by the method of selection used, as others had proven not to be the best in the reduction of apoptotic cells (Lachaud *et al.*, 2004).

There are no studies on phosphatidylserine translocation rates in human testicular spermatozoa, with previous

analyses in the human seminiferous epithelium being restricted to the determination of testicular apoptotic indexes by quantifying apoptosis in Sertoli and germ cells. They showed increased rates of phosphatidylserine externalization in meiotic arrest, but not in Sertoli cell-only syndrome, post-meiotic arrest at the round spermatid stage and hypospermatogenesis, as well as contradictory findings in relation to samples with post-meiotic arrest at the late spermatid stage (Tesarik *et al.*, 1998, 2004). This study analysed the rates of phosphatidylserine translocation in testicular spermatozoa from azoospermic men in comparison with non-azoospermic samples and normozoospermic subjects. In regard to non-azoospermic samples, data showed significantly increased rates of total and late sperm apoptosis in obstructive and non-obstructive azoospermia, including in congenital bilateral absence of the vas deferens (CBAVD) and secondary inflammatory obstruction of the epididymis (IEO). CBAVD was also shown to represent the group with significantly higher rates of total and late apoptosis. In contrast, anejaculation (AN) in comparison with normozoospermic subjects revealed a significantly increased rate of total apoptosis due to early apoptosis.

These results suggest a strong negative impact in late spermiogenesis of primary excretory duct obstruction (CBAVD) and absence of sperm clearance (AN), as well as the presence of a post-testicular induction factor that could activate the apoptotic machinery, inducing the presence of deteriorated spermatozoa in the semen. The finding that testicular sperm from oligozoospermia samples showed significantly lower rates of total and late apoptosis than ejaculated spermatozoa of oligozoospermic patients and normozoospermic subjects, further strengthened the present results and confirm previous evidence that testicular spermatozoa might be advantageously used in the treatment of patients with failure of conception using ejaculated spermatozoa (Tesarik *et al.*, 2001; Alvarez, 2003, 2005; Greco *et al.*, 2005).

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PAPER IV

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Caspase-3 detection in human testicular spermatozoa from azoospermic and non-azoospermic patients

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Running title: Active caspase-3 in human testicular sperm

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Abstract

The apoptotic mechanisms underlying spermatogenesis in testis are poorly understood. In the present study, the rates of testicular spermatozoa with active caspase-3 were quantified in testicular samples with conserved and impaired spermatogenesis. Testicular spermatozoa were collected from 18 men after treatment testicular biopsy: 5 presented oligozoospermia, 4 congenital bilateral absence of the vas deferens (CBAVD), 5 secondary obstructive azoospermia (sOAZ) and 4 hypospermatogenesis. Ejaculated samples derived from 6 normozoospermic patients. Testicular spermatozoa were analysed on a fluorescence microscope and differences between groups calculated through regression logistic models. Total rates of spermatozoa with active caspase-3 were significantly higher in sOAZ (78.6 ± 13.9), followed by hypospermatogenesis (70.8 ± 5.8), CBAVD (55.9 ± 25.5), oligozoospermia (31.7 ± 31.0) and normozoospermia (20.4 ± 15.5). Distinct patterns of active caspase-3 were observed in testicular spermatozoa compartments: midpiece, equatorial region, acrosomal vesicle region, head and cytoplasm. Hypospermatogenesis showed active caspase-3 mainly in the midpiece. In CBAVD, sOAZ and oligozoospermia active caspase-3 was mainly in the head, although no differences were found between oligozoospermia and hypospermatogenesis. Additionally, in sOAZ active caspase-3 in the sperm head was 1.89-fold higher than in CBAVD. Results suggest that tubular obstruction may induce nuclear lesions and that disrupted sperm production observed in cases of hypospermatogenesis might be associated with mitochondrial lesions.

Key words: active caspase-3, apoptosis, azoospermia, testicular spermatozoa, testis

Introduction

Spermatogenesis is a dynamic process characterized by continuous germ cell maturation towards the centre of the seminiferous tubules. Spermatogonia, adjacent to the basal membrane, proliferate through mitotic divisions, spermatocytes undergo two meiotic divisions, and spermatids differentiate and are released as spermatozoa into the lumen (Shalet, 2009). During germ cell maturation, Sertoli cells play an important role of support and nutrition. These germ cell-Sertoli cell interactions are crucial to normal spermatogenesis (Syed *et al.*, 2002).

Caspases are the central components of the programmed cell death machinery. These proteins are synthesized as inactive zymogens containing a prodomain, a large and a small subunit. Activation occurs through proteolytic cleavage with separation of the subunits and prodomain removal. In response to pro-apoptotic signals, these proteins can initiate the apoptotic cascade (caspase-8 and caspase-9 for the death receptor and the mitochondrial pathways, respectively) and activate effector caspases (caspases-3, -6, -7) that, in turn, cleave different cellular substrates or activate endonucleases that trigger the cellular collapse (Li *et al.*, 2008).

Caspases have an important role on male infertility (Said *et al.*, 2004). In fact, increased caspase activation in human spermatozoa was related to decreased sperm parameters (Weng *et al.*, 2002; Wang *et al.*, 2003; Marchetti *et al.*, 2004; Taylor *et al.*, 2004; Almeida *et al.*, 2005), decreased sperm penetration capacity (Grunewald *et al.*, 2007b) and decreased capacity to enter the capacitation process (Grunewald *et al.*, 2009a).

In testis, however, the apoptotic mechanisms underlying normal and abnormal spermatogenesis are poorly understood. In normal human testis the BCL-2 family proteins seems to play an important role in differentiation and maturation of germ cells (Oldereid *et al.*, 2001). Caspases were also found to be expressed by germ cells from fertile men. Procaspase-9 and active caspase-3 increased in an age-dependent manner (El-Domyati *et al.*, 2009) and caspase-8 and procaspases-3 and -6, were significantly less expressed than in abnormal human testicular tissue (Bozec *et al.*, 2008). In abnormal human testis, activation of the apoptotic cascade in cases of Sertoli cell-only syndrome and maturation arrest was related to the Fas-FasL system and involves the activation of caspases (Tesarik *et al.*, 2004; Kim *et al.*, 2007). However, in obstructive azoospermia and in cases of secretory azoospermia while higher levels of DNA fragmentation were showed to be present in elongated spermatids/spermatozoa (Ramos *et al.*, 2002), others failed to show the presence of the executioner step of apoptosis in these cases (Bozec *et al.*, 2008).

Little is known about the significance of apoptosis in conditions of testicular failure. The point of no return in the apoptotic cascade occurs with caspase-3 activation (Grunewald *et al.*, 2009c), an important and reliable apoptotic marker. Therefore, the aims of the present work were to determine the rates of testicular spermatozoa with active caspase-3, in samples retrieved from treatment testicular biopsies of men with oligozoospermia, obstructive and non-obstructive azoospermia, and then compare the rates between conserved and impaired spermatogenesis.

Material and Methods

Patients

In accordance with the Medical Ethics Committee, and under patients' informed and signed consent, testicular tissue was obtained from 18 men: 5 with severe oligozoospermia and needing testicular biopsy, 9 with obstructive azoospermia (4 with congenital bilateral absence of the vas deferens - CBAVD, and 5 with secondary obstructive azoospermia due to epididymal inflammation - sOAZ) and 4 with non-obstructive azoospermia (hypospermatogenesis - HP). All patients were undergoing an assisted reproduction attempt and a testicular biopsy was performed to recover testicular spermatozoa. Histological diagnosis at treatment testicular biopsy was performed in an inverted Nikon microscope equipped with Hoffman optics on a heated stage (33°C). Observations revealed samples with a good number of germ cells, which were classified as conserved spermatogenesis, and samples with reduced cell number that were considered to correspond to hypospermatogenesis (McLachlan *et al.*, 2007). Only a limited number of men was analysed because not all open testicular biopsies allowed sperm cells for analysis, as they were needed for treatments. Additionally, only a few men agreed with giving a small sample for the study. Semen samples data from six men with normal semen parameters (normozoospermic: $\geq 20 \times 10^6$ /ml sperm concentration; $\geq 15\%$ normal morphology; $\geq 25\%$ rapid progressive motility) (Almeida *et al.*, 2005), were also included. In all cases males had normal karyotypes and absence of Y microdeletions.

Samples preparation

Testicular sperm aspiration (TESA) or testicular sperm extraction by open biopsy (TESE) were used according to the pathology (Sousa *et al.*, 2002). In order to release testicular spermatozoa, testicular samples were processed as previously described (Sá *et al.*, 2008). Briefly, fragments were gently squeezed with surgical scalpels, avoiding spermatozoa damage, and the resultant fluid was washed twice (300 x g, 5 min) with sperm preparation medium (SPM; Medicult, Copenhagen, Denmark). After incubation (5 min, 37°C, 5% CO₂, filtrated humidified air) in 2 ml erythrocyte-lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, 2 mM EDTA, in water, endotoxin free, embryo and cell culture tested, pH 7.2 with KOH, 0.2 µm filtered; Sigma, Barcelona, Spain), the suspension was then washed in SPM (2 x 5 min, 500 g) and, when necessary, cells were enzymatically digested (20 min, 37°C) with 25 µg/ml DNase and 1000 U/ml collagenase-IV (Sigma) in SPM. Collagenase-IV was shown

to be efficient in testicular tissue dissociation and, additionally with DNase addition to the incubation medium to prevent clotting of the resulting cell suspension, was shown to be secure in maintaining testicular spermatozoa integrity (Crabbé *et al.*, 1997). Samples were washed twice (1000 x g, 5 min) and under informed consent, a small part was removed for the present study.

Semen samples were left to liquefy at 37°C for 30 min and a small fraction was taken for active caspase-3 activity detection and another aliquot was used for spermogram analysis (WHO, 2010).

Active caspase-3 detection in spermatozoa

Testicular spermatozoa were incubated for 30 min (37°C, 5% CO₂ in humidified air) in previously warmed medium containing (1:1) phosphate buffered saline solution (PBS, Sigma, St. Louis, USA) and PhiPhiLux® D2G2 (membrane permeant profluorogenic caspase-3 substrate; Oncolmmunin Inc., USA; stock solution: 10000 nmol/l in Roswell Park Memorial Institute (RPMI) 1640 medium with 25 mmol/l HEPES, kept in aliquots at -20°C). After two washes with 500 µl of PBS (86 x g, 10 min), cells were smeared in a slide and air dried. In order to counterstain the DNA, slides were mounted with Vectashield antifade medium (Vector Laboratories, Burlingame, USA), containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI). Active caspase-3 detection in spermatozoa from semen was performed similarly, as previously described (Almeida *et al.*, 2005). Briefly, ejaculated samples were centrifuged at 1300 x g for 2 min at room temperature. The pellet was resuspended and incubated for 30 min (37°C, 5% CO₂ in humidified air) in previously warmed 50 µl of medium containing (1:1) SPM and PhiPhi-Lux® D2G2. Samples were then washed with 500 µl of PBS (1300 x g, 2 x 1 min, room temperature), the pellet spread onto glass slides and mounted with DAPI after air dried. Differences in protocols between testicular and semen spermatozoa were due to differences in sperm maturation and, so, resistance.

Quantification and image analysis were performed with a fluorescence microscope (Axio Imager Z1, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) fitted with a CCD camera (AxioCam MRm, Zeiss) and an automated image software (FISH Imaging System, version 5.1, MetaSystems GmbH, Altlussheim, Germany). According to availability, about 100 testicular spermatozoa and 1000 ejaculated spermatozoa were counted per patient.

In the presence of active caspase-3, the substrate PhiPhi-Lux® D2G2, coupled with a fluorophore, is cleaved and emits a red fluorescence. In ejaculated spermatozoa, and as previously described, active caspase-3 was detected only in the midpiece (Almeida *et al.*,

2005). In testicular spermatozoa, however, five different localization patterns of active caspase-3 were found in testicular compartments (Figure 1): midpiece (MP), equatorial region (ER), acrosomal vesicle region (AV), cytoplasm (CY) and head (H).

Statistical analysis

Patients were divided by categories, as a routine in all studies. Patients with the same pathology obviously differ between individual characteristics, but in all cases diagnosis was performed by clinical experts in order that the diagnosis was correct in all. It included personal history, clinical findings, physical examination, hormone assays, and ecographic analysis. All these pathologies are already known and of routine characterization in all IVF Clinics, and thus are expected to not show significant group variance. Data were compared between groups at the cellular level, considering each spermatozoon independently from the others (Zhao *et al.*, 2001). For each cell the outcome, caspase-3 positive or caspase-3 negative spermatozoa, corresponds to a binary variable. To control the possibility of committing a type I error, hypotheses were tested at a probability of 0.01 rather than 0.05, and thus a stringent significance level of 1% was chosen for statistical significance. The presence or absence of active caspase-3 positive spermatozoa was studied with logistic regression models and the odds ratio (OR) for risk of presence and their associated 99% confidence interval (CI) are presented. Firstly, a comparative analysis between groups for the total rates of active caspase-3 spermatozoa was performed. To find any possible association between the presence of positive spermatozoa for active caspase-3 and the different testicular sperm compartments observed, a comparison between groups was performed for all compartments. All analyses were performed with the SPSS statistical package (version 16.0) for Windows.

Results

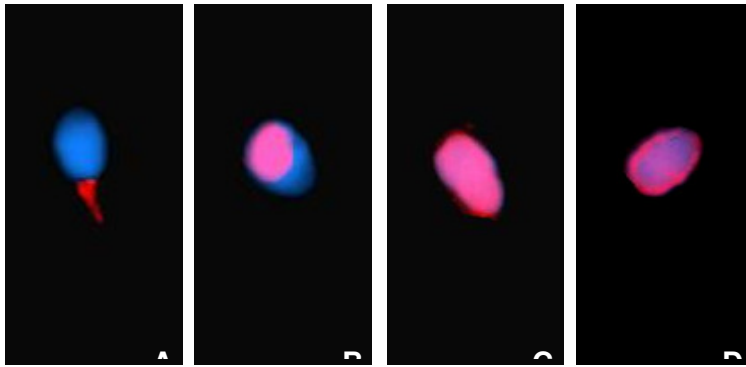


Figure 1. Active caspase-3 in sperm compartments of testicular spermatozoa: midpiece (A), acrosomal vesicle region (B), head (C), cytoplasm (D)

Total active caspase-3 positive sperm

In the normozoospermic group, a mean of $20.4\% \pm 15.5$ caspase-3 positive sperm (casp3+ sperm) was found. Testicular groups (Table 1) showed all a mean percentage of casp3+ sperm higher than the normozoospermic group ($P < 0.001$). There was, indeed, a significantly higher probability of presence of casp3+ sperm ($P < 0.001$, OR=1.88, 4.83, 14.23, 9.28, for OZ, CBAVD sOAZ and HP, respectively). Comparisons between testicular groups showed significant differences between all ($P < 0.005$) and revealed a higher probability of presence of casp3+ sperm in sOAZ followed by HP, CBAVD and OZ (Table 2, Figure 2).

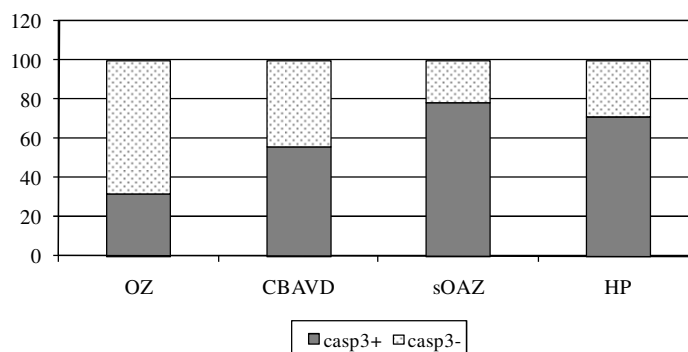


Figure 2. Percentages of active caspase-3 positive (casp3+) and negative (casp3-) testicular spermatozoa in azoospermia and non-azoospermia. (OZ) Oligozoospermia, (CBAVD) Congenital Bilateral Absence of the Vas Deferens, (sOAZ) Secondary Obstructive Azoospermia, (HP) Hypospermatogenesis.

Active caspase-3 per sperm compartment

Ejaculated spermatozoa from normozoospermic patients showed active caspase-3 only in the midpiece. Testicular spermatozoa showed distinct patterns of active caspase-3 (Figure 1) with the presence of the active protein in different sperm compartments. Comparisons for the rates of active caspase-3 per sperm compartment were then made between groups (Table 3).

Analysing each group separately, significant higher rates of spermatozoa showed active caspase-3 in the head, in cases of OZ, CBAVD and sOAZ, and in the midpiece, in cases of HP (Table 1). However, statistically significant differences were also found between groups for the presence of active caspase-3 in the equatorial region and in the cytoplasm.

Midpiece

A significantly higher probability of presence of casp3+ sperm was found for HP in relation to OZ (OR=6.45), CBAVD (OR=5.49) and sOAZ (OR=33.6), $P<0.0001$ (Table 3). No significant differences were found between OZ and CBAVD ($P=0.598$), but both showed a significantly increased risk of presence of casp3+ sperm than sOAZ ($P<0.001$).

Equatorial region

A significantly higher probability of presence of casp3+ sperm was found again for HP in relation to OZ (OR=17.26), CBAVD (OR=1.57) and sOAZ (OR=3.79), $P<0.04$ (Table 3). The probability of presence of casp3+ sperm in this region was significantly decreased in sOAZ in relation to CBAVD (OR=0.041) and was negligible for OZ that presented a mean of $1\% \pm 0.01$ of casp3+ sperm (Table 1).

Head

No significant differences were found between OZ and HP ($P=0.913$). However, a significantly higher probability of presence of casp3+ sperm was found for CBAVD and sOAZ in relation to OZ (OR=2.75 and OR=5.18, $P<0.001$, respectively) and to HP (OR=2.81 and OR=5.30, $P<0.001$, respectively). Additionally, the presence of casp3+ sperm was 1.89-fold higher in sOAZ than in CBAVD (Table 3).

Cytoplasm

Significant differences for the presence of casp3+ sperm in this sperm compartment were only found in relation to CBAVD (Table 3). sOAZ and HP showed both a higher probability of casp3+ sperm than CBAVD (OR=2.62 and OR=2.26, respectively).

Discussion

During spermatogenesis, and in order to maintain a normal architecture of seminiferous tubules, a balance between germ cell regeneration and cell death occurs. Early in development, germinal cell death by apoptosis is required for a normal spermatogenesis (Rodriguez *et al.*, 1997). Spontaneous apoptosis was also shown to occur in normal adult human testis (Oldereid *et al.*, 2001) in all male germ cell compartments, with the Bcl-2 family proteins being the core of this mechanism. This protein's family is subdivided in pro-apoptotic (Bax, Bak, Bcl-x_S, Bim, Bid e Bad) and anti-apoptotic (Bcl-2, Bcl-x_L, Bcl-w, Mcl1, A1) proteins, responsible for transmitting death and survival signals, respectively. They are localized on the outer mitochondrial, nuclear or endoplasmic reticulum membranes and are responsible to control caspase activation or inhibition. Interactions between pro- and anti-apoptotic members will determine cell fate (Borner, 2003). A selective expression of some of these proteins in human testis with normal histology was already described (Oldereid *et al.*, 2001). Additionally, germ cell apoptosis was shown to be mediated by the Fas-FasL system involving also the activation of caspases, in normal human testis (Pentikainen *et al.*, 1999) and in cases of maturation arrest and Sertoli cell-only syndrome (Kim *et al.*, 2007). However, the extent of apoptosis remains unclear either in normal human testis as in specific testicular disorders.

Caspase-3 activation is the point of no return in apoptosis so, once activated, germ cell degradation and elimination will certainly occur. The detection of active caspase-3 can be performed by different techniques (Grunewald *et al.*, 2009c), however not all allow to distinguish between the different germ cells (Brugnon *et al.*, 2009). In the present study, the presence of active caspase-3 was determined in testicular spermatozoa from testicular samples with conserved and impaired spermatogenesis, using PhiPhiLux® G2D2 which is a cell permeable fluorogenic substrate with ability to cross intact cell membranes enabling the detection of intracellular expression of active caspase-3 in live cells. Analysis was performed in a fluorescence microscope immediately following PhiPhiLux® G2D2 substrate solution incubation and sperm DNA counterstaining. Analysis within a short time after samples incubation allowed the efficient detection of the fluorescence labelling and, additionally with DNA counterstaining, the identification and analysis of only testicular spermatozoa with sperm compartments distinction.

We here quantify the presence of the final stage of apoptosis in testicular spermatozoa from azoospermic and non-azoospermic patients. We could demonstrate significant differences between the distinct pathologies investigated, as well as the presence of different patterns of cellular localization of active caspase-3. As previously described

(Almeida *et al.*, 2009), testicular samples from patients with obstructive and non-obstructive azoospermia showed increased rates of apoptosis than ejaculated and testicular samples from normozoospermic and oligozoospermic patients, respectively, a fact that reinforces the involvement of apoptosis in testicular failure (Tripathi *et al.*, 2009). We also show that the extent and origin of apoptosis depends on the testicular pathology.

Increased rates of spermatozoa with active caspase-3 were found in sOAZ, 1.53-fold higher than HP and 2.95-fold higher than CBAVD, suggesting a prominent role of apoptosis in obstructive azoospermia especially in cases of secondary obstruction.

Additionally, active caspase-3 was shown to be localized in restricted sperm compartments and a different significance was shown according to testicular disorder. In cases of sOAZ, CBAVD and OZ, active caspase-3 was mainly restricted to the head and in cases of hypospermatogenesis, mainly to the mitochondrial sheath (midpiece). Active caspase-3 had been detected in the cytoplasm of all germ cells (El-Domyati *et al.*, 2009), in the nucleus of diploid germ cells and Sertoli cells, in the cytoplasm, nucleus and acrosomal vesicle of arrested round and elongating spermatids, and in the midpiece of elongated spermatids and spermatozoa (Sá *et al.*, 2008), whereas in the semen it was only localized in the midpiece (Weng *et al.*, 2002; Wang *et al.*, 2003; Marchetti *et al.*, 2004; Almeida *et al.*, 2005; Kotwicka *et al.*, 2008) or in the postacrosomal region (Paasch *et al.*, 2004). All testicular spermatozoa analysed in the present study showed normal shape suggesting an association between the localization pattern of active caspase-3 and the different testicular pathologies.

This is the first study that shows the presence of active caspase-3 in testicular spermatozoa from hypospermatogenic patients. The increased levels present in the midpiece suggest that mitochondrial lesions may have been in the origin of apoptosis. Additionally, the increased rates of total spermatozoa with active caspase-3 (70.8%), suggest a prominent role of apoptosis in the reduced number of testicular spermatozoa production characteristic of these patients. Obstructive azoospermia, on the other hand, showed increased rates of active caspase-3 in the head suggesting that nuclear lesions might be involved in the origin of apoptosis in cases of obstruction. Total spermatozoa with active caspase-3 was, as mentioned above, 2.95-fold higher in cases of secondary obstruction (sOAZ) than primary obstruction (CBAVD), and 1.89-fold higher in the head, suggesting a strong negative impact of inflammatory duct obstruction in testicular spermatozoa development. Additionally, increased rates of active caspase-3 in the cytoplasm were shown to be present in sOAZ than in CBAVD, suggesting that a caspase-dependent apoptotic pathway although mainly activated from lesions in the nucleus, might be triggered through different stimuli. In cases of

oligozoospermia, although active caspase-3 was observed mainly in the head, this pattern was significantly lower than sOAZ and CBAVD and similar to hypospermatogenesis. So, in these cases, apoptosis in testicular spermatozoa does not seem to be associated with the pathology. Increased rates of apoptosis found in spermatozoa from semen samples rather than testicular spermatozoa from patients with oligozoospermia were, indeed, demonstrated, suggesting the presence of a post-testicular apoptotic induction factor (Almeida *et al.*, 2009).

In conclusion, results suggest a strong involvement of apoptosis in azoospermia, with significant differences being found between distinct pathologies. In comparison with primary obstruction, inflammatory duct obstruction seems to be associated with higher levels of apoptosis suggesting a strong negative impact of secondary obstruction in testicular spermatozoa development. Results additionally suggest that apoptotic events may occur essentially within the nucleus and mitochondria in cases of secondary obstruction and hypospermatogenesis, respectively.

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Caspase signalling pathways in human testicular disorders

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Running title: Active caspases in abnormal male germ line

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Abstract

Background: Increased rates of germ cell apoptosis were described in testes from men with abnormal spermatogenesis, either in cases of complete or non-complete spermatogenesis. However, little is known about the apoptotic mechanisms involved. In order to describe the significance of apoptosis in azoospermia, and to search for possible activation mechanisms, Sertoli cells and germ cells at different stages were analysed in testicular tissue from abnormal spermatogenesis. **Methods:** Under informed consent, samples were obtained from 27 testicular treatment biopsies. Five men presented oligozoospermia and the other 22 were azoospermic: 9 with obstructive azoospermia (4 congenital bilateral absence of the vas deferens; 5 secondary azoospermic) and 13 with non-obstructive azoospermia (5 hypospermatogenic; 3 with maturation arrest; 5 with Sertoli-cell-only syndrome). Immunohistochemical analysis for active caspases-3, -8 and -9 was performed using stereological tools. Results were analyzed at the seminiferous tubule and the entire testis levels. Comparisons were made per group of patients among germ cell stages and per germ cell stages among group of patients. Comparisons between active caspases-8 and -9 were also performed per group of patient and per germ cell stage. **Results:** Increased presence of active caspase-3 was found in Sertoli-cell-only syndrome. In maturation arrest, no significant differences were found between germ cells regarding active caspases. In hypospermatogenesis, primary spermatocytes were the germ cell stage with higher active caspases. In oligozoospermia and secondary obstruction, significant differences among germ cells were found for the presence of active caspases-3, -8 and -9. In oligozoospermia, spermatogonia presented significant increased active caspase-9 in relation to active caspase-8. In primary obstruction and hypospermatogenesis, germ cells presented significant increased active caspases-3 and -9. Among all groups, Sertoli cells from hypospermatogenic patients also showed with higher active caspase-3. **Conclusions:** No significance for apoptosis on maturation arrest syndrome seems to exist. On the contrary, results suggest that germ cell depletion observed in Sertoli-cell-only syndrome is related to increased casp3+. Apoptosis in secondary obstruction is suggested to be initiated due to extrinsic factors and mitochondrial lesions, whereas in primary obstruction only the intrinsic apoptotic pathway seems to be present. In hypospermatogenesis, Sertoli cell death by apoptosis and mitochondrial lesions at the meiotic stage, are suggested to be on the origin of the low numbers of germ cells observed in this pathology. Finally, oligozoospermic patients stem cell death by mitochondrial damage additionally to meiosis malfunctioning during spermatogenesis, might be on the origin of the decreased sperm output.

Key words: apoptosis, caspases, testis, obstructive azoospermia, non-obstructive azoospermia

Introduction

During normal development of an organism a balance between cell proliferation and cell apoptosis is observed. However, under certain stimuli, this balance is disrupted and cell proliferation or cell death by apoptosis deregulation occur, being on the origin of several degenerative and proliferative diseases (Thompson 1995; Jacobson *et al.*, 1997).

Caspases are a family of proteins essential in the apoptotic mechanism. Upon an apoptotic stimulus, initiator caspases are activated starting the biochemical apoptotic cascade. The initiator caspases-8 and -9 are responsible for the extrinsic and intrinsic apoptotic pathway activation, respectively, leading to the most important effector caspase activation: caspase-3. Caspase-3 activation marks the point of no return in the apoptotic process and is responsible for key proteins cleavage leading to final cell disassembly (Chowdhury *et al.*, 2008).

During spermatogenesis, there is a requirement of germ cell death by apoptosis in order to maintain normal germ cell development and to achieve a normal sperm output (Hikim *et al.*, 1999). During this process, damaged and/or excessive germ cells are phagocytised by Sertoli cells through a Fas/FasL apoptotic system dependent-manner (Pentikainen *et al.*, 1999). Apoptotic markers like active caspase-3 (Wang *et al.*, 2003; Marchetti *et al.*, 2004; Almeida *et al.*, 2005), phosphatidylserine (PS) externalization (Varum *et al.*, 2007; Almeida *et al.*, 2009) and DNA fragmentation (Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Marchetti *et al.*, 2002; Marchetti *et al.*, 2004) were described to be present in ejaculated sperm from men with normal semen parameters. However, increased rates of ejaculated sperm apoptosis were described in conditions of abnormal sperm output, mainly related to abnormal sperm concentration, normal morphology or rapid progressive motility values (Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Oosterhuis *et al.*, 2000; Marchetti *et al.*, 2002; Weng *et al.*, 2002; Wang *et al.*, 2003; Marchetti *et al.*, 2004; Almeida *et al.*, 2005; Varum *et al.*, 2007; Zhang *et al.*, 2008; Almeida *et al.*, 2009).

In cases of disrupted spermatogenesis with no ejaculated sperm production (azoospermia), testicular sperm apoptosis has also been a subject of discussion in the literature. Increased rates of testicular sperm with active caspase-3 and PS externalization were found in cases of azoospermia, either in obstructive (congenital absence of the vas deferens and secondary obstructive azoospermia) or in non-obstructive azoospermia (Hypospermatogenesis) when compared to ejaculated sperm (Almeida *et al.*, 2009; Almeida *et al.*, 2010), suggesting a relationship between testicular sperm apoptosis and testicular

failure. Additionally, increased rates of spermatids with PS externalization were described in men with incomplete spermiogenesis failure (Tesarik *et al.*, 1998). Nuclear and mitochondrial damage in cases of secondary obstructive azoospermia and hypospermatogenesis, respectively, were suggested to be on the origin of testicular sperm apoptosis (Almeida *et al.*, 2010). However, rates of germ cell apoptosis and possible activation mechanisms involved in obstructive and non-obstructive azoospermia are still poorly understood.

Although no differences were found for the presence of active caspase-3 in germ cells, increased rates of DNA fragmentation were described in cases of maturation arrest (MA) and Sertoli-cell-only syndrome (SCOS) in comparison to obstructive spermatogenesis (Kim *et al.*, 2007). On the contrary, another study showed increased rates of active caspase-3 at the spermatocyte or round spermatid level in maturation arrest, whereas in the normal testis only active caspase-8 and caspase-9 were present, in germ cells and somatic cells, respectively (Bozec *et al.*, 2008). Recently, increased apoptotic indexes were shown in hypospermatogenesis and normal spermatogenesis in comparison with maturation arrest and no DNA fragmentation was found in Sertoli cells from SCOS men (Stiblar-Martincic 2009). Overall, the results reported so far show discordant and inconclusive results for the role of apoptosis during testicular failure.

In order to avoid biased results when quantifying testicular structures in paraffin or other embedding medium sections, stereology was introduced in histological analysis of testicular sections (Petersen *et al.*, 1999). This technique overcomes possible cell distortions in number, size and orientation that would interfere with the final results. Despite several seminiferous tubules are analyzed, a testicular section only provides estimation on how the entire testis might function. Stereological analysis provide information according to the total number of cells in testis in order that final results truly represent the entire testis (Mandarim-de-Lacerda 2003).

The aims of the present study were to determine, using a stereological approach, the rates of Sertoli cell and germ cells (spermatogonia, primary and secondary spermatocyte and round spermatid) with active caspase-3 (effector), active caspase-8 (initiator; extrinsic pathway) and active caspase-9 (initiator; intrinsic pathway), in cases with complete, obstructive and non-obstructive spermatogenesis. To search for a possible apoptotic activation mechanism, comparisons between the rates of somatic and germ cells with active caspases-8 and -9 were performed.

Materials and Methods

Patients

Under patient' informed consent testicular tissue was obtained from 27 men undergoing a treatment testicular biopsy during assisted reproduction techniques. Histological diagnosis was performed during testicular biopsy procedure in an inverted Nikon microscope equipped with Hoffman optics on a heated stage (33°C).

Five patients with severe oligozoospermia needed testicular biopsy. The other 22 patients were diagnosed as azoospermic and subdivided into different groups according to testicular histology. Obstructive azoospermia was diagnosed in 9 patients based on congenital bilateral absence of the vas deferens (CBAVD, n=4) and secondary obstruction (sOAZ, n=5). Azoospermia with a non-obstructive origin was found in 13 patients and based on the presence of [1] normal histology but with reduced number of germ cells and spermatozoa, hypospermatogenesis (HP, n=5), [2] spermatogenic arrest at the secondary spermatocyte stage, maturation arrest (MA, n=3) and [3] seminiferous tubules with germ cell depletion, with only Sertoli cells, Sertoli-cell-only syndrome (SCOS, n=5) (defined according to McLachlan *et al.*, 2007). Testicular volumes varied according to pathology: 12-22 ml (OZ, HP, SCOS), 22ml (CBAVD), 15-22 (sOAZ) and 18-20ml (MA). All patients presented normal karyotypes and absence of Y chromosome microdeletions.

Testicular tissue sampling and preparation for histological analysis

Testicular tissue was obtained during treatment testicular biopsy (TESE) as previously described (Sousa *et al.*, 2002). Testicular sample biopsies volume was measured by water displacement and all presented volumes approximately of 0.016ml. Samples were fixed in 4% Paraformaldehyde (Merck, Darmstadt, Germany) in PBS (Phosphate Buffered Saline, Sigma, St. Louis, USA) for 2h at room temperature and then at 4°C overnight. After fixation, samples were washed in PBS (2x 30min), dehydrated in ethanol (Merck) series, 70° (1h), 90° (1h), 100° (4x 30min), followed by ethanol 100°/xylol (Merck) solution 1:1 for 30 min and finally only xylol (2x 1h). Testicular samples were impregnated in xylol with paraffin (Merck) 1:1 for 1h and then embedded in paraffin (2x 1h).

Histological analysis

With a manual Minot microtome (American Optical, USA), 10 histological cuts of 3µm were made in each paraffin block and transferred to Vectabound Reagent treated slides (Vector Laboratories, Burlingame, USA). In order to control tissue integrity after paraffin inclusion and confirm patient histological classification at testicular biopsy, hemalumen-eosin (He-Eo) staining was performed in all samples. Briefly, samples were deparaffinised and rehydrated in an ethanol series (90°, 70°, 50° - 3min each) and immersed in water (5min). For nuclei staining, slides were immersed in hemalumen (Hemalumen Mayer, Merck) for 10 min and then immersed in eosin solution for 3min (0.5% in water, Surgipath Medical Industries, Peterborough) for cytoplasm staining. Finally, slides were dehydrated in ethanol (50°, 70°, 90°, 95°, 2x 100°, 3min each), mounted in entellan (Merck) and observed in a light microscope.

Immunohistochemistry

Immunostaining for caspase-3 and -9 was performed using Vectastain ABC Kit (Vector Laboratories) according to manufacturer's instructions. Briefly, paraffin sections were deparaffinised in xylol (3x3min), rehydrated in ethanol series (2x100°, 95°, 70°, 50° - 3min each) and, finally, immersed in water (3min). To suppress endogenous peroxidase activity, tissue sections were treated with 3% hydrogen peroxide (Hydrogen Peroxide 30%, Merck) in distilled water for 10 min at room temperature (RT). After 1h of incubation with 5% of normal horse serum (RT, Vectastain ABC Kit), primary antibody was incubated overnight at 4°C: active caspase-3 (Monoclonal Anti-Human/Mouse Cleaved Caspase-3 Antibody, R&D Systems, Minneapolis, USA; 1:150); active caspase-9 (Monoclonal Anti-Caspase-9 Cleavage Site Specific, Sigma; 1:50). Primary antibody incubation was followed by biotinylated secondary antibody (Vectastain ABC Kit, 30min, RT) and the ABC reagent (Vectastain ABC Kit, 30min, RT). The final colorimetric reaction was performed using DAB (3,3'-Diaminobenzidine; Sigma), nuclei counterstained with hematoxylin (Vector Laboratories) and slides mounted in entellan.

Active caspase-8 immunostaining was performed with NovoLink Polymer Detection System (Novocastra Laboratories, Newcastle, UK). Similarly, paraffin sections were deparaffinised and rehydrated and endogenous peroxidase activity blocked for 5min at RT (Peroxidase block, NovoLink Polymer Detection System). Slides were then incubated for 5min with Protein Block (NovoLink Polymer Detection System) and primary antibody incubated for 1h at RT (Mouse Monoclonal Antibody Caspase-8, Novocastra; 1:1000). Primary antibody incubation was followed by Post Primary Block (NovoLink Polymer

Detection System) and Polymer (NovoLink Polymer Detection System) incubation, both for 30min at RT. The final colorimetric reaction was also performed using DAB, nuclei counterstained with hematoxylin and slides mounted with entellan.

For all immunoreactions a negative control slide was used, with the same treatment described above only omitting primary antibody labelling.

Stereological analysis

Only one testicular section per patient was analysed and, when possible, about 20 seminiferous tubules were evaluated.

Samples were analysed under light microscopy (LEITZ DMR - Leica, Germany), with digital camera (DFC480 - R2, Leica) and appropriate analysis software (Leica Image Manager 50 - IM50, Switzerland). The presence of active caspases-3, -8 and -9 was estimated per germ cells (spermatogonia - SG, primary - ST1, and secondary - ST2 spermatocytes, round spermatids - Sa) and Sertoli cells (SC), either per seminiferous tubules or per testicular volume using stereological tools.

The parameter V_v represents the relative volume occupied by a cell relatively to a specific reference space. Considering seminiferous tubules as the reference space, each V_v is determined by a manual point counting (Howard *et al.*, 1998), using a multi-system grill placed over the image obtained using an immersion oil objective (100x) (Figure 1). After counting, results were calculated with the following computation:

$V_v (\text{cell, seminiferous tubules}) = [\sum P(\text{cell}) \times 100] \div [R \cdot \sum P(\text{seminiferous tubules})]$, where “ $\sum P(\text{cell})$ ” corresponds to the total number of points counted over the cell of interest, “ $\sum P(\text{seminiferous tubules})$ ” corresponds to the total number of points over the seminiferous tubules from the testicular section in study, and “R” to the ratio of points considering the counting system used for the analysed cells and for the reference space (seminiferous tubules). This computation gives the estimation of the volume occupied per cell in seminiferous tubules. In the seminiferous tubule, results are expressed as relative volume (V_v) occupied per cell with or without active caspases.

In order to calculate the volume per cell occupied in the testis, the following computations were used:

$V_v (\text{cell, biopsy}) = V_v (\text{cell, seminiferous tubules}) \times V_v (\text{seminiferous tubules, biopsy})$ and $V (\text{cell, testis}) = V_v (\text{cell, biopsy}) \times V (\text{biopsy, testis})$, where the total volume of each biopsy was estimated by fluid displacement before preparation of samples for paraffin impregnation. The

V_v (seminiferous tubules, biopsy) is estimated by point counting with a multi-system grill (Integrationsplatte II, 100/25, Carl Zeiss) directly in the microscope ocular and with a 10x objective (Figure 1). In the entire testis, results are expressed as volume (V) occupied per cell with or without active caspases.

Statistical analysis

Different approaches were applied in the analysis of the relative (V_v) and total (V) volume occupied per cell with active caspases-8, -9 and -3 in both seminiferous tubules and testis, respectively. For each caspase, comparisons were made per group among germ cell stages and also per germ cell stage among groups. Kruskal-Wallis test with a 95% confidence interval (CI) was applied. Multiple comparisons were also performed between cells by the Mann-Whitney U Test (95% CI) and per testicular disorder. Ten different comparisons were made: SCvsSG, SCvsST1, SCvsST2, SCvsSa, SGvsST1, SGvsST2, SGvsSa, ST1vsST2, ST1vsSa, ST2vsSa. The Bonferroni method was used to adjust the significance level owing to multiple Mann-Whitney tests. The significance level of 0.05 was maintained using the formula of $0.050/k$ independent hypotheses, with $k = 10$ to reflect the ten planned analyses of interest detailed above. An adjusted significance level of 0.005 was therefore used to maintain the overall 0.05 level in the context of the multiple comparisons. Finally, comparisons between active caspase-8 and active caspase-9 relative (V_v) and total volume (V), per group and per cell, were performed using the Mann-Whitney U Test (95% CI).

In all approaches, medians were used instead of means as no normal distribution was shown by active caspases-8, -9 and -3 V_v and V values, in all groups. All comparisons were performed using Stata (version 9).

Results

In all groups, active caspases-8, -9 and -3 immunostaining were observed in SC cytoplasm and germ cell cytoplasm and nucleus (Figure 2). Cell quantification was made independently from active caspase localization.

The relative and total volume medians of Sertoli cells and germ cells with active caspases-8, -9 and -3 per testicular groups are shown in Figure 3.

Seminiferous tubules

Active Caspase-8

In all groups, increased Vv(SC) with active caspase-8 (casp8+) was shown to be present in relation to all germ cells stages, with no significant differences being found among groups ($P = 0.632$). Comparisons per group revealed significant differences among germ cell stages in cases of OZ and sOAZ. Although no significant differences were found by multiple comparisons between germ cell stages, increased Vv(ST1)casp8+ was observed in both groups. No significant differences were observed among groups, per germ cell stage.

Active Caspase-9

Increased Vv(SC) with active caspase-9 (casp9+) was observed in all groups in relation to germ cells, but with no significant differences among groups ($P = 0.554$). Comparisons per group showed significant differences among germ cell stages ($P = 0.033$). Again, increased Vv(ST1)casp9+ was found in all groups but with no significant differences in relation to the other germ cells. Contrarily to casp8+, comparisons per germ cell stage among groups showed significant differences ($P = 0.011$) with increased Vv(SG)casp9+ and Vv(ST1)casp9+ in sOAZ and increased Vv(ST2)casp9+ and Vv(Sa)casp9+ in CBAVD.

Active Caspase-3

Increased Vv(SC) with active caspase-3 (casp3+) was present in all groups in relation to germ cell and significant differences were found among groups ($P = 0.009$). Increased values were shown by SCOS and HP patients. Among germ cell stages, all groups, except MA ($P = 0.057$) presented significant differences ($P = 0.016$). Although no significant differences were observed by multiple comparisons between germ cell stages, increased

Vv(ST1)casp3+ was found in all testicular syndromes. Significant differences were observed when comparisons were made per germ cell stage and among groups ($P = 0.019$). Increased Vv(SG)casp3+, Vv(ST1)casp3+, Vv(ST2)casp3+ and Vv(Sa)casp3+ were all observed for sOAZ in relation to the other groups.

Active Caspase-8 vs Active Caspase-9

Significant increased Vv(Sa)casp8+ ($P = 0.009$) and significant increased Vv(SG)casp9+ ($P = 0.009$) were found in OZ. Hypospermatogenic patients showed significant increased Vv(Sa)casp9+ ($P = 0.028$). No significant differences were observed between casp8+ and casp9+ for the other groups ($P = 0.076$).

Testis

Active Caspase-8

Results obtained were similar to those obtained at the seminiferous tubule level. Increased V(SC)casp8+ was present in all groups but with no significant differences among them ($P = 0.706$). Significant differences among germ cell stages were observed in cases of OZ and sOAZ that presented increased but not significant V(ST1)casp8+. No significant differences were observed per germ cell stage among groups.

Active Caspase-9

Increased V(SC)casp9+ was observed in all groups in relation to germ cell stages, but with no significant differences among groups ($P = 0.102$). Comparisons per group showed significant differences for the presence of casp9+ among germ cells ($P = 0.007$) with increased but not significant V(ST1)casp9+ in all groups. An exception was observed within MA where no significant differences were found ($P = 0.058$). Contrarily to seminiferous tubules, comparisons per germ cell stage among groups showed significant differences only for V(Sa)casp9+ ($P = 0.048$) with increased but not significant V(Sa)casp9+ in sOAZ.

Active Caspase-3

Increased V(SC)casp3+ was present in all groups in relation to germ cell stages and significant differences were found among groups ($P = 0.009$) with increased values shown by SCOS and HP patients. Among germ cell stages, all groups, except MA ($P = 0.058$)

presented significant differences ($P = 0.021$). Even though multiple comparisons revealed no significant differences between germ cell stages, increased V(ST1)casp3+ was found in all testicular syndromes. Comparisons per germ cell among groups showed only significant differences for V(Sa)casp3+ ($P = 0.029$) with increased values in CBAVD.

Active Caspase-8 vs Active Caspase-9

Oligozoospermia showed significant increased V(SG)casp9+ ($P = 0.016$) and V(Sa)casp8+ ($P = 0.009$). HP showed significant increased V(Sa)casp8+ ($P = 0.047$).

Discussion

In men with normal sperm output a fine balance between germ cell proliferation and germ cell apoptosis exist (Johnson *et al.*, 2000). Sertoli cells are responsible for germ cell nurture and support, controlling the number of germ cell production (Johnson *et al.*, 2008). In the presence of excessive or damaged germ cells, Sertoli cells express FasL inducing germ cell apoptosis by the Fas/FasL system in order to maintain the equilibrium, essential to normal spermatogenesis (Pentikainen *et al.*, 1999). However, sometimes the adverse conditions are not overcome and this equilibrium is not reached. Increased rates of apoptosis have already been described in cases of azoospermia (Lin *et al.*, 1997a; Lin *et al.*, 1997b). However, apoptotic mechanisms involved are still poorly understood.

Increased rates of DNA fragmentation and active caspase-3 were found in SCOS and MA testes (Kim *et al.*, 2007) and decreased rates of TUNEL-positive germ cells were found in HP, CBAVD and sOAZ (Bozec *et al.*, 2008). However, increased apoptotic indexes were recently shown in HP in comparison with MA and no DNA fragmentation was found in Sertoli cells from SCOS men (Stiblar-Martincic 2009). Although some evidence exists for the role of apoptosis in testicular failure, different studies present discordant results.

Testicular tissue preparation for analysis is usually carried out by paraffin inclusion or other embedding media followed by 3-4µm sections cuts. This procedure can induce cell distortion in number, size and shape within the section analyzed. In order to overcome possible bias, stereological tools were introduced in histological analysis of testicular tissue (Petersen *et al.*, 1999). Stereological analysis ensures a uniformed sample where Sertoli cells and germ cells from all stages have the same probability to be sampled and where possible distortions in cell volume and orientation during histological processing will not interfere with the final results (Petersen *et al.*, 1999; Mandarim-de-Lacerda 2003). In addition, this method provides information relative to the total number of cells in testis in order that final results truly represent the entire testis (Mandarim-de-Lacerda 2003).

In order to characterize apoptosis during impaired spermatogenesis, the presence of SC and germ cells stages with active caspases-3, -8 and -9 were determined using stereological analysis. Analysis and quantification of SC and germ cells with active caspases was firstly done at the seminiferous tubule level and then results were determined for the entire testis.

In all cases, SC presented higher apoptosis in comparison to germ cells. However, no significant differences were observed among groups for the presence of active caspase-8

and active caspase-9. Significant differences were observed only for the presence of SC with active caspase-3 among groups that were shown to be higher in SCOS suggesting that Sertoli cells may undergo apoptosis. Results are in concordance with previous data where increased TUNEL-positive Sertoli cells and increased rates of active caspase-3 were found in SCOS patients and suggested to be related to germ cell depletion observed in this specific syndrome (Kim *et al.*, 2007).

Contrarily to previous studies where the FasR/FasL system with caspase-3 activation was suggested to be involved in germ cell degeneration in cases of maturation arrest (Eguchi *et al.*, 2002; Francavilla *et al.*, 2002; Kim *et al.*, 2007), our results suggest no active role for apoptosis in maturation arrest. On the contrary, another study showed increased immunostaining of active caspase-3 in spermatocytes or spermatocytes and spermatids in cases of maturation arrest. However, caspases were only evaluated according to their presence and no quantification was made (Bozec *et al.*, 2008). Additionally, increased apoptotic indexes were described in maturation arrest at the spermatocyte level in comparison to normal spermatogenesis, HP and SCOS (Kandirali *et al.*, 2008). Discordant results might be due to differences in maturation arrest histology and also to differences in germ cell quantification and analysis.

In cases of complete spermatogenesis (OZ, sOAZ, CBAVD and HP), although no significant results were observed, ST1 showed higher active caspase-3 in comparison to all other germ cell stages. Results suggest that a malfunction might exist in the meiotic phase of spermatogenesis, namely in the first meiotic phase, and that might be on the origin of some of these testicular pathologies. In sOAZ significant differences were present for active caspase-8 and active caspase-9 among germ cells but no differences were found between both. Although the intrinsic and the extrinsic apoptotic pathways seem to be present, results suggest that none of them seem to prevail. Extrinsic factors on the origin of the obstruction might activate the extrinsic apoptotic pathway with caspase-8 activation but a cross-talk between the intrinsic and the extrinsic pathways might also exist during spermatogenesis, whereas both mechanisms contribute to apoptotic germ cell death observed in these sOAZ. In cases of congenital obstruction (CBAVD), no significant differences were found among germ cells for the presence of active caspase-8. On the contrary, significant differences for the presence of germ cells with active caspase-9 and active caspase-3 were observed, with ST1 stage cells presenting higher levels. Results suggest that, in order to establish equilibrium in spermatogenesis of these patients, where no testicular spermatozoa release exists, the intrinsic apoptotic pathway might be activated through mitochondrial lesions with

consequent caspase-9 and caspase-3 activation. Meiotic germ cell death by apoptosis might be responsible for the maintenance of an appropriate germ cell number.

Previous studies on testicular spermatozoa have shown increased rates of active caspase-3 in sOAZ and CBAVD patients. However, active caspase-3 staining was observed mainly in the sperm nucleus (Almeida *et al.*, 2010). In the present study, active caspase-3 immunostaining was observed in germ cells cytoplasm and/or nucleus suggesting that external and mitochondrial lesions in cases of sOAZ and mitochondrial lesions in CBAVD patients might activate the apoptotic pathway inducing caspase-3 activation among germ cells and consequent translocation to the nucleus during spermiogenesis.

Like in CBAVD, no significant differences were found for the presence of active caspase-8 among germ cells in HP patients. Significant differences were found for the presence of active caspase-9 with higher levels at the ST1 stage. So, germ cell death due to the intrinsic apoptotic mechanism might be on the origin of the lower numbers of germ cells observed in hypospermatogenic patients. Additionally, among all syndromes and after SCOS, increased SC with active caspase-3 showed to exist. SC death by apoptosis might also lead to a lower number of germ cells due to the decreased support capacity and decreased nutrition, essential to maintain normal germ cells numbers. These results reinforce the results obtained recently where increased rates of active caspase-3 were shown to be present in the midpiece of testicular spermatozoa from HP patients (Almeida *et al.*, 2010). So, additionally with SC death, results strongly favor the hypothesis of mitochondrial damage at the ST1 stage as the origin of this testicular disorder.

Finally, OZ patients presented both significant differences for the presence of active caspase-8 and active caspase-9 among germ cells, with increased levels observed at the ST1 stage. Additionally, comparisons between both proteins showed significant increased SG with active caspase-9. Previous results have suggested no significance for apoptosis in testicular spermatozoa and the presence of a post-testicular apoptotic induction factor was suggested as the main cause of decreased ejaculated spermatozoa (Almeida *et al.*, 2009; Almeida *et al.*, 2010). The additional findings presented here suggest that stem cell death by mitochondrial damage and meiosis malfunctioning during spermatogenesis might be on the origin of decreased sperm production observed in these men. In spite of this, additional apoptosis at a post-testicular level might also contribute to ejaculated sperm apoptotic death decreasing even more the final sperm output.

In conclusion, no significance for apoptosis was found for MA syndrome whereas SCOS was related to increased levels of active caspase-3. In cases of complete spermatogenesis, meiotic germ cell apoptosis seems to be present in all pathologies.

Apoptosis in sOAZ is suggested to be initiated due to extrinsic factors and mitochondrial lesions, whereas in CBAVD only the intrinsic apoptotic pathway seems to be present. The low numbers of germ cells observed in HP are suggested to result from SC death by apoptosis and mitochondrial lesions at the ST1 stage. Finally, OZ patients where low sperm production occur, SG death by mitochondrial damage additionally to meiosis malfunctioning during spermatogenesis might be on the origin of the decreased sperm output.

The present study contributes to give some insight into possible apoptotic mechanisms involved in cases of deranged spermatogenesis.

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Figure legends

Figure 1: Grill systems used for the stereological analysis performed. A - Howard and Reed multi-scale system placed over a seminiferous tubule image on the computer (100x) in order to estimate the relative volume (V_v) occupied per cell per seminiferous tubule; B - Grill inserted in the left microscope ocular (10x) in order to estimate the volume (V) occupied per cell in the testis (Integrationsplatte II, 100/25, Carl Zeiss).

Figure 2: Active caspases-8, -9 and -3 immunohistochemical analysis for the testicular groups analyzed. OZ - Oligozoospermia; CBAVD - congenital bilateral absence of the vas deferens; sOAZ - secondary obstructive azoospermia; HP - hypospermatogenesis; MA - maturation arrest; SCOS - Sertoli-cell-only syndrome. Bar = 50 μ m.

Figure 3: Median values for relative volume (V_v) and volume (V) occupied by Sertoli cells and germ cells with active caspases-8, -9 and -3, in the seminiferous tubules and testis, respectively. OZ - Oligozoospermia; CBAVD - congenital bilateral absence of the vas deferens; sOAZ - secondary obstructive azoospermia; HP - hypospermatogenesis; MA - maturation arrest; SCOS - Sertoli-cell-only syndrome.

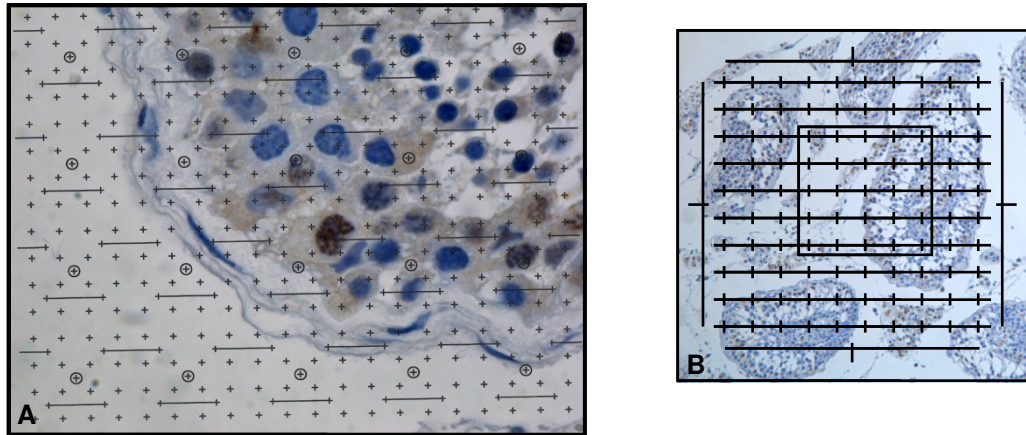
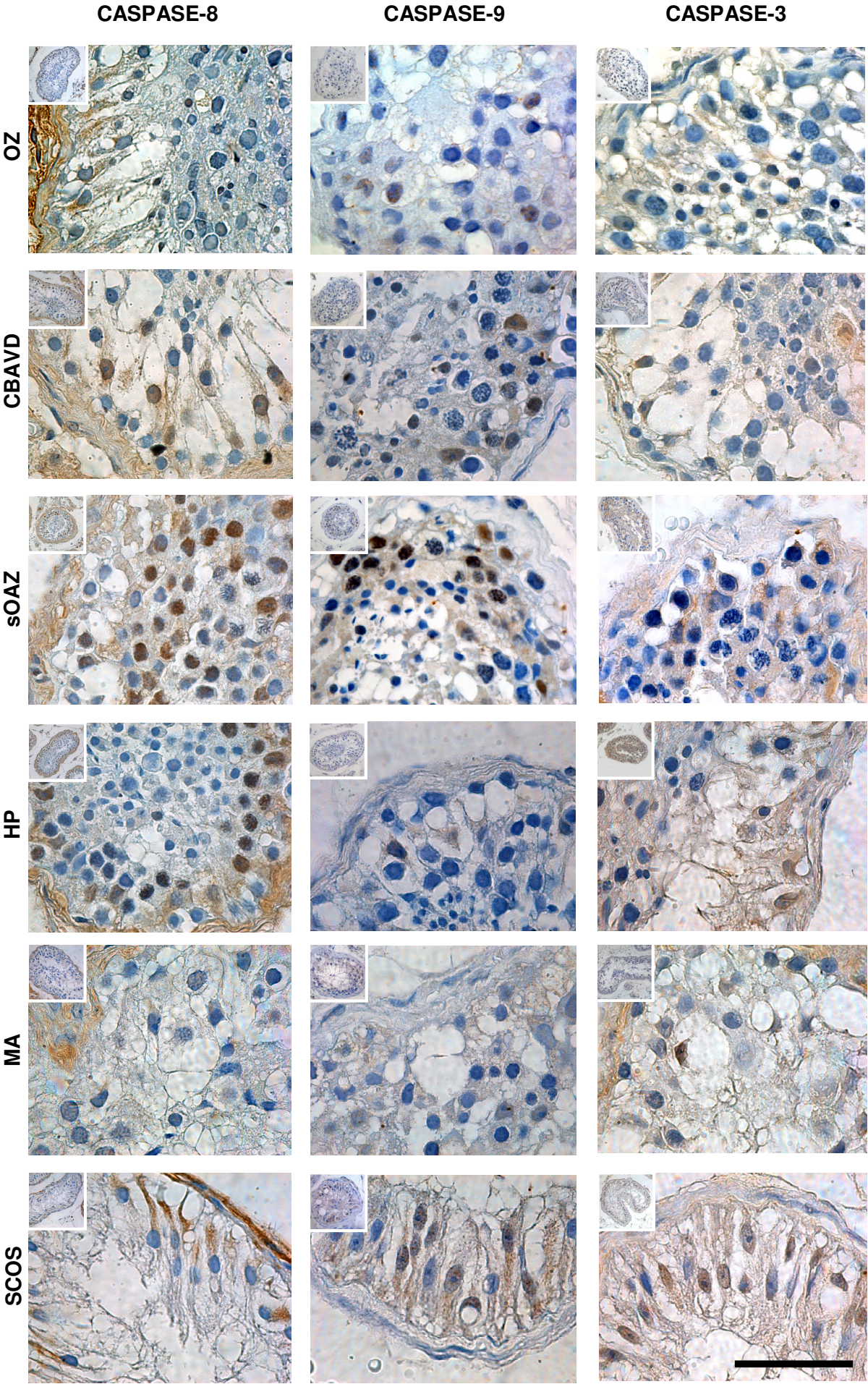
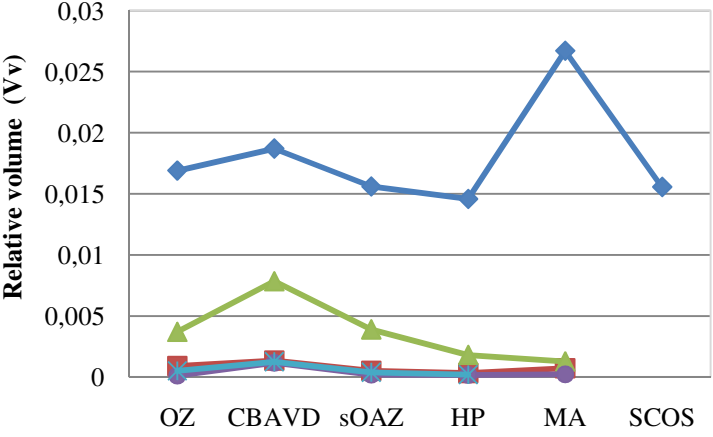


Figure 1

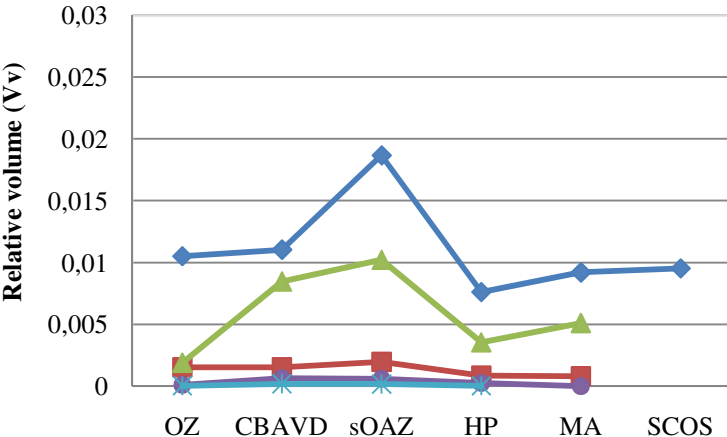


Seminiferous Tubules

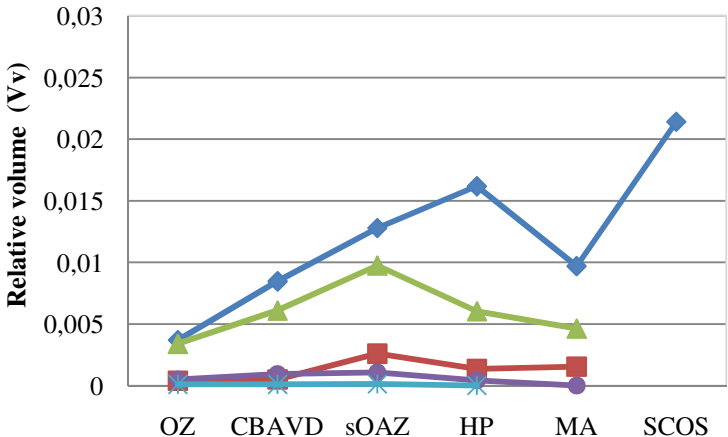
Active Caspase-8



Active Caspase-9



Active Caspase-3



Testis

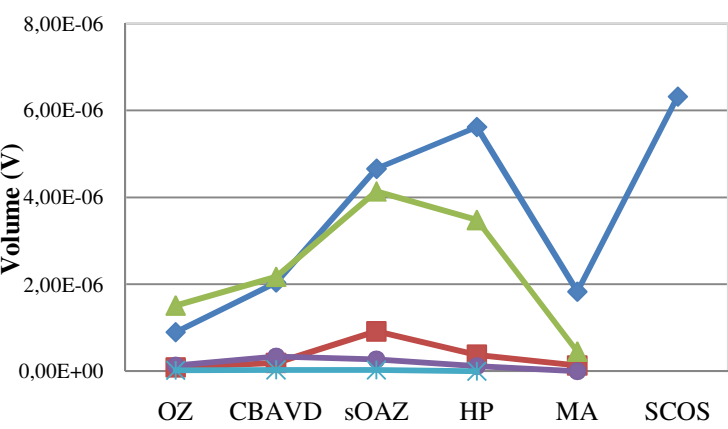
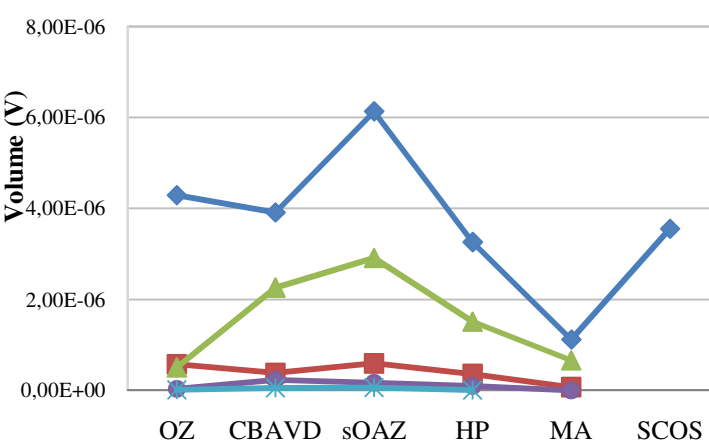
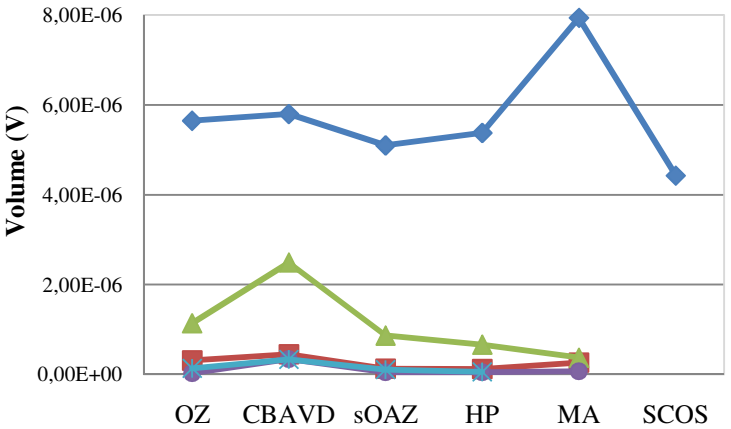


Figure 3

DISCUSSION AND CONCLUSIONS

Normal tissue development depends on a fine balance between cell proliferation and cell death (Thompson, 1995; Jacobson *et al.*, 1997) with deregulation between both mechanisms being in the origin of pathogenic conditions like neurodegenerative diseases and neoplasias (Thompson, 1995).

In spermatogenesis, spontaneous cell death by apoptosis occurs in a developmentally regulated manner controlling the final sperm output (Hikim *et al.*, 1999). In this mechanism, Sertoli cells phagocytic function is crucial for an efficient production of sperm (Nakanishi *et al.*, 2004). Germ cell apoptosis during spermatogenesis occurs according to Sertoli cell support capacity. However, apoptosis can also be induced in response to germ cell injury in order to remove damaged and potentially dangerous germ cells, assuring a normal spermatogenic development (Pentikainen *et al.*, 1999).

The presence of apoptotic markers in human ejaculated spermatozoa has been described in cases of normal and abnormal spermatogenesis and the search for the significance of apoptosis in male infertility has been the subject of many studies (Oehninger, 2003). Although some authors associate the presence of Fas-labeled spermatozoa, phosphatidylserine externalization, active caspase-3 and sperm DNA damage to a malfunction in the apoptotic mechanism, the 'abortive apoptotic mechanism' theory (Sakkas *et al.*, 1999, 2002; Shen *et al.*, 2002; Aziz *et al.*, 2007), others showed a correlation between the presence of apoptotic markers in ejaculated spermatozoa and abnormal semen parameters, suggesting an active role for apoptosis in male infertility. However, different study populations, different semen sample fractions and different apoptotic markers analyzed, gave origin to different results: sperm DNA fragmentation was negatively correlated with sperm motility and normal morphology (Gandini *et al.*, 2000), with sperm concentration and normal morphology (Sakkas *et al.*, 2002), with sperm concentration (Oosterhuis *et al.*, 2000b) and with sperm motility (Barroso *et al.*, 2000; Weng *et al.*, 2002); phosphatidylserine externalization was negatively correlated to sperm concentration and motility (Oosterhuis *et al.*, 2000b), and to sperm motility (Weng *et al.*, 2002); Fas and p53 markers were negatively correlated to sperm concentration and normal morphology (Sakkas *et al.*, 2002); active caspases-3 and -9 levels to sperm concentration, motility and morphology (Wang *et al.*, 2003), and active caspase-3 was found to be increased in the low motility fractions of semen samples from infertile men (Weng *et al.*, 2002; Taylor *et al.*, 2004). Only one study failed to show a correlation between sperm apoptosis (measured by phosphatidylserine translocation analysis) and semen quality (Ricci *et al.*, 2002).

Despite the previous studies suggesting increased rates of sperm apoptosis in cases of abnormal semen parameters, it remains to be established whether sperm defects are a

cause or consequence of increased rates of apoptosis. Spermogram is one of the essential analyses when evaluating infertile men (Shefi *et al.*, 2006). DNA fragmentation is the hallmark of apoptotic cell death. As increased rates of sperm DNA fragmentation were shown to be associated with decreased fertilization (Marchetti *et al.*, 2002) and pregnancy (Tomlinson *et al.*, 2001) rates, clarification of the relationship between sperm apoptosis and abnormal semen parameters is essential to predict clinical outcomes.

In order to define the significance of sperm apoptosis in abnormal spermatogenesis, ejaculated semen samples from 67 patients undergoing spermogram evaluation during infertility diagnosis, were analyzed for the presence of sperm with active caspase-3 (caspase-3-positive sperm) (Almeida *et al.*, 2005; Paper I). Two semen fractions were studied - neat semen and swim-up after gradient centrifugation technique -, with a total of 119 850 spermatozoa quantified (67 488 from neat fractions and 52 362 from swim-up fractions) and results analyzed according to two groups of classification criteria. In this study it was shown the importance of group classification criteria. Patients were classified according to only one semen parameter abnormality (e.g. concentration, morphology or rapid progressive motility) and according to all semen parameters abnormalities observed (e.g. concentration + morphology + motility). In the neat fraction, semen classification based in single semen parameters, revealed no relationship between caspase-3-positive sperm and semen abnormalities. In accordance with previous results (Ricci *et al.*, 2002), sperm apoptosis rates were shown to be unrelated to sperm quality. However, when groups were classified based on all semen parameters abnormalities (concentration + morphology + rapid progressive motility) in order to uncover the possible contribution of simultaneous sperm defects, increased rates of caspase-3-positive sperm were shown to be related to asthenozoospermia (<25% of sperm with rapid progressive motility). In the swim-up fraction, increased rates of caspase-3-positive sperm were related to decreased normal morphology and rapid progressive motility values when considering single semen parameters and only to teratozoospermia when considering all the other parameters normal. Additionally, and in accordance with others (Weng *et al.*, 2002; Cayli *et al.*, 2004), active caspase-3 was detected only in the midpiece suggesting that a caspase-dependent apoptotic mechanism may be originated in the cytoplasmic droplet or within mitochondria. Only one report detected the presence of active caspase-3 in the postacrosomal region (Paasch *et al.*, 2004). Caspase-3 activation is the point of no return in apoptosis after which a number of apoptotic events occur being DNA fragmentation in the end of this process (Grunewald *et al.*, 2009c). The presence of a positive correlation between active caspase-3 and DNA fragmentation (Weng *et al.*, 2002; Wang *et al.*, 2003; Marchetti *et al.*, 2004) and the additional presence of active caspase-9 in the midpiece (Paasch *et al.*, 2004) favors the hypothesis of a caspase-

dependent apoptotic mechanism activated through the mitochondrial apoptotic pathway. It was also suggested the possible involvement of other caspases in human sperm apoptosis and it was, indeed, demonstrated to be related to abnormal semen parameters (Marchetti *et al.*, 2004).

In accordance with previous studies, where different apoptotic markers were used in ejaculated samples from the raw semen and after semen preparation techniques (Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Marchetti *et al.*, 2002; Taylor *et al.*, 2004), the rates of caspase-3-positive sperm were significantly reduced after gradient centrifugation and swim-up. Caspase-3-positive sperm was 4.2-fold higher in the neat semen fraction. In the swim-up fraction, caspase-3-positive sperm was only related to teratozoospermia but patients with decreased normal sperm morphology showed only $4.5 \pm 3.5\%$ of caspase-3-positive sperm in the swim-up sample. Data thus suggest a low risk of selecting apoptotic sperm during clinical treatments. In accordance with our results (Almeida *et al.*, 2005; Paper I), semen preparation by gradient centrifugation followed by swim-up was recently shown to be efficient in reducing the rates of sperm with apoptotic features like activated caspase-3 and mitochondrial membrane potential disruption (Grunewald *et al.*, 2010). Oocyte penetration capacity had already been proved to be increased in non-apoptotic sperm (Grunewald *et al.*, 2007a). Thus, the semen preparation technique used in the present study seems to be efficient in eliminating apoptotic sperm from samples used during assisted reproduction techniques (ART) and might be essential in conception rates improvement (Grunewald *et al.*, 2007a, 2010). However, high interindividual variability was shown for this sperm selection procedure efficiency and, therefore, new selection techniques were suggested to be implemented (Grunewald *et al.*, 2010).

For the first time, it was showed that group classification criterion is essential in reveal the true relationship between apoptosis and semen parameters (Almeida *et al.*, 2005; Paper I). Results suggest that the patient population studied by previous authors, where simultaneous sperm defects were present, might be on the origin of the varied and discordant results presented until now. Using strict group classification criteria (considering all semen parameters), it was showed that caspase-3-positive sperm is related with asthenozoospermia in the neat semen fractions and with teratozoospermia in the swim-up fractions (Almeida *et al.*, 2005; Paper I). Additionally, our results suggest that these semen abnormalities are related to the presence of an active caspase-dependent apoptotic mechanism activated through the mitochondrial apoptotic pathway. In accordance with our results, sperm apoptosis induced by betulinic acid led to sperm mitochondrial membrane potential disruption followed by caspase-3 activation observed intensely in the midpiece

(Espinoza *et al.*, 2009a). Sperm caspase activity might be an important marker of poor sperm quality in cases of asthenozoospermia.

Additional data (Cayli *et al.*, 2004), suggested that the presence of immature sperm in the ejaculate (neat semen) might be due to the co-expression of active caspase-3 and the anti-apoptotic protein BCL-XL, that confers a protective effect against the apoptotic immature sperm elimination. Alternatively, caspase-3-positive sperm were shown to present morphometrical attributes typical from diminished sperm maturity, like decreased tail length and larger and amorphous heads, associated with the additional expression of creatine kinase (a marker for cytoplasmic retention). However, in our study (Almeida *et al.*, 2005; Paper I), all spermatozoa analyzed showed normal midpiece and sperm head morphology with no cytoplasmic retention, and after samples were submitted to gradient centrifugation and swim-up, caspase-3-positive sperm, although in a significant lower extent, were also present. So, it was suggested that active caspase-3 in ejaculated sperm, increased in conditions of abnormal spermiogram pathologies (astheno- and teratozoospermia), results from an active caspase-dependent apoptotic mechanism rather from immature sperm with cytoplasmic retention (Almeida *et al.*, 2005; Paper I).

Germ cell apoptosis was shown to be increased in cases of abnormal spermatogenesis not only in semen samples but also in testicular samples. Increased rates of apoptotic bodies and germ cells with DNA fragmentation were described in maturation arrest and hypospermatogenesis syndromes in relation to cases of obstructed spermatogenesis and to Sertoli Cell Only Syndrome - SCOS (Lin *et al.*, 1997a, 1997b, 1999b; Martincic *et al.*, 2001). Increased rates of DNA fragmentation were also shown to be present in the round spermatid stage of non-obstructive azoospermia cases (Jurisicova *et al.*, 1999) and in testicular and epididymal spermatozoa from men with obstructive azoospermia and anejaculation in relation to fertile donors (Ramos *et al.*, 2002). Electron microscopy and DNA fragmentation studies revealed typical apoptotic features in maturation arrest tissues, mostly in spermatogonia and primary spermatocytes. Additionally with increased Fas and FasL expression rates, previously shown to be involved in germ cell recognition by Sertoli cells in cases of normal spermatogenesis (Pentikainen *et al.*, 1999), Fas/FasL system was also suggested to be involved in the germ cell quality control mechanism in cases of maturation arrest (Francavilla *et al.*, 2000; Eguchi *et al.*, 2002). It was indeed demonstrated, through light and transmission electron microscopy, a positive correlation between the frequency of degenerative germ cells (with nuclear and cytoplasmic changes) and the frequency of Fas-labeled germ cells in meiotic arrest of spermatogenesis, namely in cases of incomplete post-meiotic arrest (Francavilla *et al.*, 2002). Testicular samples from men with non-obstructive

azoospermia have also been studied for the presence of germ cell DNA fragmentation and phosphatidylserine externalization. Results showed increased rates of DNA fragmentation associated with maturation arrest and phosphatidylserine externalization on the late stages of spermiogenesis in cases of hypospermatogenesis (Tesarik *et al.*, 1998). Other authors suggested that in cases of hypospermatogenesis, spermatogonia proliferative dysfunction rather than increased rates of apoptosis were associated with the decreased numbers of germ cells characteristic of this pathology (Takagi *et al.*, 2001).

Additional compelling evidence exists towards the involvement of apoptotic dysfunction in male infertility. After maturation arrest and hypospermatogenesis testicular biopsies, mechanical disintegration of seminiferous tubules resulted in a cell suspension of free-floating Sertoli and free-germ cells as well as Sertoli-germ cell clusters. Two different apoptotic pathways were suggested to be present: a caspase-dependent apoptotic pathway in cases of germ cells attached to Sertoli cells, where high rates of germ cells presented caspase activity, DNA fragmentation and phosphatidylserine externalization, and a caspase-independent apoptotic pathway where, despite high numbers of germ cells with DNA fragmentation, few of them presented caspase activity and, contrarily to a previous report (Tesarik *et al.*, 1998), no phosphatidylserine externalization was shown (Tesarik *et al.*, 2004). However, either in cases of SCOS, maturation arrest or hypospermatogenesis, Sertoli cells hardly expressed caspase activity and DNA fragmentation (Tesarik *et al.*, 2004). Finally, the inhibitor of apoptosis protein survivin was suggested to be involved in apoptosis control during spermatogenesis. Decreased expression rates of survivin were associated with accelerated rates of apoptosis in cases of SCOS, pre- and post-maturation arrest, and to be related to the degree of spermatogenic failure (Weikert *et al.*, 2005).

Although the presence of apoptotic markers in germ cells from men with testicular failure has been shown, the significance of apoptosis in these cases is far from being clarified. In the era of ART, where testicular spermatid retrieval has become the main procedure to overcome infertility in cases of azoospermia, testicular sperm quality needs to be assured. Studies describing the rates of apoptosis in testicular spermatozoa are still missing.

In order to describe the apoptotic rates of testicular spermatozoa in samples from patients with deranged spermatogenesis and to search for any possible relationship between both, the active form of caspase-3 was used again as a reliable apoptotic marker for the point of no return in apoptosis (Almeida *et al.*, 2010; Paper IV). The rates of testicular spermatozoa with active caspase-3 (caspase-3 positive sperm) were determined in testicular spermatozoa from patients with oligozoospermia, obstructive azoospermia (congenital bilateral absence of the vas deferens - CBAVD, and with secondary obstructive azoospermia

due to epididymal inflammation) and non-obstructive azoospermia (hypospermatogenesis) and, then, comparisons were made between complete and non-complete spermatogenesis cases. Contrarily to a study that failed to show the presence of active caspase-3 in germ cells and testicular spermatozoa from hipospermatogenic, CBAVD and secondary obstructive azoospermia testis (Bozec *et al.*, 2008), our data showed active caspase-3 positive sperm in all testicular groups with a significantly higher probability of presence of caspase-3 positive sperm in cases with secondary obstruction followed by hypospermatogenesis, CBAVD and oligozoospermia. As non-azoospermic patients (oligozoospermia) showed lower rates of caspase-3 positive sperm, results suggest the involvement of apoptosis in azoospermia. Additionally, a comparison for the presence of caspase-3 positive sperm was made with ejaculated sperm from normozoospermic men, showing significantly higher probabilities for the presence of caspase-3 positive sperm in testicular samples, reinforcing the strong involvement of apoptosis in cases of testicular failure (Almeida *et al.*, 2010a; Paper IV).

Contrarily to spermatozoa from the ejaculate (Almeida *et al.*, 2005; Paper I), active caspase-3 was detected not only in the midpiece but also in other sperm regions like equatorial region, acrosomal vesicle region, cytoplasm and nucleus. Until now, active caspase-3 had been detected in the cytoplasm of germ cells (El-Domyati *et al.*, 2009), in the nucleus of diploid germ cells and Sertoli cells, in the cytoplasm, nucleus and acrosomal vesicle of arrested round and elongating spermatids, and in the midpiece of elongated spermatids and spermatozoa (Sá *et al.*, 2008). All testicular spermatozoa analysed in the present study showed normal shape, suggesting an association between the active caspase-3 localization pattern and the different testicular pathologies. However, data suggest that among all, only the presence of active caspase-3 in testicular sperm nucleus and midpiece might be relevant (Almeida *et al.*, 2010a; Paper IV).

In cases of oligozoospermia, CBAVD and secondary obstructive azoospermia, significant increased rates of active caspase-3 were observed in the nucleus and in cases of hypospermatogenesis, in the midpiece. CBAVD and secondary obstructive azoospermia cases showed both a significantly higher probability of presence of active caspase-3 in the sperm nucleus than oligozoospermia and hypospermatogenesis, and secondary obstructive azoospermia showed 1.89-fold higher than CBAVD. However, no differences were found for the detection of active caspase-3 in the sperm nucleus between oligozoospermia and hypospermatogenesis. In cases of hypospermatogenesis, a significantly higher probability of presence of caspase-3 positive sperm was found in the midpiece in relation to oligozoospermia, CBAVD and secondary obstructive azoospermia. In cases of obstructive azoospermia, the predominant localization of active caspase-3 in testicular sperm nucleus

suggests that nuclear lesions might be on the origin of apoptosis. Additionally, as secondary obstructive azoospermia showed 2.95-fold higher total caspase-3 positive sperm than CBAVD, results also suggest a stronger negative impact of inflammatory duct obstruction in testicular spermatozoa development. In oligozoospermia, active caspase-3 in testicular spermatozoa was also observed mainly in the nucleus, in contrast to ejaculated sperm where it was restricted to the midpiece (Almeida *et al.*, 2005; Paper I). In comparison to secondary obstructive azoospermia and CBAVD, the rates of active caspase-3 in testicular spermatozoa nucleus was significantly lower and similar to hipospermatogenic cases suggesting that testicular sperm apoptosis does not seem to be associated to the pathology. Finally, in hipospermatogenesis the increased rates of active caspase-3 in the midpiece suggest that apoptosis might be related to mitochondrial lesions. Additionally, the increased rates of total caspase-3 positive sperm (70.8%) suggest a prominent role for apoptosis in the reduced sperm production associated to this testicular syndrome. In conclusion, results suggest a strong involvement of apoptosis in azoospermia, with significant differences for the extent and origin of testicular sperm apoptosis found in relation to testicular syndrome (Almeida *et al.*, 2010a; Paper IV).

After caspase-3 activation, cell death by apoptosis proceeds and key proteins are cleaved leading to final cell disassembly. One of the morphological features of apoptosis is the loss of plasma membrane asymmetry due to phosphatidylserine translocation from the inner to the external leaflet of plasma membrane. This alteration was shown to occur early during the execution phase after caspase-3 activation (Martin *et al.*, 1995; Naito *et al.*, 1997) and seems to last until the final stage, when cell breaks into apoptotic bodies (van Engeland *et al.*, 1998). Annexin-V is a protein that, in the presence of calcium, specifically binds to phosphatidylserine. When hapten-labelled, like FITC or biotin, Annexin-V can be used to identify this apoptotic morphological feature (Almeida *et al.*, 2007; Paper II; Almeida *et al.*, 2009; Paper III). Additionally, the use of propidium iodide, a membrane impermeable DNA stain, allows distinguishing live from dead cells (van Engeland *et al.*, 1998).

As active caspase-3, phosphatidylserine translocation was shown to be present in human ejaculated spermatozoa. However, results from the literature are still controversial and the presence of sperm disrupted plasma membrane was related not only to apoptosis (Oosterhuis *et al.*, 2000b; Shen *et al.*, 2002; Weng *et al.*, 2002; Barroso *et al.*, 2006; Varum *et al.*, 2007; Zhang *et al.*, 2008) but also to sperm capacitation processes (de Vries *et al.*, 2003; Martin *et al.*, 2005; Grunewald *et al.*, 2007a; Espinoza *et al.*, 2009b). Increased rates of externalized phosphatidylserine were shown in semen from infertile men and to be associated with decreased rates of sperm concentration and motility (Oosterhuis *et al.*,

2000b), decreased sperm motility and normal morphology (Shen *et al.*, 2002; Barroso *et al.*, 2006), decreased sperm concentration, motility and normal morphology (Zhang *et al.*, 2008) or only with decreased sperm motility (Weng *et al.*, 2002; Varum *et al.*, 2007). Other authors, however, failed to show any relationship between phosphatidylserine translocation and abnormal semen parameters (Barroso *et al.*, 2000; Ricci *et al.*, 2002; Lachaud *et al.*, 2004; Espinoza *et al.*, 2009b).

To better characterize the relationship between apoptosis and abnormal semen parameters, the rates of phosphatidylserine externalization with distinction among live, early and late apoptotic and necrotic spermatozoa were determined in ejaculated spermatozoa from men with normal and abnormal sperm parameters (Almeida *et al.*, 2009; Paper III). Data showed significantly decreased rates of viable sperm and significantly increased rates of total and late apoptotic sperm from the neat semen of patients with abnormal sperm concentration, motility and morphology in comparison to normozoospermic patients, thus showing a relationship with all semen parameters. Previous studies have also shown increased rates of late apoptotic sperm in ejaculated samples with decreased sperm motility (Varum *et al.*, 2007), decreased sperm motility and normal morphology (Shen *et al.*, 2002) and also decreased sperm concentration, motility and morphology (Zhang *et al.*, 2008). In the present study and when considering simultaneous semen parameters abnormalities, only oligoasthenoteratozoospermic (OAT) men showed increased rates of total and late apoptosis in comparison with normozoospermia, whereas pure teratozoospermia only showed increased rates of late apoptosis and no significant differences were found for asthenoteratozoospermia. Additionally, OAT showed increased rates of total and late apoptosis than asthenoteratozoospermia and pure teratozoospermia, and decreased rates of early apoptosis in relation to asthenoteratozoospermia and normozoospermia. These results suggest that although apoptosis seems to be related to all individual semen parameters, total rates of apoptosis are only increased when the overall semen quality is reduced (Almeida *et al.*, 2009; Paper III).

As increased rates of total apoptosis were associated only with late apoptosis, and increased rates of early apoptosis were observed only in normozoospermia, data also suggest the presence of an active mechanism responsible for the removal of cells in an advanced degradation stage in cases of normozoospermia. In counterpart, such mechanism seems to be deficient in men with abnormal semen parameters, namely in OAT and pure teratozoospermia, where the apoptotic process seems to develop to later stages thus enriching the pool with dying and incapable spermatozoa. Although more studies are needed to elucidate the mechanisms responsible for phosphatidylserine externalization in ejaculated spermatozoa from infertile men with abnormal semen parameters, previous studies support

the involvement of a caspase-dependent mechanism (Weng *et al.*, 2002; Paasch *et al.*, 2003). It was, indeed, recently shown that separation of spermatozoa with phosphatidylserine externalization by **M**agnetic-activated **c**ell **s**orting combined with **a**nnexin **V**-conjugated **m**icro**b**eads (ANMBs-MACS) resulted in an annexin-V depleted semen fraction with significantly lower rates of sperm with activated caspase-3, disrupted transmembrane mitochondrial potential and increased rates of sperm motility, long term viability and fertilization after ICSI (de Vantery Arrighi *et al.*, 2009; Grunewald *et al.*, 2009b). Because phosphatidylserine translocation and caspase-3 activation occur at different time frames (Kotwicka *et al.*, 2008) results for the relationship between apoptosis and abnormal semen abnormalities differ from the previous study (Almeida *et al.*, 2005; Paper I). However, phosphatidylserine externalization combined with PI allowed the distinction between early and late phases of apoptosis giving a more accurate relationship between apoptosis and abnormal semen parameters.

As previously shown (Almeida *et al.*, 2005; Paper I) sperm selection by gradient centrifugation and swim-up significantly decreased the rates of apoptotic sperm (active caspase-3). However, our present study showed that semen samples from OAT men still presented increased rates of total apoptosis in relation to controls, pure teratozoospermia and asthenoteratozoospermia, and this was shown to be related only to increased late apoptotic sperm. Oligoasthenoteratozoospermic men maintained an increased risk of presence of total and late apoptotic sperm and only pure teratozoospermic showed significant decreased rates of total apoptosis and a significant loss of risk (Almeida *et al.*, 2009; Paper III). This could be explained by the method of selection used, as a previous study has proven not to be the best in the reduction of apoptotic sperm positive for phosphatidylserine translocation and DNA fragmentation (Lachaud *et al.*, 2004).

Since an association between abnormal semen parameters and sperm apoptosis was proven to exist, additional techniques to test sperm quality before ART or even deplete apoptotic spermatozoa, could be useful to predict and increase ART outputs. Some methodologies were already suggested to be helpful in cases of abnormal semen parameters (Marchetti *et al.*, 2002; Paasch *et al.*, 2003; Glander, 2005). Loss of mitochondrial membrane potential is an early event associated with the intrinsic apoptotic mechanism pathway and was shown to be negatively correlated with high fertilization rates after ART. Analysis of mitochondrial membrane potential was suggested to be the most sensitive test (among DNA fragmentation, cell viability and ROS generation tests) to determine sperm quality and predict ART outcomes (Marchetti *et al.*, 2002). In order to deplete apoptotic spermatozoa and increase sperm quality from infertile patients, ANMBs-MACS was shown to efficiently deplete spermatozoa with high DNA fragmentation,

deteriorated membranes, active caspase-3 and disrupted mitochondrial membrane potential (Paasch *et al.*, 2003; Said *et al.*, 2006). This technique is based on Annexin-V binding properties to phosphatidylserine externalized on the outer leaflet of sperm plasma membrane and seems to enhance sperm fertilization potential in ART (Makker *et al.*, 2008). Combination of ANMBs-MACS with density gradient centrifugation was shown to be the most effective sperm preparation technique in providing motile, viable and non-apoptotic sperm during ART (Glander, 2005), and to result in significant embryo cleavage, clinical pregnancy and implantation rates improvement in cases of men with abnormal semen parameters (Dirican *et al.*, 2008). However, sperm motility remained impaired after sperm selection from OAT samples by density gradient centrifugation followed by ANMBs-MACS (Grunewald *et al.*, 2009b). As our data showed that total and late apoptosis remained significantly increased in OAT samples in relation to normozoospermic, asthenozoospermic and pure teratozoospermic even after density gradient centrifugation followed by swim-up (Almeida *et al.*, 2009; Paper III), data strongly suggests significant decreased sperm quality in cases of overall semen parameters abnormalities.

Sperm phosphatidylserine externalization was also related to sperm capacitation rather than to sperm apoptosis, questioning the applicability of ANMBs-MACS (Espinoza *et al.*, 2009b). However, sperm capacitation and sperm apoptosis were shown to be inverse processes in human sperm (Grunewald *et al.*, 2009a).

In normal human testis, apoptotic germ cells recognition and phagocytosis by Sertoli cells occurs in a phosphatidylserine dependent manner and is essential for an efficient sperm production (Nakanishi *et al.*, 2004). Phosphatidylserine translocation was also shown to be present in testicular germ cells from azoospermic men. In hypospermatogenesis, phosphatidylserine translocation was found in germ cells associated with Sertoli cells and shown to be related to a caspase-dependent mechanism. On the contrary, Sertoli cell-free germ cells, showed no phosphatidylserine translocation neither caspase activity (Tesarik *et al.*, 2004). However, other study showed negligible rates of phosphatidylserine translocation in primary spermatocytes and round spermatids and increased rates of externalized phosphatidylserine in late elongated spermatids from cases with post-meiotic arrest at the late spermatid stage (Tesarik *et al.*, 1998).

In order to describe the rates of elongated spermatids with phosphatidylserine externalization in human azoospermia, testicular samples from men with congenital bilateral absence of the vas deferens (CBAVD), with secondary obstructive azoospermia due to epididymal inflammation and with hypospermatogenesis were analyzed (annexin-V/PI).

Samples with oligozoospermia, and anejaculation were used as controls (Almeida *et al.*, 2007; Paper II; Almeida *et al.*, 2009; Paper III). Results showed decreased rates of intact cells and increased rates of total apoptosis in anejaculation, obstructive azoospermia and hypospermatogenesis in relation to oligozoospermia, thus further suggesting that apoptosis seems to be related to azoospermia. As patients with anejaculation revealed decreased testicular sperm quality in relation to oligozoospermic patients, data suggest a negative impact of sperm release absence on testicular spermatozoa quality. Additionally, CBAVD presented increased rates of necrosis and late apoptosis in relation to secondary obstruction and of late apoptosis in relation to hypospermatogenesis, and decreased sperm quality in relation to anejaculation, being consistent with a negative impact of long-term obstruction on testicular sperm quality (Almeida *et al.*, 2007; Paper II; Almeida *et al.*, 2009; Paper III).

Testicular samples were also compared with control samples from the neat fraction (Almeida *et al.*, 2009; Paper III). Significantly decreased rates of intact cells and significantly increased rates of total apoptotic testicular spermatozoa were found in anejaculation, obstructive azoospermia, CBAVD, secondary obstruction and hypospermatogenesis in relation to ejaculated spermatozoa from normozoospermic men, suggesting, again, a relation of apoptosis with testicular disorders. However, this was due only to early apoptosis since late apoptotic sperm was significantly reduced in comparison to ejaculated controls. Testicular spermatozoa from oligozoospermic cases needing a testicular biopsy showed increased rates of intact cells due to a significant reduction in the rates of total apoptotic sperm. Additionally, testicular spermatozoa from oligozoospermic cases also showed increased rates of intact cells and decreased rates of total apoptosis in relation to ejaculated spermatozoa from oligozoospermic and OAT patients. Again, increased rates of early apoptosis were present in testicular spermatozoa and increased rates of late apoptosis were found in ejaculated sperm. Results suggest the presence of a post-testicular apoptotic induction factor in cases of oligozoospermia, responsible for the activation of apoptosis and, consequently, the presence of ejaculated sperm in a late phase of apoptosis (Almeida *et al.*, 2009; Paper III). So, as recently suggested, oligozoospermia does not seem to be related to testicular sperm apoptosis (Almeida *et al.*, 2010; Paper IV). Results strongly favor the use of testicular spermatozoa in clinical treatments of patients with previous history of conception failure using ejaculated spermatozoa, namely, in oligozoospermic patients (Tesarik *et al.*, 2001; Alvarez, 2005; Greco *et al.*, 2005).

In conclusion, testicular sperm apoptosis seems to be increased in azoospermia. In cases characterized by prolonged tubular obstruction, such as CBAVD and anejaculation, data suggest the involvement of a caspase-dependent apoptotic mechanism that might be

activated due to nuclear lesions. In cases of hypospermatogenesis, the increased rates of apoptosis were also related to a caspase-dependent apoptotic mechanism, but activated, probably, through mitochondrial lesions. In cases of oligozoospermia, results suggest the presence of a post-testicular apoptotic induction factor and the use of testicular sperm as a good alternative during clinical treatments. Although an association between apoptosis and abnormal semen parameters was described, increased rates of late apoptotic sperm were shown to be present only in cases of overall decreased sperm quality as observed in ejaculated sperm from men with OAT.

So far, the rates of apoptosis in ejaculated and testicular sperm were described and the involvement of possible apoptotic mechanisms is suggested. Apoptosis was also shown to be present in all germ cells stages and to be essential during normal spermatogenesis in order to assure a normal sperm output (Hikim *et al.*, 1999). Although increased rates of apoptosis were shown to be present in germ cells from cases with disrupted spermatogenesis (Lin *et al.*, 1997a, 1997b, 1999b; Martincic *et al.*, 2001), more information is needed in order to search for the significance of germ cell apoptosis in these cases.

Increased rates of DNA fragmentation were found in Sertoli cells from testicular tissue of men with SCOS and in Sertoli cells and primary spermatocytes in men with maturation arrest, in relation to spermatogenic cells from men with obstructive azoospermia (Kim *et al.*, 2007). The Fas/FasL system was shown to be involved and increased rates of Fas (germ cells) and FasL (Sertoli cells) were described (Francavilla *et al.*, 2000; Eguchi *et al.*, 2002; Kim *et al.*, 2007). Active caspase-3 was shown to be significantly increased in maturation arrest (Leydig, Sertoli and germ cells) and SCOS testes in relation to testes with obstructive spermatogenesis (Kim *et al.*, 2007) and in spermatocytes and round spermatids of maturation arrest testis, whereas in testis from men with normal testicular tissue histology, pro- and active caspase-8 (Leydig cells and germ cells, mostly spermatogonia) and pro- and active caspase-9 (somatic cells) were present (Bozec *et al.*, 2008). Finally, increased rates of TUNEL-positive spermatocytes and spermatids were found in maturation arrest in relation to hypospermatogenesis, CBAVD and secondary obstructive azoospermia (Bozec *et al.*, 2008). Taken together these data suggest that active caspase-3 play an important role in cases of maturation arrest. However, increased apoptotic indexes (TUNEL assay) were recently shown in hypospermatogenesis and normal spermatogenesis in comparison with maturation arrest and no DNA fragmentation was found in Sertoli cells from SCOS men (Stiblar-Martincic, 2009). Active caspase-3 expression was also found to increase in an age dependent manner in germ cells from fertile men (El-Domyati *et al.*, 2009) and to be related to increased oxidative stress environments (Kilic *et al.*, 2009).

Although some evidence exists for the role of apoptosis in testicular failure, some of the studies are not concordant. Efficient and unbiased tools should be used when analyzing and quantifying testicular sections. Testicular tissue preparation for analysis is usually carried out by paraffin inclusion or other embedding media followed by 3-4µm sections cuts. Such procedure can induce somatic cells and germ cells distortion in number, size and shape within the section analyzed. In order to overcome possible bias, stereological tools were introduced in histological analysis of testicular tissue (Petersen *et al.*, 1999). Stereological analysis ensures an uniformed sample where Sertoli cells and germ cells from all stages have the same probability to be sampled and where possible distortions in cell volume and orientation during histological processing will not interfere with the final results (Petersen *et al.*, 1999; Mandarin-de-Lacerda, 2003). In addition, even though several seminiferous tubules are analyzed, a testicular section only provides estimation on how the entire testis might function. Stereology with appropriate computations, including testicular volume value, can give information according to the total number of cells in testis in order that final results truly represent the entire testis (Mandarin-de-Lacerda, 2003).

In order to characterize apoptosis during impaired spermatogenesis, active caspases-3, -8 and -9 were determined in Sertoli cells and all germ cells in testicular tissue from patients with oligozoospermia, CBAVD, secondary obstructive azoospermia, hypospermatogenesis, maturation arrest and SCOS. Active caspases-3, -8 and -9 per Sertoli and germ cells was determined using stereological analysis. In all cases, caspase staining was observed in Sertoli cell cytoplasm and in the nucleus and cytoplasm of all germ cell stages: spermatogonia, primary and secondary spermatocytes and round spermatids. When possible, about 20 tubules were analyzed. Analysis and quantification of Sertoli cells and germ cells with active caspases was firstly done at the seminiferous tubule level and then results were determined for the entire testis (Almeida *et al.*, 2010b; Paper V).

In all cases, Sertoli cells presented higher levels of apoptosis in comparison to germ cells. However, considering active caspases-8 and -9, no significant differences were observed among groups suggesting no specific role for Sertoli cells apoptosis in the testicular pathologies analyzed. An exception was found for active caspase-3, where increased active caspase-3 in Sertoli cells from SCOS were observed, confirming previous data where increased rates of active caspase-3 in Sertoli cells were suggested to be on the origin of germ cell depletion observed in this specific syndrome (Kim *et al.*, 2007).

Contrarily to previous studies where the FasR/FasL system with caspase-3 activation was suggested to be involved in germ cell degeneration in cases of maturation arrest (Eguchi *et al.*, 2002; Francavilla *et al.*, 2002; Kim *et al.*, 2007), our results suggest no active role for apoptosis in maturation arrest. Although significant differences were found among germ cells

for the presence of active caspase-9, no significant differences were found for the presence of active caspases-3 and -8. On the contrary, another study showed increased immunostaining of active caspase-3 in spermatocytes and in spermatocytes and spermatids in cases of maturation arrest at the spermatocyte or at the round spermatid level, respectively (Bozec *et al.*, 2008). However, caspases were only evaluated according to their presence and no quantification study was made. Additionally, increased apoptotic indexes (TUNEL-assay) were described in maturation arrest testes at the spermatocyte level in comparison to normal spermatogenesis, hypospermatogenesis and SCOS (Kandirali *et al.*, 2008). Discordant results might be due not only to differences in maturation arrest histology but also to differences in germ cell quantification and analysis. In the present study we analyzed and quantified all germ cell stages separately and used a more stringent quantification method (Almeida *et al.*, 2010b; Paper V).

In complete spermatogenesis cases (oligozoospermia, CBAVD, secondary obstructive azoospermia and hypospermatogenesis), increased primary spermatocytes with active caspase-3 in comparison to the other germ cell stages were found. Results thus suggest that a malfunctioning in the meiotic phase of spermatogenesis, namely at the first meiosis, might be on the origin of some of these testicular pathologies. In spite of this, secondary obstructive azoospermia cases showed increased active caspase-3 in all germ cell stages in comparison to the other groups. Significant differences were also present for active caspases-8 and -9 levels among germ cells but no differences were found between them. Although both the intrinsic and the extrinsic apoptotic pathways seem to be present, results suggest that none of them seem to prevail. Extrinsic factors on the origin of the obstruction might have activated the extrinsic apoptotic pathway with caspase-8 activation but a cross-talk between the intrinsic and the extrinsic pathways might also exist during spermatogenesis with both mechanisms contributing to apoptotic germ cell death observed in these patients. However, in congenital obstruction (CBAVD) cases, no significant differences were found among germ cells for the presence of active caspase-8. On the contrary, significant differences for the presence of germ cells with active caspase-9 and active caspase-3 were observed, with primary spermatocytes presenting the most increased levels. Results suggest that, in order to establish equilibrium in spermatogenesis of these patients, where no testicular spermatozoa release exists, the intrinsic apoptotic pathway might be activated through mitochondrial lesions with consequent caspase-9 and caspase-3 activation. Meiotic germ cell death by apoptosis might be responsible in maintaining an appropriate germ cell number (Almeida *et al.*, 2010b; Paper V).

Previous studies on testicular spermatozoa have shown increased rates of active caspase-3 in patients with CBAVD and secondary obstructive azoospermia. However, active

caspase-3 staining was observed mainly in the sperm nucleus (Almeida *et al.*, 2010a; Paper IV). In the present study, active caspase-3 immunostaining was observed either in germ cells cytoplasm and nucleus suggesting that inflammation and mitochondrial lesions in cases of secondary obstruction and only mitochondrial lesions in CBAVD patients, might activate the apoptotic pathway, induce caspase-3 activation among germ cells with consequent translocation to the nucleus (Almeida *et al.*, 2010b; Paper V).

Testicular histological analysis of hypospermatogenic patients show significant decreased rates of germ cells in each stage of spermatogenesis (McLachlan *et al.*, 2007). Although no significant differences were found for the presence of active caspase-8 among germ cells, significant differences were found for the presence of active caspase-9 with increased levels at the primary spermatocyte stage. So, germ cell death by the intrinsic apoptotic mechanism might be on the origin of the lower numbers of germ cells. Additionally, among all syndromes and after SCOS, Sertoli cells with active caspase-3 from hypospermatogenic patients showed to be increased. Sertoli cell death by apoptosis might also lead to a lower number of germ cells due to decreased support capacity and nutrition, essential to maintain normal germ cells numbers (Almeida *et al.*, 2010b; Paper V). These results reinforce the results obtained recently where increased rates of active caspase-3 were shown to be present in the midpiece of testicular spermatozoa from hypospermatogenic patients (Almeida *et al.*, 2010a; Paper IV). So, additionally with Sertoli cell death, results strongly favor the hypothesis of mitochondrial damage at the primary spermatocyte stage as the origin of the present testicular disorder.

Contradictory results were described with active caspase-3 staining barely present in germ cells from patients with CBAVD, secondary obstruction and hypospermatogenesis (Bozec *et al.*, 2008). However, only the presence of active caspase-3 staining was analyzed, and no germ cell quantification and statistical comparisons were made.

Finally, oligozoospermic patients needing a testicular biopsy presented significant differences for the presence of active caspases-8 and -9 among germ cells, being primary spermatocytes the germ cell stage with most increased values. Comparisons between both proteins showed significant increased spermatogonia with active caspase-9. Previous results on testicular spermatozoa apoptosis from oligozoospermic patients showed significant differences for apoptosis between semen and testis, thus suggesting a post-testicular apoptotic induction factor as the main cause of decreased ejaculated spermatozoa (Almeida *et al.*, 2009; Paper III; Almeida *et al.*, 2010; Paper IV). These additional findings suggest that stem cell death by mitochondrial damage and meiosis malfunctioning during spermatogenesis might be on the origin of decreased sperm production observed in these

men. In spite of this, additional apoptosis at a post-testicular level might contribute to ejaculated sperm apoptotic death, decreasing even more the final sperm output.

After analysis of the germ cell line in seminiferous tubules, appropriate computations were made in order to determine the significance of active caspases-3, -8 and -9 in the entire testis in each testicular syndrome. Results showed no significant differences for the presence of active caspases in Sertoli cells and germ cells from maturation arrest testis strongly suggesting no role for apoptosis in this testicular syndrome. Increased active caspase-9 in primary spermatocytes and increased significant differences between active caspase-9 from active caspase-8 in spermatogonia from oligozoospermic patients, reinforce the theory defending that stem cell death is in the origin of the low sperm production. Testicular analysis underlined some differences described at seminiferous tubule level and helped to strength some theories suggested for the significance of apoptosis during impaired spermatogenesis (Almeida et al., 2010b; Paper V).

In order to strength former theories, more patients needed to be analyzed. Unfortunately, in most cases, the reduced samples obtained from testicular biopsies during clinical treatments are not enough to provide this type of research. However, the present study gives some good insight into possible apoptotic mechanisms involved in cases of deranged spermatogenesis and that should be fundamental to explore.

Apoptosis thus seems to be involved in impaired spermatogenesis, either at the testicular or at the semen level. The rates of apoptosis were described in ejaculated sperm from patients with normal and abnormal spermatogenesis and a correlation to spermiogram abnormalities was achieved. Additionally, and using different approaches, the involvement of apoptosis in somatic Sertoli cells, different germ cell stages and testicular spermatozoa was analyzed. After all, suggestions were made for possible apoptotic activation mechanisms during abnormal spermatogenesis.

In order to confirm the suggested apoptotic mechanisms involved in each testicular syndrome and search for new insights that clearly define the apoptotic proteins involved in impaired spermatogenesis, additional studies should be performed. Stereological analysis seems to be a promising approach for testicular germ cells quantitative studies. Pro- and anti-apoptotic proteins, like members from the BCL-2 family, are crucial in apoptosis regulation and little is known about their function in these pathological cases. Since apoptosis has been proved to be one of the mechanisms involved in spermatogenic impairment, future clinical therapies involving germ cell apoptotic control may arise from those studies.

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