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Stress and Cell Death in Testicular Cells

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Abstract

The success of male reproduction requires the production of a large number of spermatozoa by a unique process known as spermatogenesis. Spermatogenesis is carried out in close association with the Sertoli cells, the only somatic cells of the seminiferous epithelium which are responsible for providing structural, nutritional and endocrine support to the developing germ cells. The seminiferous epithelium of the testes is a rapid proliferation tissue, where the germinal cells, through a large number of mitotic and meiotic divisions prior to their differentiation culminate with the structural and functional formation of spermatozoa. The number of germ cells that Sertoli cells can sustain is maintained by apoptosis, which fulfills the elimination of germ cells with genetic errors, damage to DNA or excess cell production. Apoptosis can also be activated by external factors such as stress, causing alterations in spermatogenesis and testicular involution, which compromises fertility. However, death in testicular cells is not attributed only to apoptosis, as cells use different mechanisms to activate their self-elimination, such as anoikis and autophagy. All of these mechanisms are discussed.

Keywords: Spermatogenesis; Stress; Cell death; Apoptosis; Autophagy; Anoikis

Introduction

The seminiferous epithelium of the testes is a rapid proliferation tissue, where the germinal cells are produced in a clonal way, through a large number of mitotic and meiotic divisions prior to their differentiation, culminating with the structural and functional formation of spermatozoa. Alterations in this process can lead to an excess production of germ cells, exceeding the carrying capacity of the Sertoli cells. Therefore, in the seminiferous epithelium there is a mechanism in charge of regulating the number of germ cells that Sertoli cells can sustain without exceeding their capacity [1]. This mechanism is regulated via apoptosis through the Fas system activation, which is a paracrine signaling pathway proposed as an important physiological regulator of apoptosis in germ cells [2]. Apoptosis can also be activated by internal stimuli such as: decreased testosterone [3], follicle stimulating hormone (FSH) or by external factors such as stress, which can cause alterations in the spermatogenesis development and affect spermatozoa production [4-6].

Spermatogenesis

The success of male reproduction requires the production of a large number of spermatozoa by a unique process known as spermatogenesis [6]. This process is carried out in the seminiferous epithelium that covers the seminiferous tubules continuously, in close association with Sertoli cells [7]. In the seminiferous epithelium are located spermatogonia cells (stem cells), which are undifferentiated diploid germ cells (2n), located at the base of this epithelium, near the basal lamina. These cells are mitotically divided to produce more spermatogonia; some of them mature and differentiate in type A spermatogonia, which are mitotically divided into spermatogonia, and later in type B spermatogonia. The latter divide once or twice by mitosis to form primary spermatocytes, which initiate the first meiotic division to form secondary spermatocytes, which start rapidly the second meiotic division, As a result of the second meiotic division, each secondary spermatozoon forms two rounds spermatids, each with a haploid number of 23 chromosomes in the human [6-10]. Once formed, the spermatids are transformed into functional spermatozoa by series of progressive morphological changes called collectively as spermiogenesis (Figure 1) [9,10]. In the seminiferous epithelium, germ cells present associations that progress very precisely over time and are organized cyclically [7,8], according to their different stages of development and differentiation. In the rat, spermatogenesis is divided into XIV cell stages or associations that constitute the cycle of the seminiferous epithelium, which lasts from 48 to 52 days [7,9,10]. Spermatogenesis is carried out in close association with the Sertoli cells, the only somatic cells of the seminiferous epithelium; which are responsible for providing structural, nutritional and endocrine support to the developing germ cells [11,12].

These cells have specific receptors for FSH and testosterone. The FSH exerts its action through membrane receptors coupled to Gs proteins, and plays an important role in the stimulation of DNA synthesis, in the mitosis of type B spermatogonia and in the meiosis of primary spermatocytes in the preleptotenous phase [13].

Testosterone is produced by the Leydig cells, located in the interstitial space between the seminiferous tubules, and plays an important role in maintaining the development and conservation of male sexual characteristics, but also in the functioning of the male sex glands and maintaining spermatogenesis in all stages [14]. In the testes, there is a strict endocrine regulation that directly involves testosterone and FSH, both hormones acting as germ cell survival factors [14]. The decrease in the synthesis of these hormones has been shown to increase the occurrence of cell death via apoptosis in germ cells located at specific stages of the seminiferous epithelial cycle [14,15].

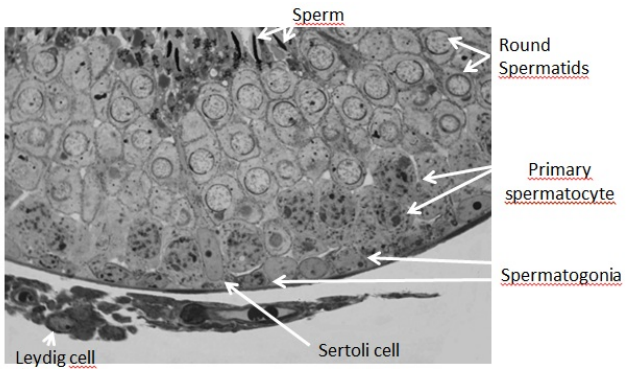

Figure 1: Cells contained in the seminiferous epithelium involved in spermatogenesis.

Apoptosis

Apoptosis is an innate and evolutionarily conserved genetically programmed process that, once activated, induces cell death through characteristic biochemical and morphological changes, culminating in cell fragmentation and elimination of apoptotic bodies by phagocytosis [16]. Apoptosis is essential during the organism development and for the maintenance of homeostasis in organs and tissues in the adult [16,17]. Two signaling pathways involved in apoptosis activation have been described: the extrinsic pathway and the intrinsic pathway [18], which converge into a common component, the activation of caspase 3; which is the main effector caspase [18,19].

As illustrated in Figure 2, the activation of the extrinsic pathway is initiated by the junction of a ligand (Fas ligand) [20] with its receptor (Fas), located on the surface of the cell membrane. While the intrinsic pathway can be activated by the changes produced in the Bax/Bcl-2 ratio, located on the outer membrane of the mitochondria, which leads to the activation of the caspases 8 and 9, respectively, which once activated, break and activate caspase 3 [19,21], initiating the cell death process.

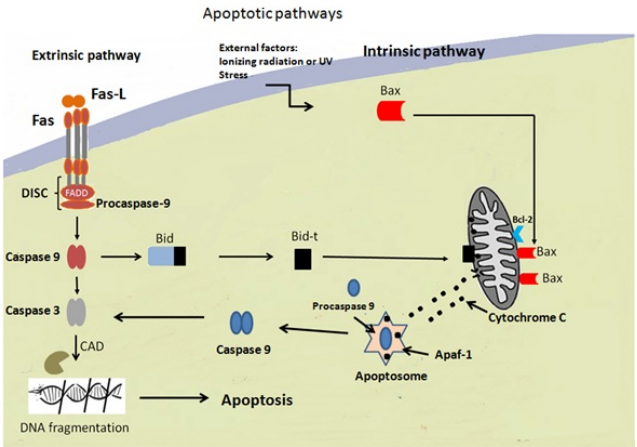

Figure 2: Components involved in the intrinsic and extrinsic apoptosis signaling pathways. Scheme modificated from [22].

During apoptosis, most cells exhibit characteristic biochemical and morphological changes that affect all cell aspects, from the plasma membrane to the nucleus [19]. The most notable changes occurring in apoptosis dying cells are: decrease in cell volume, compaction of cytoplasmic organelles, dilation of the endoplasmic reticulum, alterations in the plasma membrane, condensation and fragmentation of nuclear chromatin and the formation of apoptotic bodies [23,24], which are eliminated via phagocytosis (Table 1).

Structure	Alteration
Nucleus	Nuclear fragmentation Chromatin Condensation DNA fragmentation
Cytoplasm	Loss of cytoplasmic volume Degradation of cytoplasmic proteins
Plasma membrane	Exposure of phosphatidylserine in the extracellular space Loss of the gradient of potassium
Mitochondria	Rupture of the outer membrane Release of apoptotic proteins Loss of the gradient of the membrane

Table 1: Main biochemical and molecular changes of dying cells via apoptosis [19].

Importance of Cell Death in the Seminiferous Epithelium Germ Cells

Germ cell death is a common event that occurs during the development of spermatogenesis. During this process, a large germ cell population dies via apoptosis [23,24], mainly affecting spermatogonia, primary spermatocytes and spermatids at different stages of the seminiferous epithelial cycle [23,25]. In testes, apoptosis is an important process that fulfills several functions such as: eliminating germ cells with genetic errors, damage to DNA or excess cell production [25-27].

Several studies have shown that the Fas system is an important regulator of apoptosis in germ cells [25,28]. In the testis, the Fas receptor is mainly located on the membrane of primary spermatocytes, spermatids, and Leydig cells [25,29], whereas Fas ligand (FasL) is produced by Sertoli cells [1,26]. Fas binding with its ligand (Fas / FasL) induces a receptor trimerization, promoting the formation of the death signal inducing complex, known as DISC, by the recruitment of the adapter protein FADD (Fas associated domain of death). The DISC complex recruits the pro caspase 8, which is proteolytically processed to its active form; in this way caspase 8 can activate caspase 3 [21] and initiate death in different types of testicular cells.

In germ cells, the Fas system can be activated when germ cell overpopulation occurs in the seminiferous tubules. This occurs because Sertoli cells are unable to provide a suitable hormonal environment, so cells initiate a self-elimination process via Fas [29]. In addition, this system can also be activated by the decrease of testosterone, FSH [30] or by external stimuli such as stress or exposure to chemotherapeutic treatments, among other factors [31,32] as modulators of germ cell survival and death [32-34]. This family consists of pro-apoptotic proteins: Bax, Bad, Bak and Bid and by the anti-apoptotic proteins: Bcl-2, Bcl-XL, A1, Boo, Bcl-w, Mcl-1 [35].

There are external factors that can trigger apoptosis in germ cells through the intrinsic pathway, including heat or cold stress, exposure to radiation, or the use of chemicals that lead to an increase in the frequency of apoptosis in the germ cells of the testicular tissue, leading to a large loss of germ cells.

Stress causes Testicular Cells Death

Stress is the physiological response of the organism to a stressor. Stressors are adverse stimuli, intrinsic or extrinsic, capable of altering the body's homeostasis [36-39]. Organisms have the capacity to respond to these stimuli by activating neuroendocrine and peripheral processes in charge of the stress response [38-40]. The stress response is regulated by the stress system, which is constituted by neuroendocrine structures that are part of the central (CNS) and peripheral nervous system.

The central components include the hypothalamus and noradrenergic neurons of the locus coeruleus (LC), located in the brainstem, responsible for secreting corticotropin releasing hormone (CRH) and noreprinefrin, respectively [37,40], which are important modulators of the response of "flight or fight". Peripheral components include the pituitary and adrenal gland involving the hypothalamus-pituitary-adrenal axis (HHA). The activation of the stress system facilitates the adaptation process of the organisms and increases their survival capacity, in adverse environmental conditions [37].

There are currently animal models that are used to study the effect of stress, such as immobilization [41], unpredictable chronic stress [10], cold water immersion [42] or hot stress [43], applied in rats. These types of stressors trigger the activation of the HHA axis stimulating glucocorticoid secretion from the adrenal cortex, into the systemic circulation. Glucocorticoids are the end effectors of the HHA axis and are involved in the organism homeostasis control during the stress response [37] in situations of chronic stress, glucocorticoids inhibit the secretion of testosterone, affecting the spermatogenesis development, sperm production and inducing apoptosis in testicular cells [44].

Cell Death Activation by Stress in Testicular Germ Cells

The testes are very sensitive to stress produced by heat or cold exposure; both stimuli can promote the apoptosis activation in germ cells. Direct exposure to heat stress (43°C for 15 min) has been shown to induce the apoptosis in the germinal cells of the seminiferous epithelium, mainly affecting the primary and round spermatids located in stages IV and Xll to XIV of the seminiferous epithelial cycle [43,45]. It has been previously reported that this stressor may induce the cytoplasmic translocation of Bax to a nearby region of the outer membrane of the mitochondria [46]. The relocation of Bax is accompanied by the cytosolic release of cytochrome c and is associated with the activation of caspase 9, which in turn activates caspase 3 [23].

At the same time, Bax translocate to the endoplasmic reticulum, which is the site of localization of caspase 12. These findings indicate that both, the mitochondrial pathway and an endoplasmic reticulum-dependent pathway may be involved in the activation of apoptosis in the germ cells of the testis, induced by heat. Stress produced by thermal shock (40°C for 2 hour) as well as cold stress (40°C for 2 hour) induces apoptosis in primary spermatocytes and spermatids.

This effect is associated with increased activity of caspases 3, 8 and 9. Activation of the caspase cascade indicates that both signaling pathways (extrinsic and intrinsic) could probably be involved in the activation of apoptosis of Germ cells [47]. In recent years, the effect of chronic cold-water immersion stress on various aspects of male reproductive function, such as sexual behavior, testosterone secretion and spermatogenesis [42] has been investigated in the male rat [42].

It has been shown that this stressor has a profound inhibitory effect on testosterone plasma concentration, which significantly decreases, and at the same time causes a significant increase in plasma corticosterone concentration [42]. Cold water immersion stress (15°C for 15 min) applied to rats for 1, 20, 40 and 50 consecutive days has been shown to increase the percentage of seminiferous tubules containing apoptotic germ cells positive for TUNEL (Terminal deoxynucleotidetransferase). In addition, this stressor increases the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and peroxidation of (H_2O_2) (SOD), catalase (CAT), and glutathione peroxidase (Gpx) [48]. In the testes, H_2O_2 has the ability to induce apoptosis in testicular cells involving the Fas system and its ligand (FasL) as well as pro-apoptotic proteins: Bax, Bid and Bad [36]. This implies that the oxidative stress generated by heat or cold can induce apoptosis in testicular cells (Figure 3).

In this regard, stress by immersion in cold water increases the content of active caspase 3, from an hour after exposure to the stressor. Caspase 3 can be activated by increasing the concentration of active caspase 8 (extrinsic label), either by increasing the Bax content as well as by decreasing Bcl-2 concentration (Figure 4) [49]. Cell death is likely to start when FasL, produced by Sertoli cells, binds to the Fas receptor [30,50], located on the cytoplasmic membrane of germ cells. Although this mechanism has not been fully understood, it appears that the decrease or elimination of gonadotropins or testosterone may induce the Fas system to initiate apoptosis in germ cells via Sertoli cell specific signaling pathways [12].

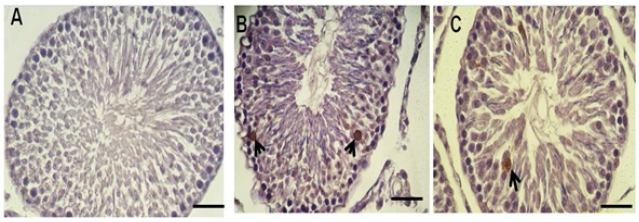

Figure 3: Testes cross-sections of control males and exposed to acute and chronic stress. In a) testis of control males, cells with apoptosis are not observed. In b) male testes exposed to acute stress, the short arrows indicate that the spermatogonia and primary spermatocytes of stages Vl-Vlll were the most susceptible to apoptosis. In c) male testes subjected to chronic stress, the long arrows indicate that the spermatogonia, primary and spermatid spermatocytes located in the Vll-Vll1 and Xll-XIV stages of the seminiferous epithelial cycle were more susceptible to dying from apoptosis. The bar: 40 μm.

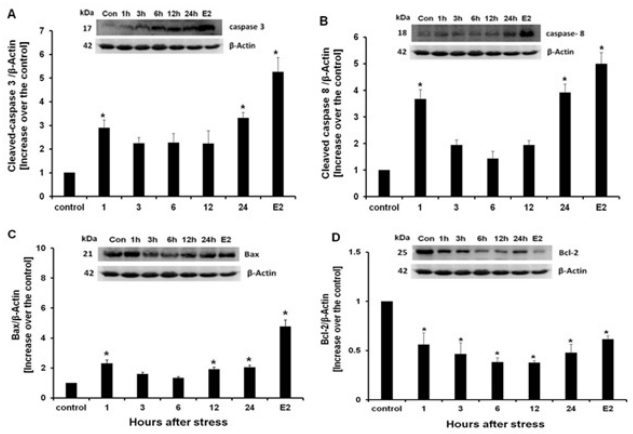

Figure 4: Western blot analysis of the proteins in: A) active caspase 3; In B) active caspase 8; In C) Bax and in D) Bcl-2. Estradiol (E2), as a positive control. The data are presented as the relative expression of protein (OD: optical density) normalized with β-actin. In E) representative gels of five proteins at each time interval. Each point represents the mean+standard error (X+E.E) (n=5) * $p<0.05$ compared to the control [49].

In the testes, both Bax and Bcl-2 have been proposed as important modulators in apoptosis of germ cells [33] Under normal conditions, Bax is located in the cytoplasm of spermatogonia, primary spermatocytes and spermatids [46]. Bcl-2 resides in the outer mitochondrial membrane of these cells, where it regulates its homeostasis and integrity. In response to external signals, Bax translocate to mitochondria and produces changes in mitochondrial membrane potential [50,51], promoting the release of cytochrome C [52]. In the cytosol, cytochrome C interacts with Apaf-1 and, in the presence of ATP, forms the complex known as apoptosome, which recruits and activates procaspase 9 [53], which in turn activates Caspase 3 [36], activating apoptosis through the intrinsic pathway.

Changes in the integrity of the outer mitochondrial membrane promote the release of molecules with proapoptotic activity, such as cytochrome C, from the intermembranal space to the cytoplasm, leading to a marked activation of apoptosis in the testicular cells of males exposed to stress by immersion in cold water [49]. It is likely that cold water immersion stress may be related to the activation of apoptosis in testicular cells through increased corticosterone concentration as well as increased ROS and oxidative stress in the testes. Changes lead to the activation of both the intrinsic and extrinsic pathway of apoptosis.

Effect of Glucocorticoids on Testicular Cells

Glucocorticoids release during the stress response may induce apoptosis in testicular cells through the binding to their cytoplasmic receptors, in primary spermatocytes, spermatids, Sertoli cells [54] and Leydig cells [55]. It has also been shown that the synthetic glucocorticoid dexamethasone can induce apoptosis in germ cells [56], located at specific stages of the seminiferous epithelial cycle (Vll-VIII), through the cytoplasmic translocation of Bax to the external mitochondrial membrane of spermatogonia and primary spermatocytes [57], promoting the activation of the intrinsic pathway of apoptosis. Glucocorticoids bind to glucocorticoid receptors (GR) in the cytoplasm and form the steroid-receptor complex. This complex is translocated to the nucleus and acts as a transcription regulator. GR-induced transcriptional regulation can activate or inactivate the transcription of multiple genes [58,59] involved in cell survival. Inactivation has been proposed to involve transcription factors such as c-jun/c-fos and NF-kB (nuclear factor kappa B), which control several survival pathways. In Leydig cells, prolonged exposure to high glucocorticoid concentrations during stress response directly inhibits the transcription of genes encoding enzymes involved in the synthesis of testosterone [60] as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase/17-20 lyase [61-63]. In addition, glucocorticoids cause apoptosis in Leydig cells [56], thus contributing to the reduction in testosterone secretion [3] observed during stress. It has been proposed that the activation of apoptosis in these cells may be related to the activation of the Fas system of procaspase 3 with the loss of mitochondrial membrane potential and the increase in the generation of ROS [3]. As mentioned above, the increase in glucocorticoid concentration may be related to the activation of apoptosis in testicular cells through an increase in ROS generation [48]. Oxidative stress may be related to various etiologies that cause infertility in various species of male mammals, including humans [64].

Effect of Stress and Apoptosis on Male Fertility

At present, the incidence of apoptosis in the germ cells of the testis is not known. However, spermatogonia, primary spermatocytes and round and elongated spermatids appear to be the most affected. Chronic exposure to stress causes alterations in the progression of spermatogenesis and decreases male fertility, as chronic exposure to water by immersion in cold water, for 20 or 50 consecutive days, causes loss of germ cells, causing a decrease in the seminiferous epithelium are and fertility, with a decrease in the number of offspring in females copulating with chronically stressed males [42]. In rats exposed to chronic stress by immobilization (1 hour) followed by forced swimming (15 min daily for 60 consecutive days), an increase was observed in the apoptosis of germ cells, mainly in type A spermatogonia, in primary spermatocytes in pachytene and in round spermatids, located in stage VII of the cycle of seminiferous epithelium. At the same time, there was a decrease in the concentration of spermatozoa in the tail of the epididymis [63]. On the other hand, a study in humans with oligozoospermia and obstructive azoospermia showed an increase in the apoptosis frequency in different cell types of the seminiferous epithelium, mainly involving primary spermatocytes, spermatids and Sertoli cells. Data obtained in this work, propose that both the intrinsic and extrinsic pathways could be involved in this process, due to the increase in expression and activity of caspases 8, 9 and 3 [64]. These alterations are frequently associated with male infertility and the increased apoptosis frequency in the germinal cells of the seminiferous epithelium [65] as well as with the decrease in the concentration of mature sperm stored in the tail of the epididymis [12,49,66]. In the epididymis, sperm undergo changes during their maturation process; they acquire motility, final condensation of the nucleus and the ability to fertilize. These processes are all androgen-dependent, mainly testosterone and dihydrotestosterone (DHT). Chronic exposure to stress causes decreased testosterone secretion as well as increased corticosterone [49,66,67]. The reduction in the sperm concentration of the epididymis may be related to the decrease in testosterone, since the decrease of this hormone affects the conversion of round to elongated spermatids during spermiogenesis in stages Vll-Vlll and causes its premature detachment, inhibiting its elongation

process [13], this may lead to an increase in the incidence of apoptosis in germ cells, leading to a large loss of germ cells [23]. Thus, eventually, the concentration of mature sperm stored in the tail of the epididymis decreases. On the other hand, stress can cause changes in the internal luminal microenvironment of the epididymis, due to the fact that an oxidizing condition is generated as a result of increased corticosterone, which causes the spermatozoa to be constantly attacked by ERO, mainly by H_2O_2, Inducing DNA fragmentation and alterations in the plasma membrane of these cells. Also, apoptosis can be activated, in order to eliminate spermatozoa that contain some type of chromosomal aberration or damage, to guarantee the production of healthy spermatozoa.

Stress Causes Germ Cell loss by other Cell Death Pathways

In the seminiferous epithelium, the degenerating spermatids that separate from the Sertoli cells, due to the loss of intercellular junctions between the two cell types, do not always present the apoptosis characteristic biochemical and morphological changes. Frequently, this cells present alteration in the cellular membrane, which is observed folded and without form, with deeply stained nuclei and with the fully condensed chromatin this process mainly affects the round spermatids. The term "anoikis" has been proposed to describe the process of cell death in spermatids that are detached from the seminiferous epithelium in response to decreased testosterone and has been observed in testicular cells as a result of chronic stress [50]. This process involves the Bcl-2 modifying factor (Bmf), a proapoptotic member of the Bcl-2 family of proteins, which is expressed in the subacrosomal space of spermatids located in step 4 to 16 of spermiogenesis [68]. However, it is necessary to expand this field of study to clarify the participation of Bmf and the mechanisms involved in this process. When germ cells undergo apoptosis, they often degenerate and may detach and release into the lumen of the tubule [69]. If this does not occur, Sertoli cells can identify and phagocyte germ cells that initiate apoptosis by recognizing phosphatidylserine that is translocated to the surface of these cells. This recognition is carried out through the scavenger type 1 receptor (SR-B1), present on the Sertoli cells surface [70]. It is likely that this mechanism will be activated to ensure that healthy germ cells continue their development and production of spermatozoa. It has recently been reported that stress can activate different mechanisms of self-destruction in germ cells. An example of this is cell death due to autophagy. In a recent study, heat stress (42°C for 15 min) in addition to triggering apoptosis in mouse germ cells was reported; this stressor may induce autophagy in these cells [71] Autophagy is a dynamic and programmed process that proceeds with the sequestration of cytoplasmic proteins and whole organelles within double membrane vacuoles, which are contacted and fused with the lysosomes, forming autolysosomes. The elements captured in the vacuoles are degraded by lysosomal proteases and removed by exocytosis [72]. The molecular mechanisms involved in the induction of autophagy are not fully understood. However, at least 30 genes related to autophagy (*Atg* genes) have been identified (72). It seems that the *Atg* genes regulate the formation of the autophagosome, which requires two conjugation systems that resemble that of ubiquitination in proteins [74], the *Atg12-Atg5* systems and the *Atg8* (LC3) system [72,73]. The formation of the autophagosome begins with the carboxyl terminal end of the glycine residue of the *Atg12* gene, which is activated by the *Atg7* gene, a gene upstream that is expressed in both systems. Subsequently, the *Atg12* gene is transferred to *Atg10* to form the *Atg12-Atg10* complex and finally, *Atg12* is covalently bound to *Atg5* [74]. The formation of the *Atg8 -LC3* system undergoes post-translational modifications prior to its binding to the membrane. Immediately after synthesis, 22 amino acids (in the rat) or 5 amino acids (in the human) are removed from their carboxyl terminal end. This process results in the formation of *LC3-I* residing in the cytosol. Following activation by *Atg7, LC3-I* binds to *Atg3* which transfers phosphoethanolamine, thus inducing the formation of *LC3-II. LC3-II* binds to the membrane of the new vesicle and remains attached to the membrane even after autophagosome formation has been completed [73]. In mice testicles exposed to heat stress, the participation of the *Atg12-Atg5* and *Atg8-LC3-1* systems involved in autophagosome formation was confirmed [74]. Since the amount *LC3-II* is related to the number of autophagosomes, it has been established as a biochemical indicator to predict the activation of autophagy in animal cells [74]. It is probable that an ubiquitin-like conjugation system is present in testis and they may be responsible for the activation of autophagy in the germ cells of the testes by the effect of heat stress. There are really few studies dedicated to assessing the effect of stress on the activation of autophagy in testicular cells, so it is necessary to expand this field of study to know the mechanisms involved in this process.

Conclusion

In the testes, the germ cells loss via apoptosis is an important process that is involved in the spermatogenesis development. However, apoptosis can be activated by external factors such as stress, causing alterations in spermatogenesis and testicular involution, which compromises fertility. Recent studies indicate that cell death in testicular cells is not attributed only to apoptosis, but cells use different mechanisms to activate their self-elimination, such as: Anoikis and autophagy. Apparently, the different cell types that make up the testicles can activate different mechanisms of cell death. This process depends on the magnitude and nature of the stimulus that triggers the death process, as well as on the physiological aspects of each cell type, including the stage of development.

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