Title: Stress response pathways in the male germ cells and gametes

Running Title: Stress response pathways in the male germ line

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ABSTRACT

The unfolded protein response (UPR) is a conserved and essential cellular pathway involved in protein quality control which is activated in response to several cellular stressors such as diseases states, ageing, infection and toxins. The cytosol, endoplasmic reticulum (ER) and mitochondria are continuously exposed to new proteins and in situations of aberrant protein folding, one of three lines of defence may be activated: (i) heat shock response; (ii) mitochondrial UPR; and (iii) endoplasmic reticulum UPR. These pathways lead to different signal transduction mechanisms that activate or upregulate transcription factors that, in turn, regulate genes that increase the cell's ability to correct the conformation of poorly folded proteins or, ultimately, lead to apoptosis. Despite the recent progress in understanding such biological processes, few studies have focused on the implications of the UPR in male infertility, highlighting the need for a first approach concerning the presence of these components in the male reproductive system. In testis there is a high rate of protein synthesis and the UPR mechanisms are well described. However, the presence of these mechanisms in spermatozoa, apparently transcriptionally inactive cells, is contentious, and it is unclear how sperm cells deal with stress. Here, we review current concepts and mechanisms of the UPR and highlight the relevance of these stress response pathways in male fertility, especially the presence and functional activation of those components in male germinal cells and spermatozoa.

Key words: cellular stress, unfolded proteins, unfolded protein response, heat shock response, spermatozoa, testis, male fertility

33 I. INTRODUCTION

34 Protein quality control is crucial to maintain the biogenesis, folding, traffic and degradation of proteins in
 35 cells, and ultimately protein and cell homeostasis. The cytosol, endoplasmic reticulum (ER) and
 36 mitochondria are exposed to nascent polypeptides and each of these compartments needs a dedicated
 37 protein-folding machinery that ensures the organelle's protein-folding capacity (Saito and Imaizumi,
 38 2018). Under cellular stress conditions, the amount of unfolded or misfolded proteins exceeds the protein-
 39 folding capacity, which can result in organelle damage (Pellegrino *et al.*, 2013). To overcome these
 40 problems, eukaryotic cells have specific signalling pathways commonly known as the unfolded protein
 41 response (UPR). UPR takes place in the cytosol (cytosolic heat shock response - HSR), in the
 42 endoplasmic reticulum (UPR^{ER}) as well as inside the mitochondria (UPR^{mt}) and requires communication
 43 with the nucleus. Any disturbance in proteostasis may result in protein aggregation and, consequently,
 44 disorders such as neurodegenerative, inflammatory and metabolic diseases (Doultsinos *et al.*, 2017;
 45 Lindholm *et al.*, 2017).

46 The male gonad (testes) is responsible for the production of spermatozoa (the male gamete), as well as
 47 testosterone (steroidogenesis). In the testis, there is a high rate of protein synthesis and the UPR
 48 mechanisms are responsible for detecting un- or misfolded proteins following exposure to heat,
 49 chemicals, drugs or pathological states. However, the presence of the proteins involved in the UPR in
 50 spermatozoa is contentious. The spermatozoon is an extremely specialized type of cell characterized by
 51 small size, lack of most typical organelles, high polarity and fast motility (Pesch and Bergmann, 2006).
 52 This type of cell is the product of a highly regulated process that takes place within the seminiferous
 53 tubules in testes called spermatogenesis, in which spermatozoa lose most of the cytoplasm and
 54 organelles (Keber *et al.*, 2012). Thus, it is not clear whether stress response pathways operate in
 55 ejaculated spermatozoa since they are virtually transcriptionally inactive and their translational machinery
 56 seems to be lost with the residual cytoplasm, during spermatogenesis. In fact, since sperm cells are
 57 deprived of ER, the UPR will probably be more prominent in cytosol and mitochondria, if present at all.

58 The present work reviews what is already known concerning the mechanisms of stress response in the
 59 male germ line, both in testis and mature spermatozoa, focusing on the UPR and its activation under
 60 specific stress conditions. The link between these defensive mechanisms and male infertility is also
 61 addressed. Additionally, an updated description of these mechanisms in somatic cells is included.

62 II. HEAT SHOCK RESPONSE (HSR)

63 One important response to stress in somatic cells is the HSR, which protects the cytosolic protein-folding
 64 environment. Under non-stress conditions heat shock factor protein 1 (HSF1) exists in an inactive,
 65 monomeric form associated with the cytosolic chaperones heat shock 70 kDa protein 1 (HSP70/HSPA1)
 66 and heat shock protein 90 (HSP90). In response to cellular stress, such as high temperatures, oxidants,
 67 heavy metals, bacterial and viral infections, this protein dissociates from the chaperones, is
 68 phosphorylated and forms homotrimers that translocate to the nucleus and bind to cis-acting DNA
 69 elements (heat shock elements - HSE) located at the promoters of the heat shock genes. It activates the
 70 transcription of heat shock protein (*HSP*) genes (Widlak *et al.*, 2007), such as HSPA1, HSP90A, and
 71 small HSPs such as HSPB1 (HSP27), to assist protein refolding. Once transcribed, HSPB1 form large
 72 oligomeric structures that after phosphorylation (P) reorganize into low-molecular-weight dimers or
 73 oligomers that bind misfolded proteins. Additionally, HSF1 induces the expression of some components
 74 of the ubiquitin-proteasome system to degrade misfolded proteins (Pellegrino *et al.*, 2013). The induction
 75 of HSP expression and accumulation by HSF1 ultimately regulates cellular homeostasis by assisting
 76 protein folding and avoid protein denaturation (Shi *et al.*, 1998; Pellegrino *et al.*, 2013) (**Figure 1**).

77 (1) HSR in the male germ line and fertility

78 The capacity to adapt to variation in temperature is essential for the viability of all living organisms. The
 79 exposure to high temperatures results in protein denaturation and the HSR constitutes the major adaptive
 80 response to proteotoxic stress (Hayashida *et al.*, 2006; Kim *et al.*, 2013a; Dokladny *et al.*, 2015).
 81 Particularly in testes, for correct spermatogenesis occurs, the testicular temperature is maintained 2 - 8°C
 82 below the body temperature (Durairajanayagam *et al.*, 2015). The deregulation of scrotal temperature
 83 results in testicular hyperthermia, conducting to genital heat stress (HS), and the effects depend on
 84 intensity, frequency and duration of heat exposure (revised in (Durairajanayagam *et al.*, 2015)). Increased
 85 testicular temperature may occur due to occupational exposure, lifestyle or clinical disorders (e.g.
 86 cryptorchidism and varicocele). The developmental stages are the most sensitive to HS with the
 87 pachytene and diplotene spermatocytes and early round spermatids being the most affected (Lue *et al.*,
 88 1999; Kim *et al.*, 2013a).
 89 As in somatic cells, in male germ cells HSF1 binds to promoter sequences of heat shock genes. Mouse
 90 testes exposed to mild (38°C) or severe temperatures (43°C) have HSF1 bound to promoters of *Hsp* genes

(e.g. *Hspa8*, *Hsp90aa1*, *Hsp90ab1*) and other stress-related genes (e.g. *Cct6a*, *Ube2g2*, *Ubqln1*) (Korfanty *et al.*, 2014). However, in spermatocytes this binding negatively regulates the transcription of genes involved in cell division and RNA processing, leading to genomic instability and thus cell death (Hayashida *et al.*, 2006; Kus-Liśkiewicz *et al.*, 2013). In fact, mice with constitutively active HSF1 in testes show spermatogenesis blockage, spermatocytes apoptosis induction and are infertile (Nakai *et al.*, 2000) and in mice lacking HSF1 there is no heat-induced apoptosis in spermatocytes (Izu *et al.*, 2004). Interestingly, Widlak *et al.* showed that prior to the onset of spermatocyte apoptosis, a marked reduction in HSPA2 mRNA and protein levels occurs (Widlak *et al.*, 2007), which is surprising since HSF1 is generally responsible for the induction of heat shock gene transcription. These data suggest that HSF1 acts as a pro-apoptotic factor in pachytene spermatocytes and that the spermatocyte specific HSPA2 may have an anti-apoptotic role in this type of germ cells. In fact, in testes this could be a mechanism to prevent the transmission of damaged material to the offspring.

In normal mouse testes, HSP90A is mainly present in the cytoplasm and to a lesser extent in the nucleus during spermatogenesis. When exposed to high temperature (42°C), the amount of this chaperone increases and it partly relocates to the nucleus in Sertoli cells, spermatogonia and primary spermatocytes, associated with perichromatin fibrils and the nucleolus (Biggiogera *et al.*, 1996). The localization in structures involved in RNA synthesis and processing suggests the protective function in this sensitive type of tissue.

Not only hyperthermia causes the activation of the HSR in testis. Various types of stress, including genotoxic stress caused by the anticancer drug doxorubicin, oxidative and nitrosative stress and thermal exposure trigger this stress response pathway (Salmand *et al.*, 2008; Rezazadeh-Reyhani *et al.*, 2015). The oxidative and nitrosative stress also results in the expression of proteins of the HSPA1 family in various germinal cell types (Rezazadeh-Reyhani *et al.*, 2015). Nanosilver particles, for example, enhance germ cell degeneration, necrosis and atrophy of the seminiferous tubules, accompanied with an increase in HSPA2 expression in groups treated with low doses. However, in groups treated with medium and high doses there is a significantly decrease in HSPA2 levels (Rezazadeh-Reyhani *et al.*, 2015).

Molecular chaperones are commonly involved in protein complex assembly and transport within cells (Jee, 2016) and, in spermatozoa, they are involved in pathological and physiological processes such as oocyte recognition, motility and capacitation (Redgrove *et al.*, 2012; Nixon *et al.*, 2015; Calle-Guisado *et al.*, 2017). HSPA1 and HSP90A, chaperones involved in sperm maturation, as well as *HSF1* transcripts

have already been identified in mature spermatozoa and infertility conditions, such as varicocele and oligozoospermia, in which they were upregulated (Ferlin *et al.*, 2010; Chan *et al.*, 2013). HSPA1 has been described in mature sperm in mouse, bull (Kamaruddin *et al.*, 2004), boar (Spinaci *et al.*, 2005), stallion, cat, dog (Volpe *et al.*, 2008) and human (Huszar *et al.*, 2000; Wenhao *et al.*, 2015). Human mature spermatozoa also seem to show a response to HS, since spermatozoa of normozoospermic men exposed to frequent scrotal hyperthermia reveal higher levels of HSF1 and HSPA1 compared with controls (Rao *et al.*, 2016). It is important to note that two testis-specific HSPA1 chaperones – HSPA2 and HSPA1L – were identified on the human sperm surface (Soren Naaby-Hansen *et al.*, 2010). However, according to more recent studies, the surface expression of HSPA2 in human sperm is contentious, being described as expressed throughout the spermatozoon, both in the head and in tail by immunofluorescence (Intasqui *et al.*, 2018). Its presence in the anterior region of the sperm head is compatible with its role in sperm-egg recognition (Redgrove *et al.*, 2012; Nixon *et al.*, 2015, 2017). HSP90A has also been found in human, boar, stallion, cat and dog (Volpe *et al.*, 2008; Wenhao *et al.*, 2015) semen extracts. Recently, Calle-Guisado *et al.* showed that HSP90A constitutes a defensive mechanisms against heat stress in boar sperm by maintaining mitochondrial membrane potential and sperm motility in non-capacitating conditions (Calle-Guisado *et al.*, 2017). The role of these chaperones in human sperm stress responses may be further investigated, and studies to elucidate proteins that may interact with HSP90A and HSPA1 and the pathways implicated in this response should be carried out. For instance, several HSPA2 interactors, involved in sperm-oocyte recognition and germ cells development, have been identified. Redgrove *et al.* showed that HSPA2 stably interacts with multimeric complexes in spermatozoa from fertile normozoospermic individuals, one of them comprising two proteins, sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ARSA), both implicated in interactions with the cumulus-oocyte complex (Redgrove *et al.*, 2012, 2013). It was showed that the complex localization changes during capacitation, to facilitate the repositioning of ARSA to the apical region of the human sperm head, allowing the mediation of sperm-zona pellucida interactions (Redgrove *et al.*, 2013). The same group demonstrated that HSPA2 interacts with HLA-B associated transcript 3 (BAT3; also known as BCL2-associated athanogene 6, BAG6) in mature human spermatozoa (Bromfield *et al.*, 2015). BAG6 seems to be a regulator of HSPA2 stability and/or function in human germ cells, being this interaction important during germ cells development (Bromfield *et al.*, 2015). More recently, the authors identified two novel potential interacting proteins of HSPA2: angiotensin converting enzyme (ACE) and protein disulphide isomerase

A6 in human spermatozoa (PDIA6) that may be involved in key elements of the fertilization cascade, specifically in acrosome reaction (Bromfield *et al.*, 2016). These studies showed that HSPs may play diverse roles in spermatozoa aside from their involvement in HSR, due to the formation of specific protein complexes.

Furthermore, it is important to consider that the alterations observed in mature spermatozoa can reflect the effect of stressful agents during spermatogenesis and not in spermatozoa itself and studies focused on this response in mature sperm are necessary. The HSR elements already identified in testes and spermatozoa are described in **Figure 1**.

III. Mitochondrial Unfolded Protein Response (UPR^{mt})

An important adaptative transcriptional response to stress in somatic cells is the UPR^{mt}. This mitochondria-to-nucleus signal transduction pathway begins once the chaperone protein-folding capacity becomes saturated, in order to re-establish mitochondrial protein-folding homeostasis (Pellegrino *et al.*, 2013; Melber and Haynes, 2018). Several factors contribute to the alterations in mitochondria protein folding microenvironment such as DNA and protein damaging reactive oxygen species (ROS), changes in temperature and exposure to toxins (Baker and Haynes, 2011).

Mitochondria are composed of four compartments, namely the outer and inner membranes, the intermembrane space (IMS) and the matrix, possibly having special machineries to deliver the proteins to their mitochondrial destination. UPR^{mt} is originally triggered by stress in the mitochondrial matrix or in the mitochondrial IMS, each one leading to distinctive signal transduction pathways and transcriptional responses. In mammals, the matrix UPR^{mt} begins with activation of the transcription factor CCAAT-enhancer-binding protein homologous protein CHOP (DDIT3), via mitogen-activated protein kinase 9 (JNK2/MAPK9) and c-JUN (Aldridge *et al.*, 2007; Melber and Haynes, 2018; Münch, 2018). CHOP stimulates the transcription of specific mitochondrial protein homeostasis genes, such as quality control protease caseinolytic peptidase P (CLPP) and mitochondrial chaperones such as HSPA1, which directly folds incoming proteins, and HSPD1-HSPE1 machinery in the matrix (Aldridge *et al.*, 2007; Pellegrino *et al.*, 2013). CLPP multimerizes and forms a macromolecular proteasome-like cylinder along with the chaperone CLPX, in the mitochondrial matrix. Additionally, double-stranded RNA-dependent protein kinase (PKR) inhibits global cytosolic protein translation by mediating phosphorylation of eIF2 α

(EIF2S1). The mechanism used by cells to sense unfolded proteins within the matrix and how the signal is transmitted across both mitochondrial membranes also remains unclear.

Recently, a separate UPR^{mt} signalling pathway was described. This particular response is triggered by unfolded protein stress within the IMS. The increase of IMS unfolded proteins induces phosphorylation of the kinase AKT1 and activation of the estrogen receptor (ESR1) causing transcriptional up-regulation of the IMS serine protease HTRA2 and the nuclear respiratory factor 1 (NRF1) (Papa and Germain, 2011), a key transcription factor involved in mitochondrial biogenesis (Mattingly *et al.*, 2008) (**Figure 2**).

(1) UPR^{mt} in the male germ line and fertility

HSPD1 is a molecular chaperone mostly present within the mitochondrial matrix and is involved in the folding of mitochondrial proteins, enabling proteolytic degradation of unfolded or denaturated proteins. It has been identified in mature spermatozoa of boar, stallion, cat, dog and human (Soren Naaby-Hansen *et al.*, 2010; Wenhao *et al.*, 2015). In mammalian sperm, the midpiece of the flagellum contains the mitochondrial sheath that surrounds the axoneme, along with outer dense fibers (Inaba, 2003). Thus, it was not surprising that in human ejaculated spermatozoa HSPD1 is localized in the midpiece (Lachance *et al.*, 2010). HSPD1 and HSPE1 were also described as being important in *Coilia nasus* testis development, participating in mitochondrial import and refolding. This study showed high HSPD1 and HSPE1 expression in primary spermatogonia, while its expression was weak or absent in mature germ cells (Fang *et al.*, 2017).

Additionally, the mRNA coding for the protein kinase MAPK9 was already identified in the nuclei of human ejaculated spermatozoa, despite its origin and fate remain to be further investigated (Dadoune *et al.*, 2005). After activation by dual phosphorylation on tyrosine and threonine residues, MAPK9 phosphorylates the transcription factors c-JUN, ATF2 and ELK1. It is well known that the nuclear transcription factor c-JUN is expressed during the premeiotic and the meiotic steps of spermatogenesis (Dadoune *et al.*, 2005).

Interestingly, CLPP null mice showed a complete male infertility phenotype with spermatogenesis disruption at the spermatid stage (Gispert *et al.*, 2013). These mice presented reduced macroscopic size of testes already at age 3 months, with a complete absence of spermatids and mature spermatozoa in the testis, spongiform changes of the seminiferous epithelium and a decrease in the number of dividing cells at the age of 6 months. These results suggested that CLPP may have an important role in male fertility and can be useful to screen male patients with non-obstructive azoospermia.

This is, by far, the most unexplored UPR pathway in testis and spermatozoa. In fact, despite several mitochondrial signalling pathways being activated in testis in response to oxidative stress, reproductive toxins and heat exposure (Vera *et al.*, 2004; Saradha *et al.*, 2009; Ji *et al.*, 2011), no studies are available regarding UPR^{mt} activation in response to stress or other physiologic process, such ageing. The UPR^{mt} elements previously identified in testis and sperm are indicated in **Figure 2**.

IV. ENDOPLASMIC RETICULUM UNFOLDED PROTEIN RESPONSE (UPR^{ER})

Most cellular proteins undergo folding during translation in ribosomes with the assistance of molecular chaperones, while transmembrane and secreted proteins fold and mature in the lumen of the ER (Hwang and Qi, 2018). The ER is the first compartment of the protein secretory pathway and is involved in the biosynthesis and processing of about one third of all proteins (Doultsinos *et al.*, 2017; Karna *et al.*, 2019). The ER chaperones, folding enzymes and proteases prevent inappropriate molecular interactions and recognize misfolded proteins, targeting them to the ER-associated degradation (ERAD) pathway (Hwang and Qi, 2018). In somatic cells, when the unfolded or misfolded protein load entering in the ER exceeds the ER folding capacity, the UPR^{ER} is activated (Hwang and Qi, 2018). Primarily, a transient adaptation through the reduction of protein synthesis and translocation of proteins into the ER occurs, followed by a transcriptional activation of UPR target genes (Ma and Hendershot, 2001; Schröder and Kaufman, 2005; Karna *et al.*, 2019). However, if homeostasis is not re-established, a third mechanism – apoptosis - is triggered, in order to protect the organism (Ron and Walter, 2007).

UPR^{ER} is a tripartite signalling pathway regulated by three proximal ER sensors: inositol requiring enzyme 1 (IRE1/ERN1), protein kinase RNA-activated (PKR)-like ER kinase (PERK/ EIF2AK3) and activating transcription factor 6 (ATF6) (Ron and Walter, 2007; Bergmann and Molinari, 2018; Almanza *et al.*, 2019). In the absence of stress, the ER chaperone BiP (GRP78/HSPA5) binds to the luminal domain of these sensors keeping them in an inactive state (Bergmann and Molinari, 2018). When stress is present, BiP is sequestered by unfolded proteins activating the ER stress transducers and, thus, activating the UPR^{ER} (Lee, 2005; Schröder and Kaufman, 2005). However, each sensor follows different signalling pathways. The first indicator of UPR is PERK activation, being responsible for the rapid reduction of protein synthesis. In response to stress, PERK dimerizes and undergoes trans-autophosphorylation activation. When activated, it recruits and phosphorylates the α -subunit of eukaryotic translation initiation factor-2 (eIF2 α /EIF2S1) at Ser51, which inhibits the guanine nucleotide exchange factor EIF2B, responsible for recovering eIF2 α active GTP-bound form, resulting in inferior levels of translation

initiation and thus, decreasing the load of synthesized proteins (Ron and Walter, 2007). However, genes with internal ribosome entry site (IRES) sequences, such as ATF4, avoid eIF2 α translational blockage. The transcription factor ATF4 controls the levels of ER chaperones, antioxidant response elements, autophagy genes and, importantly, pro-apoptotic components, such as CHOP (Bergmann and Molinari, 2018). CHOP downregulates the anti-apoptotic protein BCL2, induces the expression of essential initiators of apoptosis (Lindholm *et al.*, 2017) and upregulates DNA damage-inducible protein GADD34 (PPP1R15A). GADD34 forms a complex with protein phosphatase 1c (PPP1c) to dephosphorylate eIF2 α that, in turn, fails to prevent protein synthesis (Chakrabarti *et al.*, 2011). The oldest and most conserved arm of the UPR is mediated by IRE1, that reduces folding load by degrading mRNAs during the arrest of protein synthesis. IRE1 is a transmembrane bifunctional enzyme - a protein kinase that regulates its function as a site-specific endoribonuclease. When activated, IRE1 α processes a pre-existing mRNA that encodes the X-box binding protein-1 (XBP1) (Yoshida *et al.*, 2003; Ron and Walter, 2007; Hwang and Qi, 2018). This potent transcriptional activator regulates a subset of UPR genes that promote ERAD of misfolded proteins, ER biogenesis, autophagy and protein quality control (Yoshida *et al.*, 2003; Ron and Walter, 2007; Hwang and Qi, 2018). IRE1 can act by alternative mechanisms as well. In mammals, phosphorylated IRE1 recruits tumour necrosis factor receptor-associated factor-2 (TRAF2) activating downstream apoptosis signal-regulating kinase 1 (MAP3K5) and JNK2 (MAPK9) (Urano *et al.*, 2000), linking ER stress to cell death and dysfunction (Ron and Walter, 2007). Additionally, under ER stress, ATF6 is re-localized from the ER to the Golgi, where it is cleaved by the proteases Sphingosine-1-phosphate (S1P/MBTPS1) and Sphingosine-2-phosphate (S2P/MBTPS2), releasing the cytosolic domain (Bergmann and Molinari, 2018). This active domain translocates into the nucleus where it acts as a transcriptional factor (as ATF4) increasing the expression of ER chaperones, ERAD-related genes and proteins involved in ER expansion (Schröder and Kaufman, 2005; Ron and Walter, 2007; Kim *et al.*, 2013b) (**Figure 3**).

There are two main protein degradation pathways, that are activated by components of UPR following ER stress. The first, the ubiquitin-proteasome system (ERAD) is responsible for the degradation of misfolded proteins by retrograde transport from ER to cytosol where they are degraded by the 26S proteasome (Hwang and Qi, 2018; Almanza *et al.*, 2019). In situations of ERAD dysfunction or when the accumulation of misfolded proteins exceed the capacity of ERAD, the aggregates on the ER are degraded via lysosome-mediated protein degradation (autophagy) (Fujita *et al.*, 2007; Karna *et al.*, 2019). PERK

(eIF2 α) and IRE1 (TRAF2/JNK) are the main branches involved in ER stress-induced autophagy in mammalian cells. IRE1-JNK signalling results in the phosphorylation of Bcl-2 that dissociates from Beclin 1, a key regulator of autophagy. Free Beclin 1 becomes active and activate ATG proteins that are required for the formation of the autophagolysosome (CHENG *et al.*, 2014; Almanza *et al.*, 2019). Prolonged ER stress often culminates in apoptosis through the activation of Caspase-12 pathway (Kim *et al.*, 2013b). Overall, the mechanisms described decrease the load of proteins in the ER allowing the activation of repair mechanisms to re-establish homeostasis.

Previous studies reported the involvement of ER stress in immune response, diseases such as diabetes, Alzheimer's and Parkinson's diseases (PD) and several types of cancer (Almanza *et al.*, 2019; Karna *et al.*, 2019). The use of specific compounds to control ER stress, particularly modulating eIF2 α phosphorylation through inhibitors of eIF2 α phosphatases containing GADD34 (Salubrinal), alleviates the symptoms of several human diseases, PD (Colla *et al.*, 2012) and Amyotrophy Lateral Sclerosis (ALS) (Saxena *et al.*, 2009), in which proteostasis is deregulated (Almanza *et al.*, 2019). In PD, for instance, salubrinal increased neuronal survival of α -synuclein transgenic mice (Colla *et al.*, 2012) and, in an ALS mice model, it increased animals' lifespan (Saxena *et al.*, 2009). Unravelling the involvement of ER stress on male infertility situations may open new opportunities for the treatment of infertility.

(1) UPR^{ER} in the male germline and fertility

In testes, the UPR^{ER} seems to be triggered by several types of stress, from drugs (Cui *et al.*, 2017), heavy metals (Ji *et al.*, 2011, 2013) and particulate matter (PM) exposure (Liu *et al.*, 2017) to HS (Kim *et al.*, 2013b). Cadmium (Cd), one of the major occupational and environmental toxicants, has been associated with male infertility and poor semen quality in humans (Zhao *et al.*, 2017). Despite increasing evidence that Cd induces testicular germ cell apoptosis, the mechanisms underlying remain poorly understood. Recently, ER stress emerged as the mediator of Cd-induced germ cell apoptosis in testis. In Cd-treated mice testes, the spliced form of XBP1, IRE1's pathway target, was remarkably increased. Additionally, BiP and CHOP seem to be upregulated and acute Cd exposure significantly led to the phosphorylation of eIF2 α and MAPK9 (Ji *et al.*, 2011), which suggest that the PERK pathway is activated. The administration of N-acetylcysteine partially reverted this effect in mice testis through the attenuation of Cd-induced ER stress and UPR^{ER} (Ji *et al.*, 2013). In fact, mice testis exposed to this antioxidant reveal attenuated germ cell apoptosis and downregulation of BiP as well as inhibition of eIF2 α and PERK phosphorylation and blockage of XBP1 activation (Ji *et al.*, 2013). Li R. *et al.* also showed that Cd

activated autophagy and induced DNA damage in mouse spermatocyte-derived cells via ROS generation, through the activation of AMPK/mTOR autophagy signalling pathway (Li *et al.*, 2017). Furthermore, the exposure of rat and mice testes to endocrine-disrupting chemicals, such as bisphenol-A (BPA) and diethylstilboestrol (DES), inhibits cell proliferation and induces apoptosis and activation of ER stress. In mice, the BPA-treated testes showed fewer spermatogenic cells and spermatozoa in the seminiferous tubules and higher levels of ROS. Phosphorylation and activation of IRE1 and upregulation of CHOP in rat testis also occurred (Yin *et al.*, 2017). Knock down of the PERK/eIF2 α /CHOP pathway partially recovered BPA-induced apoptosis, making the UPR^{ER} pathway a good candidate to explain BPA/DES-induced damage in testes (Yin *et al.*, 2017).

Increasing attention is being devoted to the influence of air pollution on human health. Particulate matter (PM) is part of atmospheric dust with several chemical constituents such as inorganic ions, metals and polycyclic aromatic hydrocarbons. Recent evidences suggest that PM exposure is toxic for testis by inducing UPR^{ER} (Liu *et al.*, 2017). The exposure of Sprague Dawley rats' testis and epididymis to fine PM induces ER stress through the activation of the IRE1 α -XBP1 pathway. BIP and XBP1 were upregulated as well as CHOP and Caspase-12 levels, which suggest the activation of the UPR^{ER} and induction of testicular germ cells' apoptosis (Liu *et al.*, 2017).

As explained early in this paper, the testes of most mammals are very sensitive to temperature, adopting self-protective mechanisms. Mitochondrial signalling is an important apoptotic pathway involved in the process of heat-induced germ cell apoptosis (Vera *et al.*, 2004; Gong *et al.*, 2017), as well as HSR and UPR^{ER} (see above). In mouse testes, testicular hyperthermia (43°C, 15 min/day) leads to enhanced levels of phosphorylated eIF2 α , ATF4, GADD34 and XBP1s. Additionally, repetitive periods of hyperthermia lead to ER stress-mediated spermatocyte apoptosis associated with increased levels of CHOP, phosphorylated-MAPK9 and caspase-3 activity (Kim *et al.*, 2013b). These results showed that UPR signalling is activated with testicular hyperthermia via phosphorylated-eIF2 α /ATF4/GADD34, p90ATF6 and phosphorylated-IRE1/XBP1 in mice spermatocytes (Yoon *et al.*, 2017). Indeed, eIF2 α phosphorylation seems to act as a protective mechanism against germ cell apoptosis following heat stress. During spermiogenesis sperm cells lack ER. Consequently, some of the ER proteins can also be lost during this process. Interestingly, BiP, a resident ER chaperone involved in protein transport, folding and assembly, has already been identified in human, rat and hamster spermatozoa. In human ejaculated spermatozoa, this protein was detected in the neck (Lachance *et al.*, 2010). During the epididymal transit,

at least in hamster, BiP seems to be absent in caudal spermatozoa (Kameshwari *et al.*, 2010). The total and phosphorylated levels of BiP were described as being reduced in asthenozoospermic patients suggesting that this chaperone may also have an important role in sperm function, particularly in motility (Shen *et al.*, 2013; Lobo *et al.*, 2015). A study by Amaral *et al.* (Amaral *et al.*, 2014a) demonstrated that eIF2 α is present in the spermatozoa proteome, however, there is still no information regarding how it is activated or how it functions. Nevertheless, these proteins may play alternative roles, hence the need for further investigation in mature spermatozoa. Recently, Samanta *et al.* performed a proteomic analysis in ejaculated spermatozoa from infertile men that revealed a set of dysregulated proteins involved in posttranslational modifications and folding of proteins, specially chaperones (HSPA2, HSPA1, HSPA1L, HSP90AA1, HSP90B1, HSPA5) (Samanta *et al.*, 2019). Several other proteomic studies support this findings, suggesting that ER stress may be responsible for deficient protein formation which might lead to alterations in sperm protein content of infertile men (Intasqui *et al.*, 2018; Samanta *et al.*, 2019). These studies suggest that ER stress signalling may affect sperm maturation and even fertilization (Karna *et al.*, 2019). Thus, the study of stress response-related elements presence and functioning in human ejaculated spermatozoa emerge as a promising tool to clarify many cases of unexplained male infertility.

V. SUMMARY

When exposed to stress conditions, protein aggregation generally occurs in cells, which leads to the activation of signalling pathways commonly known as UPR. UPR fundamentally comprises HSR, UPR^{mt} and UPR^{ER} that, depending on the location of aggregates and type of stress, results in the activation of different transcription factors and molecular chaperones that correct the protein folding. Additionally, if UPR pathways cannot correct protein conformation and proteins continue to accumulate in cell, degradation pathways may be activated, which include the ubiquitin-proteasome-mediated proteolysis (ERAD) and autophagy. Although the main aim of this work does not include the discussion of these degradation pathways, is important to refer that, in sperm, there is some contention regarding the existence of an active proteasome and other mechanisms of degradation of poor quality and/or misfolded proteins. Nevertheless, some studies support the presence and activation of the mechanism of autophagy in ejaculated human spermatozoa and germ cells, and its involvement in cell survival and motility regulation (Aparicio *et al.*, 2016), and response to chemicals such as Cd (Li *et al.*, 2017). Additionally, components of the ubiquitin-proteasome system were already identified in mature spermatozoa,

particularly ubiquitin enzymes (revised by (Hou and Yang, 2013)). However, knowledge of proteasomes in spermatozoa is limited. This element seems to be involved in sperm DNA repair , sperm capacitation and acrosome reaction (Kerns *et al.*, 2016; Zigo *et al.*, 2018) and growing evidence showed that an abnormal ubiquitin-proteasome system usually leads to malformation of sperm and induces male infertility (Hou and Yang, 2013; Zigo *et al.*, 2018). Thus, its activation after stress in human spermatozoa should be further explored.

After leaving the testes, sperm cells are vulnerable to environmental changes. High temperatures can be achieved in the female reproductive tract (Ng *et al.*, 2018), and tabaco, alcohol and several environmental toxicants, such as BPA and mercury, can also impact sperm function and fertility (Mínguez-Alarcón *et al.*, 2016; Adoamnei *et al.*, 2018; Henriques *et al.*, 2019). These cellular stressors often lead to a deregulation of protein turnover and protein folding resulting in the accumulation of defective proteins or proteins usually eliminated during the maturation process.

In testis, as in somatic cells, the UPR is activated in response to the accumulation of unfolded or misfolded proteins, being the presence and function of molecular chaperones and other UPR proteins well described. However, the activation of the UPR in response to this phenomenon in ejaculated human sperm remains unclear. This is mostly due to the idea that sperm are a transcriptionally and translationally inactive type of cell and that most of these signalling pathways culminate in transcription of several genes involved in proteostasis maintenance. However, new evidence concerning the presence of nuclear-encoded protein translation in mature spermatozoa has begun to appear (Gur and Breitbart, 2006; Zhu *et al.*, 2019). Indeed, Yael Gur and Haim Breitbart (Gur and Breitbart, 2006) were the first to report the incorporation of labelled amino acids into polypeptides during sperm capacitation and showed that the use of specific inhibitors of protein translation significantly impact sperm motility, capacitation and in vitro fertilization rate. Recent studies in boar, confirmed that the incubation of sperm with mitochondrial translation inhibitor suppressed mitochondrial protein synthesis and activity, with a consequent decrease in the sperm linear motility (Zhu *et al.*, 2019). These interesting results suggest that translation may have an important role during sperm capacitation and motility and allow us to hypothesize that new proteins can also be synthesized in spermatozoa in response to cellular stress, to correct protein folding and thus prevent the cell damage. In this review we showed that most of the proteins involved in the response to stress, particularly the molecular chaperones, are present in the male germ line and mature spermatozoa (Table I). However, further studies should investigate the activation of the UPR under stress conditions in

human spermatozoa to clarify if these processes are functional in these cells. It is essential to understand if *spermatozoa are able to adapt under adverse conditions by activating other signalling pathways or by using the conventional UPR.*

The decline in sperm quality and fertilization potential associated with ageing, diseases (e.g. varicocele) and exposure to external agents may be explained by the increase in protein aggregation in these cells and consequent need for activation of stress response mechanisms. All these factors generate stress in spermatozoa that may have normal microscopic parameters, including motility and morphology, but are unable to fertilize. Thus, unravelling the mechanisms of stress response in human spermatozoa could ultimately explain some cases of idiopathic male infertility.

Additionally, changes in UPR and stress markers in biological fluids can be indicative of disease states and may constitute valuable biomarkers for several human diseases. For example, ATF4 upregulation has already been implicated in cancer progression and metastasis in patients with oesophageal squamous cells carcinoma (Almanza *et al.*, 2019). Thus, valuable non-invasive stress response-related biomarkers to evaluate sperm quality and fertility status may emerge from the study of these pathways in spermatozoa, allowing patients stratification and a better management of male (in)fertility.

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REFERENCES

- Adoamnei E, Mendiola J, Vela-Soria F, Fernández MF, Olea N, Jørgensen N, Swan SH, Torres-Cantero AM. Urinary bisphenol A concentrations are associated with reproductive parameters in young men. *Environ Res* 2018;**161**:122–128. Elsevier Inc.
- Aldridge JE, Horibe T, Hoogenraad NJ. Discovery of genes activated by the mitochondrial Unfolded Protein Response (mtUPR) and cognate promoter elements. *PLoS One* 2007;**2**..
- Almanza A, Carlesso A, Chintia C, Creedican S, Doultisinos D, Leuzzi B, Luís A, McCarthy N, Montibeller L, More S, *et al.* Endoplasmic reticulum stress signalling - from basic mechanisms to clinical applications. *FEBS J* 2019;**286**:241–278. John Wiley & Sons, Ltd (10.1111).
- Amaral A, Castillo J, Estanyol JM, Ballesca JL, Ramalho-Santos J, Oliva R. Human Sperm Tail Proteome Suggests New Endogenous Metabolic Pathways. *Mol Cell Proteomics* 2013;**12**:330–342.
- Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: Cellular pathways and implications for basic and clinical science. *Hum Reprod Update* 2014a;**20**:40–62.
- Amaral A, Paiva C, Attardo Parrinello C, Estanyol JM, Ballescà JL, Ramalho-Santos J, Oliva R. Identification of Proteins Involved in Human Sperm Motility Using High-Throughput Differential Proteomics. *J Proteome Res* 2014b;**13**:5670–5684. American Chemical Society.
- Aparicio IM, Espino J, Bejarano I, Gallardo-Soler A, Campo ML, Salido GM, Pariente JA, Peña FJ, Tapia JA. Autophagy-related proteins are functionally active in human spermatozoa and may be involved in the regulation of cell survival and motility. *Sci Rep* 2016;**6**:33647. Nature Publishing Group.
- Baker BM, Haynes CM. Mitochondrial protein quality control during biogenesis and aging. *Trends Biochem Sci* 2011;**36**:254–261.
- Baker MA, Naumovski N, Hetherington L, Weinberg A, Velkov T, Aitken RJ. Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics* 2013;**13**:61–74.
- Bergmann TJ, Molinari M. Three branches to rule them all? UPR signalling in response to chemically versus misfolded proteins-induced ER stress. *Biol Cell* 2018;**110**:197–204. John Wiley & Sons, Ltd (10.1111).
- Biggiogera M, Tanguay RM, Marin R, Wu Y, Martin TE, Fakan S. Localization of Heat Shock Proteins in Mouse Male Germ Cells: An Immunoelectron Microscopical Study. *Exp Cell Res* 1996;**229**:77–85.

- 448 Bogle OA, Kumar K, Attardo-Parrinello C, Lewis SEM, Estanyol JM, Ballescà JL, Oliva R.
 449 Identification of protein changes in human spermatozoa throughout the cryopreservation process.
 450 *Andrology* 2017;**5**:10–22.
- 451 Bromfield E, Aitken RJ, Nixon B. Novel characterization of the HSPA2-stabilizing protein BAG6 in
 452 human spermatozoa. *Mol Hum Reprod* 2015;**21**:755–769.
- 453 Bromfield EG, McLaughlin EA, Aitken RJ, Nixon B. Heat Shock Protein member A2 forms a stable
 454 complex with angiotensin converting enzyme and protein disulfide isomerase A6 in human
 455 spermatozoa. *Mol Hum Reprod* 2016;**22**:93–109.
- 456 Calle-Guisado V, Bragado MJ, García-Marín LJ, González-Fernández L. HSP90 maintains boar
 457 spermatozoa motility and mitochondrial membrane potential during heat stress. *Anim Reprod Sci*
 458 2017;0–1. Elsevier.
- 459 Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. *Biotechnol*
 460 *Bioeng* 2011;**108**:2777–2793.
- 461 Chan CC, Sun GH, Shui HA, Wu GJ. Differential spermatozoal protein expression profiles in men with
 462 varicocele compared to control subjects: Upregulation of heat shock proteins 70 and 90 in
 463 varicocele. *Urology* 2013;**81**:1379.e1-1379.e8. Elsevier Inc.
- 464 CHENG X, LIU H, JIANG C-C, FANG L, CHEN C, ZHANG X-D, JIANG Z-W. Connecting
 465 endoplasmic reticulum stress to autophagy through IRE1/JNK/beclin-1 in breast cancer cells. *Int J*
 466 *Mol Med* 2014;**34**:772–781.
- 467 Colla E, Coune P, Liu Y, Pletnikova O, Troncoso JC, Iwatsubo T, Schneider BL, Lee MK. Endoplasmic
 468 Reticulum Stress Is Important for the Manifestations of -Synucleinopathy In Vivo. *J Neurosci*
 469 2012;**32**:3306–3320.
- 470 Cui Y, Ren L, Li B, Fang J, Zhai Y, He X, Du E, Miao Y, Hua J, Peng S. Melatonin Relieves Busulfan-
 471 Induced Spermatogonial Stem Cell Apoptosis of Mouse Testis by Inhibiting Endoplasmic
 472 Reticulum Stress. *Cell Physiol Biochem* 2017;**44**:2407–2421.
- 473 Dadoune JP, Pawlak A, Alfonsi MF, Siffroi JP. Identification of transcripts by macroarrays, RT–PCR and
 474 in situ hybridization in human ejaculate spermatozoa. *MHR Basic Sci Reprod Med* 2005;**11**:133–
 475 140.
- 476 Dokladny K, Myers OB, Moseley PL. Heat shock response and autophagy—cooperation and control.
 477 *Autophagy* 2015;**11**:200–213.

- 478 Doultzinos D, Avril T, Lhomond S, Dejeans N, Guédât P, Chevet E. Control of the Unfolded Protein
479 Response in Health and Disease. *SLAS Discov Adv Life Sci R&D* 2017;**22**:787–800.
- 480 Durairajanayagam D, Agarwal A, Ong C. Causes, effects and molecular mechanisms of testicular heat
481 stress. *Reprod Biomed Online* 2015;**30**:14–27. Reproductive Healthcare Ltd.
- 482 Fang DA, Zhou YF, Zhang MY, Xu DP, Liu K, Duan JR. Developmental expression of HSP60 and
483 HSP10 in the coilia nasus testis during upstream spawning migration. *Genes (Basel)* 2017;**8**..
- 484 Ferlin A, Speltra E, Patassini C, Pati MA, Garolla A, Caretta N, Foresta C. Heat Shock Protein and Heat
485 Shock Factor Expression in Sperm: Relation to Oligozoospermia and Varicocele. *J Urol*
486 2010;**183**:1248–1252. Elsevier Inc.
- 487 Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C, Hayashi YK, Momoi T. Two
488 endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant
489 dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Hum Mol Genet*
490 2007;**16**:618–629.
- 491 Gispert S, Parganlija D, Klinkenberg M, Dröse S, Wittig I, Mittelbronn M, Grzmil P, Koob S, Hamann A,
492 Walter M, *et al.* Loss of mitochondrial peptidase clpp leads to infertility, hearing loss plus growth
493 retardation via accumulation of CLPX, mtDNA and inflammatory factors. *Hum Mol Genet*
494 2013;**22**:4871–4887.
- 495 Gong Y, Guo H, Zhang Z, Zhou H, Zhao R, He B. Heat stress reduces sperm motility via activation of
496 glycogen synthase kinase-3 α and inhibition of mitochondrial protein import. *Front Physiol*
497 2017;**8**:1–10.
- 498 Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type
499 ribosomes. *Genes Dev* 2006;**20**:411–416. Cold Spring Harbor Laboratory Press.
- 500 Hamada A, Sharma R, Plessis SS Du, Willard B, Yadav SP, Sabanegh E, Agarwal A. Two-dimensional
501 differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress.
502 *Fertil Steril* 2013;**99**:1216-1226.e2.
- 503 Hayashida N, Inouye S, Fujimoto M, Tanaka Y, Izu H, Takaki E, Ichikawa H, Rho J, Nakai A. A novel
504 HSF1-mediated death pathway that is suppressed by heat shock proteins. *EMBO J* 2006;**25**:4773–
505 4783.
- 506 Henriques MC, Loureiro S, Fardilha M, Herdeiro MT. Exposure to mercury and human reproductive
507 health: A systematic review. *Reprod Toxicol* 2019;**85**:93–103.

- 508 Hosseinifar H, Sabbaghian M, Nasrabadi D, Modarresi T, Dizaj AVT, Gourabi H, Gilani MAS. Study of
 509 the effect of varicocele on sperm proteins expression in patients with varicocele and poor
 510 sperm quality by using two-dimensional gel electrophoresis. *J Assist Reprod Genet* 2014;**31**:725–
 511 729.
- 512 Hou C-C, Yang W-X. New insights to the ubiquitin–proteasome pathway (UPP) mechanism during
 513 spermatogenesis. *Mol Biol Rep* 2013;**40**:3213–3230.
- 514 Huszar G, Stone K, Dix D, Vigue L. Putative Creatine Kinase M-Isoform in Human Sperm Is Identified as
 515 the 70-Kilodalton Heat Shock Protein HspA21. *Biol Reprod* 2000;**63**:925–932.
- 516 Hwang J, Qi L. Quality Control in the Endoplasmic Reticulum: Crosstalk between ERAD and UPR
 517 pathways. *Trends Biochem Sci* 2018;**43**:593–605. NIH Public Access.
- 518 Inaba K. Molecular architecture of the sperm flagella: molecules for motility and signaling. *Zoolog Sci*
 519 2003;**20**:1043–1056.
- 520 Intasqui P, Agarwal A, Sharma R, Samanta L, Bertolla RP. Towards the identification of reliable sperm
 521 biomarkers for male infertility: A sperm proteomic approach. *Andrologia* 2018;**50**:e12919.
- 522 Intasqui P, Camargo M, Giudice PT Del, Spaine DM, Carvalho VM, Cardozo KHM, Cedenho AP,
 523 Bertolla RP. Unraveling the sperm proteome and post-genomic pathways associated with sperm
 524 nuclear DNA fragmentation. *J Assist Reprod Genet* 2013;**30**:1187–1202. Springer.
- 525 Izu H, Inouye S, Fujimoto M, Shiraishi K, Naito K, Nakai A. Heat Shock Transcription Factor 1 Is
 526 Involved in Quality-Control Mechanisms in Male Germ Cells. *Biol Reprod* 2004;**70**:18–24.
- 527 Jee H. Size dependent classification of heat shock proteins: a mini-review. *J Exerc Rehabil* 2016;**12**:255–
 528 259.
- 529 Ji Y-L, Wang H, Zhang C, Zhang Y, Zhao M, Chen Y-H, Xu D-X. N-acetylcysteine protects against
 530 cadmium-induced germ cell apoptosis by inhibiting endoplasmic reticulum stress in testes. *Asian J*
 531 *Androl* 2013;**15**:290–296.
- 532 Ji Y-L, Wang H, Zhao X-F, Wang Q, Zhang C, Zhang Y, Zhao M, Chen Y-H, Meng X-H, Xu D-X.
 533 Crosstalk between endoplasmic reticulum stress and mitochondrial pathway mediates cadmium-
 534 induced germ cell apoptosis in testes. *Toxicol Sci* 2011;**124**:446–459.
- 535 Kamaruddin M, Kroetsch T, Basur PK, Hansen PJ, King WA. Immunolocalization of heat shock protein
 536 70 in bovine spermatozoa. *Andrologia* 2004;**36**:327–334.
- 537 Kameshwari DB, Bhande S, Sundaram CS, Kota V, Siva AB, Shivaji S. Glucose-regulated protein

- precursor (GRP78) and tumor rejection antigen (GP96) are unique to hamster caput epididymal spermatozoa. *Asian J Androl* 2010;**12**:344–355.
- Karna KK, Shin YS, Choi BR, Kim HK, Park JK. The Role of Endoplasmic Reticulum Stress Response in Male Reproductive Physiology and Pathology: A Review. *World J Mens Health* 2019;**37**..
- Keber R, Rozman D, Horvat S. Sterols in Spermatogenesis and Sperm maturation. *J Lipid Res* 2012;**54**:20–33.
- Kerns K, Morales P, Sutovsky P. Regulation of Sperm Capacitation by the 26S Proteasome: An Emerging New Paradigm in Spermatology. *Biol Reprod* 2016;**94**..
- Kim B, Park K, Rhee K. Heat stress response of male germ cells. *Cell Mol Life Sci* 2013a;**70**:2623–2636.
- Kim JH, Park SJ, Kim TS, Park HJ, Park J, Kim BK, Kim GR, Kim JM, Huang SM, Chae J Il, *et al*. Testicular hyperthermia induces unfolded protein response signaling activation in spermatocyte. *Biochem Biophys Res Commun* 2013b;**434**:861–866. Elsevier Inc.
- Korfanty J, Stokowy T, Widlak P, Gogler-Piglowska A, Handschuh L, Podkowiński J, Vydra N, Naumowicz A, Toma-Jonik A, Widlak W. Crosstalk between HSF1 and HSF2 during the heat shock response in mouse testes. *Int J Biochem Cell Biol* 2014;**57**:76–83.
- Kus-Liśkiewicz M, Polańska J, Korfanty J, Olbryt M, Vydra N, Toma A, Widlak W. Impact of heat shock transcription factor 1 on global gene expression profiles in cells which induce either cytoprotective or pro-apoptotic response following hyperthermia. *BMC Genomics* 2013;**14**:456.
- Lachance C, Fortier M, Thimon V, Sullivan R, Bailey JL, Leclerc P. Localization of Hsp60 and Grp78 in the human testis, epididymis and mature spermatozoa. *Int J Androl* 2010;**33**:33–44.
- Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* 2005;**35**:373–381.
- Li R, Luo X, Zhu Y, Zhao L, Li L, Peng Q, Ma M, Gao Y. ATM signals to AMPK to promote autophagy and positively regulate DNA damage in response to cadmium-induced ROS in mouse spermatocytes. *Environ Pollut* 2017;**231**:1560–1568.
- Lindholm D, Korhonen L, Eriksson O, Kõks S. Recent Insights into the Role of Unfolded Protein Response in ER Stress in Health and Disease. *Front cell Dev Biol* 2017;**5**:48. Frontiers Media SA.
- Liu X, Jin X, Su R, Li Z. The reproductive toxicology of male SD rats after PM2.5 exposure mediated by the stimulation of endoplasmic reticulum stress. *Chemosphere* 2017;**189**:547–555. Elsevier Ltd.
- Liu X, Liu FJ, Jin SH, Wang YW, Liu XX, Zhu P, Wang WT, Liu J, Wang WJ. Comparative proteome

- analysis of human testis from newborn, young adult, and aged men identified spermatogenesis-associated proteins. *Electrophoresis* 2015;n/a-n/a.
- Lobo V, Rao P, Gajbhiye R, Kulkarni V, Parte P. Glucose Regulated Protein 78 Phosphorylation in Sperm Undergoes Dynamic Changes during Maturation. 2015;
- Lue YH, Sinha Hikim AP, Swerdloff RS, Im P, Taing KS, Bui T, Leung A, Wang C. Single exposure to heat induces stage-specific germ cell apoptosis in rats: Role of intratesticular testosterone on stage specificity. *Endocrinology* 1999;**140**:1709–1717.
- Ma Y, Hendershot LM. The Unfolding Tale of the Unfolded Protein Response. *Cell* 2001;**107**:827–830.
- Martínez-Heredia J, Mateo S de, Vidal-Taboada JM, Ballescà JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 2008;**23**:783–791.
- Mateo S de, Castillo J, Estanyol JM, Ballescà JL, Oliva R. Proteomic characterization of the human sperm nucleus. *Proteomics* 2011;**11**:2714–2726.
- Mattingly KA, Ivanova MM, Riggs KA, Wickramasinghe NS, Barch MJ, Klinge CM. Estradiol Stimulates Transcription of Nuclear Respiratory Factor-1 and Increases Mitochondrial Biogenesis. *Mol Endocrinol* 2008;**22**:609–622.
- McReynolds S, Dzieciatkowska M, Stevens J, Hansen KC, Schoolcraft WB, Katz-Jaffe MG. Toward the identification of a subset of unexplained infertility: a sperm proteomic approach. *Fertil Steril* 2014;**102**:692–699.
- Melber A, Haynes CM. UPRmt regulation and output: a stress response mediated by mitochondrial-nuclear communication. *Cell Res* 2018;**28**:281–295. Nature Publishing Group.
- Mínguez-Alarcón L, Hauser R, Gaskins AJ. Effects of bisphenol A on male and couple reproductive health: a review. *Fertil Steril* 2016;**106**:864–870.
- Münch C. The different axes of the mammalian mitochondrial unfolded protein response. *BMC Biol* 2018;**16**:81. BioMed Central.
- Nakai A, Suzuki M, Tanabe M. Arrest of spermatogenesis in mice expressing an active heat shock transcription factor 1. *EMBO J* 2000;**19**:1545–1554. European Molecular Biology Organization.
- Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* 2018;**24**:15–34.
- Nixon B, Bromfield E, Dun M, Redgrove K, McLaughlin E, Aitken Rj. The role of the molecular

- 598 chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition. *Asian J*
 599 *Androl* 2015;**17**:568.
- 600 Nixon B, Bromfield EG, Cui J, Iuliis GN De. Heat Shock Protein A2 (HSPA2): Regulatory Roles in
 601 Germ Cell Development and Sperm Function. In D. M, editor. *Role Heat Shock Proteins Reprod*
 602 *Syst Dev Funct Adv Anatomy, Embryol Cell Biol* 2017;, p. 67–93. Springer, Cham.
- 603 Papa L, Germain D. Estrogen receptor mediates a distinct mitochondrial unfolded protein response. *J Cell*
 604 *Sci* 2011;**124**:1396–1402.
- 605 Parte PP, Rao P, Redij S, Lobo V, D’Souza SJ, Gajbhiye R, Kulkarni V. Sperm phosphoproteome
 606 profiling by ultra performance liquid chromatography followed by data independent analysis (LC–
 607 MSE) reveals altered proteomic signatures in asthenozoospermia. *J Proteomics* 2012;**75**:5861–
 608 5871.
- 609 Pellegrino MW, Nargund AM, Haynes CM. Signaling the mitochondrial unfolded protein response.
 610 *Biochim Biophys Acta - Mol Cell Res* 2013;**1833**:410–416. Elsevier B.V.
- 611 Pesch S, Bergmann M. Structure of mammalian spermatozoa in respect to viability, fertility and
 612 cryopreservation. *Micron* 2006;**37**:597–612.
- 613 Rao M, Xia W, Yang J, Hu LX, Hu SF, Lei H, Wu YQ, Zhu CH. Transient scrotal hyperthermia affects
 614 human sperm DNA integrity, sperm apoptosis, and sperm protein expression. *Andrology*
 615 2016;**4**:1054–1063.
- 616 Redgrove KA, Anderson AL, McLaughlin EA, O’Byrne MK, Aitken RJ, Nixon B. Investigation of the
 617 mechanisms by which the molecular chaperone HSPA2 regulates the expression of sperm surface
 618 receptors involved in human sperm-oocyte recognition. *Mol Hum Reprod* 2013;**19**:120–135.
- 619 Redgrove KA, Nixon B, Baker MA, Hetherington L, Baker G, Liu D-Y, Aitken RJ. The Molecular
 620 Chaperone HSPA2 Plays a Key Role in Regulating the Expression of Sperm Surface Receptors
 621 That Mediate Sperm-Egg Recognition. In Drevet JR, editor. *PLoS One* 2012;**7**:e50851.
- 622 Rezazadeh-Reyhani Z, Razi M, Malekinejad H, Sadrkhanlou R. Cytotoxic effect of nanosilver particles
 623 on testicular tissue: Evidence for biochemical stress and Hsp70-2 protein expression. *Environ*
 624 *Toxicol Pharmacol* 2015;**40**:626–638. Elsevier B.V.
- 625 Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol*
 626 *Cell Biol* 2007;**8**:519–529.
- 627 Saito A, Imaizumi K. Unfolded Protein Response-Dependent Communication and Contact among

- Endoplasmic Reticulum, Mitochondria, and Plasma Membrane. *Int J Mol Sci* 2018;**19**:3215.
- Multidisciplinary Digital Publishing Institute (MDPI).
- Salmand PA, Jungas T, Fernandez M, Conter A, Christians ES. Mouse Heat-Shock Factor 1 (HSF1) Is Involved in Testicular Response to Genotoxic Stress Induced by Doxorubicin1. *Biol Reprod* 2008;**79**:1092–1101.
- Samanta L, Sharma R, Cui Z, Agarwal A. Proteomic analysis reveals dysregulated cell signaling in ejaculated spermatozoa from infertile men. *Asian J Androl* 2019;**21**:121.
- Saradha B, Vaithinathan S, Mathur PP. Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas–FasL and mitochondria-dependent pathways. *Toxicology* 2009;**255**:131–139.
- Saraswat M, Joenväärä S, Jain T, Tomar AK, Sinha A, Singh S, Yadav S, Renkonen R. Human Spermatozoa Quantitative Proteomic Signature Classifies Normo- and Asthenozoospermia. *Mol Cell Proteomics* 2017;**16**:57–72.
- Saxena S, Cabuy E, Caroni P. A role for motoneuron subtype–selective ER stress in disease manifestations of FALS mice. *Nat Neurosci* 2009;**12**:627–636.
- Schröder M, Kaufman RJ. the Mammalian Unfolded Protein Response. *Annu Rev Biochem* 2005;**74**:739–789.
- Sharma R, Agarwal A, Mohanty G, Hamada AJ, Gopalan B, Willard B, Yadav S, Plessis S du. Proteomic analysis of human spermatozoa proteins with oxidative stress. *Reprod Biol Endocrinol* 2013;**11**:48. BioMed Central.
- Shen S, Wang J, Liang J, He D. Comparative proteomic study between human normal motility sperm and idiopathic asthenozoospermia. *World J Urol* 2013;**31**:1395–1401.
- Shi Y, Mosser DD, Morimoto RI. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev* 1998;**12**:654–666.
- Silva JV, Freitas MJ, Correia BR, Korrodi-Gregório L, Patrício A, Pelech S, Fardilha M. Profiling signaling proteins in human spermatozoa: Biomarker identification for sperm quality evaluation. *Fertil Steril* 2015;**104**:845–856e8.
- Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, Deenadayal M, Shivaji S. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. *MHR Basic Sci Reprod Med* 2010;**16**:452–462. Oxford University Press.

- 658 Soren Naaby-Hansen JCH, Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. *J*
 659 *Reprod Immunol* 2010;**84**:32–40.
- 660 Spinaci M, Volpe S, Bernardini C, Ambrogi M De, Tamanini C, Seren E, Galeati G. Immunolocalization
 661 of heat shock protein 70 (Hsp 70) in boar spermatozoa and its role during fertilization. *Mol Reprod*
 662 *Dev* 2005;**72**:534–541.
- 663 Urano F, Wang X-Z, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of Stress in the
 664 Endoplasmic Reticulum to Activation of JNK Protein Kinases by Transmembrane Protein Kinase
 665 IRE1. *Science* 2000;**287**:664–666.
- 666 Vandenbrouck Y, Lane L, Carapito C, Duek P, Rondel K, Bruley C, Macron C, Gonzalez de Peredo A,
 667 Couté Y, Chaoui K, *et al.* Looking for Missing Proteins in the Proteome of Human Spermatozoa:
 668 An Update. *J Proteome Res* 2016;**15**:3998–4019. American Chemical Society.
- 669 Vera Y, Diaz-Romero M, Rodriguez S, Lue Y, Wang C, Swerdloff RS, Sinha Hikim AP. Mitochondria-
 670 Dependent Pathway Is Involved in Heat-Induced Male Germ Cell Death: Lessons from Mutant
 671 Mice1. *Biol Reprod* 2004;**70**:1534–1540.
- 672 Volpe S, Galeati G, Bernardini C, Tamanini C, Mari G, Zambelli D, Seren E, Spinaci M. Comparative
 673 immunolocalization of heat shock proteins (Hsp)-60, -70, -90 in boar, stallion, dog and cat
 674 spermatozoa. *Reprod Domest Anim* 2008;**43**:385–392.
- 675 Wang G, Guo Y, Zhou T, Shi X, Yu J, Yang Y, Wu Y, Wang J, Liu M, Chen X, *et al.* In-depth proteomic
 676 analysis of the human sperm reveals complex protein compositions. *J Proteomics* 2013;**79**:114–
 677 122. Elsevier B.V.
- 678 Wenhao T, De-Feng L, Kai H, Lian-Ming Z, Jia-Ming M, Xin-Jie Z, Lu-Lin M, Hui J. miRNA-mediated
 679 regulation of heat shock proteins in human ejaculated spermatozoa. *Turkish J Med Sci*
 680 2015;**45**:1285–1291.
- 681 Widlak W, Vydra N, Malusecka E, Dudaladava V, Winiarski B, ??ciegli??ska D, Widlak P. Heat shock
 682 transcription factor 1 down-regulates spermatocyte-specific 70 kDa heat shock protein expression
 683 prior to the induction of apoptosis in mouse testes. *Genes to Cells* 2007;**12**:487–499.
- 684 Yin L, Dai Y, Cui Z, Jiang X, Liu W, Han F, Lin A, Cao J, Liu J. The regulation of cellular apoptosis by
 685 the ROS-triggered PERK/EIF2 α /chop pathway plays a vital role in bisphenol A-induced male
 686 reproductive toxicity. *Toxicol Appl Pharmacol* 2017;**314**:98–108. Elsevier Inc.
- 687 Yoon J, Park K, Hwang DS, Rhee K. Importance of eIF2 α phosphorylation as a protective mechanism

- 688 against heat stress in mouse male germ cells. *Mol Reprod Dev* 2017;**84**:265–274.
- 689 Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K, Mori K. A time-dependent phase shift in the
690 mammalian unfolded protein response. *Dev Cell* 2003;**4**:265–271.
- 691 Zhao L, Ru Y, Liu M, Tang J, Zheng J, Wu B, Gu Y, Shi H. Reproductive effects of cadmium on sperm
692 function and early embryonic development in vitro. In Rosenfeld CS, editor. *PLoS One*
693 2017;**12**:e0186727.
- 694 Zhu Z, Umehara T, Okazaki T, Goto M, Fujita Y, Hoque SAM, Kawai T, Zeng W, Shimada M. Gene
695 Expression and Protein Synthesis in Mitochondria Enhance the Duration of High-Speed Linear
696 Motility in Boar Sperm. *Front Physiol* 2019;**10**:252.
- 697 Zigo M, Kerns K, Sutovsky M, Sutovsky P. Modifications of the 26S proteasome during boar sperm
698 capacitation. *Cell Tissue Res* 2018;**372**:591–601.
- 699

Figure 1 - Heat shock response (HSR). Under normal conditions, monomeric HSF1 is bound to molecular chaperones (HSPA1 and HSP90). In response to several stressors that lead to defects in protein folding, HSF1 dissociates from the molecular chaperones, is phosphorylated and forms a DNA-binding trimer that is translocated into the nucleus. In the nucleus, HSF1 binds to heat-shock elements (HSE) mediating the transcription of heat shock genes. Once transcribed, small HSPs such as HSPB1 form oligomeric structures that are phosphorylated (P) and bind misfolded proteins. The binding of free HSPs, such as HSPA1, HSP90 and oligomeric HSPB1, to the misfolded or unfolded proteins results in their correct refold after stress. All the unfolded protein response related proteins present in the figure were already identified in testis and spermatozoa (yellow), except HSF1 (blue).

Figure 2 - Mitochondrial UPR (UPR^{mt}). Upon mitochondrial stress in the matrix, c-Jun N-terminal kinase (JNK2) activates the transcription factor c-Jun, which binds to AP-1 elements and activates transcription of the transcription factors CHOP and CEBPB. Dimers of CHOP and CEBPB bind to the CHOP element on the promoter of UPR^{mt} genes that encode mitochondrial quality control proteins, such as HSPD1 (HSP60) and CLPP, and mitochondrial import components. The signalling inputs that indicate stress in the mitochondrial matrix and lead to the activation of JNK2 are currently unknown, however, HSPD1 seems to be responsible for the recognition of unfolded proteins. dsRNA-activated protein kinase (PKR) inhibits global cytosolic protein translation by inducing eIF2 α phosphorylation. In the IMS, ROS activate protein kinase B (AKT1), which consequently phosphorylates estrogen receptor α (ESR1). Activated ESR1 induces IMS protein quality control by increasing the activity of proteasome 26S and inducing the transcription of IMS protease HTRA2 and mitochondrial regulator NRF1. All UPR-related proteins present in the figure were already identified in testis and proteins in yellow were also identified in spermatozoa.

Figure 3 - Endoplasmic reticulum UPR (UPR^{ER}). Upon ER stress, unfolded or misfolded proteins bind to ER chaperone BiP activating one of the three branches of UPR^{ER} . In response to stress, PERK dimerizes and is activated by trans-autophosphorylation resulting in the phosphorylation of eIF2 α and thus in global attenuation of protein synthesis. However, ATF4 is still translated and controls the levels of CHOP that in turn regulates GADD34. GADD34 mediates eIF2 α dephosphorylation through the binding and recruitment of PPP1. The oldest arm of UPR is mediated by IRE1 that when activated process the pre-mRNA encoding X-box binding protein-1 (XBP1). XBP1 is a potent transcription factor that goes to the nucleus and regulate the expression of UPR genes involved in ER biogenesis, autophagy and protein quality control. Additionally, ATF6 is re-localized from the ER to the Golgi where it is cleaved by the proteases S1P and S2P releasing the cytosolic domains. The active domain translocates to the nucleus and acts a transcription factor increasing the expression of UPR-related genes. All UPR-related proteins present in the figure were already identified in testis and proteins in yellow were also identified in spermatozoa.

Table I – UPR-related proteins already identified in mature spermatozoa, along with information concerning their identification under specific conditions, infertility-related phenotypes obtained in MGI and DisGenet databases and localization in spermatozoa.

	Protein	Identified in human sperm	Identified in spermatozoa under specific conditions	Male fertility-related phenotype	Localization in spermatozoa
HSR	HSP90A	(Baker <i>et al.</i> , 2013; Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)	Decreased ROS(up (Hamada <i>et al.</i> , 2013)) DNA fragmentation (down (Intasqui <i>et al.</i> , 2013)) Asthenozoospermia (down (Saraswat <i>et al.</i> , 2017)); (up (Parte <i>et al.</i> , 2012))	Small testis, arrest of spermatogenesis, male infertility, testicular atrophy, small scrotum, decreased testis weight, azoospermia, abnormal male meiosis, arrest of male meiosis, abnormal male germ cell apoptosis	Sperm surface
	HSP27/HSPB1	(Silva <i>et al.</i> , 2015)			
	HSP70/HSPA1	(Martínez-Heredia <i>et al.</i> , 2008; Baker <i>et al.</i> , 2013; Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)	Asthenozoospermia (down (Shen <i>et al.</i> , 2013; Saraswat <i>et al.</i> , 2017)); (up (Parte <i>et al.</i> , 2012))		Sperm surface
	HSPA2	(Martínez-Heredia <i>et al.</i> , 2008; Baker <i>et al.</i> , 2013; Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)	Asthenozoospermia (up (Martínez-Heredia <i>et al.</i> , 2008; Amaral <i>et al.</i> , 2014b)); (down (Siva <i>et al.</i> , 2010; Parte <i>et al.</i> , 2012)) Increased age (up (Liu <i>et al.</i> , 2015)) Poor blastocyst development (up (McReynolds <i>et al.</i> , 2014))	Male infertility, varicocele, Arrest of spermatogenesis, male infertility, decreased testis weight, abnormal male meiosis	Sperm surface
UPR ^m	CLPP	(Wang <i>et al.</i> , 2013; Vandenbrouck <i>et al.</i> , 2016)		Small testis, arrest of spermatogenesis, male infertility, azoospermia	
	HSPD1/HSP60	(Amaral <i>et al.</i> , 2013, 2014a; Baker <i>et al.</i> , 2013; Vandenbrouck <i>et al.</i> , 2016)	Cryopreserved semen with and without cryoprotective (Bogle <i>et al.</i> , 2017) Low-motility (down (Amaral <i>et al.</i> , 2014b))		Midpiece

UPR ^{ex}			Asthenozoospermia (up (Saraswat <i>et al.</i> , 2017))		
	HSPE1/HSP10	(Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)	Asthenozoospermia (down (Saraswat <i>et al.</i> , 2017))		
	CLPX	(Wang <i>et al.</i> , 2013; Vandenbrouck <i>et al.</i> , 2016)		Male infertility	
	AKT	(Wang <i>et al.</i> , 2013)		Abnormal testis morphology, small seminiferous tubules, seminiferous tubules degeneration, abnormal spermatogenesis, oligozoospermia	
	HTRA2	(Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)			
	NRF1	(Vandenbrouck <i>et al.</i> , 2016)			
	MAPK9/JNK2	(Wang <i>et al.</i> , 2013)			
	ERN1/IRE1	(Liu <i>et al.</i> , 2015)			
	HSPA5/GRP78	(de Mateo <i>et al.</i> , 2011; Wang <i>et al.</i> , 2013; Amaral <i>et al.</i> , 2014a; Vandenbrouck <i>et al.</i> , 2016)	Low-motility (down (Amaral <i>et al.</i> , 2014b)) Varicocele (down (Hosseinifar <i>et al.</i> , 2014)) ROS (up (Sharma <i>et al.</i> , 2013)) DNA fragmentation (down (Intasqui <i>et al.</i> , 2013)) Asthenozoospermia (down (Parte <i>et al.</i> , 2012; Shen <i>et al.</i> , 2013)); (up (Saraswat <i>et al.</i> , 2017)) Poor blastocyst development (up (McReynolds <i>et al.</i> , 2014))		Neck
	TRAF2	(Wang <i>et al.</i> , 2013; Vandenbrouck <i>et al.</i> , 2016)			
	EIF2S1/eIF2 α	(Wang <i>et al.</i> , 2013; Liu <i>et al.</i> , 2015; Vandenbrouck <i>et al.</i> , 2016)			