Biochemistry and Molecular Biology in the Post Genomic Era

HSP70s

Discovery, Structure and Functions

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Chapter 1

THE ROLE OF HSP70 IN SPERM QUALITY

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ABSTRACT

Heat shock proteins (HSP) constitute a large family of proteins that are most conserved in living organisms. The 70 kDa HSP (HSP70) is one of its most abundant members. Under normal conditions, they are constitutively expressed in cells, playing several physiological functions as molecular chaperones. HSP are generally associated with cellular response to an eclectic variety of stimuli, such as oxidative stress, hypoxia-ischemia, nutrient deprivation, and apoptosis signalling. HSP are widely distributed in mammal tissues and particularly in those associated with reproduction, such as the endometrium, trophoblast, placenta, or testicles. Two forms of testis-specific HSP70 have been localized in spermatogenic cells. They are thought crucial to germ cell differentiation and sperm production. These

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chaperone molecules have also been found in the sperm surface in different species. Situations of male infertility associated with heat or osmotic stress have been implicated in altered expression of HSP. Besides, it was demonstrated HSP70 molecules to suffer a dynamic redistribution at the sperm surface during capacitation and acrosome reaction.

It has been proposed HSP70 molecules present in semen to modulate the immune system of the female reproductive tract after ejaculation, protecting the spermatozoa against disruptive stressors. During the preparation of seminal doses, when sperm is separated from the seminal fluid, this protective effect is withdrawn, and the sperm cells became more susceptible to the effects of environmental changes. Different studies showed a decrease in the HSP70 content of sperm submitted to freezing/thawing or sperm sorting processes, in association with its redistribution at the sperm surface. These features have been associated with loss of sperm function after manipulation that mimics that of capacitation. HSP70 was also found to be disturbed in male infertility. It is running with poor semen quality, which might explain some cases of idiopathic conditions in the absence of dysfunctional sperm.

This review intends to discuss the roles of the HSP70 family in spermatogenesis and sperm function, detailing the mechanisms that may foster sperm quality and the use of HSP as markers of fertility.

Keywords: N-Cadherin, sertoli cells, germ cells, cell–cell connections, seminiferous tubules, spermatogenesis, fertility

INTRODUCTION

The discovery of heat shock proteins (HSP) is a good story of serendipity in science. In 1960 Ferruccio Ritossa began at the Genetics Institute in Pavia and the International Institute of Genetics and Biophysics in Naples. He studied what kind of nucleic acid was synthesized in puffs of Drosophila's salivary glands [1, 2]. A laboratory colleague accidentally increased the temperature of the incubator in which Ritossa was keeping the tissues and as a result, later noticed a different pattern of inhalation [3]. A calculation of time for the new RNA synthesis after the temperature change conditions showed a surprising speed of 2-3 minutes. In the beginning, he hypothesized the system to be directly correlated with energy production because a similar pattern of gene activation was observed in the presence of

mitochondrial uncoupler dinitrophenol and salicylates [1, 4]. Many years would pass, along with an enormous amount of research, before the demonstration that the heat shock response is a universal and ancient mechanism [5, 6] preserved amongst species. HSP is among the most conserved protein family in living organisms. It is widely distributed in nature and may be found either in prokaryotes or eukaryotes [7-9].

Initially reported as constitutively expressed, HSP genes are upregulated in response to a myriad of cellular stressors: temperature, gravity, osmotic pressure, pH, hypoxia-ischemia, nutrient deficiency, oxidants, radiation, xenobiotics, heavy metals, carbon monoxide and subsets of interferons and cytokines (e.g., in inflammation) [6, 9-11]. Infections by bacteria, viruses, parasites and fungi also increase the synthesis of HSP [12]. Stress-induced HSP assist the folding of nascent or misfolded proteins, intracellular transport of proteins (in the cell cytosol, endoplasmic reticulum, and mitochondria), repair or degradation of proteins and refolding of denatured proteins, preventing its aggregation, adverse metabolic effects and toxic-associated cell death [13, 14]. Stressful conditions may also derive from physiological events, such as cell growth, differentiation, development and aging, as well as during disease.

HSP are widely distributed in mammal tissues [15] and particularly in those associated with reproduction, such as the endometrium, trophoblast, placenta, or testicles [14, 16, 17]. Among the different HSP, the 70 kDa HSP (HSP70) is one of the most abundant, well studied and conserved members of these chaperone molecules [18]. Along with HSP60, HSP70 is considered a physiological alarm signal for cell trauma [14].

Testis-specific HSP70 forms have been localized within the seminiferous tubules, in spermatogenic cells [14]. It has been proposed that these molecules are crucial to germ cell differentiation and sperm production, acting as key-regulators of spermatocyte maturation and function [19]. Moreover, HSP has been found in the sperm surface in various species, and in particular, molecules belonging to HSP70 family have emerged as indispensable for sperm cell fertility [20, 21]. Situations of male infertility associated with heat or osmotic stresses have been implicated in altered expression of HSP, like those found during semen processing in

assisted reproduction. In the former, a reduced HSP expression would compromise sperm-egg interaction [22-24], while in the latter it was proposed that disturbed HSP70 expression would reflect the occurrence of capacitation-like changes in spermatozoa [21, 25].

Besides their intracellular location, different HSP may also be found in the extracellular spaces and the body fluids, where they interact with neighbour cells or enter the bloodstream [14]. HSP70 molecules have also been found in other organic fluids such as the semen. Members of the HSP70 family present in semen may modulate the immune system of the female reproductive tract after ejaculation while protecting the spermatozoa against disruptive stressors [26]. During the preparation of seminal doses, when sperm is separated from the seminal fluid, this protective effect is withdrawn, the spermatozoa become more susceptible to any detrimental environmental changes.

This review intends to discuss the information concerning the roles of HSP70 in spermatogenesis and sperm function, its contribution to male fertility and information supporting its use as markers of fertility.

HSP70 Structure and Function

Stress-inducible HSP expression is driven via the activation of heat shock responsive transcriptional factor 1 (HSF1). Under basal conditions, HSF1 exists in the cytoplasm as an inactive monomer that is repressed by its interaction with a protein complex consisting of HSP [26]. Under stress HSF1 is hyperphosphorylated in a ras-dependent manner by members of MAPK subfamilies (e.g., p38 protein kinase, ERK1) driving the dissociation of this repressive protein complex. Trimerization of hyperphosphorylated HSF1 promotes translocation from the cytoplasm to the nucleus [27, 28]. In the nucleus, activated HSF1 binds to specific heat shock elements (HSEs) in the proximal promoter region of its target genes, and induces the transcription of target genes (including several HSP) [29, 30]. Increasing levels of HSP negatively downregulate HSF trimers activity resulting in a transient generation of proteins [30, 31]. However, over the past several

years, heat shock proteins have been shown to play an essential role in the regulation of many cellular signalling pathways, both under normal physiological and pathophysiological conditions [9].

HSP are expressed constitutively under normal growth conditions in cells. They present several physiological functions acting in the cell cycle progression, replication, and transcriptional and posttranslational processes such as protein folding, stability, transportation, and degradation [11, 32, 33]. HSP assist the transport of proteins from the cell cytosol to endoplasmic reticulum, mitochondria, peroxisome and nucleus. In disorders of protein dysfunction, HSP acts as disease suppressor stimulating polypeptide unfolding isomerase and refolding of misfolded proteins or preventing their aggregation [13, 34, 35]. HSP also activate many key signal transducers in cellular response [36, 37]. Lately, they were found to be involved in processes such as autophagy, endoplasmic reticulum stress response, protein kinase and apoptosis signalling [9].

The HSP Family

HSP, a large family of proteins whose molecular weight range from 15 to 110 kDa, were initially classified in mammals according to their molecular weight as follows: a) Small heat shock protein (sHSP) family; b) HSP40; c) HSP60; d) HSP70; e) HSP90; f) HSP100 [5, 38, 39]. Few reports have also included ubiquitin (8.0 kDa) as one HSP class characteristic of the eukaryotic organisms [38, 40].

A slightly different nomenclature has been proposed for human HSP families: a) Small heat shock protein (HSPB); b) DNAJ (HSP40); c) HSPA (HSP70); d) HSPC (HSP90); e) HSPH (HSP110); f) Chaperonin family HSPD/E (HSP60/HSP10); g) CCT (TRiC) [41].

Small HSP (sHSP) presents a low molecular mass of 12–43 kDa and contain 80–100 amino acids conserved site at the C-terminus and a α/β-crystalline domain [42]. HSP40 (J-proteins) family locates in the cytosol and constitute a family with more than 40 elements in humans and assists (co-chaperoning) HSP70 in protein folding [41, 43, 44]. HSP40 contains a J

domain, which binds to the N-terminal ATPase domain of HSP70 and the adjacent linker region [43, 45].

HSP60 (Chaperonin/GroEL) family members are located in the cell cytosol and mitochondria [13, 46]. They assist in protein folding, and prevent protein aggregation and assembling of unfolding proteins via the formation of the hetero-oligomeric complex [47, 48].

The 70 kDa HSP (HSP70/DnaK) is one of the most abundant, well studied and conserved members of these chaperone molecules [18]. HSP70 plays a role in the assembly and transport of newly synthesized proteins. Additionally, it participates in the removal of denatured proteins and cares matured proteins to be folded into functional structures. GRP78 (a member of the HSP70 family) helps protein folding assembly and refolding, transporting and blocking protein degradation in the endoplasmic reticulum [39]. Some HSP70 proteins are expressed in basal levels in non-stressed cells: constitutive HSP70 or HCP70 [6].

Hsp90 is a flexible dimeric protein composed of three different domains which adopt structurally distinct conformations [49]. The ATP binding triggers the conformational change and leads to a more compact state of the protein [50, 51]. Even though not required for *de novo* folding of most proteins, Hsp90 facilitates the final maturation of selected proteins and helps to maintain the native state of the proteins [52].

HSP100 (ClpB) family are located in cytoplasm (HSP100, HSP110) and nucleus (HSP110). HSP100 cochaperones with HSP40, HSP70, and HSP90, playing a role in refolding the aggregate. HSP110 is also in working with HSP70 to fold proteins and counter stress for cell survival [53, 54].

An alternative classification uses their function to categorize HSP: a) chaperones (HSP 70 and 60); b) proteins with catalytic activity (ubiquitin, HSP100, proteases and tyrosine phosphatase); c) proteins with an obscure function (α-crystalline and secreted glycoproteins) [55].

Several high molecular weight HSP (HSP60, HSP70, HSP90 and HSP100) are ATP-dependent and show functions such as protein folding and translocation, cytoprotection, regulation of nuclear hormone receptors as well as regulation of apoptosis. On the other hand, smaller HSP (HSP10, HSP40,) are ATP-independent; they are mostly tissue-specific, playing an

essential role as a chaperone for protein folding as well as possessing a strong anti-apoptotic effect [41].

The Heat Shock Protein 70 (HSP70)

This chapter will focus on the HSP70 family, who are abundant in the cell, found in all the major subcellular compartments, ubiquitous and highly conserved ATP-dependent chaperones [56]. The molecular weight of proteins from the HSP70 family ranges from 66kDa to 78kDa [57, 58]. HSP70 are monomeric proteins with diverse localizations in mammals: the cell cytosol, nucleus, endoplasmatic reticulum, mitochondria and extracellular space [18, 59-61]. The constitutive HSP70 or HCP70 (also called HSP73 or HSPA8) and inducible (HSP72 or HSPA1) are the most studied HSP70s [10]. Non-stressed cells usually expressed basal levels of constitutive HSP70 that play a housekeeping role under normal conditions, including [18, 47, 62]:

a) assisting folding of some newly translated proteins transiently binding to incomplete protein sequences, preventing their aggregation and driving their folding into a functional state, cooperating with other protein folding and quality control machinery;
b) guiding translocating polypeptides across intracellular membranes (mitochondria, chloroplasts and the endoplasmic reticulum);
c) assembling/disassembling oligomeric protein structures and complexes;
d) facilitating proteolytic degradation of unstable proteins;
e) regulating the biological activity of folded regulatory proteins;
f) protecting proteins from proteolysis.

On the other hand, some HSP70 protein are transitorily induced (inducible HSP70) after many different stresses (oxidative, drugs…), allowing the cell to survive under lethal conditions [10]. Over-expressed

HSP70 in stress are mostly localized in the nucleolus [63]. HSP70 and plays protein protecting/recovery roles that include the prevention of protein aggregation, control of protein disaggregation, protein refolding, and protein degradation [18, 64, 65]. The levels of HSP70 hardly increases in cancer cells, suggesting that these proteins may be participating in oncogenesis and resistance to chemotherapy by interfering with cell death mechanisms of apoptosis [66, 67].

In comparison to the other HSP proteins in cellular response, HSP70 show the most accentuate response to the heat stress, hypoxia/anoxia, and chemical shocks (heavy metals, toxic chemicals, salinity stress*)* [68, 69]. It has also been shown that the expression level profile of HSP70 varies for healthy and diseased conditions [70]. HSP are widely distributed in mammalian tissues [15] and particularly in those associated with reproduction, such as the endometrium, trophoblast, placenta, or testicles [14, 16, 71].

In the case of HSP70 family members, the use of different nomenclatures leads to the use of different abbreviations. Table 1 summarizes some of those correspondences.

Table 1. Correspondence of different nomenclatures for HSP70 family members (ER - Endoplamic reticulum)

Protein name	Alternative names	Cellular location	Constitutive	Stress-Inducible	Comments
HSPA1A	HSP70-1, HSP72, HSPA1, HSP70-1A, HSP70I	Cytosol, nucleus, cell membrane, extracellular exosomes		✓	
HSPA1B	HSP70-2, HSP70-1^{B}	Cytosol, nucleus, extracellular exosomes		✓	
HSPA1L	HSP70-1L, HSP70-HOM, HSP70-1t, HUM70T	Cytosol, nucleus	✓	×	~90% homology with HSPA1A
HSPA2	HEAT SHOCK 70KD PROTEIN 2^{B}, HSP70.2	Cytosol, nucleus, cell membrane, extracellular exosomes	✓	×	~85% homology with HSPA1A

Protein name	Alternative names	Cellular location	Constitutive	Stress-Inducible	Comments
HSPA5	HSP70-5, BIP, GRP78, MIF-2	ER, extracellular exosomes	✓	×	
HSPA6	HSP70-6, HSP70B'	Cytosol, extracellular exosomes		✓	Highly homologous to HSPA1A
HSPA7	HSP70-7, HSP70B	Blood microparticles, extracellular exosomes		✓	Highly homologous to HSP6
HSPA8	HSP70-8, HSC70, HSC71, HSP71, HSP73	Cytosol, nucleus, cell membrane, extracellular exosomes	✓	×	~86% homology with HSPA1A
HSPA9	HSP70-9, GRP75, HSPA9B, MOT, MOT2, PBP74, MOT-2, MTHSP70, Mortalin	Mitochondria, nucleus	✓	×	~52% homology with HSPA1A
HSPA12A	HSP70-12A, FLJ13874, KIAA0417	Intracellular, extracellular exosomes	✓	×	
HSPA12B	HSP70-12B, RP23-32L15.1, 2700081N06Rik	Endothelial cells, intracellular, blood plasma	✓	×	
HSPA13	HSP70-13, Stch	ER, extracellular exosomes, microsomes	✓	×	
HSPA14	HSP70-14, HSP70L1, MGC131990	Cytosol, membrane		✓	

TESTICULAR HSP70

The spermatogenic process is characterized by a continuously synchronized and spacially organized sequence of events ending with the release of spermatozoa into the lumen of the seminiferous tubules [72]. Spermatogenesis involves three fundamental events: the recruitment of spermatogonia to proliferate; the reductional meiosis by spermatocytes; and

a unique maturational phase where a small round cell transforms into a highly specialized flagellated cell with very scant cytoplasmic and a tail, a tightly condensed chromatin and an acrosome – the spermatozoon [73]. In all these stages, but particularly in the maturation phase, a strong proteostasis is required to fulfil the task of producing fertile spermatozoa. HSP70 family members are molecular chaperones regulating the folding, transport, and assembly of proteins either under stress and normal physiological conditions [74]. Therefore, proteins belonging to the 70 kDa HSP family have emerged as being indispensable for male fertility [75]. More than seven different HSP70 have been detected in germ cells; either constitutively expressed or stress-induced. Two testis-specific members of the HSP70 family have been identified in the germinal epithelium. Albeit some forms have been found in the reticulum or the mitochondria, most of HSP70 were located in the cytoplasm of germ cells [76]. In testis, as in other tissues, we should be aware that HSP70 function is coupled to the action of other cochaperones HSP (like HSP90 and 60, HSP-organizing protein and HSP100) [23], in a joint process that increases its efficiency in stress-mediated conditions and thermo-tolerance. Chaperone-binding cochaperones confer functional specificity to the HSP70 chaperone system [77].

Two specific HSP70 forms have been localized in the mammalian male gonads, and thought to be critical for spermatogenesis and male fertility [61, 78]:

- Hsp70-1t (HspA1L) is constitutively expressed cytosolic protein with 90% homology to Hsp70-1.
- Hsp70.2 (HspA2), highly expressed in the testis and also the brain, presents close to 85% homology to Hsp70-1; this form is temperature-sensitive and seems particularly important for the progression of meiosis. Its expression at the sperm surface is also critical for sperm-egg interaction.

The expression pattern of these variant members of HSP70 differs between species and may relate with their roles in testicular thermoregulation mechanisms. E.g., the expression of HspA2 is down-

regulated in mammals with external testicles submitted to acute heat stress, whereas it is up-regulated in birds (with internal gonads) [79]. Considering that HSP70 forms are, in general, higly conserved among species, Padhi et al. [79] suggested that these differences represent interspecific changes, supporting the existence of adaptative mechanisms contributing to the reproductive fitness and male fertility. It would also represent an intrinsic cellular defense mechanism against stress conditions. Furthermore, HSP70 also presents tissue or developmental stage-specific expression patterns, that are suggestive of a role in tissue homeostasis and the fate and shaping of organs or structures during development [14], possibly by mediating apoptosis and other cell death pathways.

Developing male germ cells are highly susceptible to temperature. The spermatogenic process may be disrupted with only small changes of 2-3 ºC in the testicular temperature. Recently, it has been shown that HSPA1L phosphorylation mediated by MAPKAP kinase 2 protects germ cells from heat stress-induced apoptosis, enhancing HSPA1L - the constitutively express testis-specific HSP70 - chaperone activity *in vitro* [80]. HSPA1L is mainly expressed during the post-meiotic phase [81, 82].

HSPA2 is expressed in spermatocytes undergoing meiosis [83-86] and in spermatids [84-86] across mammalian species.

Disturbed HSPA2 and HSPA1L in transgenic mice impair completion of meiosis I [77, 87]. Zhu, Dix, and Eddy [87] showed that HSPA2 is required for the assembly of the CDC2/cyclin B1 complex and the activation of CDC2 kinase at the onset of meiosis (G2/M-phase transition) in mouse pachytene spermatocytes. HSPA2 has also been associated with the synaptonemal complex [76], assisting the chromosome crossover during meiosis [23]. Besides, it has also been implicated in the control of spermatid DNA packaging proteins [74]. Experiments on the identification of genome-organizing proteins in condensing spermatids reported a tight association of HSPA2 with major spermatid DNA-packaging in mice, namely the transition proteins TP1 and TP2. Thereby, it was hypothesized the existence of a shift in the HSPA2 chaperone role in post-meiotic cells. In those cells, HSPA2 spreading into the nucleus shadows the histone removal, replacing the histones before being themselves replaced by protamines [23].

Production of HSP is transcriptionally regulated by heat shock transcription factors (HSFs) [88]. These factors are also expressed during spermatogenesis, mainly in spermatocytes and round spermatids (i.e., in cells suffering extense chromatin remodelling). Impaired HSP expression originates meiosis arrest and apoptosis in the spermatocyte, and the abnormal repackaging of the DNA during spermatid differentiation [89]. All HSP are expressed in the mammalian testis. Two members of these proteins - HSF5 and HSFY – were exclusively identified in the testicular tissue, showing a specific expression pattern [89].

HSF1 is constitutively expressed in the spermatogenic cells. It acts as a cell-survival factor only in somatic and premeiotic germ cells [90]. When activated under heat-stress conditions, HSF1 downregulates HSPA2, driving the activation of the cell caspase-3-dependent apoptosis pathways in spermatocytes [90]. Conversely, in undifferentiated spermatogonia, it promotes the stem germ cells survival [88]. The dual role may serve the protection of stem spermatogenic cells while ensuring that developing cells that potentially may be functionally defective are destroyed [91].

In rodents, HSF2 presents a tight regulated stage-specific expression profile during the cycle of the seminiferous epithelium, whereas the levels of HSF1 remain relatively unchanged. In rats and mice, HSF2 locate in the nuclei of early pachytene spermatocytes and round spermatids, as well as in the intercellular cytoplasmic bridges between cells derived from a spermatogonial precursor. Testicular hypoplasia associated with vacuolisation of seminiferous tubules, increased apoptotic rate in meiotic spermatocytes and low sperm counts have been reported in HSF2 deficiency. Contrastingly, increased expression of HSF1 would trigger cell death in germ cells, particularly in pachytene spermatocytes, while seemly protective against apoptosis in the spermatogonia. These data suggest the existence of an interplay role for HSF1 and HSF2 in regulating the spermatogenic process which could encompass the use of commun targets regulators [92].

HSP70 present age-related changes in several tissues, even in the absence of stress [93]. A disruption in the balance of the HSP70 system in the ageing gonad may be responsible for the increased predisposition to develop cancer or age-related infertility.

Compared to normal conditions, HSP70 is changed in cases of exposure to acute heat-stress conditions [86, 94], toxicological stressors [95] and exogenous androgen treatment [96]. Moreover, in infertility conditions associated with deteriorated spermatogenesis, the expression of HSP70 [75] and HSFs [97] were found downregulated. Deletion of HSPA2 genes, as shown in studies with transgenic mice, originates the arrest of meiosis in prophase 1, with the subsequent absence of spermatids [23].

In cryptorchidism, the failure of the testicles to reach its normal scrotal position (in the case of mammals having external, scrotal testes) imposes elevated temperatures compromising the normal spermatogenesis.When exposed to chronically elevated temperature, the HSP70 protective mechanism is insufficient to avoid damage, and the male lost fertility. Studies in experimentally induced cryptorchidism showed a small decline in HSPA2 leading to increased apoptosis, even if this is not to be the sole pathway involved in the disruption of spermatogenesis [98, 99]. Widlak et al. [90] showed that an increase in HSF1 activity leads to the downregulation of HSPA2. This, in turn, triggers the activation of caspase-3-dependent apoptotic mechanism. When comparing the immunohistochemistry location of HSP70[1] in scrotal and spontaneously canine cryptorchidism, the expression of HSP70 protein increased abdominal retained testes, while it was decreased in early spermatocytes in sub-cutaneous retained testis. In either case spermatids were absent (Rita Payan-Carreira, unpublished observations).

HSP70 IN SPERM

Male fertility largely depends on the quality of sperm production, and on the ability of spermatozoa to survive and interact with the female gamete to produce the egg. Production of defective spermatozoa during spermatogenesis will compromise the subsequent male fertility. Even if no

[1] Using a primary polyclonal antibody (Ab 31010, Abcam, UK) directed against the human HSPA1A/HSPA2 molecule - at 1:100 V/V.

morphological defects are visible, nonetheless functional defects prejudice the spermatozoon survival and competence to fertilize. The ability to survive the impact of external stressors, in particular, the thermal, is strongly related to the presence of HSP in both the seminal fluid and sperm.

It is believed that sperm has a finite transcript capacity as its mRNAs are silenced after spermiogenesis. Most abundant rRNAs in spermatozoa are highly fragmented, rendering them transcriptionally inert [100, 101]. Therefore, sperm cytosol must contain HSP at the time of the insult to be able to respond to the situation [102] and maintain its integrity. In mature sperm cells, transcription and translation occur in the mitochondria, not in the cytoplasm [103]. Therefore, sperm survival to stressful conditions largely depends on their molecular content when leaving the testis, and in the protection provided by the seminal plasma.

HSP proteins have been detected in sperm cell surface various species. In particular, HSP70 protein is an abundant component of the sperm membrane (Table 2). HSPA2, HSPA1L and HSPA5 have all been identified in mature sperm [23]. The HS70 reported to exists in mature sperm are most likely incorporated in the plasma membrane during spermatogenesis or epididymal transit, the latter also contributing to the HSP70 forms found in the seminal plasma [23].

The location of HSP70 forms at the membrane levels is of utmost importance for the interaction between the sperm and egg [22]. A reduction in HSPA2 has been associated with impaired sperm-zona pellucida interaction [22], entailing a lost of sperm fertilizing ability. According to Dun, Aitken, and Nixon [23], HSPA2 integrates the composition of a particular subset of human sperm protein complexes expressed on the sperm surface, which harbour known zona adhesion receptors.

Table 2. Summary of some studies on HSP70 in the spermatozoa or seminal plasma

Species	Matrices [conditions]	Type of techniques			Targeted molecule	Main findings	Reference
		Transcription	Proteomics/WB	IHC, IF or equivalent			
Human	Ejaculated spermatozoa		✓	✓	HSP70	Localization in non-capacitated spermatozoa Changes in the amount and pattern in acrosome-reacted spermatozoa	[106]
	Epididymal and Ejaculated spermatozoa [ejaculates and testicular and epididymal biopsies]		✓	✓	HSPA2	Localization in non-capacitated spermatozoa Changes in the pattern in capacitated sperm	[113]
Mice	Semen		✓	✓	HSPC1, HSPA8, HSPA1L, HSPA2	HSP located at the sperm surface HSPA2 also in the spermatozoa tail	[14]
Bull	Ejaculated spermatozoa	✓			HSP70	Comparison of expression levels in fresh samples and after equilibration and freezing	[114]
	Ejaculated and epididymal spermatozoa		✓	✓	HSP70	Differences in the amount and pattern of HSP among epidydimal and ejaculated sperm Description of HSP70 pattern in capacitated spermatozoa	[105]
Boars	Semen (spermatozoa and seminal fluid) 3 industrial breeds		✓		HSP70	Protein profile and qualitative characterization of heat shock protein 70 in boar spermatozoa Found a correlation between the level of HSP70 and semen quality and season	[115]

Table 2. (Continued)

Species	Matrices [conditions]	Type of techniques			Targeted molecule	Main findings	Reference
		Transcription	Proteomics/WB	IHC, IF or equivalent			
	Ejaculated sperm		✓	✓	HSP70	Description of the differences between capacitated and non-capacitated spermatozoa	[116]
	Ejaculated sperm	✓			HSP70	Presence of HSP70 transcripts in spermatozoa	[117]
Sheep	Seminal plasma		✓		HSP70	Level of HSP70 changes between normal and acute heat-stressed semen (scrotal insulation)	[112]
Dog	Ejaculated sperm [fresh, refrigerated and frozen semen]			✓	HSP70	Characterization of the normal localization in fresh, non-capacitated spermatozoa Changes with freezing/thawing suggestive of the capacitated pattern	[25]
Boar, Dog, Stallion and Cats	Ejaculated sperm			✓	HSP70	Characterization of the normal pattern of distribution in the different species Reported changes in the pattern following capacitation	[107]

HSPA5 may mediate the intracellular calcium pathways associated with capacitation, hypermotility and predispose to acrosome reaction [23]. The association of this HSP70 form with other chaperone proteins involved in sperm capacitation strengthens this idea. Some members of the HSP70 family, e.g., HSP70A2, show a decrease and dynamic redistribution in sperm cells during sperm capacitation and the acrosome reaction. These changes allowed to establish different location patterns according to the spermatozoa status [25, 104-106]. HSP70A2 subcellular distribution may change with the species, just like it happens with its normal location in the uncapacitated spermatozoa [107].

HSP70 have a crucial role to play in semen homeostasis, contributing to thermotolerance in extreme climatic temperature variations. The level of HSP70 isoforms in sperm has been shown to change with season (hot *vs*. cold) in bulls [108, 109] and water buffalo [110], and in heat-stressed rabbits [111], as well as in the seminal plasma of rams after scrotal insulation [112].

The existence of HSP70 polymorphisms may affect innate semen´ stress tolerance [118, 119], thereby contributing to changes in spermatozoa fertility, as well as to explain the variations among male regarding the tolerance to freezing/thawing and sorting sperm procedures. These polymorphisms may also affect the potential of semen for ART. Nevertheless, they also may represent an adaptative mechanism of species or breeds native to hot climate areas. This hypothesis sought to be explored in more detail.

During the preparation of seminal doses, when spermatozoa are separated from the seminal fluid, this protective effect is withdrawn, and the sperm cells became more susceptible to the effects of environmental changes. The ability to resist to sublethal stress during cryopreservation has been associated with the ability to synthesise stress-related proteins such as HSP and antioxidant enzymes to maintain cellular homeostasis [120]. Sperm resilience to cryopreservation dramatically varies with the species and may reflect differences in the content of HSP, among other molecules. It has been shown that freezing/thawing procedures disturb the balance of HSP70 forms in spermatozoa in the bull [114], buffaloes [121], goats [122] and dogs [25]. The changes in HSP70 after thawing have been associated to a redistribution

of the proteins at cellular levels [25, 121] or to a decrease in HSP70 expression [114, 122]. Moreover, such changes have been associated with loss of function in sperm cells, mostly to a loss in motility. Besides the changes encountered in conventional seminal doses, sperm submitted to staining and sorting procedures presents a notorious decrease in HSP70 expression and related increased spermatozoa damage (either regarding motility or DNA fragmentation) [104, 123]. Redistribution of HSPA2, as described after sperm sorting in boars or after freezing/thawing in dogs, match the described pattern of capacitated and acrosome-reacted HSP patterns, which suggests that a precocious acquisition of sperm functional activity may reduce its ability to reach unreacted to the oviducts and fertilise the egg.

The osmotic stress can also mobilise HSP70 in sperm and seminal fluid. Despite the cryoprotectants used, the thermal challenges occurring in freezing/thawing procedures often co-exist with hypertonic challenges that contribute to an adverse reaction to cryopreservation. Cole and Meyers [102] showed that hypertonic stress negatively affects sperm quality in macaques, by decreasing progressive motility and sperm viability. These effects have been associated to an increase in HSP70 expression and post-translational modification of phosphoproteins such as tyrosine [102].

The quest to identify molecular markers of fertility in the spermatozoa or the ejaculate has driven the attention of many researchers. The reported differences in the expression of HSP70 in healthy or infertile males [124-126], in the level and distribution of HSP70 in damaged spermatozoa after semen technology, drive the hypothesis that HSP70 family members (e.g., HSPA2) may be useful biofunctional markers for sperm [20, 117, 124]. However, disagreement still exists among the studies reporting dysregulation of HSPA2 in infertile conditions [124-128]. The conflictual information may arise from differences in the clinical conditions tested (e.g., the type of fertility or the intensity of the aggression), differences in the methods or the supplies used, among others. As in the case of other potential biomarkers in supporting medical diagnosis, new studies are needed to highlight the context and usefulness of the molecule(s) proposed.

CONCLUSION

HSP70 are a large family of chaperone proteins. They participate in an extensive network with other members of the larger HSP family. With an essential role in the cell homeostasis, they have been associated with protection against internal and external stressors, and in maintaining proteostasis. HSPs are proteins with many roles; therefore, the entirety of their roles in reproduction and male fertility is only now been tackled. In this chapter we review some of those roles. New findings suggest that they are important partners in sperm and egg recognition. Disturbed HSP70 expression in the spermatozoon compromises fertilisation and may contribute to idiopathic infertility. Thereby, its use as a molecular biomarker for spermatozoa quality has been studied. Evidence also suggests that HSP may reflect the thermotolerance of spermatozoa to acute or chronic temperature changes (environmental or associated with sperm processing). Its levels could be useful when distinguishing good from bad freezers. Still, additional studies are sough to highlight if the HSP expression is an adequated marker to support the medical diagnosis and to break new ground on the treatment of many reproductive dysfunctions.

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