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HSP47 expression in the hamster Sertoli cell: An immunohistochemical study

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Summary. HSP47, a chaperone whose main function is the maturation of collagen molecules, is considered a marker of fibrotic diseases. Increased collagen synthesis in the testis has been associated with various pathologies leading to seminiferous tubule regression. Our aim was to study whether HSP47 is expressed in hamster Sertoli cells both in the adult and in two physiological situations of seminiferous tubule atrophy: irreversible testicular ageing and testicular regression due to short photoperiod (reversible). Eighteen animals were divided as follows: a group of 6 young animals aged 6 months, a group of 6 animals aged 24 months, which were exposed to a long photoperiod, and a final group of 6 young animals subjected to a short photoperiod. Testicular samples were fixed in methacarn and an immuno-histochemical technique was used to detect HSP47. A semiquantitative study of this protein expression was performed between tubular sections of aged animals with complete spermatogenesis and arrested spermatogenesis and tubular sections with arrest spermatogenesis of photoinhibited testes. Sertoli cells were positive for HSP47, the intensity being greater in tubular sections with arrested spermatogenesis in both aged and photoinhibited animals. Semiquantitative analysis corroborated this observation in the sense that the expression of this protein differed according to the functional state of the seminiferous tubules. Thus, the ratio of immunoreactivity was significantly higher in tubular sections with arrested spermatogenesis in aged animals compared with regressed animals, and in the latter compared with those whose tubular sections showed complete spermatogenesis. In conclusion, HSP47 expression in Sertoli cells was found for the first time in mammals. Moreover, increased expression seemed to be related to the degree of seminiferous atrophy epithelium and to the reversible or non-reversible physiological state of this epithelium.

Key words: Sertoli cell, Syrian hamster, Testes, HSP47, Aging, Photoperiod

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Introduction

Heat shock protein 47 (HSP47) is a glycoprotein belonging to the serpin superfamily, also known as serpinH1. It is a collagen-specific chaperone, which is located in the endoplasmic reticulum (ER) and its main function is collagen maturation, interacting specifically with procollagen molecules (Nagata, 1996). Unlike other HSPs, HSP47 does not require ATP to exert its chaperone function, and heat shock seems to be the main stimulus for its activation (Ito and Nagata, 2017; Bellaye et al., 2021).

Although HSP47 is induced by cellular stress, it is also constitutively expressed in several cell types that show a high degree of collagen synthesis. Thus, it was first identified in chick embryo fibroblasts, which showed strong collagen expression (Nagata, 1996; 2003). Its expression has also been observed in other collagen-producing cell types, such as chondroblasts and adipocytes (Nagata, 1996; Ito and Nagata, 2017). Recent studies have shown a link between increased HSP47 expression and excessive collagen accumulation in scar tissues in various human fibrotic diseases (Taguchi and Razzaque, 2007). For example, increased HSP47 expression has been observed in glomerular cells in experimental models of renal fibrosis, such as diabetic nephropathy and hypertensive nephrosclerosis (Razzaque et al., 2005). In addition, increased HSP47 expression has also been detected in collagen-producing interstitial myofibroblasts and tubular epithelial cells in several experimental models of renal tubulo-interstitial fibrosis (Taguchi and Razzaque, 2007). HSP47 is thus

considered a potential target in fibrotic diseases (Bellaye et al., 2021). Moreover, altered levels of HSP47 expression have been correlated with several types of cancer, such as cervical, breast, pancreatic and gastric cancer. Studies have shown that HSP47 can promote tumour angiogenesis, growth, migration and metastatic capacity, although in some cases it may have a protective effect (Duarte and Bonatto, 2018). Sertoli cells are somatic cells that form the main structural component of the seminiferous tubules of the testis, where the germ cells are located. Their main function is to 'take care' of the spermatogenic cells, providing the nutrients necessary for testicular development and spermatogenesis (Griswold, 2018; Thumfart and Mansuy, 2023), while also providing immunological protection to the germ cells (Kaur et al., 2014). They also play an important role in testis development, as they are the first cells to differentiate into the XY embryonic gonadal primordium. They are involved in the regulation of testis differentiation processes, the prevention of germ cell entry into meiosis, the differentiation of peritubular myoid cells and Leydig cells, and regression of the Müllerian ducts, the uterovaginal primordium, oviducts and upper part of the vagina (Barrionuevo et al., 2011). Pre-Sertoli cells are known to be of mesenchymal origin, meaning that they must undergo a transition from a mesenchymal to an epithelial cell type to differentiate into Sertoli cells (Cupp and Skinner, 2005). The accumulation of various extracellular matrix (ECM) components, such as laminin and collagen, has been shown to induce polarisation of embryonic Sertoli cells (Cool et al., 2012). In addition, recent studies have shown similarities between Sertoli cells and mesenchymal cells (Gong et al., 2017, Porubska et al., 2021), which are starting to be used in regenerative medicine (Luca et al., 2018). HSP47 is also known to be involved in mesenchymal-epithelial transition and is expressed by mesenchymal cells (Alt et al., 2011; Tian et al., 2020).

A common observation in aged seminiferous tubules and in patients with various testicular pathologies is the accumulation of ECM in the tubular wall, contributing to the thickening of the peritubular lamina propria (LP) (Paniagua et al., 1991; Haider et al., 1999; Volkmann et al., 2011). Thus, in patients with varicocele and cryptorchidism (Santamaria et al., 1990), Kleinfelter's syndrome (Martin et al., 1992) and Sertoli cell-only syndrome (Pöllänen et al., 1985) LP thickening is apparently due to increased deposition of collagen fibrils in the extracellular space. This thickening may alter the relationship between the germ and Sertoli cell population and the interstitial tissue, affecting hormone permeability and altering the physiological activity of the seminiferous epithelium (Dobashi et al., 2003; Wang et al., 2022). In this phenomenon of extracellular material deposition, both myoid cells and Sertoli cells are thought to be responsible for its synthesis (Sato et al., 2002; Volkmann et al., 2011), which is associated with epithelial depletion, with the presence of portions of seminiferous tubules with arrested spermatogenesis or partial or complete hyalinisation (Haider et al., 1999; Morales et al., 2004; Volkmann et al., 2011). The presence of seminiferous epithelia with arrested spermatogenesis is observed both during postnatal and prepubertal development (Paniagua and Nistal, 1984; Miething, 1993) and in the testicular regression that occurs in many species during seasonal reproduction (Beltrán-Frutos et al., 2022). These epithelial depletions are not irreversible and involve a growth process that affects both the epithelium and the LP, with the role of the Sertoli cell being determinant (Rebourcet et al., 2017; Martínez-Hernández et al., 2020).

In mouse testis, HSP47 was first identified in association with collagen XXVI synthesis during the neonatal period (Sato et al., 2002), suggesting the presence of this chaperone in myoid cells. In addition, a gene expression study in rat Leydig cells during ageing found an overexpression of transcripts of this protein in aged rats with regressed testes compared to non-regressed rats (Syntin et al., 2001). HSP47 was also found in fibroblast cells of vasectomised rats in which there was fibrosis of the testicular interstitium. Its molecular overexpression correlated with a higher degree of such fibrosis (Shiraishi et al., 2003). More recently, a higher percentage of HSP47-positive Leydig cells has been detected immunohistochemically in the testes of Syrian hamsters exposed to a short photoperiod compared to testes with a long photoperiod (Beltrán-Frutos et al., 2016). Bearing in mind that, as we have said, the Sertoli cell is of mesenchymal origin, it is also possible that this cell expresses the HSP47 protein under normal conditions and, in particular, in physiological situations associated with changes in testicular collagen synthesis, such as ageing (irreversible) or testicular regression due to short photoperiod (reversible). Therefore, the aim of this work was to clarify whether HSP47 is expressed in the Sertoli cell under normal conditions and whether it is modified in the aforementioned situations, short photoperiod and testicular ageing.

Materials and methods

Animals and tissue preparation

Eighteen male Syrian hamsters (*Mesocricetus auratus*) were divided into three groups. The first group consisted of six-month-old animals (6), a second group consisted of 24-month-old non-regressed animals (6) and the third group consisted of 6-month-old photoinhibited animals (6). The young and old non-regressed groups were exposed to a long photoperiod of 16h light and 8h dark (16:8 LD). The third group was subjected to a short photoperiod of 8h light and 16h dark (8:16 LD) for 2 months. All animals were sacrificed in a closed chamber with CO_2 in accordance with the regulations concerning the protection of laboratory animals. The samples for this study came from the previously funded project

(GERM 19892/15). The use of the animals was approved by the animal experimentation service of the University of Murcia in accordance with the Spanish Royal Decree, RD53/2013. Each animal was weighed (g) and, after opening the scrotum, the testes were removed and their largest and smallest diameters (mm) were measured. The testes were weighed separately before processing. Most of the left testes and a portion of the right testes were sectioned (4 mm) and fixed with methacarn (methanol: chloroform: acetic acid, 6:3:1) for 8h before processing for light microscopy. After fixation, the samples were embedded in paraffin (Panreac AppliChem) and 5 µm thick sections were cut on a rotary microtome (Leica Biosystems) for subsequent immunohistochemical study by light microscopy.

Immunohistochemical identification of the Heat Shock Protein 47

A 5 min cycle and another 2 min cycle for antigen unmasking buffer (DAKO, sc- 2367) were performed at 95°C in a microwave. Samples were immersed in xylene, hydrated and incubated overnight at 4°C with antiHSP-47 rabbit antibody at 1:100 dilution (Santa Cruz Biotechnology; sc-8352). Samples were then washed in phosphate-buffered saline (PBS) and incubated with biotinylated goat anti-rabbit immunoglobulin (Chemicon International; AP106B) for 45 min at room temperature in a 1:300 PBS/1% BSA solution. Samples were washed in PBS (3x5 min) after incubation with peroxidase-conjugated streptavidin (DAKO; P0397) for 30 min at room temperature (diluting the streptavidin solution in 1:300 PBS/1%BSA). After washing the samples in PBS (3x5 min), the antibody-peroxidase complex was developed using PBS containing 0.05% diamino-benzidine (DAB) (Sigma Aldrich) and 0.3% H_2O_2, for 3 min at 18-24°C. Finally, the sections were washed in fresh water, counterstained with haematoxylin for 10 seconds, dehydrated by ascending grades of alcohol and mounted in dibutyl polystyrene xylene (DPX) (Merck). The specificity of the immunohistochemical techniques was confirmed on sections incubated with non-immune serum instead of being incubated with the primary antibody.

Semi-quantitative analysis of HSP47 expression in seminiferous tubules

In this study, animals belonging to the 24-month-old non-regressed (6 animals) and 6-month-old photo-inhibited (6 animals) groups were used. The first group was exposed to a 16:8 LD photoperiod, while the second group was exposed to a short 8:16 LD photoperiod. In the 24-month-old non-regressed group, 10 cross-sections of normal or hypospermatogenic seminiferous tubules and 10 cross-sections of tubules with arrested spermatogenesis in spermatocytes were selected. In the photoinhibited group, 10 tubular sections with regressed seminiferous epithelium were also selected. A semi-quantitative study of HSP47 immunoreactivity was performed in all tubular sections. For this purpose, the area of immunoreactivity (AIR) to HSP47 and the total area of seminiferous epithelium (ASE) were calculated in each section, and the AIR/ASE ratio was obtained ((AIR/ASE) x100). These areas were determined using an Olympus BX51 light microscope (Olympus, Hicksville, NY, USA) with an Olympus DP 25 camera attached (Olympus, Hicksville, NY, USA). Images were analysed with Cell D image analysis software (Olympus, Hicksville, NY, USA). Prior to the study, the intensity of HSP-47 positivity was determined in the three types of tubular sections to be used as reference for image acquisition.

Statistical analysis

Equality of means was tested by one-way analysis of variance followed by a post hoc test of equality between pairs of means using the least significant difference (LSD) and Bonferroni methods, taking into account the total number of animals and the number of components in each of the groups. Results were considered statistically significant when the p-value was less than 0.05. The statistical software package SPSS 28 (IBM, Madrid, Spain) was used.

Results

Identification of Heat Shock Protein 47 in seminiferous tubules

In young animals, weak expression of HSP47 protein was observed in the cytoplasm and along the cytoplasmic processes of Sertoli cells in tubular sections showing complete spermatogenesis (Fig. 1A). In addition, strong positivity was observed in Leydig cells and occasionally, albeit weakly, in myoid cells of seminiferous tubules.

In aged animals, as observed in previous studies, seminiferous tubules undergo a variable and gradual atrophy along the tube, and tubular sections in different degrees of involution can be observed, from normal or hypospermatogenic to tubular sections in which the epithelium is arrested in spermatocytes or, to a lesser extent, in spermatogonia. Thus, tubular sections in hypospermatogenesis, in which all spermatogenic cells are observed but in smaller proportion compared to normal tubules, showed slightly higher positivity in the cytoplasmic extensions of Sertoli cells than in normal tubules (Fig. 1B). Sections with arrested spermatogenesis showed intense localised positivity in most of the cytoplasm of Sertoli cells. In addition, they showed a flattening of their morphology associated with the absence of germ cells in the seminiferous epithelium (Fig. 1C,D). In young animals exposed to a short photoperiod, photoinhibition caused a complete regression of the seminiferous epithelium. This was

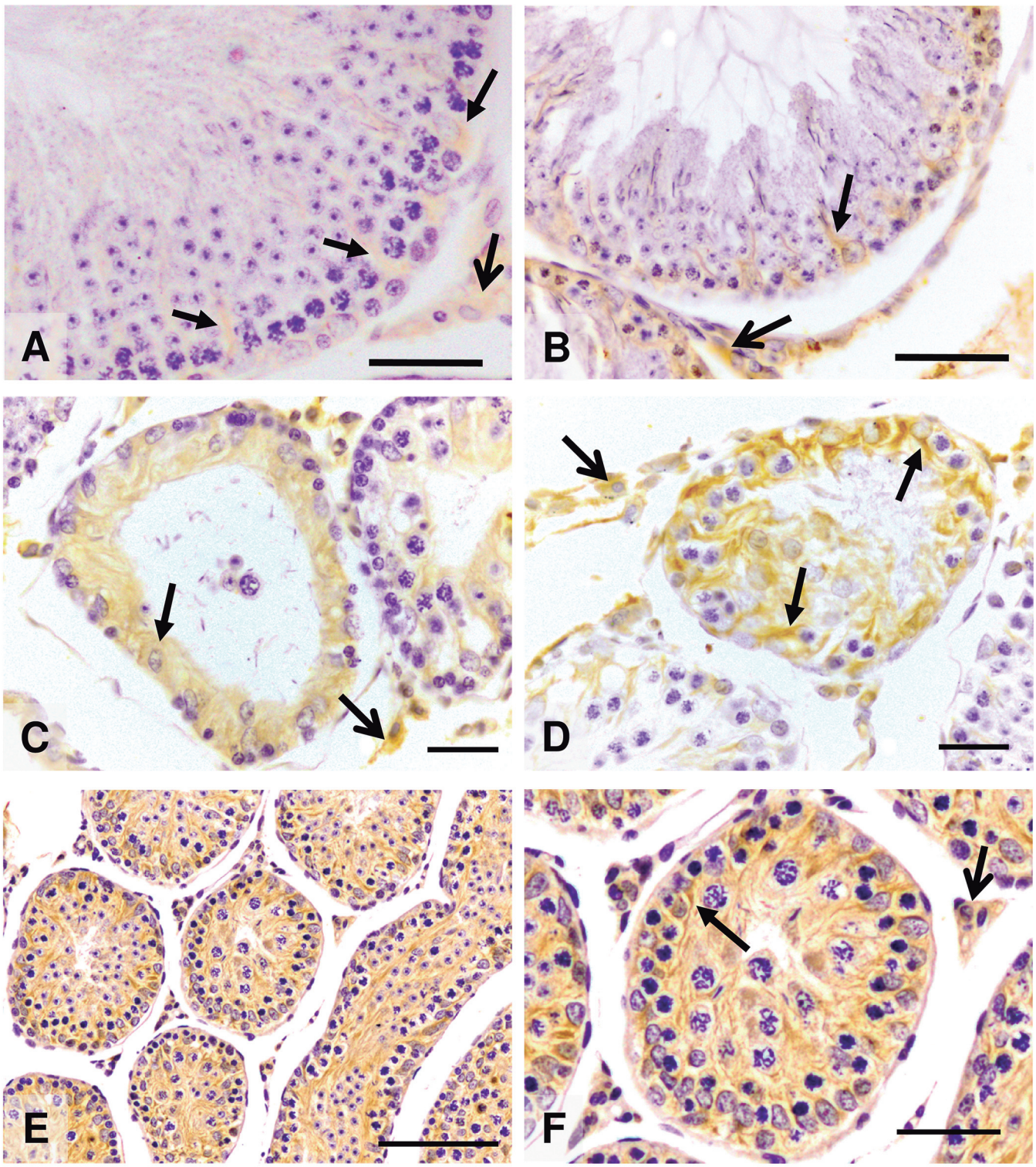

Fig. 1. Immunohistochemistry of HSP47. HSP47 immunoreactivity was observed as brown staining in the cytoplasm of Sertoli cells (closed arrow) and Leydig cells (open arrow). **A.** Section of seminiferous tubules from a 6-month-old animal with complete spermatogenesis in which HSP47 immunoreactivity was very weak in Sertoli cells (closed arrow) but more intense in Leydig cells (open arrow). **B.** Section of a seminiferous tubule from a 24-month-old animal with hypospermatogenesis. The cytoplasm of Sertoli cells (closed arrow) shows increased HSP47 immunoreactivity. **C, D.** In seminiferous tubule sections from a 24-month-old animal with arrested spermatogenesis, Sertoli cells (closed arrow) show strong HSP47 immunoreactivity. **E.** Sections of seminiferous tubules from young animals exposed to a short photoperiod with arrested spermatogenesis. **F.** Enlargement of a seminiferous tubule from image E showing strong cytoplasmic immunoreactivity in Sertoli cells (closed arrow) while immunoreactivity in Leydig cells (open arrow) is maintained. Scale bars: A, B, E, 50 μm; C, D, F, 20 μm.

observed mainly because the tubular sections were in a state of arrested spermatogenesis in both spermatocytes and round spermatids. In both types of tubular sections, the intensity of HSP47 expression was higher than that observed in animals with normal photoperiod and complete spermatogenesis (Fig. 1E-F).

Semi-quantitative analysis of HSP47 expression in seminiferous tubules

Semi-quantitative analysis of HSP47 expression (measured as area of immunoreactivity (AIR)) in Sertoli cells of the seminiferous epithelium between tubule sections of aged animals revealed important differences between the types of tubule sections analysed. On the one hand, it was observed that tubular sections that had undergone complete spermatogenesis (normal and hypospermatogenic tubules) showed significantly weaker AIR ($p<0.05$) of HSP47 compared with tubular sections that were in spermatogenic arrest in the older group. In addition, the latter group showed a significantly higher expression of HSP47 ($p<0.05$) than the young photoinhibited group. (Fig 2 Bars diagram). On the other hand, the AIR/ASE ratio of HSP47 was significantly higher ($p<0.05$) in tubular sections from photoinhibited testes than in tubular sections showing complete spermatogenesis from non-photoinhibited animals, but significantly lower ($p<0.05$) than in tubular sections showing age-related spermatogenesis arrest (Fig. 2 Dotted line).

Discussion

HSP47 protein is a chaperone directly involved in the synthesis and correct folding of collagen fibres. Thus, this chaperone is highly expressed in cells associated with high collagen production, such as fibroblasts, chondroblasts, osteoblasts and odontoblasts (Nagata, 2003). In the testis, its presence has been demonstrated in Leydig cells. Thus, there is a 2.7-fold increase in the gene expression of HSP47 in Leydig cells from aged non-regressed than in aged regressed testes (Syntin et al., 2001. Similarly, in testes regressed after a short photoperiod, immunohisto-chemistry has previously indicated a higher percentage of HSP47-positive Leydig cells, a fact that would be related to the necessary generation of new basement membrane (BM) by these cells during recrudescence (Beltrán-Frutos et al., 2016). Finally, in rats it has been shown that increased interstitial fibrosis is associated with increased HSP47 expression in fibroblasts after vasectomy (Shiraishi et al., 2003). Thus, at present, HSP 47 in the testis is thought to be associated exclusively with Leydig cells and fibroblasts of the intertubular interstitium.

It has long been known from *in vitro* studies that Sertoli cell synthesize components of the BM, such as laminin or collagen IV (Richardson et al., 1995), although the ability of these cells to synthesise collagen I has also been observed (Raychoudhury et al., 1992). In addition, α1(IV) chain was observed in the BM of seminiferous tubules in normal testes, and the BM was thicker in patients with varicocele and Sertoli cell-only syndrome. Overexpression of the α1(IV) chain correlated with abnormal BM thickening and is associated with spermatogenic dysfunction (Dobashi et al., 2003). Recently, it has been observed that the collagen α3(IV) chain, the most abundant type of collagen in the testis BM, can be cleaved, releasing the NC-1 peptide (non-collagenous (NC)1 domain), which affects blood-testis barrier (BTB) permeability by interacting with both f-actin and microtubules (Liu et al., 2021). The detection of the expression of the HSP47 protein in the cytoplasm of Sertoli cells from young animals in this work could be related to the activity of collagen synthesis, especially collagen IV, which is necessary for the maintenance of the BM on which they rest.

It is well known that ageing, an irreversible physiological phenomenon, causes significant changes in the histophysiology of the testes and leads to a decrease

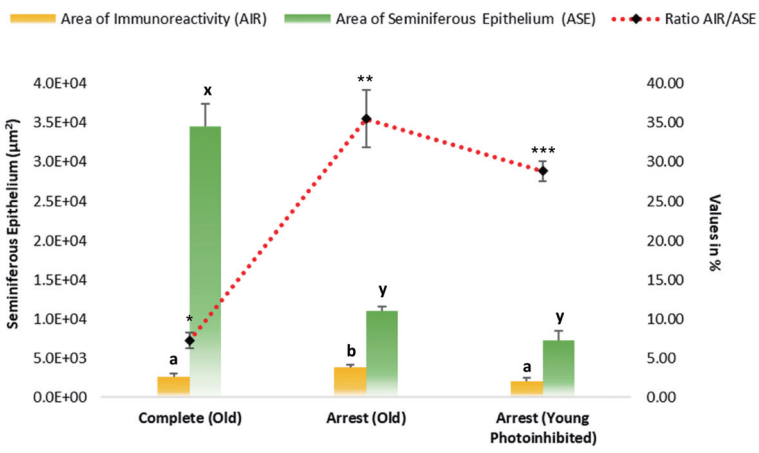

Fig. 2. HSP47 expression in Sertoli cells. The area of immunoreactivity for HSP47 expressed by Sertoli cells and the total area of seminiferous epithelium per tubular section in old hamsters with complete or arrested spermatogenesis and in young photoinhibited animals with tubular sections in spermatogenesis arrest can be observed (bars). a and b Significant differences comparing the area of immunoreactivity (AIR) between the different study groups when $p<0.05$. x and y Significant differences comparing the area of seminiferous epithelium (ASE) between the different study groups when $p<0.05$. Dotted line: Ratio, expressed in %, between the area of HSP47 immunoreactivity and the area of seminiferous epithelium (AIR/ASE) per tubular section. HSP47 expression was significantly stronger in tubular sections with arrested spermatogenesis in both old non-regressed and young photoinhibited groups compared to tubular sections having undergone complete spermatogenesis and in tubular sections of old non-regressed group compared with tubular sections of young photoinhibited group. *Significant differences ($p<0.05$) between groups.

in fertility. Thus, in humans, ageing causes both a shortening of the seminiferous tubules and a thickening of the surrounding lamina propria (LP), as well as a reduction of both germ cells and Sertoli cells (Paniagua et al., 1991; Perheentupa and Huhtaniemi, 2009). The changes in the LP are very similar to those observed in various testicular pathologies, where extracellular matrix accumulation in the LP correlates with increased deterioration of the seminiferous epithelium (Söderström, 1986; Haider et al., 1999; Volkmann et al., 2011). In hamsters, there are age-related changes on morphometric parameters and ultrastructure of seminiferous tubules, as well as in the expression of extracellular matrix proteins in the LP. In fact, a significant decrease in the percentage of normal tubules and an increase in the percentage of hypospermatogenic and arrested maturation tubules was observed (Morales et. al., 2002). The most important ultrastructural changes with ageing were the thickening of the LP, the presence of several abnormalities in the spermiogenesis process, the degeneration of germ cells and the vacuolisation and flattening of Sertoli cells showing abundant lipofucsin droplets and residual bodies. These Sertoli cell alterations were observed ultrastructurally to be more prominent in altered tubules (Morales et al., 2004). Also, decreased proliferative activity in the seminiferous epithelium and increased germinal cell apoptosis were observed. Thus, in ageing testis, a decrease in proliferation and an increase of apoptosis were observed, correlated with an increase in the number of seminiferous tubule sections with a higher histological degree of degenerated seminiferous epithelium (Morales et al., 2003; Bernal-Mañas et al., 2014). In addition, the total volume of laminin and fibronectin immunostaining per testis increased in aged hamsters (Morales et al., 2004), as well as the volume of peritubular connective tissue (Beltrán-Frutos et al., 2016). These histological changes in ageing are similar to those observed in atrophied seminiferous tubules with altered epithelia in various pathological situations (Volkmann et al., 2011). In summary, age-related changes in hamster seminiferous tubules include a thickening of the LP caused by a more extensive connective matrix between the peritubular cells and the BM associated with a significant alteration in Sertoli cell structure. During testicular regression due to a short photoperiod (reversible), no increase in peritubular connective tissue volume was observed, but there was a decrease in intertubular tissue volume, although a loss of the BM of the seminiferous tubules was also observed (Mayerhofer et al., 1989; Beltrán-Frutos et al., 2016). In addition, it has been found that Sertoli cells in fully regressed testes begin to proliferate to replenish their population and the epithelial and tubular volume lost during the regression process (Martínez-Hernández et al., 2018, 2020). Moreover, an increase in the percentage of HSP47-positive Leydig cells was observed in these animals. This has been linked to the restoration process that takes place during recrudescence and, more specifically, to the synthesis of new BM by Leydig cells (Beltrán-Frutos et al., 2016).

The results of this study suggest that HSP47 expression is more intense in tubular sections in Sertoli cells in aged animals where spermatogenesis is arrested than in sections in which spermatogenesis is fully retained. This increase in expression is also observed in tubular sections of testicular regressed animals, although to a lesser extent. As noted above, in the Syrian hamster there is a direct correlation between increased collagen, fibronectin and laminin in the peritubular space and a higher proportion of seminiferous tubule with impaired spermatogenesis. This is accompanied by ultrastructural modifications of the Sertoli cell (Morales et al., 2004). Despite studies in different mammalian species, the mechanism by which an increase in peritubular collagen occurs in ageing and in multiple testicular disorders remains unknown. Along with the possible increase in collagen IV mentioned above, which would modify the BM (Dobashi et al., 2003), a possible mechanism that has been considered for years is the dedifferentiation of myofibroblasts which is responsible for the altered spermatogenesis associated with changes in the LP (Arenas et al., 1997). In addition, evidence has recently been presented that one or two functionally external rings of myofibroblasts should be considered as an active system responsible for the formation of new collagen in the altered LP (Volkmann et al., 2011). Previously, ultrastructural studies have shown a clear association between peritubular tissue, including myoid cells, and tubular fibrosis or hyalinisation. However, intratubular collagen fibres were also found in the cytoplasm of Sertoli cells, suggesting that these cells have probably completely lost their normal function and have transformed into fibrocyte-like somatic cells (Haider et al., 1999). In relation to this modification, Sertoli cells from patients with non-obstructive azoospermia have been observed to show reduced metalloprotease synthesis. This could lead to the deposition of various extracellular matrix proteins in the peritubular space (Aydos et al., 2019), which would favour collagen deposition in the altered LP. In summary, Sertoli cells associated with thickened LP tubules in both ageing and various testicular pathologies show signs of fibroblastic differentiation. Thus, they could collaborate with myoid cells in the synthesis of both proteins and collagen during the process of LP thickening in the seminiferous tubules associated with impaired spermatogenesis. Furthermore, it is possible that this modification of Sertoli cells involves myoid cells that increase their synthesis of extracellular matrix, as this function to myoid cells is regulated by Sertoli cells (O'Donnell et al., 2022). The increased expression of HSP47 in Sertoli cells of tubules with impaired spermatogenesis observed in our study confirms the hypothesis that this protein is closely related to all fibrosis processes in the organism (Bellaye et al., 2021). It also suggests a process of dedifferentiation in Sertoli cells, making their phenotype resemble that of a

mesenchymal cell, with which they share great similarity (Gong et al., 2017; Porubska et al., 2021). The decreased expression of HSP47 by Sertoli cells in the seminiferous epithelium of the regressed testis suggests that, although the expression is higher than in Sertoli cells of normal seminiferous epithelium, the cells remain functional. The increase in this expression is probably related to the generation of new BM, necessary for both the increase in tubular volume and to support the increase in germ cells that will occur during recrudescence.

Conclusions

In conclusion, adult hamster Sertoli cells express HSP47 in their cytoplasm, pointing to a constant activity that is probably related to collagen IV synthesis. The expression of HSP47 increases in Sertoli cells in different ways depending on the atrophy of the seminiferous epithelium. In total testicular regression due to exposure to a short photoperiod (a reversible process), its increase would be related to the recovery of the epithelium and the need to increase BM synthesis during recrudescence. In contrast, in ageing (an irreversible process), the strong expression of HSP 47 in Sertoli cells is associated with portions of the tubules showing epithelial atrophy and a thickened LP, indicating a probable phenotypic change toward a fibroblastic character of this cells.

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Declaration of competing interest. The authors declare no conflicts of interest as regards this paper.

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