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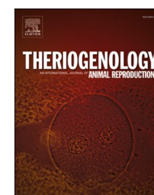

Original Research Article

Testicular expression of heat SHOCK proteins 60, 70, and 90 in cryptorchid horses

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A B S T R A C T

Heat shock proteins are the most evolutionarily conserved protein families induced by stressors including hyperthermia. In the context of pathologies of the male reproductive tract, cryptorchidism is the most common genital defect that compromises the reproductive potential of the male because it induces an increase in intratesticular temperature. In equine species, cryptorchidism affects almost 9 % of newborns and few studies have been carried out on the molecular aspects of the retained testis. In this study, the expression pattern of HSP60, 70, and 90 in abdominal and inguinal testes, in their contralateral descended normally testes, and in testes of normal horses were investigated by Western blot and immunohistochemistry. The histomorphological investigation of retained and scrotal testes was also investigated. The seminiferous epithelium of the retained testes showed a vacuolized appearance and displayed a completely blocked spermatogenesis for lacking meiotic and spermiogenetic cells. On the contrary, the contralateral scrotal testes did not show morphological damage and the seminiferous epithelium displayed all phases of the spermatogenetic cycle as in the normal testes. The morphology of Leydig cells was not affected by the cryptorchid state. Western blot and immunohistochemistry evidenced that equine testis (both scrotal and retained) expresses the three investigated HSPs. More in detail, the Western blot evidenced that HSP70 is the more expressed chaperone and that together with HSP90 it is highly expressed in the retained gonad ($P < 0.05$). The immunohistochemistry revealed the presence of the three HSPs in the spermatogonia of normal and cryptorchid testes. Spermatogonia of retained testes showed the lowest expression of HSP60 and the highest expression of HSP90. Spermatocytes, spermatids of scrotal testes, and the Sertoli cells of retained and scrotal testes did not display HSP60 whereas expressed HSP70 and HSP90. These two proteins were also localized in the nucleus of the premeiotic cells. The Leydig cells displayed the three HSPs with the higher immunostaining of HSP70 and 90 in the cryptorchid testes. The results indicate that the heat stress condition occurring in the cryptorchid testis influences the expression of HSPs.

1. Introduction

Heat shock proteins (HSPs) represent a wide class of chaperones derived from prokaryotic ancestors involved in protecting cellular proteins from stress conditions [1]. While most HSPs are induced by different stressors (oxidative stress, inflammation, hyperthermia, hypoxia) some of them are constitutively expressed and participate in complex processes in living cells including cytoplasmic protein synthesis, transport, modification, and secretion [2,3]. HSPs, which are among the most conserved protein families in evolution [4], were classified into families of different molecular sizes and the most studied, such as HSP60, 70, and 90, were identified in a wide number of tissues of eukaryotic and prokaryotic organisms [5].

The cellular activities of HSP60 are summarized in post-translational modifications of new proteins, patching up of damaged ones, and removal of excess peptides [6]. The HSP70 family exerts a primary role in cellular survival and tolerance to stress conditions but they also cooperate in the control of homeostasis in normal conditions and promote the correct folding of newly synthesized proteins [7]. Finally, the HSP90 family mainly regulates the activity of kinases, the correct folding of proteins in stress conditions, and the removal of damaged ones and as for HSP70, they are involved in stress tolerance [8].

Regarding the testicle, several evidences showed that HSPs play crucial roles in germ cell development and can be associated with male

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infertility [9].

HSP60 has been found and immunolocalized in spermatogonia, spermatocytes, spermatids, and spermatozoa of several mammals [10–19], as well as in testicular somatic cells (Sertoli and Leydig cells) [10–14,16,19].

HSP70 has also been immunolocalized in germ cells [14,16,20–23], in Sertoli [16,19], and in Leydig cells [14,19].

HSP90 has been observed in spermatogonia, spermatocytes [9,14,19,24,25], spermatids [14,16,19,24,25], Sertoli cells [9,13,24], and Leydig cells [9,13,14].

In the context of pathologies of the male reproductive tract, cryptorchidism is the most common male genital defect that compromises the reproductive potential of the subject. In equine, cryptorchidism affects almost 9 % of newborn males. Fifteen percent of the cryptorchidism-affected horses show internal localization of both testes, while in the remaining subjects, the left testis is retained more frequently due to its bigger dimension [26]. The reproductive activity of cryptorchid animals is potentially compromised because it induces an increase in intratesticular temperature. It is well known that normal testicular function is temperature-dependent, and in most mammals, the testes are maintained between 2 and 8 °C below the core body temperature by the exteriorization of the testes in the scrotum [27]. Slight increases in testicular temperature can impair spermatogenesis and induce its inhibition as well as predispose to the onset of testicular tumors [28,29]. As in other mammals, also in the horse, cryptorchidism impairs spermatogenesis [30] and predisposes to tumor development [31,32].

Several studies have demonstrated the relationship between the expression of HSPs and male fertility [9,10,22,33–35]. As for the influence of body temperature on the production of HSPs, it has been demonstrated that experimental heat stress increases the expression of HSPs in mouse [24,36,37] and rat [13,25,38] testis. The increase of HSPs under heat shock conditions has been considered a protective mechanism against cell impairment determined by a rise in the environmental temperature [24].

Currently, no reports have been published on the localization of HSPs in the "natural" cryptorchid testis of mammals. Thus, the current study was designed to determine the expression and localization of HSP60, 70, and 90 in the horse testis and investigate any difference between the scrotal and retained testes of horses. This species besides having an economic role as a farm animal has a social role as a companion and therapeutic animal.

2. Material and methods

All chemicals were purchased from Sigma-Aldrich (Milano, Italy) unless otherwise stated.

2.1. Animals

In this study, 12 unilateral cryptorchid horses (age: 2–8 years) were enrolled. Eight subjects showed abdominal (side of occurrence: left n = 4 and right n = 4) testicular retention while the remaining four horses showed inguinal (left n = 2 and right n = 2) retention. The enrolled subjects arrived at the Veterinary hospital (Section of Veterinary Clinics and Animal Production) of the University of Bari -Aldo Moro, for castration as a clinical intervention, so no license from the Departmental Bioethics Commission was necessary; anyway, written consent for the surgical procedure was released from each horse owner. Moreover, testes from three normal subjects (N) were obtained from the local abattoir.

2.2. Surgical procedure for castration

Under standing sedation, five abdominal testes (AC) were surgically removed by flank laparoscopic surgery. Under general anesthesia, the other three abdominal testes were removed via a paramedian mini-laparotomy. Under general anesthesia, the inguinal testes (IC) were removed via a scrotal approach. The scrotal approach was used to remove all the scrotal testes (AS was used to indicate the scrotal testis of the abdominal cryptorchid subjects and IS to indicate the scrotal testis of the inguinal cryptorchid subjects). After surgery, all the testes were immediately processed to obtain small fragments that were frozen in liquid nitrogen to be used for molecular analysis; while the remaining tissue was processed for histological investigations.

2.3. HSP expression

2.3.1. Western blot

Testis was cut along the sagittal plane and pieces (3–5 mm) from the inner testicular tissue were removed and pulverized in liquid nitrogen, and then homogenized in cold lysis buffer (1 *x* PBS, 0.1 % Triton-X, 50 μl/ml protease inhibitor cocktail (Santa Cruz Biotechnology, Inc. USA). After centrifugation for 30 min at 10,000×*g* total proteins were recovered in the supernatant and their concentration was determined by the Protein Assay kit (Bio-Rad, Milano, Italy). For each subject, 40 μg of the protein extract was loaded into a precast 4–20 % polyacrylamide gel (Criterion, Bio-Rad, Milano, Italy), electrophoresed under constant voltage and transferred to a PVDF membrane (Millipore, Milano, Italy) by the Trans-Blot®SD Semi-Dry Transfer cell (Bio-Rad, Milano, Italy) as previously described [39].

The membrane was hybridized to 1 μg of each primary anti-human HSP60 mouse monoclonal antibody (sc-59567), anti-human HSP70 mouse monoclonal antibody (sc-7298) (Santa Cruz Biotechnology Inc.), and anti-human HSP90 mouse monoclonal antibody (SMC-107) (StressMarq Biosciences Inc. Victoria, Canada). The antibodies were dissolved in a blocking buffer (0.1 % Tween-20 in PBS, diluted 1:600). For hybridization, the Snap i. d. System (Merck-Millipore, Germany) was used, while the immunocomplex was revealed by the Vectastain Elite ABC kit (Vector Laboratories Inc. Burlingame, CA, USA). For signal normalization and loading controls, membranes were also hybridized to anti beta-actin antibody diluted 1:1500 (A2103), (SIGMA-Aldrich, USA). Except for the anti-human HSP70 antibody, whose use in the horse was recommended by the company, for the remaining primary antibodies raised against proteins of human origin, we checked a theoretical identity between human and the equine gene sequence aligned by the BLAST software from NCBI. We found 93 % identity between the human and equine sequences for both chaperones (HSP60: sequence accession number: NM 002156-human and XM 023622284-horse; HSP90: AF275719-human and AB043676-horse). Moreover, the specificity of the obtained signals was verified by performing the following negative controls: 1) hybridizing the membrane with a solution containing the primary antibodies of the three HSPs let to react with a molar excess of their control peptides (HSP60 control peptide sc-13115P; HSP70 control peptide sc-7298P; HSP90 control peptide SPR102A) and the anti beta-actin primary antibody; 2) omitting the primary antibodies. The optical density of each protein band was quantified using Quantity One software (BioRad, Milano, Italy). The values, expressed as arbitrary units (A.U.), are presented as the ratio of the specific protein optical density to the corresponding beta-actin.

2.3.2. Histology

2.3.2.1. Tissue preparation. Tissue fragments (1 cm thick) were obtained from the abdominal, inguinal, and scrotal testis of cryptorchid horses, and testes of normal horses and fixed in 4 % (w/v) phosphate-buffered (PBS) paraformaldehyde or in Bouin's fixative at room temperature for 24 h. Then the specimens were dehydrated in an ascending ethanol series, cleared in xylene, and embedded in paraffin wax. Serial sections (5 μm thick) were cut and, after de-waxing with xylene and hydration in an ethanol series of descending concentrations, were stained with hematoxylin-eosin for morphological analysis and by

immunohistochemistry to localize the presence of HSPs.

2.3.2.2. Immunohistochemistry. De-waxed and re-hydrated tissue sections were incubated for 15 min in methanol containing 3 % H_2O_2 to inhibit endogenous peroxidase activity. After washing in PBS, non-specific binding sites were blocked by incubation in normal horse serum (NHS) (Vector-Laboratories, Burlingame, CA, USA) for 30 min at r. t. Sections were incubated overnight at 4 °C in a moist chamber with mouse anti-human HSP60, 70, and 90 monoclonal primary antibodies, the same used for the Western blot, diluted 1:200 in PBS containing 2.5 % NHS. After a washing step with PBS for 15 min, the biotinylated universal antibody (Vector-Laboratories, Burlingame, CA, USA) was added for 30 min at r. t., followed, by a new 15 min PBS wash, by 30 min incubation with Vectastain Elite ABC Reagent (Vector-Laboratories, Burlingame, CA, USA). After washing in PBS, staining was visualized by incubating the sections in DAB solution (Vector-Laboratories, Burlingame, CA, USA). Then the sections were dehydrated and mounted. The negative control was performed by replacing the primary antibodies with NHS in PBS or normal mouse serum. Under this condition, staining was abolished.

2.4. Statistical analysis

Obtained quantitative HSP values were assessed by the Kolmogorov-Smirnov test to verify the normal distribution of data. Even if normally distributed, data were analyzed by non-parametric Kruskal Wallis and U-Mann-Whitney tests, when appropriate, due to the small number of subjects. P values were two-tailed and $P \leq 0.05$ was considered significant. All values were expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Western blot and densitometric analysis

The results of the Western blot analysis revealed that the equine testis expresses all three HSPs investigated (Fig. 1, panel A). Comparing the retained and the scrotal testis, we found a statistically significant higher expression of both HSP90 and 70 in the retained gonad ($P < 0.05$; Fig. 1, panel B). HSP70 is the chaperonine expressed at a higher level in the retained testis compared to HSP90 ($P < 0.01$). HSP70 shows also the highest expression level compared to the other HSPs in the scrotal testis of the cryptorchid subjects (HSP70 *vs* HSP90 $P < 0.01$; HSP70 *vs* HSP60 $P < 0.05$). In the testis of the normal subjects, HSP90 shows the lowest expression level (HSP90 *vs* HSP70, $P = 0.05$; HSP90 *vs* HSP60 $P = 0.05$).

The expression of all three HSPs is significantly higher in the testis retained in the inguinal canal compared to the contralateral scrotal testis (HSP90, HSP70, HSP60, IC *vs* IS; $P < 0.05$; Fig. 1, panel C) and to the gonad of the normal subject (HSP90, HSP70, HSP60, IC *vs* N; $P < 0.05$). Moreover, the testis of the normal horse expresses the HSPs at a lower level when compared to the scrotal testis of the cryptorchid subjects (HSP90, HSP70, IS *vs* N; $P < 0.05$).

3.2. Histology

No microscopic lesions compatible with testicular neoplasia were observed in the scrotal or retained testes (Fig. 2A–E). The seminiferous tubules of the cryptorchid testes showed an altered morphology compared to the contralateral scrotal testes. The seminiferous epithelium of all retained testes showed a vacuolized appearance and displayed a completely blocked spermatogenesis for the presence of spermatogonial cells (Fig. 2A and B). On the contrary, the contralateral scrotal testes did not show morphological damages and their seminiferous epithelium displayed all phases of the spermatogenetic cycle (Fig. 2C and D) as in the normal testes (Fig. 2 E).

3.3. HSPs immunohistochemical localization

The immunolocalization of the HSP 60, 70, and 90 is reported in Figs. 3–5.

HSP60 positivity was detected in the cytoplasm of spermatogonia

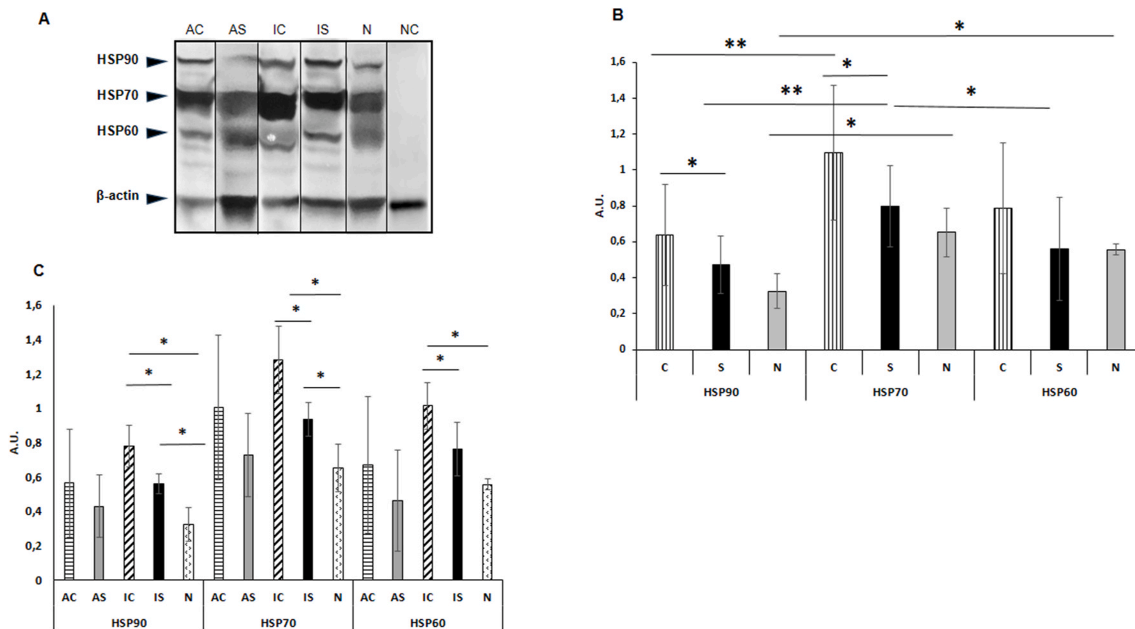

Fig. 1. The figure shows the Western blot (panel A) and the densitometric analysis of the HSP60, 70, and 90 expression pattern (panels B and C). Panel A: A representative image of three separate experiments shows the signals obtained hybridizing the electrophoresed proteins from equine testis to primary antibodies against HSP60, 70, 90. AC, IC = proteins extracted from testes retained in the abdomen and the inguinal canal respectively; AS, IS = proteins from the corresponding contralateral scrotal testis; N = proteins from normal horses; NC = negative control. Panel B: The bar graph shows for each HSP, the densitometric values obtained in cryptorchids and in normal horses. C, S = retained and scrotal testis of cryptorchid subjects; N = testis of normal horses. Panel C: as in panel B data were expressed by separating subjects with abdominal retention from those with inguinal retention. * = $P \leq 0.05$, **$P \leq 0.01$; A.U. = arbitrary units.

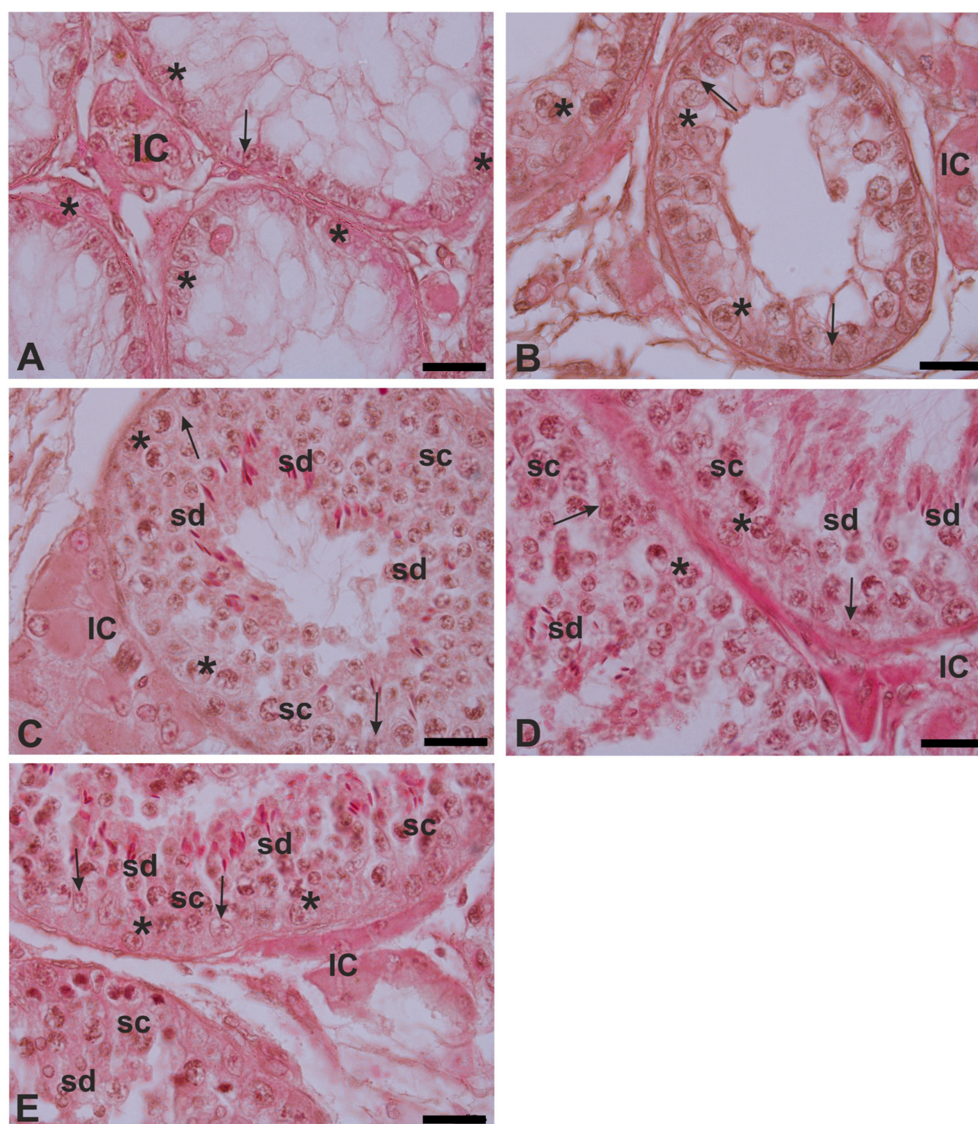

Fig. 2. Light micrographs of the abdominal (A) and inguinal (B) retained testis (B), contralateral scrotal testis of the abdominal (C) and inguinal (D) cryptorchid horses, and normal (E). IC, interstitial (Leydig) cells; sc, spermatocytes; sd, spermatids; arrow, Sertoli cells; asterisk, spermatogonium. Hematoxylin-eosin staining. Bar: 20 μm.

and in the interstitial cells (Leydig cells) of all investigated animals (Fig. 3A and B, D, E). The immunolabelled spermatogonia were low in number in the cryptorchid testes compared to the scrotal testes. Leydig cells of abdominal retained testes displayed a weaker HSP60 immunostaining when compared with the other samples. The immunoreactivity was absent in the negative controls (Fig. 3C–F).

HSP70 was localized in the cytoplasm of the Sertoli cells and the cytoplasm and nucleus of germinal cells constituting the seminiferous epithelium of both abdominal and inguinal retained testes (Fig. 4A and B). As for the seminiferous epithelium of the scrotal testes from the cryptorchid and normal animals, this protein was also expressed in the cytoplasm and nucleus of spermatogonia and primary spermatocytes as well as the cytoplasm of the other spermatogenetic cells, including elongated spermatids, and the apical cytoplasm of the Sertoli cells (Fig. 4D and E). The Leydig cells displayed HSP70 in all the retained and the scrotal testes, with the strongest immunostaining in the cryptorchid testes (in Fig. 4 compare pictures A, B with D, E). No presence of HSP70 was observed in the negative control procedures (Fig. 4C–F).

HSP90 immunoreactivity was detected in the cytoplasm and nucleus of all the germinal cells and the Sertoli cell cytoplasm constituting the seminiferous epithelium of the abdominal and inguinal retained testes (Fig. 5A and B). The scrotal testes from cryptorchid and normal horses displayed the presence of HSP90 in the cytoplasm and nucleus of spermatogonia and primary spermatocytes as well as in the cytoplasm of the other germinal cells and the Sertoli cells (Fig. 5D and E). Lastly, positive staining for HSP90 was observed in the Leydig cell cytoplasm of all retained and scrotal testes (Fig. 5A and B, D, E). No immunostaining signal was observed in the negative controls (Fig. 5C–F).

4. Discussion

This study demonstrates, for the first time, that HSP 60,70, and 90 are constitutively present in the equine testis and that cryptorchidism modifies their expression patterns. To the best of our knowledge, no report has yet been published on this issue in the naturally retained testis of other mammals.

The morphological analysis confirmed that the seminiferous tubules of the retained testicles present degenerative alterations and agree with previous reports in cryptorchid stallions [40].

It has been demonstrated that the heat stress occurring during the

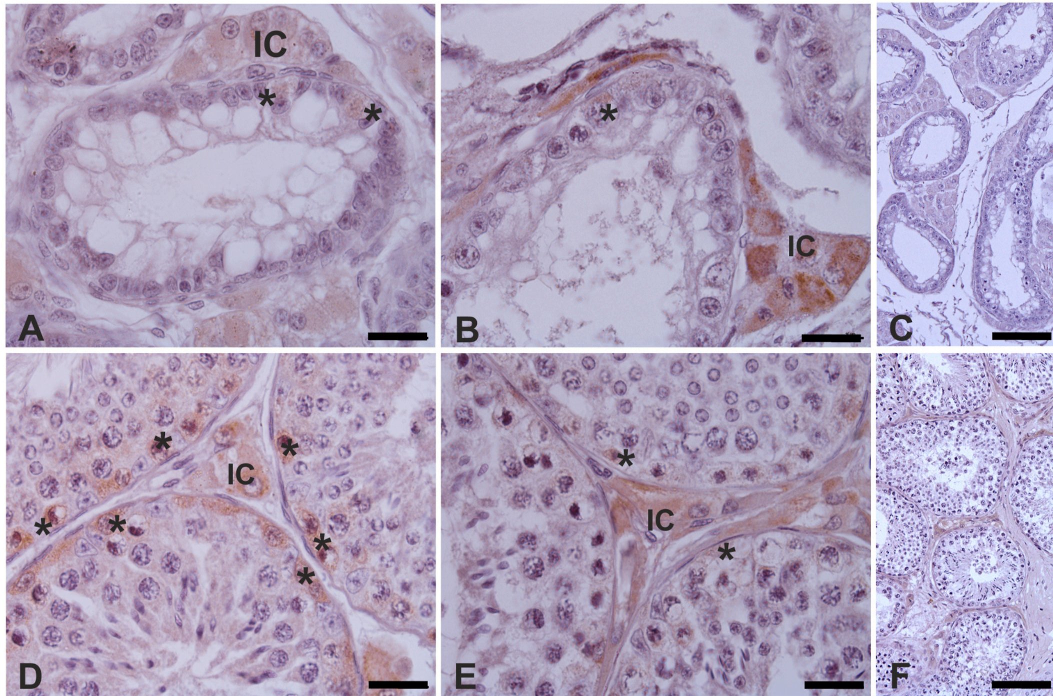

Fig. 3. Localization of the HSP60 in the abdominal retained testes (A), inguinal retained testes (B), in scrotal testis from the abdominal/inguinal (D) and normal (control) stallion (E). C, and F display the absence of immunoreactivity in negative controls in retained (C) and scrotal (F) testes. IC, interstitial (Leydig) cells; asterisk, spermatogonium. Bar: A, B, D, E, 20 μm; C, F, 100 μm.

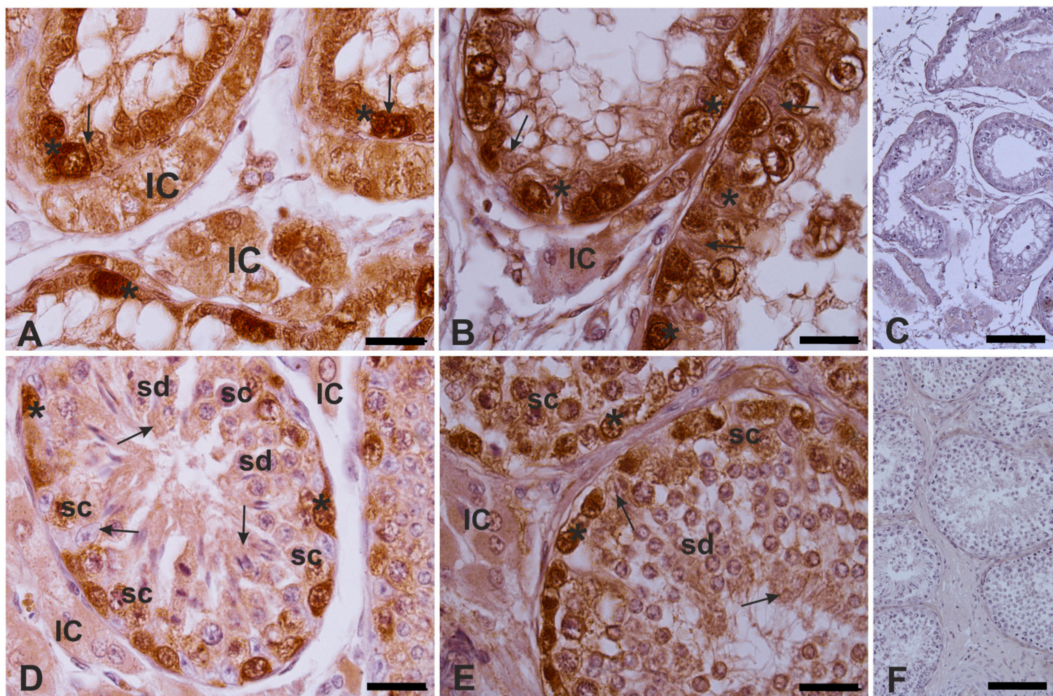

Fig. 4. Localization of the HSP70 in the abdominal retained testes (A), inguinal retained testes (B), in scrotal testis from the abdominal/inguinal (D) and normal (control) stallion (E). C, and F display the absence of immunoreactivity in negative controls in retained (C) and scrotal (F) testes. IC, interstitial (Leydig) cells; sc, spermatocytes; sd, spermatids; arrow, Sertoli cells; asterisk, spermatogonium. Bar: A, B, D, E, 20 μm; C, F, 100 μm.

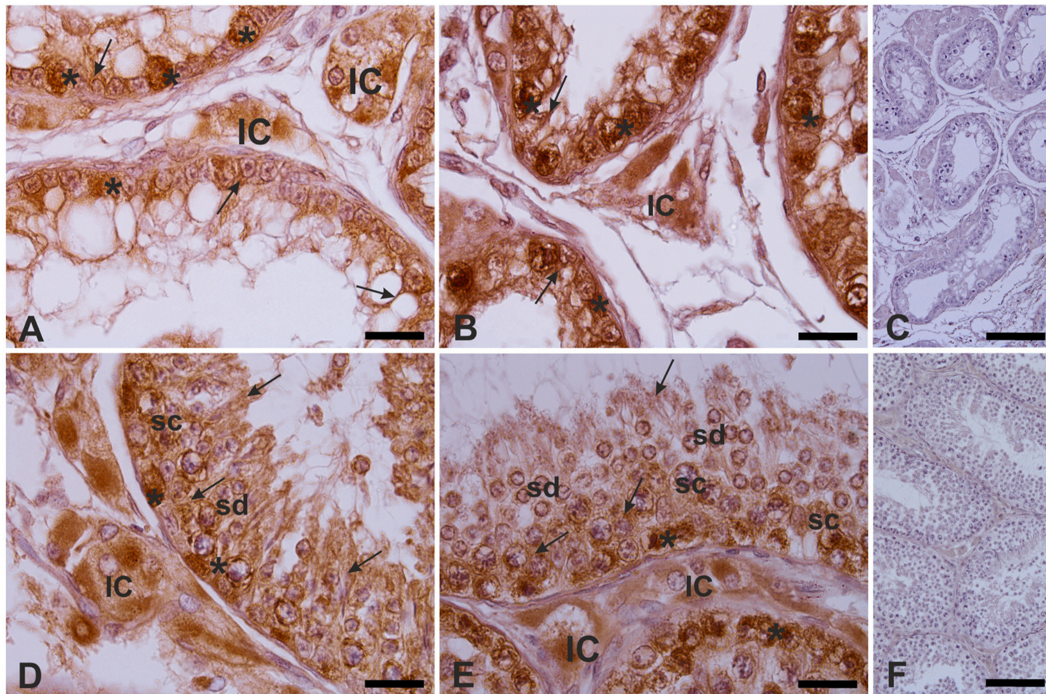

Fig. 5. Localization of the HSP90 in the abdominal retained testes (A), inguinal retained testes (B), in scrotal testis from the abdominal/inguinal (D) and normal (control) stallion (E). C and F display the absence of immunoreactivity in negative controls in retained (C) and scrotal (F) testes. IC, interstitial (Leydig) cells; sc, spermatocytes; sd, spermatids; arrow, Sertoli cells; asterisk, spermatogonium. Bar: A, B, D, E, 20 μm; C, F, 100 μm.

experimental cryptorchidism can cause an increase in some HSPs such as HSP60, 70, and 90 [13,24]. Similarly, in vitro studies have evidenced that rats' spermatocytes produced HSP70 in response to heat stress (exposition to a temperature of 44 °C for as little as 10 min) [22].

Based on the above-cited reports, this study was designed to determine the presence and localization of the previously mentioned three HSPs in the horse testis and to investigate any difference in the distribution pattern between the normal and cryptorchid stallions. In this species, the HSPs expression has so far only been reported in spermatozoa of healthy animals [15,41,42].

The molecular results demonstrated that the retained gonad expresses the three investigated HSPs at the highest level when compared to the contralateral scrotal testis and to the scrotal gonad of the normal subject (Fig. 1, panel B). Moreover, in the inguinal retention, the testicular content of each HSP is significantly higher ($P < 0.05$) than that of the normal testis (Fig. 1, panel C), it is also higher but not statistically significant, because of the high SD values recorded, than that of the abdominal testis. These findings could be the result of the different temperatures to which the testis is exposed based on its position. In mammalian testicular thermoregulation, the arterio-venous countercurrent heat exchanger is the key element that allows cooling of the blood that supplies the scrotal testicle. The more internally the testicle is located, the lower the cooling efficiency will be. It should be also considered that, based on the thermofluid-dynamic law, the increased pressure acting on the inguinal retained testis, also elevates the isobar expansion and isothermal compressibility coefficients that in turn elevates the temperature of the blood volume circulating in the testis. Moreover, early studies demonstrated that in testicondid mammals abdominal temperature does not affect spermatogenesis while in scrotal mammals it is totally inhibited and in naturally cryptorchid testicondid mammals the inguinal testis displays an intermediate response [43]. The suppression of the testicular function, in scrotal and testicondid mammals, is proportional to the temperature rise imposed experimentally, in fact in guinea pigs and rabbits if the deep-body/testis temperature differential is removed, spermatogenesis is blocked and the testis loses about 90 and 68 % of the weight respectively [43].

Lower levels of HSP expression in the abdominal retained testis compared to the inguinal and its controlateral scrotal gonad may be explained considering that the inner retained testicle could develop a kind of "heat stress tolerance condition". We hypothesize that abdominal retention compared to inguinal retention represents a more difficult condition for heat dissipation, which compromises the tissue structure more and unbalances the protective role of the HSPs system, which in turn adjusts its expression to lower levels compared to those observed in the inguinal retention. This idea is supported by evidence of decreased HSP70 gene expression in aged rats subjected to prolonged hyperthermia and children affected by varicocele respectively [44,45].

In contrast, the regulation of HSPs expression in the inguinal testis is under the effect of the continuous balance between the increase in temperature due to the mechanical pressure caused by the constriction of the inguinal canal and the more effective heat dissipation due to the more superficial localization of the testis, which prevents the "heat stress tolerance condition" of the abdominal testis from developing. Studies on humans showed that the contralateral normally descended testis of cryptorchid children displays less severe and less progressive testicular alterations than that of the retained testis [46]. This could explain the differences observed between the expression pattern of the retained testis and its contralateral gonad (Fig. 1, panel C, IC *vs* IS, $P < 0.05$). The evidenced high HSP70 expression level in the retained testis well correlates to the reported anti-tumoural immunity activity generated when intracellular HSP70 levels are increased by forced overexpression as happens in cancer [47,48]. HSP70, normally localized in the cytoplasm or nucleus, can be released from cells, thus functioning as an inter-cellular signaling ligand to activate the immune system [49]. On the other hand, one of the possible side effects of cryptorchidism is to develop testicular cancer and the overexpression of the HSP70 could be a testicular attempt to protect itself from carcinogenic initiation.

HSP60 is mainly expressed in spermatogonia and primary

spermatocytes and its expression is reduced in infertile men [10]. Therefore, it is not surprising that the heat stress condition does not significantly increase (Fig. 1, panel B, C) the level of this chaperonin in the cryptorchid testis where spermatogenesis is reduced or abolished.

HSP90 showed the same expression pattern as HSP60 in the investigated testes even if its increase appeared not statistically significant. It has been demonstrated that HSP90 is mainly involved in chromatin condensation which may be involved in the termination of several transcriptional activities including the steroid receptors [50].

The immunohistochemical results revealed the presence of HSPs 60, 70, and 90 in stallion normal and cryptorchid testis albeit with a different expression pattern in the spermatogenic and the somatic cells (Sertoli and Leydig cells).

In the seminiferous epithelium of all investigated stallion testes only the spermatogonia revealed HSP60 immunolabelling. The other spermatogenic cells (spermatocytes and spermatids) and the Sertoli cells were negative for HSP60. The same immunostaining pattern has also been observed in normal testes of rats [12], mice [51], rabbits [16], and men [10,11]. The spermatogonia represents the spermatogenetic germ cells where mitosis occurs. Thus, spermatogonial HSP60 could have a role in ensuring the renewal of the germ cell population in the seminiferous epithelium. The expression of HSP60 has been detected in the mitochondria of the rat spermatogonia [12]. It has been proposed that the disappearance of HSP60 from spermatocytes onward could depend on several proteases located in the mitochondrial matrix [52–54].

In the present study, the staining intensity and number of HSP60-immunoreactive spermatogonia were low in the cryptorchid testes compared to the scrotal testes. A decreased expression of HSP60 in spermatogonia has been reported in the testis of infertile men [10]. Therefore, our findings suggest that a decreased presence of HSP60-positive spermatogonia is correlated with the decline of spermatogenic efficiency which arrests the spermatogenesis in the cryptorchid testes.

The HSP70 family is one of the HSPs that are most abundantly expressed in the testis [55]. The scrotal testes from normal and cryptorchid stallions exhibited HSP70 immunopositivity in the cytoplasm and nucleus of spermatogonia and primary spermatocytes, in the cytoplasm of the other spermatogenetic cells, including elongated spermatids. These findings agree with the view that the expression of HSP70 is important in the development of the male germ cell lineage [56]. HSP70 is mainly localized in the cytoplasm, mitochondria, and endoplasmic reticulum [35]. The presence of HSP70 in the cytoplasm and the nucleus of spermatogonia and spermatocytes reveals its importance in the initial stages of spermatogenesis. Kotoglou et al. [57] suggested that HSP70 translocates to the nucleus and nucleoli regulating the nucleoplasmic and nucleolar integrity.

Interestingly, the expression of this protein is conserved in the head of stallion-ejaculated spermatozoa, which exhibits HSP70 positive signals in the post-acrosomal region [15]. As for equines, the presence of HSP70 has been detected in the cytoplasm of all spermatogenetic cells (from spermatogonia to elongated spermatids) in porcine [14], rabbit [16], and cat [19] testis. However, in the testis of mice [20,22]), rats [13,38], and men [33] HSP70 is expressed in spermatocytes and spermatids but not in spermatogonia.

The investigated abdominal and inguinal retained testes displayed HSP70 in the spermatogonia. The absence of the other spermatogenetic cells in these testes could depend on the level of HSP70. It has been reported that the decrease in HSP70 level is correlated with the degeneration of the spermatocytes and spermatids in the cryptorchid testis of rats [25]. The expression of HSP70 can be induced in response to heat shock in isolated germ cells [22].

HSP90 immunolabelling was observed in the cytoplasm and nucleus of spermatogonia and primary spermatocytes as well as in the cytoplasm of spermatids and Sertoli cells of the seminiferous epithelium of all scrotal testes. This chaperonin seems to be highly conserved in germ cells of stallion testis as HSP90 immunoreactivity has been observed in the ejaculated spermatozoa of this species [15]. The importance of HSP90 in the spermatogenetic process is confirmed by the presence of this protein from the spermatogonia to spermatids in mouse [24], boar [14], rat [13], and cat [19] testes. Functionally, HSP90 is related to steroid hormone receptors [58,59]. As for the expression of HSP90 in germ cells (spermatogonia) of cryptorchid stallions, the immunostaining procedure revealed a slightly increased presence compared to scrotal testes. A marked expression of HSP90 has also been reported in germ cells after heat stress in mouse [24] and rat [13] testes and in men with spermatogenic arrest [9,34]. Liu et al. [34] suggested that the high expression of HSP90 alpha may lead to a dramatic decrease in androgen receptor (AR) stability and an increase in AR ubiquitinylation.

This study revealed that stallion Sertoli cells contain HSP70 and HSP90 but not the HSP60. The absence of HSP60 has also been reported in the Sertoli cells of boars [25] and cattle [18]. However, it must be highlighted the existence of an interspecific difference in the HSP60 expression in the Sertoli cells since the immunoreactivity of HSP60 has been detected in the Sertoli cells of rats [12], rabbits [16], humans [10, 11], and cats [19].

As for the HSP70, it was detected in the apical cytoplasm of the Sertoli cells of scrotal testes from normal animals and in the entire cytoplasm of both abdominal and inguinal retained testes. Among the investigated mammalian species, HSP70 has also been immunolocalized in the Sertoli cells of boars [60], cats [19], mice [61], rabbits [16], and rats [13]. In the present study, overexpression of HSP70 in the Sertoli cells of the cryptorchid testes was observed and supports the increase found by the Western blot procedure. This finding could be a sign of resistance to the deleterious effect of overheating [23], although their morphology is strongly affected in the cryptorchid testes. The presence of HSP70 in the apical cytoplasm of scrotal Sertoli cells suggests a possible role for this protein in the interaction between Sertoli cells and spermatids during spermiogenesis.

All stallion-investigated testes displayed the presence of HSP90 in the Sertoli cells. This protein has also been localized in the Sertoli cells of mice [24], rats [13], and humans [9]. The abdominal and inguinal retained testis evidenced a slight increase of HSP90 immunostaining, suggesting that the high temperature could up-regulate the expression of this chaperonin. Although our findings agree with the reports on the effects of heat stress in the mouse Sertoli cells [24], it should be noted that the heat stress induced by the experimental cryptorchidism did not affect the expression of HSP90 in the rat Sertoli cells [13].

Leydig cells of all investigated testes displayed the presence of the HSP60, 70, and 90. The present study revealed that the Leydig cells of abdominal retained testes express a lower presence (reduced immunostaining signal) of HSP60 when compared with inguinal and scrotal testes, whose staining intensity did not appreciably vary. The reasons for this result are unclear. Probably, the heat stress affects much more the Leydig cells of the abdominal testes than the inguinal ones. It should be noted that HSP60 was not found in the testis of senile rabbits [16]. However, in experimental cryptorchidism in which one testis of fifty-day-old rats was displaced into the abdomen, the immunoreactivity of HSP60 in experimental rat Leydig cells did not differ compared to normal rats [13]. It has been reported that the expression level of HSP60 in Leydig cells is upregulated by hCG in the testis of macaque monkeys [62].

As regards HSP70, this protein was found in the Leydig cells of all stallion testes. A higher expression of HSP70 was detected in the Leydig cells of cryptorchid testes compared to the scrotal testes. This finding is in line with the Western blot results. Similar results have been observed in mice in which the expression of HSP70 increased after heat shock exposure [63,64]. These findings could agree with the proposed protective role of HSP70 against DNA damage [57]. However, studies are required to clarify this issue in the Leydig cells. The lack of HSP70 has been reported for the Leydig cells of normal and unilateral induced cryptorchidism in rhesus monkeys [65]. Long-term exposure or higher temperatures may result in generalized damage to many different germ

cell types, but Leydig cells and androgen secretion do not appear to be directly affected [66].

Leydig cells of all stallion-analyzed testes displayed HSP90. HSP90 immunoreactivity was slightly increased in cryptorchid testes compared to scrotal testes. This finding could agree with the view that Leydig cells are considered resistant to heat stress [63]. Although long-term exposure to high temperatures may result in generalized damage to many different germ cell types, Leydig cells and androgen secretion do not appear to be directly affected [66]. The thermoresistance of these cells could be explained if one considers that the temperature of the abdominal and inguinal testes is 37 °C, which should be the same as the mother's temperature during the development of the testis [63].

5. Conclusions

This study for the first time describes the expression and the distribution of HSP60, 70, and 90 in the equine cryptorchid testis. The higher temperature acting on the retained gonads compared to the scrotal ones influences the expression of HSPs, as well as their localization in the heterogeneous cell population of the testis. Although this research was carried out on horses, the results provide useful data on the expression of HSPs in the most common birth defect such as cryptorchidism.

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CRediT authorship contribution statement

Mario Cinone: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Maria Albrizio:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Conceptualization. **Antonio Ciro Guaricci:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Luca Lacitignola:** Resources, Methodology, Investigation. **Salvatore Desantis:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declarations of competing interest

The authors declare that no competing interests in this paper.

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