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RESEARCH ARTICLE

Molecular Responses of HSP60 and HSP90 to Temperature Variations in Stallion Testes during Breeding and Non-breeding Seasons

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ABSTRACT

Heat shock proteins (HSPs) are a group of molecular chaperones that are crucial for the proper folding and assembly of proteins and the protection of somatic and germ cells from damage caused by temperature changes. However, the localization and seasonal variation patterns of HSPs in stallions' testes remain unclear. Thus, we investigated the expression and seasonal variations in the localization of HSP60 and HSP90 in the testes of stallions. Testes from nine Thoroughbred stallions collected following castration during the non-breeding season (NBS) and two different environmental temperature conditions (normal temperature, NT; March–May and hot temperature, HT; June–August) during the breeding season (BS) were used. Testis tissues were subjected to Reverse transcription-quantitative polymerase chain reaction, Western Blot (WB) analysis and immunofluorescence (IF) assay. The results revealed that the relative abundance of HSP60 and HSP90 mRNA transcripts was significantly upregulated under NT and HT conditions during BS compared with that during NBS conditions. The WB analysis showed that the relative intensity of protein bands was significantly higher in NT and HT conditions during the BS than that of those during the NBS. Immunofluorescence assay showed localization of HSP60 in the cytoplasm of the Leydig cells during both seasons. HSP90 primarily showed immunostaining in undifferentiated spermatogonia and primary spermatocytes in NT and HT conditions and was additionally expressed in the spermatids during the NBS. The spermatogenesis was not monitored in the present study; however, findings of this study suggest that the localization of HSP60 and HSP90 varies in different seasons depending on the temperature, providing insight into the potential mechanisms influencing spermatogenesis during breeding and non-breeding seasons in stallions.

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INTRODUCTION

Heat shock proteins (HSPs), integral to cellular stability against environmental temperature changes, are ubiquitous in both males and females of different species, playing pivotal role in the reproductive performance (Tkacova and Angelovicova, 2012). These are involved in the transport system of nucleus, cytoplasm, endoplasmic reticulum and mitochondrial protein synthesis (Eski *et al.,* 2019). These proteins, constitutively produced in male testes, play a role in the process of spermatogenesis, in enhancing sperm motility (Huang *et al.,* 2000) and in the functionality of androgen receptors (Yue *et al.,* 1999). Their presence in stallion spermatozoa underscores their significance in germ cell development and other processes including sperm motility, capacitation, gamete interaction and mitochondrial protein folding (Volpe *et al.,* 2008).

HSP60, primarily located in the mitochondrial matrix, facilitates the folding of mitochondrial proteins and the ATP-dependent proteolysis of misfolded or denatured proteins (Shakeel and Yoon, 2023b). Its localization in mature spermatozoa of bovines (Boilard *et al.,* 2004) and

swine (Spinaci *et al.,* 2005), as well as in spermatogonia and spermatocytes of rats (Meinhardt *et al.,* 1995) and humans (Werner *et al.,* 1997), highlights its important role in reproductive biology. Similarly, the presence of HSP60 in the midpiece of stallion spermatozoa has been documented (Volpe *et al.,* 2008), further emphasizing its importance. HSP90 chaperones specialize in transforming receptor protein structures into their functional forms, aiding primarily in protein folding. Given their involvement in signal transduction and gene regulation, HSP90 chaperones are associated with modifying kinases, steroid hormone receptors, and transcription factors (Buchner and Li, 2013). In stallions, HSP90 immuno-reactivity is predominantly observed in the tail and, less frequently, in the neck or midpiece of spermatozoa, indicating its role in sperm motility, mitochondrial integrity and possibly in capacitation signaling and fertilization processes (Volpe *et al.,* 2008). The expression levels of HSP60 and HSP90 in stallion spermatozoa are influenced by temperature and semen quality, with the highest expression of HSP60 is noted during the breeding season and the lowest during the non-breeding season (Albrizio *et al.,* 2020).

The ongoing global temperature rise poses serious challenges to stallion fertility, with hot and humid conditions adversely affecting reproductive performance and, by extension, horse breeding programs. The impact of environmental factors on stallion reproduction, particularly sperm production, libido and testicular size, becomes more pronounced in regions exceeding 30° latitude (Leme *et al.,* 2012). While previous studies have explored the relationship between HSP60 and HSP90 expression and sperm kinetics in stallions (Albrizio *et al.,* 2020), there is a notable gap in understanding their expression in germ and somatic cells under various environmental conditions.

Addressing this gap, our study aimed to examine the expression of HSP60 and HSP90 in stallion testes and their seasonal expression patterns under different temperature conditions, at a specific latitude (36.4296°N) and longitude (128.0669°E). We hypothesized that the expression pattern of HSP60 and HSP90 may vary depending upon the temperature and reproductive season of the year in stallions. The findings of this study could provide invaluable insights into the molecular mechanisms underpinning stallion fertility, contributing to more effective management and breeding strategies under different environmental temperature conditions.

MATERIALS AND METHODS

Animal selection and testicular tissue preparation: This study was conducted at the Horse Science Laboratory, Kyungpook National University, South Korea, involving nine Thoroughbred stallions divided into three groups on the basis of environmental temperature: i) non-breeding season (NBS, *n*=3, aged 36 months, castrated in January-February), ii) normal temperature (NT, *n*=3, aged 36±4.0 months, castrated in March-May), and iii) hot temperature (HT, *n*=3, aged 32±4.0 months, castrated in June-August) of breeding season (BS). The average temperature during the NBS and NT and HT of BS ranged from -1.6 to 0.5°C, 6.1 to 18.3°C and 22.2 to 25°C, respectively. The study did not require any ethical approval, as castration was performed by registered veterinarians following owner's request, however a written consent for the surgical procedure was taken from each horse owner.

For immunofluorescence assay, testicular tissues (~1.0cm³) were fixed in 4% paraformaldehyde at room temperature for 24 hours, washed with PBS, dehydrated in ethanol and paraffin-embedded. For Western Blot and RT-qPCR analyses, tissues (~0.5cm³) were snap-frozen in liquid nitrogen and stored at -80°C, adapting a method previously described (Kim *et al.,* 2021).

RT-qPCR analysis: Total RNA from stallion testicular tissues was isolated using TRI-Solution (AM9738, Thermo Fisher Scientific, USA). The cDNA synthesis employed PrimeScript™ 1st Strand cDNA Synthesis Kit (6110A, Takara) with oligo-dT primers. The RT-qPCR was performed by making a minor modification to the previously reported approach (Shakeel and Yoon, 2023a). The qPCR mixture (20 µL) included 10 µL Master Mix (4367659, Thermo Fisher), 8 µL cDNA, 0.2 µL each of forward and reverse primers for HSP60 (5′-CTTTTTGCCAGGGTTGAGCC-3′ and 5′-CGTGTTGCTCCCCCTACAAT-3′) and for HSP90 (5′-CGCCTCGCGTATTTCAGATG-3′ and 5′-AGCGAATCTTGTCCAAGGCA-3′), and 1.6 µL RNase-free water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with primers 5′-CATCAAATGGGGCGATGCTG-3′ and 5′-TGCACTGTGGTCATGAGTCC-3′, served as a reference gene. RT-qPCR used the StepOnePlus™ System (Applied Biosystems) with a protocol of initial denaturation at 95°C (10 min), followed by 40 cycles of denaturation at 95°C (15 sec) and annealing at 60°C (1 min), denaturation at 95°C (15 sec), annealing at 60°C (1 min), and denaturation at 95°C (15 sec). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), presenting HSP60 and HSP90 mRNA levels normalized to GAPDH.

Western blotting: Testicular tissue samples were processed using radioimmunoprecipitation assay buffer (RIPA) and Xpert protease inhibitor (P3100-005, GeneDepot) with little modification from the previous method (Jung and Yoon, 2021). Protein concentrations were measured via a Tecan absorbance reader (560-nm filter, Switzerland). Samples, adjusted to 2 mg/mL with RIPA, were mixed equally with Bio-Rad sample buffer (161-0737, USA) and heated for 15 minutes. Proteins were separated on 10% SDS-PAGE using Mini-Protean II (Bio-Rad, USA), and transferred to nitrocellulose membranes (GE Healthcare, Germany). Membranes were blocked with blotto milk at room temperature (RT) for 45 minutes, then incubated with HSP60 and HSP90 antibodies (ADI-SPA-806-F and ADI-SPA-830-F, 1:100, Enzo Life Sciences) overnight at 4°C. As a control negative, normal IgG (sc-2025, 1:100, Santa Cruz Biotech) and for control positive mouse monoclonal β-actin antibody (sc-47778; 1:2,000; Santa Cruz Biotechnology, Inc.) was used. After three washes with phosphate-buffered saline Tween 20 (PBST), horseradish peroxidase-conjugated anti-mouse IgG, an HRP-linked antibody (7076S, 1:10,000, Cell Signaling Technology)

was applied for an hour. Enhanced chemiluminescent (ECL) detection reagents (catalog number: 34580; Thermo Fisher Scientific, USA) were used for identification, and the ImageQuant LAS 500 (Cytiva) was used to detect the protein bands.

Immunofluorescence assay: Immunofluorescence assay was performed by making a minor modification to the previously reported approach (Shakeel *et al.,* 2022). Testicular tissue slices (~5 µm thick) underwent rehydration in ethanol grades (100, 95, 80, 70, 50 and 25%) post-xylene removal (Duksan Pure Chemicals, Korea). The slides were exposed to antigen retrieval buffer (ab93678, Abcam) at 95°C for 30 min, followed by cooling at room temperature for 45 min. Slides were then PBST-washed twice, blocked with PBS containing 5% donkey serum (017-000-121, Sigma) for 30 min, and incubated with primary antibodies of HSP60 mouse monoclonal (ADI-SPA-806-F, 1:1,00; Enzo Life Sciences) and HSP90 mouse monoclonal (ADI-SPA-830-F, 1:1,00; Enzo Life Sciences). Normal mouse IgG (sc-2025, 1:1,00; Santa Cruz Biotechnology) was applied as a negative control. Following 1.5-hour room temperature incubation in a humid chamber, the slides were washed thrice with PBST. The secondary antibody, donkey anti-mouse (A21202, 1:1,000; Life Technologies), was applied for 45 min. After three more PBST washings, Vectashield® mounting solution with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) was used for mounting the tissue and sealed with a thick viscous liquid (Estée Wannabe, Korea).

Imaging: Cells labeled with HSP60 and HSP90 antibodies were analyzed using a Leica DM2500 fluorescence microscope (Leica, Wetzlar, Germany) with an EL 6000 light source and a dual-emission filter for specific fluorescence. Green-fluorescing cells indicated HSP60 and HSP90 positivity, captured using a Leica DFC 450 C camera.

Statistical analysis: Data analysis was conducted using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA). Tests for normality (Shapiro–Wilk) and variance homogeneity (Levene) were applied. Seasonal variations in HSP60 and HSP90 protein intensities were assessed via one-way Analysis of Variance (ANOVA). The least significant difference was used as a post-hoc test for one-way ANOVA. RT-qPCR data, not normally distributed, were analyzed with the Kruskal–Wallis test to compare HSP60 and HSP90 mRNA levels across seasons. All P values ≤0.05 were considered significant, and the data were presented as ±SEM.

RESULTS

Relative abundance of mRNA transcripts of HSP60 and HSP90 in stallions' testes: RT-qPCR, performed with three technical replicates of each of three biological replicates per group, revealed that the upregulation of the relative abundance of mRNA transcripts of HSP60 and HSP90 (Fig. 1) was observed at normal temperatures (NT), followed by that of those at high temperatures (HT). The relative abundance of HSP60 and HSP90 mRNA transcripts was significantly downregulated ($p<0.01$) in the non-breeding season (NBS) group. Both HSP60 and HSP90 mRNA transcripts at NT were higher than those at HT during the breeding season (BS), although the difference was statistically non-significant ($p>0.05$).

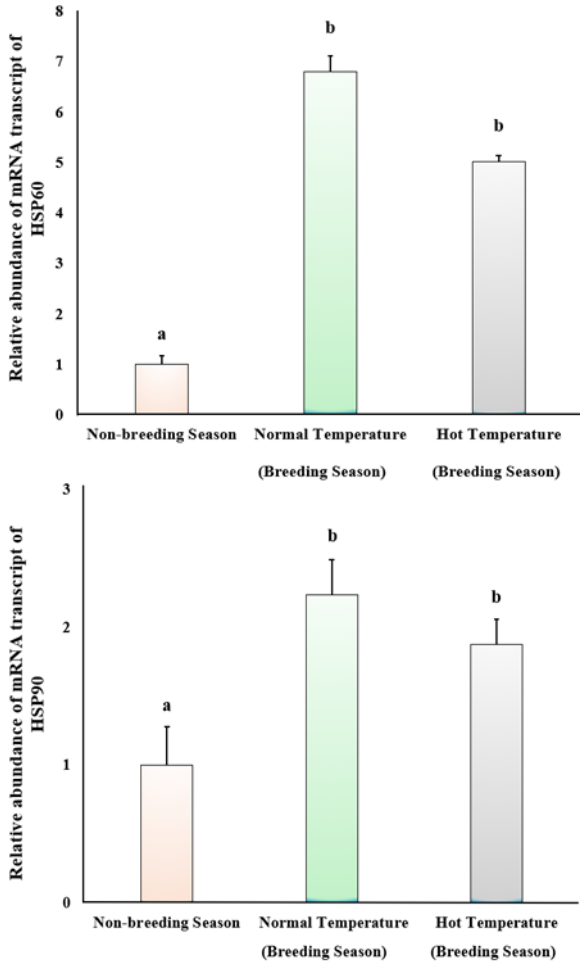

Fig. 1: Relative abundance of mRNA transcripts of heat shock proteins HSP60 and HSP90 in the testicular tissues of stallions collected during the non-breeding season and breeding season (normal temperature and hot temperature). RT-qPCR was performed with three technical replicates of each of three biological replicates per group. All data are expressed as mean±SEM. a-b different superscript = $p < 0.01$.

Cross-reactivity of HSP60 and HSP90 antibodies in stallions' testes: Testicular tissues were examined for the cross-reactivity of HSP60 and HSP90 antibodies by WB. The protein band for HSP60 in the stallions' testes was observed at 60 kDa (Fig. 2B), for HSP90 it was observed at 90 kDa (Fig. 2A), while for β-actin positive control the band was observed at 43 kDa (Fig. 2B). However, no protein band was noted for the negative control treated with normal IgG (Fig. 2C).

Season-dependent HSP60 and HSP90 expressions in stallions' testes: The seasonal expression of HSP60 and HSP90 proteins bands measured through ImageJ software revealed significantly lower levels ($p<0.05$) of HSP60 and HSP90 (Fig. 3) during the NBS as compared to the BS. Relatively higher expression was recorded during NT compared with that of HT conditions during the breeding season, but difference was not statistically significant ($p>0.05$).

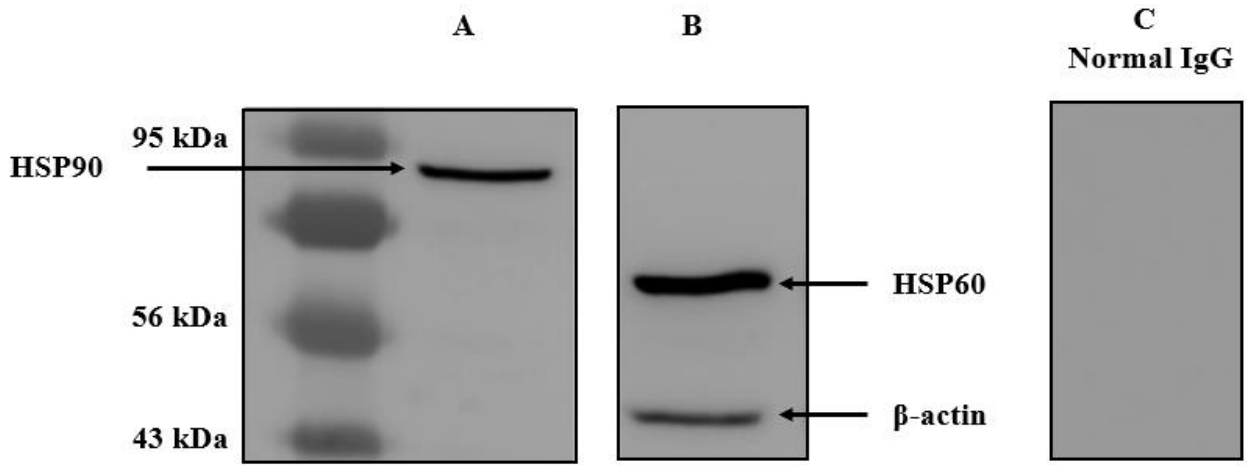

Fig. 2: Cross-reactivity of mouse monoclonal heat shock proteins HSP60 and HSP90 antibodies in stallions' testicular tissue. The protein band obtained after Western blotting for HSP90 is at 90 kDa (A) and that for HSP60 is at 60 kDa (B). The protein band of control positive, β-actin is at 43 kDa (B). In the negative control lane, no band is present (C).

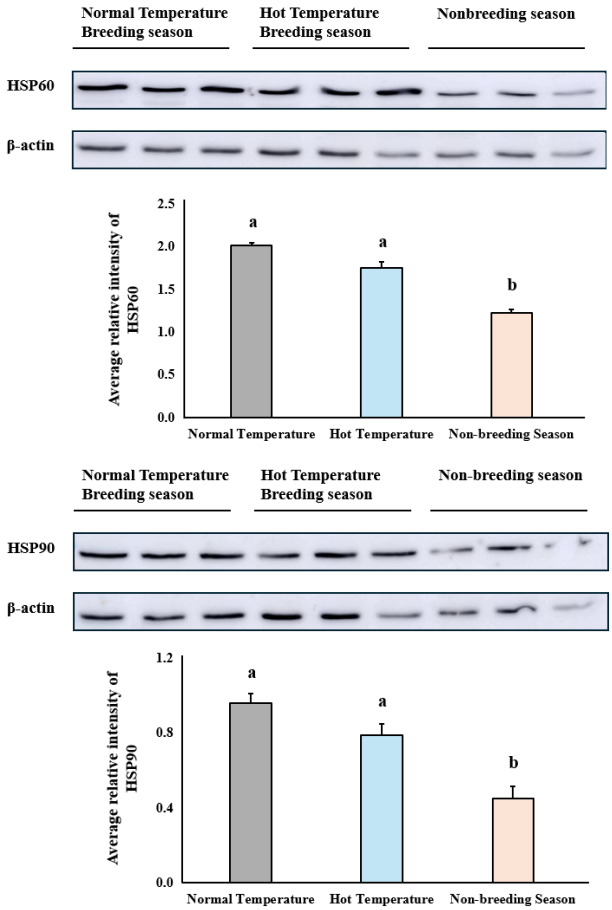

Fig. 3: Comparison of heat shock proteins HSP60 (A) and HPS90 (B) expression in stallions' testes during the non-breeding season and breeding season (normal temperature and hot temperature). Data are presented as ±SEM of three individuals per group. a-b different superscript = $p<0.05$.

Immunofluorescence staining of HSP60 in stallions' testes: Immunostaining techniques were employed to localize HSP60 within the testicular tissues collected from stallions across different seasons. HSP60 was predominantly localized within the cytoplasm of Leydig cells, irrespective of the season or temperature (Fig. 4A, 4F, 4K). Interestingly, neither Sertoli cells nor germ cells showed staining for HSP60 in any season. No immunolocalization was observed in any type of testicular cells treated with normal mouse IgG in all three groups (Fig. 4E, 4J, 4O).

Immunofluorescence staining of HSP90 in stallions' testes: The localization of HSP90 was observed primarily in the spermatogonia at NT and HT (Fig. 5A and 5F). The germ cells localized around the lumen and primary spermatocytes in the NT group were also stained (Fig. 5A). Only a few undifferentiated spermatogonia were stained with HSP90. The localization of HSP90 was also observed in the primary spermatocytes, secondary spermatocytes, and round spermatids in the NBS group (Fig. 5K, 5N). The Sertoli and Leydig cells were not stained with HSP90 in any season. Immunolocalization in any type of testicular cells treated with normal mouse IgG was not observed in all three groups (Fig. 5E, 5J, 5O).

DISCUSSION

Horses are susceptible to overheating and rising global temperatures pose a serious concern to stallion fertility. Heat and humidity impair reproductive capabilities, crucial for horse breeding. Heat shock proteins (HSPs), as molecular chaperones, safeguard somatic and germ cells during spermatogenesis, accommodating protein folding amidst temperature shifts (Nelson *et al.*, 2022). This study explores the expression patterns of HSP60 and HSP90 by analyzing the relative abundance of mRNA transcripts, protein expressions, and season-dependent immunostaining in stallion testes during NBS and varying temperature conditions (NT and HT) of BS.

Our study observed an upregulation in the relative intensity and abundance of HSP60 and HSP90 mRNA transcripts during the BS and a downregulation during the NBS. This is supported by the findings of Albrizio *et al.* (2020), who found similar seasonal variations in stallion spermatozoa. This suggests that temperature fluctuations across seasons elicit rapid genetic responses in HSPs, crucial for protecting gap-associated proteins critical for spermatogenesis (Calle-Guisado *et al.*, 2017). Furthermore, the well-known effect of photoperiod on

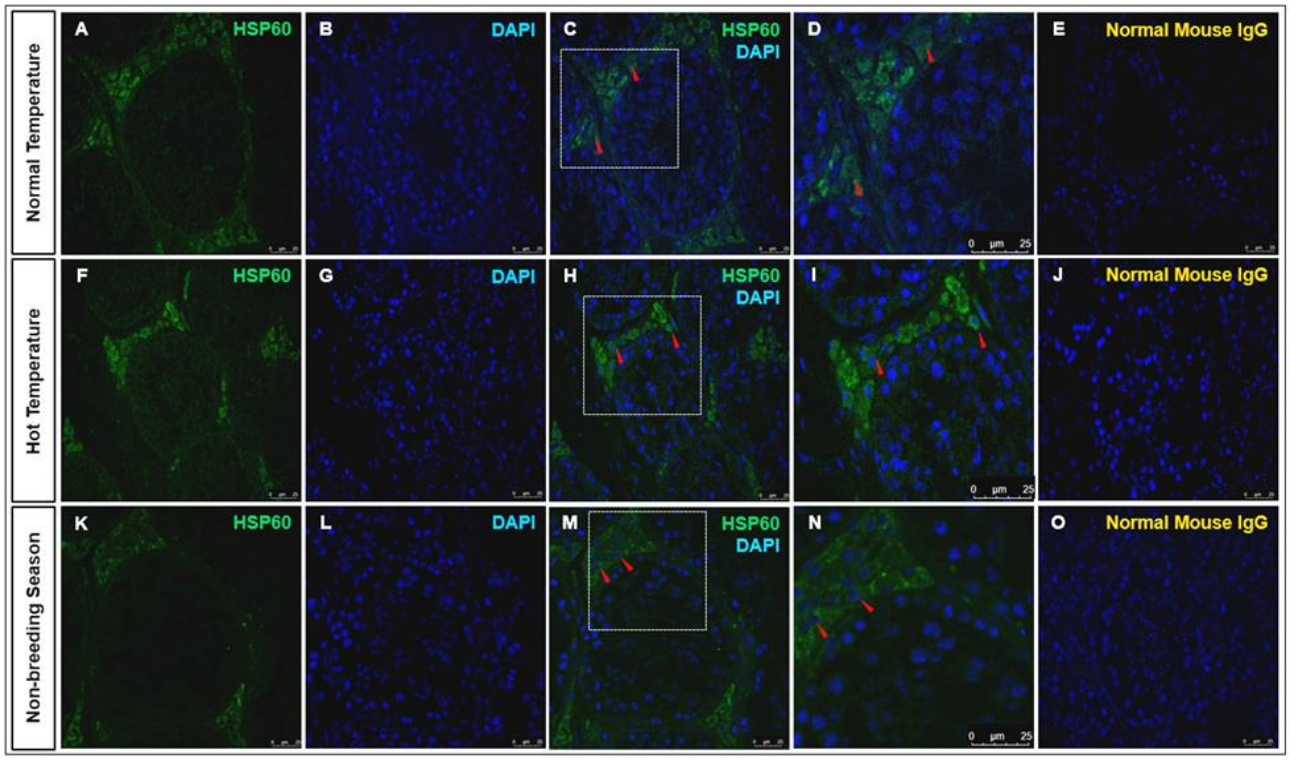

Fig. 4: Immunofluorescence staining of heat shock protein HSP60 in the testicular tissues of stallions collected during the non-breeding season and breeding season (normal temperature and hot temperature). HSP60 localization is observed in the cytoplasm of the Leydig cells in all seasons. A, F and K show the HSP60 expression; B, G and L show the DAPI staining; C, H and M show the HSP60 with DAPI; D, I and N are enlarged areas of white-line boxes present in C, H and M; E, J and O show staining with normal mouse IgG during normal and hot temperature conditions of breeding season and non-breeding season respectively. Red arrowhead indicates HSP60-positive Leydig cells. Scale bar=25 µm.

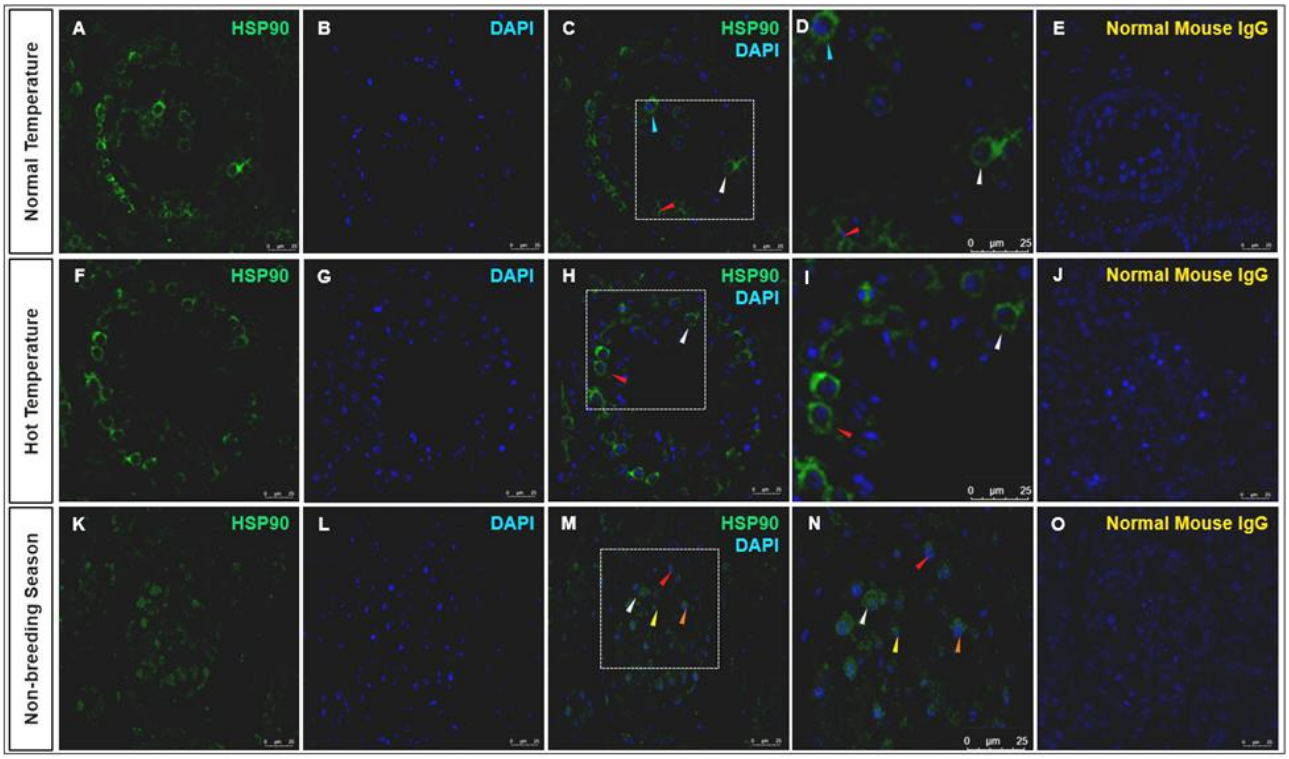

Fig. 5: Immunofluorescence staining of heat shock protein HSP90 in stallions' testicular tissues collected during the non-breeding season and breeding season (normal temperature and hot temperature). HSP90 localization is observed in spermatogonia and primary spermatocytes at normal and hot temperatures. The germ cells located at the center of the lumen are stained at normal temperature. HSP90 localization is observed in the round spermatids during the non-breeding season. A, F and K show the HSP90 expression; B, G and L show the DAPI staining; C, H and M show the HSP90 with DAPI; D, I and N are enlarged areas of white-line boxes present in C, H and M; E, J and O show staining with normal mouse IgG during normal and hot temperature conditions of breeding season and non-breeding season, respectively. Red arrowhead indicates HSP90-positive spermatogonia; white arrowhead, HSP90-positive primary spermatocytes; blue arrowhead, HSP90-positive germ cell close to center of lumen; yellow arrowhead, HSP90-positive secondary spermatocytes; and orange arrowhead, HSP90-positive round spermatids. Scale bar=25 µm.

equine reproduction (Palmer and Guillaume, 1992), alongside reduced HSP levels in the NBS marked by shorter days, implies a potential link between daylight duration and HSP expression. We propose that increasing day length towards the end of NBS decreases melatonin levels, thus raising GnRH levels, which in turn increases FSH and LH production (Coelho *et al.,* 2023), leading to increased germ cell count in the testes (O'Shaughnessy, 2014). This period likely necessitates higher HSP levels for optimal protein folding and cellular stability essential for spermatogenesis. Conversely, the NBS is characterized by a lower need for HSP60, correlating with reduced testicular activity, as evidenced by the notable downregulation of HSP mRNA transcripts. In this study, testes samples were collected across different seasons, with NBS in January-February, and NT in March-May and HT in June-August during BS. Interestingly, while HSP expression in stallion testes rose at NT and declined at HT, though the difference was not statistically significant, studies on other species like bulls (Deb *et al.,* 2014) and pigs (Calle-Guisado *et al.,* 2017) have shown increased HSP expression at higher temperatures, indicating species-specific responses to heat. Moreover, in South Korea, July and August are considered the most humid months of the year (Shin *et al.,* 2021), and the expression patterns of HSPs in stallions are negatively correlated to humidity. Similarly, Albrizio *et al.* (2020) showed that sperm concentration, viability and percentage of progressively motile spermatozoa in stallions are negatively correlated with humidity. Based on our results and those of previous studies, it can be speculated that horses in temperate zones are not heat tolerant.

Our findings revealed the localization of HSP60 in the cytoplasm of Leydig cells across all stallion groups, which is in line with previous studies on pigs (Huang *et al.,* 2005) and monkeys (Meinhardt *et al.,* 1998), but differs from those in humans (Lachance *et al.,* 2010) and rats (Meinhardt *et al.,* 1995). In stallions, data regarding the immunoreactivity of HSP60 in germ cells could not be traced; however, the midpiece exactly coincides with the mitochondrial location in stallion spermatozoa stained with HSP60 (Volpe *et al.,* 2008). In contrast to the localization of HSP60 in the Leydig cells of stallions in this study, HSP60 marks spermatogonia and primary spermatocytes in humans (Lachance *et al.,* 2010) and mice (Asquith *et al.,* 2005), while in rats (Meinhardt *et al.,* 1995), it was seen in spermatogonia, primary spermatocytes, and Sertoli and Leydig cells. This species-specific distribution of HSP60 underlines the varied role of this protein in testicular functions, development and spermatogenesis.

The HSP90 family, comprising isoforms like HSP90α, HSP90β and others, exhibits distinct localization patterns in testicular cells. Primordial and mature germ cells express HSP90α predominantly, whereas HSP90β is mainly found in Sertoli cells (Vanmuylder *et al.,* 2002; Ohsako *et al.,* 1995). Our findings demonstrated HSP90 localization in the cytoplasm of various germ cells including spermatogonia, spermatocytes and spermatids. Similar observations have been recorded in cattle yaks (Cui *et al.,* 2022), pigs (Huang *et al.,* 2005) and mice (Kajiwara *et al.,* 2012). This underscores the essential role of HSP90 in spermatocyte survival and spermatogenesis. According to Kajiwara *et al.* (2012), apoptosis resulting from the induced ablation of the HSP90 gene entirely halted spermatogenesis in mice testes. Intriguingly, in stallions, HSP90 localization was primarily seen in the sperm tail, with its implications for fertilization capability and capacitation signaling (Volpe *et al.,* 2008). Our study noted HSP90 expression variations during the breeding season (under different temperatures) and non-breeding season, suggesting its involvement in adapting spermatogenesis to environmental conditions. The distinct localization of HSP60 in the sperm midpiece and HSP90 in the tail, as previously identified (Volpe *et al.,* 2008), highlights their critical roles in germ cell DNA protection and maintenance for successful spermatogenesis, particularly during the breeding season.

In the present study, the expression patterns of HSP60 and HSP90 showed obvious seasonality, and there were significant differences between the non-breeding season and the breeding season, indicating that HSP60 and HSP90 were involved in the seasonal breeding of stallions. However, the evidence provided so far is not enough to prove that the seasonal expression patterns of HSP60 and HSP90 are caused by temperature. When the temperature is lower than 25ºC, the change of ambient temperature has little effect on testicular temperature, so it is difficult to be convinced that the expression changes of HSP60 and HSP90 are corresponding to temperature. Moreover, the data presented in this paper do not seem to be sufficient to show that the seasonal variation of HSP60 and HSP90 is related to temperature. Therefore, more evidence needs to be provided to support the findings of the present study.

Conclusion: In conclusion, these findings suggest that the expression of HSPs varies in different seasons depending on the temperature. The results of this study could help determine the mechanisms behind the effects of heat shock on stallion fertility and provide a fundamental point of reference for research regarding the roles of HSPs in the reproductive performance of stallions.

Authors' contributions: MS conceived the idea, designed and executed the study, evaluated the results, analyzed the data and wrote the manuscript. SY also executed the study. MY provided resources and critically reviewed and edited the manuscript.

Declaration of interest: No conflicts of interest have been disclosed by the authors.

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