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Effect of boar semen supplementation with recombinant heat shock proteins during summer

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ABSTRACT

Artificial insemination (AI) in pigs is mainly performed with refrigerated boar semen. There is a marked negative seasonal effect on the quality of boar sperm, mainly due to relatively greater ambient temperatures; to counteract this thermal stress, sperm cells possess natural defensive mechanisms such as Heat Shock Proteins (HSPs) that prevent protein denaturation. Thus, the objective of this research was to improve the quality of commercial boar semen collected during the summer when ambient temperatures are greater using recombinant HSPs. For this purpose, different concentrations (0.1, 0.5 and 1 μg/ml) of recombinant heat shock proteins (HSPD1, HSPA8 or HSP86) were added to commercial boar semen and there was cooling for 48 h at 17 °C. After this storage period, sperm quality was assessed by analyzing sperm viability, mitochondrial membrane potential and plasma membrane lipid organization using flow cytometry; additionally, sperm motility was examined using a CASA system. Also, in vitro fertilization (IVF) using HSP-supplemented boar semen was performed and the quality of the embryos produced was evaluated using quantitative real-time polymerase chain reaction (qPCR) analyzing the relative abundance of mRNA transcripts for genes encoding for embryo quality-related proteins (*BAX*, *TFAM*, *POLG* and *POG2*). Sperm quality variables, blastocyst rates and the abundance of mRNA transcripts for the selected genes were not affected by the presence of recombinant HSPs at any concentration. These results indicate that the supplementation of commercial seminal doses with recombinant HSPs does not improve boar sperm quality or fertility during the summer months when ambient temperatures are greater.

1. Introduction

In Spain, the pork industry is a primary agricultural sector as it accounts for 39% of livestock production and 14% of total agricultural production (Spanish Ministry of Agriculture, Fisheries and Food, 2018, https://www.mapa.gob.es/va/ganaderia/temas/produccion-y-mercados-ganaderos/sectores-ganaderos/porcino/ (Accessed 25 September 2019)). The pork sector has evolved greatly

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to reduce production costs with a primary aim being greater efficiency of pork yields. Currently, over 99% of the commercial seminal doses marketed for artificial insemination (AI) are cooled and used the same day or stored at 15–20 °C for 1–5 days (Paulenz et al., 2000; Johnson et al., 2000; Knox, 2016). The quality of semen doses depends on the initial quality of the ejaculate for which there are several factors that affect this variable, including the environmental factors. In this regard, there is a seasonal effect that detrimentally affects the quantity and quality of boar sperm in ejaculates mainly due to relatively greater ambient temperatures (Wettemann et al., 1976; Colenbrander and Kemp, 1990). This seasonal effect results in a significant decrease in the number of semen doses obtained per ejaculate, as well as a reduction in the sperm fertilizing capacity with a marked economic impact on the boar AI industry.

Even when boar studs are equipped with ventilation and cooling systems to maintain a constant temperature, in southern European countries temperatures during the summer increase to greater than 40 °C at times; hence, inevitable fluctuations in the barn temperature occurs and a decrease in the quality of commercial boar semen is often an outcome (Pinart et al., 2013; Peña et al., 2018). Because heat stress during the summer is a major concern for the boar AI industry, there have been different approaches to prevent the negative effects that heat stress exerts on sperm, including enriched diets with L-Arginine (Chen et al., 2018), betaine (Cabezon et al., 2016) or multi-antioxidant supplements (Peña et al., 2019) with limited success. To alleviate heat stress, the cells have other natural defensive mechanisms such as Heat Shock Proteins (HSPs). The HSPs are a family of chaperones that are activated in response to different type of stress, including heat stress, maintaining the proteins' structure and avoiding non-functional aggregation between proteins (Ellis, 1987; Welch, 1993; Ellis, 1996; Buchner, 1996; Bukau and Horwich, 1998). In addition, the HSPs have an essential function in cell survival, metabolism, cell growth and development or DNA repair among other functions in somatic cells (Sottile and Nadin, 2018). Specifically, recombinant HSPA8 has been used to increase sperm viability in vitro in bulls (Moein-Vaziri et al., 2014), rams (Lloyd et al., 2012) and bears (Alvarez-Rodriguez et al., 2013). Also, the addition of recombinant HSPA8 to boar sperm prior to conducting in vitro fertilization (IVF) procedures enhances monospermy and embryo yield (Elliott et al., 2009; Moein-Vaziri et al., 2014). The mechanism by which these recombinant proteins exert functions on spermatozoa is unclear, but it has been postulated that these functions could be mediated by interactions with the spermatozoa plasma membrane (Elliott et al., 2009).

With consideration of all the previous findings, the aim of the present study was to assess whether supplementation of boar semen produced during the summer with recombinant HSPD1, HSPA8 or HSP86 could help to improve sperm quality and IVF outcomes. To achieve this aim, commercial boar semen was purchased during the summer (June-July) and separately supplemented with the previously described recombinant HSPs. Sperm motility, viability, plasma membrane lipid organization and mitochondrial membrane potential were assessed. In addition, IVF experiments were performed, and the relative abundances of specific mRNAs of genes encoding the proteins of interest related to embryo quality were evaluated.

2. Materials and methods

2.1. Chemicals and sources

Propidium iodide (PI), SYBR-14, Merocyanine-540 (M540), YoPro-1 and Mitotracker® orange probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA); the recombinant proteins were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) at 1 mg/ml for HSPD1, 1.1 mg/ml for HSPA8 and 2.2 mg/ml for HSP86 in phosphate buffer saline (PBS); equine chorionic gonadotropin (eCG; Foligon) was purchased from Intervet International BV (Boxmeer, Holland); human chorionic gonadotropin (hCG; VeterinCorion) was purchased from Divasa Farmavic (Barcelona,Spain) and colloidal silica suspension (SpermFilter®) was purchased from Nidacon International AB (Mölndal, Sweden).

2.2. Semen collection and processing

There was purchasing of boar semen from a commercial boar station (Tecnogenext, S.L, Mérida, Spain) during June and July. Duroc boars were maintained according to institutional and European regulations. Six seminal doses diluted in Vitasem extender (Magapor S.L., Spain) at 30×10^6 spermatozoa/ml from six different boars were used ($n = 6$). After transport of the semen samples to the laboratory, the semen samples were supplemented or were not supplemented (control) with different concentrations of recombinant HSPD1, HSPA8 or HSP86 (0.1, 0.5 or 1 μg/ml) and were stored at 17 °C for 48 h.

2.3. Evaluation of sperm motility

Sperm motility was evaluated objectively using a CASA system (ISAS®, Proiser R + D, Paterna, Valencia, Spain). Aliquots of boar semen from the samples obtained from the boar commercial stud were incubated for 15 min at 38.5 °C, and 2 μl of the sample were placed in a pre-warmed counting chamber (Leja; Luzernestraat, The Netherlands) and analyzed using a microscope (Nikon Eclipse 50i) equipped with a 10 x negative-phase contrast objective and a heated stage at 38.5 °C. Analysis was based on the examination of 25 consecutive digitalized images and at least 300 spermatozoa per sample were analyzed. After assessing sperm in four representative fields in a random distribution, total motility (TM) and progressive motility (PM) were recorded. Spermatozoa with an average path velocity (VAP) > 15 μm/s were considered motile and spermatozoa with a straightness (STR) of greater than 80% were considered progressively motile.

2.4. Flow cytometry

Flow cytometry analysis was performed using an ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) equipped with a blue/red laser (488/640 nm) and three detection channels: BL-1 channel (530 ± 30 nm band pass filter); BL-2 channel (572 ± 28 nm band pass filter) and BL-4 channel (675 ± 30 nm band pass filter). Flow cytometer performance was ensured using fluorescent validation particles (NovoCyte™ Quality Control (QC) Particles; ACEA Biosciences, Inc., San Diego, CA, USA) to assess the mean fluorescence intensity (MFI) and coefficient of variance (CV) of FSC, BL-1 channel, BL-2 channel and BL-4 channel. Forward scatter (FSC) and side scatter (SSC) were used to gate the sperm population and to exclude debris. Spermatozoa were analyzed at 400–800 cells/s, and data were collected for 10,000 cells in each treatment. The data are represented in a logarithmic scale. Flow cytometry experiments and data analyses were performed using ACEA Novo Express® software (ACEA Biosciences, Inc., San Diego, CA, USA).

2.4.1. Analysis of sperm viability

Sperm viability was performed as described previously (Calle-Guisado et al., 2017). There was dilution of semen in PBS at 3×10^6 spermatozoa/ml and SYBR-14 and PI were added (SYBR-14 20 nM and PI 5 μM) and incubated for 15 min at room temperature (RT) in a darkened area. After excitation at 488 nm, fluorescence was detected using a 530 ± 30 nm band pass filter for SYBR-14 and 675 ± 30 nm band pass filter for PI. Viable spermatozoa were considered to be the average of the percentage of SYBR14-positive and PI-negative spermatozoa.

2.4.2. Evaluation of the plasma membrane lipid organization

Sperm plasma membrane lipid organization was assessed by staining with M540 and YoPro-1 as described previously (González-Fernández et al., 2018); YoPro-1 (75 nM) and M540 (6 μM) were added to 500 μl of diluted semen and incubated at 38.5 °C for 15 min. After excitation at 488 nm, fluorescence was detected using a 572 ± 28 nm band pass filter for M540 and 530 ± 30 nm band pass filter for YoPro-1. Results were expressed as the average of the percentage of viable cells with plasma membrane lipid disorganization: M540-positive and YoPro-1-negative.

2.4.3. Analysis of mitochondrial membrane potential

The mitochondrial membrane potential was evaluated using the specific probe Mitotracker® orange. The probe (50 nM final concentration) was added to 500 μl of diluted semen and incubated at 38.5 °C for 10 min. After excitation at 488 nm, fluorescence was determined using a 572 ± 28 nm band pass filter. Results are expressed as the average of the percentage of spermatozoa having relatively greater mitochondrial membrane potential (orange-stained cells).

2.5. In vitro fertilization

The IVF experiments were performed only with boar semen supplemented with 1 μg/ml of HSPs and incubated at 38.5 °C for 48 h. Sow ovaries were collected at an abattoir and were maintained in a saline solution (0.9% NaCl) supplemented with 70 mg/l of kanamycin sulfate at 37 °C. After transport of the ovaries to the laboratory, the ovaries were rinsed with PBS at 37 °C. Cumulus-oocyte complexes (COCs) were aspirated from 3- to 6-mm-diameter follicles using an aspiration pump. Oocytes with three or more layers of compact cumulus cells were washed in NCSU-37 medium (Petters and Wells, 1993) supplemented with 0.57 mM cysteine, 50 μM B-mercaptoethanol, 10 IU/ml eCG, 10 IU/ml hCG, 1 mM dibutyryl cAMP, 5 mg/l insulin and 10% porcine follicular fluid (Romero-Aguirregomezcorta et al., 2014). The COCs were transferred to a four-well Nunc® plate in groups of 50 oocytes in 500 μl of supplemented NCSU-37 medium (Petters and Wells, 1993) placed under mineral oil and incubated at 38.5 °C for 20 to 22 h in a 5% CO_2/95% air incubator with humidified atmosphere. After 20 to 22 h of incubation, COCs were transferred to NSCU-37 medium without eCG, hCG and cAMP and culture for an additional 20 to 22 h of incubation (Funahashi et al., 1997). After oocyte maturation, cumulus cells were removed using a vortex instrument and then, oocytes were washed in the fertilization medium (TALP medium) (Rath et al., 1999) and were transferred in groups of 20 to 30 oocytes to 500 μl of TALP medium in a four-well Nunc® plate. The boar semen for each treatment group (1 ml) was centrifuged at room temperature in 1 ml of colloidal silica suspension (40%) for 10 min at 600 *g*. The pellet was re-suspended in TALP medium and spermatozoa were added to the well containing the oocytes at a final concentration of 1×10^5 spermatozoa/ml. Gametes were co-incubated for 4 h at 38.5 °C in a 5% CO_2/95% air atmosphere and presumptive zygotes from each treatment were washed in NCSU-23 medium (Petters and Wells, 1993) and transferred into 500 μl of the same medium in a four-well Nunc® plate and covered with mineral oil. Incubation of presumptive zygotes was performed in a humidified atmosphere at 38.5 °C in a 5% CO_2/95% air incubator. Development rate to the blastocyst stage was recorded on day 7 post-insemination. Three replicates were conducted, and the embryos obtained were placed in a minimum volume of sterile PBS in a 500 μl tube, plunged into liquid nitrogen and frozen at −80 °C in PBS until RNA extraction.

2.6. Analysis of mRNA abundance for genes of interest

The abundances of mRNA transcripts for the genes of interest were determined using the quantitative real-time polymerase chain reaction (qPCR) utilizing specific primers previously designed by Lloyd et al. (2009). The amplification efficiency of the primers was optimized. For all target genes, there were efficiencies of about 100% and these were similar for targeted and reference genes.

The RNA was extracted from the stored embryos at −80 °C (nine for control, five for HSPA8 and five for HSP86) using

RNAqueous-Micro RNA Isolation kit according to the manufacturer's instructions (Ambion, Austin, TX, USA), followed by reverse transcription using the PrimeScript™ RT reagent kit according to the manufacturer's protocol (Takara Bion Inc., Shiga, Japan) and stored at −20 °C until use. The cDNA was diluted 1:10 for the subsequent real-time PCR analysis. The SYBR green real-time quantitative assay was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bion Inc., Shiga, Japan) and an Applied Biosystems® Stepone PCR System. The qPCR program included an initial denaturation process for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s with both annealing and extension steps at 56 °C for 1 min. Each cDNA sample was analyzed in duplicate. Control reactions lacking reverse transcriptase did not result in any significant amplification from any of the primer pairs used in this study indicating that chromosomal DNA was not present in a significant amount in the RNA preparations. The specificity for each pair of primers was verified by analyzing the melting curve. Relative abundance of mRNA transcripts from: Transcription Factor A, Mitochondrial (*TFAM)*; Bcl-2 associated X protein (*BAX)*; DNA polymerase subunit gamma *(POLG)* and *POLG2* genes were obtained using the $2-\Delta\Delta Ct$ calculation method (Livak and Schmittgen, 2001) with the abundance of nuclear small-subunit ribosomal RNA (*18S rRNA*) gene used as endogenous control.

2.7. Statistical analysis

Data were assessed for normality using a Saphiro-Wilk test and analyzed for equal variances. A one-way ANOVA was used to compare values when normality was confirmed and if the data did not have a gaussian distribution, a Kruskal-Wallis ANOVA on ranks test was used. Blastocyst rates relative to initial oocyte number were compared among groups using a Chi-Square test. The level of significance was set at $P < 0.05$. Analyses were performed using SigmaPlot software (ver. 12.0) for windows (Systat Software, Chicago, IL).

3. Results

3.1. Effect of recombinant HSPs supplementation on sperm viability from seminal doses preserved at 17 °C

Initially there was assessment of whether supplementation of boar semen with recombinant HSPs improved sperm viability after cooled storage at 17 °C for 48 h. The concentrations of the recombinant proteins used were selected based on a previous report where there was a study conducted with bull sperm (Moein-Vaziri et al., 2014). Supplementation of boar semen with different concentrations of recombinant HSPD1, HSPA8 or HSP86 during 48 h at 17 °C did not affect the percentage of viable spermatozoa compared to the control group (Fig. 1).

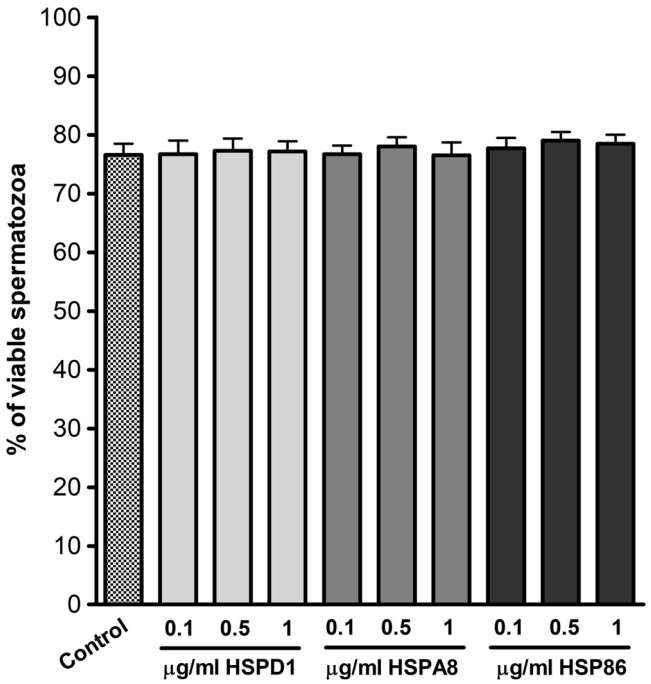

Fig. 1. Effect of different concentrations of recombinant HSPD1, HSPA8 and HSP86 on sperm viability; Spermatozoa from boar semen supplemented or not supplemented with different concentrations of recombinant HSPs were analyzed after 48 h of storage at 17 °C; Values are expressed as the mean of the percentage of viable spermatozoa (mean % ± SEM; $n = 6$).

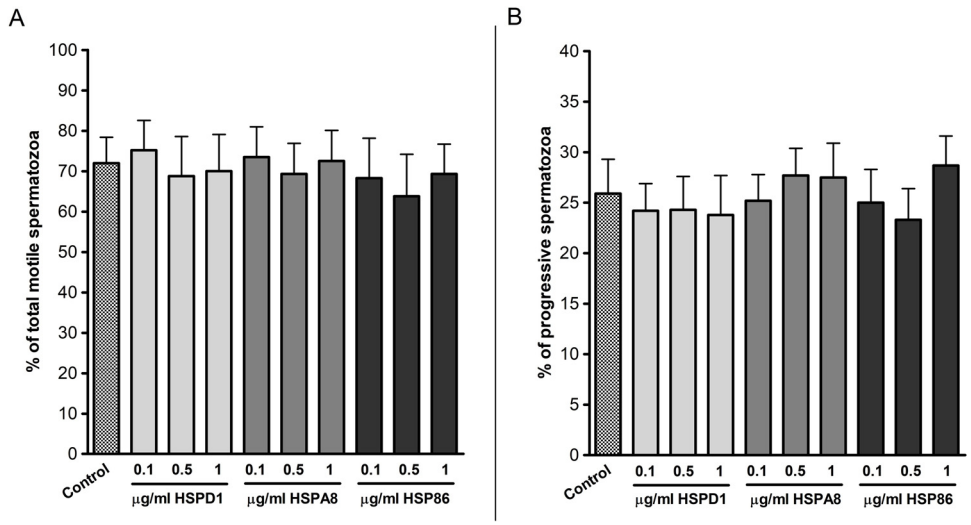

Fig. 2. Effect of different concentrations of recombinant HSPD1, HSPA8 and HSP86 on sperm motility; Spermatozoa from boar semen supplemented or not supplemented with different concentrations of recombinant HSPs were analyzed after 48 h of storage at 17 °C; Values are expressed as the mean of (A) total or (B) progressive sperm motility (mean % ± SEM; $n = 6$).

3.2. Effect of recombinant HSPs supplementation on sperm motility from seminal doses preserved at 17 °C

There was a subsequent study of the motility of boar spermatozoa of semen aliquots supplemented with recombinant HSPs using a CASA system. Incubation of boar semen with recombinant HSPs did not result in changes of total (TM) or progressive motility (PM) when compared with the control group after 48 h of storage at 17 °C (Fig. 2A and 2B).

3.3. Effect of recombinant HSPs supplementation on sperm mitochondrial membrane potential and plasma membrane lipid organization of boar sperm preserved at 17 °C

Other sperm quality variables were assessed such us mitochondrial membrane potential and plasma membrane lipid organization. Spermatozoa from semen aliquots incubated with recombinant HSPs were not different compared to the control for the percentage of spermatozoa having a relatively greater mitochondrial membrane potential (Fig. 3) or enhanced plasma membrane lipid disorganization (Fig. 4).

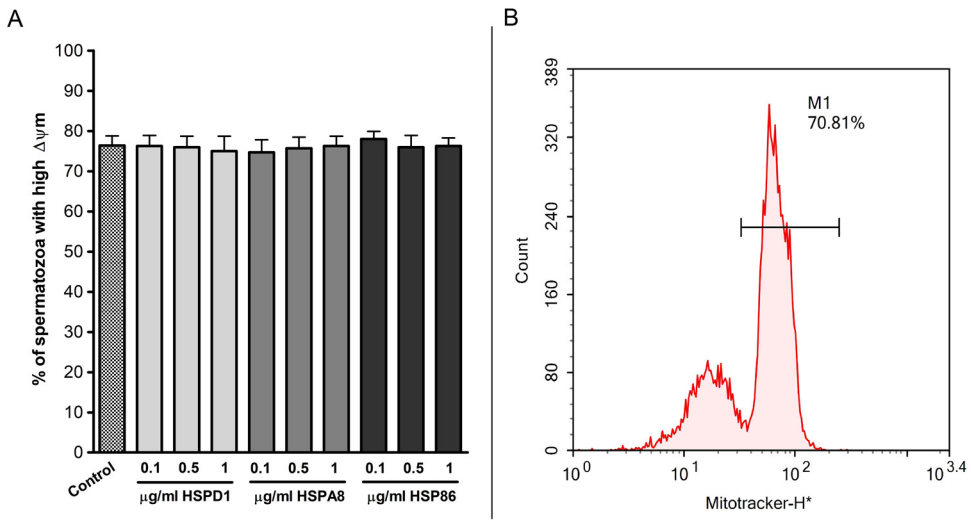

Fig. 3. Effect of different concentrations of recombinant HSPD1, HSPA8 and HSP86 on sperm mitochondrial membrane potential (ΔΨm); (A) Spermatozoa from boar semen supplemented or not supplemented with recombinant HSPs were analyzed after 48 h at 17 °C; Values are expressed as the mean of the percentage of spermatozoa with high ΔΨm (mean % ± SEM; $n = 6$); (B) Representative fluorescence histogram is shown; M1: spermatozoa with relatively greater mitochondrial membrane potential.

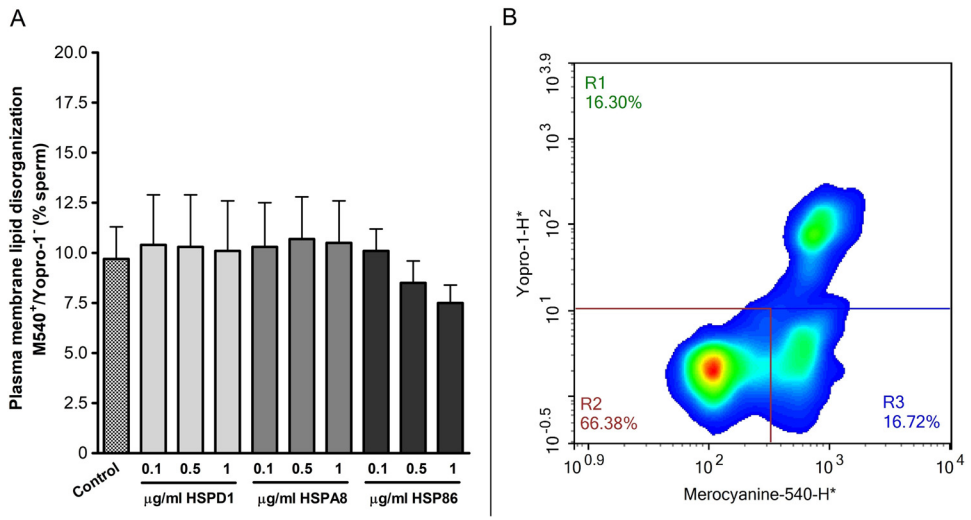

Fig. 4. Effect of different concentrations of recombinant HSPD1, HSPA8 and HSP86 on sperm plasma membrane lipid organization; Sperm from boar semen supplemented or not supplemented with recombinant HSPs were analyzed after 48 h of storage at 17 °C; (A) Values are expressed as the mean of the percentage of spermatozoa with plasma membrane lipid disorganization (mean % ± SEM; $n = 6$); (B) Representative dot plot is shown; R1: dead spermatozoa; R2: live spermatozoa with a relatively lesser M540 fluorescence; R3: live spermatozoa with a relatively greater M540 fluorescence.

3.4. Effect of supplementation of boar semen with recombinant HSPs on in vitro fertilization

Aliquots of boar semen supplemented for 48 h with recombinant HSPs were used for IVF assays and the embryo development to the blastocyst stage was assessed. The addition of recombinant HSPs at 1 µg/ml to boar semen resulted in a decrease in blastocyst yield, but this drop was not statistically significant (Table 1).

3.5. Effect of boar semen supplementation with recombinant HSPs on embryo quality

In addition, boar semen aliquots supplemented with recombinant HSPs was used for IVF experiments and the quality of the embryos produced was assessed. To conduct this experiment, the relative abundance of mRNA transcripts of different genes related to embryo quality in pigs were assessed (i.e., apoptosis - *BAX*; mtDNA transcription and replication - *TFAM*, *POLG* and *POLG2*) (Lloyd et al., 2009). The RNA of IVF-produced embryos was extracted and analyzed using qPCR, except for HSPD1 treatment that was not analyzed, as there was only one embryo for this experimental group. There were no differences in the mRNA abundance of selected genes when HSPA8 or HSP86 were added to the semen used for IVF procedures as compared with that of the control group (Fig. 5).

4. Discussion

The results of this study indicate that supplementation of commercial boar semen produced during the summer with recombinant HSPs (HSPD1, HSPA8 or HSP86) does not improve sperm quality or fertility. In the conditions that prevailed during the present study, supplementation of semen with recombinant HSPA8 for 48 h at 17 °C did not result in improved sperm viability (Fig. 1). Results of previous studies indicated there was an increase in sperm viability when bull spermatozoa were incubated with recombinant HSPA8 at 1 µg/ml for 48 h at 39 °C (Moein-Vaziri et al., 2014) or when ram spermatozoa were supplemented with recombinant HSPA8 at 4 µg/ml for 48 h at 17 °C (Lloyd et al., 2012). Furthermore, bear spermatozoa incubated with 0.5 µg/ml recombinant HSPA8 at 5 °C for 48 h had an improved viability (Alvarez-Rodriguez et al., 2013). The results from these previous reports are inconsistent with those of the present study indicating there may be a species-specific effect of recombinant HSPA8 on spermatozoa, a selective effect of

Table 1
Blastocyst rate after IVF using porcine seminal doses supplemented with recombinant HSPs.

Treatment	n° oocytes	Blastocyst (%)
Control	105	9 (8.6)
HSPD1 (1 µg/ml)	111	1 (0.9)
HSPA8 (1 µg/ml)	108	5 (4.6)
HSP86 (1 µg/ml)	108	5 (4.6)

Values are represented as total number and (percentage of total oocytes incubated) ($n = 3$). No significant differences were found compared to control ($P > 0.05$).

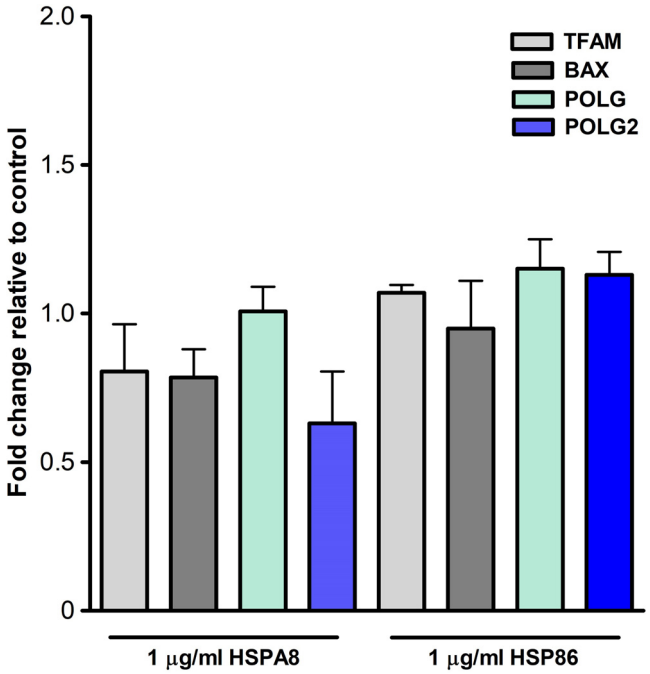

Fig. 5. Effect of boar semen supplementation with recombinant HSPs on the relative abundance of mRNA transcripts of genes of interest related to embryo quality; IVF experiments were performed using boar semen supplemented with recombinant HSPA8 or recombinant HSP86; relative abundances of mRNA transcripts of *BAX*, *TFAM*, *POLG* and *POLG2* genes expressed as a fold change relative to the control value are shown. Data are presented as mean ± SEM.

this recombinant protein depending upon the incubation conditions used (5 or 39 °C) or a significant effect of the concentration of recombinant HSPA8 used.

Other key variables related to sperm quality such as sperm motility, mitochondrial membrane potential or plasma membrane lipid organization remained unchanged when there was treatment with the varying concentrations of recombinant HSPA8 used in the present study (Figs. 2, 3 and 4). These results indicate that sperm quality, as assessed in vitro, does not improve when recombinant HSPA8 is added to cooled commercial boar semen. The lack of effect of recombinant HSPA8 on boar sperm motility is consistent with the findings of Alvarez-Rodriguez et al. (2013) where there was not changes in total motility of bear spermatozoa incubated at 5 °C for 48 h. Consistent with the results of the present study and the study with bear spermatozoa, results of other studies indicate that supplementation of boar semen with 0.5 μg/ml of recombinant HSPA8 for 24 h at 17 °C resulted in a decrease in sperm motility when bicarbonate was added to the semen (Holt and Satake, 2018).

Regarding recombinant HSPD1, there is only one previous study by Lachance et al. (2007) for which it was reported that human spermatozoa incubated for 4 h at 37 °C with recombinant HSPD1 did not affect sperm viability or motility, which is consistent with results from the present study. Even though HSP86 has been previously reported to have regulation actions on sperm motility and in maintenance of membrane mitochondrial potential during heat stress (Calle-Guisado et al., 2017), to the best of our knowledge, the present study is the first in which this recombinant protein has been added to cooled stored spermatozoa of any species.

In the conditions that prevailed in the present study, for none of the selected sperm variables analyzed were there significant changes when recombinant HSPA8, HSPD1 or HSP86 were added during cooled storage (Figs. 1–4). Nonetheless, it has to be noted that the classical variables used to assess sperm quality such as those evaluated in the present study may not accurately predict the fertilizing capacity of spermatozoa (Jung et al., 2015), and other variables could be affected by the supplementation with recombinant HSPs. To address this possibility, IVF experiments were conducted using boar semen supplemented with recombinant HSPs, however, there were no changes in the rates of development of embryos to the blastocyst stage among groups (Table 1). Interestingly, there was a tendency for a decrease the rate of embryo development to the blastocyst stage when spermatozoa were incubated with recombinant HSPD1. In this regard, supplementation of mice semen with HSPD1 at 10 ng/ml had a positive effect on development of mouse embryos to the two-cell stage, however, there were adverse effects on fertilization and cleavage rates when there was supplementation at greater concentrations (50 and 100 ng/ml; Abdi et al., 2019). Hence, the concentration of HSPD1 used in present study (1 μg/ml) could, in part, explain the decrease observed in the blastocyst rate when boar semen was supplemented with recombinant HSPD1.

Regarding the associations between expression of genes related to embryo quality, there was a lesser abundance of *TFAM*, *POLG* and *POLG2* and an upregulation of *BAX* genes, as indicated by relative abundance of mRNA transcript for these genes, resulting in lesser quality pig embryos compared to what occurred in high quality pig embryos (Lloyd et al., 2009). Nevertheless, in the experimental conditions imposed in the present study, there were no differences in the mRNA abundance of selected genes in IVF-

derived embryos of the HSP-treated groups compared with the control group (Fig. 5). Based on this finding, the use of semen supplemented with recombinant HSPs did not improve the quality of the embryos produced.

When there was comparison of the semen used in the present study (summer) to that collected during winter months using the same procedures, the percentage of total sperm motility after 48 h of storage at 17 °C (approx. 75%; Fig. 2) was slightly less compared to the sperm total motility when semen is collected during the winter months (approx. 84%; data not shown). These results are not consistent with those reported by Pinart et al. (2013) where there was a marked decrease in the percentage of total sperm motility from 85% (doses obtained in March) to 45% (doses obtained in June) after 48 h of storage at 17 °C. These inconsistent results between the two studies could be attributed, at least partially, to the distinct geographical localization of the two studies with intrinsic and important seasonal temperature differences. Also, the breed of swine used in the two studies could have affected the results obtained, because Duroc boars were used for the comparisons in the present study while Pinart et al. (2013) used Piétrain boars, considering the reported variations in sperm quality among swine breeds (Yeste et al., 2010). In this regard, Huang et al. (2000) reported that the Landrace boars had greater semen quality than Yorkshire and Duroc boars from an AI center during the season of the year when ambient temperatures are greater in which this temperature ranged between 23 to 32 °C. It has been recently reported that the ambient temperature increases that of the barn especially from mid June to the end of July and this increase has marked effects on boar sperm quality, particularly in breeds highly susceptible to heat stress (Lugar et al., 2019). It was classically assumed that 27 °C was the greatest temperature of the thermoneutral zone for pigs (Stone, 1982), however results from more recent research indicate this upper thermoneutral limit should not exceed 23 °C (Brown-Brandl et al., 2013), and hence, the likelihood of heat stress in boar barns during summer is notably increased when there are high ambient temperatures. The temperature variations in the barn together with the use of different breeds could explain the differences observed in sperm quality among studies.

Even though the male factor is important when considering heat stress, heat stress also impairs female fertility (Einarsson et al., 2008). Heat stress affects embryo development to the blastocyst stage in other domestic species such as cattle, in which there is a decrease in the rate of development to the blastocyst stage when IVF yields are evaluated during the summer compared to winter months (Pavani et al., 2015). Accordingly, in the present study the rate of development of embryos to the blastocyst stage in the absence of recombinant HSPs supplementation during summer (control) was less than the blastocyst yield obtained during winter (8.6% compared with 14.4%, respectively; data not shown). Hence, results from the present study indicate that the decrease observed in swine fertility during the summer months is not reversible by addition of recombinant HSPs to cooled semen. Instead, to counteract the adverse effect of high temperatures on boar fertility of the Duroc breed, the efforts should focus specifically on the female.

5. Conclusions

Results of the present study indicate that supplementation of boar semen during summer with recombinant HSPD1, HSPA8 or HSP86 does not lead to improvements in sperm quality or fertility. In addition, the quality of the embryos produced using IVF is not enhanced with use of recombinant HSP supplementations of boar semen during cooled storage.

Declaration of Competing Interest

The authors declare no conflict of interest.

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