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Relative abundance of heat shock proteins and clusterin transcripts in spermatozoa collected from boar routinely utilised in an artificial insemination centre: preliminary results

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**Relative abundance of heat shock proteins and clusterin transcripts in spermatozoa collected from boar routinely utilised in an artificial insemination centre: preliminary results**

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## Abstract

It is widely accepted that mature sperm contains RNA. The first hypothesis was that sperm RNAs have no functions of their own but are simply residues of spermatogenesis reflecting the events that occurred during their formation in the testes. More recently new discoveries have essentially expanded these views, showing that sperm mRNAs constitute a population of stable full-length transcripts, many of which are selectively retained during spermatogenesis and delivered to oocytes contributing to early embryo development. It is well known that semen quality can be influenced by occasional physical stress, infection, and variation in temperature and the definition of new markers for evaluation of semen could offer knowledge about the fertility potential of a semen sample. The aim of the present study was to evaluate the presence and the relative quantity of transcripts and protein of heat shock protein 70 (HSP70), 90 (HSP90) and clusterin (CLU) in Percoll-selected spermatozoa collected from seven adult boars of proven fertility routinely employed for artificial insemination. Our results showed the presence of HSP70, HSP90 and CLU transcripts with different level of expression: high for HSPs and low for CLU transcripts. The transcript level of both HSPs are similar among selected spermatozoa derived from high quality sperm with the exception of one boar that showed a reduced content of HSP70 and HSP90 mRNA together with a lower semen quality. At protein level, both HSPs were detected with similar amount among all seven boars whilst no band was evidenced for CLU protein.

Keywords: boar; clusterin; HSP70; HSP90; sperm

## 1. Introduction

It is widely accepted that mature sperm contains RNA. As male gametes have been considered transcriptionally inert cells because of the substitution of histones with protamines, the first hypothesis was that sperm RNAs have no functions of their own but are simply residues of spermatogenesis reflecting the events that occurred during their formation in the testes (Krawetz 2005). However, other discoveries have essentially expanded these views, showing that sperm mRNAs constitute a population of stable full-length transcripts, many of which are selectively retained during spermatogenesis (Ostermeier et al. 2002; Hamatani 2012). Although the functions of sperm RNAs await full clarification, it has been suggested that sperm transcriptional profiles provide a biomarker for male infertility and an increasing number of studies in humans have demonstrated that the sperm mRNA profile may serve as a molecular diagnostic marker for evaluating male fertility (Jodar et al. 2013).

Messenger RNAs have been found in the mature mammalian spermatozoa of several species (Dadoune et al. 2005; Gilbert et al. 2007; Yang et al. 2009). In swine, transcriptome analysis of ejaculated spermatozoa has been performed, identifying many mRNAs involved in biological processes, molecular functions and cellular components (Yang et al. 2009). More recently, the presence of various transcripts has been investigated in order to describe mRNA involvement in capacitation and early embryo development in pigs (Hwang et al. 2013). It is well known that semen quality can be influenced by occasional physical stress, infection, and variation in temperature (Althouse et al. 2000; Althouse and Lu 2005; Kunavongkrit et al. 2005; Knox et al. 2008; Althouse 2008) and the definition of new markers for evaluation of semen could offer knowledge about the fertility potential of a semen sample. Semen quality assessment represents a fundamental step for obtaining successful artificial insemination. In addition to the conventional semen parameter evaluation, an increasing number of innovative molecular markers, at proteomic and transcriptomic level, have been used to identify more reliable markers of sperm quality and fertility. In bulls, mRNA abundance of specific sperm proteins has been related to a fertility index, allowing the determination of biomarkers able to predict the fertility of subjects (Feugang et al. 2010; Kasimanickam et al. 2012).

In order to contribute to define new potential biomarkers of porcine semen quality, we selected three proteins whose presence is well documented in sperm cells, heat shock protein (HSP) 70, HSP90 and clusterin (CLU). The presence of HSP70 and HSP90 proteins in porcine spermatozoa has been described

as playing a role in sperm-oocyte interaction and sperm motility respectively (Huang et al. 2000b; Spinaci et al. 2005; Volpe et al. 2008). No correlation between HSP70 and semen quality was found (Turba et al. 2007) while HSP90 was investigated only in relation to the quality of cryopreserved semen (Wang et al. 2014; Zhang et al. 2015). Clusterin (CLU), a pleiotropic glycoprotein mainly present in seminal plasma has also been found to be associated with spermatozoa in various species (Sylvester et al. 1991; Ibrahim et al. 1999; Ibrahim et al. 2001; Han et al. 2012). In human and bull sperm, clusterin mainly exists on the surface of immature, low motile or morphologically abnormal spermatozoa and therefore its presence has been proposed as a marker of poor sperm quality (O'Bryan et al. 1994; Ibrahim et al. 2000; Muciaccia et al. 2010).

Until now the presence and the levels of the transcripts of these important proteins (HSP70, HSP90 and CLU) in pig sperm cells have not been clearly described. Only a single paper reported the presence of CLU transcript, suggesting a role of the paternal mRNA in early embryo development (Kempisty et al. 2008).

Thus, the objectives of this study were to evaluate first the presence of HSP70, HSP90 and CLU transcripts in ejaculated spermatozoa of seven boars of proven fertility, and to verify if the relative abundance of transcripts and their related proteins.

## **2. Materials and methods**

### **2.1 Animal semen collection and quality evaluation parameters**

Seven healthy Large White boars, (2-7-year-olds), housed under the same condition (temp.  $20 \pm 2^\circ\text{C}$ ) on a commercial farm and routinely employed for artificial insemination (AI) were used in this study. The semen parameters were evaluated in order to confirm the quality of the ejaculated routinely. The average farrowing rate was 86% with a range of 84% to 89%.

Semen was collected every 3 to seven days, but only every fortnight was it used for analysis.

For this study, three ejaculated/boar (21 ejaculated in total) were collected and evaluated; all the samples were collected from December 2013 to January 2014, under favourable environmental conditions.

### **2.2 Semen collection and evaluation of semen quality**

Sperm samples (n= 21) were collected by an experienced technician using the gloved-hand technique and a dummy. The sperm-rich fraction was collected in a pre-warmed thermos and was diluted 1:1 (v:v) with swine fertilization medium (SFM - home made extender as previously reported (Fantinati et al. 2009)) and immediately transported to the laboratory for evaluation of semen parameters.

The concentration of each ejaculate was assessed after spermatozoa immobilization in 1.5 M NaCl solution under a phase-contrast microscope at 400x using a Thoma counting chamber. Three replicates for each ejaculate were analysed.

The spermatozoa viability was evaluated by eosin-nigrosin staining. A working solution was prepared with two parts of 10% (w/v) eosin and one part of 5% (w/v) nigrosin. The sperm suspension and the working solution were mixed 1:1 (v:v) and the viability was evaluated. At least 200 cells were observed (Nikon Eclipse E600, Nikon Corporation, Japan). Three replicates for each ejaculate were analysed.

The sperm motility was subjectively assessed by visual estimation under a phase contrast microscope equipped with a heated plate. Three replicates for each ejaculate were analysed. A total of 10 fields were evaluated.

### **2.3 Percoll density gradient of swine spermatozoa**

In order to collect highly motile and morphologically normal spermatozoa, without somatic cell contaminants or debris, the spermatozoa samples, previously diluted with SFM, were separated by centrifugation through a discontinuous Percoll (Sigma-Aldrich St Louis, MO, USA) density gradient (45/90, v/v; (Matàs et al. 2011)). A bilayer of 2 ml each of Percoll solution with different density (45 and 90 in the bottom) was prepared in 15 ml conic centrifuge tubes; then diluted semen samples ( $5-6 \times 10^7$  sperms/2ml/tube) were added at the top, care being taken to maintain the integrity of the layers.

Samples were then centrifuged for 30 min at 700g. The sperm pellet was collected from the 45/90 interfaces, and then washed twice with DPBS (1ml). Sperm sample concentration, viability and motility was then evaluated, as described above. Aliquots of  $5 \times 10^6$  spermatozoa for each ejaculate were frozen and stored at -80°C until RNA and protein analysis.

### **2.4 RNA isolation and quantitative real-time PCR (qRT-PCR) for HSP70, HSP90 and CLU**

Total RNA from purified spermatozoa samples ( $5 \times 10^6$ ) was extracted using NucleoSpin RNA II (Macherey Nagel, Düren, Germany) and triplicates for each ejaculate were analysed. The lysis of the cells was carried out with Tissue Lyser TL (QIAGEN, Hilden, Germany) in the presence of two beads (50Hz

for 5 min). Total RNA was extracted according to the manufacturer's instructions, except for double treatment with DNase (instead of single DNase treatment as suggested). RNA was spectrophotometrically quantified (DeNovix Inc, Wilmington, DE, USA) and its quality was determined by gel electrophoresis on 1% agarose. Then 1 µg of RNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA), in a final volume of 20 µl. Real time quantitative PCR was performed using an iCycler Thermal Cycler (Bio-RAD). Primers for swine HSP70, HSP90 and CLU were designed using Beacon Designer 2.07 software (Premier Biosoft International, Palo Alto, CA, USA). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize mRNA levels across different samples. Primer sequences are shown in Table 1.

A master mix of the following reaction components was prepared in the end-concentrations indicated: 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 9.5 µl water and 12.5 µl IQ SYBR Green BioRad Supermix (Bio-RAD). Two µl of cDNA were added to 23 µl of the master mix. All samples were analysed in duplicate. The real-time PCR protocol employed was: initial denaturation for 3 min at 95°C, 40 cycles at 95°C for 15 sec and at 60°C for 30 sec, followed by a melting step with slow heating from 55°C to 95°C at a rate of 0.5°C/sec. Primer specificity as well as real-time efficiency was evaluated in previously collected porcine testicular tissue (positive control).

Contamination of epithelial cells, germ cells and leucocyte (CDH1, KIT and CD4) in spermatozoa RNA was preliminary verified using specific primers (table 1); the positive control used for the assay was porcine testicular cDNA or cDNA synthesized from RNA purified from blood (Kumar et al., 2010).

The gene expression level of HSP70, HSP90 and CLU was evaluated as  $\Delta C_t$  (threshold Cycle) = ( $C_t$  reference gene –  $C_t$  gene of interest) which directly correlates with the expression level.

## **2.5 Western blot for HSP70, HSP90 and CLU**

The spermatozoa samples ( $5 \times 10^6$  /sample) were resuspended in SDS buffer (Tris-HCl 62.5 mM pH 6.8; SDS 2%, glycerol 20%) and duplicates for each ejaculate were analysed. Proteins from spermatozoa ( $1 \times 10^6$  for HSP 70 and HSP90,  $2 \times 10^6$  for CLU) were separated by NuPage 10% Bis-Tris Gel (Gibco-Invitrogen, Paisley, UK) for 50 min at 200 V, and then electrophoretically transferred onto a nitrocellulose membrane. HSP70, HSP90 detection was performed as previously reported (Volpe et al. 2008), while CLU western blotting was performed as previously reported (Zannoni et al. 2015). Briefly, the nitrocellulose membranes were blocked with 3% milk powder in PBS-T20 (phosphate buffer saline-0.1% tween-20) for one hour at room temperature. The membranes were then incubated overnight at 4°C



with a 1:1000 dilution of monoclonal anti-human HSP70 (C92F3A-5 Stressgen Biotechnologies Corp. Victoria, BC, Canada), monoclonal anti-human HSP90 (AC88 Stressgen) and monoclonal anti-human CLU (05-354 Upstate Millipore Corp. Temecula, CA, USA), in Tris Buffered Saline-T20 (TBS-T20 20mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). After several washings with PBS-T20 the membranes were incubated with the secondary biotin-conjugate antibody and then with a 1:1000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody. All western blots were developed using chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. All membranes were stripped and reprobed for  $\beta$ -tubulin in order to normalize the results. The intensity of the resultant bands was acquired using Fluor-S<sup>TM</sup> Multimager and quantified by Quantity One software (Bio-RAD). The relative protein content was determined by the density of the resultant bands and expressed in arbitrary units (AU) relative to the  $\beta$ -tubulin content, using the Quantity One software (Bio-RAD).

## **2.6 Statistical analysis**

Data were analysed by one-way analysis of variance (ANOVA) and post hoc Tukey HSD test. The statistical significance was set at  $P < 0.05$ .

## **3. Results**

### **3.1 Sperm parameters: viability, motility and concentration**

Data on viability, motility and spermatozoa concentration are shown in Fig.1. All the ejaculates collected from six out of the seven boars showed ejaculates of high sperm quality. One boar (B2) showed seminal parameters significantly different from the others and below acceptability for use in artificial insemination (AI) (Fig. 1). After Percoll separation the values of motility ( $92 \pm 3.7 \%$ ) and viability ( $93 \pm 2.4 \%$ ) were similar for all boars.

### **3.2 Quantitative real-time PCR (qRT-PCR) for HSP70, HSP90 and CLU**

Quantitative PCR experiments performed to verify the presence of contaminants mRNA showed that the selected mRNA markers were not detectable.

Quantitative PCR data demonstrated that the HSP70 and HSP90 mRNAs were abundant in all samples whilst CLU mRNA was very low. No statistically significant differences were observed in the gene

expression level of HSP70, HSP90 and CLU among boars, with the exception of one boar (B2) that showed a reduced content of HSP70 and HSP90 mRNA (Fig.2).

### 3.3 Western blots for HSP70, HSP90 and CLU

Western blot analysis for HSPs showed in all samples a specific band (Fig. 3B) as previously reported by us (Volpe et al. 2008). No statistically significant differences were observed in the expression levels of the protein among the seven boars (Fig. 3A).

No signal was observed for CLU protein in sperm cell samples, but only in the positive control of aortic porcine endothelial cells (data not shown).

## 4. Discussion

A growing interest is directed towards the presence of mRNA in mammalian ejaculated spermatozoa. Some findings suggested that these transcripts can code for proteins, which are essential in early embryo development or for cellular function. Other studies reported that a semen quality evaluation could be derived from RNA profiling which might provide clinical markers for male infertility (Jodar et al. 2013). In swine species, over the past 10 years, transcriptome analysis has identified many mRNAs involved in different biological processes (Yang et al. 2009). More recently, the presence of various transcripts has been investigated in order to clarify their involvement in early embryo development (Hwang et al. 2013).

The present study aimed to evaluate the expression of HSP70, HSP90 and CLU in spermatozoa of boar routinely employed in AI.

Our study considered seven boars of different ages. One of these boars (B2), at the time of the study, presented seminal parameters unsuitable for AI because of transient genitourinary infection but was nonetheless included in the study to obtain Percoll-selected spermatozoa from low-quality semen.

Results showed the presence of HSP70, HSP90 and CLU transcripts. In the literature a CLU transcript has been reported (Kempisty et al. 2008) while only the presence of a transcript member of the HSP70 family (HSP70.2) has been described in porcine spermatozoa (Yang et al. 2009). The number of transcripts differed, with HSP70 and HSP90 relatively highly abundant with respect to the chosen housekeeping gene while CLU was very low or undetectable in some samples.

At the protein level, we detected similar amounts of HSP70 and 90 among all seven boars, but the CLU specific band was not detectable in any sample. A previous paper showed how an aggressive manipulation such as cell sorting does not affect the quantity of the expressed protein in boar

spermatozoa (Spinaci et al.,2010), therefore we are confident that a much more mild procedure, such as Percoll selection, will not influence the protein levels.

Even if our data may be considered preliminary, it's interesting to note that the HSPs transcript seem to be more informative than HSP protein in relation to the sperm quality. Indeed, six boars with high sperm quality showed high and similar level of HSPs transcripts while the boar (B2), with low sperm quality, showed a reduced level of HSPs transcripts. The existence of the correlation, if confirmed in future studies, allows us to speculate that, even if selected and able to fertilize, sperm cells deriving from a poor-quality semen sample have characteristics that make them unfit to sustain embryonic development.

Previous findings about the absence or low correlation between HSP70 protein level and sperm motility and viability were reported (Huang et al. 2000a; Turba et al. 2007) being that HSP70 seems to be more correlated with morphological abnormalities (Huszar et al. 2000; Turba et al. 2007).

It is important to underline that, in the present work, protein detection was performed after Percoll selection of spermatozoa, and, therefore the lack of differences in HSPs content among the different boars may be related to the selection of only highly motile, good-quality spermatozoa, probably all rich in HSPs. The opposite may obtain concerning the absence of CLU protein. CLU is reported in the literature as a marker of poor sperm quality and is essentially detectable only in low motile and morphologically abnormal spermatozoa in humans and in bulls (O'Bryan et al. 1994; Ibrahim et al. 2000; Martínez-Heredia et al. 2008; Muciaccia et al. 2010) moreover the low number of CLU transcripts detected in our Percoll-selected sample agrees with a recent paper that showed a higher content of the transcript in motility-impaired bull semen compared with a good-quality one (Kumar et al. 2015).

Since sperm HSPs and CLU, like other proteins, are probably synthesized prior to chromatin condensation, their presence cannot be related to the residual mRNA present in the mature cells. Although the presence of transcripts should be representative of past cellular events during spermatogenesis (Ostermeier et al. 2002; Lambard et al. 2004; Dadoune et al. 2005) the presence of relatively abundant amounts of HSP70 and HSP90 mRNA in Percoll-selected spermatozoa could also be related to the ability to perform active translation of new proteins in developing embryos before maternal genome activation. The importance of heat shock proteins in developing embryos is well known (Neuer et al. 2000; Zhang et al. 2011; Driver and Khatib 2013), though the paternal RNA contribution to protein synthesis must be more precisely defined. A further study with the employment of an higher number of boars and with the possibility to utilize a CASA system to evaluate semen parameters, will be useful to confirm our preliminary data and verify the correlation between the transcripts and semen quality and to

evaluate if these transcripts are active and related to embryonic development after fertilization, as hypothesized by some authors (Ostermeier et al. 2004; Kempisty et al. 2008; Hwang et al. 2013).

## **Conclusion**

This is the first report on differential abundance of HSP70, 90 and clusterin transcripts in selected porcine spermatozoa: the level of expression of HSP was high while CLU expression was very low. The quantitative analysis of mRNA of HSPs that selected spermatozoa derived from high quality of the semen show similar and high level of expression suggesting a potential role of these transcripts as reliable markers of semen quality.

## **Compliance with ethical standards**

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** All applicable international and institutional guide-lines for the care and use of animals were followed

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**Table caption**

**Table 1** Forward and reverse primer sequences, RT-PCR length (bp) and accession numbers (Acc. No.) in the NCBI database.

### Figure legends

**Fig. 1** Percentage of viability and motility and concentration values of ejaculated spermatozoa from different boars (B1-B7) evaluated before density gradient. The data are expressed as mean  $\pm$  S.D. Different letters indicate statistically significant differences ( $P < 0.05$ , ANOVA post hoc Tukey test) among boars.

**Fig. 2** Relative gene expression of HSP70, HSP90 and clusterin (CLU) in ejaculated spermatozoa, after Percoll selection, of different boars (B1-7). mRNA data are presented as  $\Delta C_t$  method (reference gene Ct – gene of interest Ct). Error bars represent the range of expression. Different lower case or different capital letters indicate statistically significant differences among boars ( $P < 0.05$ , ANOVA post hoc Tukey test) within HSP70 (white histograms, lower case) or within HSP90 (grey histograms, capital letters) gene expression. No statistically significant differences were observed in CLU gene expression (black histograms).

GI = interest gene (HSP70 or HSP90 or CLU).

**Fig. 3** A) Expression of HSP70 (white histograms) and HSP90 (grey histograms) in ejaculated spermatozoa, after Percoll selection, of different boars (B1-7). The data are represented as mean  $\pm$  SEM of relative protein content (AU = arbitrary units). B) Representative images of western blotting of HSP70 and 90 in ejaculated spermatozoa. MWM molecular weight marker.