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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

62

63

64 **1. Introduction**

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

75 Messenger RNAs have been found in the mature mammalian spermatozoa of several species (Dadoune et
76 al. 2005; Gilbert et al. 2007; Yang et al. 2009). In swine, transcriptome analysis of ejaculated
77 spermatozoa has been performed, identifying many mRNAs involved in biological processes, molecular
78 functions and cellular components (Yang et al. 2009). More recently, the presence of various transcripts
79 has been investigated in order to describe mRNA involvement in capacitation and early embryo
80 development in pigs (Hwang et al. 2013). It is well known that semen quality can be influenced by
81 occasional physical stress, infection, and variation in temperature (Althouse et al. 2000; Althouse and Lu
82 2005; Kunavongkrit et al. 2005; Knox et al. 2008; Althouse 2008) and the definition of new markers for
83 evaluation of semen could offer knowledge about the fertility potential of a semen sample. Semen quality
84 assessment represents a fundamental step for obtaining successful artificial insemination. In addition to
85 the conventional semen parameter evaluation, an increasing number of innovative molecular markers, at
86 proteomic and transcriptomic level, have been used to identify more reliable markers of sperm quality
87 and fertility. In bulls, mRNA abundance of specific sperm proteins has been related to a fertility index,
88 allowing the determination of biomarkers able to predict the fertility of subjects (Feugang et al. 2010;
89 Kasimanickam et al. 2012).

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

93 as playing a role in sperm-oocyte interaction and sperm motility respectively (Huang et al. 2000b; Spinaci
94 et al. 2005; Volpe et al. 2008). No correlation between HSP70 and semen quality was found (Turba et al.
95 2007) while HSP90 was investigated only in relation to the quality of cryopreserved semen (Wang et al.
96 2014; Zhang et al. 2015). Clusterin (CLU), a pleiotropic glycoprotein mainly present in seminal plasma
97 has also been found to be associated with spermatozoa in various species (Sylvester et al. 1991; Ibrahim
98 et al. 1999; Ibrahim et al. 2001; Han et al. 2012). In human and bull sperm, clusterin mainly exists on the
99 surface of immature, low motile or morphologically abnormal spermatozoa and therefore its presence has
100 been proposed as a marker of poor sperm quality (O'Bryan et al. 1994; Ibrahim et al. 2000; Muciaccia et
101 al. 2010).

102 Until now the presence and the levels of the transcripts of these important proteins (HSP70, HSP90 and
103 CLU) in pig sperm cells have not been clearly described. Only a single paper reported the presence of
104 CLU transcript, suggesting a role of the paternal mRNA in early embryo development (Kempisty et al.
105 2008).

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

109

110 **2. Materials and methods**

111

112 **2.1 Animal semen collection and quality evaluation parameters**

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

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

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

120

121 **2.2 Semen collection and evaluation of semen quality**

122 Sperm samples (n= 21) were collected by an experienced technician using the gloved-hand technique and
123 a dummy. The sperm-rich fraction was collected in a pre-warmed thermos and was diluted 1:1 (v:v) with
124 swine fertilization medium (SFM - home made extender as previously reported (Fantinati et al. 2009))
125 and immediately transported to the laboratory for evaluation of semen parameters.
126 The concentration of each ejaculate was assessed after spermatozoa immobilization in 1.5 M NaCl
127 solution under a phase-contrast microscope at 400x using a Thoma counting chamber. Three replicates
128 for each ejaculate were analysed.
129 The spermatozoa viability was evaluated by eosin-nigrosin staining. A working solution was prepared
130 with two parts of 10% (w/v) eosin and one part of 5% (w/v) nigrosin. The sperm suspension and the
131 working solution were mixed 1:1 (v:v) and the viability was evaluated. At least 200 cells were observed
132 (Nikon Eclipse E600, Nikon Corporation, Japan). Three replicates for each ejaculate were analysed.
133 The sperm motility was subjectively assessed by visual estimation under a phase contrast microscope
134 equipped with a heated plate. Three replicates for each ejaculate were analysed. A total of 10 fields were
135 evaluated.
136
137 **2.3 Percoll density gradient of swine spermatozoa**
138 In order to collect highly motile and morphologically normal spermatozoa, without somatic cell
139 contaminants or debris, the spermatozoa samples, previously diluted with SFM, were separated by
140 centrifugation through a discontinuous Percoll (Sigma-Aldrich St Louis, MO, USA) density gradient
141 (45/90, v/v; (Matàs et al. 2011)). A bilayer of 2 ml each of Percoll solution with different density (45 and
142 90 in the bottom) was prepared in 15 ml conic centrifuge tubes; then diluted semen samples (5- 6 x 10^7
143 sperms/2ml/tube) were added at the top, care being taken to maintain the integrity of the layers.
144 Samples were then centrifuged for 30 min at 700g. The sperm pellet was collected from the 45/90
145 interfaces, and then washed twice with DPBS (1ml). Sperm sample concentration, viability and motility
146 was then evaluated, as described above. Aliquots of $5x10^6$ spermatozoa for each ejaculate were frozen
147 and stored at -80°C until RNA and protein analysis.
148
149 **2.4 RNA isolation and quantitative real-time PCR (qRT-PCR) for HSP70, HSP90 and CLU**
150 Total RNA from purified spermatozoa samples ($5x10^6$) was extracted using NucleoSpin RNA II
151 (Macherey Nagel, Düren, Germany) and triplicates for each ejaculate were analysed. The lysis of the cells
152 was carried out with Tissue Lyser TL (QIAGEN, Hilden, Germany) in the presence of two beads (50Hz

153 for 5 min). Total RNA was extracted according to the manufacturer's instructions, except for double
154 treatment with DNase (instead of single DNase treatment as suggested). RNA was spectrophotometrically
155 quantified (DeNovix Inc, Wilmington, DE, USA) and its quality was determined by gel electrophoresis
156 on 1% agarose. Then 1 µg of RNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis
157 Kit (Bio-RAD Laboratories Inc., California, USA), in a final volume of 20 µl. Real time quantitative PCR
158 was performed using an iCycler Thermal Cycler (Bio-RAD). Primers for swine HSP70, HSP90 and CLU
159 were designed using Beacon Designer 2.07 software (Premier Biosoft International, Palo Alto, CA,
160 USA). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize mRNA
161 levels across different samples. Primer sequences are shown in Table 1.
162 A master mix of the following reaction components was prepared in the end-concentrations indicated: 0.5
163 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
164 BioRad Supermix (Bio-RAD). Two µl of cDNA were added to 23 µl of the master mix. All samples were
165 analysed in duplicate. The real-time PCR protocol employed was: initial denaturation for 3 min at 95°C,
166 40 cycles at 95°C for 15 sec and at 60°C for 30 sec, followed by a melting step with slow heating from
167 55°C to 95°C at a rate of 0.5C/sec. Primer specificity as well as real-time efficiency was evaluated in
168 previously collected porcine testicular tissue (positive control).
169 Contamination of epithelial cells, germ cells and leucocyte (CDH1, KIT and CD4) in spermatozoa RNA
170 was preliminary verified using specific primers (table 1); the positive control used for the assay was
171 porcine testicular cDNA or cDNA synthetized from RNA purified from blood (Kumar et al., 2010).
172 The gene expression level of HSP70, HSP90 and CLU was evaluated as ΔCt (threshold Cycle) = (Ct
173 $_{reference\ gene}$ – $Ct_{gene\ of\ interest}$) which directly correlates with the expression level.
174

175 **2.5 Western blot for HSP70, HSP90 and CLU**

176 The spermatozoa samples ($5x10^6$ /sample) were resuspended in SDS buffer (Tris-HCl 62.5 mM pH 6.8;
177 SDS 2%, glycerol 20%) and duplicates for each ejaculate were analysed. Proteins from spermatozoa
178 ($1x10^6$ for HSP 70 and HSP90, $2x10^6$ for CLU) were separated by NuPage 10% Bis–Tris Gel (Gibco-
179 Invitrogen, Paisley, UK) for 50 min at 200 V, and then electrophoretically transferred onto a
180 nitrocellulose membrane. HSP70, HSP90 detection was performed as previously reported (Volpe et al.
181 2008), while CLU western blotting was performed as previously reported (Zannoni et al. 2015). Briefly,
182 the nitrocellulose membranes were blocked with 3% milk powder in PBS-T20 (phosphate buffer saline-
183 0.1% tween-20) for one hour at room temperature. The membranes were then incubated overnight at 4°C

184 with a 1:1000 dilution of monoclonal anti-human HSP70 (C92F3A-5 Stressgen Biotechnologies Corp.
185 Victoria, BC, Canada), monoclonal anti-human HSP90 (AC88 Stressgen) and monoclonal anti-human
186 CLU (05-354 Upstate Millipore Corp. Temecula, CA, USA), in Tris Buffered Saline-T20 (TBS-T20
187 20mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). After several washings with PBS-T20 the
188 membranes were incubated with the secondary biotin-conjugate antibody and then with a 1:1000 dilution
189 of an anti-biotin horseradish peroxidase (HRP)-linked antibody. All western blots were developed using
190 chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology,
191 Inc., Rockford, IL, USA) according to the manufacturer's instructions. All membranes were stripped and
192 reprobed for β-tubulin in order to normalize the results. The intensity of the resultant bands was acquired
193 using Fluor-S^{TM} Multimager and quantified by Quantity One software (Bio-RAD). The relative protein
194 content was determined by the density of the resultant bands and expressed in arbitrary units (AU)
195 relative to the β-tubulin content, using the Quantity One software (Bio-RAD).
196
197 **2.6 Statistical analysis**
198 Data were analysed by one-way analysis of variance (ANOVA) and post hoc Tukey HSD test. The
199 statistical significance was set at $P < 0.05$.
200
201 **3. Results**
202
203 **3.1 Sperm parameters: viability, motility and concentration**
204 Data on viability, motility and spermatozoa concentration are shown in Fig.1. All the ejaculates collected
205 from six out of the seven boars showed ejaculates of high sperm quality. One boar (B2) showed seminal
206 parameters significantly different from the others and below acceptability for use in artificial
207 insemination (AI) (Fig. 1). After Percoll separation the values of motility (92 ± 3.7 %) and viability (93
208 ± 2.4 %) were similar for all boars.
209
210 **3.2 Quantitative real-time PCR (qRT-PCR) for HSP70, HSP90 and CLU**
211 Quantitative PCR experiments performed to verify the presence of contaminants mRNA showed that the
212 selected mRNA markers were not detectable.
213 Quantitative PCR data demonstrated that the HSP70 and HSP90 mRNAs were abundant in all samples
214 whilst CLU mRNA was very low. No statistically significant differences were observed in the gene

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

217 **3.3 Western blots for HSP70, HSP90 and CLU**

218 Western blot analysis for HSPs showed in all samples a specific band (Fig. 3B) as previously reported by
219 us (Volpe et al. 2008). No statistically significant differences were observed in the expression levels of
220 the protein among the seven boars (Fig. 3A).
221 No signal was observed for CLU protein in sperm cell samples, but only in the positive control of aortic
222 porcine endothelial cells (data not shown).
223

224 **4. Discussion**

225 A growing interest is directed towards the presence of mRNA in mammalian ejaculated spermatozoa.
226 Some findings suggested that these transcripts can code for proteins, which are essential in early embryo
227 development or for cellular function. Other studies reported that a semen quality evaluation could be
228 derived from RNA profiling which might provide clinical markers for male infertility (Jodar et al. 2013).
229 In swine species, over the past 10 years, transcriptome analysis has identified many mRNAs involved in
230 different biological processes (Yang et al. 2009). More recently, the presence of various transcripts has
231 been investigated in order to clarify their involvement in early embryo development (Hwang et al. 2013).
232 The present study aimed to evaluate the expression of HSP70, HSP90 and CLU in spermatozoa of boar
233 routinely employed in AI.
234 Our study considered seven boars of different ages. One of these boars (B2), at the time of the study,
235 presented seminal parameters unsuitable for AI because of transient genitourinary infection but was
236 nonetheless included in the study to obtain Percoll-selected spermatozoa from low-quality semen.
237 Results showed the presence of HSP70, HSP90 and CLU transcripts. In the literature a CLU transcript
238 has been reported (Kempisty et al. 2008) while only the presence of a transcript member of the HSP70
239 family (HSP70.2) has been described in porcine spermatozoa (Yang et al. 2009). The number of
240 transcripts differed, with HSP70 and HSP90 relatively highly abundant with respect to the chosen
241 housekeeping gene while CLU was very low or undetectable in some samples.
242 At the protein level, we detected similar amounts of HSP70 and 90 among all seven boars, but the CLU
243 specific band was not detectable in any sample. A previous paper showed how an aggressive
244 manipulation such as cell sorting does not affect the quantity of the expressed protein in boar

245 spermatozoa (Spinaci et al.,2010), therefore we are confident that a much more mild procedure, such as
246 Percoll selection, will not influence the protein levels.
247 Even if our data may be considered preliminary, it's interesting to note that the HSPs transcript seem to
248 be more informative than HSP protein in relation to the sperm quality. Indeed, six boars with high sperm
249 quality showed high and similar level of HSPs transcripts while the boar (B2), with low sperm quality,
250 showed a reduced level of HSPs transcripts. The existence of the correlation, if confirmed in future
251 studies, allows us to speculate that, even if selected and able to fertilize, sperm cells deriving from a poor-
252 quality semen sample have characteristics that make them unfit to sustain embryonic development.
253 Previous findings about the absence or low correlation between HSP70 protein level and sperm motility
254 and viability were reported (Huang et al. 2000a; Turba et al. 2007) being that HSP70 seems to be more
255 correlated with morphological abnormalities (Huszar et al. 2000; Turba et al. 2007).
256 It is important to underline that, in the present work, protein detection was performed after Percoll
257 selection of spermatozoa, and, therefore the lack of differences in HSPs content among the different boars
258 may be related to the selection of only highly motile, good-quality spermatozoa, probably all rich in
259 HSPs. The opposite may obtain concerning the absence of CLU protein. CLU is reported in the literature
260 as a marker of poor sperm quality and is essentially detectable only in low motile and morphologically
261 abnormal spermatozoa in humans and in bulls (O'Bryan et al. 1994; Ibrahim et al. 2000; Martínez-
262 Heredia et al. 2008; Muciaccia et al. 2010) moreover the low number of CLU transcripts detected in our
263 Percoll-selected sample agrees with a recent paper that showed a higher content of the transcript in
264 motility-impaired bull semen compared with a good-quality one (Kumar et al. 2015).
265 Since sperm HSPs and CLU, like other proteins, are probably synthesized prior to chromatin
266 condensation, their presence cannot be related to the residual mRNA present in the mature cells.
267 Although the presence of transcripts should be representative of past cellular events during
268 spermatogenesis (Ostermeier et al. 2002; Lambard et al. 2004; Dadoune et al. 2005) the presence of
269 relatively abundant amounts of HSP70 and HSP90 mRNA in Percoll-selected spermatozoa could also be
270 related to the ability to perform active translation of new proteins in developing embryos before maternal
271 genome activation. The importance of heat shock proteins in developing embryos is well known (Neuer
272 et al. 2000; Zhang et al. 2011; Driver and Khatib 2013), though the paternal RNA contribution to protein
273 synthesis must be more precisely defined. A further study with the employment of an higher number of
274 boars and with the possibility to utilize a CASA system to evaluate semen parameters, will be useful to
275 confirm our preliminary data and verify the correlation between the transcripts and semen quality and to

276 evaluate if these transcripts are active and related to embryonic development after fertilization, as
277 hypothesized by some authors (Ostermeier et al. 2004; Kempisty et al. 2008; Hwang et al. 2013).
278
279 **Conclusion**
280 This is the first report on differential abundance of HSP70, 90 and clusterin transcripts in selected porcine
281 spermatozoa: the level of expression of HSP was high while CLU expression was very low. The
282 quantitative analysis of mRNA of HSPs that selected spermatozoa derived from high quality of the semen
283 show similar and high level of expression suggesting a potential role of these transcripts as reliable
284 markers of semen quality.
285
286
287 **Compliance with ethical standards**
288
289 **Conflict of interest** The authors declare that there is no conflict of interest.
290
291 **Ethical approval** All applicable international and institutional guide-lines for the care and use of animals
292 were followed
293
294
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298
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398 **Table caption**
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400 **Table 1** Forward and reverse primer sequences, RT-PCR length (bp) and accession numbers (Acc. No.) in
401 the NCBI database.
402
403 **Figure legends**
404
405 **Fig. 1** Percentage of viability and motility and concentration values of ejaculated spermatozoa from different
406 boars (B1-B7) evaluated before density gradient. The data are expressed as mean ± S.D. Different letters
407 indicate statistically significant differences (P < 0.05, ANOVA post hoc Tukey test) among boars.
408
409 **Fig. 2** Relative gene expression of HSP70, HSP90 and clusterin (CLU) in ejaculated spermatozoa, after
410 Percoll selection, of different boars (B1-7). mRNA data are presented as ΔCt method (reference gene Ct –
411 gene of interest Ct). Error bars represent the range of expression. Different lower case or different capital
412 letters indicate statistically significant differences among boars (P < 0.05, ANOVA post hoc Tukey test)
413 within HSP70 (white histograms, lower case) or within HSP90 (grey histograms, capital letters) gene
414 expression. No statistically significant differences were observed in CLU gene expression (black
415 histograms).
416 GI = interest gene (HSP70 or HSP90 or CLU).
417
418 **Fig. 3** A) Expression of HSP70 (white histograms) and HSP90 (grey histograms) in ejaculated spermatozoa,
419 after Percoll selection, of different boars (B1-7). The data are represented as mean ± SEM of relative protein
420 content (AU = arbitrary units). B) Representative images of western blotting of HSP70 and 90 in ejaculated
421 spermatozoa. MWM molecular weight marker.