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A. Zannoni *, C. Bernardini, A. Zaniboni, E. Ferlizza, D. Ventrella, M. L. Bacci, M. Forni

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Department of Veterinary Medical Sciences – DIMEVET- University of Bologna, Via Tolara di Sopra 50,

Ozzano dell'Emilia, 40064 Bologna, Italy

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Corresponding author A. Zannoni

Tel.: +39.051.2097911; fax +39.051.2097899; e-mail: augusta.zannoni@unibo.it (A. Zannoni)

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32 **Abstract**

33 It is widely accepted that mature sperm contains RNA. The first hypothesis was that sperm RNAs have

34 no functions of their own but are simply residues of spermatogenesis reflecting the events that occurred

35 during their formation in the testes. More recently new discoveries have essentially expanded these

36 views, showing that sperm mRNAs constitute a population of stable full-length transcripts, many of

37 which are selectively retained during spermatogenesis and delivered to oocytes contributing to early

38 embryo development. It is well known that semen quality can be influenced by occasional physical stress,

39 infection, and variation in temperature and the definition of new markers for evaluation of semen could

40 offer knowledge about the fertility potential of a semen sample. The aim of the present study was to

41 evaluate the presence and the relative quantity of transcripts and protein of heat shock protein 70

42 (HSP70), 90 (HSP90) and clusterin (CLU) in Percoll-selected spermatozoa collected from seven adult

43 boars of proven fertility routinely employed for artificial insemination. Our results showed the presence

44 of HSP70, HSP90 and CLU transcripts with different level of expression: high for HSPs and low for CLU

45 transcripts. The transcript level of both HSPs are similar among selected spermatozoa derived from high

46 quality sperm with the exception of one boar that showed a reduced content of HSP70 and HSP90 mRNA

47 together with a lower semen quality. At protein level, both HSPs were detected with similar amount

48 among all seven boars whilst no band was evidenced for CLU protein.

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52 Keywords: boar; clusterin; HSP70; HSP90; sperm

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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

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 ± 2C) 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 x 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 5×10^6 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 (5×10^6) 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 0.5C/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 synthetized from RNA purified from blood (Kumar et al., 2010).

The gene expression level of HSP70, HSP90 and CLU was evaluated as ΔCt (threshold Cycle) = ($Ct_{\text{reference gene}} - Ct_{\text{gene of interest}}$) which directly correlates with the expression level.

2.5 Western blot for HSP70, HSP90 and CLU

The spermatozoa samples (5×10^6 /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 (1×10^6 for HSP 70 and HSP90, 2×10^6 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

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.

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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.

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397

398   **Table caption**

399

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.