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Beta-mercaptoethanol supplementation of in vitro maturation medium does not influence nuclear and cytoplasmic maturation of equine oocytes

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11 influence nuclear and cytoplasmic maturation of equine oocytes

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19

20 Running head: Beta-mercaptoethanol and horse oocyte maturation

21

22 **Abstract**

23 *In vitro* embryo production in the horse is still not as efficient as in other species.
24 Oxidative stress negatively affect oocyte and embryo culture. To attenuate/minimize the
25 oxidative stress, antioxidants such as low molecular thiol compounds can be added to
26 culture media. Beta-mercaptoethanol (BME) has been shown to improve maturation and
27 embryo development in different species. The aim of this study was to investigate
28 whether the addition to maturation medium of BME at common (0.1mM) and high (0.7
29 mM) concentration could improve oocyte maturation also in the horse. Equine oocytes
30 recovered from slaughterhouse ovaries were used. Meiotic configuration after *in vitro*
31 maturation (IVM) and early embryo production after intra cytoplasmic sperm injection
32 (ICSI) were considered as criteria for assessing nuclear and cytoplasmic maturation
33 respectively. A total of 1076 oocytes were analyzed over 2 experiments: 848 (control
34 n=293, BME 0.1 n=270, BME 0.7 n=285) were stained with Hoechst 33342 and
35 examined for nuclear stage after 26 h of IVM, and 228 MII oocytes were fertilized by
36 ICSI (control n=83, BME 0.1 n=65, BME 0.7 n=80). Cleavage rates were determined
37 after 60 h of culture. Unlike results obtained in other species, the addition of BME did
38 not influence maturation rates (51.9 % control vs 55.6% BME 0.1 mM and 55.1% BME
39 0.7 mM), nor cleavage rates after ICSI (38.6% vs 38.5% and 41.3% respectively). In
40 conclusion, the addition of BME at 0.1 and 0.7 mM to the maturation medium, in our
41 culture conditions, has no effect on nuclear and cytoplasmic maturation of equine
42 oocytes.

43

44 **Keywords:** equine, oocyte maturation, beta-mercaptoethanol, ICSI

45

46 **Introduction**

47 Despite assisted reproductive technologies over past decades have improved
48 reproductive performances in domestic animals, and in some countries embryo
49 technologies found extended practical application in livestock production, *in vitro*
50 embryo production (IVEP) in the horse is still not as efficient as in other species.
51 Conventional *in vitro* fertilization (IVF) is inadequate and *in vitro* blastocyst production
52 can be achieved only by ICSI.
53 Compared to the *in vivo* environment, *in vitro* culture conditions entail higher oxygen
54 concentrations, producing an increased level of reactive oxygen species (ROS), with
55 oxidative damage to DNA, lipids, proteins and consequently result in cell growth arrest
56 and cell death of preimplantation embryos (Tsunoda et al., 2014). Glutathione (GSH), a
57 tripeptide thiol (c-L-glutamyl-L-cysteinylglycine), is the major non-protein sulfhydryl
58 compound in mammalian cells that plays an important role in the maintenance and
59 regulation of the thiol redox status of the cell, thus protecting cell from oxidative
60 damage (Deleuze and Goudet, 2010). Moreover, it is known that sperm nuclear
61 decondensation is related to the amount of GSH in the cytoplasm of oocytes
62 (Yanagimachi, 1994).
63 The addition of low molecular thiol compound, such as beta-mercaptoethanol (BME),
64 cysteamine, cysteine and cystin, to *in vitro* maturation (IVM) medium increases GSH
65 synthesis (de Matos et al., 1995; de Matos and Furnus, 2000) and leads to low oxidative
66 stress in many species (Abeydeera et al., 1998; de Matos et al., 2002; Kim et al., 2004;
67 Songsasen and Apimeteetumrong, 2002; Rodriguez-Gonzalez et al., 2003).
68 Nevertheless, not all the concentrations and the different thiol compounds influenced
69 positively maturation and embryo production in different species (de Matos et al., 2002;
70 Songsasen et al., 2002).

71 In the horse, the addition of cysteamine to maturation medium did not affect GSH
72 content and maturation rate (Luciano et al., 2006) nor the ability of the oocyte to
73 undergo *in vitro* and *in vivo* fertilization (Deleuze et al., 2010). Anyway, no further
74 reports are available on the efficacy of other thiol compounds in the equine IVEP
75 system.

76 The aims of this study were to investigate whether the addition to maturation medium of
77 BME improves: 1) nuclear maturation of horse oocytes, 2) early embryonic
78 developmental capability after ICSI.

79

80 **Materials and methods**

81 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless
82 otherwise stated.

83

84 *Collection and culture of cumulus oocyte complexes*

85 Mare ovaries were collected, at a slaughterhouse during October-May, and transported
86 to the laboratory within 2-3 h at 25°C in a thermos case (Cell Incubator, IMV
87 Technologies, Italy). Upon arrival, the ovaries were dissected free from connective
88 tissue, washed with 30°C tap water and transferred to 0.9% (w/v) saline solution
89 supplemented with 0.1% (v/v) penicillin/streptomycin. The cumulus-oocyte complexes
90 (COCs) were recovered by aspirating the contents of 5–30 mm follicles, using a 19-
91 gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg). The fluid
92 containing the COCs was collected into 250 ml glass flasks (Duran Group, Germany)
93 and filtered through a 65 µm mesh nylon filter (EmSafe, Minitube, Germany). COCs
94 with at least 3-5 layers of cumulus investment were classified as compact (having a
95 tight, complete compact cumulus with a distinct, smooth hillock), expanded (having a
96 granular or expanded cumulus), or denuded (having a partial cumulus or only corona

97 radiata present) (Hinrichs et al., 1993). For IVM, groups of 25-30 compact COCs were
98 cultured for 26 h in 500 µl maturation medium without (n=452) or with beta-
99 mercaptoethanol (0.1 mM n=390 and 0.7 mM n=428) in four-well plates (Scientific
100 Plastic Labware, EuroClone, Italy) at 38.5°C, in a humidified atmosphere of 5% CO₂ in
101 air. Maturation medium consisted of Dulbecco Modified Eagle Medium Nutrient
102 Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10%
103 (v/v) heat-inactivated fetal calf serum (FCS; Gibco, Life Technologies, Italy), 25 µl/ml
104 ITS (insulin, transferrin, sodium selenite) supplement, 50 ng/ml epidermal growth
105 factor, 100 ng/ml insulin-like growth factor 1, 10 IU/mL equine chorionic gonadotropin
106 (Folligon, Intervet, Italy), and 10 IU/mL human chorionic gonadotropin (Corulon,
107 Intervet, Italy).

108

109 *Experiment 1: Effect of BME on nuclear maturation*

110 At the end of the maturation period oocytes were partially denuded by incubation in
111 HEPES Synthetic Oviductal Fluid (HSOF) containing 25 µg/ml hyaluronidase followed
112 by aspiration through a pipette tip. Remaining cumulus cells were removed by
113 incubating the oocytes for 1.5 min in a 0.25% (w/v) solution of trypsin in HSOF before
114 transfer to HSOF supplemented with 10% FCS and repeated aspiration through a fine
115 glass pipette.

116 Oocytes were stained with 1 µg/mL bisbenzimidazole fluorescent dye (Hoechst 33342) in
117 PBS (Phosphate Buffered Solution) for 30 min at room temperature, washed in PBS and
118 observed under a fluorescent microscope (Nikon Eclipse E 400, Japan) to assess
119 chromatin configuration.

120 The meiotic stage of each oocyte after Hoechst staining was characterized as previously
121 described (Bezard et al., 2002): GV: diffuse fluorescence of the oocyte nucleus often
122 presenting a cloudy or partly filamentous chromatin pattern (Fig. 1A); diakinesis (Dia):

123 distinct fine filamentous pattern of often loop-shaped slightly condensed chromatin,
124 forming an irregularly shaped web (Fig. 1B); dense chromatin (dCh): one spot of
125 condensed chromatin, occasionally with an irregular outline; no filaments visible (Fig.
126 1C); MI: one set of chromosomes oriented in an equatorial plate (Fig. 1D); MII: one set
127 of chromosomes oriented in an equatorial plate and another more irregularly arranged
128 (Fig. 1E); degenerated (Deg): no or small spots of condensed chromatin sometimes
129 dispersed throughout the oocyte (Fig. 1E). Only oocytes that reached the MII stage were
130 considered matured.

131

132 *Experiment 2: Effect of BME on early embryo development after ICSI*

133 Denuded oocytes with a normal MII appearance, including an extruded first polar body
134 (PB), were considered suitable for ICSI. Frozen-thawed semen from a stallion of proven
135 fertility was used. After thawing in a water bath at 37°C for 30 sec, the sperm was
136 prepared as describe for IVF and diluted in SOF-IVF to a final concentration of 4×10^6
137 spermatozoa/ml. Finally, the sperm suspension was diluted 1:1 (v/v) with a 12%
138 solution of polyvinylpyrrolidone (PVP) in PBS. Pipettes produced using a glass
139 micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA) were used for
140 holding oocytes and for sperm injection. ICSI was performed at 37°C using a
141 micromanipulator (Narishige Co. Ltd, Tokyo, Japan) equipped with a Piezo
142 micropipette-driving unit (Prima Tech, Ibaraki, Japan) and mounted on an inverted
143 microscope (Nikon TE 300: Nikon, Kawasaki, Japan). A motile sperm was immobilized
144 by applying two or three piezo-pulses to its tail-midpiece region, and it was then
145 aspirated into the tip of the injection needle. The oocyte for injection was immobilized
146 using the holding pipette and orientated with its PB at 06:00 or 12:00 h. The ICSI
147 needle was then advanced through the zona pellucida and oolemma at 15:00 h using the
148 piezo-drilling motion, and the sperm was released into the ooplasm.

149 Following ICSI, oocytes were denuded and groups of ten were cultured in 20 µl droplets
150 of SOF supplemented with MEM amino acids and 16 mg/ml FAF- BSA (SOF-IVC)
151 under mineral oil at 38.5°C in an atmosphere of 5% CO₂, 7% O₂, and 88% N₂ for 60 h,
152 before cleavage assessment.

153 Cleaved embryos were stained with Hoechst 33342 (1 µg/mL PBS) for 30 min at room
154 temperature then transferred on a glass slide, covered with a coverslip, and examined
155 under a the above described fluorescent microscope to confirm the presence of cell
156 nuclei.

157

158 *Experimental design*

159 Experiment 1 was performed in 12 replicates. Horse oocytes were matured in standard
160 medium (Control group, n=293) and in the same medium supplemented with 0.1 mM
161 (BME 0.1 group, n=270) and 0.7 mM (BME 0.7 group, n=285) beta-mercaptoethanol.
162 After maturation oocytes were denuded, stained and observed for meiotic configuration.

163 Experiment 2 was performed in 15 replicates. IVM oocytes (Control n=159, BME 0.1
164 group n=120, BME 0.7 group n=143) were fertilized by ICSI (Control n=83, BME 0.1
165 n=65, BME 0.7 group n=80), then cultured for 60 h before cleavage assessment.

166

167 *Statistical analysis*

168 Data were analyzed using Chi Square test (IBM SPSS Statistics 23, IBM Corporation,
169 Milan, Italy). Significance has been assessed for P<0.05.

170

171 **Results**

172

173 *Experiment 1: Effect of BME on nuclear maturation*

174 A total of 848 oocytes were analysed. Overall maturation rate was 54.1%. Nuclear
175 maturation and all the other meiotic configurations were not statistically different
176 ($P>0.05$) between oocytes cultured with or without BME (Table. 1).

177

178 *Experiment 2: Effect of BME on early embryo development after ICSI*

179 A total of 228 oocytes were evaluated after ICSI. Overall cleavage rate was 39.5%
180 (range 0-80%). No significant differences ($P>0.05$) were observed in cleavage rate nor
181 in early embryo development between oocytes cultured with or without BME (Table.
182 2).

183

184 **Discussion**

185

186 The aim of the present study was to improve nuclear and cytoplasmic maturation by
187 adding BME to a chemically defined IVM medium for mare oocytes. The results
188 showed that there was no significant difference in the proportions of *in vitro* matured
189 oocytes among treatments. The overall nuclear maturation rate was 54.1%, and it is
190 similar to previous reports in the horse (Hall et al., 2013).

191 Studies in other species, such as buffalo (Songsasen and Apimeteetumrong, 2002) and
192 bovine (Takahashi et al., 1993; de Matos et al., 1996; Lim et al., 1996), demonstrated
193 that, although supplementation of BME to maturation medium did not increase nuclear
194 maturation and pronuclear formation, it improves quality and developmental
195 competence of embryos produced from oocytes matured in its presence.

196 Supplementation of BME improved buffalo (100 μ M) (Sadeesh et al., 2014) and pre-
197 pubertal goat (5 μ M) oocyte *in vitro* maturation (Ly et al., 2010) while 200 μ M BME
198 had no effect in sheep (de Matos et al., 2002).

199 Methaphase II rates, after *in vitro* maturation of canine oocytes, increased by adding
200 100 μ M BME (Kim et al., 2004) but not 25 μ M BME (Songsasen et al., 2002).
201 Reported concentrations of BME in various species range from 5 μ M to 50 mM (Ly et
202 al., 2010); Yadav et al., 2013). In buffalo, 25 μ M BME reduced the degeneration rate
203 during IVM (Ullah et al., 2006), 100 μ M BME plus 20 ng/ml EGF was the best
204 combination increasing both MII and cleavage rates (Sadeesh et al., 2014), 0.5 mM
205 BME increased maturation rate and decreased degeneration (Nasiri and Beheshti, 2012),
206 while 10 mM BME did not influenced nuclear maturation and blastocyst production rate
207 but improved the proportion of oocytes that exhibited synchronous pronuclei formation
208 and the embryo quality (Songsasen and Apimeteetumrong, 2002).
209 Therefore it seems that effect of BME supplementation to maturation medium is highly
210 species-specific and concentration dependent. In the horse this is the first report on the
211 use of BME during IVM, so a standard concentration (0.1 mM) and a higher one (0.7
212 mM) were chosen. In the few studies using a thiol compound during equine IVM, 100
213 μ M cysteamine was added to maturation medium failing to positively affect the
214 maturation rate of equine oocytes (Luciano et al., 2006; Deleuze et al., 2010) and GSH
215 content in MII oocytes (Luciano et al., 2006).
216 It has been demonstrated that GSH synthesis is influenced by other substances
217 contained in maturation media, such as serum, that inhibited it, and FSH, that induced a
218 higher GSH content in equine oocytes (Luciano et al., 2006). It is likely that maturation
219 conditions used in our research influenced the GSH synthesis, and consequently the
220 possible BME influence, since FBS and eCG instead of FSH were used. Anyway, even
221 when GSH levels after IVM were similar to those of *in vivo* matured oocytes, *in vitro*
222 maturation, fertilization and early developmental competencies of equine oocytes were
223 not enhanced (Luciano et al., 2006). It seems that other factors beyond oxidative stress
224 are involved in equine oocyte maturation, fertilization and early embryo development.

225 Nevertheless, further studies are needed to investigate if a beneficial effect of adding
226 antioxidants is evident at a later embryo developmental stage, in terms of blastocyst
227 yield and embryo quality.

228 After ICSI we observed a similar cleavage rate after 60 h of culture, and also a similar
229 embryo development beyond the 2 cell stage, confirming that the developmental
230 capability was not affected by the addition of BME, as already observed for cysteamine
231 after ICSI (Luciano et al., 2006) and *in vivo* fertilization (Deleuze et al., 2010).

232 In the present study, the efficiency of ICSI (overall 39.5% cleavage rate) was lower as
233 compared to others studies (Luciano et al., 2006; Hinrichs et al., 2005; Galli et al.,
234 2007), but our percentage ranged from 0% to 80%. Beyond the operator ability, that is
235 crucial in ICSI procedures, such a wide range of variability could be related to the
236 source of oocytes. It has been recently demonstrated that the developmental stage of the
237 originating follicle population has a significant impact on chromosomal and
238 cytoplasmic properties of oocytes at the time of recovery *in vivo* (Vernunft et al., 2013).

239 Therefore, scheduling of recovery in relation to the follicle wave could potentially be
240 used to increase the quality of oocytes recovered for equine assisted reproduction
241 techniques (Vernunft et al., 2013). Ovaries collected at abattoir over a long period, as in
242 the present study, provide every time diverging material, and there is lack of
243 information about the estrous cycle stage and follicular growth. There is no repeatability
244 for oocyte quality at each replicate.

245 In conclusion, under our conditions, the addition of 0.1 and 0.7 mM beta-
246 mercaptoethanol to maturation medium do not influence nuclear maturation of horse
247 oocytes, nor the cytoplasmic maturation, assessed by their ability to undergo
248 fertilization and early embryo development after ICSI.

249

250 **Aknowlegments**

251 The Authors wish to thank Mrs Cinzia Cappannari for her precious technical support.

252

253 **Conflict of interest**

254 There is no conflict of interest

255

256 **References**

257

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341

342 **Table 1** Meiotic configuration of horse oocytes matured in vitro in absence (Control) or
 343 presence of 0.1 and 0.7 mM beta-mercaptoethanol (BME 0.1 and BME 0.7)

Group	N° oocytes	GV (%)	Dia (%)	dCh (%)	MI (%)	MII (%)	Deg (%)
Control	293	49 (16.7)	0 (0.0)	5 (1.7)	50 (17.1)	152 (51.9)	37 (12.6)
BME 0.1	270	48 (17.8)	0 (0.0)	4 (1.5)	36 (13.3)	150 (55.6)	32 (11.8)
BME 0.7	285	52 (18.2)	2 (0.7)	4 (1.4)	43 (15.1)	157 (55.1)	27 (9.5)

344 GV = germinal vesicle; Dia = diakinesis; dCh = dense chromatine; MI = metaphase I;
 345 MII = metaphase II, including oocytes from anaphase I through metaphase II; Deg =
 346 degenerate
 347

348 **Table 2** Cleavage and early embryo development after ICSI of horse oocytes matured in
349 vitro in absence (Control) or presence of 0.1 and 0.7 mM beta-mercaptoethanol (BME
350 0.1 and BME 0.7)

Group	N° oocytes	Cleavage (%)	2 cells (%)	4cells (%)	6-8 cells (%)
Control	83	32 (38.6)	12 (14.5)	10 (12.0)	10 (12.0)
BME 0.1	65	25 (38.5)	7 (10.7)	11 (16.9)	7 (10.7)
BME 0.7	80	33 (41.3)	8 (10.0)	18 (22.5)	7 (8.8)

351

352

353 **Fig. 1.** Nuclear maturation stages of equine oocytes after staining with Hoechst. (A) GV
354 with a cloudy staining of fluorescent chromatin X600, (B) Dia with staining of
355 chromatin filaments X600, (C) dCh with highly fluorescent DNA, no filaments are
356 visible X600, (D) MI with staining of chromosomes oriented in an equatorial plate
357 X600, (E) MII with staining of chromosomes oriented in an equatorial plate and of
358 irregularly arranged chromosomes of the polar body X600, (F) Deg small spots of
359 dispersed condensed chromatin.