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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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Running head: Beta-mercaptoethanol and horse oocyte maturation

Abstract

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

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

Introduction

Despite assisted reproductive technologies over past decades have improved reproductive performances in domestic animals, and in some countries embryo technologies found extended practical application in livestock production, *in vitro* embryo production (IVEP) in the horse is still not as efficient as in other species. Conventional *in vitro* fertilization (IVF) is inadequate and *in vitro* blastocyst production can be achieved only by ICSI.

Compared to the *in vivo* environment, *in vitro* culture conditions entail higher oxygen concentrations, producing an increased level of reactive oxygen species (ROS), with oxidative damage to DNA, lipids, proteins and consequently result in cell growth arrest and cell death of preimplantation embryos (Tsunoda et al., 2014). Glutathione (GSH), a tripeptide thiol (c-L-glutamyl-Lcysteinylglycine), is the major non-protein sulfydryl compound in mammalian cells that plays an important role in the maintenance and regulation of the thiol redox status of the cell, thus protecting cell from oxidative damage (Deleuze and Goudet, 2010). Moreover, it is known that sperm nuclear decondensation is related to the amount of GSH in the cytoplasm of oocytes (Yanagimachi, 1994).

The addition of low molecular thiol compound, such as beta-mercaptoethanol (BME), cysteamine, cysteine and cystin, to *in vitro* maturation (IVM) medium increases GSH synthesis (de Matos et al., 1995; de Matos and Furnus, 2000) and leads to low oxidative stress in many species (Abeydeera et al., 1998; de Matos et al., 2002; Kim et al., 2004; Songsasen and Apimeteetumrong, 2002; Rodriguez-Gonzalez et al., 2003). Nevertheless, not all the concentrations and the different thiol compounds influenced positively maturation and embryo production in different species (de Matos et al., 2002; Songsasen et al., 2002).

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

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

Materials and methods

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

Collection and culture of cumulus oocyte complexes

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

radiata present) (Hinrichs et al., 1993). For IVM, groups of 25-30 compact COCs were cultured for 26 h in 500 µl maturation medium without (n=452) or with beta-mercaptoethanol (0.1 mM n=390 and 0.7 mM n=428) in four-well plates (Scientific Plastic Labware, EuroClone, Italy) at 38.5°C, in a humidified atmosphere of 5% CO_2 in air. Maturation medium consisted of Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco, Life Technologies, Italy), 25 µl/ml ITS (insulin, transferrin, sodium selenite) supplement, 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 10 IU/mL equine chorionic gonadotropin (Folligon, Intervet, Italy), and 10 IU/mL human chorionic gonadotropin (Corulon, Intervet, Italy).

Experiment 1: Effect of BME on nuclear maturation

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

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

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

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

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

Denuded oocytes with a normal MII appearance, including an extruded first polar body (PB), were considered suitable for ICSI. Frozen-thawed semen from a stallion of proven fertility was used. After thawing in a water bath at 37°C for 30 sec, the sperm was prepared as describe for IVF and diluted in SOF-IVF to a final concentration of $4x10^6$ spermatozoa/ml. Finally, the sperm suspension was diluted 1:1 (v/v) with a 12% solution of polyvinylpyrrolidone (PVP) in PBS. Pipettes produced using a glass micropipette puller (Model P-87, Sutter Instruments, Novato, CA, USA) were used for holding oocytes and for sperm injection. ICSI was performed at 37°C using a micromanipulator (Narishige Co. Ltd, Tokyo, Japan) equipped with a Piezo micropipette-driving unit (Prima Tech, Ibaraki, Japan) and mounted on an inverted microscope (Nikon TE 300: Nikon, Kawasaki, Japan). A motile sperm was immobilized by applying two or three piezo-pulses to its tail-midpiece region, and it was then aspirated into the tip of the injection needle. The oocyte for injection was immobilized using the holding pipette and orientated with its PB at 06:00 or 12:00 h. The ICSI needle was then advanced through the zona pellucida and oolemma at 15:00 h using the piezo-drilling motion, and the sperm was released into the ooplasm.

Following ICSI, oocytes were denuded and groups of ten were cultured in 20 µl droplets of SOF supplemented with MEM amino acids and 16 mg/ml FAF- BSA (SOF-IVC) under mineral oil at 38.5°C in an atmosphere of 5% CO_2, 7% O_2, and 88% N_2 for 60 h, before cleavage assessment.

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

Experimental design

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

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

Statistical analysis

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

Results

Experiment 1:Effect of BME on nuclear maturation

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

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

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

Discussion

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

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

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

Methaphase II rates, after *in vitro* maturation of canine oocytes, increased by adding 100 µM BME (Kim et al., 2004) but not 25 µM BME (Songsasen et al., 2002). Reported concentrations of BME in various species range from 5 µM to 50 mM (Ly et al., 2010); Yadav et al., 2013). In buffalo, 25 µM BME reduced the degeneration rate during IVM (Ullah et al., 2006), 100 µM BME plus 20 ng/ml EGF was the best combination increasing both MII and cleavage rates (Sadeesh et al., 2014), 0.5 mM BME increased maturation rate and decreased degeneration (Nasiri and Beheshti, 2012), while 10 mM BME did not influenced nuclear maturation and blastocyst production rate but improved the proportion of oocytes that exhibited synchronous pronuclei formation and the embryo quality (Songsasen and Apimeteetumrong, 2002).

Therefore it seems that effect of BME supplementation to maturation medium is highly species-specific and concentration dependent. In the horse this is the first report on the use of BME during IVM, so a standard concentration (0.1 mM) and a higher one (0.7 mM) were chosen. In the few studies using a thiol compound during equine IVM, 100 µM cysteamine was added to maturation medium failing to positively affect the maturation rate of equine oocytes (Luciano et al., 2006; Deleuze et al., 2010) and GSH content in MII oocytes (Luciano et al., 2006).

It has been demonstrated that GSH synthesis is influenced by other substances contained in maturation media, such as serum, that inhibited it, and FSH, that induced a higher GSH content in equine oocytes (Luciano et al., 2006). It is likely that maturation conditions used in our research influenced the GSH synthesis, and consequently the possible BME influence, since FBS and eCG instead of FSH were used. Anyway, even when GSH levels after IVM were similar to those of *in vivo* matured oocytes, *in vitro* maturation, fertilization and early developmental competencies of equine oocytes were not enhanced (Luciano et al., 2006). It seems that other factors beyond oxidative stress are involved in equine oocyte maturation, fertilization and early embryo development.

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

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

In the present study, the efficiency of ICSI (overall 39.5% cleavage rate) was lower as compared to others studies (Luciano et al., 2006; Hinrichs et al., 2005; Galli et al., 2007), but our percentage ranged from 0% to 80%. Beyond the operator ability, that is crucial in ICSI procedures, such a wide range of variability could be related to the source of oocytes. It has been recently demonstrated that the developmental stage of the originating follicle population has a significant impact on chromosomal and cytoplasmic properties of oocytes at the time of recovery *in vivo* (Vernunft et al*.,* 2013). Therefore, scheduling of recovery in relation to the follicle wave could potentially be used to increase the quality of oocytes recovered for equine assisted reproduction techniques (Vernunft et al*.,* 2013). Ovaries collected at abattoir over a long period, as in the present study, provide every time diverging material, and there is lack of information about the estrous cycle stage and follicular growth. There is no repeatability for oocyte quality at each replicate.

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

Aknowlegments

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Conflict of interest

There is no conflict of interest

References

Abeydeera LR, Wang WH, Cantley TC, Prather RS and Day BN 1998: Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured *in vitro* and the rate of blastocyst development after *in vitro* fertilization. Theriogenology **50**, 747-756.

Bézard J, Bøgh IB, Duchamp G, Hyttel P and Greve T 2002: Comparative evaluation of nuclear morphology of equine oocytes aspirated *in vivo* and stained with Hoechst and orcein. Cells Tissues Organs **170**, :228-236.

de Matos DG and Furnus CC 2000: The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. Theriogenology **53**, 761-771.

de Matos DG, Furnus CC, Moses DF and Baldassarre H 1995: Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. Mole Reprod Dev **42**, 432-436.

de Matos DG, Furnus CC, Moses DF, Martinez AG and Matkovic M 1996: Stimulation of glutathione synthesis of *in vitro* matured bovine oocytes and its effect on embryo development and freezability. Mol Reprod Dev **45**, 451–457.

de Matos DG, Gasparrini B, Pasqualini SR and Thompson JG 2002: Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology 57, 1443-1451.

277 Deleuze S and Goudet G 2010: Cysteamine supplementation of *in vitro* maturation
278 media: a review. Reprod Dom Anim **45**, 476-482.
279 Deleuze S, Dubois CS, Caillaud M, Bruneau B, Goudet G and Duchamp G 2010:
280 Influence of cysteamine on *in vitro* maturation, *in vitro* and *in vivo* fertilization of
281 equine oocytes. Reprod Dom Anim **45**, 1-7.
282 Galli C, Colleoni S, Duchi R, Lagutina I and Lazzari G 2007: Developmental
283 competence of equine oocytes and embryos obtained by *in vitro* procedures ranging
284 from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell
285 nuclear transfer. Anim Reprod Sci **98**, 39-55.
286 Hall V, Hinrichs K, Lazzari G, Betts DH nad Hyttel P 2013. Early embryonic
287 development, assisted reproductive technologies, and pluripotent stem cell biology in
288 domestic mammals. Vet J **197**, 128-142.
289 Hinrichs K, Choi YH, Love LB, Varner DD, Love CC and Walckenaer BE 2005:
290 Chromatin configuration within the germinal vesicle of horse oocytes: Changes post
291 mortem and relationship to meiotic and developmental competence. Biol Reprod **72**,
292 1142-1150.
293 Hinrichs K, Schmidt AL, Friedman PP, Selgrath JP and Martin MG 1993: *In vitro*
294 maturation of horse oocytes: characterization of chromatin configuration using
295 fluorescence microscopy. Biol Reprod **48**, 363-370.
296 Kim MK, Fibrianto YH, Oh HJ, Jang G, Kim HJ, Lee KS, Kang SK, Lee BC and
297 Hwang WS 2004: Effect of beta-mercaptoethanol or epidermal growth factor
298 supplementation on *in vitro* maturation of canine oocytes collected from dogs with
299 different stages of the estrus cycle. J Vet Sci **5**, 253-258.
300 Lim JM, Liou SS and Hansel W 1996: Intracytoplasmic glutathione concentration and
301 the role of ß-mercaptoethanol in preimplantation development of bovine embryos.
302 Theriogenology **46**, 429–439.

303 Luciano AM, Goudet G, Perazzoli F, Lahuec C and Gérard N 2006: Glutathione content
304 and glutathione peroxidase expression in *in vivo* and *in vitro* matured equine oocytes.
305 Mol Reprod Dev **73**, 658-666.
306 Lv L, Yue W, Liu W, Ren Y, Li F, Lee KB and Smith GW 2010: Effect of oocyte
307 selection, estradiol and antioxidant treatment on *in vitro* maturation of oocytes collected
308 from prepubertal Boer goats. Ital J Anim Sci 9, 50-54.
309 Nasiri Y and Beheshti R 2012: Effect of β-mercaptoethanol on *in vitro* maturation on
310 oocyte of Murrah buffalo. ROAVS **2**, 377-380.
311 Rodríguez-González E, López-Bejar M, Mertens MJ and Paramio MT 2003: Effects on
312 *in vitro* embryo development and intracellular glutathione content of the presence of
313 thiol compounds during maturation of prepubertal goat oocytes. Mol Reprod Dev **65**,
314 446-453.
315 Sadeesh EM,. Shah F, Balhara AK, Thirumaran SMK., Yadav S and Yadav PS 2014:
316 Effect of growth factor and antioxidant on *in vitro* maturation of oocytes and cleavage
317 rates of *in vitro* produced Indian buffalo (Bubalus bubalis) embryos. Vet Arhiv **84**, 459-
318 474.
319 Songsasen N and Apimeteetumrong M 2002: Effects of beta-mercaptoethanol on
320 formation of pronuclei and developmental competence of swamp buffalo oocytes. Anim
321 Reprod Sci **71**, 193-202.
322 Songsasen N, Yu I and Leibo SP 2002: Nuclear maturation of canine oocytes cultured
323 in protein-free media. Mol Reprod Dev **62**, 407-415.
324 Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N and Okano A 199: Effect
325 of thiol compounds on *in vitro* development and intracellular glutathione content of
326 bovine embryos. Biol Reprod **49**, 228–232.
327 Tsunoda S, Kimura N and Fujii J 2014: Oxidative stress and redox regulation of
328 gametogenesis, fertilization, and embryonic development. Reprod Med Biol **13**, 71-79.

329 Ullah I, Jalali S, Shami SA, Farooq K and Khan MI 2006: Effect of the 2-
330 Mercaptoethanol on Nili Ravi Buffalo Oocytes During *In vitro* Maturation. J Anim Vet
331 Adv **5**, 380-385.
332 Vernunft A, Alm H, Tuchscherer A, Kanitz W, Hinrichs K and Torner H 2013:
333 Chromatin and cytoplasmic characteristics of equine oocytes recovered by transvaginal
334 ultrasound-guided follicle aspiration are influenced by the developmental stage of their
335 follicle of origin. Theriogenology **80**, 1-9.
336 Yadav P, Kharche SD, Goel AK, Jindal SK and Goel P 2013: Assessment of nuclear
337 maturation and subsequent *in vitro* embryo development of caprine oocytes with
338 different supplementations in maturation medium. Indian J Anim Sci **83**, 1048-1052.
339 Yanagimachi R 1994: Mammalian fertilization. In: Knobil E and Neil JD (eds) The
340 Physiology of Reproduction, Raven Press New York, pp. 189–317
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 vescicle; Dia = diakinesis; dCh = dense chromatine; MI = metaphase I;
345 MII = metaphase II, including oocytes from anaphase I through metaphase II; Deg =
346 degenerate
347

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

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