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54

ABSTRACT

The demand for equine *in vitro* produced embryos has increased over the last decade. The aim of this study was to compare the effects of an extended IVM or a prolonged period before fertilization, including holding time, on equine immature oocyte developmental competence. Oocytes, collected from abattoir-derived ovaries, were divided into 4 groups: H0/24 (n=165) 0 h holding + standard 24-26 h IVM; H8/36 (n=160) 8 h holding + 36 h IVM; H20/24 (n=187) 20 h holding + 24 h IVM; H0/44 (n=164) 0 h holding + 44 h IVM. Oocytes matured to MII were fertilized by intracytoplasmic sperm injection (ICSI) and cultured for 10 days. The oocyte degeneration rate was higher (P<0.05) for H20/24 than the other groups (H0/24 38.2%, H8/36 43.1%, H20/24 54.5%, H0/44 32.9%). Cleavage was higher (P<0.05) in H20/24 (70%) compared to H0/24 (45%) and H8/36 (54%) but not to H0/44 (63%). No differences among groups were observed in the number of blastocysts per oocyte. Injected oocytes that reached the blastocysts stage were higher (P<0.05) for H20/24 (20%) than H0/24 (7%) and H0/44 (7%) but not H8/36 (12%). For cleaved oocytes, a higher blastocyst rate (P<0.05) was observed for H20/24 (28%) than H0/44 (11%), while H0/24 (15%) and H8/36 (21%) were not different from any group (P>0.05). Timing of blastocyst development was not different among groups. Overnight holding of equine immature oocytes followed by a standard IVM interval may induce a pre-selection of the most competent oocytes thereby improving cleavage and embryo development rates after ICSI.

Keywords: Oocyte; In vitro maturation; Intracytoplasmic sperm injection; Embryo development

75 **1. Introduction**

76 Demand for *in vitro* equine embryo production by intracytoplasmic sperm injection (ICSI) has
77 increased over the last decade. In commercial programs, equine oocytes are usually obtained by
78 ovum pick up (OPU) from immature follicles, and transported to ICSI laboratories for *in vitro*
79 maturation (IVM), ICSI and embryo culture (Morris, 2018). Depending on the morphology of the
80 cumulus oocyte complex (COCs) at the time of recovery, 24-30 hours of IVM are required to reach
81 the metaphase II (MII) stage (Hinrichs et al., 2005). Holding immature oocytes allows not only for
82 the transportation, but also for the facilitation of timing the procedures in the laboratory.Indeed,
83 shipment and preservation of oocytes avoid the risks and expense of animal transport, while allow
84 the mare owner to take advantage of the most recent advances in assisted reproductive technologies
85 (Hinrichs, 2020). Particularly, the ability to hold equine immature oocytes overnight has contributed
86 greatly to the widespread use of ICSI as a clinical tool because oocytes may be recovered from
87 mares locally and then shipped overnight to central laboratories for *in vitro* embryo production
88 (Hinrichs, 2020). Overnight holding of horse oocytes at room temperature does not affect
89 maturation and embryo development to the blastocyst stage (Choi et al., 2006a). Furthermore, it
90 maintains meiotic arrest, viability, and mitochondrial potential of equine oocytes (Martino et al.,
91 2014). Different holding media have been successfully used at room temperature for 18-24 h (Foss
92 et al., 2013; Diaw et al., 2018) before an IVM of at least 22 hours (Dini et al., 2016). More recently,
93 the overnight exposure of oocytes to temperatures below room temperature (16°C) appeared to
94 optimise equine *in vitro* embryo production (IVEP) (Metcalf et al., 2020). In addition, holding of
95 equine immature oocytes at 15°C for 2 days slowed some morphokinetic parameters of embryo
96 development, but did not affect blastocyst production (Martino et al., 2019).

97 Oocyte nuclear and cytoplasmic maturation are necessary for normal embryo development. Galli
98 et al. in 2018 (Galli et al., 2018) observed that their current IVM protocols entailed a 24 to 28 h
99 maturation time assessed by the appearance of the first polar body, despite the fact that the time

100 from hCG administration (considered the trigger for the resumption of meiosis) to ovulation *in vivo*
101 is 36 to 40 h. Furthermore, they observed that some oocytes matured to MII after 24-28 hours of
102 IVM did not undergo cleavage after ICSI, indicating a possible uncoupling between cytoplasmic
103 and nuclear maturation (Galli et al., 2018). Thus, the maturation time was extended from 24-28 h to
104 36 h, following 7 to 8 h holding at room temperature for logistical reasons, with a beneficial effect
105 on the number of blastocysts produced (Galli et al., 2018). It was concluded that the additional
106 maturation time improved the developmental competence (Galli et al., 2018). However, similar high
107 blastocyst rates were obtained using overnight holding at room temperature from abattoir derived
108 (Diaw et al., 2018; Brom-de-Luna et al., 2019) or OPU derived immature oocytes (Brom-de-Luna et
109 al., 2018; Salgado et al., 2018; Metcalf et al., 2020) with IVM protocols lasting 24-30 h. There is no
110 study comparing the effect of overnight holding and prolonged IVM in order to clarify which of the
111 two different conditions mainly affects oocyte developmental competence. The objective of this
112 study was to compare the effects of an extended IVM or a prolonged period before fertilization,
113 including holding time, on equine immature oocyte developmental competence. For this purpose,
114 different combination of holding/IVM protocols (44 h in total) were compared to control IVM (24-
115 26 h) or direct prolonged IVM (44 h).
116
117 **2. Materials and methods**
118 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise
119 stated.
120
121 *2.1 Collection and in vitro maturation of cumulus oocyte complexes*
122 Ovaries from mares of unknown origin were collected at the slaughterhouse and transported to
123 the laboratory within 2 to 3 h at 25°C in an insulate container. Oocytes were collected as previously
124 described (Merlo et al., 2018). Briefly, the ovaries were dissected free from connective tissue,
125 washed with demineralized water, and oocytes were recovered by aspirating the contents of 10 to 30

126 mm follicles, using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100
127 mmHg). The aspirated follicular fluid was collected into 250 ml glass flasks and filtered through a
128 65 µm mesh nylon filter (EmSafe, Minitube, Germany). Oocytes were then divided into four
129 groups: 1) H0/24 (control group) (*n*=165): no holding before standard IVM for 24-26 h; H8/36
130 (*n*=160): 8 h holding at room temperature (22 to 25°C) then IVM for 36 h; 3) H20/24 (*n*=187): 20 h
131 holding at room temperature then IVM for 24 h; 4) H0/44 (extended IVM group) (*n*=164): no
132 holding before IVM for 44 h. All groups except H0/24 had a total interval of 44h from oocyte
133 collection to injection.

134 For oocyte holding, sterile 0.5 mL Eppendorf tubes with screw caps (Sarstedt, Verona, Italy)
135 were filled with HSOF (Holding Synthetic Oviductal Fluid, SOF (Tervit et al., 1972) supplemented
136 with 20mM HEPES, 1 mM glutamine, 10mM glycine, minimum essential medium (MEM) essential
137 (50x) and non-essential (100x) amino acids solutions, 6 mg/mL BSA). After oocytes deposition,
138 tubes were closed and wrapped with aluminium foil to prevent the exposure to light. For IVM,
139 oocytes were cultured in 500 µL maturation medium in four-well plates (Scientific Plastic Labware,
140 EuroClone, Italy) at 38.5°C, in a humidified atmosphere of 5% CO_2 in air. Maturation medium
141 consisted of Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life
142 Technologies, Italy) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco,
143 Life Technologies, Italy), 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1,
144 0.1 IU/mL porcine FSH-LH (Pluset, Calier, Italy).

145 At the end of IVM, oocytes were incubated for 1.5 min in a 0.25% (w/v) solution of trypsin in
146 HSOF and pipetted to mechanically remove cumulus cells. Then they were transferred to HSOF
147 supplemented with 10% FCS for trypsin inactivation. After denuded of cumulus cells, oocytes with
148 an extruded first polar body were considered suitable for ICSI. Oocytes with a damaged oolemma
149 were considered degenerate, while oocytes with an intact oolemma but no polar body were
150 considered immature.

151

152 *2.2 Intracytoplasmic sperm injection and in vitro embryo culture*

153 Frozen-thawed semen from a stallion of *in vitro* proven fertility was used. After cutting a piece
154 of straw under liquid nitrogen, semen was thawed by placing the cut section of straw into 1 ml of
155 SOF-IVF [SOF supplemented with 6 mg/ml fatty-acid-free BSA (FAF-BSA), 20mM HEPES, 1
156 mM glutamine, 10mM glycine, MEM essential (50x) and non essential (100x) amino acids
157 solutions, 1 mg/mL heparin, 20 mM penicillamine, 1 mM epinephrine, and 10 mM hypothaurine]
158 pre warmed at 37°C and centrifuged at 500 g for 2 min. Supernatant was discarded leaving only 0.1
159 mL of medium. The sperm suspension was subsequently diluted 1:1 (v/v) with a 12% solution of
160 polyvinylpyrrolidone (PVP) in PBS (phosphate buffered saline) to a final concentration of $2x10^6$
161 spermatozoa/ml. Manufactured ICSI pipettes (Biomedical Instruments, Zöllnitz, Germany) were
162 used. ICSI was performed at 37°C using a micromanipulator (Narishige Co. Ltd, Tokyo, Japan)
163 equipped with a Piezo micropipette-driving unit (Prime Tech, Ibaraki, Japan) and mounted on an
164 inverted microscope (Nikon TE 300: Nikon, Kawasaki, Japan). A motile sperm was immobilized by
165 applying two or three piezo-pulses to the tail or midpiece regions, and it was then aspirated into the
166 tip of the injection pipette. The oocyte was immobilized using the holding pipette and orientated
167 with its polar body at 06:00 or 12:00 h. The injection pipette was then used to penetrate through the
168 zona pellucida and oolemma at the 15:00 h position using the piezo-drilling motion, and the
169 spermatozoon was released into the ooplasm.

170 Following ICSI, oocytes were cultured in 20 µL droplets of SOF supplemented with 20mM
171 HEPES, 1 mM glutamine, 10mM glycine, MEM essential (50x) and non essential (100x) amino
172 acids solutions and 16 mg/ml FAF-BSA (SOF-IVC) under mineral oil at 38.5°C in an atmosphere
173 of 5% CO_2, 7% O_2, and 88% N_2 for 10 days. Culture medium was refreshed every 3 days by adding
174 20 µL of fresh SOF-IVC into each droplet and thereafter aspirating the same volume. On day 6 of
175 IVC, 5% FBS was added. Presumptive zygotes were monitored for cleavage 48 hours after injection
176 and development to the blastocyst stage was evaluated daily at day 7 through 10. At the end of the

177 culture period, blastocysts were evaluated by staining with Hoechst 33342 (bisbenzimide 10 µg/mL
178 in PBS) to confirm the presence of nuclear material.
179
180 *2.3 Statistical analysis*
181 The study was done in 10 replicates, in order to obtain an appropriate sample size of equine
182 immature oocytes. Some oocytes were included in all groups per replicate. Data were analysed
183 using a Chi Square test (IBM SPSS Statistics 25, IBM Corporation, Milan, Italy). Significance was
184 assessed at $P<0.05$.
185
186 **3. Results**
187 From the 676 recovered oocytes, 288 (42.6%) were degenerate after IVM, 367 (54.3%) reached
188 the metaphase II stage and 21 (3.1%) were determined to be immature. The highest rate of
189 degenerate oocytes (54.5%) and the lowest maturation rate (43.3%) ($P<0.05$) were observed in
190 H20/24 (Tab. 1). On the other hand, H0/24 group showed an higher rate of immature oocytes
191 (6,7%) compared to H8/36 (1.3%) and H20/24 (2.1%) ($P<0.05$) (Tab. 1). Consequently, analysing
192 maturation rates without considering degenerate oocytes, the percentage of MII oocytes was lower
193 in H0/24 group (89.2%) than H8/36 (97.8%) and H0/44 (96.4%) ($P<0.05$), while H20/24 (95.3%)
194 was statistically similar to all groups ($P>0.05$) (Tab. 1).
195 Even though no differences were observed in the proportion of blastocysts per oocyte (prior to
196 maturation) ($P>0.05$), cleavage was significantly higher ($P<0.05$) in H20/24 (70%) compared to
197 H0/24 (45%) and H8/36 (54%) (Tab. 2). Furthermore, H0/24 cleavage rate was lower ($P<0.05$) than
198 H0/44 (Tab. 2). The number of injected oocytes that developed to the blastocyst stage was higher
199 ($P<0.05$) for H20/24 (20%) than H0/24 (7%) and H0/44 (7%) (Tab. 2). Considering the embryo
200 developmental ability of cleaved oocytes, the only significant difference in blastocyst rate was
201 observed between H20/24 (28%) and H0/44 (11%) ($P<0.05$) (Tab. 2).

202 No statistically significant differences were observed in timing of blastocyst development after
203 ICSI (P>0.05) (Tab. 3).
204
205 **4. Discussion**
206 *In vitro* embryo production is becoming more popular in commercial equine breeding
207 programmes. Shipping stored oocytes to ICSI laboratories is a requirement in ovum pick up/ICSI
208 clinical programmes. Holding immature oocytes before the onset of IVM simplifies not only oocyte
209 transport but also scheduling of subsequent manipulations. Although *in vivo* follicle maturation
210 after induction requires approximately 36 h, the process *in vitro* is different. When standard IVM
211 time (24-28 h) was extended to 36 h, including also 7 to 8 h holding period at room temperature for
212 logistical reasons, the number of blastocysts produced more than doubled (Galli et al., 2018). On
213 the other hand, similar blastocyst rates were obtained using overnight holding at room temperature
214 followed by 24-30 h IVM (Brom-de-Luna et al., 2018; Diaw et al., 2018; Salgado et al., 2018;
215 Brom-de-Luna et al., 2019; Metcalf et al., 2020). It remains unclear if the beneficial effect on
216 equine immature oocyte developmental competence is related to an extended IVM or to a prolonged
217 period before fertilization. To our knowledge, this is the first study combining different holding
218 period and maturation times in the attempt to elucidate this aspect.
219 In the present study, the overall maturation rate was 54.3%, similarly to other previous reports in
220 the horse (Hall et al., 2013; Merlo et al., 2016, 2018). Extending the IVM length (both 36 h and 44
221 h) increased nuclear maturation compared to direct 24-26 h IVM, but was similar to delayed (after
222 20 h holding) 24 h IVM. In addition, a longer IVM did not increase blastocyst production. On the
223 other hand, comparing developmental competence of injected oocytes matured for 24 h with or
224 without overnight holding, cleavage rate and blastocyst production were improved by 20 h holding,
225 in spite of similar ability of cleaved oocytes to reach the blastocyst stage. Furthermore, overnight
226 holding and 24 h IVM enhanced blastocyst rates compared to immediate placement of the oocytes
227 in IMV for the same total interval of 44 h, even if similar cleavage ability was observed. It is likely

228 that 24 h IVM may be shorter and 44 h IVM may be longer than optimal, depending on oocytes.
229 Finally, short holding time (8 h) followed by extended IVM (36 h) achieved a lower cleavage rate
230 than overnight holding followed by 24 h IVM, even if blastocyst production was not statistically
231 lower.

232 Different aspects need to be considered to in order to try to explain what was observed. Firstly,
233 meiotic competence of horse oocytes is dependent upon initial cumulus configuration, size of the
234 follicle from which the oocyte was recovered, and the period of time the oocyte is in the ovary
235 before recovery (Hinrichs, 2010). Oocytes recovered after being held within the ovary 5-9 h
236 matured in larger proportion at 24 h of culture compared with oocytes recovered immediately and
237 cultured for the same period (Hinrichs et al., 2005). Furthermore, ovary storage was associated with
238 an increase in developmental competence of horse oocytes, as reflected in the higher blastocyst
239 development for oocytes collected after a delay (Hinrichs et al., 2005). This suggest that pre-
240 maturational changes may occur in horse oocytes held at room temperature within the ovary
241 (Hinrichs et al., 2005). In the present study, oocytes were recovered after being held within the
242 ovary 4-5 h, and this could have influenced the subsequent effects of IVM duration on oocyte
243 developmental competence. In fact, oocytes might have matured in lower proportion at 24 h of
244 culture.

245 Another important aspect is the pre-maturation period after oocyte collection. Both maturation-
246 promoting factor (MPF) and microtubule-associated protein (MAP) kinase have been identified in
247 horse oocytes, and MPF activity is higher in mature than in immature horse oocytes (Goudet et al.,
248 1998a, 1998b). Several studies investigated meiotic arrest in the horse and its effect on oocyte
249 developmental competence and blastocyst production. Cycloheximide (Alm and Hinrichs, 1996), 6-
250 dimethylaminopurine (6-DMAP), and butyrolactone I (Hinrichs et al., 2002) have been used to
251 maintain meiotic arrest in equine oocytes. Roscovitine was also effective in suppressing meiosis
252 (Hinrichs et al., 2002), but decreased cleavage rates for expanded COCs (Franz et al., 2003), while
253 compact COCs could be held in roscovitine before maturation without any harmful effect on

254 blastocyst formation (Choi et al., 2006b). Nonetheless, when cycloheximide or roscovitine were
255 added to the pre-IVM medium of equine expanded oocytes, although they were successful at
256 holding oocytes at the germinal vesicle stage and maintaining the meiotic competence, a lower
257 number of blastocysts resulted from the treated oocytes than from those held at room temperature
258 overnight in the absence of meiotic inhibitors (Choi et al., 2006a). More recently, meiotic
259 competence of COCs from follicles 1–2 cm in diameter was not affected by prematuration in
260 cilostamide, whereas they yielded blastocysts with a higher number of cells than oocytes that
261 underwent direct IVM (Lodde et al., 2019). Furthermore, the addition of forskolin and 3-isobutyl-1-
262 methylxanthine to overnight holding medium before maturation improved blastocyst production,
263 suggesting that management of oocyte and cumulus cell cAMP levels before IVM may be an
264 effective means to enhance equine oocyte developmental competence and blastocyst development
265 (Metcalf et al., 2020).

266 The successful method for overnight holding of equine oocytes in the absence of meiotic
267 inhibitors was first reported in 2006 (Choi et al., 2006a). Since then, different holding periods,
268 media, and temperatures were tested (Choi et al., 2007; Foss et al., 2013; Galli et al., 2014; Martino
269 et al., 2014; Dini et al., 2016; Diaw et al., 2018; Martino et al., 2019; Campos-Chillon et al., 2019;
270 Metcalf et al., 2020). Temperature sensitivity of equine oocytes differs depending upon the
271 follicular stage. Oocytes recovered from subordinate follicles are less sensitive than those collected
272 from pre-ovulatory follicles (Choi et al., 2006a; Foss et al., 2013), in which meiosis has been
273 initiated (Mortensen et al., 2010). The progression of pre-meiotic chromatin configuration and
274 mitochondrial status are dependent upon temperature, and resumption of meiosis is suppressed
275 when the oocytes are held overnight at 25°C but not at increased temperatures (30°C and 38°C)
276 (Martino et al., 2014). Immature oocytes had higher developmental ability to the blastocyst stage
277 when held overnight at room temperature (23°C) versus body temperature (37°C) (Foss et al.,
278 2013). On the other hand, when holding immature oocytes below room temperatures (17°C and
279 4°C), maturation rates were not affected (Dini et al., 2016), but blastocyst production rates were

280 lower for oocytes held overnight at 7°C than at room temperature (Diaw et al., 2018). On the
281 contrary, a 2 day holding at 15°C led to similar blastocyst rates as compared to overnight holding at
282 room temperature (Martino et al., 2019). Finally, a cooler temperature of 16°C versus 20°C for
283 overnight holding of immature oocytes resulted in a similar maturation rate but in a higher cleavage
284 rate, while dropping the temperature to 5°C was not appropriate (Metcalf et al., 2020). The ideal
285 temperature for the overnight holding of immature COCs is yet to be thoroughly investigated and
286 determined. Moreover, the underlying mechanisms of maintaining meiotic arrest in the absence of
287 inhibitors have not been clarified.

288 Our results employing a short holding period (8 h) and an extended maturation (36 h) compared
289 to standard 24-26 h maturation are only partially in line with what observed by Galli et al. (Galli et
290 al., 2018), where the difference with the control was significant. Data from a retrospective analysis
291 on OPU-ICSI in the same laboratory showed that increasing IVM from 26-28 h to 36-38 h after a 2-
292 12 holding at 22-24°C enhanced embryo production, and the same trend was observed for shipped
293 oocytes, matured for 26-30 h after an overnight holding (Lazzari et al., 2020). It is likely that the
294 present results vary from some of the clinical ICSI work because of some holding in ovaries and
295 more variables in slaughterhouse animals. The findings that maturation kinetics and oocyte
296 developmental competence vary with initial cumulus morphology, time of collection of oocytes
297 from the ovary, and duration of maturation make it difficult to compare results among laboratories
298 (Hinrichs, 2010). Different maturation media are used, and even with the same IVM medium
299 opposite results were obtained (Hinrichs, 2018). Moreover, even when ICSI is successfully
300 performed, the culture of equine embryos up to the blastocyst stage appears to be much more
301 challenging, with wide differences between laboratories (Stout, 2020). An important difference that
302 might have greatly influenced the results could be in part dependent on the use of the piezo drill.
303 Using the Eppendorf Piezo Expert, where the settings and the functionality are reproducible each
304 time, increasing the intensity of piezo pulses enhanced cleavage and embryo development[10]. In the
305 present study, the Prime Tech piezo was used, for which the settings depend on how the piezo

306 pipette is mounted. Such difference could have introduced more variability in the efficiency of ICSI
307 and reduced the significance of the differences. Indeed, in our conditions, the differences in the
308 number of blastocysts per oocyte (prior to maturation) were not significant. Nevertheless, the
309 developmental competence of MII oocytes matured for 24 h after overnight holding was superior to
310 those directly matured, proving an effect of the pre-maturational holding period. As also previously
311 observed (Galli et al., 2014), more oocytes were degenerate after 20 h holding and IVM, with a
312 decrease of the number of matured oocytes, indicating that oocytes that are already partially
313 compromised may be less tolerant to this treatment. Considering that a lower number of injected
314 oocytes had a better overall development to the blastocyst stage, it is likely that the holding period
315 may pre-select the oocytes, and the oocytes that degenerate include those that have impaired
316 developmental competence. On the other hand, maturing the oocytes for the entire 44 h period
317 decreases oocyte developmental competence, possibly because of oocyte aging.
318

319 **5. Conclusion**

320 Overall, an extended period of IVM is not responsible for an increased oocyte developmental
321 competence, while an overnight holding followed by a standard IVM may induce a pre-selection of
322 the most competent oocytes thereby improving their developmental competence after ICSI.
323 Different protocols can be used for holding and IVM without changing embryo production per
324 oocyte. This allows more flexibility in a clinical program.
325

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443 **Table 1**

444 Percentage of equine oocytes determined to be mature (MII), immature (IM) or degenerate (DEG)
445 and percentage of MII oocytes per nondegenerate (MII/nonDEG) for holding and maturation
446 groups. H0/24 = 0 h holding + 24-26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24 = 20 h
447 holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

448

Group	Oocytes	MII (%)	IM(%)	DEG(%)	MII /nonDEG
H0/24	165	91 (55.2)[a]	11 (6.7)[a]	63 (38.2)[b]	89.2[b]
H8/36	160	89 (55.6)[a]	2 (1.3)[b]	69 (43.1)[b]	97.8[a]
H20/24	187	81 (43.3)[b]	4 (2.1)[b]	102 (54.5)[a]	95.3[a,b]
H0/44	164	106 (64.6)[a]	4 (2.4)[a,b]	54 (32.9)[b]	96.4[a]

449 [a,b] Different superscript letters within the same column differed at $P<0.05$.
450

451 **Table 2**

452 Cleavage and blastocyst development after ICSI of equine oocytes among holding and *in vitro*
453 maturation groups. H0/24 = 0 h holding + 24-26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24
454 = 20 h holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

455

Group	Oocytes	Injected	Cleavage (%)	Bl/oocyte (%)	Bl/injected (%)	Bl/cleaved (%)
H0/24	165	91	41 (45)[c]	6/165 (3.6)	6/91 (7)[b]	6/41 (15)[a,b]
H8/36	160	87	47 (54)[b,c]	10/160 (6.25)	10/87 (12)[a,b]	10/47 (21)[a,b]
H20/24	187	81	57 (70)[a]	16/187 (8.6)	16/81 (20)[a]	16/57 (28)[a]
H0/44	164	104	65 (63)[a,b]	7/164 (4.2)	7/104 (7)[b]	7/65 (11)[b]

456 [a,b] Different superscript letters within the same column differed at $P<0.05$.
457 Bl = blastocyst

458

459

460 **Table 3**

461 Timing of blastocyst development after ICSI of equine oocytes among holding and *in vitro*
462 maturation groups. H0/24 = 0 h holding + 24-26 h IVM; H8/36 = 8 h holding + 36 h IVM; H20/24
463 = 20 h holding + 24 h IVM; H0/44 = 0 h holding + 44 h IVM.

464

Group	Bl day 7 (%)	Bl day 8 (%)	Bl day 9 (%)	Bl day 10 (%)	Total Bl
H0/24	1 (16.7)	3 (50.0)	2 (33.3)	0 (0.0)	6
H8/36	2 (20.0)	2 (20.0)	5 (50.0)	1 (10.0)	10
H20/24	5 (31.2)	2 (12.5)	6 (37.5)	3 (18.8)	16
H0/44	2 (28.6)	3 (42.9)	1 (14.3)	1 (14.3)	7

465 Bl = blastocyst

466