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PERMISSIONS

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1 **Reactive Oxygen Species Production and Redox state in**

2 **Parthenogenetic and Sperm mediated bovine oocyte activation**

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5 **Short title:** ROS and redox in bovine oocyte activation

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- 1 -

ABSTRACT

The knowledge concerning redox and reactive oxygen species (ROS) mediated regulation of early embryo development is scarce and remains controversial. The aim of this work was to determine ROS production and redox state during early *in vitro* embryo development in sperm mediated and parthenogenetic activation of bovine oocytes. Sperm mediated oocyte activation was carried out in IVF-mSOF with frozen-thawed semen. Parthenogenetic activation was performed in TALP plus ionomycin and then in IVF-mSOF with 6-dimethylaminopurine plus cytochalasin B. Embryos were cultured in IVF-mSOF. ROS and redox state were determined at each 2-h interval (7–24h from activation) by 2´,7´-dichlorodihydrofluorescein diacetate and RedoxSensor Red CC-1 fluorochromes, respectively. ROS levels and redox state differed between activated and non-activated oocytes ($p<0.05$). In sperm activated oocytes an increase was observed between 15 and 19h ($p<0.05$). Conversely, in parthenogenetically activated oocytes, we observed a decrease at 9h ($p<0.05$). In sperm activated oocytes, ROS fluctuated throughout the 24h, presenting peaks around 7, 19 and 24h ($p<0.05$), while in parthenogenetic activation, peaks were detected at 7, 11 and 17h ($p<0.05$). In the present work, we found clear distinctive metabolic patterns between normal and parthenogenetic zygotes. Oxidative activity and ROS production are an integral part of bovine zygote behavior, and defining a temporal pattern of change may be linked with developmental competence.

INTRODUCTION

Oxidative stress has been widely reported in biological sciences to describe an enhanced state of oxidants in cells, a situation in which the concentration of reactive oxygen species (ROS) increases above its biologically normal levels (Sikka *et al.*, 2001). Oxidative stress, mediated by ROS results in an imbalance of the intracellular redox potential towards an oxidized potential (Balaban *et al.*, 2005). The role of ROS in biological processes is still controversial. It was found that the oxidative modification of cell components due to the action of ROS is one of the most potentially damaging processes for normal cell function, leading to inactivation of proteins, lipid membrane peroxidation and DNA alterations (Yang *et al.*, 1998). However, it has been observed that at physiological concentrations, ROS participate in normal cell processes as major factors in growth and development regulation (Hancock *et al.,* 2001).

The procedure for producing embryos *in vitro* in cattle is still unsatisfactory, with results ranging from 35 to 50% blastocyst rate at day 7/8 of development (Lim *et al.*, 2007, Shirazi *et al.*, 2009). Chemical activation presented significantly different success rates and

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74 blastocyst formation compared with IVF (Ruggeri *et al.*, 2012), demonstrating that the process
75 of oocyte activation is a major factor for successful production of reconstructed embryos by
76 somatic cell nuclear transfer (Wells *et al.*, 1999). ROS generation has been implicated as a
77 major cause of poor development of bovine embryo *in vitro*. ROS has been suggested to
78 participate in meiotic arrest in oocytes (Nakamura *et al.*, 2002), and embryonic block and cell
79 death (Hashimoto *et al.*, 2000). It has been advanced that high levels of ROS may cause oocyte
80 meiotic arrest (Downs and Mastropolo, 1994). Within the oocyte, a critical intracellular
81 concentration of ascorbic acid is necessary for normal cytoplasmic maturation and embryo
82 developmental competence (Tatemoto *et al.*, 2001). It has been observed that an excessive
83 amount of glucose in the maturation medium produces high ROS concentrations and exerts a
84 negative effect on subsequent bovine embryo development to the blastocyst stage (Hashimoto *et*
85 *al.*, 2000). The importance of regulating ROS levels is revealed by the observation that
86 cumulus–oocyte complexes (COCs) have developed significant antioxidant strategies to control
87 ROS production (Cetica *et al.*, 2001, Tatemoto *et al.*, 2001, Dalvit *et al.*, 2005a).

88   On the other hand, some evidence exists demonstrating that ROS are important to
89 spermatozoa in regulating every aspect of sperm function examined, including their movement
90 characteristics, capacitation, sperm-zona interaction, acrosome reaction and sperm-oocyte
91 fusion (Baker and Aitken, 2004, Rivlin *et al.*, 2004). Some studies have also documented that
92 the addition of natural antioxidants to oocyte maturation medium failed to modify the
93 percentage of bovine embryos produced *in vitro* (Blondin *et al.*, 1997) or even diminished the
94 rate of embryo production (Dalvit *et al.*, 2005b). Other cell-permeable antioxidants inhibited the
95 precocious resumption of meiosis in rat oocytes, suggesting a regulatory function of ROS in the
96 maturation process (Takami *et al.*, 1999).

97   Several transcription factors involved in diverse developmental processes are now
98 known to be regulated by the intracellular redox potential (Dickinson and Forman, 2002; Funato
99 *et al.*, 2006; Imai *et al.*, 2000; Liu *et al.*, 2005; Rahman *et al.*, 2004; Zhang *et al.*, 2002). The
100 recent discovery that these factors can be sensitive to oxidation by ROS or S-glutathionylation,
101 or require NAD(P)H (the reduced form) or NAD(P)$^+$ (the oxidised form) is opening new
102 insights in the regulation of embryonic development (Dumollard *et al.*, 2007). It has been
103 observed that redox state and ROS levels are negatively associated within the cell. A high
104 cellular oxidative activity (eg. increased mitochondrial oxygen consumption rate) is usually
105 associated with lower ROS production and vice verse (Boveris and Cadenas, 1982). In the early
106 mouse embryo, the fundamental importance of redox state and ROS regulation of early embryo
107 development has also been demonstrated (Dumollard *et al.*, 2007).

108   Previous studies from our group have documented variations in ROS production
109 attributable to oocyte and early embryo metabolic activities during bovine *in vitro* maturation
110 (IVM) and embryo development (Dalvit *et al.*, 2005a; Morado *et al.*, 2009). In addition,

- 3 -

111 temporal changes in oxygen consumption were detected in bovine oocytes undergoing the
112 transition from oocyte to zygote (Lopes et al., 2010). Accordingly, the aim of this work was to
113 determine the production of ROS and redox state during early *in vitro* embryo development in
114 sperm mediated and parthenogenetic activation of bovine oocytes.
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116
117 **MATERIALS AND METHODS**
118
119 The materials used in these experiments were obtained from Sigma-Aldrich (St. Louis,
120 Missouri), unless otherwise indicated.
121
122 **Recovery and classification of cumulus-oocyte complexes**
123
124 Bovine ovaries were obtained from an abattoir within 30 min after slaughter and kept
125 warm (30-33°C) until they were brought to the laboratory. Ovaries were washed in
126 physiological saline containing 100000 IU/l penicillin and 100 mg/l streptomycin. COCs were
127 recovered by aspiration of antral follicles (2–5 mm in diameter) and classified according to
128 cumulus morphology under a stereomicroscope. Only oocytes completely surrounded by
129 compact and multiple layers of cumulus cells were employed.
130
131 **Oocyte *in vitro* maturation**
132
133 Groups of 50 COCs were cultured in 500 µl of medium 199 (GIBCO, Grand Island,
134 NY, USA) supplemented with 0.2 mg L^{-1} porcine follicle-stimulating hormone (FSH;
135 Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg L^{-1} porcine luteinizing hormone (LH;
136 Lutropin-V; Bioniche), 5% (v/v) fetal bovine serum (FBS, Internegocios, Mercedes, Buenos
137 Aires, Argentina) and 50 mg/l gentamycin sulphate under mineral oil (Squibb & Sons Inc.,
138 Princeton, NJ, USA) at 39ºC for 22 h in an atmosphere of 5% CO_2 in humidified air.
139
140 **Sperm mediated activation of matured oocytes**
141
142 *In vitro* fertilization (IVF) was carried out using frozen–thawed Holstein bull semen
143 from a male of proven fertility. Semen was thawed at 37ºC in modified synthetic oviductal fluid
144 (mSOF) (Takahashi and First, 1992) with 10 mmol/l theophylline, centrifuged at 500 xg twice
145 for 5 min and then resuspended in fertilization medium to a final concentration of 2 $\times 10^6$ motile
146 spermatozoa/ml. Co-incubation of COCs and spermatozoa was performed in IVF–mSOF
147 medium, consisting of mSOF supplemented with 10 IU/ml heparin and 5 mg/ml BSA, under

- 4 -

148 mineral oil at 39ºC, 5% CO_2 in humidified air during 24 h. Different nuclear early embryo
149 development stages were evaluated within 24 h of culture by the fluorescent stain Hoescht
150 33342 as described below.
151
152 **Parthenogenetic activation of matured oocytes**
153
154 Oocytes matured *in vitro* were denuded in phosphate buffer saline (PBS) supplemented
155 with 3 mg/ml bovine serum albumin (BSA) by gentle pipetting with a Pasteur pipette. Oocytes
156 were considered mature when the first polar body was present.
157 Only mature oocytes were incubated in TALP supplemented with 3 mg/ml BSA with 5
158 µM ionomycin for 5 min and then in mSOF added with 2 mM 6-dimethylaminopurine (6-
159 DMAP) + 7.5 µg/ml cytochalasin B for 3 h as described by Grupen *et al.* (2002). They were
160 then washed and placed in IVF-mSOF under mineral oil at $90\%N_2$: $5\%CO_2$: $5\%O_2$ and 100%
161 humidity for 21 h.
162
163 **Determination of redox state and nuclear stage**
164
165 To determine redox state and nuclear stage, matured oocytes, putative zygotes and
166 parthenotes were collected from culture media at 2-h interval from 7 to 24 h post-
167 activation/insemination. They were then denuded and incubated in PBS supplemented with 3
168 mg/ml BSA in the presence of 1 nM RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR,
169 USA) plus 1 µM of Hoechst 33342 for 10 min in the dark at 39.5°C.
170 All oocytes were then washed in PBS supplemented with 3mg/ml BSA and mounted on
171 glass slides. Fluorescence was measured by means of digital microphotographs using a Jenamed
172 II epifluorescence microscope with an x12 objective using 450-490 nm (excitation) and 570 nm
173 (emission) filters for RedoxSensor Red CC-1. Pixel intensity within microphotographs of each
174 oocyte/zygote/parthenote was determined using Image J 1.240 software (National Institutes of
175 Health, Federal Government of the United States). To normalise measurements between
176 different replicates, the fluorescence of matured oocytes was set at a consistent level. Nuclear
177 stage was evaluated at x400 using 330-380 nm (excitation) and 420 nm (emission) filters for
178 Hoechst 33342.
179 Redox state measurements were expressed as Arbitrary Units/oocyte or
180 zygote/parthenote.
181
182 **Determination of ROS production and nuclear stage**
183

184   To measure ROS production and nuclear stage, matured oocytes, putative zygotes and
185 parthenotes were collected from culture media at 2-h interval from 7 to 24 h post
186 activation/insemination, denuded and incubated in PBS supplemented with 3 mg/ml BSA for 30
187 min in the presence of 5 μM 2´,7´-dichlorodihydrofluorescein diacetate (DCHFDA) (Le Bel *et*
188 *al*., 1992) and 1 μM of Hoechst 33342. To measure esterase activity, 25% of the cells of each
189 sample were incubated in the dark at 39.5˚C in PBS supplemented with 3 mg/ml BSA for 15
190 min in the presence of 0.12 μM fluorescein diacetate (FDA).

191   After exposure to DCHFDA plus Hoechst 33342 or FDA, all oocytes were washed in
192 PBS supplemented with 3 mg/ml BSA and mounted on glass slides. Fluorescence was measured
193 as described above using 450-490 nM (excitation) and 520 nM (emission) filters for DCHFDA
194 and FDA.

195   Both DCHFDA and FDA fluorescence are dependent on the endogenous esterase
196 activity, therefore, a pixel intensity ratio between DCHFDA fluorescence and the mean FDA
197 fluorescence (for the subset measured) at each time point for each oocyte was determined as
198 justified by Lane *et al.* (2002). ROS levels were expressed as Arbitrary ROS Units/oocyte or
199 zygote/parthenote.

200

201 **Experimental design and statistical analysis**

202

203   Data were expressed as mean ± SEM. Values at different time points were compared
204 using ANOVA. A p-value < 0.05 was considered significant.

205

206

207 **RESULTS**

208

209 **Sperm mediated oocyte activation**

210

211   In sperm mediated activation, following insemination, pronuclei formation began by 9 h
212 and peaked at 13-15 h (around 80% of the zygotes), then slightly decreased until 24 h. Syngamy
213 began around 13 h and reached a plateau at 17-24 h (around 40% of the zygotes). First cleavage
214 of embryos began at 21 h (Figure 1).

215

216 **Parthenogenetic oocyte activation**

217

218   In parthenogenetically activated oocytes, by the first observation at 7h, all oocytes were
219 at pronuclear stage and maintained up to 17 h from the initiation of activation, when they

- 6 -

220 abruptly decreased. Chromosomal fusion and cleavage began at 17 h, with most parthenotes
221 (around 76%) appearing about 19-21 h (Figure 2).

222

223 **Redox state in non-activated, sperm and parthenogenetically activated oocytes**

224

225 In sperm activated oocytes, oxidative activity presented an increase between 15 and 19
226 h ($p<0.05$) (Figure 1; Figure 3, a - d). On the other hand, in parthenogenetically activated
227 oocytes, we observed a decrease at 9 h ($p<0.05$) which did not alter until 24 h (Figure 2, Figure
228 3, e – ch).

229 To determine whether the oxidative activity detected during early oocyte activation
230 depended on activation or simply reflected the length of time since maturation, *in vitro* matured
231 non-activated oocytes were cultured *in vitro* for 24 h, observing a significant decrease between
232 11 and 17 h ($p<0.05$) (Figure 4).

233

234 **Reactive oxygen species production in non-activated and sperm and parthenogenetically**
235 **activated oocytes**

236

237 ROS production was compared between sperm-activated oocytes and
238 parthenogenetically activated oocytes. In sperm-activated oocytes, ROS levels fluctuated
239 throughout the 24 h of development, presenting clearly discernable peaks around 7, 19 and 24 h
240 ($p<0.05$) (Figure 1; Figure 5, a - d), while in parthenogenetically activated oocytes, peaks were
241 detected at 7, 11 and 17 h ($p<0.05$) (Figure 2, Figure 5, e - h).

242 To determine whether the rise in ROS levels depended on oocyte activation or reflected
243 ROS production in the aging matured oocyte, non-activated oocytes were cultured *in vitro* for
244 24 h, in which we observed a significant decrease after 0 h ($p<0.05$) (Figure 4).

245
246

247 **DISCUSSION**

248

249 To our knowledge, this is the first time significant shifts in both ROS production and
250 redox state have been observed in association with temporal developmental events in bovine
251 oocyte sperm mediated and parthenogenetic activation.

252 Different temporal patterns of nuclear events were observed between both types of
253 activation. Within putative zygotes stemming from sperm mediated activation, there was a
254 temporal spread in the major developmental events after fertilization. In contrast, and not
255 surprisingly, parthenogenetically activated oocytes behaved in a highly synchronized manner
256 throughout development to the first cleavage division. The temporal sequence of developmental

257 events observed with both types of activation are similar to those previously reported for bovine
258 zygotes (Gordon, 1994). The difference in the patterns observed is attributed to the asynchrony
259 of sperm penetration, which lasts about 4 h in bovine (Jiang, 1991). Nevertheless, the
260 synchronous nuclear progression observed in parthenogenetic activation does not necessarily
261 equate to improved embryo development *in vitro* (Monaghan, 1993).

262 RedoxSensor Red CC-1 is a fluorescent dye that has been used as an indicator of
263 oxidative activity in living cells (Chen and Gee, 2000). The increase in oxidative activity
264 observed in sperm-activated oocytes corresponds with the initiation of pronuclear formation and
265 first mitotic division in putative zygotes, suggesting increased demands of energy for these
266 events. It has been observed that one and two cell bovine embryos are dependent on
267 mitochondrial oxidative phosphorylation for energy supply, consuming oxidative substrates to
268 produce ATP (Kim *et al.*, 1993, Thompson *et al.*, 1996). Coincidently, a higher oxygen
269 consumption rate was detected prior to cleavage in bovine zygotes (Lopes *et al.*, 2010).

270 In contrast, parthenotes initially have a high oxidative activity, which then declines from
271 7 h following activation and remains low thereafter during the developmental process with some
272 small, non-significant, oscillations despite the events of chromosomal fusion and first cleavage.

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273 To our knowledge, there is no published data concerning the metabolism of bovine parthenotes,
274 but an increase in the metabolic activity is expected in any type of embryo which undergoes cell
275 division. In coincidence with our findings, in mouse, parthenogenetic 1- to 2-cell embryos

Comment [a6]: changed

276 present a lower glucose metabolism, glycogen content, ATP content and adenylate kinase
277 activity than fertilized embryos (Han *et al.*, 2008). This difference in metabolic behavior
278 between both groups of activated oocytes could in part be responsible for the markedly lower
279 developmental competence of the parthenogenetically activated oocytes.

280 It has been shown that the DCFHDA probe is oxidized by hydrogen peroxide, its
281 derived oxidants, other peroxides and indirectly by the superoxide anion when generating
282 hydrogen peroxide, thus providing a useful test to evaluate ROS production (LeBel *et al.*, 1992).
283 In sperm activated oocytes, ROS peaks appear before and/or during structural events associated
284 with early embryo cleavage. The first peak occurs during preparative stages prior to pronuclear
285 formation (7 h), such as sperm penetration and sperm head decondensation and the second and
286 third peaks with association of pronuclei (19 h) and first mitotic division (24 h), respectively.
287 On the contrary, in non-activated oocytes ROS levels dropped after 7 h of culture and remained
288 low until 24 h. These results are in agreement with those reported for murine zygotes, in which
289 only fertilized oocytes showed a rise in ROS production, while unfertilized oocytes presented
290 declining levels over the same period (Nasr-Esfahani and Johnson, 1991). It has been suggested
291 that certain levels of ROS are needed for the interaction between the spermatozoa and oocytes
292 during bovine IVF, indicating that they may play different roles depending on the moment and
293 the quantity in which they are present (Blondin *et al.*, 1997). Very recently, a new class of

294 dioxigenases have been identified, the Ten-eleven translocation proteins (Tet 1-3) that are key to

295 the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine, thereby initiating the first

296 steps towards DNA de-methylation (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009). As

297 DNA demethylation is a key process within early embryo development immediately following

298 fertilization and pro-nuclear formation, perhaps the rise we have observed here in ROS

299 production and elsewhere in oxygen consumption (Lopes *et al*., 2010) reflects Tet protein

300 activity. In support of this, bovine pro-nuclear zygotes have significant levels of

301 hydroxymethylcytosine in the male pronucleus, but not the female pronucleus, which appears

302 largely due to the activity of Tet 3 (Wossidlo *et al*., 2011).

303 In parthenotes, high levels of ROS were observed, coinciding with a high rate of

304 oocytes at the pro-nuclear stage (7 and 11 h) and prior to cleavage (17 h). Once again, ROS

305 production seems to be associated with structural events related to early embryo development.

306 Coincidently, an increase in ROS production in parthenogenetically activated murine oocytes

307 was also observed (Nasr-Esfahani and Johnson, 1991).

308 Of interest was the lack of synchronicity between the peaks in ROS levels and those of

309 oxidative activity in both types of activation, indeed their behavior were contrasting during

310 development. It is known in somatic eukaryotic cells that mitochondria in a resting respiration

311 state (state 4) produce higher levels of ROS than those with active oxygen consumption (state

312 3); some of the electrons passing through the mitochondrial electron transport chain are

313 transferred to molecular oxygen to form superoxide anion, which can then derive hydrogen

314 peroxide (Boveris and Cadenas, 1975 and 1982).

315 During fertilization, the stimulation of mitochondrial respiration by sperm-triggered

316 Ca^{+2} oscillations has been observed (Campbell and Swann, 2006; Dumollard *et al.*, 2003, 2004;

317 Schomer and Epel, 1998). Thus, the lack of oxidative burst observed in parthenotes could also

318 be related to the single Ca^{+2} peak induced by parthenogenetic activation, which would not be

319 efficient to stimulate mitochondria consistently.

320 There is scarce information about metabolic changes which occur in early bovine

321 zygotes, especially in parthenogenetically activated oocytes. In the present work, we found clear

322 and distinctive metabolic patterns between non-activated oocytes, *in vitro* fertilized and

323 parthenogenetically activated oocytes. Characteristic behaviors in redox activity and

324 fluctuations of ROS production during early development could be integrated in our

325 understanding of measurements of oocyte and early embryo competence. The differences

326 observed in parthenogenetic zygotes with respect to these oxidative patterns could in part

327 explain their impaired developmental competence. Further studies into the metabolic control of

328 parthenogenetic activation could contribute to improve the performance of these embryos for

329 different biotechnological applications, such as somatic cell nuclear transfer for genetic

330 improvement through cloning and transgenesis.

- 9 -

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489 **LEGEND OF FIGURES**

490

491 **Figure 1:**

492   **A)** Nuclear stage of putative zygotes in sperm mediated oocyte activation, n= 1612
493   zygotes.

494   **B)** Redox state in sperm mediated oocyte activation. Values are expressed as mean
495   arbitrary units/oocyte or zygote $\pm$ SEM. n = 134 putative zygotes. [a,b] Values with
496   different superscripts are significantly different ($p<0.05$).

497   **C)** Reactive oxygen species production/total esterase activity in sperm mediated oocyte
498   activation. Values are the ratio between DCHFDA and FDA assays; they are expressed
499   as mean Arbitrary ROS Units/oocyte or zygote $\pm$ SEM. n = 1478 putative zygotes. [a,b,c,d,e]
500   Values with different superscripts are significantly different ($p<0.05$).

501

502 **Figure 2:**

503   **A)** Nuclear stages observed in parthenogenetic oocyte activation, n= 630 parthenotes.

504   **B)** Redox state in parthenogenetically activated oocytes. Values are expressed as mean
505   arbitrary units/oocyte or parthenote $\pm$ SEM. n = 172 parthenotes. [a,b,c] Values with
506   different superscripts are significantly different ($p<0.05$).

507   **C)** Reactive oxygen species production/total esterase activity in parthenogenetically
508   activated oocytes. Values are the ratio between DCHFDA and FDA assays; they are
509   expressed as mean Arbitrary ROS Units/oocyte or parthenote $\pm$ SEM. n = 458
510   parthenotes. [a,b,c] Values with different superscripts are significantly different ($p<0.05$).

511

512 **Figure 3:** Representative activated oocytes or putative zygotes stained with RedoxSensor Red
513 CC1 (x120). (a) to (d) Sperm activated oocytes at 0, 7, 11 and 19 h from activation and (e) to (h)
514 Parthenogenetically activated oocytes at 0, 7, 11 and 19 h from activation. Bar = 50 μm.

515

516　**Figure 4:**

517　　**A)** Redox state in non-activated oocytes. Values are expressed as mean arbitrary
518　　　units/oocyte $\pm$ SEM. n = 180 oocytes. [a,b] Values with different superscripts are
519　　　significantly different ($p<0.05$).

520　　**B)** Reactive oxygen species production/total esterase activity in non-activated oocytes.
521　　　Values are the ratio between DCHFDA and FDA assays; they are expressed as mean
522　　　Arbitrary ROS Units/oocyte $\pm$ SEM. n = 200 oocytes. [a,b,c] Values with different
523　　　superscripts are significantly different ($p<0.05$).

524

525　**Figure 5:**　Representative activated oocytes or putative zygotes stained with 2,7 –
526　dichlorodihydrofluorescein diacetate (x120). (a) to (d) Sperm activated oocytes at 0, 7, 11 and
527　19 h from activation and (e) to (h) Parthenogenetically activated oocytes at 0, 7, 11 and 19 h
528　from activation. Bar = 50 μm.

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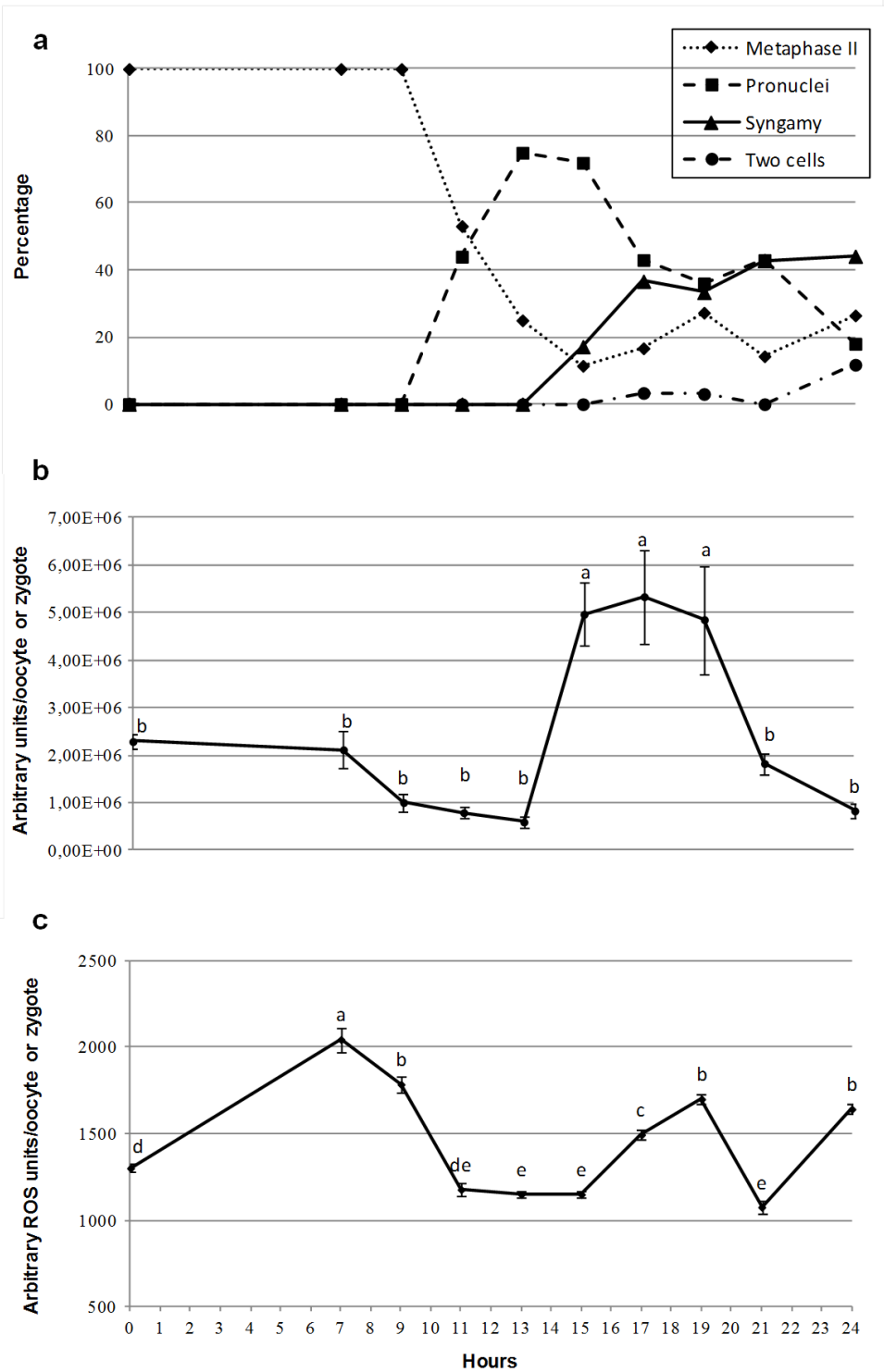537
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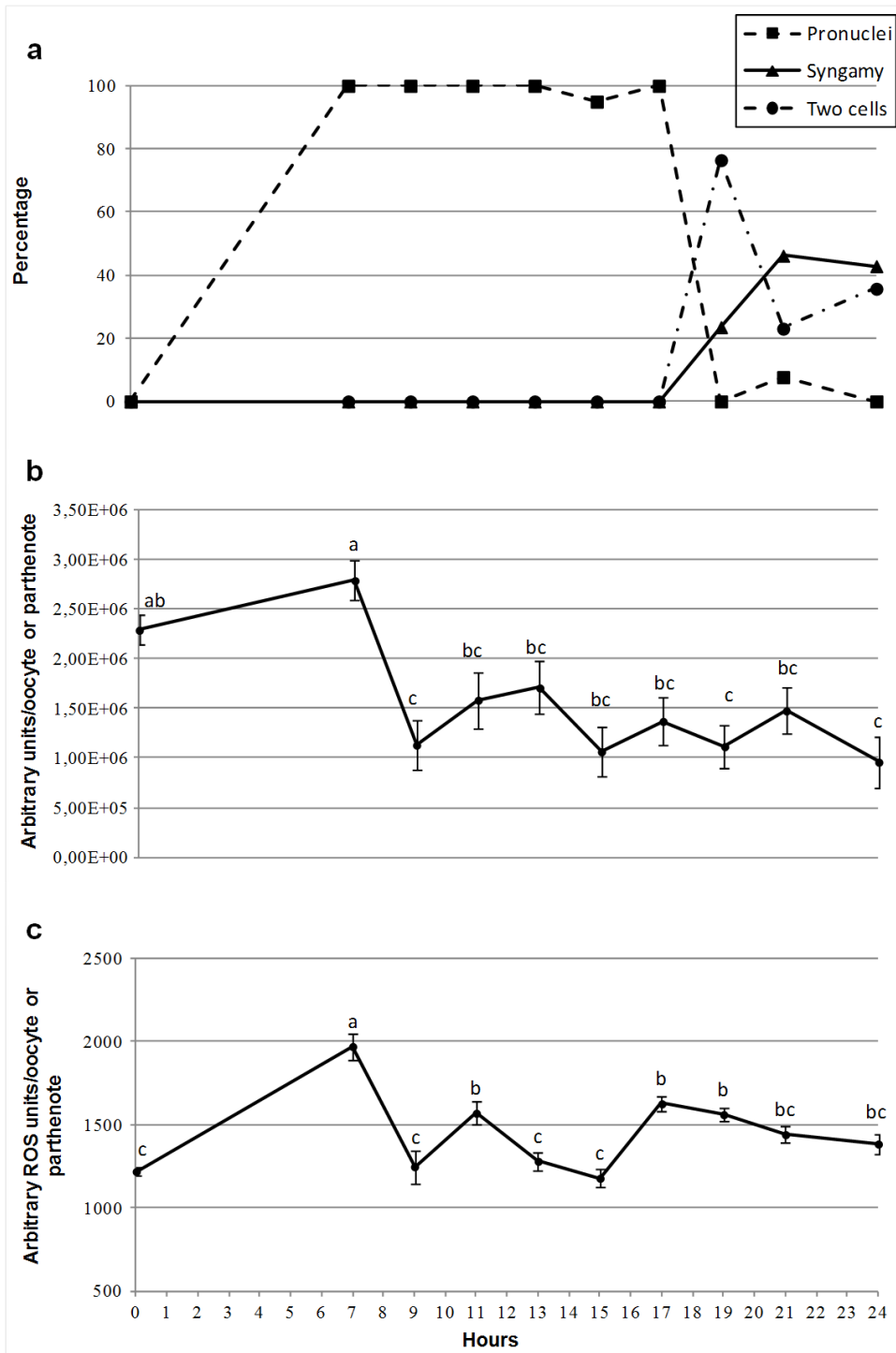
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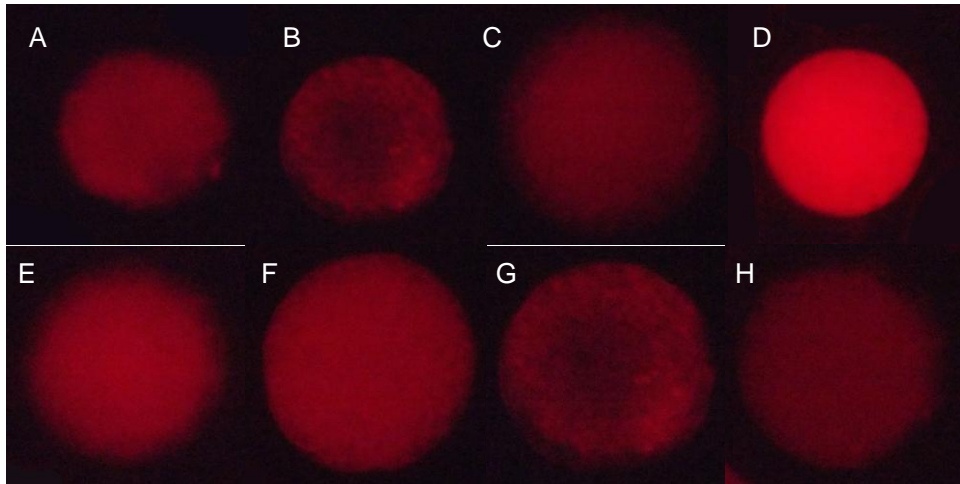556
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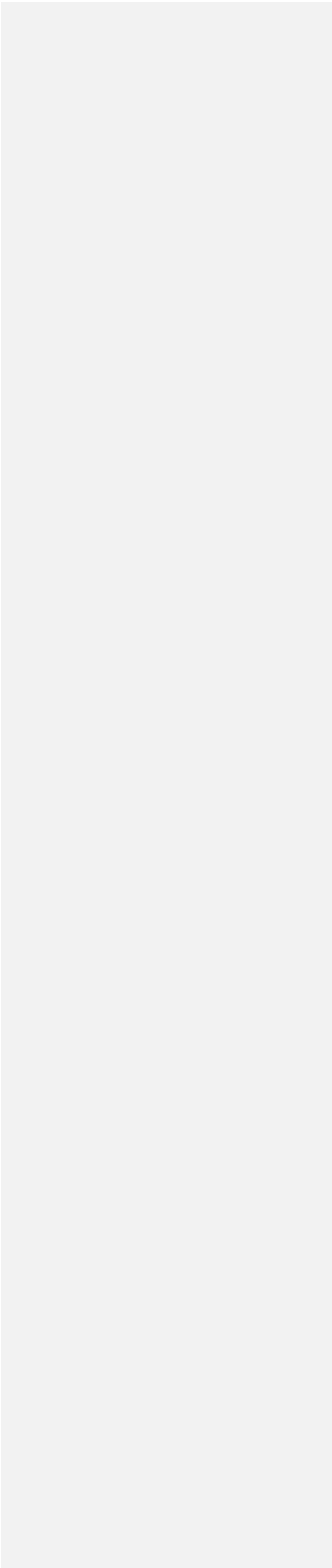
577 **Figure 3**

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606 **Figure 4**
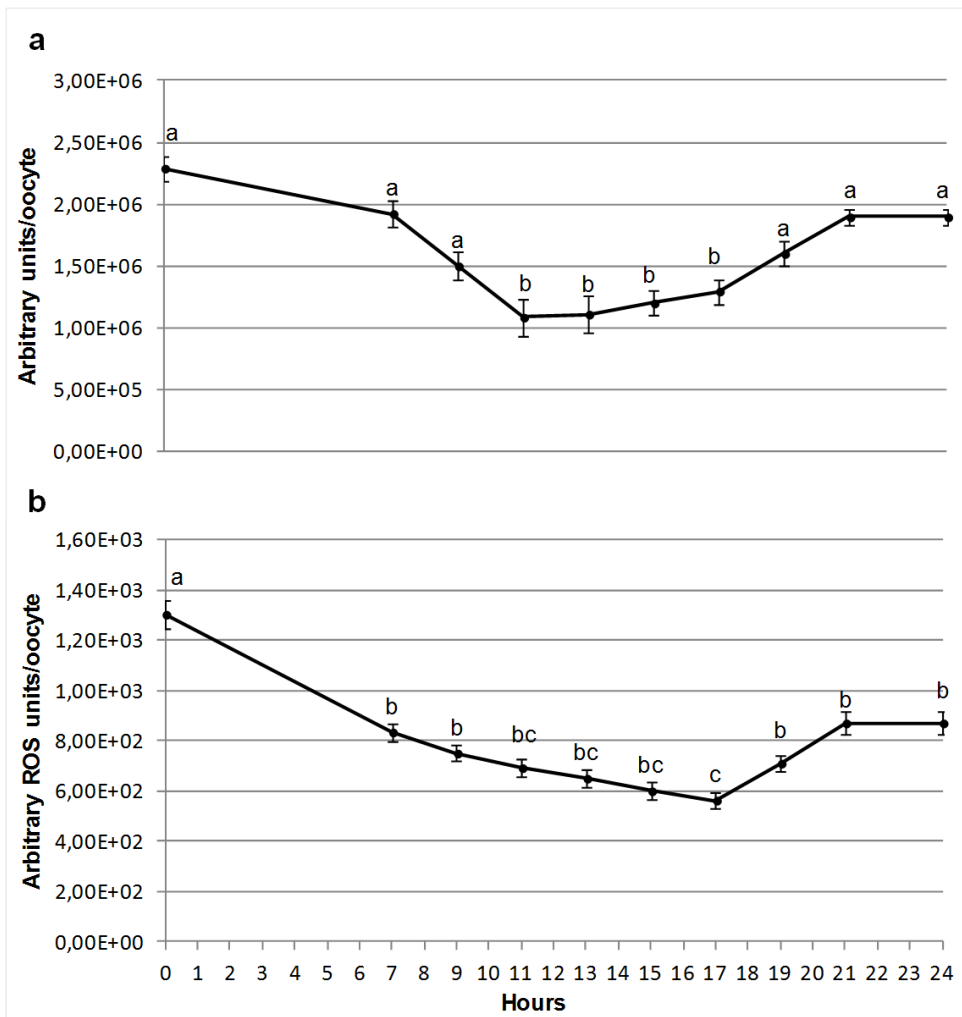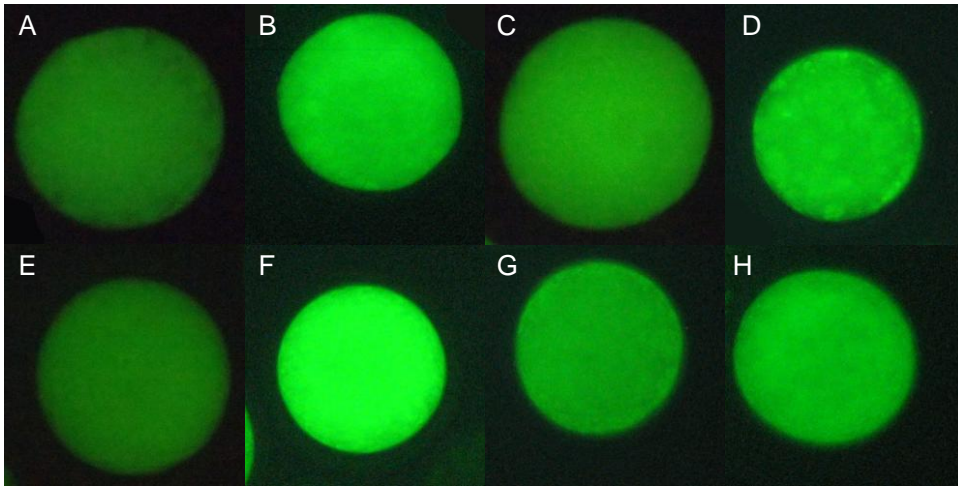
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620 **Figure 5**

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