

This is the final peer-reviewed accepted manuscript of:

Spinaci M, Bucci D, Muccilli V, Cardullo N, Nerozzi C, Galeati G. A polyphenol-rich extract from an oenological oak-derived tannin influences in vitro maturation of porcine oocytes. *Theriogenology* 2019;129:82-89.

The final published version is available online at:

<https://doi.org/10.1016/j.theriogenology.2019.02.017>

© [2019]. This manuscript version is made available under the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) 4.0 International License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

A POLYPHENOL-RICH EXTRACT FROM AN OENOLOGICAL OAK-DERIVED TANNIN INFLUENCES IN VITRO MATURATION OF PORCINE OOCYTES

Marcella Spinaci¹, Diego Bucci¹, Vera Muccilli², Nunzio Cardullo², Chiara Nerozzi¹, Giovanna Galeati¹

¹Dipartimento di Scienze Mediche Veterinarie, Università di Bologna, Bologna, Italy

²Dipartimento di Scienze Chimiche, Università di Catania, Catania, Italy

Dedicated to the memory of Carmela Spatafora

Corresponding author: Marcella Spinaci

Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia-Bologna, University of Bologna, Italy

e-mail address: marcella.spinaci@unibo.it

19 **ABSTRACT**

20 Tannins have been demonstrated to have antioxidant and various health benefit properties. The
21 aim of this study was to determine the effect of an ethanol extract (TRE) of a commercial oenological
22 tannin (*Quercus robur* toasted oak wood, Tan'Activ R[®]) on female gamete using an in vitro model of
23 pig oocyte maturation (IVM) and examining nuclear maturation, cytoplasmic maturation,
24 intracellular GSH and ROS levels and cumulus cell steroidogenesis.

25 To this aim, during IVM performed in medium either supplemented (IVM A) or not supplemented
26 (IVM B) with cysteine and β -mercaptoethanol, TRE was added at different concentrations (0, 1, 5,
27 10, 20 $\mu\text{g/ml}$).

28 The addition of TRE at all the concentration tested to either IVM A or IVM B, did not influence oocyte
29 nuclear maturation. When IVM was performed in IVM A, no effect was induced on cytoplasmic
30 maturation by TRE at the concentration of 1, 5 and 10 $\mu\text{g/ml}$, while TRE 20 $\mu\text{g/ml}$ significantly
31 reduced the penetration rate after IVF ($p<0.05$) and the blastocyst rate after parthenogenetic
32 activation ($p<0.01$). Oocyte maturation in IVM B, compared to IVM A group, decreased GSH
33 ($p<0.001$) and increased ROS ($p<0.01$) intracellular levels and in turn impaired oocyte cytoplasmic
34 maturation reducing the ability to sustain male pronuclear formation after IVM ($p<0.001$) and the
35 developmental competence after parthenogenetic activation ($p<0.001$). TRE supplementation to
36 IVM B significantly reduced ROS production (5, 10, 20 $\mu\text{g/ml}$ TRE) to levels similar to IVM A group,
37 and increased GSH levels (10, 20 $\mu\text{g/ml}$ TRE) compared to IVM B ($p<0.05$) without reaching those of
38 IVM A group. TRE supplementation to IVM B at the concentrations of 1, 5 and 10 $\mu\text{g/ml}$ significantly
39 improved ($p<0.001$) oocyte cytoplasmic maturation enhancing the ability to sustain male pronuclear
40 formation without reaching, however, IVM A group levels.

41 TRE addition at all the concentration tested to both IVM A and IVM B, did not induce any effect on
42 E2 and P4 secretion by cumulus cells suggesting that the biological effect of the ethanol extract is
43 not exerted through a modulation of cumulus cell steroidogenesis.

44 In conclusion, TRE, thanks to its antioxidant activity, was partially able to reduce the negative effect
45 of the absence of cysteine and β -mercaptoethanol in IVM B, while TRE at high concentration in IVM
46 A was detrimental for oocyte cytoplasmic maturation underlying the importance of maintaining a
47 balanced redox environment during oocyte maturation.

48

49 **KEYWORDS**

50 Antioxidants, IVM, IVF, oocyte developmental competence, GSH, ROS

51

52

53 **1. INTRODUCTION**

54 Tannins are a broad class of bioactive compounds that are present not only in red wine but also in
55 tea, cocoa, chocolates, coffee, herbal preparations, grapes and fruits like blackberries and
56 cranberries.

57 Wine aging process in oak barrels, due to soluble polyphenols diffusion into the wine, plays a crucial
58 role not only in improving organoleptic properties, such as color, flavor and aroma but also in
59 acquiring health protective properties [1].

60 Many studies reported beneficial effects of tannins and their extracts in somatic cells, in fact tannins
61 proved to have various health protective activities, especially antioxidant, anticarcinogenic,
62 cardioprotective, antiinflammatory [2-5]. Nevertheless, there is limited information regarding the
63 effects of tannins and its extracts on reproduction.

64 Recently, extracts of commercial oenological tannins from *Quercus robur* [6] and *Castanea sativa*
65 [7] have been evaluated for their hypoglycemic and antioxidant activities. The ethanol extract (TRE)
66 of *Quercus robur* toasted oak wood (Tan'Activ R[®]) and its fractions have been demonstrated to exert
67 a powerful biological effect on male gametes finely modulating sperm capacitation and in turn
68 sperm fertilizing ability [8]. However, no information is available on the biological effect of TRE on
69 the female gamete counterpart.

70 The objective of this study was to examine the possible biological effect of TRE on female gamete
71 using an "in vitro" model of pig oocyte maturation (IVM) performed in medium either supplemented
72 or not with cysteine and β -mercaptoethanol, both of these molecules known to improve pig oocyte
73 maturation inducing a reduction of ROS levels and an increase in GSH content of the oocyte [9-13].

74 To that purpose, at the end of the maturation period we evaluated nuclear and cytoplasmic
75 maturation of oocytes, steroidogenic activity of cumulus cells, intracellular levels of glutathione

76 (GSH) and ROS of oocytes, as well as blastocyst formation after parthenogenetic activation of IVM
77 oocytes.

78

79 **2. MATERIALS AND METHODS**

80 Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy).

81 The ethanol extract of the commercial Tan'Activ R[®] (TRE) was obtained as previously reported (QR2E
82 extract) [6].

83

84 **2.1. In vitro maturation of porcine oocytes (IVM)**

85 Porcine cumulus–oocyte complexes (COCs) were aspirated using a 18 gauge needle attached to a
86 10 mL disposable syringe from 4 to 6 mm follicles of ovaries collected at a local abattoir and
87 transported to the laboratory within 1 h. Under a stereomicroscope, intact COCs were selected and
88 transferred into a petri dish (35 mm, Nunclon,Denmark) prefilled with 2 mL of modified PBS
89 supplemented with 0.4% BSA.

90 The maturation media used were: NCSU 37 [14] supplemented with 1mM glutamine, 5.0 µg/mL
91 insulin, 10 ng/mL epidermal growth factor (EGF), 10% porcine follicular fluid, 0.57 mM cysteine and
92 50 µM β-mercaptoethanol (IVM A) and the same medium (IVM A) without cysteine and β-
93 mercaptoethanol supplementation (IVM B).

94 After three washes in IVM A or IVM B, groups of 45-50 COCs were transferred to a Nunc 4-well
95 multidish containing 500 µL the same maturation medium per well and cultured at 39 °C in a
96 humidified atmosphere of 5% CO₂ in air. For the first 22 h of in vitro maturation the medium was
97 supplemented with 1.0 mM db-cAMP, 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands)
98 and 10 IU/mL hCG (Corulon, Intervet). For the last 22 h COCs were transferred to fresh maturation

99 medium (IVM A or IVM B). At the end of the maturation period the oocytes were denuded by gentle
100 repeated pipetting.

101

102 **2.2. Evaluation of nuclear maturation**

103 At the end of the maturation period oocytes were mounted on microscope slides, fixed in acetic
104 acid/ ethanol (1:3) for 24 h and stained with lacmoid. The oocytes were observed under a phase
105 contrast microscope in order to evaluate the meiotic stage achieved and those with a nuclear
106 morphology corresponding to MII were considered mature.

107

108 **2.3. Evaluation of cytoplasmic maturation**

109 At the end of the maturation period cytoplasmic maturation was assessed by evaluating:

110 a) the ability of oocytes to decondense sperm head and sustain male pronucleus formation after in
111 vitro fertilization.

112 Frozen boar semen was purchased from a commercial company (Inseme S.P.A., Modena, Italy).

113 Straws were thawed in a water-bath at 37°C under agitation for 30 s and immediately diluted, at the
114 same temperature, in Beltsville Thawing Solution (BTS) at a dilution rate 1:3.

115 After 1 h semen was washed twice with BTS and finally resuspended with Brackett & Oliphant's
116 medium [15] supplemented with 12% fetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/ml
117 caffeine (IVF medium). 45–50 in vitro matured oocytes freed from cumulus cells were transferred
118 to 500 µl IVF medium containing 1×10^6 sperm/ml. After 1 h of coculture, oocytes were transferred
119 to fresh IVF medium previously equilibrated under 5% CO₂ in air and cultured until fixation, as above
120 described, 18-19 h post-insemination.

121 Oocytes were considered penetrated when containing two polar bodies, one female pronucleus and
122 one or more sperm heads and/or male pronuclei with their corresponding sperm tails. Oocytes were

123 considered cytoplasmically mature when at least one decondensed sperm head or male pronucleus
124 could be identified [13]. Degenerated and immature oocytes were not counted. Parameters
125 evaluated were:

- 126 - penetration rate (number of oocytes penetrated/total inseminated),
- 127 - monospermy rate (number of oocytes containing only one sperm head - male
128 pronucleus/total penetrated)
- 129 - percentage of penetrated oocytes with one female pronucleus and at least one
130 decondensed sperm head
- 131 - percentage of penetrated oocytes with one female and at least one male pronucleus

132

133 *b) the developmental competence of parthenotes after 7 days of in vitro culture*

134 At the end of maturation period oocytes were denuded as described above, washed three times in
135 IVF medium and then parthenogenetically activated according to the method described by Boquest
136 et al. [16] slightly modified [17]. Briefly, the oocytes were transferred to IVF medium containing 5
137 μ M ionomycin for 5 min, then washed twice and incubated in NCSU-23 [14] containing 2 mM 6-
138 dimethylaminopurine (6-DMAP) for 3 h at 39°C. Presumptive parthenotes were washed twice in
139 NCSU-23 and cultured in groups of 45–50 in 500 ml of the same medium. On Day 5 postactivation,
140 250 μ l of the medium were replaced with fresh pre-equilibrated NCSU-23 containing 20% (v/v) FCS
141 to reach a final FCS concentration of 10% (v/v). At Day 7 postactivation, percent of blastocysts and
142 number of blastocyst nuclei were determined by fixing and staining parthenotes as above described
143 for oocytes. Embryos with at least 20 blastomeres and a clearly visible blastocoel were considered
144 as blastocysts.

145

146 **2.4. Evaluation of cumulus cell steroidogenesis**

147 IVM media of both the first and the second day of culture were collected, centrifuged at 900xg for
148 5 min and the supernatants were stored at -20 °C until assayed for progesterone (P4) and estradiol-
149 17 β (E2) by validated radioimmunoassays [18]. At the end of the maturation period, cumulus cells
150 were counted using a Thoma's hemocytometer, after being freed from matured oocytes by gentle
151 repeated pipetting. For P4, intra and interassay coefficients of variation were 7.8% and 10.1%,
152 respectively; assay sensitivity was 3,3 pg/tube. Intra and interassay coefficients of variation for E2
153 were 4% and 12%, respectively; assay sensitivity was 1.1 pg/tube. Steroid concentrations are
154 expressed as ng/10⁶ cells.

155

156 **2.5. Evaluation of intracellular ROS and GSH levels**

157 Intracellular GSH and ROS levels of oocytes at the end of maturation period were determined using
158 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen, Italy) or
159 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Italy), respectively. From each
160 treatment group, oocytes were incubated in the dark for 30 min at 39 °C in PBS/0.1% (wt/vol) PVA
161 supplemented with 10 μ M H2DCFDA or 10 μ M CellTracker Blue. Following incubation, the oocytes
162 were washed in PBS/0.1% (wt/vol) PVA, placed into 10- μ l droplets, and fluorescence was evaluated
163 under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoevedop, The
164 Netherlands). The fluorescence images were analysed with Image J software (public domain).
165 Relative oocyte fluorescence was measured by normalizing the oocyte fluorescence with the
166 background and with each oocyte area.

167

168 **2.6 Experimental design**

169 To examine the biological effect of the addition of an ethanol extract (TRE) of a commercial
170 oenological tannin (Tan'Activ R®) during in vitro maturation of pig oocytes, different concentrations
171 of TRE (0, 1, 5, 10, 20 µg/ml) were added to in vitro maturation medium supplemented (IVM A) or
172 non-supplemented (IVM B) with cysteine and β-mercaptoethanol.

173 A total of 2729 oocytes were fixed at the end of the maturation period to evaluate their nuclear
174 maturation [seven replicates; oocytes examined in each replicate for each experimental group:
175 median (interquartile range, IQR) = 40 (10)].

176 In order to examine TRE effect on cytoplasmic maturation, at the end of the maturation period a
177 total of 2613 oocytes were inseminated to evaluate their ability to decondense sperm head and
178 sustain male pronucleus formation [seven replicates; oocytes examined in each replicate for each
179 experimental group: median (IQR) = 38 (9)]; moreover a total 3102 oocytes were
180 parthenogenetically activated to evaluate their developmental competence [seven replicates;
181 oocytes examined in each replicate for each experimental group: median (IQR) = 47 (5)].

182 IVM media from the different experimental groups (seven replicates) were collected the first and
183 the second day of culture and assayed for steroid production (P4 and E2) by cumulus cells.

184 The effect of TRE on intracellular levels of GSH and ROS was investigated in three independent
185 experiment with 15-20 oocytes each time for each experimental group (GSH samples, n= 570
186 oocytes; ROS samples, n= 601 oocytes).

187

188 **2.7. Statistical analyses**

189 Statistical analyses were performed using R (version 3.4.0)[19]. Values are expressed as
190 mean ± standard deviation (SD) and level of significance was at $p < 0.05$ unless otherwise specified.

191 Data on nuclear maturation, IVF trials, blastocyst formation and cumulus cell steroidogenesis were
192 analysed using a general linear model with binomial distribution and a Tukey post-hoc test was
193 subsequently run to determine differences between treatments.

194 Data on blastomere number were analysed using a Poisson distribution and a Tukey post-hoc test
195 was subsequently run to determine differences between treatments.

196 Data on GSH and ROS intracellular levels, after being tested for normality and homogeneity of
197 variances through Shapiro-Wilk test, were analysed using Non-parametric Kruskal-Wallis Test and
198 Wilcoxon test was subsequently used to assess differences between treatments.

199

200 **3. RESULTS**

201 **3.1. Effect of TRE on nuclear maturation**

202 When COCs were cultured in IVM A or IVM B in presence of increasing concentrations of TRE (0, 1,
203 5, 10, 20 µg/ml), no significant variations in the proportion of oocytes completing nuclear
204 maturation showing a MII nuclear morphology were recorded (Fig. 1).

205

206 **3.2. Effect of TRE on cytoplasmic maturation**

207 *a) Effect of TRE on the ability of oocytes to decondense sperm head and sustain male pronucleus*
208 *formation after in vitro fertilization.*

209 After in vitro fertilization, when oocytes were matured in IVM A, TRE at the concentrations of 1
210 µg/ml induced a slight not significant increase in penetration rate compared to control.
211 Concentrations of 5 and 10 µg/ml significantly ($p < 0.01$) decreased the percentage of penetrated
212 oocytes compared to TRE 1 µg/ml, while 20 µg/ml showed a significantly lower penetration rate
213 compared to both IVM A ($p < 0.05$) and TRE 1 µg/ml ($p < 0.01$). No significant change in the other

214 fertilization parameters assessed (monospermy rate, ability of oocytes to decondense sperm head
215 and sustain male pronucleus formation) were observed (Fig. 2A).

216 Oocytes maturation in IVM B did not influence penetration rate and monospermy rate compared to
217 IVM A, moreover the addition of TRE at all the concentration tested (1, 5, 10, 20 $\mu\text{g}/\text{ml}$) did not
218 induce any significant change in these parameters (Fig. 2B, left panel).

219 However, oocytes matured in IVM B, as compared to IVM A group, showed a dramatic decrease
220 ($p<0.001$) in the percentage of penetrated oocytes with at least one male pronucleus while the
221 percentage of penetrated oocytes with at least one decondensed sperm head was significantly
222 increased (Fig. 2 B, right panel). The percentage of the oocyte considered cytoplasmically mature
223 significantly dropped ($p<0.001$) in IVM B as compared to IVM A group.

224 TRE addition to IVM B at the concentrations of 1, 5, and 10 $\mu\text{g}/\text{ml}$ induced a significant increase of
225 the percentage of penetrated oocytes with male pronucleus compared to IVM B ($p<0.001$ for TRE 5
226 $\mu\text{g}/\text{ml}$ and $p<0.01$ for TRE 1 and 10 $\mu\text{g}/\text{ml}$) remaining, however, significantly lower ($p<0.001$)
227 compared to IVM A. The addition of TRE to IVM B did not increase the percentage of oocytes with
228 decondensed sperm head/s. As a consequence, TRE addition to IVM B at the concentrations of 1,
229 5, and 10 $\mu\text{g}/\text{ml}$ significantly ($p<0.001$) improved the percentage of cytoplasmically mature oocytes
230 compared to IVM B without reaching, however, the level of oocyte matured in IVM A (Fig. 2B, right
231 panel).

232

233 *b) Effect of TRE on the developmental competence of parthenotes after 7 days of in vitro culture.*

234 TRE addition to IVM A during oocyte maturation period did not modify, after parthenogenic
235 activation, the percentage of oocytes that developed to blastocyst stage and the mean blastomere
236 number per blastocyst except for a reduction in blastocyst rate observed in TRE 20 group ($p<0.01$)
237 (Fig.3, left panel).

238 Oocytes matured in IVM B, regardless of TRE supplementation, showed a significant lower ($p < 0.001$)
239 blastocyst formation rate as compared to IVM A group. The mean blastomere number per blastocyst
240 was significantly lower in IVM B group compared to IVM A group ($p < 0.05$). However TRE at the
241 concentration of 5 and 10 $\mu\text{g}/\text{ml}$ increased the blastomere number reaching values similar to IVM A
242 group (Fig.3, right panel)

243

244 **3.3. Effect of TRE on cumulus cell steroidogenesis**

245 Basal steroid production by cumulus cells after 22 and 44 h of culture is shown in Fig. 4.

246 E2 and P4 outputs when COCs were cultured in IVM A were 14.1 ± 3.0 and 14.1 ± 5.2 $\text{ng}/10^6$ cells,
247 121.0 ± 7.6 and 1252.8 ± 349.7 $\text{ng}/10^6$ cells after 22 and 44 h, respectively. E2 and P4 outputs when
248 COCs were cultured in IVM B were 13.1 ± 5.0 and 12.7 ± 5.2 $\text{ng}/10^6$ cells, 118.8 ± 40.5 and $1154.3 \pm$
249 509.8 $\text{ng}/10^6$ cells after 22 and 44 h, respectively. No differences were recorded in E2 and P4
250 production between IVM A and IVM B.

251 None of the TRE concentrations tested induced any effect on both E2 and P4 production, both on
252 the first and the second day of culture compared to control group.

253

254 **3.4. Effect of TRE on ROS and GSH levels**

255 The levels of ROS were not statistically influenced by the addition of TRE when oocytes were
256 matured in IVM A (Fig. 5A). Oocytes matured in IVM B showed significantly higher ($p < 0.01$)
257 intracellular ROS levels compared to IVM A group. TRE addition to IVM B at the concentrations of 5,
258 10 and 20 $\mu\text{g}/\text{ml}$ induced a significant reduction of intracellular ROS levels to values similar to IVM
259 A group (Fig. 5B).

260 The addition of TRE to IVM A did not induce any statistical modification of intracellular GSH levels
261 (Fig. 6A). Oocytes matured in IVM B, as compared to IVM A group, showed a significant decrease

262 (p<0.001) in the intracellular GSH levels. TRE addition to IVM B at the concentrations of 5 and 10
263 µg/ml induced a significant increase of intracellular GSH levels without reaching those of IVM A
264 group (Fig. 6B).

265

266 **4. DISCUSSION**

267 The aim of the present study was to examine the possible biological effect on female gamete of an
268 ethanol extract of a commercial oenological tannin (*Quercus robur* toasted oak wood, Tan'Activ
269 R®)(TRE) with antioxidant properties [6], using an in vitro model of pig oocyte maturation evaluating
270 nuclear and cytoplasmic maturation, intracellular levels of ROS and GSH and cumulus cell
271 steroidogenesis.

272 The process of oocyte maturation requires a rigorous supply of energy in the form of adenosine
273 triphosphate. The ATP generation by the mitochondrial electron transport chain during the
274 maturation process results in the production of ROS. Increased levels of ROS beyond the
275 physiological range which may lead to oxidative stress, can result in deterioration of oocyte quality
276 [20]. In vivo oocytes are protected from the harmful effects of ROS by anti-oxidant enzymes which
277 are present in the follicular fluid [21]. However, during in vitro maturation, besides the endogenous
278 ROS production and the lack of physiological defense mechanisms present in the follicular fluid,
279 multiple exogenous factors can act as potential sources of ROS (i.e. exposure to visible light, pH and
280 temperature, oxygen concentration, handling of gamete).

281 In order to evaluate the activity of antioxidant molecules during IVM, several studies have induced
282 an oxidative stress by H₂O₂ supplementation or adding to the culture medium exogenous ROS
283 generating systems such as hypoxanthine-xanthine oxidase system [12,13,23]. In this study, instead
284 of exogenous inducing oxidative stress, we evaluated the effect of TRE supplementation during

285 porcine IVM performed either in presence (IVM A) or in absence (IVM B) of cysteine and β -
286 mercaptoethanol, molecules often added to pig IVM media as have been demonstrated to induce a
287 reduction of ROS levels and an increase in GSH content of porcine oocyte, improving the cytoplasmic
288 maturation of this gamete [9-13].

289 As a first step of this study, we investigated the biological effect of the ethanol extract TRE on
290 nuclear maturation of pig oocytes. All the TRE concentration tested (1, 5, 10, 20 $\mu\text{g/ml}$), in both IVM
291 A and IVM B media, did not modify the percentage of oocytes reaching MII stage. Other studies have
292 reported similar findings after antioxidant addition during pig IVM [23-25]. It has to be stressed that
293 the addition of cysteine and β -mercaptoethanol to the culture medium (IVM A) did not modify
294 meiotic progression compared to IVM B, where these molecules were absent. The lack of effect of
295 cysteine and β -mercaptoethanol on pig oocyte nuclear maturation was previously reported [11,26]
296 even in presence of ROS production systems (xanthine + xanthine oxidase) [13]. All together, these
297 results seems to suggest that the meiotic progression is not strongly influenced by oxidative stress.

298 However when a very high concentration of TRE (500 $\mu\text{g/ml}$) was added to IVM A, a significant
299 reduction in the percentage of oocytes reaching MII stage was observed (data not shown). This
300 result agrees well with those of other authors who recorded that the polar body extrusion rate was
301 negatively influenced in presence of high concentration of taxifolin or quercetin (50 $\mu\text{g/ml}$), plant-
302 derived flavonoids with antioxidant properties [23,27]. These results suggest the possibility of
303 inducing, by excessive antioxidants supplementation, a dangerous condition called "antioxidant
304 paradox" leading to "reductive stress" [28,29] that in turn may impair nuclear maturation. In fact,
305 controlled and physiological ROS amounts seem to be required for meiotic resumption and nuclear
306 maturation of oocytes [30-32].

307 The ability of oocytes to decondense sperm head and sustain male pronucleus formation after in
308 vitro fertilization and the developmental competence of parthenotes after 7 days of in vitro culture
309 were used as parameters of proper cytoplasmic maturation.

310 When oocytes matured in presence of cystein and β -mercaptoethanol (IVM A) were fertilized in
311 vitro, TRE 20 was detrimental to penetration rate possibly due to a surplus of antioxidant molecules
312 at the cellular level with the creation of an environment that was too reduced; in fact oocytes
313 matured in IVM A showed a tendency, although not significant, to a decrease of ROS levels with
314 increasing concentration of TRE. The adverse effect of TRE addition at high concentration (20 $\mu\text{g}/\text{mL}$)
315 during maturation in IVM A was also evident after partenogenetic activation leading to a significant
316 reduction of blastocyst rate. These results suggest a possible toxic effect of excessive amount of
317 antioxidants and confirm the need of a proper balance between pro and antioxidant during oocyte
318 in vitro maturation [30].

319 In vitro maturation of pig oocyte in absence of cysteine and β -mercaptoethanol (IVM B) did not
320 affect the penetration rate. However maturation in IVM B halved the percentage of cytoplasmically
321 mature oocytes, significantly reducing the percentage of oocytes able to sustain male pronuclear
322 formation compared to IVM A group; this decrease is likely a consequence of the reduction of
323 intracellular GSH levels and the increase of ROS, which was recorded in this study, confirming the
324 findings obtained by other authors [9-13]. Adequate oocyte GSH levels are in fact needed in order
325 to reduce sperm nuclear disulfide bonds that represent the first step in the induction of sperm
326 nuclear decondensation and hence male pronucleus formation after in vitro fertilization [33].

327 Interestingly, while no significant differences on penetration rate were recorded, the addition of TRE
328 at the concentrations of 1, 5, and 10 $\mu\text{g}/\text{ml}$ to IVM B medium improved oocyte cytoplasmic
329 maturation promoting male pronuclear formation. The percentages of cytoplasmically mature

330 oocytes, however, did not reach those of IVM A group and this result could be due to the absence
331 in IVM B of β -mercaptoethanol and, in particular, of cystein, a required substrate for GSH synthesis
332 in maturing pig oocytes [33]. In fact, while TRE addition at the concentration of 5, 10, 20 $\mu\text{g}/\text{ml}$ to
333 IVM B reduced intracellular ROS levels to values comparable to those found in IVM A group, TRE 5
334 and 10 supported a level of GSH synthesis significantly higher compared to IVM B without reaching,
335 however, that of oocytes matured in presence of cystein and β -mercaptoethanol (IVM A). The
336 improvement of GSH levels observed in TRE 5 and TRE 10 groups matured in IVM B was probably
337 still insufficient to fully support the subsequent embryonic development after parthenogenetic
338 activation as the blastocyst rate did not differ compared to IVM B group and was significantly lower
339 compared to IVM A group. However when in vitro maturation was performed in IVM B in presence
340 of 5 and 10 $\mu\text{g}/\text{ml}$ TRE, a significant increase of blastomere number per blastocyst up to that of IVM
341 A group was observed suggesting a certain beneficial effect of the extract.

342 Irrespective of IVM medium used and TRE treatments, P4 production dramatically increased during
343 the second half of culture, likely due to cumulus cell differentiation/luteinization [34]. In our model,
344 TRE at all the concentration tested did not induce any effect on E2 and P4 secretion by cumulus cells
345 after 22 and 44 hours of culture suggesting that the biological effect of the ethanol extract is not
346 exerted through a modulation of cumulus cell steroidogenesis.

347 To our knowledge, the results of this study represent the first evaluation of biological activities of
348 an ethanol extract of an oenological commercial oak-derived tannin on female gametes. TRE exerted
349 a beneficial biological effect during oocyte maturation performed in absence of cysteine and β -
350 mercaptoethanol (IVM B) reducing intracellular ROS levels, increasing GSH levels and in turn
351 improving cytoplasmic maturation, particularly in term of oocyte ability to promote male pronucleus
352 formation. No positive effect of TRE supplementation was observed when maturation was
353 performed in medium IVM A in which the presence cysteine and β -mercaptoethanol, probably

354 saturating oocyte's antioxidant requirement, may have masked TRE activity. TRE addition at high
355 concentration to IVM A, and therefore an excessive antioxidant capacity, seems even detrimental
356 for oocyte cytoplasmic maturation underlying the importance of maintaining a balanced redox
357 environment during oocyte maturation.

358

359 **Funding**

360 Work supported by "Piano della Ricerca di Ateneo 2016-2018, Linea di Intervento 2, Università
361 degli Studi di Catania, Italy.

362 **Acknowledgement**

363 The Authors wish to thank Mrs Cinzia Cappannari and Mr Danilo Matteuzzi for their precious
364 support.

365 **Conflicts of interest**

366 The authors have declared no conflicts of interest.

367

368

369 **5. REFERENCES**

370

371 [1] Zhang B, Cai J, Duan CQ, Reeves MJ, He F. A review of polyphenolics in oak woods. *Int J Mol Sci*
372 2015;16:6978-7014. <https://doi.org/10.3390/ijms16046978>.

- 373 [2] Koleckar V, Kubikova K, Rehakova Z, Kuca K, Jun D, Jahodar L, Opletal L. Condensed and
374 hydrolysable tannins as antioxidants influencing the health. *Mini Rev Med Chem* 2008;8:436-47.
375 <https://doi.org/10.2174/138955708784223486>.
- 376 [3] Fernandes,A. Fernandes I, Cruz L, Mateus N, Cabral M, de Freitas V. Antioxidant and biological
377 properties of bioactive phenolic compounds from *Quercus suber* L. *Journal of Agricultural and*
378 *Food Chemistry* 2009;57:11154–60. <https://doi.org/10.1021/jf902093m>.
- 379 [4] Okuda T, Ito H. Tannins of Constant Structure in Medicinal and Food Plants—Hydrolyzable
380 Tannins and Polyphenols Related to Tannins. *Molecules* 2011;16: 2191-217.
381 <https://doi.org/10.3390/molecules16032191>.
- 382 [5] Martinez J P, Sasse F, Broenstrup M, Diez J, Meyerhans A. Antiviral drug discovery: Broad-
383 spectrum drugs from nature. *Natural Product Reports* 2015;32:29–48.
384 <https://doi.org/10.1039/c4np00085d>.
- 385 [6] Muccilli V, Cardullo N, Spatafora C, Cunsolo V, Tringali C. α -Glucosidase inhibition and antioxidant
386 activity of an oenological commercial tannin. Extraction, fractionation and analysis by HPLC/ESI-
387 MS/MS and $(1)H$ NMR. *Food Chem* 2017;215: 50-60.
388 <https://doi.org/10.1016/j.foodchem.2016.07.136>.
- 389 [7] Cardullo N, Muccilli V, Saletti R, Giovando S, Tringali C. A mass spectrometry and 1H NMR study
390 of hypoglycemic and antioxidant principles from a *Castanea sativa* tannin employed in
391 oenology. *Food Chem* 2018; 268: 585-593. <https://doi.org/10.1016/j.foodchem.2018.06.117>
- 392 [8] Spinaci M, Muccilli V, Bucci D, Cardullo N, Gadani B, Tringali C, Tamanini C, Galeati G. Biological
393 effects of polyphenol-rich extract and fractions from an oenological oak-derived tannin on in vitro

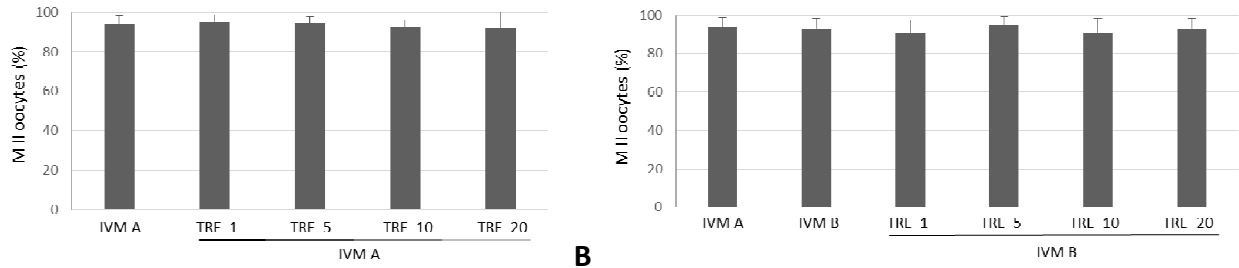
- 394 swine sperm capacitation and fertilizing ability. *Theriogenology* 2018;108:284-90.
395 <https://doi.org/10.1016/j.theriogenology.2017.12.015>.
- 396 [9] Sawai K, Funahashi H, Niwa K. Stage-specific requirement of cysteine during in vitro maturation
397 of porcine oocytes for glutathione synthesis associated with male pronuclear formation. *Biol*
398 *Reprod* 1997;57(1):1-6. <https://doi.org/10.1095/biolreprod57.1.1>.
- 399 [10] Abeydeera LR, Wang WH, Cantley TC, Prather RS, Day BN. Presence of beta-mercaptoethanol
400 can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst
401 development after in vitro fertilization. *Theriogenology* 1998;50:747-56.
402 <https://doi.org/10.1017/S0967199499000581>
- 403 [11] Abeydeera LR, Wang WH, Cantley TC, Prather RS, Day BN. Glutathione content and embryo
404 development after in vitro fertilisation of pig oocytes matured in the presence of a thiol
405 compound and various concentrations of cysteine. *Zygote* 1999;7:203-10.
406 <https://doi.org/10.1017/S0967199499000581>.
- 407 [12] Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in
408 vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during
409 in vitro maturation. *Biol Reprod* 2001;65:1800-6. <https://doi.org/10.1095/biolreprod65.6.1800>.
- 410 [13] Alvarez GM, Morado SA, Soto MP, Dalvit GC, Cetica PD. The control of reactive oxygen species
411 influences porcine oocyte in vitro maturation. *Reprod Domest Anim* 2015;50:200-5.
412 <https://doi.org/10.1111/rda.12469>.
- 413 [14] Petters RM, Wells KD. Culture of pig embryos. *J Reprod Fertil* 1993;48:61-73.
- 414 [15] Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. *Biol Reprod* 1975;12:260-
415 74. <https://doi.org/10.1095/biolreprod12.2.260>.

- 416 [16] Boquest AC, Grupen CG, Harrison SJ, McIlpatrick SM, Ashman RJ, D'Apice AJF, Nottle MB.
417 Production of cloned pigs from cultured fetal fibroblast cells. *Biol Reprod* 2002;66:1283–7.
418 <https://doi.org/10.1095/biolreprod66.5.1283>.
- 419 [17] Spinaci M, Volpe S, De Ambrogi M, Tamanini C, Galeati G. Effects of epigallocatechin-3-gallate
420 (EGCG) on in vitro maturation and fertilization of porcine oocytes. *Theriogenology* 2008;69:877-
421 85. <https://doi.org/10.1016/j.theriogenology.2008.01.005>.
- 422 [18] Tamanini C, Bono G, Cairoli F, Chiesa F. Endocrine responses induced in anestrus goats by the
423 administration of different hormones after fluorogestone acetate treatment. *Anim Reprod Sci*
424 1985;9:357– 64. [https://doi.org/10.1016/0378-4320\(85\)90064-8](https://doi.org/10.1016/0378-4320(85)90064-8).
- 425 [19] R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R
426 Foundation for Statistical Computing, 2017.
- 427 [20] Kala M, Shaikh MV, Nivsarkar M. Equilibrium between anti-oxidants and reactive oxygen
428 species: a requisite for oocyte development and maturation. *Reprod Med Biol* 2017;16:28-35.
429 <https://doi.org/10.1002/rmb2.12013>.
- 430 [21] Ambekar AS, Nirujogi RS, Srikanth SM, Chavan, S, Kelkar DS, Hinduja I, Zaveri K, Prasad TSK,
431 Harsha HC, Pandey A, Mukherjee S. Proteomic analysis of human follicular fluid: a new
432 perspective towards understanding folliculogenesis. *J Proteomics* 2013;87:68–77.
433 <https://doi.org/10.1016/j.jprot.2013.05.017>.
- 434 [21] Ishikawa S, Machida R, Hiraga K, Hiradate Y, Suda Y, Tanemura K. Hanging drop monoculture
435 for selection of optimal antioxidants during in vitro maturation of porcine oocytes. *Reprod*
436 *Domest Anim* 2014;49:e26-30. <https://doi.org/10.1111/rda.12289>.

- 437 [23] Kang JT, Kwon DK, Park SJ, Kim SJ, Moon JH, Koo OJ, Jang G, Lee BC. Quercetin improves the in
438 vitro development of porcine oocytes by decreasing reactive oxygen species levels. *J Vet Sci.*
439 2013;14:15-20. <https://doi.org/10.4142/jvs.2013.14.1.15>.
- 440 [24] Kere M, Siriboon C, Lo NW, Nguyen N T, Ju JC. Ascorbic acid improves the developmental
441 competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear
442 transplantation. *J Reprod Dev* 2013;59, 78–84.
- 443 [25] Wu GQ, Jia BY, Li JJ, Fu XW, Zhou GB, Hou YP, Zhu SE. L-carnitine enhances oocyte maturation
444 and development of parthenogenetic embryos in pigs. *Theriogenology* 2011;76:785–93.
445 <https://doi.org/10.1016/j.theriogenology.2011.04.011>.
- 446 [26] Oh SA, Chung YC, Kim CK, Shin HA, Choi SS, Yeon SE, Park JS, Hwang SS and Yoon YH. Effect of
447 β -Mercaptoethanol during in vitro maturation on in vitro fertilization and glutathione
448 concentration in porcine oocytes. *Asian Aust J Anim Sci* 2000;13: 221.
- 449 [27] Kang JT, Moon JH, Choi JY, Park SJ, Kim SJ, Saadeldin IM, Lee BC. Effect of Antioxidant Flavonoids
450 (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes. *Asian-Australas J Anim Sci*
451 2016;29:352-8. <https://doi.org/10.5713/ajas.15.0341>.
- 452 [28] Agarwal A, Majzoub A. Role of Antioxidants in Assisted Reproductive Techniques. *World J Mens*
453 *Health* 2017;35:77-93. <https://doi.org/10.5534/wjmh.2017.35.2.77>.
- 454 [29] Fisher D, Mentor S. Antioxidant-induced reductive stress has untoward consequences on the
455 brain microvasculature. *Neural Regeneration Research* 2017;12:743-4.
456 <https://doi.org/10.4103/1673-5374.206640>.
- 457 [30] Combelles CM, Gupta S, Agarwal A. Could oxidative stress influence the in-vitro maturation of
458 oocytes? *Reprod Biomed Online*. 2009 Jun;18(6):864-80.

- 459 [31] Pandey AN, Tripathi A, Premkumar KV, Shrivastav TG, Chaube SK. Reactive oxygen and nitrogen
460 species during meiotic resumption from diplotene arrest in mammalian oocytes. *J Cell Biochem*
461 2010;111:521-8. <https://doi.org/10.1002/jcb.22736>.
- 462 [32] Tiwari M, Chaube SK. Moderate increase of reactive oxygen species triggers meiotic resumption
463 in rat follicular oocytes. *J Obstet Gynaecol Res* 2016;42:536–46.
464 <https://doi.org/10.1111/jog.12938>.
- 465 [33] Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during
466 maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male
467 pronucleus. *Biol Reprod* 1993;49:89-94. <https://doi.org/10.1095/biolreprod49.1.89>.
- 468 [34] Galeati G, Vallorani C, Bucci D, Bernardini C, Tamanini C, Parmeggiani A, Spinaci M. Daidzein
469 does affect progesterone secretion by pig cumulus cells but it does not impair oocytes IVM.
470 *Theriogenology* 2010;74:451-7. <https://doi.org/10.1016/j.theriogenology.2010.02.028>.
- 471

472



473

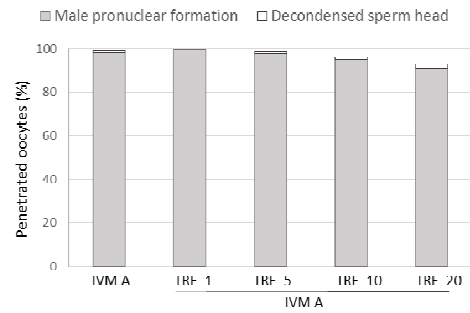
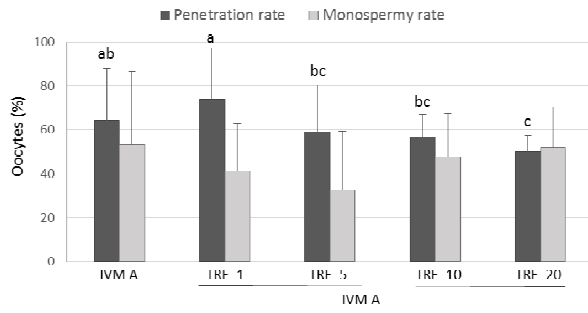
474

475

476 Fig. 1. Effect of TRE addition to either IVM A or IVM B on nuclear maturation. Data represent the
477 mean \pm SD of seven replicates repeated in different experiments [oocytes examined in each
478 replicate for each experimental group: median (interquartile range, IQR) = 40 (10)].

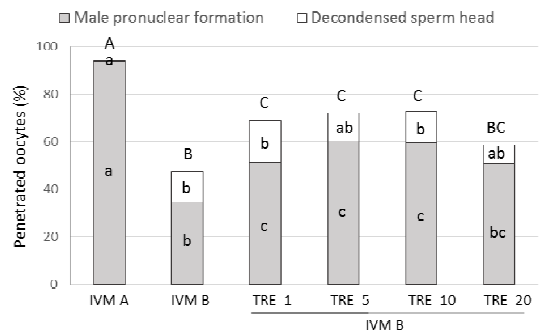
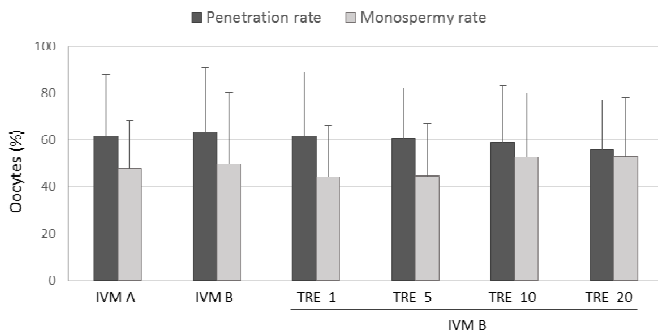
479

480



481 **A**

482



483

484 **B**

485

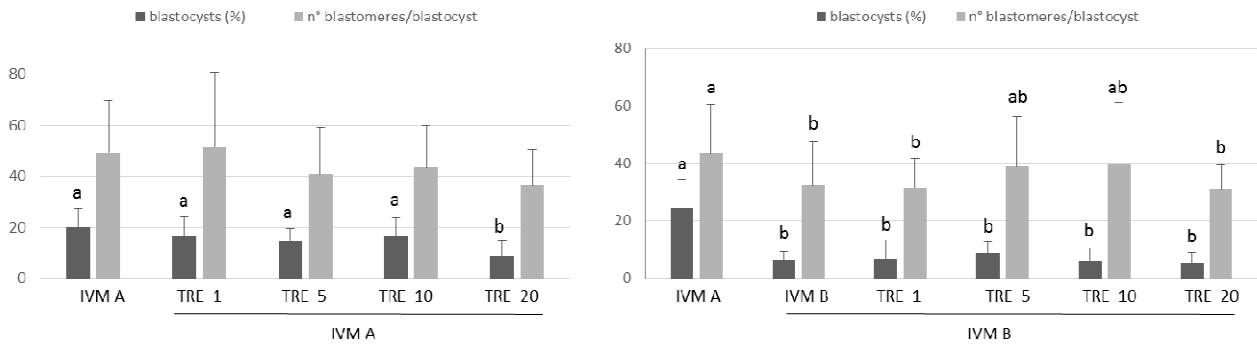
486

487 Fig. 2. Effect of TRE addition, at concentrations of 1, 5, 10, 20 µg/ml, to IVM A (panel A) or IVM B
 488 (panel B) on fertilization rate and monospermy rate (left panels) and on the ability of oocytes to
 489 decondense sperm head and sustain male pronucleus formation after in vitro fertilization (right
 490 panels).

491 Data represent the mean ± SD of seven replicates repeated in different experiments [oocytes
 492 examined in each replicate for each experimental group: median (IQR) = 38 (9)]. Different letters on
 493 the same bar type represent significant difference for p < 0.05 between treatments.

494

495



496

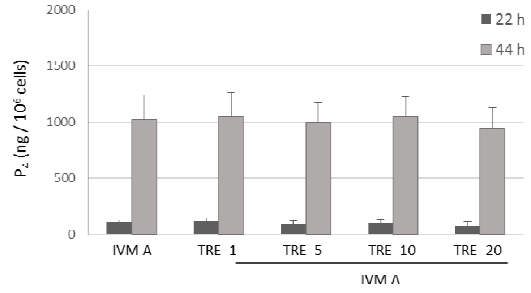
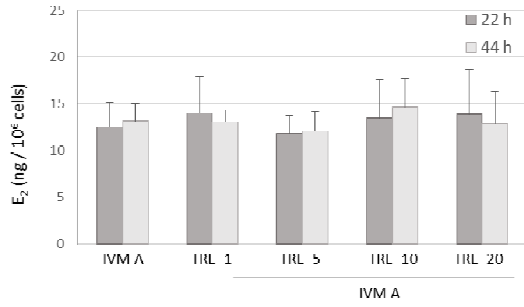
497

498

499 Fig. 3. Effect of TRE addition, at concentrations of 1, 5, 10, 20 $\mu\text{g}/\text{ml}$ to IVM A (left panel) or IVM B
500 (right panel) on blastocyst rate and blastomere number per blastocyst after parthenogenic
501 activation.

502 Data represent the mean \pm SD of seven replicates repeated in different experiments [oocytes
503 examined in each replicate for each experimental group: median (IQR) = 47 (5)]. Different letters on
504 the same bar type represent significant difference for $P < 0.05$ between treatments.

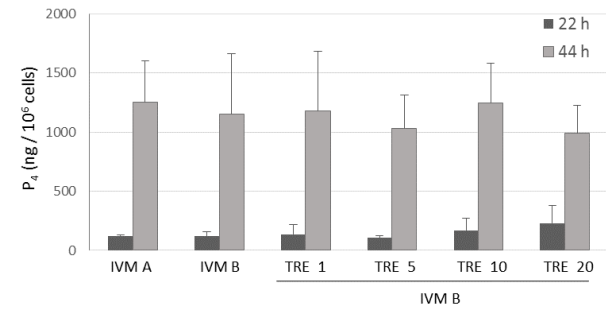
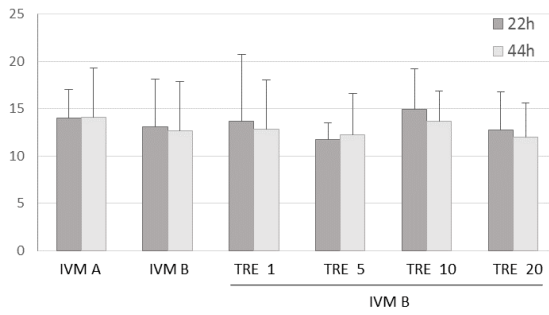
505



506

507

A



508

509

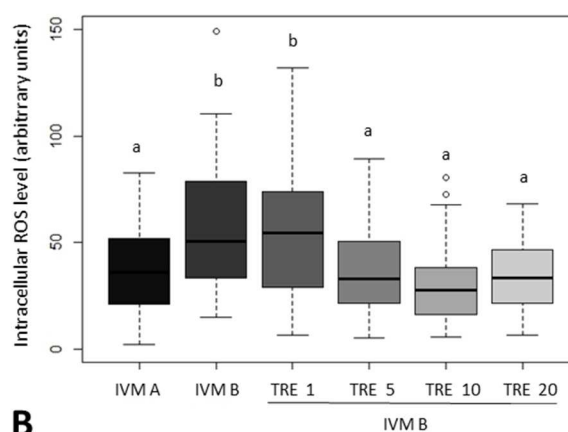
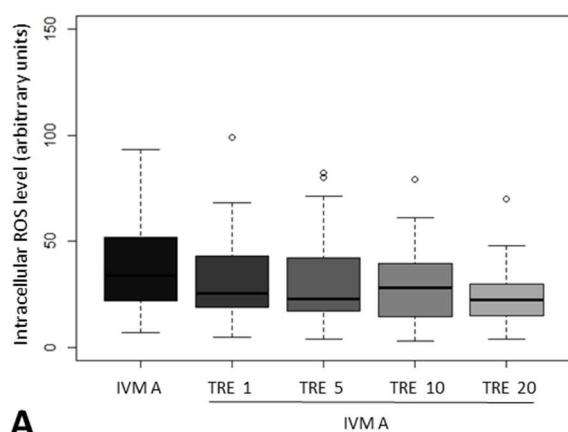
B

510

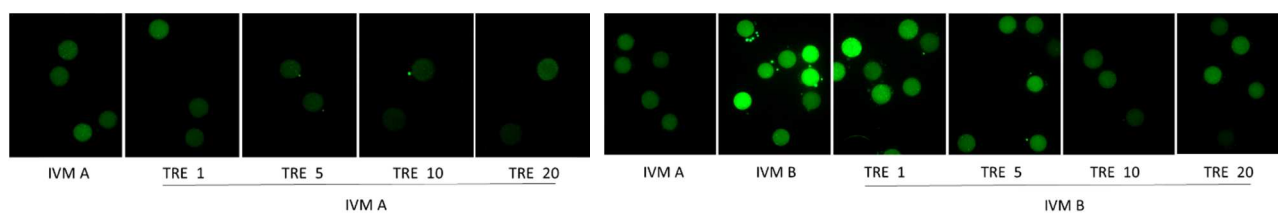
511

512 Fig. 4. E2 and P4 production by cumulus cells after 22 and 44 h of in vitro maturation of oocytes in
 513 IVM A (panel A) or IVM B (panel B) in absence or in presence of TRE at concentrations of 1, 5, 10, 20
 514 µg/ml. Data represent the mean ± SD of seven replicates repeated in different experiments.

515



516



517

518 **C**

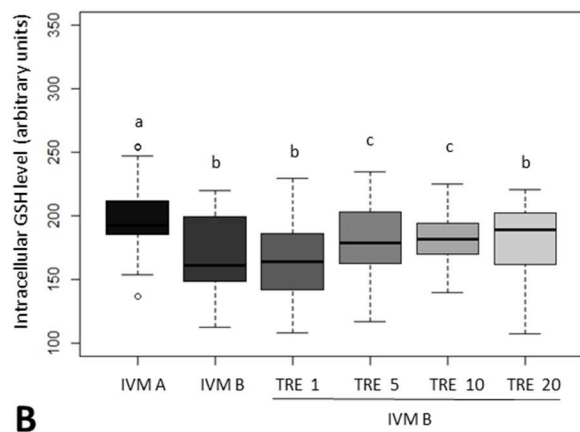
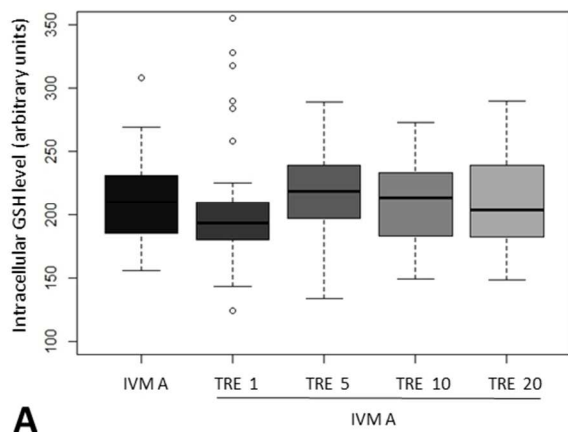
519

520 Fig. 5. Box plots for intracellular ROS levels of oocytes matured in either IVM A (panel A) or IVM B
 521 (panel B) supplemented with 1, 5, 10, 20 $\mu\text{g}/\text{ml}$ of TRE. Oocytes were dyed with H2DCFDA. Central
 522 line represent median; boxes represent 25-75 percentile; whiskers represent minimum and
 523 maximum; dots represent outliers. Different letters within same graph represent significant
 524 difference for $P < 0.05$ between treatments. The experiment was replicated 3 times with 15-20
 525 oocytes each time.

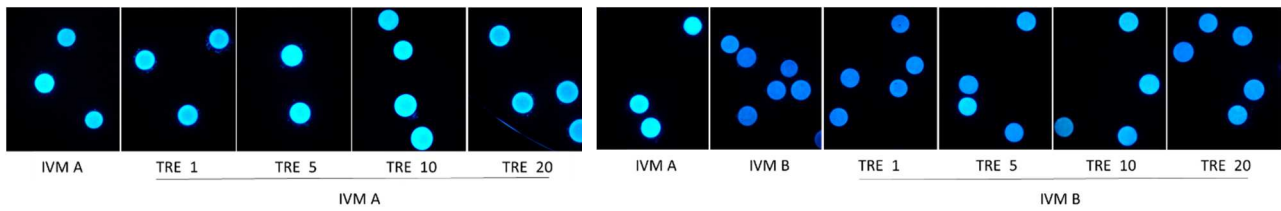
526 Panel C: Representative epifluorescent microphotographic images of in vitro matured porcine
 527 oocytes from the different experimental groups stained with H2DCFDA to detect intracellular ROS
 528 levels.

529

530



531



532

533 **C**

534

535 Fig. 6. Box plots for intracellular GSH levels of oocytes matured in either IVM A (panel A) or IVM B
 536 (panel B) supplemented with 1, 5, 10, 20 µg/ml of TRE. Oocytes were dyed with CellTracker Blue.
 537 Central line represent median; boxes represent 25-75 percentile; whiskers represent minimum and
 538 maximum; dots represent outliers. Different letters within same graph represent significant
 539 difference for $P < 0.05$ between treatments. The experiment was replicated 3 times with 15-20
 540 oocytes each time.

541 Panel C: Representative epifluorescent microphotographic images of in vitro matured porcine
 542 oocytes from the different experimental groups stained CellTracker Blue to detect intracellular GSH
 543 levels.

544