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

REVISED

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

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Dedicated to the memory of Carmela Spatafora

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ABSTRACT

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

To this aim, during IVM performed in medium either supplemented (IVM A) or not supplemented (IVM B) with cysteine and ß-mercaptoethanol, TRE was added at different concentrations (0, 1, 5, 10, 20 µg/ml).

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

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

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

KEYWORDS

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

1. INTRODUCTION

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

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

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

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

The objective of this study was to examine the possible biological effect of TRE on female gamete using an "in vitro" model of pig oocyte maturation (IVM) performed in medium either supplemented or not with cysteine and ß-mercaptoethanol, both of these molecules known to improve pig oocyte maturation inducing a reduction of ROS levels and an increase in GSH content of the oocyte [9-13]. To that purpose, at the end of the maturation period we evaluated nuclear and cytoplasmic maturation of oocytes, steroidogenic activity of cumulus cells, intracellular levels of glutathione

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

2. MATERIALS AND METHODS

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy). The ethanol extract of the commercial Tan'Activ R® (TRE) was obtained as previously reported (QR2E extract) [6].

2.1. In vitro maturation of porcine oocytes (IVM)

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

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

After three washes in IVM A or IVM B, groups of 45-50 COCs were transferred to a Nunc 4-well multidish containing 500 µL the same maturation medium per well and cultured at 39 °C in a humidified atmosphere of 5% CO_2 in air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands) 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 $1x10^6$ 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

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

- penetration rate (number of oocytes penetrated/total inseminated),
- monospermy rate (number of oocytes containing only one sperm head - male pronucleus/total penetrated)
- percentage of penetrated oocytes with one female pronucleus and at least one decondesend sperm head
- percentage of penetrated oocytes with one female and at least one male pronucleus

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

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

2.4. Evaluation of cumulus cell steroidogenesis

IVM media of both the first and the second day of culture were collected, centrifuged at 900xg for 5 min and the supernatants were stored at -20 °C until assayed for progesterone (P4) and estradiol-17β (E2) by validated radioimmunoassays [18]. At the end of the maturation period, cumulus cells were counted using a Thoma's hemocytometer, after being freed from matured oocytes by gentle repeated pipetting. For P4, intra and interassay coefficients of variation were 7.8% and 10.1%, respectively; assay sensitivity was 3,3 pg/tube. Intra and interassay coefficients of variation for E2 were 4% and 12%, respectively; assay sensitivity was 1.1 pg/tube. Steroid concentrations are expressed as ng/10^6 cells.

2.5. Evaluation of intracellular ROS and GSH levels

Intracellular GSH and ROS levels of oocytes at the end of maturation period were determined using 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen, Italy) or 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Italy), respectively. From each treatment group, oocytes were incubated in the dark for 30 min at 39 °C in PBS/0.1% (wt/vol) PVA supplemented with 10 µM H2DCFDA or 10 µM CellTracker Blue. Following incubation, the oocytes were washed in PBS/0.1% (wt/vol) PVA, placed into 10-µl droplets, and fluorescence was evaluated under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). The fluorescence images were analysed with Image J software (public domain). Relative oocyte fluorescence was measured by normalizing the oocyte fluorescence with the background and with each oocyte area.

2.6 Experimental design

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

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

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

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

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

2.7. Statistical analyses

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

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

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

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

3. RESULTS

3.1. Effect of TRE on nuclear maturation

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

3.2. Effect of TRE on cytoplasmic maturation

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

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

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

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

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

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

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

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

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

3.3. Effect of TRE on cumulus cell steroidogenesis

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

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

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

3.4. Effect of TRE on ROS and GSH levels

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

The addition of TRE to IVM A did not induce any statistical modification of intracellular GSH levels (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 H_2O_2 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 exogenoulsy inducing oxidative stress , we evaluated the effect of TRE supplementation during

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

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

However when a very high concentration of TRE (500 μg/ml) was added to IVM A, a significant reduction in the percentage of oocytes reaching MII stage was observed (data not shown). This result agrees well with those of other authors who recorded that the polar body extrusion rate was negatively influenced in presence of high concentration of taxifolin or quercetin (50 μg/ml), plant-derived flavonoids with antioxidant properties [23,27]. These results suggest the possibility of inducing, by excessive antioxidants supplementation, a dangerous condition called “antioxidant paradox” leading to “reductive stress” [28,29] that in turn may impair nuclear maturation. In fact, controlled and physiological ROS amounts seem to be required for meiotic resumption and nuclear 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 μg/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 Interestigly, while no significant differences on penetration rate were recorded, the addition of TRE
328 at the concentrations of 1, 5, and 10 μg/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 syntesis
332 in maturing pig oocytes [33]. In fact, while TRE addition at the concentration of 5, 10, 20 μg/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 comparted to IVM A group. However when in vitro maturation was performed in IVM B in presence
340 of 5 and 10 μg/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 thought 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

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

Funding

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

Acknowledgement

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

Conflicts of interest

The authors have declared no conflicts of interest.

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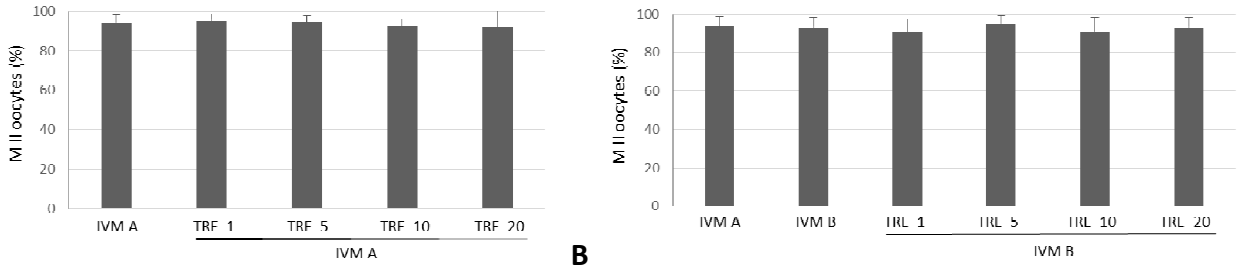
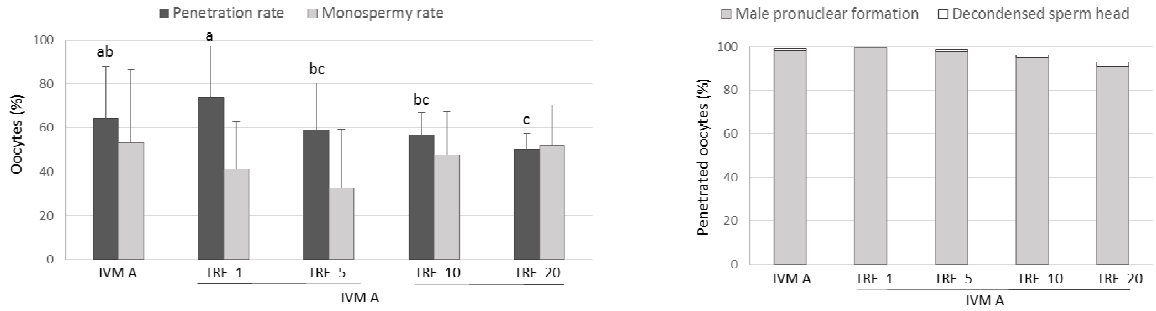


Fig. 1. Effect of TRE addition to either IVM A or IVM B on nuclear maturation. Data represent the mean ± SD of seven replicates repeated in different experiments [oocytes examined in each replicate for each experimental group: median (interquartile range, IQR) = 40 (10)].



481 **A**

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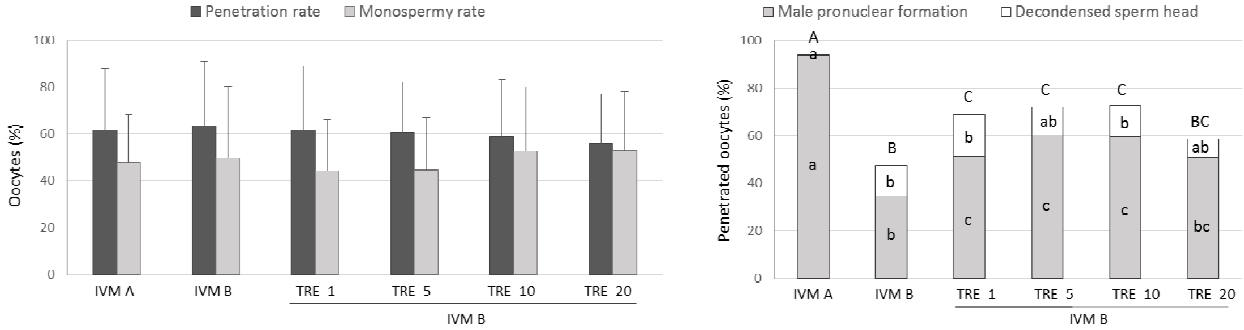

483

484 **B**

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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

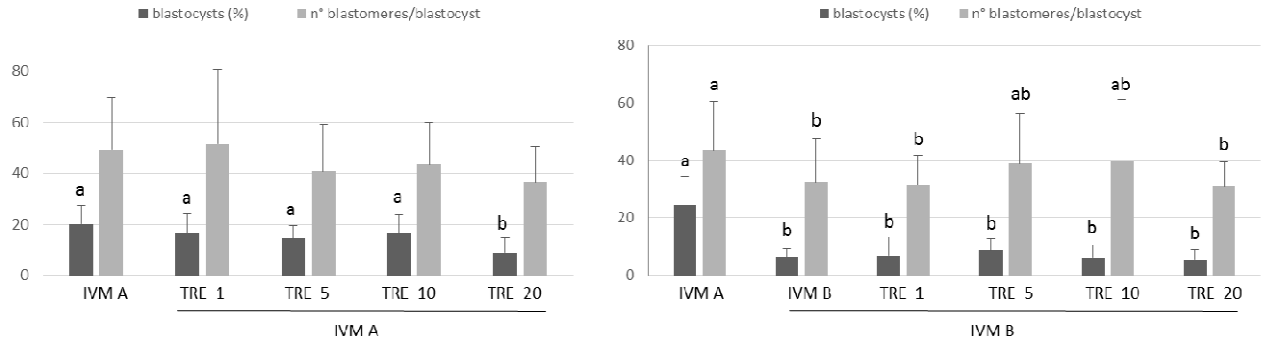
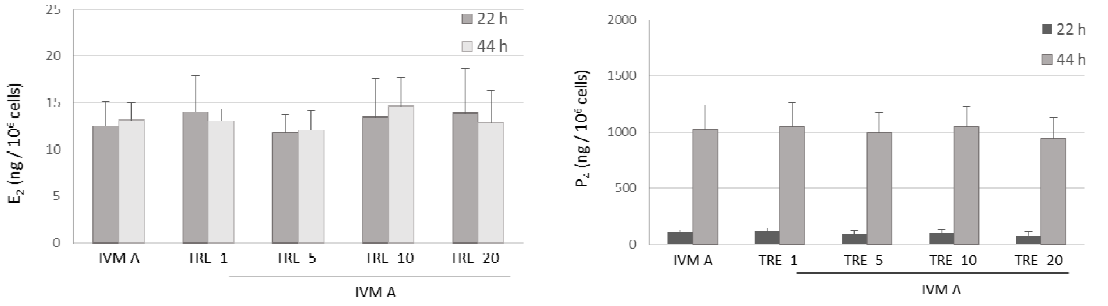


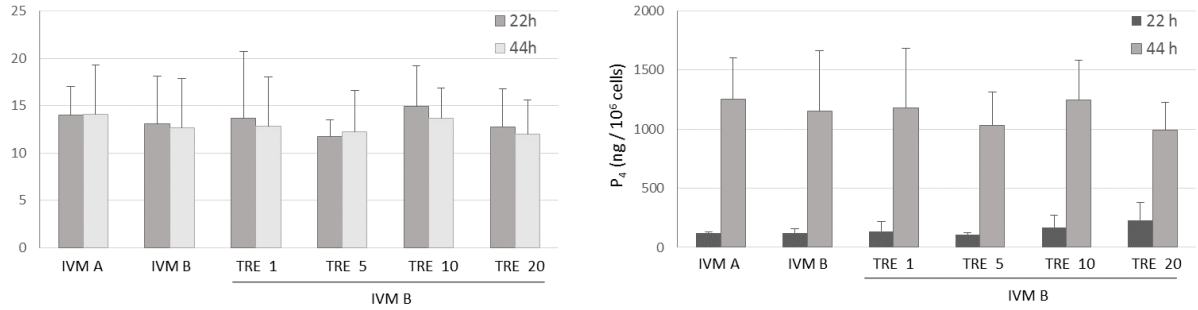
Fig. 3. Effect of TRE addition, at concentrations of 1, 5, 10, 20 µg/ml to IVM A (left panel) or IVM B (right panel) on blastocyst rate and blastomere number per blastocyst after parthenogenic activation.

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



506

507 **A**



508

509 **B**

510

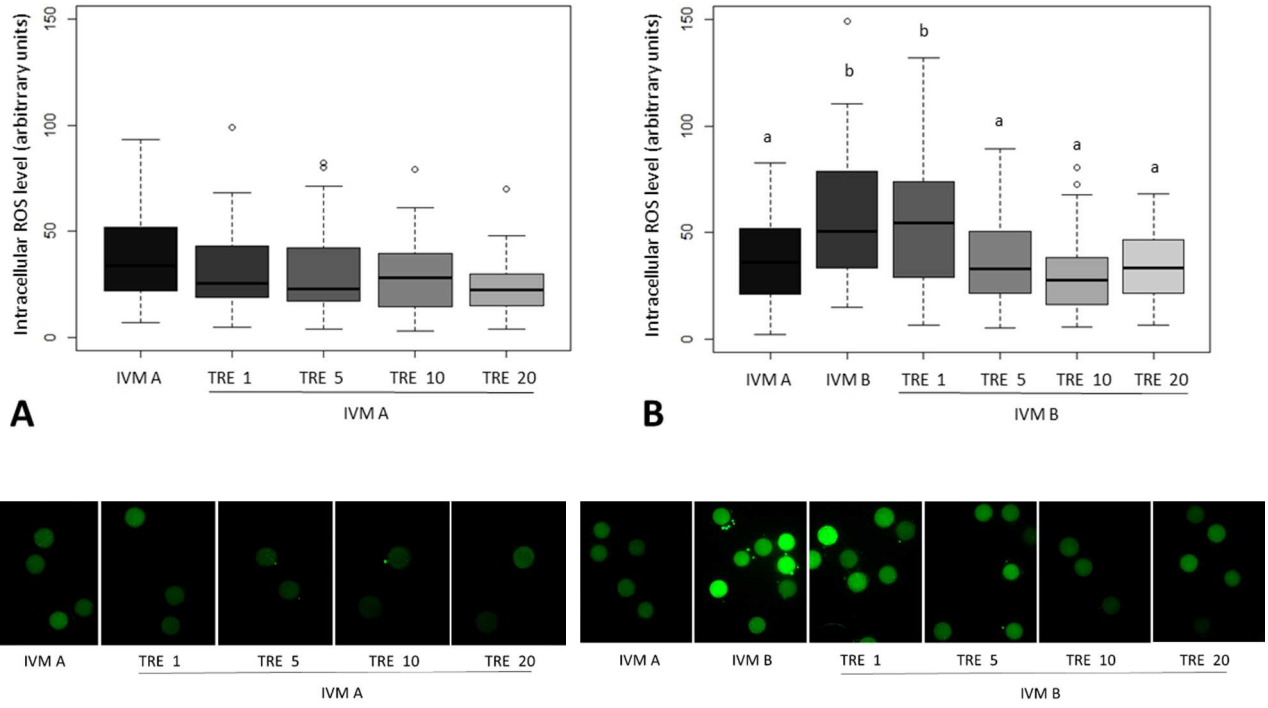
511

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 µg/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.

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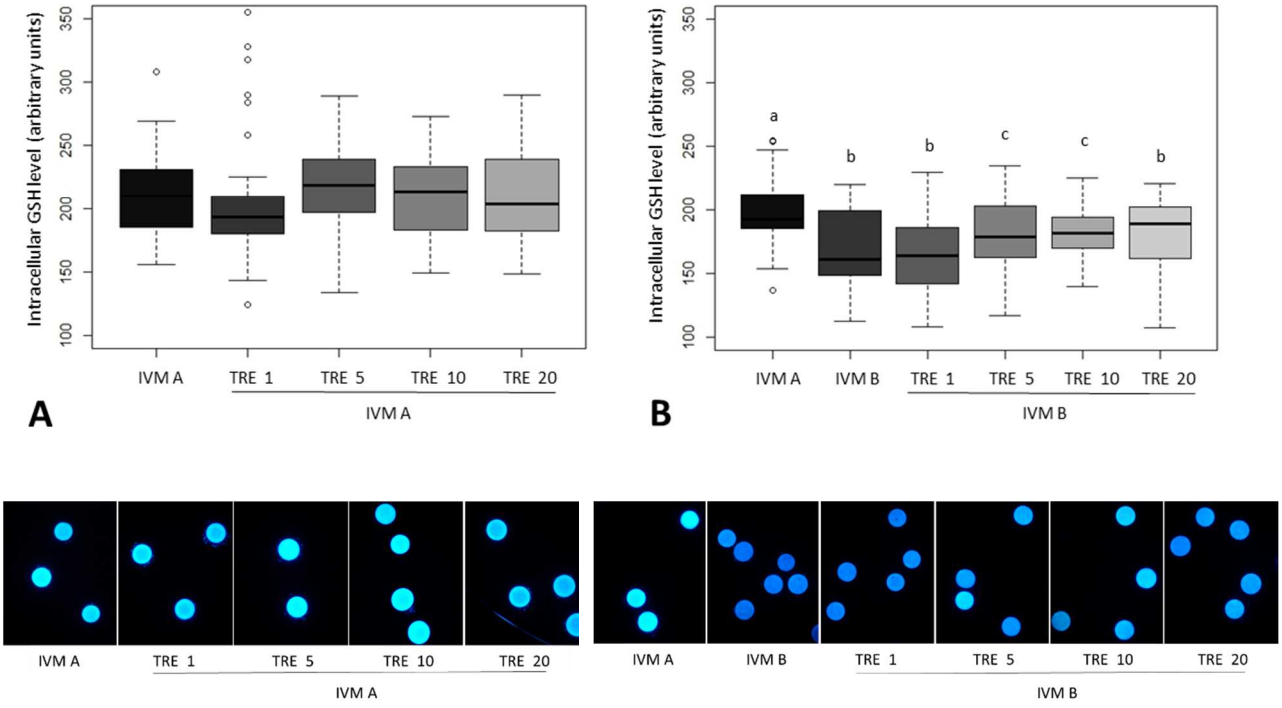

C

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

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