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1

2 **Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro**
3 **fertilization**

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5

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12

13 **Abstract**

14 Thawing is one of the most delicate process after semen cryopreservation as spermatozoa pass from
15 a dormant metabolic stage to a sudden awakening in cellular metabolism. The rapid oxygen utilization
16 leads to an overproduction of reactive oxygen species that can damage sperm cells, thus causing a
17 significant decrease of fertilizing potential of frozen-thawed spermatozoa. Resveratrol (Res) is a
18 natural grape-derived phytoalexin and Epigallocatechin-3-gallate (EGCG) is the major polyphenol in
19 green tea (*Camellia sinensis*); both molecules are known to possess high levels of antioxidant activity.
20 The objective of the present study was to assess the effect of different concentrations of Res (0.5, 1
21 or 2 mM; Experiment 1) or EGCG (25, 50 or 100 μ M; Experiment 2) supplementation to thawing
22 boar semen extender on sperm quality parameters (viability and acrosome integrity) and in vitro
23 fertilization (IVF).

24 Semen after thawing and dilution with three volumes of Beltsville Thawing Solution (BTS), was
25 immediately divided in control group without antioxidants addition (CTR) and either Res or EGCG
26 groups. Sperm viability and acrosome integrity were evaluated in CTR, Res or EGCG groups after 1
27 h of incubation at 37°C.

28 The addition of different doses of Res or EGCG to thawing extender for 1 h did not induce any effect
29 on boar sperm viability and acrosome integrity. However, both Res and EGCG treated samples
30 exhibited a significantly higher penetration rate compared with CTR when used for IVF. In particular
31 the treatment with all the EGCG concentrations increased the penetration rate ($P < 0.01$) while only
32 Res 2 mM induced a significant increase of this parameter ($P < 0.01$). In addition, EGCG 25 and 50
33 μ M supplementation significantly increased total fertilization efficiency as compared to control
34 (EGCG 25 μ M: 40.3 ± 8.2 vs 26.8 ± 9.5 , $P < 0.05$; EGCG 50 μ M: 40.4 ± 7.8 vs 26.8 ± 9.5 , $P < 0.01$).
35 The same effect was observed with Res 2 mM (51.0 ± 7.6 vs 29.6 ± 11.3 , $P < 0.01$).

36 In conclusion, our results indicate that the addition of different doses of the two antioxidants to
37 thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome
38 integrity, efficiently improves in vitro penetration rate. Moreover, both molecules (EGCG 25 and 50
39 μ M and Res 2 mM) significantly increases the total efficiency of fertilization.

40

41 **Key words:** boar semen, cryopreservation, antioxidants, fertilization, Resveratrol, Epigallocatechin-
42 3-gallate

43

44 **1. Introduction**

45 Sperm cryopreservation is the most efficient method for long term sperm storage (reviewed in [1]).
46 However, frozen-thawed boar semen is not routinely used because of the high performance of long-
47 term extenders for liquid storage and the non optimal quality of thawed boar spermatozoa. Anyhow
48 it is important to create an efficient cryopreserved semen gene bank, planning insemination at
49 artificial insemination centers, maintaining genetic diversity and promoting the rapid growth of swine
50 models [2,3].

51 During the cryopreservation process, spermatozoa undergo a variety of harmful cellular alterations
52 called “cold shock”, mainly induced by the increase of reactive oxygen species (ROS) levels [4,5].
53 ROS, such as hydrogen peroxide (H₂O₂), superoxide anions (O₂⁻), hydroxyl radicals (OH⁻), generated
54 during intermediate steps of oxygen reduction, are known for their ability to damage cellular proteins,
55 DNA and plasma membrane lipids, due to their free radical nature [6]. Even if very low and controlled
56 concentrations of ROS are required for sperm hyperactivation, capacitation, acrosome reaction and
57 zona binding events [7–9], when ROS are overproduced, spermatozoa cannot easily adapt to this
58 condition and oxidative stress occurs leading to cell damage [6].

59 On these bases, frozen-thawed boar spermatozoa may present nucleoprotein-DNA structural
60 alterations [8,10–12] and capacitation-like changes that could lead to an important reduction in
61 fertilizing potential of frozen-thawed sperm [13]. In order to reduce oxidative damage, one approach
62 is to supplement semen extender with enzymatic and non-enzymatic antioxidant compounds during
63 freeze-thawing.

64 Resveratrol (3,4',5-trihydroxy-trans-stilbene) (Res) is a polyphenolic natural product with a stilbene
65 structure isolated at first from the roots of white hellebore in 1940 [14] and later from *Polygonum*
66 *cuspidatum*, a medicinal plant. Today it is widely consumed in the Mediterranean diet in the form of
67 peanuts, grapes and wine. Res shows many biological activities such as anti-inflammatory,
68 cardioprotective, chemopreventive and antiapoptotic [15,16]. Moreover, Res has been reported to
69 possibly act as antioxidant thanks to its ability to reduce mitochondria ROS production, scavenge
70 superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors
71 and enzymes [16]. It has been reported that mouse [17], bovine [18] and human spermatozoa [19] can
72 be protected by Res from experimentally induced oxidative stress. A protective effect of Res against
73 membrane oxidative damage but not against the loss of motility induced by the cryopreservation of
74 human semen, has been observed [20]. Furthermore, Res is effective in minimizing post-thawing
75 DNA damage in human spermatozoa [21] and in improving post-thaw bull sperm quality in terms of
76 sperm motility, high mitochondrial activity and DNA integrity [22]. In frozen-thawed ram sperm the

77 addition of Res to the Tris-egg yolk-glycerol extender has been shown to reduce sperm mitochondrial
78 membrane potential [23].

79 Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*) and is
80 reported to possess a high level of antioxidant activity [24,25]. The supplementation of canine sperm
81 with green tea polyphenol extracts (PFs) as been demonstrated to improve motility and viability of
82 spermatozoa during long-term liquid storage [26,27]. Moreover, pre-treatment of boar spermatozoa
83 with PFs prior to freezing exhibited significantly higher degrees of post-thaw sperm viability and
84 acrosomal integrity [28]. The beneficial effect of EGCG has been observed during liquid storage at
85 15°C of sorted boar semen: it increased the percentage of viable spermatozoa and inhibited caspase
86 activation [29].

87 On these bases, the objective of the present study was to assess whether Res or EGCG
88 supplementation of thawing boar semen extender is effective in influencing sperm quality parameters
89 (viability and acrosome integrity) and in vitro fertilization (IVF).

90

91 **2. Materials and Methods**

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

93

94 *2.1 Sperm thawing*

95 The study was performed using commercial frozen semen from 3 Large White boars purchased by
96 Suiseme Srl (Saliceta San Giuliano, Modena, Italy). Straws (0.5 mL/straw) were thawed for 30 sec
97 in water bath at 37°C and immediately diluted, at the same temperature, with three volumes of
98 Beltsville Thawing Solution (BTS).

99 Semen was immediately divided in the following experimental groups: CTR (control: without
100 antioxidant addition), and either Res (addition of 0.5, 1, 2 mM R to BTS thawing extender;
101 Experiment 1) or EGCG (addition of 25, 50, 100 µM EGCG to BTS thawing extender; Experiment
102 2). Only sperm samples with viability > 40% as assessed immediately after thawing were used for
103 the experiments.

104 Sperm viability and acrosome integrity were evaluated 1 h after thawing in CTR and Res or EGCG
105 groups. After 1 h of incubation at 37°C in either absence or presence of different doses of Res or
106 EGCG, semen samples were washed and used for in vitro fertilization (IVF).

107

108 *2.2 Post-thaw spermatozoa evaluation*

109 *2.2.1 Sperm viability assessment*

110 Sperm viability was evaluated by incubating 25 μ L of semen with 2 μ L of a 300 μ M Propidium Iodide
111 (PI) stock solution and 2 μ L of a 10 μ M SYBR-14 stock solution (LIVE/DEAD®Sperm Viability kit,
112 Molecular Probes, Invitrogen), for 5 min at 37°C in the dark. After incubation, 10 μ L of sperm
113 suspensions were analyzed with a Nikon Eclipse epifluorescence microscope using a double-band-
114 pass filter for green and red fluorescence. The spermatozoa with green or red fluorescence on the
115 head were considered live or dead, respectively (see supplementary file, panel A). At least 200 cells
116 were counted in each analysis.

117

118 *2.2.2 Acrosome integrity assessment*

119 Acrosome integrity was measured with a FITC conjugated lectin from *Pisum Sativum* (FITC-PSA)
120 which labels acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended
121 with ethanol 95% and fixed at 4°C for 30 min. Samples were dried in heated slides and incubated
122 with FITC-PSA solution (5.0 μ g PSA-FITC/1 mL H₂O) for 20 min in darkness. After staining
123 samples were washed in PBS and mounted with Vectashield mounting medium with PI (Vector
124 Laboratories, Burlingame, CA, USA). The slides were then observed with the above described
125 fluorescence microscope. The presence of a green acrosomal fluorescence was considered indicative
126 of an intact acrosome, while a partial or total absence of fluorescence was considered to indicate
127 acrosome disruption or acrosome reaction (see supplementary file, panel B).

128

129 *2.3 In vitro maturation (IVM) of cumulus-oocyte-complexes*

130 Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with
131 physiological saline at 30-35°C. Cumulus oocyte complexes (COCs) from follicles 3-6 mm in
132 diameter were aspirated using 18 gauge needle attached to a 10 mL disposable syringe. Under a
133 stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon,
134 Roskilde, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs
135 with complete and dense cumulus oophorus were used. After three washes in NCSU 37 [30]
136 supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 μ M
137 β -mercaptoethanol and 10% PCV2-PCR-negative porcine follicular fluid (IVM medium), groups of
138 50 COCs were transferred to a Nunc 4-well multidish containing 500 μ l of the same medium per well
139 and cultured at 39°C in humidified atmosphere of 5% CO₂/7% O₂ in air. For the first 22 h of *in vitro*
140 maturation, the IVM medium was supplemented with 1.0 mM dibutyryl cyclic adenosine
141 monophosphate (db-cAMP), 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10

142 IU/mL hCG (Corulon, Intervet). For the last 22-24 h COCs were transferred to fresh maturation
143 medium [31].

144

145 *2.4 In vitro fertilization (IVF)*

146 For in vitro fertilization, thawed semen after 1 h of incubation at 37°C with or without antioxidants,
147 was washed twice with BTS and finally resuspended with Brackett & Oliphant's [32] medium
148 supplemented with 12% foetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/mL caffeine (IVF
149 medium). Sperm concentrations were evaluated and 45–50 matured oocytes, freed from cumulus cells
150 by gentle repeated pipetting, were transferred to 500 mL IVF medium containing 1×10^6 sperm/mL.
151 After 1 h of co-culture, oocytes were transferred to fresh IVF medium and cultured for 19 h until
152 fixation in acetic acid/ethanol (1 : 3) for 24 h and stained with Lacmoid.

153 The oocytes were observed under a phase-contrast microscope and the following parameters were
154 assessed:

- 155 (1) penetration rate (number of fertilized oocytes/ number of inseminated oocytes);
- 156 (2) monospermy rate (number of oocytes containing only one sperm head–male pronucleus/
157 number of penetrated oocytes);
- 158 (3) total efficiency of fertilization (number of monospermic oocytes/number of inseminated
159 oocytes).

160 Degenerated and immature oocytes were not counted.

161

162 *2.5 Statistical analysis*

163 Data were analyzed using R version 3.0.3 [33]. Significance was set at $p < 0.05$ unless otherwise
164 specified. Results are expressed as mean \pm standard deviation. One-way ANOVA and Tukey post
165 hoc test were performed to assess the difference in sperm viability and acrosome integrity percentages
166 between treatments after arcsine transformation. In vitro fertilization data were analyzed by a
167 generalized linear model (GLM) and a Tukey post hoc test was used to assess difference between
168 treatments.

169

170 **3. Results**

171 *3.1 Experiment 1: Effect of Res supplementation to thawed boar sperm on viability, acrosome* 172 *integrity and IVF parameters*

173 The addition of Res 0.5, 1 or 2 mM to thawed sperm for 1 h (Res 0.5, Res 1, Res 2) did not induce
174 any significant effect on sperm viability as compared to semen without Res (CTR) ($51.7\% \pm 9.4\%$,
175 $48.5\% \pm 5.3$, $48.4\% \pm 8.6$, $43.8\% \pm 10.7$ in CTR, Res 0.5, Res 1, Res 2 respectively) (Fig.1A).

176 Moreover, the supplementation of different concentrations of Res to thawed sperm for 1 h did not
 177 improve the percentage of spermatozoa with intact acrosome compared to CTR group (86.4 % ±
 178 6.1 %, 82.5% ± 3.2 %, 81.9 % ± 2.6 %, 82.4% ± 1.0 %, in CTR, Res 0.5, Res 1, Res 2 respectively)
 179 (Fig. 1B).

180 When Res 2 mM treated samples were used for IVF a significantly (P<0.01) higher penetration rate
 181 (number of oocytes penetrated/total inseminated) compared with CTR was observed (Table 1). Res
 182 2 mM also exerted a positive effect (P<0.01) on the total efficiency of fertilization as compared to
 183 CTR group but did not induce any effect on monospermy rate (Table 1).

184

185 Table 1. Effect of Res (0.5, 1 and 2 mM) supplementation to thawed boar sperm on IVF parameters.

186

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	237	33.8 ± 12.4 ^a	87.3 ± 5.5	29.6 ± 11.3 ^a
Res 0.5	152	42.3 ± 2.7 ^a	73.2 ± 11.7	30.9 ± 5.1 ^a
Res 1	263	46.8 ± 5.0 ^{a§}	82.3 ± 12.5	38.5 ± 6.8 ^a
Res 2	192	68.8 ± 6.4 ^b	74.4 ± 12.3	51.0 ± 7.6 ^b

187

188 Penetration rate (number of fertilized oocytes / number of inseminated oocytes).

189 Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

190 Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

191 Values are expressed as the mean ± SD of six replicates (three boars).

192 Different letters indicate significant difference for P < 0.01 in column between treatments.

193 § indicates significant difference in column for P = 0.05 compared with control.

194

195

196

197

198 3.2 Experiment 2: Effect of EGCG supplementation to thawed boar sperm on viability, acrosome 199 integrity and IVF parameters

200 The addition of different concentrations of EGCG (25, 50 and 100 µM) to thawed sperm for 1 h did
 201 not exert any significant effect on sperm viability (45.1% ± 3.8 %, 46.6 % ± 7.0 %, 45.4 % ± 10.1 %,
 202 48.2 % ± 5.8 %, in CTR, EGCG 25, EGCG 50, EGCG 100 µM respectively) and acrosome integrity
 203 (86.2 % ± 5.5 %, 84.3% ± 6.3 %, 87.1 % ± 1.5 %, 85.4 % ± 1.5 %, in CTR, EGCG 25, EGCG 50,
 204 EGCG 100 µM respectively) (Fig. 2A and 2B). Oocytes inseminated with thawed spermatozoa

205 pretreated with all the different EGCG concentrations tested presented a significantly ($P < 0.01$)
 206 increased penetration rate compared to CTR (Table 2). In addition, 25 and 50 μM EGCG
 207 supplementation exerted a positive effect ($P < 0.01$) on the total efficiency of fertilization without
 208 inducing any effect on monospermy rate (Table 2).

209

210

211

212 Table 2. Effects of EGCG (25, 5 and 100 μM) supplementation to thawed boar sperm on IVF
 213 parameters.

214

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	247	$31.9 \pm 9.4^{\text{a}}$	83.7 ± 8.8	$26.8 \pm 9.5^{\text{aA}}$
EGCG 25	158	$55.0 \pm 5.3^{\text{b}}$	74.5 ± 19.5	$40.3 \pm 8.2^{\text{aB}}$
EGCG 50	234	$54.8 \pm 9.0^{\text{b}}$	74.5 ± 12.3	$40.4 \pm 7.8^{\text{bB}}$
EGCG 100	244	$48.6 \pm 10.4^{\text{b}}$	72.6 ± 18.7	$34.6 \pm 9.9^{\text{aA}}$

215

216 Penetration rate (number of penetrated oocytes / number of inseminated oocytes).

217 Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

218 Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

219 Values are expressed as the mean \pm SD of six replicates (three boars).

220 Different lowercase letters indicate significant difference for $P < 0.01$ in column between treatments.

221 Different capital letters indicate significant difference for $P < 0.05$ in column between treatments.

222

223

224

225 4. Discussion

226 Sperm cryopreservation is the best technology to store boar semen for long periods for planning
 227 artificial insemination and preserving genetic material through time; nevertheless, frozen-thawed
 228 sperm is not routinely used in pig industry (less than 1%) [34]. The main reason is that freezing and
 229 thawing procedures lead to a reduced sperm fertilizing ability and reproductive performance [3]
 230 because of two important events occurring during cryopreservation procedure: the vast production of
 231 ROS and the parallel decrease in antioxidants defenses [35]. On this basis, in order to improve post-

232 thaw quality of boar sperm, various antioxidants are routinely added during freezing protocols and
233 new molecules are continuously studied [36].

234 In our study, Res supplementation did not induce any effect on sperm viability as already reported by
235 other Authors even if in different experimental conditions and species: liquid storage of boar [37] and
236 stallion [38] semen or cryopreservation of bull sperm [22].

237 Thawed boar spermatozoa can show membrane rearrangements and consequent lipid packing faults
238 [39]. In this way, efficiency of calcium channels could be compromised, leading to an increase in
239 calcium concentration inside the cell that could lead to capacitation like changes [40]. Liu et al. [41]
240 reported that Res affects intracellular calcium release, so it could be important in preventing
241 premature sperm capacitation and, consequently, acrosome reaction; in this study, however, Res
242 supplementation to thawing media for one hour did not preserve sperm acrosome integrity. Similar
243 results have been obtained by Martín-Hidalgo et al. [37] who reported that storage at 17°C of fresh
244 boar semen with Res did not exert any effect on this parameter and by Silva et al. [23] who added
245 Res to ram cryopreserved semen.

246 In our study, no protective effect of EGCG was observed on acrosome integrity in agreement with
247 the results obtained by Vallorani et al. [29] on liquid storage of boar sexed semen. Different results
248 have been shown by Kitaji et al. [28] who observed a higher post-thaw viability and acrosome
249 integrity of boar spermatozoa incubated prior to freezing in a semen extender supplemented with
250 0.01% of green tea polyphenol extracts.

251 When either Res 2 mM or EGCG 25, 50 and 100 µM treated samples were used for IVF we observed
252 a significantly higher penetration rate ($P<0.01$) compared with control; in addition, Res 2 mM and
253 EGCG 25 and 50 µM supplementation exerted a positive effect ($P<0.01$, $P< 0.05$ and $P<0.01$
254 respectively) on total efficiency of fertilization.

255 Our results agree well with those from several studies examining the effect of EGCG during IVF in
256 different experimental conditions. Pre-incubation of boar spermatozoa with green tea polyphenol
257 extracts prior to freezing has been shown to increase both the efficiency of IVF (rates of monospermic
258 oocyte) and blastocyst formation [28]. Kaedei et al. [42] demonstrated that penetration rate improves
259 when boar frozen-thawed spermatozoa are co-incubated with oocyte in IVF medium supplemented
260 with 50 µM EGCG. A positive influence of EGCG has also been recorded on fresh boar and stallion
261 spermatozoa under capacitating conditions, in which a significant increase in the number of sperm
262 bound to oocyte zona pellucida was observed [43,44]. Moreover, the presence of this polyphenol
263 during pig IVF using fresh semen was able to increase, in a dose response manner, the fertilization
264 rate [43].

265 Therefore, EGCG could likely modulate sperm capacitation process probably thanks to its
266 antioxidant activity; in fact under capacitating conditions EGCG has been demonstrated to reduce
267 H₂O₂ production in boar spermatozoa [43] and to be able, in stallion spermatozoa, to reverse the
268 inhibition of mitochondrial complex I by rotenone, a molecule known to induce mitochondrial ROS
269 production [44].

270 To our knowledge, Res treated semen has never been used for IVF so far, while it is known that Res
271 supplementation during IVM and IVC improves developmental potential of porcine oocytes and
272 porcine embryo development [45–47].

273 Studies performed on mouse, human, bovine and ram spermatozoa demonstrated that Res could
274 effectively protect spermatozoa from oxidative stress induced by cryopreservation or pro-oxidant
275 agents supplementation [17,19–23]. In our work, although Res 2 mM added to thawing sperm media
276 for 1 hour did not exert any effect on parameters assessed (viability and acrosome integrity), it
277 significantly (P<0.01) increased the penetration rate and total efficiency of fertilization. The
278 encouraging beneficial effect of Res and EGCG addition to the thawing extender was evident during
279 IVF and therefore after washing away the tested molecules: the two molecules were left with semen
280 for 1 hour after thawing and then the medium was discarded and spermatozoa washed with fresh
281 Bracket and Oliphant's medium. This suggests that the protective action during thawing can lead to
282 positive effects on sperm function that, in turn, are responsible for the subsequent increased fertilizing
283 ability even if the molecules are no more present. Therefore, it cannot be excluded (and it should
284 certainly be tested) that Res or EGCG pretreatment of thawed semen could lead to positive effects
285 also *in vivo*.

286 In conclusion, our results indicate that the addition of Res 2 mM or EGCG 25, 50 and 100 μM to
287 thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome
288 integrity, improves *in vitro* penetration rate; in addition, EGCG 50 μM and Res 2 mM increases the
289 total efficiency of fertilization. These results could be possibly important not only *in vitro*, but also
290 *in vivo* as the addition of one of these two antioxidants in the commercial thawing solution might
291 enhance sperm fertilizing ability and reproductive performance during porcine AI with frozen-thawed
292 boar semen.

293

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297

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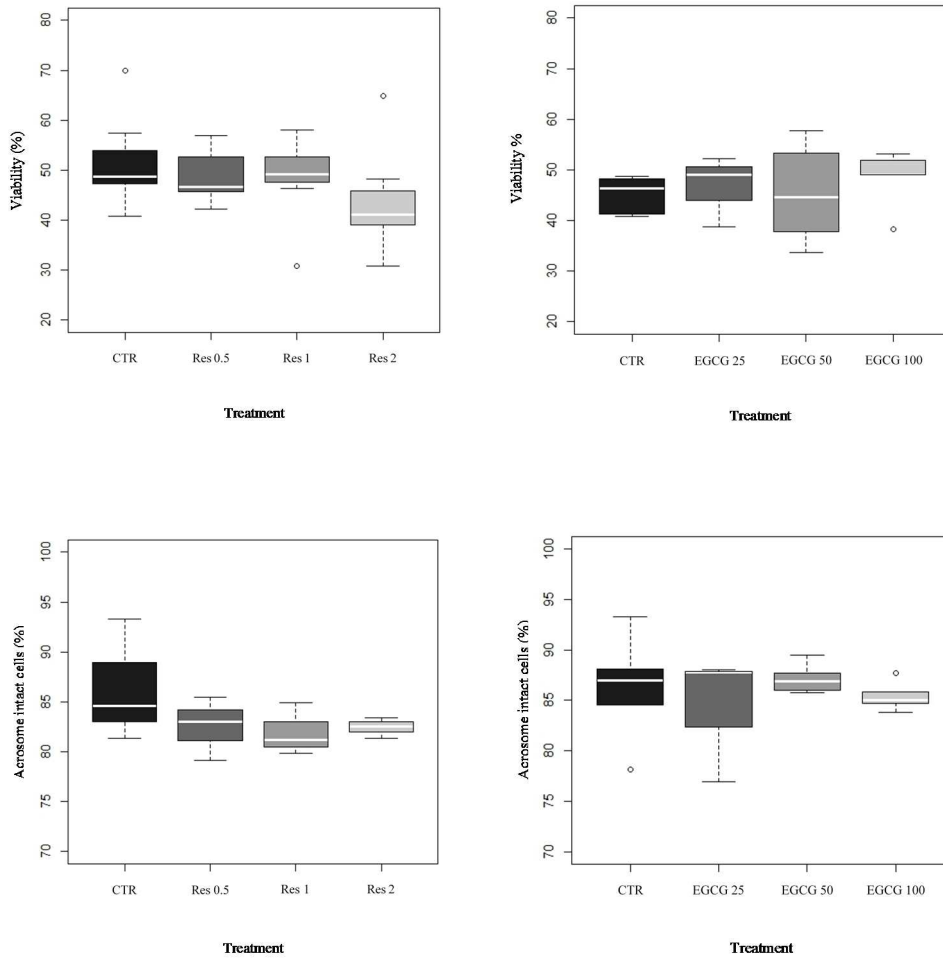
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415 Fig. 1 Boxplots representing sperm viability (upper panels) and acrosome integrity (lower panels) of
416 frozen-thawed spermatozoa after 1 h of incubation at 37°C without (CTR) or with Res (0.5, 1 or 2
417 mM)(left panels) and EGCG (25, 50 or 100 μ M) (right panel). Each experiment was repeated six
418 times (three boars).

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425 Fig. 1
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