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# EPIGALLOCATECHIN-3-GALLATE (EGCG) REDUCES ROTENONE EFFECT ON STALLION SPERM ZONA PELLUCIDA HETEROLOGOUS BINDING

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**Running title:** Effect of rotenone and EGCG on stallion sperm-ZP binding

19 **CONTENTS**

20 Stallion spermatozoa are highly dependent on oxidative phosphorylation for ATP
21 production to achieve normal sperm function and to fuel the motility. The aim of this
22 study was to evaluate the response of equine sperm under capacitating conditions to the
23 inhibition of mitochondrial complex I by rotenone and to test if epigallocatechin-3-gallate
24 (EGCG), a natural polyphenol component of green tea, could counteract this effect. After
25 2 h incubation of stallion spermatozoa in modified Tyrodes medium, rotenone (100 nM,
26 500 nM, 5 μM) and EGCG (10 μM, 20 μM, 60 μM), alone or in association, did not
27 induced any significant difference on the percentage of viable cells, live sperm with active
28 mitochondria and spermatozoa with intact acrosome. The inhibition of complex I of
29 mitochondrial respiratory chain of stallion sperm with rotenone exerted a negative effect
30 on heterologous ZP-binding ability. EGCG at the concentrations of 10 μM and 20 μM
31 (but not of 60 μM) induced a significant increase in the number of sperm bound to the
32 ZP compared with that for control. Moreover when stallion sperm were treated with
33 rotenone 100 nM, the presence of ECGC at all the concentration tested (10 μM,20 μM,
34 60 μM) significantly increased the number of sperm bound to the ZP up to control levels
35 suggesting that this green tea polyphenol is able to reduce the toxicity of rotenone.

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40 **Keywords:** horse, spermatozoa, mitochondria, oxidative phosphorylation.

41

42

43 **INTRODUCTION**

44 Spermatozoa require ATP to achieve normal sperm function and to fuel the
45 motility. Mammalian sperm rely mainly on two metabolic pathways to produce ATP
46 which are localized to different regions of the cell: oxidative phosphorylation (OXPHOS)
47 occurs in mitochondria localized in the sperm mid piece while anaerobic glycolysis takes
48 place mainly in the fibrous sheath of the flagellum where glycolytic enzymes are tightly
49 anchored (Feramosca and Zara, 2014; Tourmente et al., 2015).
50 While human sperm rely mainly on glycolysis for ATP production, bull spermatozoa are
51 characterized by both high respiration and glycolysis. On the other hand stallion
52 spermatozoa are highly dependent on OXPHOS for ATP production (Cummins, 2009;
53 Gibb et al., 2014). The great importance of sperm mitochondrial functionality in horse is
54 confirmed by the observation that the most fertile stallion ejaculates exhibit the highest
55 levels of OXPHOS activity (Gibb et al., 2014).
56 The inhibition of electron transport chain (ETC) along the respiratory complexes
57 produces free radicals that damages the functionality of the mitochondria, decreases the
58 intracellular ATP content resulting in a decrease in stallion sperm motility (Gibb et al,
59 2014), even in presence of glucose (Plaza Dávila et al., 2015).
60 One of the most active inhibitors of mitochondrial respiratory chain (MRC) is rotenone,
61 a lipophilic isoflavonoid that inhibits complex I (NADH reductase) (Singer and Ramsay,
62 1994). Rotenone reduces ATP production by mitochondria, leading to increased
63 formation of free radicals besides a deregulation of cell homeostasis and ROS release into
64 the mitochondrial matrix, where they can overwhelm the intra-mitochondrial antioxidant
65 defense enzymes. This would account for the ability of rotenone to induce peroxidative
66 damage in the midpiece of the spermatozoa. The peroxidative damage, in turn, induces a
67 progressive loss of motility in terms of the percentage of motile and progressive

spermatozoa (Koppers et al., 2008). The presence of antioxidants, such as α-tocopherol, can prevent these negative effects of rotenone (Koppers et al., 2008).

Different natural antioxidants can help to reverse the negative effect of inhibitors of mitochondrial respiratory chain (MRC). Among Green tea catechins the principal polyphenolic compound is epigallocatechin-3-gallate (EGCG) (Stewart et al., 2005) which can act as eliminator of free radical by reaction with hydrogen, alkoxyl or peroxyl radicals (Wang et al., 2000) and as an iron chelator (Grinberg et al., 1997). In addition, its antioxidant capacity by removing free radicals can indirectly increase endogenous antioxidants activity (Quiong et al., 1996; Skrzydlewska et al., 2002). Moreover EGCG accumulates within the mitochondria and preserve catalase activity (Schroeder et al., 2008). Valenti et al. (2013) demonstrated that EGCG restores the overall rate of mitochondrial ATP synthesis of cells from subjects with Down´s syndrome, in which the deficit of complex I and ATP synthase results in depressed energy production by mitochondrial OXPHOS.

Sperm mitochondria are organelles that greatly suffer due to damage induced by reproductive technologies, such as cryopreservation and sex sorting (Ortega-Ferrusola et al., 2009; Balao da Silva et al., 2014; Peña et al, 2015). Attempts to protect mitochondria can be an attractive strategy to improve the quality of stallion sperm that underwent such biotechnical procedures.

The aim of our study was to evaluate the response of equine sperm under capacitating conditions to the inhibition of mitochondrial complex I by rotenone and to test if EGCG, could counteract this effect.

## MATERIAL AND METHODS

### Experimental design

93 Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy).
94 Three ejaculates from each of three stallions of proven fertility were used. Fifteen
95 different experimental groups for each ejaculate in the basis of the additions were
96 considered: control group (CTR), rotenone 100 nM (R100), rotenone 500 nM (R500),
97 rotenone 5 μM (R5), EGCG 10 μM (E10), EGCG 20 μM (E20), EGCG 60 μM (E60),
98 rotenone 100 nM + EGCG 10μM (R100+E10), rotenone 100 nM + EGCG 20μM
99 (R100+E20), rotenone 100 nM + EGCG 60μM (R100+E60), rotenone 500 nM + EGCG
100 10μM (R500+E10), rotenone 500 nM + EGCG 20μM (R500+E20), rotenone 500 nM +
101 EGCG 60μM (R500+E60), rotenone 5 μM + EGCG 10μM (R5+E10), rotenone 5 μM +
102 EGCG 20μM (R5+E20), rotenone 5 μM + EGCG 60μM (R5+E60).
103 The evaluation of viability, acrosome status and mitochondrial membrane potential were
104 performed on fresh semen (CTR), and after 2 h of incubation in modified Tyrodes
105 medium pH 7.4 (Rathi et al., 2001).
106 The heterologous binding assay was performed co-incubating for 1 h in vitro matured
107 porcine oocytes with semen previously pre-incubated 1h in presence or absence of
108 different concentrations of Rotenone and EGCG.
109

110 **Semen collection and preparation**

111 The experiment was approved by the Ethic-scientific Committee of Alma Mater
112 Studiorum, University of Bologna

113 Semen was obtained from 3 different stallions of proven fertility (14, 15 and 18 years
114 old) individually housed at the National Institute of Artificial Insemination, University of
115 Bologna, Italy, from October to November 2013. Stallions jumped on a breeding phantom
116 and ejaculates were collected with a Missouri artificial vagina equipped with a disposable
117 liner and aniline filter (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately

118 evaluated for volume and concentration (NucleoCounterSP 100, Chemometec,
119 Denmark), diluted 1:1 in Kenney's extender (Kenney et al., 1975) and sent to the
120 laboratory within 1 h, maintained at 22ºC.
121 Aliquots of the ejaculates were centrifuged twice for 2 min at 900 × g. The supernatants
122 were removed and the pellets resuspended in modified Tyrodes solution (96 mM NaCl,
123 3.1 mM KCl, 2 mM $CaCl_2$. $2H_20$, 0.4 mM $MgSO_4.7H_{2,}$ 0,0.3 mM $KHPO_4$, 20 mM
124 HEPES, 5 mM glucose, 21.7mM NaLactate, 1 mM Na Pyruvate, 15 mM $NaH_2CO_3$, 7
125 mg/mL BSA, 50 μg/mL Kanamicin) pH 7.4 (Rathi et al., 2001) to obtain $20x10^6$
126 spermatozoa/mL.
127 For the evaluation of viability, acrosome status and mitochondrial membrane potential
128 500μL of semen suspensions were incubated for 2 h in Nunc 4-well multidish at 38°C in
129 95% humidity 5% CO2 in presence or absence of different concentrations of Rotenone
130 and EGCG.
131
132 **Viability assessment with SYBR-PI**
133 Twenty-five microliters of semen were incubated with 2 μL of a 300 μM solution of
134 propidium iodide (PI) and 2 μL of a 10 μM solution of SYBR green-14, both obtained
135 from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA) for 5
136 min at 37 °C in the dark. Aliquots of the stained suspensions were placed on clean
137 microscope slides, carefully overlaid with coverslips, and at least 200 spermatozoa per
138 sample were scored under a Nikon Eclipse E 600 epifluorescence microscope (Nikon
139 Europe BV, Badhoeverdop, The Netherlands). Spermatozoa stained with SYBR-14 and
140 not stained with PI were considered as viable. Spermatozoa both SYBR-14+ and PI+ and
141 those SYBR-14–/PI+ were considered with damaged membranes or dead.
142

143 **Evaluation of mitochondrial membrane potential**

144 For each sample, an aliquot (25µL) of semen was incubated with 2 µL of a 300 µM
145 propidium iodide (PI) stock solution, 2 µL of a 10 µM SYBR green-14 stock solution and
146 2 µL of a 150 µM 5,5',6,6'-tetrachloro-1,1',3,3'
147 tetraethylbenzimidazolylcarbocyanineiodide (JC-1) solution for 20 min at 37°C in the
148 dark. Ten microliters of the sperm suspension were then placed on a slide and at least 200
149 spermatozoa per samples were scored using the above described microscope. JC-1
150 monomers emit a green fluorescence in mitochondria with low potential, while emitting
151 a bright red-orange fluorescence in case of multimer formation (J-aggregates) in
152 mitochondria with high membrane potential. Sperm cells SYBR+/PI- with an orange
153 fluorescence in the mid piece were considered as live spermatozoa with high
154 mitochondrial membrane potential.

155

156 **Evaluation of acrosome status**

157 Acrosome integrity was evaluated by using a FITC-conjugated lectin from Pisum
158 Sativum (FITC-PSA) which label acrosomal matrix glycoproteins. Spermatozoa were
159 washed twice in PBS, resuspended in ethanol 95% and fixed/permeabilized at 4°C for at
160 least 30 min. Samples were dried in heated slides and incubated with FIC-PSA solution
161 (5 µg PSA-FITC/1 mL H2O) for 20 min in darkness. After staining, samples were washed
162 in PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories,
163 Burlingame, CA, USA). The slides were then observed with a fluorescence microscope.
164 The presence of a green acrosomal fluorescence was considered indicative of an intact
165 acrosome, whereas a partial or total absence of fluorescence was considered to indicate
166 acrosome disruption or acrosome reaction.

167

168 **In vitro maturation (IVM)**

169 Porcine cumulus–oocyte complexes (COCs) were aspirated using an 18 gauge needle
170 attached to a 10 mL disposable syringe from 4 to 6 mm follicles of ovaries collected at a
171 local abattoir and transported to the laboratory within 1 h. Under a stereomicroscope,
172 intact COCs were selected and transferred into a petri dish (35 mm, Nunclon,Denmark)
173 prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. After three washes
174 in NCSU 37 (Petters and Wells, 1993) supplemented with 5.0 mg/mLinsulin, 0.57 mM
175 cysteine, 10 ng/mL epidermal growth factor (EGF), 50 mM ß-mercaptoethanol and 10%
176 porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-
177 well multidish containing 500 µL of the same medium per well and cultured at 39 ºC in
178 a humidified atmosphere of 5% $CO_2$/7% $O_2$ in air. For the first 22 h of in vitro maturation
179 the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL, eCG (Folligon,
180 Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, IntervetBoxmeer, The
181 Netherlands). For the last 22 h COCs were transferred to fresh maturation medium
182 (Funahashi et al., 1997). At the end of the maturation period the oocytes were denuded
183 by gentle repeated pipetting in maturation medium containing 0.4% hyaluronidase.

184

185 **Heterologous binding assay**

186 For the binding assay, the semen was centrifuged twice for 2 min at 900 × g, resuspended
187 in modified Tyrodes medium to obtain $1\times10^6$ spermatozoa /ml and 500 µL of the sperm
188 suspensions were preincubated for 1 h in presence or absence of different concentrations
189 of Rotenone and EGCG. After oocyte maturation 30-35 denuded oocytes were added in
190 each well and after 1 h of gamete co-incubation at 38 °C in 95% humidity and 5% CO2
191 in air the oocytes were washed four times in PBS 0.4% BSA with a wide bore glass pipette
192 in order to remove the spermatozoa loosely attacked to zona pellucida. The oocytes were

193 then fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated
194 with 8.9 μM Hoechst 33342 for 10 min in PBS 0.4% BSA in the dark, washed twice in
195 the same medium, and individually placed in droplets of Vectashield (Vector
196 Laboratories, Burlingame, CA, USA) on a slide, and covered with a coverslip. The
197 number of spermatozoa attached to the zona pellucida of each oocyte was assessed by
198 using the above described microscope and was expressed as standard deviation units (see
199 Statistical analysis).
200

201 **Statistical analysis**

202 Statistical analysis was performed using R version 3.1.1 ,(R Core Team Computing,
203 2014).
204 Sperm analysis data are expressed as mean ± SD. Significance was set for $p < 0.05$. Data
205 were checked for normality with the Shapiro-Wilk test; differences between treatments
206 were analyzed by an ANOVA test.
207 As for heterologous binding assay, data were standardized by dividing the number of
208 bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed
209 as standard deviation units. Data were analyzed by linear mixed effect model.
210 Significance was set at $p<0.05$.
211

212 **RESULTS**

213 **Evaluation of viability, mitochondrial membrane potential and acrosome status**

214 Rotenone treatment of stallion semen at all the concentrations tested (100 nM, 500 nM, 5
215 μM) during a 2 h in modified Tyrodes medium did not induced any significant difference
216 on the percentage of viable cells, live sperm with active mitochondria and spermatozoa
217 with intact acrosome (Fig. 1 A,B,C). EGCG at all the concentrations tested (10 μM, 20

218 μM, 60 μM) did not exert any significant effect on the parameter analyzed when
219 supplemented either alone or in presence of rotenone (Fig. 1 A,B,C).
220

221 **Heterologous binding assay**

222 To evaluate the effect of rotenone and EGCG on equine sperm capability to bind to swine
223 ZP, denuded in vitro matured porcine oocytes were co-incubated for 1h with semen
224 previously pre-incubated 1h in presence or absence of different concentrations of
225 Rotenone and EGCG (around 100 oocytes per treatment). The results are expressed as
226 number of sperm bound per oocyte normalized to the daily standard deviation (Figure 2).
227 Rotenone at all the concentrations tested (100 nM, 500 nM, 5 μM) induced a significant
228 decrease in the number of sperm bound to the ZP compared with that for control.
229 EGCG at the concentrations of 10 μM and 20 μM (but not of 60 μM) induced a significant
230 increase in the number of sperm bound to the ZP compared with that for control.
231 When stallion sperm were treated with rotenone 100 nM, the presence of ECGC at all the
232 concentration tested (10 μM,20 μM, 60 μM) significantly increased the number of sperm
233 bound to the ZP up to control levels. However, ECGC at concentration of 20 and 60 μM
234 did not significantly increase the number of sperm bound to the ZP compared with R 100.
235 EGCG at all the concentrations tested (10 μM, 20 μM, 60 μM) did not induce any
236 increase in the number of spermatozoa bound when added in presence of the higher
237 concentration of rotenone (500 nM, 5 μM).
238

239 **DISCUSSION**

240 The aim of our study was to evaluate the response of equine sperm after inhibiting
241 mitochondrial complex I by rotenone during in vitro capacitation for two hours and to test

242 if EGCG, a natural polyphenol component of green tea, could counteract the effect of
243 rotenone.
244 The evaluation of stallion sperm viability, acrosomal membrane integrity and
245 mitochondrial activity did not evidence any significant effect of rotenone at all the
246 concentrations tested (100 nM, 500 nM, 5µM). The absence of significant differences on
247 the percentage of viable stallion sperm agree well with the data obtained by Gibb at al.
248 (2014) and Plaza Dávila et al., (2015) who observed a sperm viability similar to control
249 even using a higher rotenone concentration (10µM) for 1h; only after 3h of incubation,
250 rotenone 10µM induced a significant reduction of the percentage of intact sperm (Plaza
251 Dávila et al., 2015). In contrast with the results of those authors we did not observe a
252 significant decrease in the percentage of live spermatozoa with high mitochondrial
253 membrane potential. This discrepancy could be due to different reasons: Gibb et al. (2014)
254 and Plaza Dávila et al. (2015) evaluated JC1 positivity by flow cytometry while we used
255 fluorescence microscopy possibly overestimating JC1 positive cells classifying as JC1
256 positive also those cells with only partial or spot like JC1 positive mitochondria. A further
257 explanation could be the lower rotenone concentrations used in our work and the different
258 conditions of the incubation with rotenone: capacitating in our study and non capacitating
259 in Gibb et al. (2014) and Plaza Dávila et al. (2015) studies.
260 To evaluate the effect of rotenone and EGCG on the in vitro function of equine
261 spermatozoa, an heterologous binding assay was performed co-incubating denuded IVM
262 porcine oocytes for 1h with semen previously pre-incubated 1h in presence or absence of
263 different concentrations of rotenone and EGCG. It has been demonstrated that sperm-
264 oocyte binding assays offer a good reliability in the prediction of horse in vivo fertility
265 (Fazeli et al., 1993; Fazeli et al., 1995; Meyers et al., 1996). Due to the low availability
266 of equine oocytes, in our study a heterologous binding assay was performed as the

267 efficiency/reliability of using bovine or swine oocytes has been demonstrated (Sinowatz
268 et al., 2003; Balao da Silva et al., 2013; Clulow et al., 2010). As in the case of the
269 homologous assay, the process of capacitation is needed for stallion spermatozoa to bind
270 to heterologous oocytes (Clulow et al., 2010).
271 The results obtained in this study demonstrate for the first time that inhibition of complex
272 I of MRC of stallion sperm with rotenone exerts a negative effect on ZP binding ability.
273 In fact rotenone at all the tested concentrations (100 nM, 500 nM, 5μM) significantly
274 decreased the number of bound sperm per oocyte in comparison with control group.
275 When stallion spermatozoa were treated under capacitating condition with 10 μM and 20
276 μM EGCG stallion ZP-binding activity was improved compared with control semen. A
277 positive influence of EGCG addition on both fresh and frozen-thawed spermatozoa
278 during IVF on ZP-binding and oocyte penetration was already recorded in pig (Spinaci et
279 al., 2008; Kaedei et al., 2012) suggesting a modulating action of this polyphenol on sperm
280 capacitation. This effect could be exerted thanks to the antioxidant ability of EGCG that
281 can act on the balance between excessive ROS production, that overwhelms the limited
282 capacity of these cells to protect themselves from oxidative stress, and mild intracellular
283 ROS generation, which stimulates intracellular cAMP generation, inhibits tyrosine
284 phosphatase activity and enhances the formation of oxysterols, thus inducing a
285 physiological capacitation (Aitken et al., 2015).
286 ECGC at 10 μM concentration significantly blunt the negative effect on stallion ZP-
287 binding activity of rotenone at the lower dose tested (100 nM). EGCG at the higher doses
288 tested (20 μM, 60 μM), even if it was not able to completely reverse the inhibitory effect
289 of rotenone 100 nM, increased the number of sperm bound to ZP up to levels of the
290 control group. However EGCG was not able to reduce the negative effect on heterologous
291 binding induced by higher concentration of rotenone (500 nM and 5 μM).

292 Our results agree with the ability of epicatechin and ECGC (but not of other flavonoids
293 such as gebistein and baicain) demonstrated by Kamalden et al. (2012) in protecting a
294 transformed cell line (RGC-5 cells) from rotenone-induced toxicity. This positive effect,
295 as suggested by the authors, could be mainly, but not exclusively, attributed to the
296 antioxidant activity of these flavonoids. The ability of ECGC to counteract mitochondrial
297 energy deficit due to impaired activities of complex I has been demonstrated by Valenti
298 et al. (2013) in cultured fibroblasts and lymphoblasts from Down's syndrome subjects.
299 This effect was associated with EGCG-induced promotion of cAMP and PKA-dependent
300 phosphorylation of complex I.
301 Rotenone inhibits oxidative glycolysis and ATP production in stallion spermatozoa
302 inducing a reduction of sperm motility parameters (Plaza Dávila et al., 2015). It could be
303 hypothesized that EGCG, counteracting rotenone-induced deficit in mitochondrial ATP
304 synthesis, may ensure under capacitating conditions the adequate energy supply. In this
305 way the spermatozoa can sustain changes occurring during capacitation, such as
306 hyperactivated motility and protein phosphorylation (Feramosca and Zara, 2014).
307 In conclusion, the inhibition of complex I by rotenone results in a decreased ZB-binding
308 ability of stallion spermatozoa and the presence EGCG is able to reduce the toxicity of
309 rotenone at the lower dose (100 nM). Moreover, spermatozoa treated with EGCG attach
310 better than non-treated ones, suggesting that they have a more advanced capacitation-like
311 status.
312

313 **Conflict of interest**

314 None of the authors have conflict of interest to declare

315

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412 Figure 1. Viability (A), mitochondrial membrane potential (B) and acrosome status (C)

413 of stallion spermatozoa after 2 h incubation in capacitating condition in presence of

414 rotenone and/or EGCG.

415 R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 μM; E10, EGCG 10

416 μM; E20, EGCG 20 μM; E60, EGCG 60 μM.

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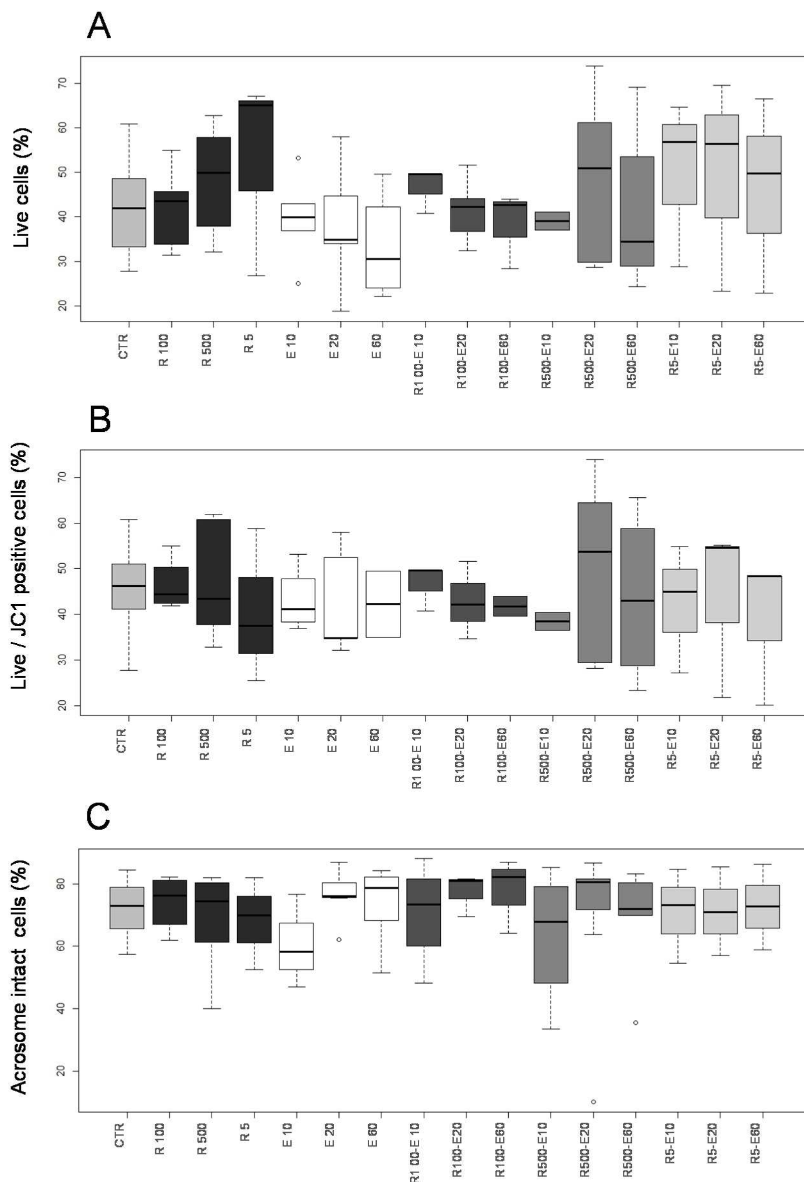


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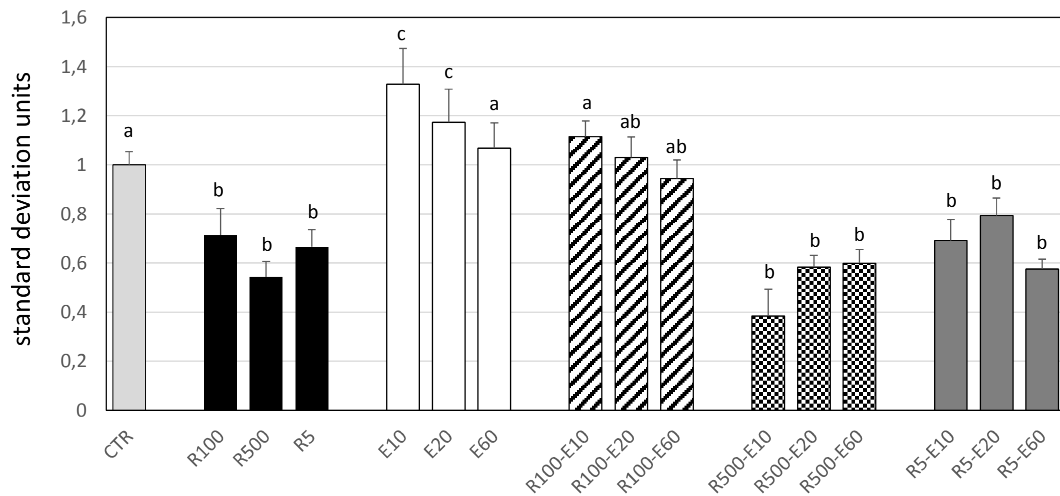
420 Figure 2. Effect of rotenone and/or EGCG on stallion heterologous binding ability. Data
421 were standardized by dividing the number of bound spermatozoa/oocyte by the daily
422 standard deviation, and are therefore expressed as standard deviation units.
423 R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 μM; E10, EGCG 10
424 μM; E20, EGCG 20 μM; E60, EGCG 60 μM.
425 Different letters on the bars indicate a significant difference.
426

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