

This is the peer reviewed version of the following article:

Plaza Dávila M, Bucci D, Galeati G, Peña FJ, Mari G, Giaretta E, Tamanini C, Spinaci M. Epigallocatechin-3-Gallate (EGCG) Reduces Rotenone Effect on Stallion Sperm-Zona Pellucida Heterologous Binding. *Reprod Domest Anim* 2015;50(6):1011-6.

which has been published in final form at

<https://doi.org/10.1111/rda.12628>

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

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

4

5 Plaza Dávila M.<sup>1</sup>, Bucci D.<sup>2</sup>, Galeati G.<sup>2</sup>, Peña F.J.<sup>1</sup>, Mari G.<sup>2,3</sup>, Giaretta E.<sup>2</sup>, Tamanini  
6 C.<sup>2</sup>, Spinaci M<sup>2\*</sup>.

7 <sup>1</sup> Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching  
8 Hospital, University of Extremadura, Cáceres, Spain, <sup>2</sup>Department of Veterinary Medical  
9 Sciences (DIMEVET), <sup>3</sup>AUB INFA, University of Bologna, Bologna, Italy.

10

11 \*Corresponding author: Marcella Spinaci,

12 Department of Veterinary Medical Sciences - DIMEVET, University of Bologna, Via  
13 Tolara di Sopra 50, Ozzano dell'Emilia, 40064 Bologna, Italy

14 marcella.spinaci@unibo.it

15

16

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

18

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  $\mu$ M) and EGCG (10  $\mu$ M, 20  $\mu$ M, 60  $\mu$ M), 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  $\mu$ M and 20  $\mu$ M  
31 (but not of 60  $\mu$ M) 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  $\mu$ M,20  $\mu$ M,  
34 60  $\mu$ M) 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.

36

37

38

39

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

68 spermatozoa (Koppers et al., 2008). The presence of antioxidants, such as  $\alpha$ -tocopherol,  
69 can prevent these negative effects of rotenone (Koppers et al., 2008).

70 Different natural antioxidants can help to reverse the negative effect of inhibitors of  
71 mitochondrial respiratory chain (MRC). Among Green tea catechins the principal  
72 polyphenolic compound is epigallocatechin-3-gallate (EGCG) (Stewart et al., 2005)  
73 which can act as eliminator of free radical by reaction with hydrogen, alkoxyl or peroxy  
74 radicals (Wang et al., 2000) and as an iron chelator (Grinberg et al., 1997). In addition,  
75 its antioxidant capacity by removing free radicals can indirectly increase endogenous  
76 antioxidants activity (Quiong et al., 1996; Skrzydlewska et al., 2002). Moreover EGCG  
77 accumulates within the mitochondria and preserve catalase activity (Schroeder et al.,  
78 2008). Valenti et al. (2013) demonstrated that EGCG restores the overall rate of  
79 mitochondrial ATP synthesis of cells from subjects with Down's syndrome, in which the  
80 deficit of complex I and ATP synthase results in depressed energy production by  
81 mitochondrial OXPHOS.

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

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

90

## 91 **MATERIAL AND METHODS**

### 92 **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  $\mu$ M (R5), EGCG 10  $\mu$ M (E10), EGCG 20  $\mu$ M (E20), EGCG 60  $\mu$ M (E60),  
98 rotenone 100 nM + EGCG 10 $\mu$ M (R100+E10), rotenone 100 nM + EGCG 20 $\mu$ M  
99 (R100+E20), rotenone 100 nM + EGCG 60 $\mu$ M (R100+E60), rotenone 500 nM + EGCG  
100 10 $\mu$ M (R500+E10), rotenone 500 nM + EGCG 20 $\mu$ M (R500+E20), rotenone 500 nM +  
101 EGCG 60 $\mu$ M (R500+E60), rotenone 5  $\mu$ M + EGCG 10 $\mu$ M (R5+E10), rotenone 5  $\mu$ M +  
102 EGCG 20 $\mu$ M (R5+E20), rotenone 5  $\mu$ M + EGCG 60 $\mu$ M (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 22°C.

121 Aliquots of the ejaculates were centrifuged twice for 2 min at  $900 \times g$ . The supernatants  
122 were removed and the pellets resuspended in modified Tyrodes solution (96 mM NaCl,  
123 3.1 mM KCl, 2 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.4 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 mM  $\text{KH}_2\text{PO}_4$ , 20 mM  
124 HEPES, 5 mM glucose, 21.7mM NaLactate, 1 mM Na Pyruvate, 15 mM  $\text{NaH}_2\text{CO}_3$ , 7  
125 mg/mL BSA, 50  $\mu\text{g/mL}$  Kanamicin) pH 7.4 (Rathi et al., 2001) to obtain  $20 \times 10^6$   
126 spermatozoa/mL.

127 For the evaluation of viability, acrosome status and mitochondrial membrane potential  
128 500 $\mu\text{L}$  of semen suspensions were incubated for 2 h in Nunc 4-well multidish at 38°C in  
129 95% humidity 5%  $\text{CO}_2$  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  $\mu\text{L}$  of a 300  $\mu\text{M}$  solution of  
134 propidium iodide (PI) and 2  $\mu\text{L}$  of a 10  $\mu\text{M}$  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, Badhoevedop, 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 (25 $\mu$ L) of semen was incubated with 2  $\mu$ L of a 300  $\mu$ M  
145 propidium iodide (PI) stock solution, 2  $\mu$ L of a 10  $\mu$ M SYBR green-14 stock solution and  
146 2  $\mu$ L of a 150  $\mu$ M 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  $\mu$ g PSA-FITC/1 mL H<sub>2</sub>O) 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  $\beta$ -mercaptoethanol and 10%  
176 porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-  
177 well multidish containing 500  $\mu$ L of the same medium per well and cultured at 39 °C in  
178 a humidified atmosphere of 5% CO<sub>2</sub>/7% O<sub>2</sub> 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  $\times$  g, resuspended  
187 in modified Tyrodes medium to obtain  $1 \times 10^6$  spermatozoa /ml and 500  $\mu$ L 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% CO<sub>2</sub>  
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 attached 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  $\mu$ M 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  $\pm$  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  $\mu$ M) 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  $\mu$ M, 20

218  $\mu\text{M}$ ,  $60 \mu\text{M}$ ) 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,  $5 \mu\text{M}$ ) 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  $10 \mu\text{M}$  and  $20 \mu\text{M}$  (but not of  $60 \mu\text{M}$ ) 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 EGCG at all the  
232 concentration tested ( $10 \mu\text{M}$ ,  $20 \mu\text{M}$ ,  $60 \mu\text{M}$ ) significantly increased the number of sperm  
233 bound to the ZP up to control levels. However, EGCG at concentration of 20 and  $60 \mu\text{M}$   
234 did not significantly increase the number of sperm bound to the ZP compared with R 100.

235 EGCG at all the concentrations tested ( $10 \mu\text{M}$ ,  $20 \mu\text{M}$ ,  $60 \mu\text{M}$ ) 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,  $5 \mu\text{M}$ ).

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, 5 $\mu$ M). The absence of significant differences on  
247 the percentage of viable stallion sperm agree well with the data obtained by Gibb et al.  
248 (2014) and Plaza Dávila et al., (2015) who observed a sperm viability similar to control  
249 even using a higher rotenone concentration (10 $\mu$ M) for 1h; only after 3h of incubation,  
250 rotenone 10 $\mu$ M 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, 5 $\mu$ M) 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  $\mu$ M and 20  
276  $\mu$ M 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 EGCG at 10  $\mu$ M 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  $\mu$ M, 60  $\mu$ M), 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  $\mu$ M).

292 Our results agree with the ability of epicatechin and ECGC (but not of other flavonoids  
293 such as gallocatechin and baicalin) 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 ECGC-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 ECGC, 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 (Ferasmosca 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 ECGC is able to reduce the toxicity of  
309 rotenone at the lower dose (100 nM). Moreover, spermatozoa treated with ECGC 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

316 **REFERENCES**

- 317 Aitken RJ, Baker MA, Nixon B, 2015. Are sperm capacitation and apoptosis the opposite  
318 ends of a continuum driven by oxidative stress? *Asian J Androl* doi: 10.4103/1008-  
319 682X.153850. [Epub ahead of print]
- 320 Balao da Silva C, Ortega Ferrusola C, Gallardo Bolanos J, Plaza Dávila M, Martin-Munoz  
321 P, Morrell J, Rodriguez Martinez H, Peña F, 2014: Effect of overnight staining on  
322 the quality of flow cytometric sorted stallion sperm: comparison with traditional  
323 protocols. *Reprod Domest Anim* **49**,1021–1027.
- 324 Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Peña FJ, Mari G, Galeati G, 2013:  
325 Effect of sex sorting on stallion spermatozoa: Heterologous oocyte binding, tyrosine  
326 phosphorylation and acrosome reaction assay. *Anim Reprod Sci* **141**, 68-74.
- 327 Clulow JR, Evans G, Maxwell WM, Morris LH, 2010: Evaluation of the function of fresh  
328 and frozen-thawed sex-sorted and non-sorted stallion spermatozoa using a  
329 heterologous oocyte binding assay. *Reprod Fertil Dev* **22**, 710–717.
- 330 Cummins JM, 2009: Sperm motility and energetics.in *Sperm biology, an evolutionary*  
331 *perspective* (Birkhead, T. R., Hosken, D. J., and Pitnick, S. eds.), Academic Press,  
332 San Diego, CA., pp. 185-206
- 333 Fazeli AR, Steenweg W, Bevers MM, de Loos FA, van den Broek J, Colenbrander B,  
334 1993: Development of a sperm zonapellucida binding assay for bull semen. *Vet Rec*  
335 **132**, 14–16.
- 336 Fazeli AR, Steenweg W, Bevers MM, van den Broek J, Bracher V, Parlevliet J,  
337 Colenbrander B, 1995: Relation between stallion sperm binding to homologous  
338 hemizonae and fertility. *Theriogenology* **44**, 751–760

339 Ferramosca A, Zara V, 2014: .Bioenergetics of mammalian sperm capacitation. *Biomed*  
340 *Res Int* 2014:902953

341 Gibb Z, Lambourne SR, Aitken RJ, 2014: The paradoxical relationship between stallion  
342 fertility and oxidative stress. *Biol Reprod* **91**, 1-10

343 Grinberg LN, Newmark H, Kitrossky N, Rahamim E, Chevion M, Rachmilewitz EA,  
344 1997: Protective effects of tea polyphenols against oxidative damage to red blood  
345 cells. *Biochem Pharmacol* **54**, 973–978

346 Kaedei Y, Naito M, Naoi H, Sato Y, Taniguchi M, Tanihara F, Kikuchi K, Nagai T, Otoi  
347 T, 2012: Effects of (-)-epigallocatechingallate on the motility and penetrability of  
348 frozen-thawed boar spermatozoa incubated in the fertilization medium. *Reprod*  
349 *Domest Anim* **47**, 880-6.

350 Kamalden TA, Ji D, Osborne NN, 2012: Rotenone-induced death of RGC-5 cells is  
351 caspase independent, involves the JNK and p38 pathways and is attenuated by  
352 specific green tea flavonoids. *Neurochem Res* **37**, 1091-101.

353 Kenney, R.M., Bergman, R.V., Cooper, W.L., Morse, G.W, 1975: Minimal  
354 contamination techniques for breeding mares: techniques and preliminary findings.  
355 In: 21st Annual Convention of the American Association of Equine Practitioners,  
356 327.

357 Koppers AJ, De luliis GN, Finnie JM, McLaughlin EA , Aitken RJ, 2008: Significance  
358 of mitochondrial reactive oxygen species in the generation of oxidative stress in  
359 spermatozoa. *J Clin Endocrinol Metab* **93**, 3199-207.

360 Meyers SA, Liu IK, Overstreet JW, Vadas S, Drobnis EZ, 1996. Zona pellucida binding  
361 and zona-induced acrosome reactions in horse spermatozoa: comparisons between  
362 fertile and subfertile stallions. *Theriogenology* **46**, 1277–1288.



363 Ortega Ferrusola C, González Fernández L, Morrell JM, Salazar Sandoval C,  
364 MacíasGarcía B, Rodríguez-Martinez H, Tapia JA, Peña FJ, 2009: Lipid  
365 peroxidation, assessed with BODIPY-C11, increases after cryopreservation of  
366 stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes.  
367 *Reproduction* **138**, 55-63

368 Peña FJ, Plaza Dávila M, Ball BA, Squires EL, Martin Munoz P, Ortega Ferrusola C,  
369 Balao da Silva C, 2015: The Impact of Reproductive Technologies on Stallion  
370 Mitochondrial Function *Reprod Dom Anim* doi: 10.1111/rda.12551

371 Plaza Dávila M, Martín Muñoz P, Tapia JA, Ortega Ferrusola C, Balao da Silva C, Peña  
372 FJ (2015) Inhibition of mitochondrial complex I leads to decreased motility and  
373 membrane integrity related to increased hydrogen peroxide and reduced ATP  
374 production, while the inhibition of glycolysis has less impact on sperm motility *Plos*  
375 *One* DOI 10.1371/journal.pone.0138777 .

376 Petters, R.M., Wells, K.D, 1993. Culture of pig embryos. *J Reprod Fertil Suppl* **48**, 61–  
377 73.

378 Qiong G, Baolu Z, Meifen L, Shengrong S, Wenjuan X, 1996: Studies on protective  
379 mechanisms of four components of Green tea polyphenols against lipid peroxidation  
380 in synaptosomes. *Biochim Biophys Acta* **1304**, 210–222

381 Rathi, R., Colenbrander, B., Bevers, M.M., Gadella, B.M, 2001: Evaluation of in vitro  
382 capacitation of stallion spermatozoa. *Biol Reprod* **65**, 462–470.

383 Schroeder EK, Kelsey NA, Doyle J, Breed E, Bouchard RJ, Loucks A, HarbisonA,  
384 Linseman DA, 2008: Green tea epigallocatechin 3-gallate accumulates in  
385 mitochondria and displays a selective anti-apoptotic effect against inducers of  
386 mitochondrial oxidative stress in neurons. *Antioxid Redox Signal* **11**, 469-80.

387 Sinowatz F, Wessa E, Neumuller C, Palma G, 2003: On the species speci- ficity of sperm  
388 binding and sperm penetration of the zona pellucida. *Reprod Domest Anim* **38**, 141–  
389 146.

390 Skrzydlewska E, Ostrowska J, Farbiszewski R, Michalak K, 2002: Protective effect of  
391 green tea against lipid peroxidation in the rat liver, blood serum and the brain.  
392 *Phytomedicine* **9**, 232–238

393 Spinaci M, Volpe S, De Ambrogi M, Tamanini C, GaleatiG, 2008: Effects of  
394 epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine  
395 oocytes. *Theriogenology* **69**, 877-85

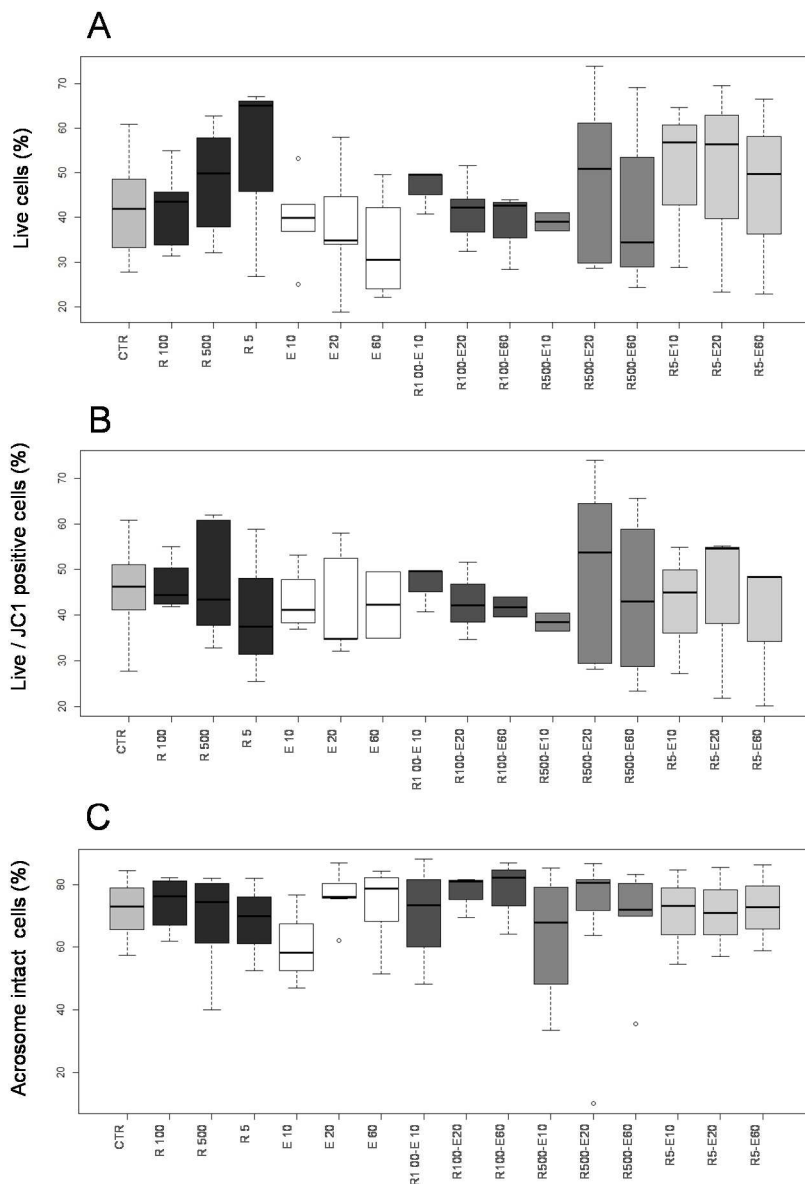
396 Stewart AJ, Mullen W, Crozier A, 2005: On-line high-performance liquid  
397 chromatography analysis of the antioxidant activity of phenolic compounds in green  
398 and black tea. *Mol Nutr Food Res* **49**, 52–60

399 Tourmente M, Villar-Moya P, Rial E, Roldan ER. Differences in ATP Generation Via  
400 Glycolysis and Oxidative Phosphorylation, and Relationships with SpermMotility,  
401 in Mouse Species. *J Biol Chem*. 2015 Jun 5. pii: jbc.M115.664813. [Epub ahead of  
402 print]

403 Valenti D, De Rasmio D, Signorile A, Rossi L, de Bari L, Scala I, Granese B, Papa S,  
404 Vacca RA, 2013: Epigallocatechin-3-gallate prevents oxidative phosphorylation  
405 déficit and promotes mitochondrial biogénesis in human cells from subjects with  
406 Down´s syndrome. *Biochim Biophys Acta* **1832**, 542-52.

407 Wang LF, Kim DM, Lee CY, 2000: Effects of heat processing and storage on flavanols  
408 and sensory qualities of green tea beverage. *J Agric Food Chem* **48**, 4227–4232  
409  
410  
411

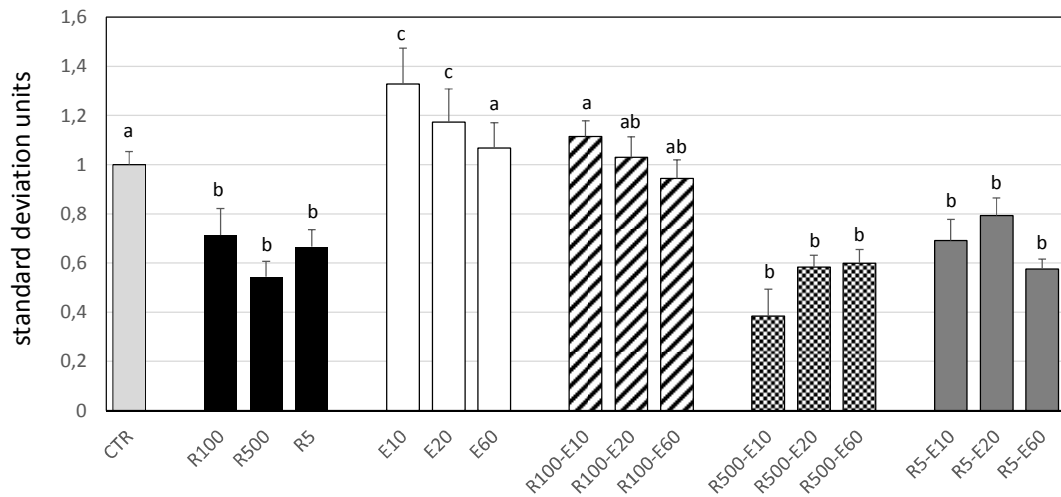
412 Figure 1. Viability (A), mitochondrial membrane potential (B) and acrosome status (C)  
 413 of stallion spermatozoa after 2 h incubation in capacitating condition in presence of  
 414 rotenone and/or EGCG.  
 415 R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5  $\mu$ M; E10, EGCG 10  
 416  $\mu$ M; E20, EGCG 20  $\mu$ M; E60, EGCG 60  $\mu$ M.  
 417



418

419

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  $\mu$ M; E10, EGCG 10  
 424  $\mu$ M; E20, EGCG 20  $\mu$ M; E60, EGCG 60  $\mu$ M.  
 425 Different letters on the bars indicate a significant difference.  
 426



427