

## Epigallocatechin-3-Gallate (EGCG) Reduces Rotenone Effect on Stallion Sperm–Zona Pellucida Heterologous Binding

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### Contents

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

### Introduction

Spermatozoa require ATP to achieve normal sperm function and to fuel the motility. Mammalian sperm rely mainly on two metabolic pathways to produce ATP which are localized to different regions of the cell: oxidative phosphorylation (OXPHOS) occurs in mitochondria localized in the sperm mid-piece, while anaerobic glycolysis takes place mainly in the fibrous sheath of the flagellum where glycolytic enzymes are tightly anchored (Ferramosca and Zara 2014; Tourmente et al. 2015).

While human sperm rely mainly on glycolysis for ATP production, bull spermatozoa are characterized by both high respiration and glycolysis. On the other hand, stallion spermatozoa are highly dependent on OXPHOS for ATP production (Cummins 2009; Gibb et al. 2014). The great importance of sperm mitochondrial functionality in horse is confirmed by the observation that the most fertile stallion ejaculates exhibit the highest levels of OXPHOS activity (Gibb et al. 2014).

The inhibition of electron transport chain (ETC) along the respiratory complexes produces free radicals

that damage the functionality of the mitochondria and decrease the intracellular ATP content resulting in a decrease in stallion sperm motility (Gibb et al. 2014), even in the presence of glucose (Plaza Dávila et al. 2015).

One of the most active inhibitors of mitochondrial respiratory chain (MRC) is rotenone, a lipophilic isoflavonoid that inhibits complex I (NADH reductase) (Singer and Ramsay 1994). Rotenone reduces ATP production by mitochondria, leading to increased formation of free radicals besides a deregulation of cell homeostasis and ROS release into the mitochondrial matrix, where they can overwhelm the intramitochondrial antioxidant defence enzymes. This would account for the ability of rotenone to induce peroxidative damage in the mid-piece of the spermatozoa. The peroxidative damage, in turn, induces a progressive loss of motility in terms of the percentage of motile and progressive spermatozoa (Koppers et al. 2008). The presence of antioxidants, such as  $\alpha$ -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 an eliminator of free radical by reaction with hydrogen, alkoxyl or peroxy 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 (Guo et al. 1996; Skrzydlewska et al. 2002). Moreover, EGCG accumulates within the mitochondria and preserves 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 whether EGCG could counteract this effect.

## Material and Methods

### Experimental design

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Three ejaculates from each of three stallions of proven fertility were used. A control group (CTR) and fifteen different experimental groups for each ejaculate on the basis of the additions were considered: rotenone 100 nM (R100), rotenone 500 nM (R500), rotenone 5  $\mu$ M (R5), EGCG 10  $\mu$ M (E10), EGCG 20  $\mu$ M (E20), EGCG 60  $\mu$ M (E60), rotenone 100 nM + EGCG 10  $\mu$ M (R100 + E10), rotenone 100 nM + EGCG 20  $\mu$ M (R100 + E20), rotenone 100 nM + EGCG 60  $\mu$ M (R100 + E60), rotenone 500 nM + EGCG 10  $\mu$ M (R500 + E10), rotenone 500 nM + EGCG 20  $\mu$ M (R500 + E20), rotenone 500 nM + EGCG 60  $\mu$ M (R500 + E60), rotenone 5  $\mu$ M + EGCG 10  $\mu$ M (R5 + E10), rotenone 5  $\mu$ M + EGCG 20  $\mu$ M (R5 + E20) and rotenone 5  $\mu$ M + EGCG 60  $\mu$ M (R5 + E60).

The evaluation of viability, acrosome status and mitochondrial membrane potential was performed on fresh semen (CTR), and after 2 h of incubation in modified Tyrode's medium pH 7.4 (Rathi et al. 2001).

The heterologous binding assay was performed co-incubating for 1 h *in vitro*-matured porcine oocytes with semen previously pre-incubated for 1 h in the presence or absence of different concentrations of rotenone and EGCG.

### Semen collection and preparation

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

Semen was obtained from three different stallions of proven fertility (14, 15 and 18 years old) individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, from October to November 2013. Stallions jumped on a breeding phantom and ejaculates were collected with a Missouri artificial vagina equipped with a disposable liner and aniline filter (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately evaluated for volume and concentration (NucleoCounter SP-100; Chemometec, Allerød, Denmark), diluted 1 : 1 in Kenney's extender (Kenney et al. 1975) and sent to the laboratory within 1 h, maintained at 22°C.

Aliquots of the ejaculates were centrifuged twice for 2 min at 900  $\times$  g. The supernatants were removed and the pellets resuspended in modified Tyrode's solution (96 mM NaCl, 3.1 mM KCl, 2 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.4 mM

MgSO<sub>4</sub>·7H<sub>2</sub>, 0.3 mM KHPO<sub>4</sub>, 20 mM HEPES, 5 mM glucose, 21.7 mM Na lactate, 1 mM Na pyruvate, 15 mM NaH<sub>2</sub>CO<sub>3</sub>, 7 mg/ml BSA, 50  $\mu$ g/ml kanamycin) pH 7.4 (Rathi et al. 2001) to obtain 20  $\times$  10<sup>6</sup> spermatozoa/ml.

For the evaluation of viability, acrosome status and mitochondrial membrane potential, 500  $\mu$ l of semen suspensions was incubated for 2 h in Nunc 4-well multidish at 38°C in 95% humidity 5% CO<sub>2</sub> in the presence or absence of different concentrations of rotenone and EGCG.

### Viability assessment with SYBR-PI

Twenty-five microlitres of semen was incubated with 2  $\mu$ l of a 300  $\mu$ M solution of propidium iodide (PI) and 2  $\mu$ l of a 10  $\mu$ M solution of SYBR green-14, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA), for 5 min at 37°C in the dark. Aliquots of the stained suspensions were placed on clean microscope slides and carefully overlaid with coverslips, and at least 200 spermatozoa per sample were scored under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, the Netherlands). Spermatozoa stained with SYBR-14 and not stained with PI were considered as viable. Spermatozoa both SYBR-14+ and PI+ and those SYBR-14-/PI+ were considered with damaged membranes or dead.

### Evaluation of mitochondrial membrane potential

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

### Evaluation of acrosome status

Acrosome integrity was evaluated using a FITC-conjugated lectin from *Pisum sativum* (FITC-PSA) which label acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended in ethanol 95% and fixed/permeabilized at 4°C for at least 30 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5  $\mu$ g PSA-FITC/1 ml H<sub>2</sub>O) for 20 min in the dark. After staining, samples were washed in PBS and mounted with Vectashield mounting

medium with PI (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with a fluorescence microscope. The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absence of fluorescence was considered to indicate acrosome disruption or acrosome reaction.

### ***In vitro* maturation (IVM)**

Porcine cumulus–oocyte complexes (COCs) were aspirated using an 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, Roskilde, Denmark) prefilled with 2 ml of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 (Petters and Wells 1993) supplemented with 5.0 mg/ml insulin, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 50 mM  $\beta$ -mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500  $\mu$ l of the same medium per well and cultured at 39°C in 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, the medium was supplemented with 1.0 mM db-cAMP, 10 IU/ml, eCG (Folligon; Intervet, Boxmeer, the Netherlands) and 10 IU/ml hCG (Chorulon; Intervet). For the last 22 h, COCs were transferred to fresh maturation medium (Funahashi et al. 1997). At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting in maturation medium containing 0.4% hyaluronidase.

### **Heterologous binding assay**

For the binding assay, the semen was centrifuged twice for 2 min at 900  $\times g$  and resuspended in modified Tyrode's medium to obtain  $1 \times 10^6$  spermatozoa/ml, and 500  $\mu$ l of the sperm suspensions was pre-incubated for 1 h in the presence or absence of different concentrations of rotenone and EGCG. After oocyte maturation, 30–35 denuded oocytes were added in each well, and after 1 h of gamete co-incubation at 38°C in 95% humidity and 5% CO<sub>2</sub> in air, the oocytes were washed four times in PBS/0.4% BSA with a wide bore glass pipette to remove the spermatozoa loosely attached to zona pellucida. The oocytes were then fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated with 8.9  $\mu$ M Hoechst 33342 for 10 min in PBS/0.4% BSA in the dark, washed twice in the same medium, and individually placed in droplets of Vectashield (Vector Laboratories) on a slide and covered with a coverslip. The number of spermatozoa attached to the zona pellucida of each oocyte was assessed using the above-described microscope and was expressed as standard deviation units (see Statistical analysis).

### **Statistical analysis**

Statistical analysis was performed using R version 3.1.1 (R Core Team 2012).

Sperm analysis data are expressed as mean  $\pm$  SD. Significance was set at  $p < 0.05$ . Data were checked for normality using the Shapiro–Wilk test; differences between the treatments were analysed using an ANOVA test.

As for heterologous binding assay, data were standardized by dividing the number of bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed as standard deviation units. Data were analysed using a linear mixed-effect model. Significance was set at  $p < 0.05$ .

## **Results**

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

Rotenone treatment of stallion semen at all the concentrations tested (100 nM, 500 nM and 5  $\mu$ M) during a 2-h incubation in modified Tyrode's medium did not induce any significant difference on the percentage of viable cells, live sperm with active mitochondria and spermatozoa with intact acrosome (Fig. 1a–c). EGCG at all the concentrations tested (10, 20 and 60  $\mu$ M) did not exert any significant effect on the parameter analysed when supplemented either alone or in the presence of rotenone (Fig. 1a–c).

### **Heterologous binding assay**

To evaluate the effect of rotenone and EGCG on equine sperm capability to bind to swine ZP, denuded *in vitro*-matured porcine oocytes were co-incubated for 1 h with semen previously pre-incubated 1 h in the presence or absence of different concentrations of rotenone and EGCG (approximately 100 oocytes per treatment). The results are expressed as the number of sperm bound per oocyte normalized to the daily standard deviation (Fig. 2).

Rotenone at all the concentrations tested (100 nM, 500 nM and 5  $\mu$ M) induced a significant decrease in the number of sperm bound to the ZP compared with that for control.

EGCG at the concentrations of 10 and 20  $\mu$ M (but not of 60  $\mu$ M) induced a significant increase in the number of sperm bound to the ZP compared with that for control.

When stallion sperm were treated with rotenone 100 nM, the presence of EGCG at all the concentrations tested (10, 20 and 60  $\mu$ M) significantly increased the number of sperm bound to the ZP up to control levels. However, EGCG at the concentrations of 20 and 60  $\mu$ M did not significantly increase the number of sperm bound to the ZP compared with R 100.

EGCG at all the concentrations tested (10, 20 and 60  $\mu$ M) did not induce any increase in the number of spermatozoa bound when added in the presence of the higher concentration of rotenone (500 nM, 5  $\mu$ M).

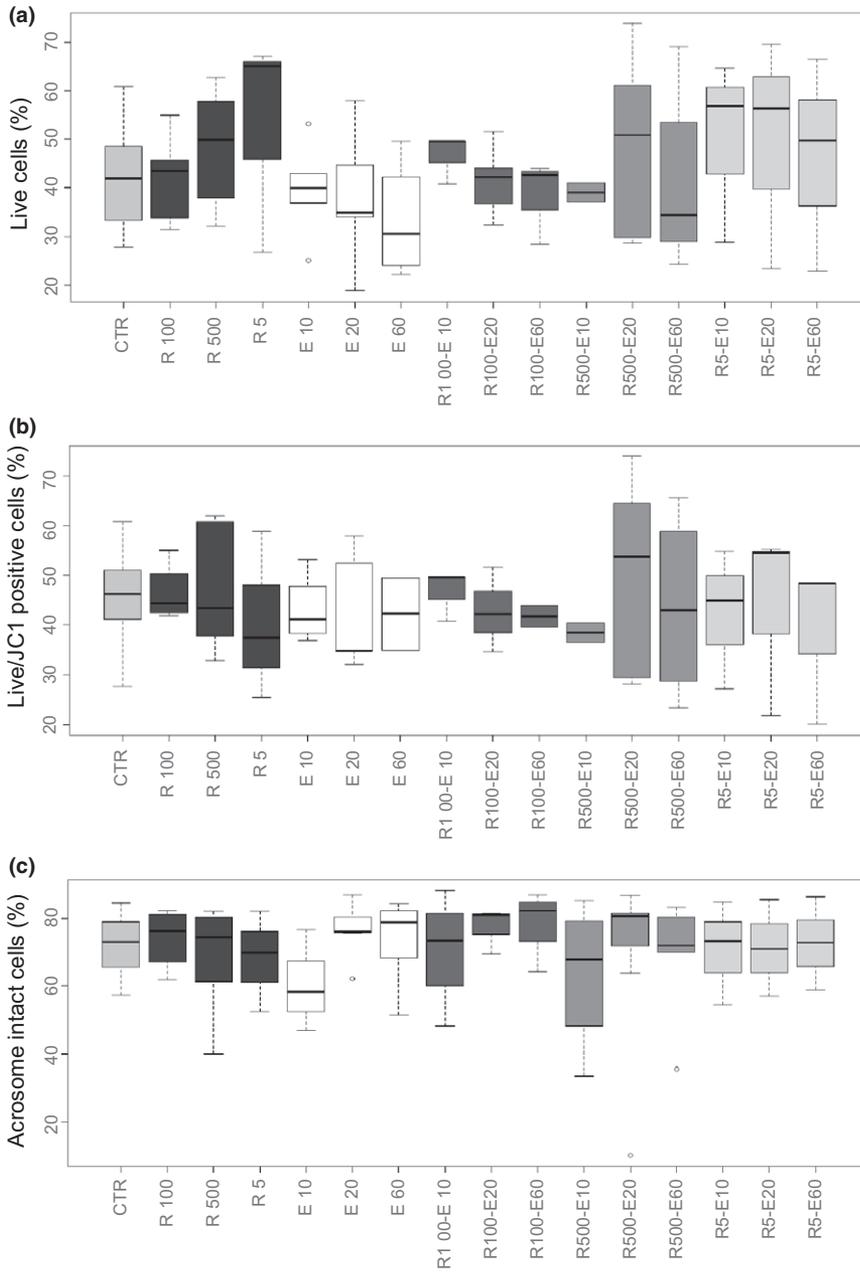


Fig. 1. Viability (a), mitochondrial membrane potential (b) and acrosome status (c) of stallion spermatozoa after 2-h incubation under capacitating condition in the presence of rotenone and/or EGCG. R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5  $\mu$ M; E10, EGCG 10  $\mu$ M; E20, EGCG 20  $\mu$ M; E60, EGCG 60  $\mu$ M

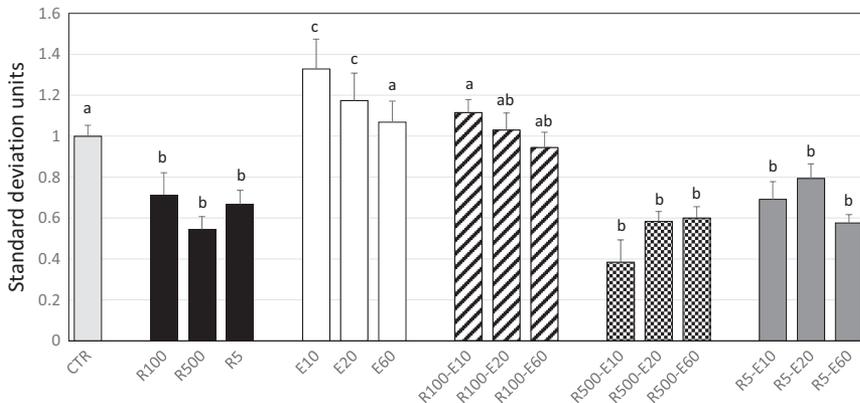


Fig. 2. Effect of rotenone and/or EGCG on heterologous zona pellucida binding ability of stallion sperm. Data were standardized by dividing the number of bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed as standard deviation units. R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5  $\mu$ M; E10, EGCG 10  $\mu$ M; E20, EGCG 20  $\mu$ M; E60, EGCG 60  $\mu$ M. Different letters on the bars indicate a significant difference

## Discussion

The aim of our study was to evaluate the response of equine sperm after inhibiting mitochondrial complex I by rotenone during *in vitro* capacitation for 2 h and to test whether EGCG, a natural polyphenol component of green tea, could counteract the effect of rotenone.

The evaluation of stallion sperm viability, acrosomal membrane integrity and mitochondrial activity did not evidence any significant effect of rotenone at all the concentrations tested (100 nM, 500 nM and 5  $\mu$ M). The absence of significant differences on the percentage of viable stallion sperm agrees well with the data obtained by Gibb et al. (2014) and Plaza Dávila et al. (2015) who observed a sperm viability similar to that in control even using a higher rotenone concentration (10  $\mu$ M) for 1 h; only after 3 h of incubation, rotenone at the concentration of 10  $\mu$ M induced a significant reduction in the percentage of intact sperm (Plaza Dávila et al. 2015). In contrast to the results of those authors, we did not observe any significant decrease in the percentage of live spermatozoa with high mitochondrial membrane potential. This discrepancy could be due to different reasons: Gibb et al. (2014) and Plaza Dávila et al. (2015) evaluated JC-1 positivity by flow cytometry, while we used fluorescence microscopy possibly overestimating JC-1-positive cells classifying as JC-1 positive also those cells with only partial or spot-like JC-1-positive mitochondria. A further explanation could be the lower rotenone concentrations used in our work and the different conditions of the incubation with rotenone: capacitation in our study and non-capacitation in Gibb et al. (2014) and Plaza Dávila et al. (2015) studies.

To evaluate the effect of rotenone and EGCG on the *in vitro* function of equine spermatozoa, an heterologous binding assay was performed co-incubating denuded IVM porcine oocytes for 1 h with semen previously pre-incubated 1 h in the presence or absence of different concentrations of rotenone and EGCG. It has been demonstrated that sperm–oocyte binding assays offer a good reliability in the prediction of horse *in vivo* fertility (Fazeli et al. 1993, 1995; Meyers et al. 1996). Due to the low availability of equine oocytes, in our study a heterologous binding assay was performed as the efficiency/reliability of using bovine or swine oocytes has been demonstrated (Sinowatz et al. 2003; Clulow et al. 2010; Balao da Silva et al. 2013). As in the case of the homologous assay, the process of capacitation is needed for stallion spermatozoa to bind to heterologous oocytes (Clulow et al. 2010).

The results obtained in this study demonstrate for the first time that inhibition of complex I of MRC of stallion sperm with rotenone exerts a negative effect on ZP binding ability. In fact, rotenone at all the concentrations tested (100 nM, 500 nM and 5  $\mu$ M) significantly decreased the number of sperm bound per oocyte in comparison with control group.

When stallion spermatozoa were treated under capacitating condition with 10 and 20  $\mu$ M EGCG, stallion sperm–ZP binding activity was improved compared with control semen. A positive influence of EGCG addition on both fresh and frozen–thawed spermatozoa during IVF on ZP binding and oocyte penetration was already recorded in pig (Spinaci et al. 2008; Kaedei et al. 2012), suggesting a modulating action of this polyphenol on sperm capacitation. This effect could be exerted thanks to the antioxidant ability of EGCG that can act on the balance between excessive ROS production, which overwhelms the limited capacity of these cells to protect themselves from oxidative stress, and mild intracellular ROS generation, which stimulates intracellular cAMP generation, inhibits tyrosine phosphatase activity and enhances the formation of oxysterols, thus inducing a physiological capacitation (Aitken et al. 2015).

EGCG at 10  $\mu$ M concentration significantly blunted the negative effect on stallion sperm–ZP binding activity of rotenone at the lower dose tested (100 nM). EGCG at the higher doses tested (20, 60  $\mu$ M), even if it was not able to completely reverse the inhibitory effect of rotenone 100 nM, increased the number of sperm bound to ZP up to the levels of the control group. However, EGCG was not able to reduce the negative effect on heterologous binding induced by higher concentration of rotenone (500 nM and 5  $\mu$ M).

Our results agree with the ability of epicatechin and EGCG (but not of other flavonoids such as genistein and baicalin) demonstrated by Kamalden et al. (2012) in protecting a transformed cell line (RGC-5 cells) from rotenone-induced toxicity. This positive effect, as suggested by the authors, could be mainly, but not exclusively, attributed to the antioxidant activity of these flavonoids. The ability of EGCG to counteract mitochondrial energy deficit due to impaired activities of complex I has been demonstrated by Valenti et al. (2013) in cultured fibroblasts and lymphoblasts from subjects with Down's syndrome. This effect was associated with EGCG-induced promotion of cAMP and PKA-dependent phosphorylation of complex I.

Rotenone inhibits oxidative glycolysis and ATP production in stallion spermatozoa inducing a reduction in sperm motility parameters (Plaza Dávila et al. 2015). It could be hypothesized that EGCG, counteracting rotenone-induced deficit in mitochondrial ATP synthesis, may ensure under capacitating conditions the adequate energy supply. In this way, the spermatozoa can sustain changes occurring during capacitation, such as hyperactivated motility and protein phosphorylation (Ferramosca and Zara 2014).

In conclusion, the inhibition of complex I by rotenone results in a decreased ZP binding ability of stallion spermatozoa, and the presence of EGCG is able to reduce the toxicity of rotenone at the lower dose (100 nM). Moreover, spermatozoa treated with EGCG attach better than non-treated ones, suggesting that they have a more advanced capacitation-like status.

## Conflict of interest

None of the authors have conflict of interest to declare.

## Author contributions

All the authors contributed to the research.

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