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

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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

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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 if epigallocatechin-3-gallate (EGCG), a natural polyphenol component of green tea, could counteract this effect. After 2 h incubation of stallion spermatozoa in modified Tyrodes medium, rotenone (100 nM, 500 nM, 5 µM) and EGCG (10 µM, 20 µM, 60 µM), alone or in association, did not induced 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 µM 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 ECGC at all the concentration tested (10 µM,20 µM, 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.

Keywords: horse, spermatozoa, mitochondria, oxidative phosphorylation.

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 (Feramosca 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 damages the functionality of the mitochondria, decreases the intracellular ATP content resulting in a decrease in stallion sperm motility (Gibb et al, 2014), even in 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 intra-mitochondrial antioxidant defense enzymes. This would account for the ability of rotenone to induce peroxidative damage in the midpiece 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 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

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Milan, Italy). Three ejaculates from each of three stallions of proven fertility were used. Fifteen different experimental groups for each ejaculate in the basis of the additions were considered: control group (CTR), rotenone 100 nM (R100), rotenone 500 nM (R500), rotenone 5 µM (R5), EGCG 10 µM (E10), EGCG 20 µM (E20), EGCG 60 µM (E60), rotenone 100 nM + EGCG 10µM (R100+E10), rotenone 100 nM + EGCG 20µM (R100+E20), rotenone 100 nM + EGCG 60µM (R100+E60), rotenone 500 nM + EGCG 10µM (R500+E10), rotenone 500 nM + EGCG 20µM (R500+E20), rotenone 500 nM + EGCG 60µM (R500+E60), rotenone 5 µM + EGCG 10µM (R5+E10), rotenone 5 µM + EGCG 20µM (R5+E20), rotenone 5 µM + EGCG 60µM (R5+E60).

The evaluation of viability, acrosome status and mitochondrial membrane potential were performed on fresh semen (CTR), and after 2 h of incubation in modified Tyrodes 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 1h in 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 3 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 (NucleoCounterSP 100, Chemometec, Denmark), diluted 1:1 in Kenney's extender (Kenney et al., 1975) and sent to the laboratory within 1 h, maintained at 22ºC.

Aliquots of the ejaculates were centrifuged twice for 2 min at 900 × g. The supernatants were removed and the pellets resuspended in modified Tyrodes solution (96 mM NaCl, 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 HEPES, 5 mM glucose, 21.7mM NaLactate, 1 mM Na Pyruvate, 15 mM NaH_2CO_3, 7 mg/mL BSA, 50 µg/mL Kanamicin) pH 7.4 (Rathi et al., 2001) to obtain $20x10^6$ spermatozoa/mL.

For the evaluation of viability, acrosome status and mitochondrial membrane potential 500µL of semen suspensions were incubated for 2 h in Nunc 4-well multidish at 38°C in 95% humidity 5% CO2 in presence or absence of different concentrations of Rotenone and EGCG.

Viability assessment with SYBR-PI

Twenty-five microliters of semen were incubated with 2 µL of a 300 µM solution of propidium iodide (PI) and 2 µL of a 10 µM 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, carefully overlaid with coverslips, and at least 200 spermatozoa per sample were scored under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, 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 (25μL) of semen was incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution, 2 μL of a 10 μM SYBR green-14 stock solution and 2 μL of a 150 μM 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanineiodide (JC-1) solution for 20 min at 37°C in the dark. Ten microliters of the sperm suspension were 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 by 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 4ºC for at least 30 min. Samples were dried in heated slides and incubated with FIC-PSA solution (5 μg PSA-FITC/1 mL H2O) for 20 min in darkness. 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,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/mLinsulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 mM ß-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL of the same medium per well and cultured at 39 ºC in a humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL, eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, IntervetBoxmeer, The Netherlands). 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 × g, resuspended in modified Tyrodes medium to obtain $1{\times}10^6$ spermatozoa /ml and 500 µL of the sperm suspensions were preincubated for 1 h in 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% CO2 in air the oocytes were washed four times in PBS 0.4% BSA with a wide bore glass pipette in order to remove the spermatozoa loosely attacked to zona pellucida. The oocytes were then fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated with 8.9 µM 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, Burlingame, CA, USA) on a slide, and covered with a coverslip. The number of spermatozoa attached to the zona pellucida of each oocyte was assessed by 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 Computing, 2014).

Sperm analysis data are expressed as mean ± SD. Significance was set for $p < 0.05$. Data were checked for normality with the Shapiro-Wilk test; differences between treatments were analyzed by 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 analyzed by 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, 5 µM) during a 2 h in modified Tyrodes medium did not induced any significant difference on the percentage of viable cells, live sperm with active mitochondria and spermatozoa with intact acrosome (Fig. 1 A,B,C). EGCG at all the concentrations tested (10 µM, 20 µM, 60 µM) did not exert any significant effect on the parameter analyzed when supplemented either alone or in presence of rotenone (Fig. 1 A,B,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 1h with semen previously pre-incubated 1h in presence or absence of different concentrations of Rotenone and EGCG (around 100 oocytes per treatment). The results are expressed as number of sperm bound per oocyte normalized to the daily standard deviation (Figure 2). Rotenone at all the concentrations tested (100 nM, 500 nM, 5 µM) induced a significant decrease in the number of sperm bound to the ZP compared with that for control. EGCG at the concentrations of 10 µM and 20 µM (butnot of 60 µM) 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 ECGC at all the concentration tested (10 µM,20 µM, 60 µM) significantly increased the number of sperm bound to the ZP up to control levels. However, ECGC at concentration of 20 and 60 µM did not significantly increase the number of sperm bound to the ZP compared with R 100. EGCG at all the concentrations tested (10 µM, 20 µM, 60 µM) did not induce any increase in the number of spermatozoa bound when added in presence of the higher concentration of rotenone (500 nM, 5 µM).

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 two hours and to test if 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, 5µM). The absence of significant differences on the percentage of viable stallion sperm agree well with the data obtained by Gibb at al. (2014) and Plaza Dávila et al., (2015) who observed a sperm viability similar to control even using a higher rotenone concentration (10µM) for 1h; only after 3h of incubation, rotenone 10µM induced a significant reduction of the percentage of intact sperm (Plaza Dávila et al., 2015). In contrast with the results of those authors we did not observe a 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 JC1 positivity by flow cytometry while we used fluorescence microscopy possibly overestimating JC1 positive cells classifying as JC1 positive also those cells with only partial or spot like JC1 positive mitochondria. A further explanation could be the lower rotenone concentrations used in our work and the different conditions of the incubation with rotenone: capacitating in our study and non capacitating 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 1h with semen previously pre-incubated 1h in 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; Fazeli et al., 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; Balao da Silva et al., 2013; Clulow et al., 2010). 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 tested concentrations (100 nM, 500 nM, 5µM) significantly decreased the number of bound sperm per oocyte in comparison with control group.

When stallion spermatozoa were treated under capacitating condition with 10 µM and 20 µM EGCG stallion 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, that 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).

ECGC at 10 µM concentration significantly blunt the negative effect on stallion ZP-binding activity of rotenone at the lower dose tested (100 nM). EGCG at the higher doses tested (20 µM, 60 µM), 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 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 µM).

Our results agree with the ability of epicatechin and ECGC (but not of other flavonoids such as gebistein and baicain) 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 ECGC 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 Down's syndrome subjects. 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 of 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 (Feramosca and Zara, 2014).

In conclusion, the inhibition of complex I by rotenone results in a decreased ZB-binding ability of stallion spermatozoa and the presence 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

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Figure 1. Viability (A), mitochondrial membrane potential (B) and acrosome status (C) of stallion spermatozoa after 2 h incubation in capacitating condition in presence of rotenone and/or EGCG.

R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 µM; E10, EGCG 10 µM; E20, EGCG 20 µM; E60, EGCG 60 µM.

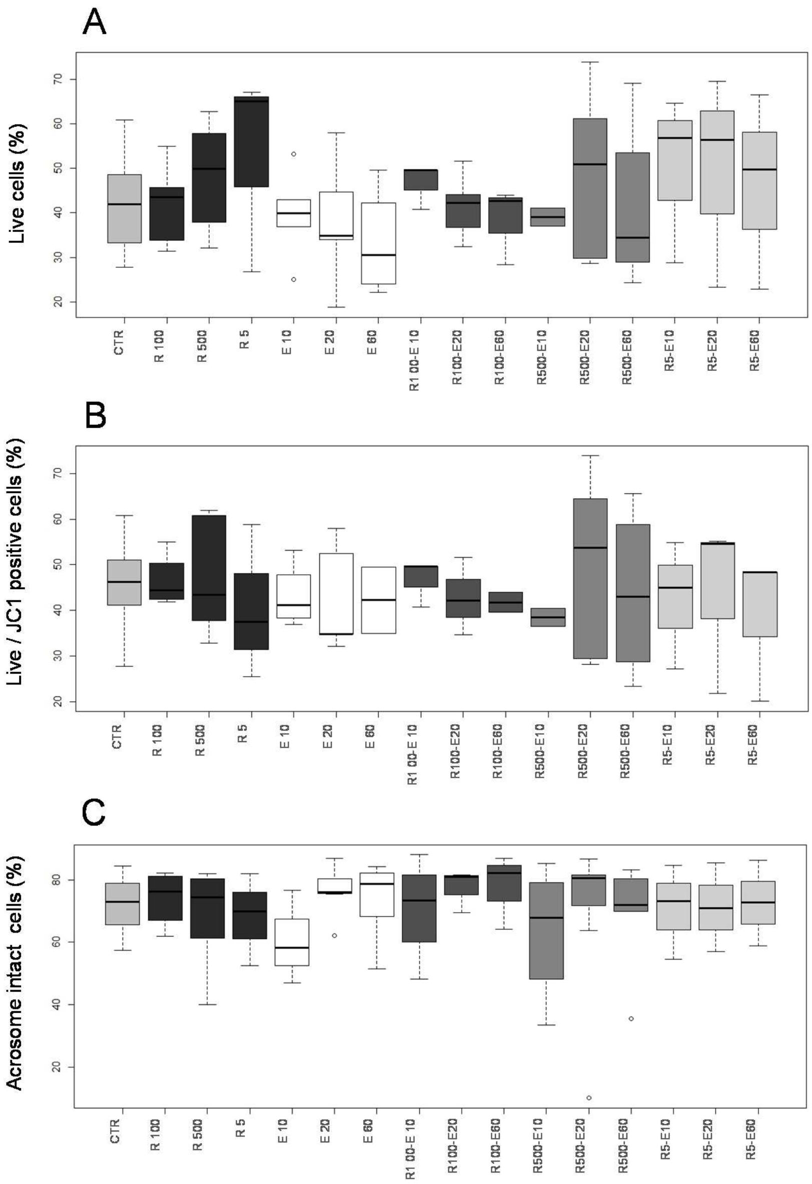

Figure 2. Effect of rotenone and/or EGCG on stallion heterologous binding ability. Data were standardized by dividing the number of bound spermatozoa/oocyte by the daily standard deviation, and are therefore expressed as standard deviation units.

R100, rotenone 100 nM; R500, rotenone 500 nM; R5, rotenone 5 µM; E10, EGCG 10 µM; E20, EGCG 20 µM; E60, EGCG 60 µM.

Different letters on the bars indicate a significant difference.