Epigallocatechin-3-gallate added after thawing to frozen dog semen: Effect on sperm parameters and ability to bind to oocytes' zona pellucida

EPIGALLOCATECHIN-3-GALLATE ADDED AFTER THAWING TO FROZEN DOG SEMEN: EFFECT ON SPERM PARAMETERS AND ABILITY TO BIND TO OOCYTES' ZONA PELLUCIDA

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Abstract

Dog sperm cryopreservation is gaining importance both in breeding dogs for commercial purposes and for pet animals. Anyway, cryopreservation of mammalian spermatozoa, including dog ones, induces some negative effect on sperm fertility, leading to a lower use of this technique and limiting its widespread use. Therefore, studies to improve the quality of canine semen after cryopreservation could have a relevant impact on both the scientific advancement and the clinical practice.

The aim of the present work was to investigate the putative ameliorative effect of Epigallochatechin-3-gallate (EGCG) addition to post thawing medium on dog sperm motility, mitochondrial activity, acrosome integrity and on zona-binding ability (zona binding assay).

Spermatozoa were thawed in Tris-fructose-citrate medium supplemented with EGCG (0, 25 and 50 μM) and sperm motility, mitochondrial activity and acrosome integrity were assayed at 0.5, 1.5, 3 and 6 h after post thawing incubation at 37°C. An aliquot of semen from each treatment group after 1.5 h post thawing incubation was washed and used to perform heterologous (using porcine oocytes) or homologous zona binding assay.

The results obtained showed that no significant effect is exerted by EGCG on sperm parameters analysed neither at 0.5, 1.5, 3 or 6 h after thawing excepting for the reduction of the percentage of live cells with active mitochondria at the higher dose at 6 h; furthermore, both homologous or heterologous zona binding ability, was not influenced by EGCG.

In conclusion, EGCG supplementation to thawing medium does not improve dog sperm quality or zona binding capacity.

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1. Introduction

Sperm cryopreservation importance is increasing in the last decades in various mammalian species, as it permits preservation of male gametes for a mid to long term and their use in assisted reproduction techniques. In dog assisted reproduction the use of artificial insemination is mainly related to breeds or subjects with objective difficulty to breed or for trading purposes [1]; semen freezing permits the increase in dog semen international trade volume, the preservation of male gametes of valuable sires and to the organization of plans for endangered wild canine species [1].

Implementing cryopreserved spermatozoa quality is one of the main goal in many species such as porcine [2] and equine [3]. In those species, several attempts were made to avoid detrimental effects of cryopreservation (reviewed by Yeste 2016), that can affect membrane integrity, mitochondrial function, DNA integrity and motility. In dog some different approaches aimed at better define and improve the outcome of the freezing process, as reviewed by Peña et al. (2006): extenders, mainly based on Tris-glucose or Tris-fructose, added with egg yolk and glycerol are commonly used, and different protocols are applied to canine sperm [1]. Anyway, dog semen quality, as that from other mammalians, decreases after freezing [5].

Several substances were used to increase the quality of cryopreserved sperm cells in canine species to mitigate the adverse effect of cryopreservation: ascorbic acid and glutathione, alone or in association, were added to the freezing medium and exerted a positive effect on post thawing sperm longevity and viability [6]. In addition, a more recent study showed positive effects of glutathione on sperm quality and fertility when added to the freezing medium [7]. Vitamin C, N-acetyl-L-cystein, taurine, catalase, vitamin E and vitamin B12 were added to the freezing

extender separately [5]; that study demonstrated that antioxidant exerted positive effects on dog semen but the most effective one was catalase. A recent study involved spermine supplementation of freezing medium [8] giving encouraging results at the 5 mM dose. These studies demonstrate that cryopreserved canine sperm quality could be enhanced using some antioxidants and many others could be profitably applied to this technique.

Green tea extracts, for example, were used in dog sperm liquid storage at 5°C [9,10] alone or in association with vitamin C, showing some positive effects on motility after a prolonged storage.

Recently in our laboratories we performed some studies regarding the effect of supplementation of thawing extender with Epigallocatechin-3-gallate (EGCG) and resveratrol [11,12] in porcine species, obtaining promising results on IVF trials.

EGCG is the main polyphenol in green tea (*Camellia sinensis*) and it possess a high level of antioxidant activity exerted by removing free radical and by enhancing catalase activity [13].

Basing on the previous results on different species, the addition of molecules to the thawing medium could be interesting in enhancing the metabolism and resistance of sperm cells surviving the cryopreservation process.

The aims of the present study were to evaluate the effects of EGCG when added to thawing medium on canine sperm quality and zona binding ability.

2. Materials and methods

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

2.1. Semen collection, evaluation and freezing

Semen from 3 fertile dogs was collected and frozen as described below. In all cases, dogs were referred to Department of Veterinary Medical Sciences of Alma Mater Studiorum – University of Bologna for sperm evaluation and informed consent of the owner was taken. An informed consent of the owner of bitches subjected to ovariectomy/ovariohysterectomy of was also taken.

The three fractions of the ejaculates were collected in calibrated plastic vials by digital manipulation. The sperm-rich fraction was evaluated before freezing for volume, concentration, and rate of morphologically normal spermatozoa. The volume was measured by a calibrated micropipette, and sperm concentration was determined with a Bürker chamber, after dilution of the sperm suspension 1:40 with 10% formol buffered saline to immobilize spermatozoa and counted using a phase contrast microscope (400x; Axiolab; Zeiss, Italy) equipped with a warming plate (37°C; Thermo Plate; Tokai Hit, Japan) as reported in [14]. Percentages of morphologically normal spermatozoa were determined at the same microscope (1000x) after dilution of semen 1:1 with 10% formol buffered saline, and at least 200 spermatozoa per sample were examined.

For the study only samples with total motility (TM) ≥ 90%, rate of morphologically normal spermatozoa ≥ 90% were used.

2.1.1. Extenders and freezing procedure

After centrifugation at 300 g for 10 min [14], performed to concentrate sperm, the supernatant was removed and sperm pellets were resuspended in two steps in freezing extenders reaching a final concentration of 200×10^6 spermatozoa/mL. Freezing extenders for the first step was Tris-glucose-citrate (TGC) (2.4% wt/vol

Tris, 1.4% wt/vol citric acid, 0.8% wt/vol glucose, 0.06% wt/vol sodium benzylpenicillin, 0.1% wt/vol streptomycin sulfate in distilled water) supplemented with 20% (v:v) egg yolk, 3% (v:v) glycerol (EYTGC1), whereas for the second step TGC was supplemented with 20% (v:v) egg yolk, 7% (v:v) glycerol, and 1% (v:v) Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA; EYTGC2).

In particular, first the spermatozoa were diluted slowly at room temperature (20°C–25°C) by dropwise addition of a volume of EYTGC1 to reach 400 × 10^6 spermatozoa/mL. After that, sperm suspension was cooled to 4°C for 60 min in a controlled temperature refrigerator (about −0.3°C/min). Then the second dilution with a volume of precooled EYTGC2 to reach 200 × 10^6 spermatozoa/mL was made at 4°C. After an additional equilibration period of 15 minutes at 4°C, the samples were loaded in plastic straws (0.5 mL) and the straws were heat sealed. Sperm suspensions were frozen on liquid nitrogen vapors into a polystyrene box (36 × 33 × 30 cm) keeping straws at 7 cm from liquid nitrogen for 10 minutes, before plunging straws directly into liquid nitrogen. Finally, the straws were transferred into a Dewar flask at −196°C.

2.2. Experimental design

Two straws (0.5 mL/straw) were thawed for 30 sec in water bath at 37°C and immediately diluted, at the same temperature, with 2.33 mL of thawing medium (Tris-glucose-citrate, TGC). The sample was immediately divided in three experimental groups (1.0 mL each): CTR (control: without antioxidant addition), EGCG25 (addition of 25μM EGCG to TGC extender) and EGCG50 (50 μM EGCG). EGCG concentrations used in this study were chosen on the basis of previous researches of our group [11,12].

153 Samples from two dogs (2 ejaculates/dog) were used to assess sperm viability and
154 mitochondrial activity, acrosome integrity and motility in CTR, EGCG25 and
155 EGCG50 groups after 0.5, 1.5, 3 and 6 h post thawing incubation at 37°C.
156 Samples from three dogs (2 ejaculates/dog) were used for zona binding assays:
157 aliquots of semen after 1.5 h post thawing incubation at 37°C in absence or
158 presence of EGCG (25μM or 50 μM), were washed and used for heterologous or
159 homologous zona binding assay as below described.
160

161 ### *2.2.* Post-thaw sperm evaluation

162 2.3.1. Acrosome integrity assessment
163 Acrosome integrity was measured with a FITC conjugated lectin from Pisum
164 Sativum (FITC-PSA) which labelled acrosomal matrix glycoproteins. Spermatozoa
165 were washed twice in PBS, resuspended with ethanol 95% and fixed at 4°C for 30
166 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5
167 μg PSA-FITC/1 mL H2O) for 20 min in darkness. After staining slides were washed
168 in PBS and mounted with Vectashield mounting medium with propidium iodide
169 (PI) counterstain for DNA (Vector Laboratories, Burlingame, CA, USA). The slides
170 were then observed using a Nikon Eclipse E 600 epifluorescence microscope (Nikon
171 Europe BV, Badhoeverdop, The Netherlands) and at least 200 spermatozoa per
172 sample were scored. The presence of a green acrosomal fluorescence was
173 considered indicative of an intact acrosome, while a partial or total absence of
174 fluorescence was considered to indicate acrosome disruption or acrosome reaction
175 [14].
176

177 2.3.2. Mitochondrial activity and membrane integrity evaluation

For each sample, an aliquot (25 µL) of sperm suspension (30 x 10^6 spermatozoa/mL) 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, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc.) and 2 µL of a 150 µM JC-1 solution for 20 min at 37°C in the dark. Ten µL of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using the above described fluorescence microscope. Spermatozoa stained with SYBR-14 but not with PI were considered as viable (SYBR-14+/PI-), whereas SYBR-14+/PI+ and SYBR-14-/PI+ spermatozoa were considered as non-viable. JC-1 monomers emit green fluorescence in mitochondria with low membrane potential (JC-1-) and form aggregates in mitochondria with high membrane potential (JC-1+), then emitting a bright red-orange fluorescence. Therefore, those viable sperm showing orange-red fluorescence in the mid piece (JC-1+) were considered as viable sperm with high mitochondrial membrane potential (SYBR-14+/PI-/JC-1+) [15].

The results are focused on JC1 positive and negative living cells.

2.3.3. Sperm motility

Motility was measured by a computer-assisted sperm analysis system, using the open source Image J BGM plugin as described by Giaretta et al. [15]. Briefly, a Leitz diaplan microscope (Wild Leitz GmbH, D6330, Wetzlar, Germany) with a 10x plan objective with negative phase-contrast was used. The microscope was equipped with a Z31A Ascon technologic heated stage (Ascon technologic, PV – IT). The video camera, 3.1-megapixel CMOS USB 2.0 Infinity1-3 Camera (Lumenera corporation, Ottawa, ON, Canada), was coupled to the microscope by a c-mount adapter and videos were registered for three seconds at a resolution of 800x600 pixel and 60

frames/sec (fps). Images were recorded on a hard drive using the Infinity analysing and capture software 6.4 (Lumenera corporation) and converted to avi format for subsequent analysis using the BGM Image J plugin.

Prior to any observation, spermatozoa ($30x10^6$ sperm/mL) were loaded onto a fixed height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of separate fields and lasting three seconds each were recorded per sperm sample. Sperm motility endpoints assessed were: percent of total motile spermatozoa (TM), percent of progressive spermatozoa (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The setting parameters of the program were the followings (taken from the Hamilton-Thorne IVOS-12 CASA system): frames per second 60, number of frames 45, threshold path minimum VSL for motile sperm 9µm/sec; min VAP for motile 20 µm/sec; min VCL for motile 25 µm/sec; VAP cutoff for Progressive cells 60 µm/sec; STR cutoff for Progressive cells 40%.

2.3. **Zona Pellucida Binding Assays** (ZBA)

2.4.1. Heterologous ZBA

Porcine oocytes maturation was performed as reported by [16]. Briefly, ovaries were obtained from pre-pubertal gilts at a local abattoir and transported to the laboratory within 1 h. Cumulus–oocyte complexes (COCs) were aspirated from 4 to 6 mm follicles using a 18 gauge needle attached to a 10 mL disposable syringe. 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 supplemented with

228 5.0 µg/mL insulin, 1.0 mM glutamine, 0.57 mM cysteine, 10 ng/mL epidermal
229 growth factor (EGF), 50 µM ß-mercaptoethanol and 10% porcine follicular fluid
230 (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish
231 containing 500 µL of the same medium per well and cultured at 39 ◦C in a
232 humidified atmosphere of 5% CO2 in air. For the first 22 h of in vitro maturation
233 the medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL, eCG (Folligon,
234 Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, Intervet). For
235 the last 22 h COCs were transferred to fresh maturation medium.
236 Heterologous zona binding assay was conducted as described by [17]. Briefly,
237 semen thawed in presence or absence of EGCG (25µM or 50 µM) was kept for 1 h
238 incubation at 37°C; subsequently spermatozoa were washed twice with canine
239 capacitation medium (CCM) and incubated ($1x10^6$ sperm/mL) for 1.5 h at 37°C,
240 5% CO_2 [18] with slight modification with 20-25 matured denuded oocytes. After
241 co-incubation the oocytes were washed three times in PBS 0.4% BSA with a wide
242 bore glass pipette, fixed in 4% paraformaldehyde for 15 min at room temperature
243 in the dark and stained with 8,9 µM Hoechst 33342. Cells were washed twice in
244 PBS, and individually placed in droplets of Vectashield (Vector Laboratories,
245 Burlingame, CA, USA) on a slide, and covered with a coverslip. The number of
246 spermatozoa attached to each oocyte was assessed by using the above described
247 microscope and was expressed as mean number of spermatozoa per oocyte.
248 A total number of 241 oocytes (79 for CTR; 83 for EGCG25 ;79 for EGCG50) were
249 used in five repetitions
250
251 2.4.2. Homologous ZBA

252 Ovaries were obtained from healthy crossbred bitches, subjected to
253 ovariectomy/ovariohysterectomy at different ages and at various stages of the
254 oestrus cycle. The ovaries were washed and frozen in physiological saline and then
255 stored at -80°C until use. On each experimental day, 4-6 ovaries (randomly chosen)
256 were used. After thawing at room temperature, the ovaries were sliced in a Petri
257 dish with PBS containing 0.4 % BSA, under stereomicroscope. The oocytes
258 recovered were incubated for two hours in PBS at room temperature and then
259 mechanically denuded by using a finely drawn pipette. The denuded oocytes were
260 washed with CCM medium and divided in three groups of 20-25 oocytes per group
261 in 300 µl of CCM medium. To each group spermatozoa ($1x10^6$/mL) thawed in
262 presence or absence of EGCG (same treatments of heterologous ZBA) were added
263 and co-incubated for 1.5 h at 37°C and 5% CO_2.[19] Then the oocytes were treated
264 as in heterologous ZBA. A total number of 201 oocytes (69 for CTR; 65 for EGCG25;
265 67 for EGCG50) were used in four repetitions.
266
267 **3. Statistical analysis**
268 Data were analysed using R version 3.4.0 (2017-04-21) (Copyright © 2017, The R
269 Foundation for Statistical Computing) and significance was set at $p<0.05$ unless
270 otherwise specified.
271 Results are expressed as mean ± standard deviation for descriptive statistics. Data
272 were analysed by a mixed effect model setting the treatment and time as fixed
273 effects and the single repetition as random effect. The Tukey Honest Significant
274 Difference test was applied when due.

As for the quantification of the effect of EGCG on zona binding assay, a mixed effect model with Poisson distribution was set up setting the treatment as a fixed effect and the repetition as a random effect.

4. Results

Mean data from the evaluation of sperm rich fraction of two ejaculates from three mixed-breed dogs after collection were volume, 1.17 ± 0.53 mL; concentration, 935 ± 201 x 10^6 spermatozoa/mL; rate of morphologically normal spermatozoa 93.83 ± 1.83%.

The percentage of live cells with active or non-active mitochondria and sperm acrosome integrity after thawing and incubation with different doses of EGCG is presented in Table 1. A time dependent decrease in the percentage of live spermatozoa with active mitochondria was evident after 6 h incubation in all groups ($p<0.05$), being more evident in the EGCG treated ones, in fact a significant difference ($p<0.05$) between control and EGCG50 groups at 6 h was recorded. Concomitantly, the percentage of living JC1 negative cells showed a time-dependent increase, even if not significant. The only significant difference registered is present between time 0.5 h and 6 h in the EGCG treated groups (Table 1).

Regarding acrosome intact cells, a time dependent reduction in all groups was evident: starting from 3 h incubation the percentage of acrosome intact cells decreased in all groups compared to 0.5 h.

Motility parameters are presented in Table 2 and supplementary file 1. Incubation time exerted a negative effect on sperm motility (total and progressive) at 6 h

299 incubation, significant ($p<0.05$) in the EGCG50 group. The other kinematic
300 parameters were slightly influenced (not significantly) by time and treatments.
301 Data on zona binding assay clearly showed that EGCG supplementation to thawing
302 medium does not exert nor positive nor negative effects on binding capacity of dog
303 sperm independently from the kind of assay (heterologous or homologous zona
304 binding assay) (Fig. 1 and 2). The mean numbers of bound sperm (mean ± SD)
305 recorded for heterologous zona binding assays were: 3.4±3.7 for CTR (79 oocytes),
306 4.5±4.6 for EGCG25 (83 oocytes) and 4.2±5.0 for EGCG 50 (79 oocytes) groups
307 respectively, while for homologous zona binding assays were: 9.4±8.9 for CTR (69
308 oocytes); 8.8±8.6 for EGCG25 (65 oocytes); 9.6±8.1 for EGCG50 (67 oocytes).
309

310 **5. Discussion**

311 Cryopreservation of canine spermatozoa is covering an emergent part of research
312 on assisted reproduction techniques in this species. Several studies in canine and
313 other species showed a possible detrimental role of Reactive Oxygen Species (ROS)
314 during and after sperm cryopreservation [20,21]. The addition of antioxidant before
315 freezing has represented and still represent one of the most promising technique
316 for improving post-thaw sperm parameters, as reported by various Authors [1,5–
317 7]. In recent works from our laboratories [11,12] we showed that it is possible to
318 achieve good results in term of pig semen fertility *in vitro* by adding EGCG into the
319 thawing medium and incubating sperm cells for one hour at 37°C.
320 The same protocol was applied in this experimental work, with some modifications:
321 in fact, we thawed canine spermatozoa with or without addition of 25 and 50 μM
322 EGCG and analysed sperm quality at different time points: 0.5, 1.5, 3 h and 6 h.
323 All sperm parameters analysed (acrosome integrity, mitochondrial activity and

motility parameters) were not significantly influenced by the addition of EGCG at both concentrations, excepting live cells with active mitochondria percentage , that, after 6 h, was negatively affected in EGCG50 group. In the previous works on boar semen [11,12] we did not register any change on viability or acrosome integrity after 1 h incubation with EGCG; the present study results demonstrate that a slight negative effect is present when semen is exposed to EGCG for 1 h after thawing. Even if sperm quality was overall maintained up to 3 hours, all the parameters were negatively affected by incubation time. However, all the parameters analysed resulted slightly worst when EGCG at higher dosage was used. This result should induce prudence in the use of EGCG in dog thawing medium: while no positive effect is evident at the lower dose tested, the high dose seems to exert a negative impact.

Other Authors incubated canine spermatozoa with green tea polyphenols [9,10] in different preservation conditions: in fact those Authors did not froze semen but preserved it in liquid state for a long time. Evidently, the different conditions and the presence of seminal plasma (that, in our case, was eliminated) influenced the difference in results Those Authors demonstrated that polyphenols exerted a positive role in preserving sperm motility, even if this effect was obtained after a very long storage, that is not applicable in routine AI procedures. Moreover, it must be taken into account that in that work a mixture of green tea polyphenols, and not the purified molecule EGCG, was used.

Other studies, in which antioxidants were added during the cooling (pre-freezing) procedure [5] demonstrated some positive effects on sperm motility and viability by catalase, while N-acetyl-L-cysteine, taurine and tocopherol only exerted positive effects on sperm viability; as in our experiments, acrosome integrity was not

influenced by the addition of antioxidants. Spermine showed positive effect on viability, intra and extra-cellular ROS formation and some sperm kinematics parameters, while total motility, as in our case, was not affected [8].

An interesting study by Ogata and colleagues [7] showed that glutathione supplementation to canine spermatozoa during cooling exerted a positive effect on sperm motility (up to 24 h after thawing) and on sperm viability, acrosome integrity and lipid peroxidation. Those Authors used the cryopreserved semen for transcervical insemination, demonstrating that no negative effect was exerted by glutathione on embryo development.

Zona binding assays were used to assess the functionality of spermatozoa after thawing and incubation with EGCG for 1.5 h.

Heterologous zona binding assay was already used in other works from our and other laboratories [17,22–24] in equine species. Availability of many porcine oocytes from the abattoir represent a good chance to perform this functional test also in species in which the availability of homologous oocytes is not so wide. In this work we had the possibility to preform both heterologous and homologous zona binding assay using porcine and canine oocytes. The results from these assays showed no effect of EGCG at all the concentrations tested on heterologous zona binding. Using horse semen we detected some difference between homologous and heterologous zona binding assay [25] in the effect exerted by alkaline phosphatase, thus demonstrating that the assay is useful to detect any change in sperm function. In this study anyway, the effect of EGCG was not present, and the same was registered for the homologous zona binding assay. It is therefore evident that EGCG does not act (nor positively or negatively) on the interaction between dog gametes in our experimental conditions.

This functional assay demonstrates, on a very large number of oocytes in both heterologous or homologous conditions, that EGCG does not stimulate spermatozoa binding to zona pellucida. This is consistent with the results on sperm quality and membrane intactness, as EGCG does not seem to influence membrane stability.

In conclusion, EGCG does not implement canine frozen sperm characteristics or zona binding capacity when added after thawing.

The lack of effect of EGCG in dog seems not to be in agreement with previous results obtained in porcine frozen semen suggesting that the effect of this molecule is species specific and information obtained in one species cannot be borrowed to another one. Moreover, the different effect of each antioxidant supplementation could also be attributed to the extender and concentration used. This is why it is necessary to test different concentrations of each antioxidant molecule in each species.

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