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.

51

52

53 **1. Introduction**

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

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

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

78 extender separately [5]; that study demonstrated that antioxidant exerted positive
79 effects on dog semen but the most effective one was catalase. A recent study
80 involved spermine supplementation of freezing medium [8] giving encouraging
81 results at the 5 mM dose. These studies demonstrate that cryopreserved canine
82 sperm quality could be enhanced using some antioxidants and many others could
83 be profitably applied to this technique.
84 Green tea extracts, for example, were used in dog sperm liquid storage at 5°C [9,10]
85 alone or in association with vitamin C, showing some positive effects on motility
86 after a prolonged storage.
87 Recently in our laboratories we performed some studies regarding the effect of
88 supplementation of thawing extender with Epigallocatechin-3-gallate (EGCG) and
89 resveratrol [11,12] in porcine species, obtaining promising results on IVF trials.
90 EGCG is the main polyphenol in green tea (*Camellia sinensis*) and it possess a high
91 level of antioxidant activity exerted by removing free radical and by enhancing
92 catalase activity [13].
93 Basing on the previous results on different species, the addition of molecules to the
94 thawing medium could be interesting in enhancing the metabolism and resistance
95 of sperm cells surviving the cryopreservation process.
96 The aims of the present study were to evaluate the effects of EGCG when added to
97 thawing medium on canine sperm quality and zona binding ability.
98

99 **2. Materials and methods**

100 Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan,
101 Italy).
102

103 **2.1. Semen collection, evaluation and freezing**

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

109 The three fractions of the ejaculates were collected in calibrated plastic vials by
110 digital manipulation. The sperm-rich fraction was evaluated before freezing for
111 volume, concentration, and rate of morphologically normal spermatozoa. The
112 volume was measured by a calibrated micropipette, and sperm concentration was
113 determined with a Bürker chamber, after dilution of the sperm suspension 1:40
114 with 10% formol buffered saline to immobilize spermatozoa and counted using a
115 phase contrast microscope (400x; Axiolab; Zeiss, Italy) equipped with a warming
116 plate (37°C; Thermo Plate; Tokai Hit, Japan) as reported in [14]. Percentages of
117 morphologically normal spermatozoa were determined at the same microscope
118 (1000x) after dilution of semen 1:1 with 10% formol buffered saline, and at least
119 200 spermatozoa per sample were examined.

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

122

123 2.1.1. Extenders and freezing procedure

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

128 Tris, 1.4% wt/vol citric acid, 0.8% wt/vol glucose, 0.06% wt/vol sodium
129 benzylpenicillin, 0.1% wt/vol streptomycin sulfate in distilled water) supplemented
130 with 20% (v:v) egg yolk, 3% (v:v) glycerol (EYTGC1), whereas for the second step
131 TGC was supplemented with 20% (v:v) egg yolk, 7% (v:v) glycerol, and 1% (v:v)
132 Equex STM Paste (Nova Chemical Sales Inc., Scituate, MA, USA; EYTGC2).
133 In particular, first the spermatozoa were diluted slowly at room temperature (20°C–
134 25°C) by dropwise addition of a volume of EYTGC1 to reach 400 × 10^6
135 spermatozoa/mL. After that, sperm suspension was cooled to 4°C for 60 min in a
136 controlled temperature refrigerator (about −0.3°C/min). Then the second dilution
137 with a volume of precooled EYTGC2 to reach 200 × 10^6 spermatozoa/mL was made
138 at 4°C. After an additional equilibration period of 15 minutes at 4°C, the samples
139 were loaded in plastic straws (0.5 mL) and the straws were heat sealed. Sperm
140 suspensions were frozen on liquid nitrogen vapors into a polystyrene box (36 × 33
141 × 30 cm) keeping straws at 7 cm from liquid nitrogen for 10 minutes, before
142 plunging straws directly into liquid nitrogen. Finally, the straws were transferred
143 into a Dewar flask at −196°C.
144

145 **2.2. Experimental design**

146 Two straws (0.5 mL/straw) were thawed for 30 sec in water bath at 37°C and
147 immediately diluted, at the same temperature, with 2.33 mL of thawing medium
148 (Tris-glucose-citrate, TGC). The sample was immediately divided in three
149 experimental groups (1.0 mL each): CTR (control: without antioxidant addition),
150 EGCG25 (addition of 25μM EGCG to TGC extender) and EGCG50 (50 μM EGCG).
151 EGCG concentrations used in this study were chosen on the basis of previous
152 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

178 For each sample, an aliquot (25 µL) of sperm suspension (30 x 10^6
179 spermatozoa/mL) was incubated with 2 µL of a 300 µM propidium iodide (PI) stock
180 solution, 2 µL of a 10 µM SYBR green-14 stock solution, both obtained from the
181 live/dead sperm viability kit (Molecular Probes, Inc.) and 2 µL of a 150 µM JC-1
182 solution for 20 min at 37°C in the dark. Ten µL of the sperm suspension were then
183 placed on a slide and at least 200 spermatozoa per sample were scored using the
184 above described fluorescence microscope. Spermatozoa stained with SYBR-14 but
185 not with PI were considered as viable (SYBR-14+/PI-), whereas SYBR-14+/PI+ and
186 SYBR-14-/PI+ spermatozoa were considered as non-viable. JC-1 monomers emit
187 green fluorescence in mitochondria with low membrane potential (JC-1-) and form
188 aggregates in mitochondria with high membrane potential (JC-1+), then emitting a
189 bright red-orange fluorescence. Therefore, those viable sperm showing orange-red
190 fluorescence in the mid piece (JC-1+) were considered as viable sperm with high
191 mitochondrial membrane potential (SYBR-14+/PI-/JC-1+) [15].
192 The results are focused on JC1 positive and negative living cells.
193
194 2.3.3. Sperm motility
195 Motility was measured by a computer-assisted sperm analysis system, using the
196 open source Image J BGM plugin as described by Giaretta et al. [15]. Briefly, a Leitz
197 diaplan microscope (Wild Leitz GmbH, D6330, Wetzlar, Germany) with a 10x plan
198 objective with negative phase-contrast was used. The microscope was equipped
199 with a Z31A Ascon technologic heated stage (Ascon technologic, PV – IT). The video
200 camera, 3.1-megapixel CMOS USB 2.0 Infinity1-3 Camera (Lumenera corporation,
201 Ottawa, ON, Canada), was coupled to the microscope by a c-mount adapter and
202 videos were registered for three seconds at a resolution of 800x600 pixel and 60

203 frames/sec (fps). Images were recorded on a hard drive using the Infinity analysing
204 and capture software 6.4 (Lumenera corporation) and converted to avi format for
205 subsequent analysis using the BGM Image J plugin.
206 Prior to any observation, spermatozoa ($30x10^6$ sperm/mL) were loaded onto a fixed
207 height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos
208 of separate fields and lasting three seconds each were recorded per sperm sample.
209 Sperm motility endpoints assessed were: percent of total motile spermatozoa (TM),
210 percent of progressive spermatozoa (PM), curvilinear velocity (VCL) and mean
211 velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat
212 cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The
213 setting parameters of the program were the followings (taken from the Hamilton-
214 Thorne IVOS-12 CASA system): frames per second 60, number of frames 45,
215 threshold path minimum VSL for motile sperm 9µm/sec; min VAP for motile 20
216 µm/sec; min VCL for motile 25 µm/sec; VAP cutoff for Progressive cells 60 µm/sec;
217 STR cutoff for Progressive cells 40%.
218
219 ***2.3.* Zona Pellucida Binding Assays** (ZBA)
220 2.4.1. Heterologous ZBA
221 Porcine oocytes maturation was performed as reported by [16]. Briefly, ovaries were
222 obtained from pre-pubertal gilts at a local abattoir and transported to the
223 laboratory within 1 h. Cumulus–oocyte complexes (COCs) were aspirated from 4 to
224 6 mm follicles using a 18 gauge needle attached to a 10 mL disposable syringe.
225 Under a stereomicroscope, intact COCs were selected and transferred into a petri
226 dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS
227 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.

275 As for the quantification of the effect of EGCG on zona binding assay, a mixed effect
276 model with Poisson distribution was set up setting the treatment as a fixed effect
277 and the repetition as a random effect.
278

279 **4. Results**

280 Mean data from the evaluation of sperm rich fraction of two ejaculates from three
281 mixed-breed dogs after collection were volume, 1.17 ± 0.53 mL; concentration, 935
282 ± 201 x 10^6 spermatozoa/mL; rate of morphologically normal spermatozoa 93.83 ±
283 1.83%.
284 The percentage of live cells with active or non-active mitochondria and sperm
285 acrosome integrity after thawing and incubation with different doses of EGCG is
286 presented in Table 1. A time dependent decrease in the percentage of live
287 spermatozoa with active mitochondria was evident after 6 h incubation in all
288 groups ($p<0.05$), being more evident in the EGCG treated ones, in fact a significant
289 difference ($p<0.05$) between control and EGCG50 groups at 6 h was recorded.
290 Concomitantly, the percentage of living JC1 negative cells showed a time-
291 dependent increase, even if not significant. The only significant difference
292 registered is present between time 0.5 h and 6 h in the EGCG treated groups (Table
293 1).
294 Regarding acrosome intact cells, a time dependent reduction in all groups was
295 evident: starting from 3 h incubation the percentage of acrosome intact cells
296 decreased in all groups compared to 0.5 h.
297 Motility parameters are presented in Table 2 and supplementary file 1. Incubation
298 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

324 motility parameters) were not significantly influenced by the addition of EGCG at
325 both concentrations, excepting live cells with active mitochondria percentage , that,
326 after 6 h, was negatively affected in EGCG50 group. In the previous works on boar
327 semen [11,12] we did not register any change on viability or acrosome integrity
328 after 1 h incubation with EGCG; the present study results demonstrate that a slight
329 negative effect is present when semen is exposed to EGCG for 1 h after thawing.
330 Even if sperm quality was overall maintained up to 3 hours, all the parameters
331 were negatively affected by incubation time. However, all the parameters analysed
332 resulted slightly worst when EGCG at higher dosage was used. This result should
333 induce prudence in the use of EGCG in dog thawing medium: while no positive
334 effect is evident at the lower dose tested, the high dose seems to exert a negative
335 impact.
336 Other Authors incubated canine spermatozoa with green tea polyphenols [9,10] in
337 different preservation conditions: in fact those Authors did not froze semen but
338 preserved it in liquid state for a long time. Evidently, the different conditions and
339 the presence of seminal plasma (that, in our case, was eliminated) influenced the
340 difference in results Those Authors demonstrated that polyphenols exerted a
341 positive role in preserving sperm motility, even if this effect was obtained after a
342 very long storage, that is not applicable in routine AI procedures. Moreover, it must
343 be taken into account that in that work a mixture of green tea polyphenols, and
344 not the purified molecule EGCG, was used.
345 Other studies, in which antioxidants were added during the cooling (pre-freezing)
346 procedure [5] demonstrated some positive effects on sperm motility and viability by
347 catalase, while N-acetyl-L-cysteine, taurine and tocopherol only exerted positive
348 effects on sperm viability; as in our experiments, acrosome integrity was not

349 influenced by the addition of antioxidants. Spermine showed positive effect on
350 viability, intra and extra-cellular ROS formation and some sperm kinematics
351 parameters, while total motility, as in our case, was not affected [8].
352 An interesting study by Ogata and colleagues [7] showed that glutathione
353 supplementation to canine spermatozoa during cooling exerted a positive effect on
354 sperm motility (up to 24 h after thawing) and on sperm viability, acrosome integrity
355 and lipid peroxidation. Those Authors used the cryopreserved semen for
356 transcervical insemination, demonstrating that no negative effect was exerted by
357 glutathione on embryo development.
358 Zona binding assays were used to assess the functionality of spermatozoa after
359 thawing and incubation with EGCG for 1.5 h.
360 Heterologous zona binding assay was already used in other works from our and
361 other laboratories [17,22–24] in equine species. Availability of many porcine
362 oocytes from the abattoir represent a good chance to perform this functional test
363 also in species in which the availability of homologous oocytes is not so wide. In
364 this work we had the possibility to preform both heterologous and homologous zona
365 binding assay using porcine and canine oocytes. The results from these assays
366 showed no effect of EGCG at all the concentrations tested on heterologous zona
367 binding. Using horse semen we detected some difference between homologous and
368 heterologous zona binding assay [25] in the effect exerted by alkaline phosphatase,
369 thus demonstrating that the assay is useful to detect any change in sperm function.
370 In this study anyway, the effect of EGCG was not present, and the same was
371 registered for the homologous zona binding assay. It is therefore evident that EGCG
372 does not act (nor positively or negatively) on the interaction between dog gametes
373 in our experimental conditions.

374 This functional assay demonstrates, on a very large number of oocytes in both
375 heterologous or homologous conditions, that EGCG does not stimulate
376 spermatozoa binding to zona pellucida. This is consistent with the results on sperm
377 quality and membrane intactness, as EGCG does not seem to influence membrane
378 stability.
379 In conclusion, EGCG does not implement canine frozen sperm characteristics or
380 zona binding capacity when added after thawing.
381 The lack of effect of EGCG in dog seems not to be in agreement with previous results
382 obtained in porcine frozen semen suggesting that the effect of this molecule is
383 species specific and information obtained in one species cannot be borrowed to
384 another one. Moreover, the different effect of each antioxidant supplementation
385 could also be attributed to the extender and concentration used. This is why it is
386 necessary to test different concentrations of each antioxidant molecule in each
387 species.
388
389 **6. References**
390 [1] Peña FJ, Núñez-Martínez I, Morán JM. Semen technologies in dog breeding:
391 An update. Reprod Domest Anim 2006;41:21–9. doi:10.1111/j.1439-
392 0531.2006.00766.x.
393 [2] Yeste M, Rodríguez-Gil JE, Bonet S. Artificial insemination with frozen-
394 thawed boar sperm. Mol Reprod Dev 2017:1–38. doi:10.1002/mrd.22840.
395 [3] Battut IB, Kempfer A, Lemasson N, Chevrier L, Camugli S. Prediction of the
396 fertility of stallion frozen-thawed semen using a combination of computer-
397 assisted motility analysis, microscopical observation and flow cytometry.
398 Theriogenology 2017;97:186–200.

399 doi:10.1016/j.theriogenology.2017.04.036.
400 [4] Yeste M. Sperm cryopreservation update: Cryodamage, markers, and factors
401 affecting the sperm freezability in pigs. Theriogenology 2016;85:47–64.
402 doi:10.1016/j.theriogenology.2015.09.047.
403 [5] Michael A, Alexopoulos C, Pontiki E, Hadjipavlou-Litina D, Saratsis P, Boscos
404 C. Effect of antioxidant supplementation on semen quality and reactive
405 oxygen species of frozen-thawed canine spermatozoa. Theriogenology
406 2007;68:204–12. doi:10.1016/j.theriogenology.2007.04.053.
407 [6] Monteiro JC, Gonçalves JSA, Rodrigues JA, Lúcio CF, Silva LCG, Assumpção
408 MEOA, et al. Influence of ascorbic acid and glutathione antioxidants on
409 frozen-thawed canine semen. Reprod Domest Anim 2009;44:359–62.
410 doi:10.1111/j.1439-0531.2009.01434.x.
411 [7] Ogata K, Sasaki A, Kato Y, Takeda A, Wakabayashi M, Sarentonglaga B, et al.
412 Glutathione supplementation to semen extender improves the quality of
413 frozen-thawed canine spermatozoa for transcervical insemination. J Reprod
414 Dev 2015;61:116–22. doi:10.1262/jrd.2014-130.
415 [8] Setyawan EMN, Kim MJ, Oh HJ, Kim GA, Jo YK, Lee SH, et al. Spermine
416 reduces reactive oxygen species levels and decreases cryocapacitation in
417 canine sperm cryopreservation. Biochem Biophys Res Commun
418 2016;479:927–32. doi:10.1016/j.bbrc.2016.08.091.
419 [9] Wittayarat M, Kimura T, Kodama R, Namula Z, Chatdarong K, Techakumphu
420 M, et al. Long-term preservation of chilled canine semen using vitamin C in
421 combination with green tea polyphenol. Cryo-Letters 2012;33:318–26.
422 [10] Wittayarat M, Ito A, Kimura T, Namula Z, Luu VV, Do LTK, et al. Effects of
423 green tea polyphenol on the quality of canine semen after long-term storage

424 at 5°C. Reprod Biol 2013;13:251–4. doi:10.1016/j.repbio.2013.07.006.
425 [11] Gadani B, Bucci D, Spinaci M, Tamanini C, Galeati G. Resveratrol and
426 Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro
427 fertilization. Theriogenology 2017;90:88–93.
428 doi:10.1016/j.theriogenology.2016.11.020.
429 [12] Bucci D, Spinaci M, Yeste M, Mislei B, Gadani B, Prieto N, et al. Combined
430 effects of resveratrol and epigallocatechin-3-gallate on post thaw boar sperm
431 and IVF parameters. Theriogenology 2018;117:16–25.
432 doi:10.1016/j.theriogenology.2018.05.016.
433 [13] Schroeder EK, Kelsey N a, Doyle J, Breed E, Bouchard RJ, Loucks FA, et al.
434 Green tea epigallocatechin 3-gallate accumulates in mitochondria and
435 displays a selective antiapoptotic effect against inducers of mitochondrial
436 oxidative stress in neurons. Antioxid Redox Signal 2009.
437 doi:10.1089/ars.2008.2215.
438 [14] Merlo B, Zambelli D, Cunto M, Iacono E, Nasi L, Giaretta E, et al. Sex-sorted
439 canine sperm cryopreservation: Limits and procedural considerations.
440 Theriogenology 2015;83:1121–7. doi:10.1016/j.theriogenology.2014.12.018.
441 [15] Giaretta E, Munerato M, Yeste M, Galeati G, Spinaci M, Tamanini C, et al.
442 Implementing an open-access CASA software for the assessment of stallion
443 sperm motility: Relationship with other sperm quality parameters. Anim
444 Reprod Sci 2017;176:11–9. doi:10.1016/j.anireprosci.2016.11.003.
445 [16] Spinaci M, Bucci D, Gadani B, Porcu E, Tamanini C, Galeati G. Pig sperm
446 preincubation and gamete coincubation with glutamate enhance sperm-
447 oocyte binding and in vitro fertilization. Theriogenology 2017;95:149–53.
448 doi:10.1016/j.theriogenology.2017.03.017.

449 [17] Balao da Silva CM, Spinaci M, Bucci D, Giaretta E, Peña FJ, Mari G, et al.
450 Effect of sex sorting on stallion spermatozoa: Heterologous oocyte binding,
451 tyrosine phosphorylation and acrosome reaction assay. Anim Reprod Sci
452 2013;141:68–74. doi:10.1016/j.anireprosci.2013.07.008.
453 [18] Bucci D, Isani G, Spinaci M, Tamanini C, Mari G, Zambelli D, et al.
454 Comparative immunolocalization of GLUTs 1, 2, 3 and 5 in boar, stallion and
455 dog spermatozoa. Reprod Domest Anim 2010;45:315–22.
456 doi:10.1111/j.1439-0531.2008.01307.x.
457 [19] Hermansson U, Ponglowhapan S, Forsberg CL, Holst BS. A short sperm-
458 oocyte incubation time ZBA in the dog. Theriogenology 2006;66:717–25.
459 doi:10.1016/j.theriogenology.2006.01.043.
460 [20] Tselkas K, Saratsis P, Karagianidis A, Samouilidis S. Extracellular presence
461 of reactive oxygen species (ROS) in fresh and frozen-thawed canine semen
462 and their effects on some semen parameters. Dtsch Tierarztl Wochenschr
463 2000;107:69–72.
464 [21] Yeste M. State-of-the-art of boar sperm preservation in liquid and frozen state.
465 Anim Reprod 2017;14:69–81. doi:10.21451/1984-3143-AR895.
466 [22] Plaza Dávila M, Bucci D, Galeati G, Peña F, Mari G, Giaretta E, et al.
467 Epigallocatechin-3-Gallate (EGCG) Reduces Rotenone Effect on Stallion
468 Sperm-Zona Pellucida Heterologous Binding. Reprod Domest Anim
469 2015;50:1011–6. doi:10.1111/rda.12628.
470 [23] Bucci D, Giaretta E, Merlo B, Iacono E, Spinaci M, Gadani B, et al. Alkaline
471 phosphatase added to capacitating medium enhances horse sperm-zona
472 pellucida binding. Theriogenology 2017;87:72–8.
473 doi:10.1016/j.theriogenology.2016.08.003.

[24] Rathi R, Colenbrander B, Bevers MM, Gadella BM. Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod 2001;65:462–70. doi:10.1095/biolreprod65.2.462.

[25] Bucci D, Giaretta E, Spinaci M, Rizzato G, Isani G, Mislei B, et al. Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-thawed stallion spermatozoa. Theriogenology 2015. doi:10.1016/j.theriogenology.2015.09.007.