Combined effects of resveratrol and epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters

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Abstract

Frozen-thawed boar semen suffer a fertility decrease that negatively affects its widespread use. In recent years supplementing frozen-thawed boar sperm with different antioxidants gave interesting and promising results; the aim of the present work was to study the effect of supplementing boar sperm thawing medium for 1 h with combination of epigallocatechin-3-gallate (EGCG, 50 μM) and Resveratrol (R, 2mM), on boar sperm motility (assessed by CASA), viability, acrosome integrity, mitochondrial function, lipid peroxidation and DNA integrity (assessed by flow cytometry), protein tyrosine phosphorylation (assessed by immunofluorescence) and on in vitro fertilization (IVF).
Our results demonstrate that sperm motility is negatively affected by R (alone or associated with EGCG, $p<0.05$) in comparison to control and EGCG groups both at 1h and 4 h; this effect is evident both in average motility parameters and in single cells kinematics, studied by cluster analysis, that showed the presence of a specific cell population with simil-hyperactivated features in R group ($p<0.01$).
Viability, acrosome integrity, mitochondrial functionality and lipid peroxidation are not influenced by the addition of the antioxidants; finally, DNA integrity is negatively influenced by R (both alone or associated with EGCG) both at 1h and 4h incubation ($p<0.05$). Finally, tyrosine phosphorylated protein immunolocalization, used as capacitation parameter, is not affected by the different treatments.
Penetration rate is strongly enhanced by R, both alone or associated with EGCG ($p<0.05$); EGCG increases penetration rate as well but to a lower extent.
Our findings demonstrate that the combination of R and EGCG could positively affect frozen-thawed boar sperm fertility in vitro; the effect is evident also in R groups, thus demonstrating that this antioxidant is predominant, and no synergic effect is present. Some insights are needed to understand if, in particular R (that showed the strongest effect) could be profitably used for artificial insemination in vivo, given the detrimental effect of this molecule on both sperm motility and DNA integrity.

Keywords

Resveratrol
Epigallocatechin-3-gallate
Pig
Frozen-thawed spermatozoa

1. Introduction

Cryopreservation is a useful technique to store germinal cells, and in particular spermatozoa; in boar sperm production, this particular field represent an important challenge for commercial farm or AI centres as nowadays is not yet suitable for a widespread use and it is of utmost importance in gene banking, research and advanced reproductive biotechnologies [1,2].
During cryopreservation, sperm cells undergo some changes which are mainly concentrated in the cooling-freezing passage [3]. Sperm membrane is particularly susceptible to cryodamage, as in pig the cholesterol:lipid ratio is lower than in other species [4–6], moreover freeze-thawing can affect

sperm nuclear proteins (histones and protamine) and DNA [7–9], and can lower mitochondrial activity [10]. Cryodamage also induces structural modifications in several proteins, such as membrane transporters (e.g. Ca^{2+} channels, glucose transporters) [10–12], impairs sperm motility and affects the distribution of the different motile subpopulations [13,14].

Attention has been given to damages derived from reactive oxygen species (ROS). These molecules have a recognized and important role in sperm function, as they are involved in sperm activation and capacitation [15–18]. These substances (e.g. anion superoxide, hydrogen peroxide and nitric oxide), when produced at controlled rates have a stimulatory role on some events related to capacitation, sperm-oocyte interaction and acrosome reaction [15,17], via the activation of some internal pathways (cyclic AMP-Protein Kinase A ; mitogen activated protein kinase/extracellular regulated Kinases; protein tyrosine phosphorylation) that are also linked to hyperactivated motility and acrosome reactivity. However, the excessive accumulation of some of these molecules into the cell or in the surrounding environment may represent a danger for sperm cells [16,19–21]. In fact, and as previously reported [1,21], ROS can affect sperm integrity (i.e. sperm plasma membrane, acrosome, DNA, mitochondria), thereby impairing their function (motility, hyperactivation, sperm-oocyte interaction). Specific features of boar sperm cells are potential targets for ROS such as: polyunsaturated fatty acid (PUFA) and phospholipase A present in the membrane [21].

Several antioxidants (L-cysteine, α- tocopherol, lutein, butylated hydroxytoluene, Trolox, ascorbic acid, epigallocatechin-3-gallate , reduced glutathione, resveratrol) have been added to preservation media to mitigate the adverse effects of ROS and cryopreservation in boar spermatozoa and to better maintain the sperm function [1,22–24]. While, some molecules, such as ascorbic acid and reduced glutathione, have been demonstrated to be active and powerful, either alone or combined [24,25], others, such as resveratrol , have not been found to exert a positive impact [26]. The latter, anyway, showed an interesting effect on vitrified porcine oocytes [27] and may be much efficient in protecting from the freezing process.

In a recent study, we supplemented the thawing medium with natural antioxidants (epigallo catechin 3-gallate and resveratrol) and observed an increase in vitro fertilization rate of boar frozen semen and no change in either sperm viability and acrosome integrity [22].

Based on the above-mentioned researches the aim of the present study was to test the effects of the combination of resveratrol (R) and epigallocatechin-3-gallate (EGCG) added to thawing medium on both sperm parameters and fertilizing ability.

2. Materials and Methods

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

2.1 Experimental design

All the experiments were conducted following the guidelines of the Ethic committee when applicable. Boar semen frozen in 0.5 mL straws was purchased from a commercial company (Inseme S.P.A., Modena, Italy).

Three straws from the same ejaculate were thawed in a water bath at 37°C under agitation for 30 seconds and subsequently pooled and diluted in Beltsville Thawing Solution (BTS) at a dilution rate

1:3. Only thawed samples with sperm viability higher than 40%, as evaluated by SYBR14/PI test (see below), were used. Thereafter, each sample was divided into four aliquots, each corresponding to one of the following treatments: control (CTR), resveratrol 2 mM (R), Epigallocatechin-3-gallate 50 µM (EGCG) and R+EGCG at the same concentrations.
The antioxidants doses were chosen on the basis of the results of our previous work [22].
Sperm suspensions were kept for 1 h at 37°C in the dark and subsequently used in Experiment 1 or 2.

2.1.1 Experiment 1. Assessment of sperm parameters

An aliquot (500 µL) of each sperm suspension incubated at 37°C for 1 h in the dark was used to evaluate: sperm motility, viability, acrosome integrity, mitochondrial activity, lipid peroxidation and DNA integrity (See below for description of the methods). The remaining part of sperm suspension was kept at 37°C for an additional 3-h period in the dark and the same analyses were subsequently performed as reported by other Authors [28]. Four different animals (1 ejaculate each) were used and each experiment was repeated three times.
For tyrosine phosphorylation immunostaining analyses, an aliquot of spermatozoa incubated for 1 h in the dark at 37°C from each experimental group was fixed, while another aliquot was washed twice in Brackett and Oliphant's (BO) medium [29] supplemented with 12% foetal calf serum (FCS) and 0.7g/L caffeine (IVF medium) and then resuspended in the same medium at a final concentration of $30x10^6$ spermatozoa/mL and incubated 1 h under capacitating condition (5% CO2, 39° C). Finally samples were fixed for immunolocalization of tyrosine phosphorylated proteins. Three different animals (1 ejaculate each) were used and each experiment was repeated twice (n=6).

2.1.2 Experiment 2. Effects of R and EGCG on sperm fertilizing ability

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After 1 h incubation in BTS with the different antioxidants, aliquots of sperm cells were used for in-vitro fertilization (IVF) trials and final sperm concentration was adjusted to $1x10^6$ spz/mL (See Section 2..4). Oocytes were treated as described in Section 2.4.
Three different animals (1 ejaculate each) were used and each experiment was repeated three times for IVF (n=9).

2.2 Sperm motility assessment

Sperm motility was assessed using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12); the standard boar setup was used (60 frame per sec; 45 n. of frames; min contrast 49; min cell size 6 pixels; progressive cells: VAP 20.1 µm/sec; straightness percentage 75; static cell cutoff: VAP 20 µm/sec, VSL 5 µm/sec). Approximately one thousand cells at 30×10^6 sperm/mL were evaluated for each sample using a fixed-height Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Parameters assessed were percentages of total motile spermatozoa (TM), percentages of progressively motile spermatozoa (PM), curvilinear velocity (VCL µm/sec), average path velocity (VAP µm/sec), straight line velocity (VSL µm/sec), percentages of straightness (STR) and linearity (LIN), average lateral head displacement (ALH µm) and beat cross frequency (BCF Hz).

Together with global sample analysis, individual sperm tracks were assessed and VCL, VAP, VSL, STR, LIN, ALH and BCF were recorded for each motile spermatozoon. These parameters were used to study the distribution of sperm subpopulations in all treatments (See statistical analysis – cluster analysis for sperm motion).

2.3 Flow cytometry analysis

Information about flow cytometry analyses is reported taking into account the recommendations of the International Society for Advancement of Cytometry [30]. Flow cytometry analyses were conducted to evaluate sperm viability, acrosome integrity, mitochondrial function, lipid peroxidation levels and DNA fragmentation. In each assay, sperm concentration was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL BTS, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below. Samples were evaluated through a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser. Emission measurements were made by means of three different filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2) and >670 long pass (far red/FL3) filters. Data were acquired using the BD CellQuest Pro software (Becton Dickinson).
Signals were logarithmically amplified and photomultiplier settings were adjusted to each particular staining method. FL1 was used to detect green fluorescence from SYBR14, fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA), low mitochondrial membrane potential (JC1 negative), and BODIPY 581/591, whereas FL2 was used to detect orange fluorescence from high mitochondrial membrane potential (JC1 positive) and FL3 was used to detect orange-red fluorescence from propidium iodide (PI).
Side scatter height (SS-h) and forward scatter height (FS-h) were recorded in logarithmic mode (in FS vs. SS dot plots) and sperm population was positively gated based on FS and SS while other events were gated out. A minimum of 10,000 sperm events were evaluated per replicate.
In FITC-conjugated PSA flow cytometric assessment, percentages of non-DNA–containing particles (alien particles), (*f*) were determined to avoid an overestimation of sperm particles in the first quadrant (q_1) as described by [31], according to the following formula:

$$q'_1 = \frac{q_1 - f}{100 - f} \times 100$$

where q'_1 is the percentage of non-stained spermatozoa after correction.

2.3.1. Sperm membrane integrity (SYBR14/PI)

Sperm viability was assessed by checking the membrane integrity using two separate fluorochromes SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). SYBR-14 is a membrane-permeable dye, which stains the head of viable spermatozoa in green, while PI is a membrane-impermeable dye that only penetrates through disrupted plasma membrane, staining the sperm heads of non-viable cells in red. Sperm samples were diluted with BTS to a concentration of 1×10^6 spermatozoa/mL and aliquots of 500 µL were stained with 5 µL SYBR-14 working solution (final concentration: 100 nM) and with 2.5 µL of PI (final concentration: 12 µM) for 10 min at 37°C in darkness. Viable spermatozoa exhibited a positive staining for SYBR-14 and negative staining for

PI (SYBR-14+/PI-). Single-stained samples were used for setting the voltage gain for FL1 and FL3 photomultipliers.

2.3.2 Acrosome integrity analysis (PSA-FITC/PI)

Sperm acrosome intactness was assessed by Pisum sativum agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) (2.5 mg/mL stock solution; 0.5mg/mL working solution) coupled with Propidium Iodide (2.4mM stock solution). Sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL and aliquots of 500 µL were stained with 10 µL FITC-PSA (final concentration: 10 µg/mL) and with 3 µL PI (final concentration: 14 µM) for 10 min at 37 °C in darkness. Four different sperm subpopulations were distinguished: a) viable acrosome-intact spermatozoa were those cells that did not stain with either FITC-PSA or PI and appeared in the lower left quadrant of FL1 *vs.* FL3 plots; b) viable spermatozoa with disrupted acrosome stained only in green with FITC-PSA and were found in the lower right panel; c) non-viable spermatozoa with intact acrosome stained with PI only and appeared in the upper left quadrant; and d) non-viable spermatozoa with disrupted acrosomes were found in the upper right quadrant and stained positively with both stains.

2.3.3 Mitochondrial membrane potential analysis (JC1/PI)

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was used to evaluate mitochondrial membrane potential. When it comes in contact with mitochondria with high membrane potential, JC-1 forms multimers (known as J-aggregates) and emits orange fluorescence at 590 nm, which is detected by FL-2 photomultiplier. In contrast, when mitochondria have low membrane potential, JC-1 maintains its monomeric form (M-band) and emits green fluorescence at 530 nm, which is detected by FL-1 photomultiplier.
Sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL and aliquots of 500 µL were stained with 5 µL JC1 (at a final concentration of 1µg/mL) and 3 µL of PI (at a final concentration of 14 µM); samples were successively incubated at 37 °C for 30 min in the dark.
PI positive cells were gated out in a FL-1/FL-3 dot plot; PI negative cells were gated and analysed in a FL-1/FL-2 plot. High mitochondrial membrane potential cells (HMMP) stained orange (higher FL-2) and low mitochondrial membrane potential cells (LMMP) stained green (higher FL-1).

2.3.4 Lipid peroxidation analysis (Bodipy/PI)

BODIPY 581/591 (Molecular Probes Eugene, CA, USA) stock solution was prepared diluting 1 mg of the molecule in 1980 µL DMSO. For analysis, sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL; aliquots of 500 µL were centrifuged at 900x g for 2 min at room temperature; the supernatant was discarded, and sperm pellet resuspended with 492 µL BTS and stained with 5 µL BODIPY stock solution (final concentration 0.01 ug/mL and 3 µL of PI (at a final concentration of 14 µM). Cells were incubated for 30 min at 37 °C in the darkness and subsequently analysed.
As no separate sub-populations in FL1-FL3 plots were detectable, a relative fluorescence quantification method was used, as described by [19]. Briefly, the instrument was set with 10

242 references of the same ejaculate of frozen-thawed boar semen and the mean FL1 signal was registered.
243 For each analysis, one sample of the same reference was used to set the voltage and gain of the
244 instrument to get the same reference value; subsequently the experimental samples were run.
245
246 2.3.5 Sperm chromatin structure assay (SCSA)
247
248 Sample preparation and processing, as well as flow cytometer adjustments, were performed as
249 previously described [32–34]. Briefly, 50 µl of each semen sample were immediately frozen and
250 stored at −80°C until analysis (maximum 2 weeks). Sperm samples were handled individually and
251 were thawed in a 37°C water bath. Immediately after thawing (30–60 s), aliquots of thawed semen
252 were added with 200 µL of a buffer solution (0.186 g disodium EDTA, 0.790 g Tris–HCl and 4.380
253 g NaCl in 500 mL deionized water; pH adjusted to 7.4). This was mixed with 400 µL of an acid
254 detergent solution (2.19 g NaCl, 1.0 ml of 2 N HCl solution, 0.25 ml Triton X, and deionized water
255 quantum sufficit to a final volume of 250 ml). After 30 sec, 1.2 ml of the acridine orange solution
256 were added Cell flow rate was set on low which resulted in an actual flow rate of 100–200 cells/sec.
257 A total of 5,000 events was evaluated for each sample. Sperm from a single control boar were used
258 as a biologic control to standardize instrument settings between days of use. The flow cytometer was
259 adjusted such that the mean green fluorescence was set at the 500 channel (FL-l at 500) and mean red
260 fluorescence at the 150 channel (FL-3 at 150). Data were acquired in a list mode (linear scale), and
261 analysis was performed using winlist software (Verity Software House). The percentage of sperm
262 with abnormal DNA was defined by the parameter DNA fragmentation index (DFI).
263
264
265 2.4 In vitro fertilization (IVF) trials
266
267 Oocytes were cultured as already described [22]; briefly, ovaries were obtained from pre-pubertal
268 gilts at a local abattoir. Cumulus oocyte complexes (COCs) from follicles 3–6 mm in diameter were
269 aspirated using a 18-gauge needle attached to a 10-mL disposable syringe. Intact COCs were selected
270 under a stereomicroscope and only COCs with more than two layers of intact cumulus oophorus and
271 with uniform cytoplasm were used. Next, COCs were washed three times with NCSU 37 [35]
272 supplemented with 5.0 µg/mL insulin, 1mM glutamine, 0.57 mM cysteine, 10 ng/mL epidermal
273 growth factor (EGF), 50 µM ß -mercaptoethanol and 10% porcine follicular fluid (IVM medium).
274 Groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL of the same
275 medium per well and in vitro matured at 39°C and 5% CO_2 in a humidified atmosphere. During the
276 first 22 h of in vitro maturation, IVM medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL
277 equine chorionic gonadotropin (eCG) (Folligon; Intervet, Boxmeer, The Netherlands) and 10 IU/mL
278 human chorionic gonadotropin (hCG) (Chorulon; Intervet). For the last 22–24 h of IVM, COCs were
279 transferred to fresh maturation medium [36].
280 Groups of 50 matured oocytes, freed from cumulus cells by repeated gentle pipetting, were transferred
281 to 500 µL IVF medium containing 1×10^6 spz/mL. After 1 h of co-culture, oocytes were transferred
282 to fresh IVF medium and cultured for 20 h. The oocytes were then mounted on microscope slides,
283 fixed in acetic acid/ethanol (1:3; v:v) for 24 h and stained with Lacmoid. Oocytes were observed
284 under a phase-contrast microscope and parameters evaluated were: penetration rate (number of
285 oocytes penetrated/total inseminated), monospermy rate (number of oocytes containing only one

sperm head–male pronucleus/total fertilized) and total efficiency (number of oocytes containing only one sperm head–male pronucleus/total inseminated). Degenerated and immature oocytes were not counted.

2.5 Immunolocalization of tyrosine phosphorylated proteins in spermatozoa

Sperm cells preparation were analysed as described by [37]; sperm cells ($30x10^6$ spermatozoa/mL) were spread onto poly-L-lysine-coated slides, fixed with absolute methanol at -20°C for 10 min and then with acetone for 30 sec. Slides were washed with phosphate buffered saline solution (PBS), let dry and then blocked with 10% (v/v) FCS in PBS for 30 min. A primary antibody against tyrosine phosphorylated proteins (Upstate Millipore, Watford, UK) was added at a 1:200 dilution (v:v) in PBS 10% FCS. Incubation was carried out overnight at 4°C in humid chambers. After three washings in PBS, slides were incubated with a goat anti-mouse (dilution 1:800), FITC-conjugated secondary antibody for 1 h in the dark. Next, slides were washed again three times in PBS and mounted with anti-fading Vectashield mounting medium with PI (Vector Laboratories) to counterstain the nuclei. Negative controls were treated similarly with the omission of the primary antiserum. Images were obtained using a Nikon digital camera installed on a Nikon epifluorescence microscope (Nikon Inc., Melville, NY, USA). Two-hundred cells were counted for each replicate.
Different patterns of tyrosine phosphorylated sperm proteins were identified, following the description by [37] with some modifications: A: spermatozoa with acrosome and equatorial sub segment positivity; B: spermatozoa with tail positivity (whole tail or the principal piece only) and acrosome and/or equatorial sub-segment positivity; C: spermatozoa with tail positivity (whole tail or principal piece only); and NEG: spermatozoa with no positive signal.

2.6. Statistical analyses

Statistical analyses were performed using R (version 3.4.0). Values are expressed as mean ± standard deviation (SD), unless otherwise specified and level of significance was at $P \leq 0.05$.

2.6.1 Effects of treatment upon sperm function parameters, tyrosine phosphorylation and IVF outcomes

Motility and post thawing parameters assessed by flow cytometry expressed as percentages were transformed with arcsine square root. Subsequently all variables (both motility and post thawing parameters) were tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests. Then, a mixed effect model was set to determine treatment and time effects (1 and 4 h at post-thaw) and their interaction, with the boar ejaculate and repetition as random effects.
As for IVF trials, the variables (i.e. penetration rates and monospermy) were analysed using a general linear model with binomial distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.
Finally, tyrosine phosphorylation data were arcsin square root-transformed. Normality of the residues was checked by Shapiro Wilk test and homogeneity of the variances was tested with Levene test. One-way ANOVA and Tukey post hoc test were used to assess differences between treatments.

2.6.2 Cluster analysis for motility parameters

Sperm kinematics parameters were registered for each individual motile sperm cell from three boars, each analysed twice at 1 and 4 h post-thaw; ALH, BCF, VCL, VAP, VSL, LIN and STR were recorded and used for cluster analysis.
As reported by [38], data were first normalized, then a principal component analysis was performed to reduce the total number variables. Finally, a hierarchical clustering using the Ward's method and Euclidean distances was applied to the Principal components and 4 clusters were identified. Finally, a chi square analysis was used to assess difference in the composition of the different clusters in relation to treatment. In total, 17,953 cells were analysed.

3. Results

EGCG did not affect sperm motility in comparison with CTR, while R and R+EGCG affected all sperm parameters. TM, VAP and VCL in R and R+EGCG treatments were significantly ($P<0.05$) lower than CTR and EGCG at both 1 and 4 h post-thaw. In addition, the overall motility parameters were found to decrease throughout post-thaw incubation time (i.e. 1 h *vs.* 4 h post-thaw; Supplementary file 1 and Fig.1 panels A, B).
On the other hand, whereas PM, VSL, BCF, LIN and STR were also significantly lower in R and R+EGCG treatments than in CTR and EGCG, the effects of post-thaw incubation time were less apparent (Supplementary file 1 and Fig.1). Finally, ALH showed a significant decrease due to treatment (with resveratrol) and post-thaw incubation time and their interaction (Supplementary file1).

The principal component analysis resulted in four principal components that were used for subsequent cluster analysis (Supplementary file 2). The resulting analysis showed four distinct clusters. These different clusters showed different motion characteristics (Table 1) that allowed us to identify them as: 1) slow non-progressive; 2) average; 3) rapid progressive; 4) rapid non-progressive.
Spermatozoa were assigned to the different clusters and, as reported in Table 2, significant differences in the percentages of spermatozoa belonging to each subpopulation were found between treatments (CTR, R, EGCG, R+EGCG).

Sperm viability, evaluated through SYBR14/PI test, was not influenced by treatments, but a significant reduction in this parameter was observed in all the treatments throughout post-thaw incubation time (1 and 4 h of incubation after thawing) (Fig. 1, panel C).
Percentages of viable spermatozoa with an intact acrosome, evaluated by PSA-FITC/PI test, were not influenced by treatment or post-thaw incubation time (Fig. 1, panel D).
With regard to mitochondrial membrane potential, and as shown in Fig 1, panel E, percentages of viable spermatozoa displaying high mitochondrial membrane potential were not influenced by the treatment. However, those percentages were significantly reduced in all the treatments throughout post-thaw incubation time.
Live sperm membranes lipid peroxidation measurement showed no significant differences between treatments and times of post-thaw incubation (Fig. 1, panel F).

Finally, sperm DNA integrity, measured by DNA fragmentation index (DFI%), showed significant ($p<0.05$) differences between treatments (R and R+EGCG compared to CTR and EGCG) and for R and R+EGCG groups, times of incubation (Fig. 1, panel G).

Immunolocalization of tyrosine phosphorylated proteins after 1 h incubation in BTS showed no significant difference between groups (CTR, EGCG, R, EGCG+R) with around 95% spermatozoa showing A-pattern and the remaining 5% no positivity. Although the percentage of cells displaying the different patterns changed after 1 h of incubation in capacitating conditions (incubation in IVF medium for 1h at 39° C in a humified chamber 5% CO_2), no significant effect between treatments was observed (Fig. 1, panels H-M).

Penetration rates were found to be increased when R and/or EGCG were added. Notwithstanding, the extent of that increase compared to control was even higher when both R and EGCG (R+EGCG) were added in combination ($P<0.001$; Fig.2, panel A). Monospermy (number of oocytes penetrated by only one spermatozoon divided by the total number of fertilized oocytes) was also affected by treatments, as R and R+EGCG exhibited significantly ($P<0.05$) lower monospermy rates than CTR and EGCG (Fig. 2, panel B).
Total efficiency was not influenced by the different treatments (Fig.2 panel C).
The total number of oocytes analysed was 1594, divided into the different groups as follow: 395 oocytes CTR, 435 EGCG, 378 R, 386 R+EGCG.

4. Discussion

Cryopreservation induces some alterations on boar spermatozoa that bring about a loss of fertility [39], due to numerous effects strictly related to the process [1,2]. One concern is the excessive generation of ROS that tends to induce hyper-oxidation damage in various sperm structures, such as plasma membrane, DNA, nucleus and mitochondria [17,20,40].
The present work aimed at limiting the negative effects of ROS generation by adding, in combination, two different natural antioxidants, R and EGCG after thawing boar spermatozoa. A previous study [22], demonstrated that 50 µM EGCG and 2 mM R were the most effective doses and resulted in the highest IVF rates of *in vitro* matured oocytes following IVF [22]. The same effect was observed also in the present work. However, the most noticeable finding of this work after evaluating the single and combined effects of both antioxidants, was that R is more powerful than EGCG in enhancing penetration rates in vitro. In fact, whereas ECGC induced a significant increase in the percentage of fertilized eggs when compared to the control, the extent of that increase was even higher in the case of R. However, no synergic effect was observed when the two antioxidants (R+EGCG) were supplemented in combination. The effects on fertilization were promising: the increase in penetration rates could be very encouraging for the addition of these substances to post-thawing media for boar spermatozoa, being that post-thawing fertility seems to be one of the major limitations for the use of frozen-thawed boar semen in a wide scale [39,41]. In this regard, artificial insemination trials should be performed to verify whether the strong effect observed in IVF is maintained *in vivo*, which would involve an effect on both farrowing rates and litter sizes. The selection of "good freezing" boars is a

418 central strategy for the application of frozen-thawed boar semen on a large scale; the males we used
419 for IVF trials were chosen because of their good semen quality after thawing and their ability to
420 fertilize oocytes *in vitro*.
421 One of the main questions raised from these results is which mechanism could explain the strong
422 effect on IVF.
423 The first trial we performed, together with IVF, was aimed to study the localization of tyrosine
424 phosphorylated proteins in sperm cell; as we did in other reports [37,42]. This parameter, known to
425 be related to sperm capacitation, was used to detect different sperm subpopulations patterns (A, B, C
426 and Neg) [37] and we studied the changes in their distribution due to the aforementioned treatments.
427 Immunolocalization results clearly showed no difference between groups. Evidently, this parameter
428 could be retained too "downstream" in the capacitation events to be thoroughly changed by the
429 different treatments [37]. It should also be remarked that, after 1 h of incubation in BTS medium, no
430 difference was found between groups; in particular, almost 95% of the sperm cells showed the A
431 pattern, typical of non-capacitated spermatozoa.
432 To better determine whether any change on sperm metabolism and membrane composition resulted
433 from the addition of R and ECGC to frozen-thawed spermatozoa, we studied a wider panel of sperm
434 parameters, while only viability and acrosome integrity were evaluated in our previous work [22].
435 We assessed sperm viability, acrosome integrity, mitochondrial membrane potential, lipid
436 peroxidation and DNA integrity through flow cytometry and sperm motility (including motile
437 subpopulations) by CASA. All the flow cytometric analyses were performed both at 1 and 4 h after
438 thawing, keeping the semen at 37°C.
439 Sperm viability and acrosome integrity were not influenced by the addition of antioxidants. These
440 results match with those obtained in our previous study [22] even if the current study evaluated those
441 parameters by flow cytometry and our previous work used epifluorescence microscope. It is worth
442 noting that there was a decrease in viable spermatozoa throughout post-thaw incubation time (i.e.
443 between 1 h and 4 h of incubation).
444 Regarding the metabolic status of spermatozoa, we checked mitochondria functionality of viable
445 cells, with a particular emphasis on the percentages of viable spermatozoa with high mitochondrial
446 membrane potential (HMMP) as reported by [43]. These percentages did not change between
447 treatments thus indicating that these two natural antioxidants have no impact on mitochondrial
448 function. In addition, a significant, even if not dramatic, drop in HMMP cells was recorded between
449 1 and 4 h of incubation. In this context, one should note that the control semen after 1 h of incubation
450 exhibited a very high percentage of viable cells with HMMP (around 85%), and it would therefore
451 have been difficult to further increase this percentage. Other reports indicate that mitochondrial
452 function of boar spermatozoa during and after cryopreservation is impaired [10]; however, no
453 distinction between viable and non-viable spermatozoa was made by the aforementioned work, which
454 could explain why their data were different compared to ours.
455 From our results, it is reasonable to affirm that those cells that survive to cryopreservation are
456 effectively functionally intact, both after 1 and 4 h of incubation at 37°C. As natural antioxidants
457 were not effective in increasing the high percentage of cells with HMMP, the key point in
458 ameliorating freezing protocols is to maintain as many viable spermatozoa as possible after freeze-
459 thawing procedures. It should be also taken into account that mitochondrial activity in boar
460 spermatozoa does not seem to be impaired by ROS (that could be formed in excess during the
461 cryopreservation process) [44]. This information is supported by other reports [8,45–48] that clearly

show that the main energy source for boar spermatozoa is not the oxidative phosphorylation (taking place in the mitochondria), but anaerobic glycolysis [47,49].

Lipid peroxidation was investigated through Bodipy; also, for this parameter we did not find any difference between treatments and no changes between 1 and 4 h of incubation were observed. We checked the mean fluorescent intensity of Bodipy fluorochrome exhibited by viable spermatozoa and it was evident that viable spermatozoa surviving to cryopreservation did not undergo a dramatic change in lipid peroxidation. These data confirm that the oxidative process after cryopreservation is not so heavily detrimental for porcine sperm cells [23,50] and, thus, that impact of the addition of antioxidants may only be marginal.

Together with the high positive impact of R and EGCG on IVF, a very strong effect of R is evident on sperm motility: both total and progressive motility were negatively affected by R Also, the other motility parameters followed the same trend, with a detrimental effect caused by R. The reason for this drop in motility is not clear. Whereas some Authors showed a positive effect of R on rat, bull and human sperm motility [51–53] during liquid storage, other reports on equine [54] and porcine species [26] indicated that this molecule exerts detrimental effects on sperm motility. However, cryopreserved sperm cells seem to be more sensitive to this antioxidant. The key point is that although spermatozoa motility (checked at 1 h incubation at 37°C, at the beginning of the incubation time for IVF) is evidently compromised by R, their fertilizing ability was highest. This surprising and, to some extent, paradoxical effect deserves further research. Therefore, future studies should verify whether frozen-thawed boar sperm treated with R also exhibits higher reproductive performance *in vivo*. While the *in vitro* system is standardized and allows understanding some features of the fertilization process, there is great difference between in vivo and in vitro environment, the first one being more "selective" towards spermatozoa and the second one being fitted to obtain a good fertilization rate. In this context, another question that arises is whether R-treated sperm cells are able to pass the selection operated by the female genital tract and to fertilize a higher number of oocytes if compared to untreated frozen-thawed spermatozoa. At present, we have no data to answer this question, as no experimental evidence is provided from in vivo trials yet.

To better delineate the features of R-treated sperm motility, we performed cluster analysis using kinematics parameters of single sperm cells [13,55,56]. On the basis of cluster analysis sperm were classified into four clusters, with the following characteristics: one included sperm cells with low VSL, VCL, VAP and linearity and was considered as "slow non-progressive"; the second showed average parameters' values; the third showed high velocity and highly linear cells (high VSL, LIN and STR) and was considered to be "rapid progressive"; finally, the last one showed high velocities but low linearity and was defined as "rapid non-progressive". The effects of R on the proportions of each sperm subpopulation were very apparent, as those treatments in which R was present (R and R+EGCG) showed a significant increase in the percentage of slow non-progressive cells, which was concomitant with a dramatic decrease in the percentage of "rapid progressive" cells. In addition, at 1 h, there was a significant increase in the percentage of "rapid non-progressive" cells, more evident in the R groups, but also present in the EGCG one, while at 4 h this cluster is equally represented in both treatments. If we consider "rapid non-progressive" cells as hyperactivated-like spermatozoa [57], this could provide a proof of the ameliorative effect of EGCG and particularly of R on IVF.. Therefore, under the controlled IVF environment and the small IVF volume of the incubation dish, it is likely that a higher number of "hyperactivated" cells could reach the oocytes.

The last result deserving discussion regards DNA integrity. SCSA assay showed a noticeable effect of R, as the presence of this antioxidant in the thawing medium significantly increased the percentage of spermatozoa with fragmented DNA both at 1 and 4 h post-thaw. This fact was really surprising, as other articles reported that freeze-thawing induces slight some detrimental changes on boar sperm nucleus, in particular regarding the protamine-histone-DNA structure and the integrity of disulphide bonds between nucleoproteins [7,41]. Other reports evaluated DNA fragmentation in boar spermatozoa using SCSA and found no difference between fresh and frozen-thawed semen [58,59]. The levels DFI reported in those studies were similar to those obtained in our control group. Therefore, our results on R effects should be interpreted taking into account that [59,60] found negative correlations between DFI and farrow rate and average total number of pigs born, thus suggesting that a spermatozoon with a fragmented DNA can fertilize an egg, but the outcome is lower than that obtained with intact spermatozoa.

In conclusion, R and EGCG showed a positive effect on in vitro fertility of boar spermatozoa if added after thawing, as they both increased penetration rate, with R being much influent on this parameter, so that it masked the effect of EGCG when the two antioxidants were used together showing no synergic effect. Anyway, R showed a negative impact on boar frozen thawed spermatozoa because it negatively affects sperm motility and DNA integrity. All other parameters indicate that both the molecules are, as other antioxidants, almost ineffective (viability, acrosome integrity, lipid peroxidation, mitochondrial function). Taken together these results are difficult to be explained: from one side boar sperm characteristics are poorly enhanced or negatively affected by the molecules, but on the other side, a positive effect on sperm function is evident.

On the basis of our results it is necessary to understand if the positive effect is also maintained in vivo; in addition, further studies are needed to understand the effective mechanism by which the molecules act and to verify whether the development of the *in vitro* fertilized zygotes is normal or could be negatively affected.

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