Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization

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Abstract

Thawing is one of the most delicate process after semen cryopreservation as spermatozoa pass from a dormant metabolic stage to a sudden awakening in cellular metabolism. The rapid oxygen utilization leads to an overproduction of reactive oxygen species that can damage sperm cells, thus causing a significant decrease of fertilizing potential of frozen-thawed spermatozoa. Resveratrol (Res) is a natural grape-derived phytoalexin and Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*); both molecules are known to possess high levels of antioxidant activity. The objective of the present study was to assess the effect of different concentrations of Res (0.5, 1 or 2 mM; Experiment 1) or EGCG (25, 50 or 100 μM; Experiment 2) supplementation to thawing boar semen extender on sperm quality parameters (viability and acrosome integrity) and in vitro fertilization (IVF).

Semen after thawing and dilution with three volumes of Beltsville Thawing Solution (BTS), was immediately divided in control group without antioxidants addition (CTR) and either Res or EGCG groups. Sperm viability and acrosome integrity were evaluated in CTR, Res or EGCG groups after 1 h of incubation at 37°C.

The addition of different doses of Res or EGCG to thawing extender for 1 h did not induce any effect on boar sperm viability and acrosome integrity. However, both Res and EGCG treated samples exhibited a significantly higher penetration rate compared with CTR when used for IVF. In particular the treatment with all the EGCG concentrations increased the penetration rate ($P<0.01$) while only Res 2 mM induced a significant increase of this parameter ($P< 0.01$). In addition, EGCG 25 and 50 μM supplementation significantly increased total fertilization efficiency as compared to control (EGCG 25 μM: 40.3 ± 8.2 vs 26.8 ± 9.5, $P< 0.05$; EGCG 50 μM: 40.4 ± 7.8 vs 26.8 ± 9.5, $P < 0.01$). The same effect was observed with Res 2 mM (51.0 ± 7.6 vs 29.6 ± 11.3, $P<0.01$).

In conclusion, our results indicate that the addition of different doses of the two antioxidants to thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome integrity, efficiently improves in vitro penetration rate. Moreover, both molecules (EGCG 25 and 50 μM and Res 2 mM) significantly increases the total efficiency of fertilization.

Key words: boar semen, cryopreservation, antioxidants, fertilization, Resveratrol, Epigallocatechin-3-gallate

1. Introduction

Sperm cryopreservation is the most efficient method for long term sperm storage (reviewed in [1]). However, frozen-thawed boar semen is not routinely used because of the high performance of long-term extenders for liquid storage and the non optimal quality of thawed boar spermatozoa. Anyhow it is important to create an efficient cryopreserved semen gene bank, planning insemination at artificial insemination centers, maintaining genetic diversity and promoting the rapid growth of swine models [2,3].

During the cryopreservation process, spermatozoa undergo a variety of harmful cellular alterations called “cold shock”, mainly induced by the increase of reactive oxygen species (ROS) levels [4,5]. ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radicals (OH^-), generated during intermediate steps of oxygen reduction, are known for their ability to damage cellular proteins, DNA and plasma membrane lipids, due to their free radical nature [6]. Even if very low and controlled concentrations of ROS are required for sperm hyperactivation, capacitation, acrosome reaction and zona binding events [7–9], when ROS are overproduced, spermatozoa cannot easily adapt to this condition and oxidative stress occurs leading to cell damage [6].

On these bases, frozen-thawed boar spermatozoa may present nucleoprotein-DNA structural alterations [8,10–12] and capacitation-like changes that could lead to an important reduction in fertilizing potential of frozen-thawed sperm [13]. In order to reduce oxidative damage, one approach is to supplement semen extender with enzymatic and non-enzymatic antioxidant compounds during freeze-thawing.

Resveratrol (3,4’,5-trihydroxy-trans-stilbene) (Res) is a polyphenolic natural product with a stilbene structure isolated at first from the roots of white hellebore in 1940 [14] and later from Polygonum cupsidatum, a medicinal plant. Today it is widely consumed in the Mediterranean diet in the form of peanuts, grapes and wine. Res shows many biological activities such as anti-inflammatory, cardioprotective, chemopreventive and antiapoptotic [15,16]. Moreover, Res has been reported to possibly act as antioxidant thanks to its ability to reduce mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes [16]. It has been reported that mouse [17], bovine [18] and human spermatozoa [19] can be protected by Res from experimentally induced oxidative stress. A protective effect of Res against membrane oxidative damage but not against the loss of motility induced by the cryopreservation of human semen, has been observed [20]. Furthermore, Res is effective in minimizing post-thawing DNA damage in human spermatozoa [21] and in improving post-thaw bull sperm quality in terms of sperm motility, high mitochondrial activity and DNA integrity [22]. In frozen-thawed ram sperm the

addition of Res to the Tris-egg yolk-glycerol extender has been shown to reduce sperm mitochondrial membrane potential [23].

Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*) and is reported to possess a high level of antioxidant activity [24,25]. The supplementation of canine sperm with green tea polyphenol extracts (PFs) as been demonstrated to improve motility and viability of spermatozoa during long-term liquid storage [26,27]. Moreover, pre-treatment of boar spermatozoa with PFs prior to freezing exhibited significantly higher degrees of post-thaw sperm viability and acrosomal integrity [28]. The beneficial effect of EGCG has been observed during liquid storage at 15°C of sorted boar semen: it increased the percentage of viable spermatozoa and inhibited caspase activation [29].

On these bases, the objective of the present study was to assess whether Res or EGCG supplementation of thawing boar semen extender is effective in influencing sperm quality parameters (viability and acrosome integrity) and in vitro fertilization (IVF).

2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Sigma–Aldrich (Milan, Italy).

2.1 Sperm thawing

The study was performed using commercial frozen semen from 3 Large White boars purchased by Suiseme Srl (Saliceta San Giuliano, Modena, Italy). Straws (0.5 mL/straw) were thawed for 30 sec in water bath at 37ºC and immediately diluted, at the same temperature, with three volumes of Beltsville Thawing Solution (BTS).

Semen was immediately divided in the following experimental groups: CTR (control: without antioxidant addition), and either Res (addition of 0.5, 1, 2 mM R to BTS thawing extender; Experiment 1) or EGCG (addition of 25, 50, 100 µM EGCG to BTS thawing extender; Experiment 2). Only sperm samples with viability > 40% as assessed immediately after thawing were used for the experiments.

Sperm viability and acrosome integrity were evaluated 1 h after thawing in CTR and Res or EGCG groups. After 1 h of incubation at 37°C in either absence or presence of different doses of Res or EGCG, semen samples were washed and used for in vitro fertilization (IVF).

2.2 Post-thaw spermatozoa evaluation

2.2.1 Sperm viability assessment

Sperm viability was evaluated by incubating 25 µL of semen with 2 µL of a 300 µM Propidium Iodide (PI) stock solution and 2 µL of a 10 µM SYBR-14 stock solution (LIVE/DEAD®Sperm Viability kit, Molecular Probes, Invitrogen), for 5 min at 37°C in the dark. After incubation, 10 µL of sperm suspensions were analyzed with a Nikon Eclipse epifluorescence microscope using a double-band-pass filter for green and red fluorescence. The spermatozoa with green or red fluorescence on the head were considered live or dead, respectively (see supplementary file, panel A). At least 200 cells were counted in each analysis.

2.2.2 Acrosome integrity assessment

Acrosome integrity was measured with a FITC conjugated lectin from Pisum Sativum (FITC-PSA) which labels acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended with ethanol 95% and fixed at 4°C for 30 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5.0 µg PSA-FITC/1 mL H2O) for 20 min in darkness. After staining samples were washed in PBS and mounted with Vectashield mounting medium with PI (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with the above described fluorescence microscope. The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, while a partial or total absence of fluorescence was considered to indicate acrosome disruption or acrosome reaction (see supplementary file, panel B).

2.3 In vitro maturation (IVM) of cumulus-oocyte-complexes

Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with physiological saline at 30-35°C. Cumulus oocyte complexes (COCs) from follicles 3-6 mm in diameter were aspirated using 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, Roskilde, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs with complete and dense cumulus oophorus were used. After three washes in NCSU 37 [30] supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 µM ß-mercaptoethanol and 10% PCV2-PCR-negative porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µl of the same medium per well and cultured at 39°C in humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 h of *in vitro* maturation, the IVM medium was supplemented with 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10

IU/mL hCG (Corulon, Intervet). For the last 22-24 h COCs were transferred to fresh maturation medium [31].

2.4 In vitro fertilization (IVF)

For in vitro fertilization, thawed semen after 1 h of incubation at 37°C with or without antioxidants, was washed twice with BTS and finally resuspended with Brackett & Oliphant's [32] medium supplemented with 12% foetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/mL caffeine (IVF medium). Sperm concentrations were evaluated and 45–50 matured oocytes, freed from cumulus cells by gentle repeated pipetting, were transferred to 500 mL IVF medium containing 1 x 10^6 sperm/mL. After 1 h of co-culture, oocytes were transferred to fresh IVF medium and cultured for 19 h until fixation in acetic acid/ethanol (1 : 3) for 24 h and stained with Lacmoid.

The oocytes were observed under a phase-contrast microscope and the following parameters were assessed:

(1) penetration rate (number of fertilized oocytes/ number of inseminated oocytes);

(2) monospermy rate (number of oocytes containing only one sperm head–male pronucleus/ number of penetrated oocytes);

(3) total efficiency of fertilization (number of monospermic oocytes/number of inseminated oocytes).

Degenerated and immature oocytes were not counted.

2.5 Statistical analysis

Data were analyzed using R version 3.0.3 [33]. Significance was set at $p<0.05$ unless otherwise specified. Results are expressed as mean ± standard deviation. One-way ANOVA and Tukey post hoc test were performed to assess the difference in sperm viability and acrosome integrity percentages between treatments after arcsine transformation. In vitro fertilization data were analyzed by a generalized linear model (GLM) and a Tukey post hoc test was used to assess difference between treaments.

3. Results

3.1 Experiment 1: Effect of Res supplementation to thawed boar sperm on viability, acrosome integrity and IVF parameters

The addition of Res 0.5, 1 or 2 mM to thawed sperm for 1 h (Res 0.5, Res 1, Res 2) did not induce any significant effect on sperm viability as compared to semen without Res (CTR) (51.7% ± 9.4%, 48.5% ± 5.3, 48.4% ± 8.6, 43.8% ± 10.7 in CTR, Res 0.5, Res 1, Res 2 respectively) (Fig.1A).

Moreover, the supplementation of different concentrations of Res to thawed sperm for 1 h did not improve the percentage of spermatozoa with intact acrosome compared to CTR group (86.4 % ± 6.1 %, 82.5% ± 3.2 %, 81.9 % ± 2.6 %, 82.4% ± 1.0 %, in CTR, Res 0.5, Res 1, Res 2 respectively) (Fig. 1B).

When Res 2 mM treated samples were used for IVF a significantly ($P<0.01$) higher penetration rate (number of oocytes penetrated/total inseminated) compared with CTR was observed (Table 1). Res 2 mM also exerted a positive effect ($P<0.01$) on the total efficiency of fertilization as compared to CTR group but did not induce any effect on monospermy rate (Table 1).

Table 1. Effect of Res (0.5, 1 and 2 mM) supplementation to thawed boar sperm on IVF parameters.

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	237	33.8 ± 12.4[a]	87.3 ± 5.5	29.6 ± 11.3[a]
Res 0.5	152	42.3 ± 2.7 [a]	73.2 ± 11.7	30.9 ± 5.1 [a]
Res 1	263	46.8 ± 5.0 [a §]	82.3 ± 12.5	38.5 ± 6.8 [a]
Res 2	192	68.8 ± 6.4 [b]	74.4 ± 12.3	51.0 ± 7.6 [b]

Penetration rate (number of fertilized oocytes / number of inseminated oocytes).

Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

Values are expressed as the mean ± SD of six replicates (three boars).

Different letters indicate significant difference for $P < 0.01$ in column between treatments.

§ indicates significant difference in column for $P = 0.05$ compared with control.

3.2 Experiment 2: Effect of EGCG supplementation to thawed boar sperm on viability, acrosome integrity and IVF parameters

The addition of different concentrations of EGCG (25, 50 and 100 μM) to thawed sperm for 1 h did not exert any significant effect on sperm viability (45.1% ± 3.8 %, 46.6 % ± 7.0 %, 45.4 % ± 10.1 %, 48.2 % ± 5.8 %, in CTR, EGCG 25, EGCG 50, EGCG 100 μM respectively) and acrosome integrity (86.2 % ± 5.5 %, 84.3% ± 6.3 %, 87.1 % ± 1.5 %, 85.4 % ± 1.5 %, in CTR, EGCG 25, EGCG 50, EGCG 100 μM respectively) (Fig. 2A and 2B). Oocytes inseminated with thawed spermatozoa

pretreated with all the different EGCG concentrations tested presented a significantly ($P < 0.01$) increased penetration rate compared to CTR (Table 2). In addition, 25 and 50 µM EGCG supplementation exerted a positive effect ($P<0.01$) on the total efficiency of fertilization without inducing any effect on monospermy rate (Table 2).

Table 2. Effects of EGCG (25, 5 and 100 µM) supplementation to thawed boar sperm on IVF parameters.

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	247	31.9 ± 9.4^{a}	83.7 ± 8.8	26.8 ± 9.5^{aA}
EGCG 25	158	55.0 ± 5.3^{b}	74.5 ± 19.5	40.3 ± 8.2^{aB}
EGCG 50	234	54.8 ± 9.0^{b}	74.5 ± 12.3	40.4 ± 7.8^{bB}
EGCG 100	244	48.6 ± 10.4^{b}	72.6 ± 18.7	34.6 ± 9.9^{aA}

Penetration rate (number of penetrated oocytes / number of inseminated oocytes).

Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

Values are expressed as the mean ± SD of six replicates (three boars).

Different lowercase letters indicate significant difference for $P < 0.01$ in column between treatments.

Different capital letters indicate significant difference for $P < 0.05$ in column between treatments.

4. Discussion

Sperm cryopreservation is the best technology to store boar semen for long periods for planning artificial insemination and preserving genetic material through time; nevertheless, frozen-thawed sperm is not routinely used in pig industry (less than 1%) [34]. The main reason is that freezing and thawing procedures lead to a reduced sperm fertilizing ability and reproductive performance [3] because of two important events occurring during cryopreservation procedure: the vast production of ROS and the parallel decrease in antioxidants defenses [35]. On this basis, in order to improve post-

thaw quality of boar sperm, various antioxidants are routinely added during freezing protocols and new molecules are continuously studied [36].

In our study, Res supplementation did not induce any effect on sperm viability as already reported by other Authors even if in different experimental conditions and species: liquid storage of boar [37] and stallion [38] semen or cryopreservation of bull sperm [22].

Thawed boar spermatozoa can show membrane rearrangements and consequent lipid packing faults [39]. In this way, efficiency of calcium channels could be compromised, leading to an increase in calcium concentration inside the cell that could lead to capacitation like changes [40]. Liu et al. [41] reported that Res affects intracellular calcium release, so it could be important in preventing premature sperm capacitation and, consequently, acrosome reaction; in this study, however, Res supplementation to thawing media for one hour did not preserve sperm acrosome integrity. Similar results have been obtained by Martín-Hidalgo et al. [37] who reported that storage at 17°C of fresh boar semen with Res did not exert any effect on this parameter and by Silva et al. [23] who added Res to ram cryopreserved semen.

In our study, no protective effect of EGCG was observed on acrosome integrity in agreement with the results obtained by Vallorani et al. [29] on liquid storage of boar sexed semen. Different results have been shown by Kitaji et al. [28] who observed a higher post-thaw viability and acrosome integrity of boar spermatozoa incubated prior to freezing in a semen extender supplemented with 0.01% of green tea polyphenol extracts.

When either Res 2 mM or EGCG 25, 50 and 100 µM treated samples were used for IVF we observed a significantly higher penetration rate ($P<0.01$) compared with control; in addition, Res 2 mM and EGCG 25 and 50 µM supplementation exerted a positive effect ($P<0.01$, $P< 0.05$ and $P<0.01$ respectively) on total efficiency of fertilization.

Our results agree well with those from several studies examining the effect of EGCG during IVF in different experimental conditions. Pre-incubation of boar spermatozoa with green tea polyphenol extracts prior to freezing has been shown to increase both the efficiency of IVF (rates of monospermic oocyte) and blastocyst formation [28]. Kaedei et al. [42] demonstrated that penetration rate improves when boar frozen-thawed spermatozoa are co-incubated with oocyte in IVF medium supplemented with 50 µM EGCG. A positive influence of EGCG has also been recorded on fresh boar and stallion spermatozoa under capacitating conditions, in which a significant increase in the number of sperm bound to oocyte zona pellucida was observed [43,44]. Moreover, the presence of this polyphenol during pig IVF using fresh semen was able to increase, in a dose response manner, the fertilization rate [43].

Therefore, EGCG could likely modulate sperm capacitation process probably thanks to its antioxidant activity; in fact under capacitating conditions EGCG has been demonstrated to reduce H_2O_2 production in boar spermatozoa [43] and to be able, in stallion spermatozoa, to reverse the inhibition of mitochondrial complex I by rotenone, a molecule known to induce mitochondrial ROS production [44].

To our knowledge, Res treated semen has never been used for IVF so far, while it is known that Res supplementation during IVM and IVC improves developmental potential of porcine oocytes and porcine embryo development [45–47].

Studies performed on mouse, human, bovine and ram spermatozoa demonstrated that Res could effectively protect spermatozoa from oxidative stress induced by cryopreservation or pro-oxidant agents supplementation [17,19–23]. In our work, although Res 2 mM added to thawing sperm media for 1 hour did not exert any effect on parameters assessed (viability and acrosome integrity), it significantly ($P<0.01$) increased the penetration rate and total efficiency of fertilization. The encouraging beneficial effect of Res and EGCG addition to the thawing extender was evident during IVF and therefore after washing away the tested molecules: the two molecules were left with semen for 1 hour after thawing and then the medium was discarded and spermatozoa washed with fresh Bracket and Oliphant's medium. This suggests that the protective action during thawing can lead to positive effects on sperm function that, in turn, are responsible for the subsequent increased fertilizing ability even if the molecules are no more present. Therefore, it cannot be excluded (and it should certainly be tested) that Res or EGCG pretreatment of thawed semen could lead to positive effects also in vivo.

In conclusion, our results indicate that the addition of Res 2 mM or EGCG 25, 50 and 100 µM to thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome integrity, improves in vitro penetration rate; in addition, EGCG 50 µM and Res 2 mM increases the total efficiency of fertilization. These results could be possibly important not only *in vitro*, but also *in vivo* as the addition of one of these two antioxidants in the commercial thawing solution might enhance sperm fertilizing ability and reproductive performance during porcine AI with frozen-thawed boar semen.

Acknowledgements

The work was supported by "Fondazione Sfameni" and "Fondazione del Monte di Bologna e Ravenna" grants.

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Fig. 1 Boxplots representing sperm viability (upper panels) and acrosome integrity (lower panels) of frozen-thawed spermatozoa after 1 h of incubation at 37°C without (CTR) or with Res (0.5, 1 or 2 mM)(left panels) and EGCG (25, 50 or 100 µM) (right panel). Each experiment was repeated six times (three boars).

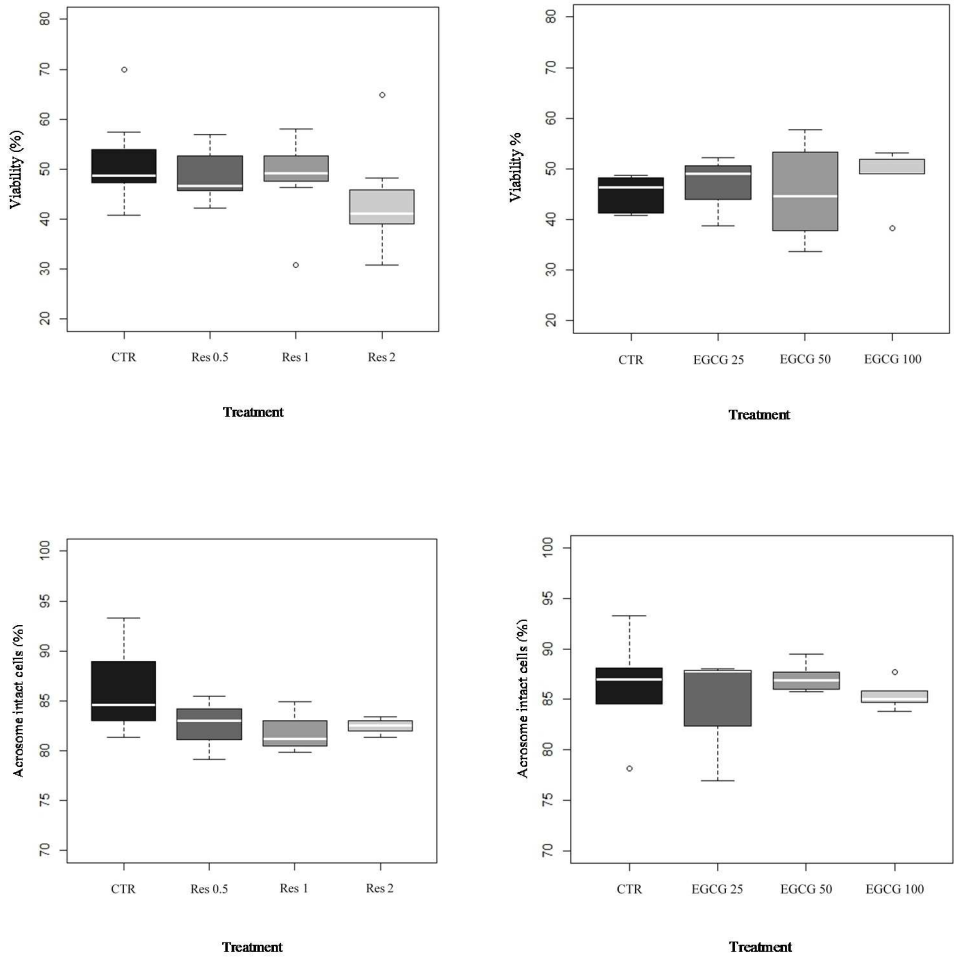

Fig. 1