Theriogenology 90 (2017) 88–93

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

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

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form
18 November 2016
Accepted 20 November 2016
Available online 22 November 2016

Keywords:
Boar semen
Cryopreservation
Antioxidants
Fertilization
Resveratrol
Epigallocatechin-3-gallate

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.

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

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http://dx.doi.org/10.1016/j.theriogenology.2016.11.020
0093-691X/

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 anti-apoptotic [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 37 °C 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 °C–35 °C. Cumulus oocyte complexes (COCs) from follicles 3 to 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% fetal calf serum (Gibco, Invitrogen, Italy) and