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Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells

G. Collodel[a,b], M.G. Federico[a], M. Geminiani[a], S. Martini[c,e], C. Bonechi[c,e], C. Rossi[c,e], N. Figura[d], E. Moretti[a,b,*]

[a] Department Biomedical Sciences, Applied Biology Section, Italy
[b] Interdepartmental Centre for Research and Therapy of Male Infertility, Italy
[c] Department of Pharmaceutical and Applied Chemistry, University of Siena, Italy
[d] Department of Internal Medicine, Endocrine-Metabolic Sciences and Biochemistry, University of Siena, Italy
[e] Centre for Colloid and Surface Science (CSGI), University of Florence, Via della Lastruccia, 3, Sesto Fiorentino, FI, Italy

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ABSTRACT

Resveratrol is a phytoalexin with antioxidant properties. We evaluated resveratrol toxicity in swim-up selected human sperm and in rat spermatocytes and spermatids separated by the STAPUT method. Resveratrol antioxidant activity was tested against lipid peroxidation (LPO) induced by *tert*-butyl hydroperoxide. LPO was evaluated using the C11-BODIPY$^{581/591}$ probe and transmission electron microscopy in samples incubated with and without resveratrol. LD50 for human sperm and rat spermatids was 50 μM; spermatocytes were more sensitive to resveratrol cytotoxicity. Sperm motility increased progressively at 30 μM, 15 μM and 6 μM. 15 μM resveratrol acts against LPO, preserving sperm chromatin and plasma membranes. LPO were more marked in spermatocytes than in spermatids and the effect of resveratrol was more evident in spermatocytes. In this study, the scavenger properties of resveratrol were demonstrated *in vitro* in human sperm and rat germ cells, thus resveratrol could be added to the media used in assisted reproduction techniques and cryopreservation when oxidative stress is exacerbated.

1. Introduction

Reactive oxygen species (ROS) are products of normal cellular metabolism and they play a functional role in many cell types as second messengers. When ROS are produced at a very low concentration, they trigger cell signaling events and regulate physiological function [1,2].

Sperm were the first type of cell reported to produce free radicals and MacLeod [3] noted that the incubation of sperm under high oxygen tension leads to a rapid loss of sperm motility. Low level production of ROS by sperm supports some main functions, such as capacitation, acrosome reaction, zona pellucida binding and oocyte fusion [4]. Nevertheless, uncontrolled ROS production can play an important role in causing sperm aberration, leading to infertility. What is usually called "oxidative stress" appears to be the result of defects in the balance between the concentration of ROS and the antioxidant scavenging system. Sperm membranes are rich in polyunsaturated fatty acid, which makes them very susceptible to oxygen-induced damage mediated by lipid peroxidation (LPO) [5]. Oxidative stress has been suggested to be an important factor in the aetiology of poor sperm function through peroxidative damage to the cell membrane, to DNA (inducing single- and double-strand DNA breaks) and to proteins [6–8].

Seminal plasma and sperm are endowed with an array of protective antioxidants, such as the glutathione peroxidase/reductase system, superoxide dismutase, catalase and low-molecular weight antioxidants, vitamin E, vitamin C, urate, and albumin, which scavenge ROS in order to prevent possible cellular damage [9]. One of the rational strategies for counteracting oxidative stress is to increase the scavenging capacity of seminal plasma. However, the use of antioxidant supplementation to reverse the effect of ROS is still being debated [2,10].

Resveratrol (RSV) is a natural phytoalexin with antioxidant properties that is widely consumed in the Mediterranean diet in the form of peanuts, grapes and wine. Interest in the compounds present in wine has increased since epidemiological studies indicated an inverse correlation between red wine consumption and the incidence of cardiovascular disease [11]. In addition, RSV has exhibited a broad range of biological activities, including antiinflammatory, antiviral and antitumoral properties [12].

* Corresponding author at: Department of Biomedical Sciences, Applied Biology Section, University of Siena, Policlinico Santa Maria alle Scotte, Viale Bracci, 53100 Siena, Italy. Tel.: +39 577 233511; fax: +39 0577 233527.
E-mail address: moretti@unisi.it (E. Moretti).

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Regarding the male reproductive system, some recent *in vivo* studies in animal models demonstrated that RSV administration enhances sperm production in rats by stimulating the hypothalamic–pituitary–gonadal axis without inducing adverse effects [13]. Moreover, RSV may decrease germ cell apoptosis in mice and rats [14,15] and have a positive effect by triggering penile erection and by enhancing blood testosterone levels, testicular sperm count and epididymal sperm motility, as demonstrated in rabbits [16].

A protective effect of RSV, against oxidative damages but not against the loss of motility induced by the cryopreservation of human semen, has recently been observed [17].

This study was designed to evaluate, *in vitro*, the effect of RSV in ejaculated human sperm in order to assess the possible toxicity of such a compound. RSV was then used to investigate its antioxidant properties against LPO induced in human sperm by *tert*-butyl hydroperoxide (TBHP). The oxidative damage in samples incubated with and without RSV was evaluated using the C11-BODIPY$^{581/591}$ probe and transmission electron microscopy (TEM). The study was also extended to two populations of spermatocytes and round spermatids from rat testis separated with the STAPUT method to assess the analysed properties of RSV, also in cells of seminiferous epithelium.

2. Materials and methods

2.1. Semen analysis

Semen samples were obtained from men recruited at the Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena.

Semen samples were collected by masturbation after 3–5 days of sexual abstinence and examined after liquefaction for 30 min at 37 °C. Volume, pH, concentration and motility were evaluated according to World Health Organization guidelines [18]. Patients were informed of and gave written consent for the procedures related to the study.

2.2. Sperm selection: swim-up

Motile sperm fraction was selected by the swim-up technique. A direct swim-up of sperm from semen was performed: 1 ml of each semen sample was placed in a sterile conical centrifuge tube and gently layered with 1.2 ml of Quinn's® Sperm Washing Medium (Sage, *In vitro* fertilization, Inc., Trumbull, CT, USA). The tubes, inclined at an angle of 45°, were incubated for 45 min at 37 °C with 5% CO_2. The uppermost 1 ml of medium was then recovered, which contained highly motile sperm cells.

2.3. Rat germ cells isolation

Pachytene spermatocytes and round spermatids were obtained from three 35-day-old Wistar rats (100 g of body weight) (Charles River, Calco, Italy). Rats were sacrificed by cervical dislocation and testes were collected and placed in Minimum Essential Medium supplemented with pyruvate and lactate. After removal of the tunica albuginea, the testes were placed in culture medium containing collagenase (activity 0.450 unit/ml) and incubated for 10 min to remove the interstitium, which was discarded. A second incubation in a shaking water bath at 32 °C for 45 min with the same enzymes and 0.1% Bovine Serum Albumin and DNase was performed to partially digest the basal lamina of the tubules. The cell suspension obtained after enzymatic digestion was centrifuged and fractionated by velocity sedimentation at unit gravity on 0.5–3% albumin gradient (STAPUT method). The two collected and purified cellular fractions were made up of pachytene spermatocytes and spermatids. All experiments were approved by the Ethics Committee of the University of Siena.

2.4. RES preparation and determination of cytotoxicity

Trans-RSV (3,5,4′-trihydroxy-*trans*-stilbene) was purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland) and used without further purification. 100 μM RSV solutions were prepared by dissolving the appropriate amount of RSV in phosphate buffered saline (PBS) solution (pH 7.4). 10 μL of 0.1 M NaOH solution was added to help dissolve the RSV. The RSV solution was stored under nitrogen.

In order to test the possible cytotoxicity of the compound used, the swim-up selected sperm and the two isolated fractions of rat germ cells were added to RVS dissolved in Biggers, Whitten, Whittingham (BWW) bicarbonate-free medium at different concentrations: 100 μM, 50 μM, 15 μM, 6 μM. Mixtures were incubated at 37 °C and 5% CO_2 for 1 h. The sperm and the germ cells were then stained with 0.5% eosin Y (CI 45380) in 0.9% aqueous sodium chloride solution. A few minutes after staining, the samples were observed by light microscope (Leica, Wetzlar, Germany) and stained (dead) cells and unstained (living) cells were scored. The control was an aliquot of selected semen or cell fractions devoid of RSV and treated in the same conditions.

Sperm motility was evaluated in swim-up selected sperm incubated with RSV, using a Burker counting chamber and categorizing them by the different grades of motility (rapid and slow progressive motility, nonprogressive motility, and immotile sperm; WHO [18]. The control was an aliquot of selected semen devoid of RSV and treated in the same conditions.

All experiments were carried out in triplicate and the results are reported as mean values.

2.5. LPO induction and RSV treatment

Selected human sperm, rat pachytene spermatocytes and round spermatids were incubated with 5 μM of a fluorescent fatty acid probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4-adiaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$, Molecular Probes, Eugene, USA) at 37 °C for 30 min. Excess probe was removed by washing the cells twice with BWW bicarbonate free medium for 10 min.

C11-BODIPY$^{581/591}$ is able to intercalate in the phospholipids bilayer and LPO was localized to observe the changes in C11BODIPY$^{581/591}$ fluorescence. The intact probe fluoresces red when it is intercalated into the membrane (λ excitation = 590 and λ emission = 635 nm) and shifts to green (λ excitation = 485 and λ emission = 535 nm) after oxidative radicals attack.

After incubation with this probe, each sample was then divided into two aliquots as follows: sperm or cells treated with TBHP (45 μM) and RSV (6 μM and 15 μM) and sperm or cells treated with TBHP without RSV. Both aliquots were incubated at 37 °C and 5% CO_2 for 1 h. The staining was examined by Leitz Aristoplan Microscope fluorescence (Leica, Wetzlar, Germany). Images were taken using Leica Q Fluoro Standard, Leica Chantal software. The presence of the green signal was estimated by the same researcher in two degrees: high and low fluorescence. For each sperm sample and for each rat germ cell fraction, 300 cells were examined. All experiments were carried out in triplicate and the results are reported as mean values.

2.6. Transmission electron microscopy (TEM)

Human sperm and rat cell fractions treated with TBHP and RSV (15 μM) and with TBHP without RSV were processed for transmission electron microscopy (TEM).

Sperm samples were fixed in cold Karnovsky fixative and maintained at 4 °C for 2 h and then washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1 h at 4 °C, dehydrated and embedded in Epon Araldite. Ultra-thin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate and observed and photographed with a Philips EM208 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands). Three hundred ultra-thin sperm sections were analysed for each experiment. Major submicroscopic characteristics were recorded by trained examiners who were blind to the experiment.

For human sperm, a minimum of 300 sperm sections were analysed for each sample and the anomalies related to the acrosome (reacted, swollen), the chromatin (disrupted), the axoneme (disorganized) and the plasma membrane (broken) were quantified.

For isolated rat germ cell fractions, 300 cell sections were analysed for each sample and the percentage of normal and necrotic cells (altered) was calculated. A cell is considered necrotic when shows broken plasma membrane, disrupted chromatin and vacuolated cytoplasm.

All experiments were carried out in triplicate and the results are reported as mean values.

3. Results

We tested the effect of the possible cytotoxicity of RSV and its scavenger properties in selected human sperm and rat germ cells fractioned as a meiotic population composed of pachytene spermatocytes and a post meiotic fraction of early spermatids.

3.1. Determination of cytotoxicity

The effect of RSV on the viability of human sperm, rat spermatocytes and spermatids was determined by eosin Y dye exclusion test.

RSV at 100 μM exerts a cytotoxic activity against human sperm and against rat spermatocytes and spermatids (Figs. 1–3) and it acts