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Essential rosemary oil protects testicular cells against DNA-damaging effects of H_2O_2 and DMNQ

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ABSTRACT

Rosemary oil (RO) is popular in the Mediterranean region as a culinary additive which has the ability to protect delicate organs such as liver, brain and heart. We examined the effect of RO consumption on resistance of rat testicular cells (TCs) against DNA-damaging effects of the oxidative agents H_2O_2 and DMNQ and on the activity of the antioxidant enzymes glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). DNA lesions were detected by conventional and modified comet assay and the activities of GSH-Px and SOD were measured spectrophotometrically. Since TCs represent a mixture of haploid, diploid and tetraploid cells, we used flow cytometry for their differentiation and calculation of DNA-damaging effects of H_2O_2 and DMNQ in cells of different ploidy. The results showed that the oxidative DNA lesions were significantly reduced in TCs from rats administered RO; however, the activity of antioxidant enzymes did not differ in TCs from control and RO-supplemented rats.

1. Introduction

A significant decrease in fertility of men connected with a deteriorating quality of sperm as well as increased incidence of testicular cancer (Forman & Møller, 1994) initiated a growing interest in research of human and rodent male germ cells over the last 20 years. Much evidence has implicated oxidative agents as an etiological factor in the development of male infertility. Increase in oxidative stress probably plays a critical role in the induction of sperm abnormalities (Aitken, 1995). In somatic cells, unrepaired oxidative DNA damage is considered to be the stimulus for initiation of a variety of biological ramifications, including inhibition of transcription and replication, apoptosis, mutagenesis and carcinogenesis. Moreover, the potential consequences of the damage to male germ cell DNA may be deleterious for the offspring, since mutations in the paternal genome may be passed onto the progeny and lead to malformations and genetic defects in the offspring.

Oxidative agents remove electrons from another substance and are thus themselves reduced. They induce tissue injury mediated by intracellular generation of potentially deleterious reactive oxygen species – ROS (Farber, Kyle, & Coleman, 1990). ROS are formed in cells as byproducts of normal cellular metabolism or by external sources and they react with DNA to form genotoxic lesions, such as 8-oxo-7,8-dihydroguanine (8-oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy), thymine glycol (TG), and 5-hydroxycytosine (5-ohC). While 8-oxoG residues are mutagenic, FaPy and TG are cytotoxic. Both testicular cells (TCs) and sperm contain oxidised DNA lesions, which increase following exposure to a variety of drugs, irradiation and environmental factors. The level of DNA damage in terms of 7,8-dihydro-8-oxo-2′-deoxyguanosine has been closely correlated to male fertility and sperm parameters. However, male germ cells from humans and rats are highly proficient in base excision repair (Olsen et al., 2003). Nearly all oxidatively induced DNA lesions (except double strand breaks) can be repaired *via* the DNA base excision repair (BER) pathway in organisms ranging from *Escherichia coli* to mammals (Hazra et al., 2007).

In our study we used two strong oxidants, hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). H_2O_2 induces predominantly DNA breaks *via* the formation of ·OH radicals by the Haber–Weiss reaction, which is catalysed by ferric ion. As a reactive oxygen species, it is generated from nearly all sources of the oxidative cycle and has the ability to diffuse in and out of cells and tissues (Barbouti, Doulias, Nousis, Tenopoulou, & Galaris, 2002). Oxidative stress induced by physiological concentrations of H_2O_2 decreased significantly both testosterone production and 3 β-hydroxysteroid dehydrogenase activity in adult interstitial Leydig cells (Gautam, Misro, Chaki, & Sehgal, 2006). Among ROS, ·OH

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has an extremely high reactivity with DNA, lipids, and proteins, leading to cellular injury (Dröge, 2002). The second oxidant used, DMNQ, belongs to redox cycling quinones. The toxicity of quinones is induced by two principal mechanisms. They (1) react covalently with thiols (reducing glutathione-GSH) or the cysteine residues of proteins to form arylation products that eventually cause cellular damage (Tapper, Sheedy, Hammermeister, & Schmieder, 2000), or (2) induce oxidative stress *via* redox cycling. Quinones undergo one-electron reduction to yield semiquinone radicals, which are then reoxidised in cooperation with molecular oxygen to form superoxide anions (Kappus & Sies, 1981). These are converted into ROS (hydrogen peroxide, hydroxyl radical, singlet oxygen) through various pathways within cells.

Under normal conditions, the rate and magnitude of oxidation is balanced by the rate of oxidant elimination. However, an imbalance between prooxidants and antioxidants results in oxidative stress, which is the pathogenic outcome of oxidant overproduction that overwhelms the cellular antioxidant capacity. It was found (Jones, Mann, & Sherins, 1979) that testes, being rich in polyunsaturated fatty acids and having rather poor antioxidant defences, are much more vulnerable to peroxidation injury than other tissues. In individual testicular cell types, Bauché, Fouchard, and Jégou (1994) observed small but statistically significant differences among the activity of enzymes involved in antioxidant defence. Natural antioxidants might be helpful in preventing oxidative stress related to functional abnormalities in testes. An example of a natural antioxidant which possesses various health benefits and therapeutic effects is essential oil of rosemary. A detailed description of antioxidant, antibacterial, anticarcinogenic and further properties of rosemary oil was included in our recent paper (Horváthová, Slameňová, & Navarová, 2010). In the present investigation we examined the effects of 14-day consumption of RO (given to rats in drinking water) on the resistance of freshly isolated rat TCs against DNA-damaging effects of H_2O_2 and DMNQ. Conventional and modified comet assay techniques were used for evaluation of DNA lesions in cells. As TCs isolated from whole testes represent a mixture of different stages of developing germ cells, as well as somatic cells characterised by different ploidy, flow cytometric analysis of ploidy was used as a supplementary test for differentiation of haploid, diploid and tetraploid cells. The sensitivity of all categories of cells against the DNA-damaging effects of the oxidative agents studied was compared. In addition to the sensitivity of DNA against H_2O_2 and DMNQ, also the activities of two important antioxidant enzymes, i.e., glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), were evaluated in TCs isolated from both control and RO-fed rats.

2. Materials and methods

2.1. Rosemary oil (RO) and chemicals

RO, used in our experiments was prepared from the above-ground parts of *Rosmarinus officinalis* (by steam distillation) and chromatographically analysed by Calendula Inc. (Nová Ľubovňa, Slovakia). It contains approximately 25% 1,8-cineole, 19% α-pinene, 19% camphor, 17% *p*-cymene, 9% camphene, 5% β-pinene, 2% borneol, and 4% unidentified compounds (Lot 5-014-009-12-06). It was kept at room temperature, dissolved in Cremophor® EL (Fluka, Sigma–Aldrich Co., Steinheim, Germany) and 70 °C drinking water to 1.25%, 2.5% and 5% stock solutions that were diluted in drinking water to final concentrations of 0.125‰, 0.25‰ and 0.50‰ RO. Control rats drank Cremophor EL-supplemented water (1‰).

Hydrogen peroxide (H_2O_2; Sigma, Sigma–Aldrich Co., Steinheim, Germany) was stored at 4 °C and diluted in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free) to final concentrations of 100 and 250 μM immediately before treatment of TCs. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ; Sigma, Sigma–Aldrich Co., Steinheim, Germany) was kept at a concentration of 50 mM at −20 °C. Isolated TCs (1×10^6) were treated with individual concentrations of DMNQ (0, 50, 100 and 300 μM) in centrifugation tubes in complete RPMI medium in a water bath at 32 °C for 30 min with shaking. From each sample 300 μL (3×10^5 cells) were taken, added to 700 μL of PBS in Eppendorf tubes and centrifuged (1200 rpm, 5 min). After centrifugation the supernatants were removed from each sample and to the sediments of cells was added low-melting-point agarose (800 μL). The cells were mixed and spread (2×10^4) on a base layer of 1% normal-melting-point agarose placed on microscopic slides and covered with cover slips.

Endonuclease III (EndoIII) and formamidopyrimidine-DNA glycosylase (Fpg) were obtained from Prof. A.R. Collins (University of Oslo, Norway). The crude extracts of EndoIII and Fpg were diluted in 40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin (BSA; Sigma), pH 8.0 (1:1000 and 1:3000, respectively) immediately before use.

Glutathione, NADPH and glutathione reductase were purchased from Sigma–Aldrich Co., and RANSOD kit from Randox Laboratories Ltd. Crumlin, UK. Media and other chemicals used for cell isolation and cultivation were purchased from Gibco-BRL, Paisley, UK. All chemicals were of analytical grade from commercial suppliers.

2.2. Animals and rosemary oil-supplementation

Adult male Sprague–Dawley (SD) rats weighing 207.5–310 g were used. The animals were obtained from an in-house strain and maintained at one per cage in a temperature- and humidity-controlled room with a 12-h light–dark cycle. They were given standard diet (MP, PD Horné Dubové-Naháč, Slovakia) *ad libitum*. Four groups (0‰, 0.125‰, 0.25‰ and 0.50‰ RO) containing 3–5 rats were used for experiments.

Water consumption for each rat was recorded daily and individual body weights were measured at the beginning and at the end of experiments. After 14 days of supplementation, the average total body weight gain was 30–52.5 g. No degeneration of inner organs or worsening of the overall health condition was observed either in Cremophor EL- or in RO-supplemented rats. The workstation of the Cancer Research Institute of the Slovak Academy of Sciences (CRI SAS) is authorised to perform scientific research on animals. The Ethics Committee of CRI SAS approved the rat experiments, which were performed in full compliance with the European Community Guidelines concerning principles for the care and use of laboratory animals. At the end of the experiments, the rats were ethically sacrificed by an i.p. dose of thiopental and used for the isolation of TCs.

2.3. Isolation of testicular cells

TCs were isolated from rat testes of sexually mature male SD rats by enzymatic digestion as described by Bradley and Dysart (1985), with some modifications suggested by Søderlund et al. (1988). Briefly, the testes were decapsulated and incubated at 32 °C in RPMI medium without foetal bovine serum (FBS) with collagenase (100 U/mL) in a water bath for 20 min with shaking. Trypsin (2100 U/mL) was then added and the tubular suspension was further incubated for 8 min. The resulting cell suspension was filtered, washed and resuspended in RPMI medium with 10% FBS, centrifuged four times (1200 rpm, 5 min) and filtered through a nylon mesh (100 μm). Viability of isolated TCs measured by trypan blue exclusion exceeded 95%.