ORIGINAL ARTICLE

Propolis and Caffeic Acid Phenethyl Ester Attenuates Nitrosative Stress in Lens Tissue in Radiation-induced Cataracts in Rats

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ABSTRACT

Objective: *Eye morbidity is widely observed in patients receiving total body irradiation prior to bone marrow transplantation or radiotherapy for ocular or head and neck cancers. Cataract blindness is the major cause of preventable blindness worldwide, especially in the developing countries. The aim of this study was to investigate whether propolis and caffeic acid phenethyl ester (CAPE) prevent radiation-induced cataractogenesis.*

Methods: *Fifty-four Sprague–Dawley rats were randomly divided into six groups. Group 1 (irradiation (IR) + propolis) received total cranium irradiation and propolis was given orally through an orogastric tube daily. Group 2 (IR+CAPE) received total cranium irradiation plus CAPE intraperitoneally every day. Group 3 (IR) received 5 Gy of gamma irradiation as a single dose to total cranium plus 1 ml saline daily. Group 4 received daily plain saline. Group 5 received daily plain dimethyl sulfoxide. Group 6 (normal control group) did not receive anything.*

Results: *At the end of the 10-day time period, cataracts developed in 80% of the rats in group 3 (IR group). After irradiation, cataract rate drop to 30% and 40% in groups treated with propolis and CAPE, respectively. Nitric oxide synthase activity, nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) levels were significantly higher in group 3 compared to all other groups.*

Conclusion: *The results suggest that propolis and CAPE have free radical scavenging activities in the irradiation-induced cataractogenesis, and reduced nitrosative stress markers. Propolis was found to be more effective in anticataractogenic effect than CAPE.*

Keywords: Caffeic acid phenethyl ester, cataract, irradiation, nitrosative stress, propolis.

INTRODUCTION

Nitrosative stress is induced by a reaction of superoxide anion radical ($O_2^{\bullet-}$) with nitric oxide ($NO^{\bullet}$), which generates peroxynitrite ($ONOO^{-}$) (1), and has been implicated in the pathogenesis of a large number of diseases such as diabetes mellitus, cataract, cancer, Behçet's disease, and rheumatoid arthritis (2–5). Increased nitrosative stress could (a) induce protein nitration; (b) damage membrane proteins and fatty acids, leading to changes of cellular signalling transduction; (c) upregulate inflammatory response; and (d) extensively activate apoptotic pathway (1).

Radiation therapy has been used effectively in treating head and neck cancers for many decades, which is a common and important tool for cancer treatment (6, 7). The killing action of ionizing radiation is mainly mediated through the free radicals generated from the radiolytic decomposition of cellular water, including $O_2^{\bullet-}$ and hydroxyl radical ($OH^{\bullet}$), which can cause damage in most major cellular macromolecules such as DNA, proteins, lipids, membrane, etc. These reactions take place in tumour as well as normal cells when exposed to radiation (8–10). Eye morbidity is widely observed in patients receiving total body irradiation

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prior to bone marrow transplantation or radiotherapy for ocular or head and neck cancers (11).

Cataract blindness is the major cause of preventable blindness worldwide, especially in the developing countries (12). Cataracts may occur in response to a variety of different agents and environmental stresses, and obviously damage seems to become a result of the oxidative damage component in almost all cases. Although cataract is a known late effect of ionizing radiation exposure, most of the experimental studies have concentrated on single, acute high doses or multiple, fractionated and chronic exposure of radiation (8, 13).

Propolis and caffeic acid phenethyl ester (CAPE), an active component of propolis extract, have immunomodulatory, antitumoural, cytotoxic, antimetastatic, anti-inflammatory, and antioxidant properties and have been shown to inhibit lipoxygenase activities as well as suppress lipid peroxidation (14–16).

To our knowledge, there is no experimental study that simultaneously investigates the effects of propolis and CAPE supplementations on nitric oxide synthase (NOS) activity, nitric oxide ($NO^{\bullet}$) and $ONOO^{-}$ in the lens tissue of rats receiving ionizing radiation. Therefore, our main objective is to investigate the effects of these supplementations on nitrosative stress parameters in the lens tissue of rats with or without exposure to total cranium irradiation in the present study.

MATERIALS AND METHODS

Animals and experiments

Fifty-four rats, 10–12 weeks old, weighing 200 ± 25 g at the time of radiation, bred at Gaziantep University Medical School, Experimental Animal Laboratory, were used for the experiment. The animals were purchased from Gaziantep University Medical School, Experimental Animal Laboratory, and housed in cages 1 week before the start of the experiments. All animals received humane care in compliance with the guidelines of Gaziantep University Research Council's criteria. Food and tap water were available *ad libitum*. The laboratory was windowless with automatic temperature (22 ± 1°C) and lighting controls (14 hours light/10 hours dark). Rats were randomly divided into six groups (eight rats from control groups and ten rats from other groups) and placed in separate cages during the study. This study was approved by the local ethics committee of the Gaziantep University.

The groups were as follows:

Group A: The irradiation (IR) plus propolis group received both 5 gray (Gy) of gamma irradiation as a single dose to total cranium and propolis (80 mg/kg/day) starting 1 hour before irradiation and continuing for 10 days through an orogastric tube. Propolis was dissolved in dimethyl sulfoxide (DMSO) just before giving to the rats.

Group B: The IR plus CAPE group received both 5 Gy of gamma irradiation as a single dose to total cranium and CAPE (10 µmol/kg/day, intraperitoneal [IP]) injection, starting 30 minutes before the radiation dose and subsequently daily for 10 days after irradiation. Caffeic acid phenethyl ester was dissolved in DMSO just before giving to the rats. The final concentration of DMSO was 0.1%.

Group C: The IR group received total cranium 5 Gy of gamma irradiation as a single dose. Also, physiological saline solution (1 ml/kg/day, IP injection) was administered daily for 10 days.

Group D1: The control group for group A received neither propolis and CAPE nor irradiation, but received daily physiological saline solution (1 ml/kg/day, orally) for 10 days.

Group D2: The control group for group B did not receive propolis, CAPE and irradiation, but received daily IP injection of DMSO at an equal volume to that of TQ used in group B for 10 days.

Group D3: The normal control group did not receive neither propolis, CAPE, TQ and irradiation nor oral/IP physiological saline solutions.

Prior to total cranium irradiation, all rats were anaesthetized with 80 mg/kg ketamine hydrochloride (Pfizer Ilac, Istanbul, Turkey) and placed on a tray in the prone position. The rats in the IR and the IR plus TQ groups received irradiation via a Cobalt-60 teletherapy unit (Picker, C9, Maryland, NY, USA) from a source-to-surface distance of 80 cm by 5 × 5 cm anterior fields, with the total cranium gamma irradiation being a single dose of 5 Gy, while the rats in the control and sham control groups received sham irradiation. The dose rate was 0.49 Gy/min. The central axis dose was calculated at a depth of 0.5 cm.

Determination of clinical cataract

In this study, the Lens Opacities Classification System, Version III (LOCS III) was used in the cataract classification (17). Before the cataracts were graded, the pupils were dilated with tropicamide (1.0%) and phenylephrine hydrochloride (2.5% drops), and proparacaine (0.5%) was used as a topical anaesthetic. The lenses were graded by slit-lamp biomicroscopy (Keeler PSL Classic, Keeler Ltd, Windsor, UK) as follows: The features of nuclear

opacification and brunescence were graded according to one set of six photographs. The brightness of scatter from the nuclear region was designated nuclear opalescence (NO) and the intensity of brunescence, nuclear colour (NC). The amount of cortical cataract (C) was determined by comparing the estimated aggregate of cortical spoking with that seen in five separate photographs. Similarly, the estimated amount of posterior subcapsular cataract (P) was determined by comparing it with another five photographs depicting increasing amounts of posterior subcapsular cataract. At the beginning of the experiment, all rats were examined biomicroscopically and were only included in this study if their lenses had been NO_{0-}, NC_{0-}, C_{0-} and P_{0-}.

Biochemical analysis

At the end of the study, all rats were anaesthetized with 80 mg/kg ketamine hydrochloride (Pfizer Ilac, Istanbul, Turkey) and then all animals were killed by decapitation, and their eyes were enucleated, and the lenses were dissected immediately. Lenses were homogenized by an IKA-NERKE (Staufen, Germany) homogenizer in physiological saline solution (20-fold) on ice for 10 seconds in the first speed level. The homogenate was centrifuged at 10 000 g for 1 hour to remove debris. The clear upper supernatant was collected and all assays were conducted on this fraction. All of the procedures were performed at 4°C.

Nitric oxide synthase activity determination

Nitric oxide synthase activity assay is based on the diazotization of sulfanilic acid by $NO^{\bullet}$ at acid pH and subsequent coupling to N-(1-naphthyl) ethylenediamine. To 0.1 ml of sample, 0.2 ml of 0.2 M arginine was added and incubated at 37°C for 1 hour. Then, the combination 0.2 ml of 10 mM HCl, 100 mM sulfanilic acid and 60 mM N-(1-naphthyl) ethylenediamine was added. After 30 minutes, the absorbance of the sample tube was measured against a blank tube at 540 nm (18). Results are expressed as U/mg protein.

Nitric oxide determination

$NO^{\bullet}$ levels in lens tissue were measured using the Griess reagent as previously described (19, 20). Griess reagent, the mixture (1:1) of 0.2% N-(1-napthyl) ethylenediamine and 2% sulphanilamide in 5% phosphoric acid, gives a red-violet diazo dye with nitrite, and the resultant colour was measured at 540 nm. First, nitrate was converted to nitrite using nitrate reductase. The second step was the addition of Griess reagent, which converts nitrite to a deep purple azocompound; photometric measurement, the absorbance of 540 nm, determines the nitrite concentration. Results were expressed as μmol/g wet tissue.

Peroxynitrite determination

$ONOO^{-}$ assay was determined as described (21, 22). Ten microlitres of samples was added to 5 mM phenol in 50 mM sodium phosphate buffer (pH 7.4) to get a final volume of 2 ml. After 2 hours of incubation in a dark place at 37°C, 15 μL of 0.1 MNaOH was added and the absorbance, at wavelength of 412 nm, of the samples was immediately recorded. The yield of nitrophenol was calculated from $\varepsilon = 4400/M/cm$. Results were expressed as μmol/g wet tissue. The protein content was determined as described (23). Biochemical measurements were carried out using a visible/UV spectrophotometer (Shimadzu UV 1601, Tokyo, Japan).

Statistical analyses

Statistical and correlation analyses were undertaken using a one-way variance analysis and Spearman's rank correlation test, respectively. Following the analysis of variance, the significance of differences between groups was tested using least significant difference multiple range test procedure. Acceptable significance was recorded when p values were < 0.05. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS, version 11.5, Chicago, IL, USA).

RESULTS

In this study, the LOCS III was used in the cataract classification (17). The lenses were graded by slit-lamp biomicroscopy (Keeler PSL Classic, Keeler Ltd, Windsor, UK). All the rat lenses at the beginning were graded as 0. After irradiation, cataract developed in 80% of the rats in the radiotherapy group. After irradiation, cataract rate drop to 30% in propolis and 40% in CAPE groups which treated with these substances, respectively, and was limited at grade 1 and grade 2. Nitric oxide synthase activity and levels of $NO^{\bullet}$ and $ONOO^{-}$ in the radiotherapy group were significantly higher than all other groups (Figure). In the current study, while the cataract ratio was highly observed in the radiotherapy group, it decreased in the other groups in which propolis and CAPE were given after radiation.

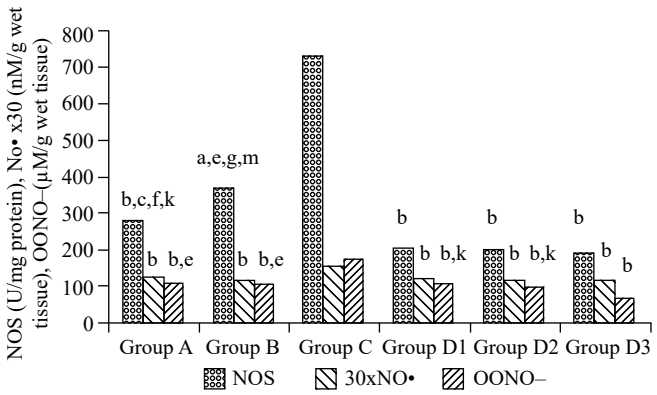

Figure: Mean ± SD of nitric oxide synthase activity, nitric oxide and peroxynitrite levels in the rat lenses. Group A: irradiation (IR) plus propolis group, group B: IR plus caffeic acid phenethyl ester (CAPE) group, group C: IR group only, group D1 (the control group of A group), group D2 (the control group of B group), group D3 (normal control group): nitric oxide synthase (NOS): nitric oxide ($NO^{\bullet}$): peroxynitrite ($ONOO^{-}$). a: $p < 0.01$, b: $p < 0.0001$ *vs* group C, c: $p < 0.05$, d: $p < 0.01$, e: $p < 0.001$ *vs* group D3, f: $p < 0.05$, g: $p < 0.001$ *vs* group D2, k: $p < 0.01$ *vs* group D1.

DISCUSSION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive species generated by biochemical redox reactions as a part of normal cell metabolism. Oxygen is a critical element for the living organisms and has some advantages. In view of the generation of ROS, certain adverse effects also occur. Basically, all the essential biomolecules can undergo oxidative reactions mediated by ROS. The ROS, RNS and their suggested effects on biological systems have become an important area of biomedical research in recent years (24–26). The increase in ROS and RNS production or the decrease in antioxidant mechanisms generates a condition called oxidative and nitrosative stress, respectively, defined as the imbalance between pro- and antioxidants in favour of the oxidants. These highly reactive compounds are dangerous for the cells when their cellular production exceeds the antioxidant capacity (27, 28).

Nitrosative stress is caused by overproduction of RNS. Some studies have shown that nitrosative stress is increased in the diabetic retina and radiation (1, 10, 29). Although $NO^{\bullet}$ itself, a free radical produced by NOS, is a weak oxidant, biochemical studies have shown that $NO^{\bullet}$ rapidly interacts with superoxide anion radical to yield $ONOO^{-}$ and its conjugate acid, peroxynitrous acid, which then decomposes to form highly reactive oxidant species, such as hydroxyl and nitrogen dioxide ($NO_2^{\bullet}$).

The $NO_2^{\bullet}$ radical is an important RNS in biological systems. $NO_2^{\bullet}$ is a strong one-electron oxidant and has a rich chemistry, including reacting rapidly with other radical species and $1e^{-}$ oxidation of reductants, and slower addition to double bonds and H atom abstraction. $NO_2^{\bullet}$ reacts with lipids, DNA and proteins, and plays a key role in cellular nitrosative stress. Either directly or indirectly, it plays a key role in the cellular oxidative and nitrosative stress. Both $NO^{\bullet}$ and $ONOO^{-}$, directly or via reactive intermediates, may induce cytotoxicity via a number of mechanisms including tyrosine nitration, lipid peroxidation, DNA strand breaks and the consequent activation of poly-ADP ribose synthase (1). There is a balance between production and scavenging of ROS/RNS. If the balance changes in support of production of ROS/RNS, oxidative or nitrosative stress takes place and may result in a variety of diseases: cancer, cardiovascular, neurological diseases and cataract (10, 29, 30).

Irradiation (IR) is one of the most common therapies for treating human cancers. Ionizing radiation interacts with living cells and produces different cytotoxic effects that are mediated through the production of ROS/RNS. Cumulative results involving animals exposed to either non-lethal or lethal doses of X-radiation show that the biological effects of the IR are dependent on the radiation dose and post-irradiation time. A judicious balance should be between the total dose of IR received and the threshold limit of the normal tissue near the tumour. Normal tissue should be protected against the side effects of radiation to obtain better tumour control with a higher dose (6, 29). Radiation is a known producer of free radicals, contributing to radiation injury in cells and formed in cells. Biological tissues that are exposed to ionizing radiation may have oxidative damage in biological molecules such as nucleic acids, proteins and lipids, and they form free radicals in aqueous solutions and may result in cataracts. One of the mechanisms proposed to explain lens opacification is the oxidation of crystallins, either by radiation or ROS and RNS. The other mechanism is the formation of opacities with increased calcium release by mitochondrial damage. Ionizing radiation has been shown to enhance the production of these free radicals in cells (3, 9, 31). For this reason, to prevent injury caused by radiation on healthy tissue, many investigations related natural products that have antiviral, anticancer, immunostimulant and antioxidant effects have been constructed. Several studies on radioprotective agents are ongoing. To our knowledge, this is the first report demonstrating lens radioprotection by propolis and CAPE.

We found that NOS activity, $NO^{\bullet}$ and $ONOO^{-}$ levels were significantly higher in lens tissues of irradiated rats that were exposed to total cranium irradiation, compared to other groups. Similar results were obtained in different studies (10, 12). The nitrosative stress parameters in the groups receiving irradiation plus propolis or CAPE treatment were significantly decreased compared to the group that received irradiation only. These results revealed that propolis and CAPE clearly decreased the nitrosative stress in lens tissue of rats exposed to total cranium irradiation. $NO^{\bullet}$ is synthesized endogenously by the enzyme, NOS, via its precursor arginine. Endogenous $NO^{\bullet}$ plays a dual role in specialized tissues and cells, where it is not only an essential physiological signalling molecule mediating various cell functions but also induces cytotoxic and mutagenic effects when present excessively under oxidative stress conditions induced by irradiation (4). $NO^{\bullet}$ is recognized by two distinct categories in the chemical biology, such as direct and indirect effects. Direct effects are those chemical reactions where $NO^{\bullet}$ reacts directly with its biological target. The direct effects are very rapid reactions that occur at low $NO^{\bullet}$ concentrations and generally involve haem proteins such as guanylate cyclase, cytochrome P450 and haemoglobin, whereas indirect effects are mediated by RNS, which are derived from $NO^{\bullet}$ metabolism. It requires that $NO^{\bullet}$ is first activated by $O_2^{\bullet-}$, or oxygen to form RNS which then undergoes further reactions with the respective biological target (32, 33). Also, $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet-}$ to form $ONOO^{-}$, which may be cytotoxic by itself or easily decomposed to the highly reactive and toxic $OH^{\bullet}$ and $NO_2^{\bullet}$ (4). These RNS are highly reactive with major cellular macromolecules such as DNA, proteins, lipids, membrane, *etc*, and are thought to be responsible for $NO^{\bullet}$-mediated cell deaths (32, 33).

Some studies have reported that $NO^{\bullet}$ levels in rats that are exposed to irradiation were significantly higher (10, 12, 29, 33). In the current study, NOS activity, $NO^{\bullet}$ and $ONOO^{-}$ levels in lens tissue of rats exposed to radiation were also increased before the treatment of rats with propolis and CAPE. Our results showing low nitrosative stress parameters in the propolis and CAPE treatment groups suggest that propolis and CAPE have protective effects against toxicity to irradiation.

CONCLUSION

In this study, we found that NOS activity, $NO^{\bullet}$ and $ONOO^{-}$ levels in the radiotherapy group were higher than those of all other groups. This is the first study that simultaneously investigates the effects of propolis and CAPE on the nitrosative stress in the lens tissue of the irradiated rats. The results suggest an important role of nitrosative stress in the irradiation-induced cataractogenesis. Propolis and CAPE clearly have antioxidant properties, free radical scavenging activities and decreased nitrosative stress.

AUTHORS' NOTE

The authors report no declarations of interest.

None of the authors have a commercial interest, financial interest, and/or other relationship with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices or with commercial providers of medically related services.

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