Eugenol as An *In Vivo* Radioprotective Agent

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**Eugenol/Micronucleus test/Radiation/Radioprotection/Oxidative stress**

In the present work, an attempt has been made to evaluate the possible *in vivo* radioprotection by eugenol. Swiss albino mice were administered different doses of eugenol (75, 150 and 300 mg/kg) before exposure to 1.5 Gy of gamma radiation. The micronucleus test was carried out to determine the genetic damage in bone marrow. Our results demonstrated significant reduction in the frequencies of micronucleated polychromatic erythrocytes (MnPCEs) with all three eugenol doses. Eugenol (150 mg/kg) was also tested against different doses of radiation (0.5, 1, 1.5, and 2 Gy) and was found to afford significant radioprotection. Reduction in the incidence of MnPCEs could be noticed up to 72 h postirradiation (1.5 Gy). Moreover, the level of peroxidative damage and the specific activities of lactate dehydrogenase (LDH) and methylglyoxalase I (Gly I) were observed in the liver of mice treated with eugenol for seven days in comparison to untreated mice. The results revealed that eugenol exerted significant protection against oxidative stress. This possibility was further supported by the enhanced response of Gly I and the lowered activity of LDH. The present findings suggested that eugenol has a radioprotective potential.

**INTRODUCTION**

The radiosensitivity of cells is known to be directly proportional to their reproductive activity and inversely proportional to their degree of differentiation. Although these findings have laid the foundation of the radiation therapy of cancer, the presence of hypoxic cells in tumors limits success in a clinical situation. The drug, which can differentially protect the surrounding normal tissue and/or sensitize hypoxic tumor cells, is a desirable strategy to improve the therapeutic index. It is important to note that in radiation therapeutic practice, besides tumor control, the total radiation dose is also chosen on the basis of maximum dose tolerated by normal tissues with the aim of optimizing tumor cure. Therefore any chemical agent that can improve the tolerance of normal tissue to radiation is of paramount interest. Several chemical agents have been tested for their radiomodifying properties. Some of these agents showed promising effect, but they were not widely acceptable because of their toxicity and nondifferential effect. There is growing interest to find new chemical agents to overcome this limitation, particularly of the origin of plants that have been consumed by human beings and are known to have medicinal properties.

Sizable evidence is now on hand to show that phytochemicals may play an important role as *in vivo* radioprotective agents. Many are known to be antioxidants found in small quantities in our diet. The antioxidants of plant origin, such as chlorogenic acid, curcumin, chlorophyll, β-carotene, garlic, and chlorella, were reported to provide protection against radiation-induced genetic damage in mice. Curcumin was shown to have a preventive effect against the radiation-induced initiation of mammary tumorgenesis in rats. The protective effect of aqueous extracts of chili, black pepper, and turmeric has been demonstrated against the gamma-radiation induced inactivation of bacteria. Further, the spice extracts, curcumin and piperine, have been shown to protect plasmid DNA against radiation and to prevent its degradation.

In the present work, the radiomodulatory potential of the naturally occurring alkenylbenzene eugenol (4 allyl-1-hydroxy-2-methoxy benzene) has been evaluated. Eugenol is a main constituent of the essential oil obtained from commonly consumed spices such as clove, cinnamon, basil, and nutmeg. It is used in pharmaceutical and food products and in beverages as a flavoring agent. In several studies, eugenol was reported to be nongenotoxic and noncarcinogenic. It is important that eugenol was found to be protective against many genotoxins and carcinogens. Biochemical studies in mice and rats have demonstrated its role as an effective inducer of detoxifying phase II enzymes.
Furthermore, eugenol was reported to inhibit lipid peroxidation by acting as a chain-breaking antioxidant \cite{16,22-24}. These findings suggest that eugenol is very likely to have radioprotective potential. Therefore it was administered to mice orally for seven consecutive days before exposure to gamma radiation. Genetic damage was assessed in the mouse by a bone marrow micronucleus test. Together with this test, biochemical studies were carried out to assess the influence of eugenol on the peroxidation and the specific activity of lactate dehydrogenase (LDH) and glyoxalase I (Gly I) in the liver of mice.

**MATERIAL AND METHODS**

**Animals**

Male Swiss albino mice 7–8 weeks old, weighing 26 ± 3g, were used for the present study. These animals were maintained in a university animal house at 25 ± 2°C in polypropylene cages. They were provided with standard mouse diet (pellets) and water ad libitum.

**Pretreatment with Eugenol**

Eugenol obtained from Aldrich (USA) was diluted in peanut oil and administered to the experimental animals by gavage. Three test doses of 75, 150 and 300 mg kg⁻¹ were administered to the experimental animals for seven days before irradiation. Each pretreatment group consisted of at least 10 mice. Control animals received the same volume of peanut oil (vehicle). Another group with no treatment served as negative control. The animals were regularly observed for their general health during an experimentation period of 7 days. No signs of toxicity could be seen in the eugenol-treated group of animals. No appreciable difference in body weight between the eugenol-treated animals and the untreated group of animals was noticed. Further, no death was reported during the treatment period. It may be mentioned that the doses of eugenol used in the present work were quite low compared to its LD 50 dose in mice (3,000 mg/kg body weight). The duration of treatment was also short.

**Irradiation of animals**

The experimental animals were whole-body irradiated at room temperature in a gamma chamber (⁶⁰Co source, 240 TBq, Model 4000A) obtained from Bhaba Atomic Research Center, Mumbai, India. The mice were restrained within individual compartments in a well-ventilated acrylic cylinder and placed in the gamma chamber. The dose rate was estimated by Fricke dosimetry and was found to be 0.015 Gy/sec. The experiments were conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals, Government of India.

**Micronucleus test**

The micronucleus test was carried out according to Schmid\cite{25}. The animals were killed humanely by cervical dislocation under mild anesthesia. The bone marrow cells were flushed out into 1 ml of fetal calf serum (Gibco, Scotland) 24, 48, and 72h after irradiation. Briefly, the cell suspension was centrifuged at 2,000 rpm for 10 min, and the supernatant removed. The pellet was resuspended in a drop of serum before being used for preparing slides. The air-dried slides were stained with May-Grunwald and Giemsa. The permanent slides were coded, and 2500 polychromatic erythrocytes were scored for determining the frequency of micronucleated polychromatic erythrocytes (MnPCEs). The same person scored all the slides.

**Biochemical assay**

Biochemical studies were carried out in a separate set of experiments. The animals used in these experiments were not exposed to radiation. After the animals were sacrificed, their livers were excised and perfused with ice-cold saline. A 10% homogenate of liver was prepared by the use of Potter Elvehjem homogenizer in 0.1M phosphate buffer (pH 7.0). The pellet was discarded and the supernatant recenterfuged at 105,000 g for 60 min in Beckman-Type 50 Ti rotor. The resulting supernatant was used for an assay of enzymes.

**Determination of oxidative damage and specific activity of LDH**

Lipid peroxidation was estimated in the cytosolic fraction by the Thiobarbituric acid (TBA) method and expressed in terms of Thiobarbituric acid reactive species (TBARS) formed per mg protein\cite{26}. LDH was assayed by measuring the rate of oxidation of NADH at 340 nm, following the method of Bergmeyer and Bernt\cite{27}. The enzyme activity was calculated by the use of an extinction coefficient of 6.22 m² M⁻¹ and expressed in units/mg protein.

**Determination of Glyoxalase I activity**

Gly I was assayed spectrophotometrically at 25°C as described by Sharma-Luthra and Kale\cite{28}. The activity was calculated by use of the extinction coefficient value 2.86 mM⁻¹ cm⁻¹. One unit of Gly I is defined as the amount of enzyme catalyzing the formation of 1 µmol S-D- lactoylglutathione/min under assay conditions. The enzyme activity is given as units/mg of protein.

**Protein determination**

We determined the protein concentration by using the method of Lowry\cite{29}, using bovine serum albumin as a standard.

**Statistical analysis**

The significance of difference between the data pairs was evaluated by an analysis of variance (ANOVA) followed by the Mann-Whitney U–t- test.
RESULTS

Micronucleus test

The irradiation of animals with 1.5 Gy resulted in an increase in the incidence of micronuclei in the bone marrow. However, the administration of eugenol (75, 150, and 300 mg/kg body wt) for seven days before irradiation inhibited MnPCEs significantly in a dose-dependent manner (Table 1). Eugenol has no adverse effect on the animals. There was no significant increase in the incidence of MnPCEs in the groups of animals treated with these three doses of eugenol. Since sufficient radioprotection was provided by 150 mg/kg dose of eugenol, separate experiments were designed to examine the modulation of chromosome damage induced by different doses of radiation (0.5, 1.0, 1.5, and 2 Gy), using this dose of eugenol (Fig. 1). Eugenol (150 mg/kg body wt) was able to provide significant protection against all the radiation doses studied. The time course (24, 48, and 72 h) of radomodulation of chromosome damage was also studied by means of 1.5 Gy of radiation and 150 mg/kg body weight dose of eugenol. It was important that eugenol lowered the expected chromosomal damage. It may also be mentioned that the levels of Mn PCEs were declined with time, both in the irradiated controls and in the treated group of animals (Fig. 2).

Table 1. Effect of 7-day pretreatment with eugenol on the gamma-ray-induced chromosomal damage in the bone marrow of mice

| Dose (mg/kg) | Gamma radiation (Gy) | Mn PCEs/2500 PCEs |
|-------------|----------------------|------------------|
| 0           | 0                    | 2.4 ± 0.24       |
| (Vehicle peanut oil) | 0        | 3.2 ± 0.58       |
| 75          | 0                    | 3.4 ± 0.52       |
| 150         | 0                    | 2.4 ± 0.51       |
| 300         | 0                    | 2.2 ± 0.37       |
| 0           | 1.5                  | 77.8 ± 3.51      |
| 75          | 1.5                  | 37.6 ± 3.41**    |
| 150         | 1.5                  | 36.4 ± 3.61**    |
| 300         | 1.5                  | 29.4 ± 1.75**    |

The mice were sacrificed 24 hours after irradiation. PCEs: Polychromatic Erythrocytes; Mn PCEs: Micronucleated polychromatic erythrocytes. Values are means ± SEM from 5 mice.

** Significantly different from irradiated controls. (P<0.01)

Fig. 1. Effect of Eugenol pretreatment on MnPCEs (micronucleated polychromatic erythrocytes) induced by different doses of Gamma radiation (0.5, 1.0, 1.5, and 2 Gy) in bone marrow of Swiss albino mice. Values are mean ± SD obtained from 5 mice/point. * Significantly different from respective irradiated control P<0.05.

Fig. 2. Effect of different postirradiation sampling time (24, 48, 72 h and Eugenol pretreatment (150 mg/kg body weight) on Mn PCEs (Micronucleated polychromatic erythrocytes) induced by 1.5 Gy of Gamma radiation in bone marrow of Swiss albino mice. Values are mean ± SD obtained from 5 mice/point. * Significantly different from respective irradiated control P<0.05.

Fig. 3. Effect of different doses of eugenol feeding for seven days on the level of peroxidative damage in TBARS (Thiobarbituric acid-reactive substances) per mg protein in the liver of mice. Each value represents mean ± SD obtained from five animals. * Significantly different from untreated group (P<0.05).
Biochemical assay

In a separate set of experiments, the levels of peroxidation and the specific activities of LDH and Gly I in the liver of mice treated with eugenol (75, 150, and 300 mg/kg body weight) were examined. It is quite clear from the results depicted in Fig. 3 that the oxidative stress was lower in animals, which received eugenol for seven days, compared to the untreated group of animals. The change was significant, especially with 150 and 300 mg/kg body weight of eugenol and was supported by the similar findings with LDH (Fig. 4), a biochemical indicator of cellular damage. The glyoxalase system is present in cytosol of all living cells and is intimately linked to cellular damage. The results in Fig. 5 show an increase in the activity of Gly I at all the three doses of eugenol.

DISCUSSION

The present work has demonstrated perhaps for the first time that eugenol can ameliorate gamma radiation induced in vivo clastogenic effects. All three doses of eugenol tested have reduced the frequencies of MnPCEs by more than 50% (Table 1). The protective effect of eugenol against different radiation doses ranging from 0.5 to 2 Gy was also a significant observation (Fig. 1). In other experiments, the bone marrow cells were sampled at 48 and 72 h after irradiation to ascertain whether the reduction observed in the 24 h sample is due to a delay in cell cycle progression following pretreatment of mice with eugenol. However, the reductions observed in these samples were less pronounced when compared to the 24 h sample (Fig. 2). This could be because of cell death and dilution that occurred during erythroblast multiplication. The present findings are suggestive of the protective ability of eugenol against radiation damage.

The radioprotectors are known to exert their action through various mechanisms, such as scavenging of free radicals, detoxification of radiation induced species, target stabilization, and enhancement of repair and recovery processes\(^6\). An antioxidant group of agents mainly provides protection by the removal of free radicals. On the irradiation of animals, free radicals are generated from the radiolytic decomposition of cellular water. These free radicals interact with biomolecules and bring about the changes in their structure and function leading to damage and/or cell death. The scavenging of free radicals is known to result in protection against the detrimental effects of radiation. Therefore it is quite possible that eugenol, being an antioxidant\(^{22,23}\), might have scavenged the radiolytically generated free radicals and in turn provided protection against radiation induced chromosome damage.

It is interesting that the seven-day pretreatment with eugenol showed no significant genotoxicity. Similar nontoxic effects of eugenol were also reported earlier\(^{15,20}\). Some doses of eugenol used in those studies were even higher than the ones used in the present work. It may also be mentioned that the doses of eugenol used in the present work were relatively quite low compared to its LD 50 dose in mice i.e., 3,000 mg/kg body weight. Moreover the duration of treatment was also short.

A decrease in the level of the peroxidation in the liver of mice treated with eugenol, compared to the untreated controls (Fig. 3), is suggestive of the lowered oxidative stress. The peroxidation is a free radical chain reaction\(^5\). The scavenging of free radicals involved in initiation as well as the propagation of peroxidation could result in its inhibition. Therefore eugenol might have scavenged these free radicals and lowered the peroxidation resulting in decreased oxidative stress in animals. The peroxidation is an important effect on membranes.
Because of membrane damage, LDH is quite likely to be released in the cytosol. The low level of the activity of LDH in the cytosolic fractions prepared from liver of the mice treated with eugenol (Fig. 4) support the idea that treated mice experienced reduced oxidative stress.

The methylglyoxalase system is known to play a vital role in biological function because it regulates cell division and differentiation. It is important that it was suggested to have an antioxidant function as the electrophiles and that cytotoxic alpha oxoaldehydes are converted to less reactive chemical species. The increased response of Gly I (Fig. 5) because of treatment with eugenol is expected to help in the removal of toxic species and to reduce oxidative stress. Eugenol has also been reported to induce the detoxifying enzymes. Therefore it could be inferred that eugenol probably enhanced the antioxidant function of animals.

It could be concluded that eugenol has the ability to provide protection against radiation-induced chromosomal damage in Swiss albino mice. The enhanced activity of Gly I and the lowered levels of peroxidative damage and specific activity of LDH support the possibility that eugenol could reduce the oxidative stress. The toxicity and inability to differentiate between tumor and normal cells are the main reasons for the failure of radioprotectors at clinical level. Further work on eugenol is therefore needed to be focused to overcome these difficulties. To reduce the toxicity, it could be used in nontoxic amounts in combination with other protectors or made nontoxic by chemically modifying its structure. The work from our laboratory has clearly shown that the same radiomodifier could behave as a protector and a sensitizer. It is of paramount interest to know those conditions, which make radiomodifiers protectors and sensitizers. One approach is suggested in our hypothesis. Eugenol could be tested for such ability.

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