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Antimutagenicity of rosmarinic acid in Swiss mice evaluated by the micronucleus assay

Michelle Andrade Furtado, Lenita Caetano Fernandes de Almeida, Ricardo Andrade Furtado, Wilson Roberto Cunha, Denise Crispim Tavares *

Universidade de Franca, Avenida Dr. Armando Salles de Oliveira, 201 – Parque Universitário, 14404-600, Franca, São Paulo, Brazil

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ABSTRACT

Rosmarinic acid (RA) is a natural phenolic compound which presents different biological activities such as antitumor, antibacterial, anti-inflammatory, hepatoprotective and cardioprotective properties. In view of its important biological activities, the study of the effects of RA on genetic material becomes relevant. Thus, the objective of the present study was to evaluate the mutagenic and/or antimutagenic potential of RA on peripheral blood cells of Swiss mice using the micronucleus assay. Three doses of RA (50, 100 and 200 mg/kg body weight, b.w.) were used for the evaluation of its mutagenic potential. In the antimutagenicity assays, the different concentrations of RA were combined with the chemotherapeutic agent doxorubicin (DXR, 15 mg/kg b.w.). Peripheral blood samples were collected 24, 48 and 72 h after treatment for the evaluation of micronucleated polychromatic erythrocytes (MNPCEs). The results of the mutagenicity assays showed no increase in the frequency of micronuclei in animals treated with different concentrations of RA when compared to the negative controls. Treatment with different concentrations of RA combined with DXR revealed a significant reduction in the frequency of micronuclei compared to animals treated with DXR only. Although the mechanisms underlying the protective effect of RA are not completely understood, the putative antioxidant activity of RA might explain its effect on DXR mutagenicity.

1. Introduction

Free radicals can exert noxious effects on cell components such as membranes, lipoproteins, proteins, carbohydrates, DNA and RNA. These radicals are generally produced by various endogenous processes that can be stimulated by external factors such as air pollution, irradiation, smoking, stress and toxins present in food and/or drinking water [1]. The implication of oxidative and nitrosative stress in the etiology and progression of several acute and chronic disorders suggests that antioxidants can have health benefits as prophylactic agents. Epidemiological studies have shown that diets rich in fruit and vegetables and other plant foods are associated with a decreased risk of premature death and mortality from oxidation-related diseases, including cancer and cardiovascular and neurodegenerative diseases [2,3]. Phenolic compounds, which are characterized by high antioxidant activity, are believed to be responsible, at least in part, for these effects [4].

Rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyl lactic acid; RA) is a naturally occurring hydroxylated compound that consists of two phenolic rings, both of them containing two *ortho*-position hydroxyl groups. A carbonyl group, an unsaturated double bond and a carboxylic acid group are located between the two rings (Fig. 1). In 1958, two Italian chemists, Scarpati and Oriente [5], isolated RA for the first time as a pure compound and named it according to the plant it was isolated from, *Rosmarinus officinalis*. RA is mainly found in species of the family Boraginaceae and in the subfamily Nepetoidae (family Lamiaceae) [6].

A number of bioactivities have been assigned to RA, such as antidepressive [7], hepatoprotective [8], anti-inflammatory [9], antiangiogenic [10], antitumor [11], and HIV-1-inhibiting properties [12]. RA is also known to possess marked antioxidant properties as a reactive species scavenger and lipid peroxidation inhibitor [13].

RA has a broad range of applications, including products ranging from food preservatives and cosmetics to medications [14]. Therefore, the evaluation of a possible mutagenic activity of RA is important to guarantee its safe use in humans. As part of our ongoing research regarding the biological activities of natural compounds [15–18], we studied the potential mutagenic effect of RA and its influence on the mutagenicity induced by the chemotherapeutic agent doxorubicin (DXR) using the in vivo mouse peripheral blood micronucleus assay.

* Corresponding author. Tel.: +55 16 3711 8871; fax: +55 16 3711 8878.
E-mail address: denisecrispim2001@yahoo.com (D.C. Tavares).

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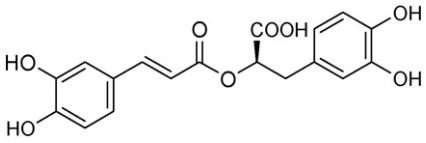

Fig. 1. Chemical structure of rosmarinic acid.

2. Materials and methods

2.1. Chemicals

RA at 97% purity (CAS 20283-92-5) was obtained from Sigma–Aldrich (St. Louis, MO, USA) and was dissolved in distilled water. The DXR ampoule containing 50 mg at 98% purity was purchased from Pharmacia Brasil Ltda. (São Paulo, Brazil). This compound dissolved in distilled water was used as an inducer of micronuclei in mouse peripheral blood cells (positive control). The DXR dose (15 mg/kg body weight, b.w.) was selected based on its effectiveness in inducing chromosome damage [19].

2.2. Animals

Male Swiss mice (*Mus musculus*), 6–8 weeks old and weighing approximately 30 g, were supplied by the Animal House of the Faculty of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto (São Paulo, Brazil) and acclimated for a period of 1 week before the beginning of the experiment. The animals were kept in plastic boxes with wood chip bedding, maintained in an experimental room under controlled conditions of temperature (22 ± 2 °C) and humidity (50 ± 10%) under a 12-h light–dark cycle, with standard rat chow (Labina, São Paulo, SP, Brazil) and water being available *ad libitum*. The study protocol was approved by the Ethics Committee for Animal Care of the University of Franca (process 042/07A).

2.3. Experimental design

The animals were divided into eight experimental groups of six animals each, as shown in Table 1. Preliminary studies were conducted to select the doses of RA, using the citotoxicity as a criterion [20]. The tested doses ranged from 2 to 400 mg/kg b.w. The animals treated with doses up to 200 mg/kg b.w. RA showed a significant reduction in the polychromatic erythrocyte (PCE)/PCE + normochromatic erythrocytes (NCE) ratio. Therefore, the doses of RA selected to our study were 50, 100 and 200 mg/kg b.w. The different doses were administered to the animals by gavage in a volume of 16.7 mL/kg b.w., 10 min before intraperitoneal (i.p.) injection of DXR (10.0 mL/kg b.w.). Peripheral blood samples were collected 24, 48 and 72 h after treatment.

2.4. Micronucleus assay

The peripheral blood micronucleus assay was performed according to the protocol described by MacGregor et al. [21]. A total of 2000 polychromatic erythrocytes were analyzed per animal for the determination of the frequency of micronucleated polychromatic erythrocytes (MNPCEs). The PCE/PCE + NCE ratio was calculated by the analysis of 400 erythrocytes in order to determine the cytotoxicity of RA [22]. Slides were scored blindly using a light microscope with a 100× immersion objective.

The percent reduction in the frequency of MNPCEs was calculated according to Waters et al. [23], using the following formula:

$$\%\text{reduction} = \frac{\text{frequency of MNPCEs in A} - \text{frequency of MNPCEs in B}}{\text{frequency of MNPCES in A} - \text{frequency of MNPCEs in C}} \times 100$$

Table 1
Experimental groups and treatment protocol.

Treatment	Group[a]	Dose
Negative control	1	No treatment (distilled water)
RA 50	2	50 mg/kg b.w.
RA 100	3	100 mg/kg b.w.
RA 200	4	200 mg/kg b.w.
DXR	5	15 mg/kg b.w.
RA 50 + DXR	6	As in (2) and (5)
RA 100 + DXR	7	As in (3) and (5)
RA 200 + DXR	8	As in (4) and (5)

Rosmarinic acid (RA) was administered by gavage simultaneously with an i.p. injection of doxorubicin (DXR).

[a] There were six males per group.

Table 2
Frequencies of micronucleated polychromatic erythrocytes (MNPCEs) in peripheral blood of Swiss mice treated with rosmarinic acid (RA) and doxorubicin (DXR) and their respective controls 24 h post-treatment.

Treatment	PCE/PCE + NCE[a]	MNPCEs[b]		Reduction (%)
	Mean ± S.D.	Number	%	
Negative control	0.11 ± 0.02	14	0.11	–
RA 50	0.10 ± 0.01	20	0.17	–
RA 100	0.11 ± 0.01	20	0.17	–
RA 200	0.11 ± 0.02	22	0.18	–
DXR	0.09 ± 0.03	107[c]	0.89	–
RA 50 + DXR	0.10 ± 0.02	61[c,d]	0.51	49.5
RA 100 + DXR	0.11 ± 0.02	45[c,d]	0.37	66.7
RA 200 + DXR	0.10 ± 0.01	45[c,d]	0.37	66.7

[a] Four-hundred erythrocytes were analyzed per animal, for a total of 2400 cells per treatment (PCE/PCE + NCE).

[b] Two-thousand PCEs were analyzed per animal, for a total of 12,000 cells per group.

[c] Significantly different from control ($P < 0.05$).

[d] Significantly different from the DXR group ($P < 0.05$).

where A corresponds to the group treated with DXR (positive control), B corresponds to the group treated with RA plus DXR, and C corresponds to the group treated with water (negative control).

2.5. Statistical analysis

All data were analyzed statistically by analysis of variance for fully randomized experiments, with calculation of F statistics and respective P values. In cases in which $P < 0.05$, treatment means were compared by the Tukey test and the minimum significant difference was calculated for $P = 0.05$.

3. Results

The frequencies of MNPCEs in peripheral blood of Swiss mice treated with RA, alone or in combination with DXR, at the different times of blood sampling are summarized in Tables 2–4. No significant difference in the induction of micronuclei was observed between the groups treated with the different doses of RA and the negative control. These findings indicate the absence of a mutagenic effect of the different concentrations of RA used in the present study. The frequency of MNPCEs increased in animals receiving i.p. DXR as expected, and a statistically significant difference was observed compared to the negative control.

Administration of a single oral dose of each concentration of RA simultaneously with DXR resulted in a significant reduction in the frequency of DXR-induced MNPCEs when compared to the group

Table 3
Frequencies of micronucleated polychromatic erythrocytes (MNPCEs) in peripheral blood of Swiss mice treated with rosmarinic acid (RA) and doxorubicin (DXR) and their respective controls 48 h post-treatment.

Treatment	PCE/PCE + NCE[a]	MNPCEs[b]		Reduction (%)
	Mean ± S.D.	Number	%	
Negative control	0.10 ± 0.01	18	0.15	–
RA 50	0.11 ± 0.01	27	0.22	–
RA 100	0.11 ± 0.01	31	0.25	–
RA 200	0.11 ± 0.01	27	0.22	–
DXR	0.10 ± 0.02	142[c]	1.18	–
RA 50 + DXR	0.11 ± 0.01	80[c,d]	0.67	50.0
RA 100 + DXR	0.10 ± 0.01	66[c,d]	0.55	61.3
RA 200 + DXR	0.10 ± 0.01	79[c,d]	0.66	50.8

[a] Four-hundred erythrocytes were analyzed per animal, for a total of 2400 cells per treatment (PCE/PCE + NCE).

[b] Two-thousand PCEs were analyzed per animal, for a total of 12,000 cells per group.

[c] Significantly different from control ($P < 0.05$).

[d] Significantly different from the DXR group ($P < 0.05$).