# PROTECTIVE EFFECTS OF CARNOSIC ACID AGAINST CHROMOSOMAL DAMAGE INDUCED IN HUMAN LYMPHOCYTES BY RADIATION GAMMA

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**The protective effects of carnosic acid (CA), carnosol (COL) and rosmarinic acid (RO) against chromosomal damage induced by □-ray, compared with those of the L-ascorbic acid (AA) and the S-containing compound dimethylsulphoxide (DMSO), were determined by use the micronucleus test for antimutagenic activity, evaluating the reduction in the frequency of micronuclei (MN) in cytokinesis-blocked cells of human lymphocytes before and after -ray irradiation. With treatment before -irradiation, the most effective compounds were, in order, *CA > RO = COL > AA > DMSO*. The radioprotective effects (antimutagenic) with treatment after -irradiation were lower, and the most effective compounds were CA and COL. RO and AA presented small protective activity, and the sulfur-containing compound DMSO lacked -ray radioprotection capacity. Therefore, CA and COL are the only compounds that showed a significant antimutagenic activity both before and after -irradiation treatments. These results are closely related to those reported by other authors on the antioxidant activity of the same compounds, and the degree of effectiveness depends of their structure. Futhermore, the results of different treatments before and after -ray irradiation suggest the existence of different protective mechanisms in each case.**

## INTRODUCTION

It is know that ionizing radiations such X and -rays generate reactive oxygen species (ROS) in organisms and induced cellular DNA damage, which leads to mutations and chromosomal aberrations[1, 2]. Recently, scavenging ability of certains plant extracts containing several polyphenols, flavones, catechins and procyanidins against ROS and their inhibitory effects against X-ray and -ray induced cell transformation were reported, both in vivo and in vitro[1-5].

At present, the micronucleus assay in human lymphocytes irradiated and treated with cytokinesis-block, as described by Fenech and Morley, is the most widely used test for analyzing the mutagenic capacity of chemical substances and physical agents[6, 7].

The objective of the present work was to study of the protective effects (antimutagenic activity) of carnosic acid, against the chromosomal damage induced in human lymphocytes by γ-rays, using the micronucleus test.

## MATERIALS AND METHODS

Heparinized samples of human peripheral blood were obtained from two healthy young non-smoking female donors. The rosemary phenolics used in this study, CA, COL and RO were dissolved in 5% aqueous DMSO at the ratio of 1 mg/ml, and AA was dissolved in 5% aqueous DMSO at the ratio of 2,5 mg/ml. For the before -irradiation treatments, 20 l of these solutions were added to 2 ml of human blood to obtain 25 M concentration and the samples were homogenized just before -irradiation. For the after -irradiation treatments, also 20 l of these solutions were added to 2 ml of irradiated human blood (25 M) and homogenized 15 min after -irradiation.

The blood samples were exposed to 137-Cs -rays with an Irradiator IBL 437 C (CIS, France) at dose of 2 Gy 3%. The irradiation was performed at room temperature for 40 s with a dose rate of 5 cGy/s in the moment of the study. The -ray exposure was established by means of thermoluminescent

dosimeters (TLDs) (GR-200, Conqueror Electronics Technology Co. Ltd, China).

**Culture technique.** After -irradiation treatments, the micronucleus assay was carried out on human lymphocyte culture according to the method of Fenech and Morley. Whole blood (1ml) was cultured at 37 °C for 72 hours in 9 ml of F-10 medium (Sigma Co.), containing 15% fetal bovine serum (Sigma Co.), 1.6% phytohaemagglutinin (Sigma Co.), and 1% penicillin/streptomycin (Sigma Co.). Forty-four hours after initiation of lymphocyte culture, cytochalasin B (Cyt. B) (Sigma Co.) was added in concentration of 3 $\mu$g/ml. At 72 hours the lymphocytes were treated with hypotonic solution (KCL, 0.075 M) for 3 min and fixed using metanol/acetic acid (3:1). Air-dried preparations were made, and slides stained with May-Grunwald Giemsa.

**Scoring of Micronuclei.** The number of micronuclei (MN) in at least 500 cytokinesis-blocked cells (CB cells) was scored using a Zeiss light microscope (Oberkochem, Germany) with 400 X magnification for surveying the slides and 1000 X magnification to confirm the presence or absence of MN in the cells. **Statistical analysis.** The degree of dependence and correlation between variables was assessed using analysis of variance, complemented by a contrast of means using p value ($p < 0.05$). Quantitative means were compared by regression and lineal correlation analysis. Also, was assessed the magnitude of protection: magnitude of protection (%) = [(Fcontrol - Ftreated) / Fcontrol] x 100. (where Fcontrol = frequency of MN in irradiated blood lymphocytes and Ftreated = frequency of MN in blood lymphocytes treated before and after the -ray irradiation[8]).

## RESULTS

Figure 1 shows the influence of treatments before -ray irradiation on the frequency of micronuclei (MN) in non-irradiated and irradiated human lymphocytes, which permits a comparison of the potential genotoxicity (non irradiated) of each compound versus its antimutagenic capacity (irradiated). In non irradiated human lymphocytes, all phenolic compounds show the same level of MN than control, whereas the sulfur-compound DMSO shows higher toxicity than the others. In irradiated human lymphocytes, the order of treatments, from lowest to highest level of MN generated after irradiation was: **CA < RO = COL < AA < DMSO** ($p< 0,001$).

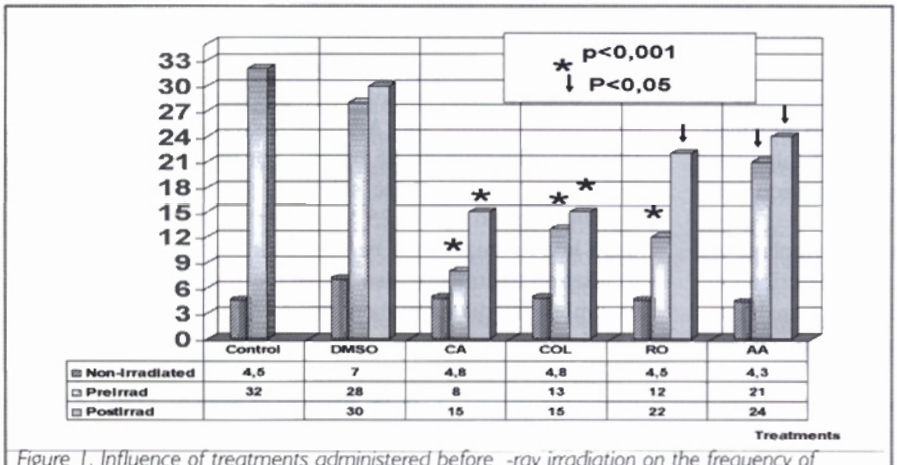


*Figure 1. Influence of treatments administered before -ray irradiation on the frequency of micronuclei in irradiated and non irradiated human lymphocytes.*

Figure 1 shows the influence of treatments after -ray irradiation on the frequencies of MN. The frequencies are higher than obseved in the treatments before -ray irradiation. It is clear that only CA and COL show a significant antimutagenic activity ($p< 0,001$). RO and AA present a low degree of radioprotective activity, and the sulfur-containing compound DMSO lack of -ray protection capacity. The order of treatments, from lowest to highest level of MN induced by irradiation, was **CA = COL < RO < AA < DMSO** ($p< 0,05$).

The protective effects and, consequently, the antimutagenic (or antigenotoxic) activity of the different compounds assayed (before and after -ray irradiation), were established according to the decrease in MN numbers according to the above equation[8] (see Material and Methods), obtaining a percentage value that determines the degree of protection of each compound. Figure 2 shows the values of these protection capacities, the orders of efficacy being **CA > RO = COL > AA > DMSO** for treatments before -ray irradiation and **CA = COL > RO > AA > DMSO** for treatments after -ray irradiation.

## DISCUSSION

In vivo, -rays cause a high generation of hydroxyl radicals, by homolytic cleavage of body water or endogenous hydrogen peroxide (formed by reduction of the superoxide anion) by two mechanisms: the Haber-Weiss and Fenton models. The hydroxyl radical is the most cytotoxic of all those so far described, with an estimated half-life of 10-9s[9, 10]. The high reactivity of this radical implies immediate reaction at the place where it is generated. When hydroxyl radical generation is masive, as with -irradiation, the cytotoxic effect is not only local but can propagate intracellularly and extracellularly, increasing the interaction of these radicals with phospholipoid structures and inducing peroxidation processes that increase the hydroxyl radical activity in DNA oxidative damage[9, 11].

In these oxidative stress conditions, when even the endogenous antioxidant systems are defective or insufficient, exogenous agents with a strong radical scavenging capacity must be used. This capacity depends on high absolute reactivity against different radicals or the high stability of the intermediate aroxyl radical formed[9]. In this study, CA, COL, RO, AA and the sulfur-containing compound DMSO were used as radioprotective agents. AA is considered to be one of the most powerful and least toxic natural antioxidant; it is water-soluble and is found in high concentrations in many tissues. On interaction with ROS, AA is oxidized to dehydroascorbate via the intermediate ascorbyl free radical and recycled back to ascorbic acid by the enzyme dehydro-ascorbate reductase. DMSO is a classic radical scavenger, with a high capacity for *in vitro* hydroxyl radical scavenging[12,13]. However, when applied in radioprotective doses, in the absence of any subsequent irradiation, is highly toxic in animals[14-16].

The results obtained concerning radioprotective effects (antimutagenic activity) of the different phenolics of *R. officinalis* in our study were closely related to those reported by other authors on the antioxidant activity of the same compounds[17]. Obviously, the degree of effectiveness depends on their structure. It is know that the capacity to inhibit hydroxyl radical is principally based on the combination of conjugate structures in the polyphenolic skeletons, mainly the o-dihydroxy-phenol or catechol structure and also the presence of carboxylic group. This greater activity of these compounds is also due to the stability of the polyphenolic radical generated in the process[9].

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