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Antioxidant Effect of Silymarin During Non-Enzymatic Peroxidation of Rat Kidney Microsomes and Mitochondria

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Abstract

Cellular metabolic changes during kidney disease may induce higher production of oxygen radicals that play a significant role in the progression of renal damage and in the onset of important comorbidities. In this study, the effect of Silymarin (SM) on chemiluminescence of mitochondria and microsomes isolated from kidney rat was analyzed and reported. After incubation of mitochondria and microsomes in an Ascorbate (0.4 mM)-Fe^{++} (2.15 µM) system (180 min at 37°C), non-enzymatic peroxidation was observed and the total cpm/mg of protein originated from light emission (chemiluminescence) was lower in kidney mitochondria and microsomes obtained from SM group than the control group (without SM). Moreover, it was observed that the SM was reduced based on concentration (6.25, 12.5, 25 and 50 µg) of chemiluminescence, and the same was measured as total cpm. The analyses of chemiluminescence indicate that SM may act as antioxidant protecting rat kidney mitochondria and microsomes from peroxidative damage.

***Keywords:** Antioxidant effect; Kidney; Peroxidation; Microsomes; Mitochondria*

Introduction

Silymarin, a standardized extract of the seeds of milk thistle (*Silybum marianum*) and its major component, silybin, is now used as an active component in a broad spectrum of dietary supplements and has received a tremendous amount of attention over the last decade as an herbal remedy [1,2]. Oxidative stress leads to deleterious processes. Therefore, restoring antioxidants is essential to maintain homeostasis. One method of restoring antioxidants is to consume natural compounds with antioxidant capacity. Antioxidant natural products and chemoprevention are considered nowadays as an effective approach against health various disorders and diseases induced by oxidative stress or free radicals [3]. SM is a potent antioxidant medicine and has been widely used for the treatment of diseases over 30 years. However, the mechanism underlying the action of Silymarin is not clarified. The production of reactive oxygen species in the kidney has been implicated as a common factor in the etiology of a number of diseases [4,5]. Present study was designed to determine whether mitochondria and microsomes from rat kidney could be a target for non-enzymatic lipoperoxidation as well as to establish the level of protection of such membranes incubated with SM. The degradative process was followed by the determination of chemiluminescence [6,7].

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Material and Methods

Female Wistar AH/HOK were obtained from Laboratory Animal Facility, Faculty of Veterinary Science, National University of La Plata. BSA (Fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. Sylimarin was kindly supplied by Vetanco S.A., 1603 Villa Martelli, Buenos Aires, Argentina. L (+) ascorbic acid was from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma.

Animals and preparation of microsomes and mitochondria

Seven-weeks-old female Wistar AH/HOK rats, weighing 120 g to 137 g were used. All rats were fed with commercial rat chow and water *ad libitum*. The rats were sacrificed by cervical dislocation and kidneys were rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30% (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4 using a Potter-Elvejhem homogenizer. The homogenate was spun at 10,000 RPM for 10 min. The supernatant (3 ml) obtained was applied to Sepharose 4B column (1.6 cm × 12 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.01% NaN_3. The microsomal fraction appearing in the void volume (10 ml to 16 ml) was brought to 0.25 M sucrose. All operations were performed at 4°C and under dim light. The quality of this microsomal preparation is of similar composition with respect to concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [8]. Mitochondria were obtained by method described by Schneider and Hogeboom [9].

Peroxidation of rat kidney microsomes and mitochondria

Chemiluminescence and peroxidation were initiated by adding ascorbate to microsomes or mitochondria [10]. The microsomes or mitochondria (1 mg of microsomal or mitochondrial protein) with addition of SM (6.25, 12.5, 25 and 50 µg-SM group) were incubated at 37°C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, final vol. 2 ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron (final concentration in the incubation mixture was 2.15 µM) for peroxidation [11]. Mitochondria or microsomal preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculated cpm/mg protein. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment with a program for chemiluminescence.

Protein determination

Proteins were determined by the method of Lowry et al. [12] using BSA as standard.

Statistical analysis

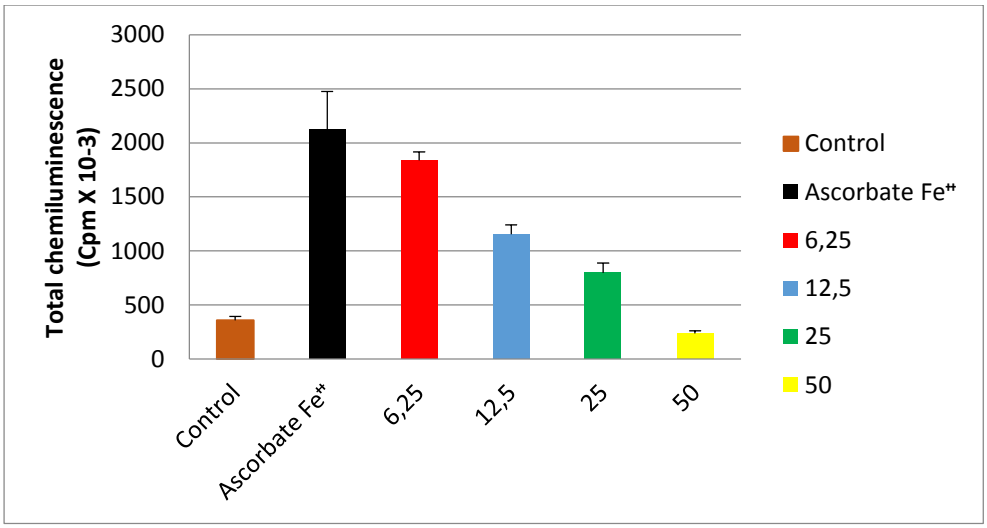
Results are expressed as means ± SD of six independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different p-values, which was indicated in each case.

Results

Light emission of rat kidney mitochondria during peroxidation

The incubation of rat kidney mitochondria in the presence of Ascorbate-Fe^{++} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of mitochondria in an Ascorbate-Fe^{++} system at 37°C

for 180 min, the cpm originated from light emission was lower (concentration dependent) in the SM group than in the control group. FIG. 1 shows the light emission obtained from SM group and control group. The values were from 2119.2 ± 312.9 in the control group to 236.7 ± 42.4 cpm with the addition of 50 µg of SM/mg prot.

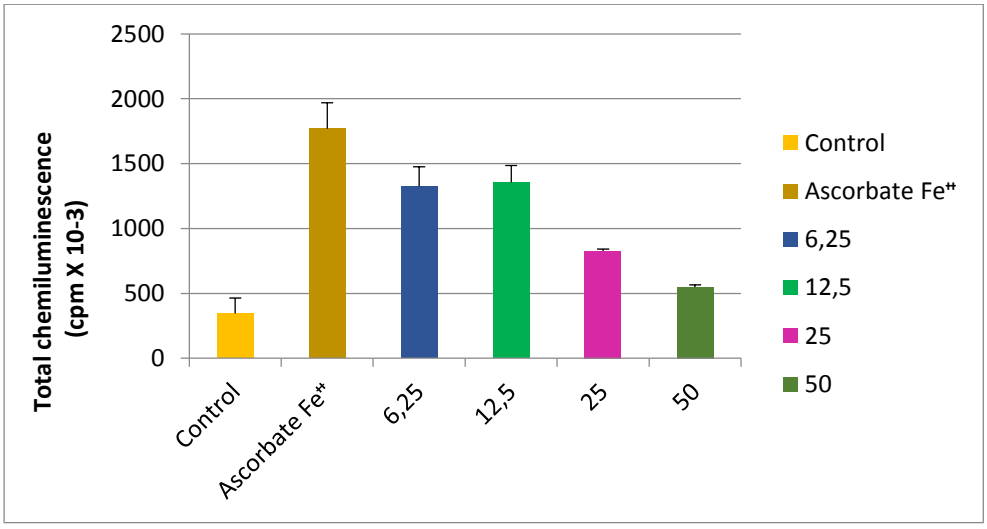


Data are given as the mean ± SD of six experiments.

FIG. 1. **Light emission of rat kidney mitochondria during peroxidation with different concentration of SM.**

Light emission of rat kidney microsomes during peroxidation

The incubation of rat kidney microsomes in the presence of Ascorbate-Fe^{++} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of microsomes in an Ascorbate-Fe^{++} system at 37°C for 180 min, the cpm originated from light emission was lower (concentration dependent) in the SM group than in the control group. FIG. 2 shows the light emission obtained from SM group and control group. The values were from 1768.3 ± 201.19 in the control group to 549.33 ± 15.27 cpm with the addition of 50 µg of SM/mg prot.



Data are given as the mean ± SD of six experiments.

FIG. 2. **Light emission of rat kidney microsomes during peroxidation with different concentration of SM.**

Comparative percentage of light emission inhibition in microsomes and mitochondria of rat kidney

After incubation of microsomes and mitochondria in an Ascorbate-Fe^{++} system (180 min at 37°C) it was observed that the percentage cpm/mg of protein originated from light emission (chemiluminescence) was lower in kidney microsomes and mitochondria with addition of SM. Thus, the percentage of peroxidation inhibition produced by SM was 88.83% in mitochondria and 68.93% in micromes with 50 µg of SM (FIG. 3).

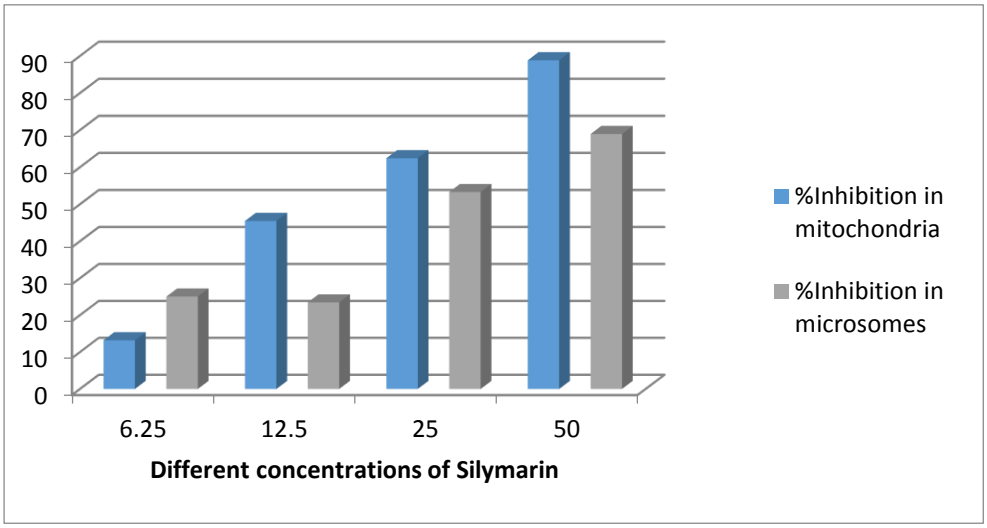


FIG. 3. **Percentage of light emission inhibition in microsomes and mitochondria of rat kidney by SM.**

Discussion

The aim of our study was evaluate the capacity of SM to protect kidney microsomes or mitochondria against peroxidation. Peroxidation studies in vitro are useful for the elucidation of possible mechanism of peroxide formation *in vivo* [13]. Possible antioxidant mechanisms of SM are evaluated by another authors: (1) direct scavenging free radicals and chelating free Fe and Cu are mainly effective in the gut; (2) preventing free radical formation by inhibiting specific ROS-producing enzymes, or improving an integrity of mitochondria in stress conditions, are of great importance; (3) maintaining an optimal redox balance in the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants [2].

Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation [14,15], it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. In the present study, we have determined that kidney microsomes or mitochondria with addition of SM are protected against peroxidation when compared with similar membranes obtained from control rats, as shown by the results obtained by chemiluminescence. In conclusion our results are consistent with the hypothesis that SM may act as an antioxidant in the cells membranes. However, further studies are needed to more adequately evaluate these observations.

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