SYNTETIC MELANOIDINS HAVE PROTECTIVE EFFECTS ON THE TOXICITY OF ADRIAMYCIN ON RAT HEPATOCYTES

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ABSTRACT

Melanoidins exhibit antioxidant properties *in vitro* either through their protective effect against reactive oxygen species or through metal chelating properties. The aim of the present study was to investigate the effect of synthetic melanoidins on adriamycin cytotoxicity in hepatocytes isolated of rats. The cellular responses to oxidative stress induced by adriamycin was examined in the form of LDH leakage, lipid peroxidation, protein oxidation and in form of the changes in the ATP levels. When the hepatocytes were incubated with melanoidins alone showed no presence of citotoxicity. The protective effect of melanoidins in hepatocytes subjected to oxidative stress with adriamycin was demonstrated because its presence resulted in a decrease of lipid peroxidation and protein oxidation and in the increase of ATP levels.

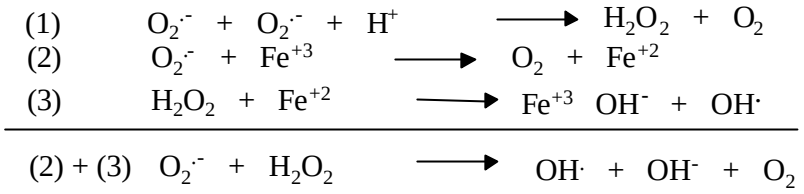
INTRODUCTION

Food is a mixture of numerous chemicals, including proteins, amino acids, carbohydrates, lipids vitamins, and minerals. It is well known that heat treatment such as cooling, promotes complex chemical reactions among food components. Among the many reactions occurring in processed foods, the non-enzymatic browing reaction or the Maillard reaction play the most important role in the formation of various chemical (Maillard, 1912). The brown and high molecular weight products of the Maillard reaction are the melanoidins (Ames, 1990; Friedman, 1996). Melanoidins are materials formed by interactions between reducing sugars and compounds possessing a free amino group, such as free amino acids and the free amino groups of peptides (Yaylayan and Kaminsky, 1998). The complex network of interactions, resulting in melanoidins as the final reaction products, is commonly referred to as the Maillard reaction products (MRPs) (Maillard, 1912).

The Maillard reaction products MRPs possess antioxidant activity (Chuyen 1998; Hayase et al., 1989) to act as scavenger of hydroxyl, superoxide and peroxyl radicals (Maillard, 1912; Wagner et al., 2002). Furthermore, antioxidant studies have included the ability of melanoidins to bind metals such as copper and zinc (Homma and Fujimaki, 1981). The cells of the human body are subjected to oxidative damage, which is associated with certain biological complications such as aging and carcinogenesis. Although living systems are protected from active oxidants by enzymatic systems, additional antioxidants are required to protect living cells from oxidation. Moreover, it is obvious that humans consume some quantity of antioxidants formed in heat-treated foods . These antioxidants may play a role in protection against oxidative damage associated with the diseases associated to oxidative stress.

The anthracycline antibiotic adriamycin have been in use as a potent antitumoral antibiotic used for the treatment of a variety of human cancers including lymphomas, leukemias, and solid tumours (Gewirtz, 1999; Hortobagyi, 1997). A number of different mechanisms have been proposed for the cytostatic and cytotoxic actions of these agents. Furthermore, it has been demonstrated that redox cycling of anthracyclines takes place in the complex I of the electronic

transport chain. The adriamycin can undergo a one-electron reduction and become a semiquinone radical. In the presence of oxygen, this semiquinone radical rapidly oxidizes, forming superoxide ion ($O_2^{\cdot -}$). This ion, after undergoing dismutation reaction gives rise to the formation of hydrogen peroxide (H_2O_2) which later, in the presence of transition metals such as iron leads to the formation of the hydroxyl radical ($^{\cdot}OH$). This reaction was proposed by Haber-Weiss in 1939, the results of which had been proposed by Fenton in 1894.



These free radicals or reactive oxygenic species stimulate lipid peroxidation and inhibit the mitochondrial function, causing cell damage which provokes cardiotoxicity, one of the side effects of the antibiotic. These include intercalation into DNA with consequent inhibition of macromolecular biosynthesis and generation of reactive oxygen species (ROS) (Hortobagyi, 1997; Valls et al., 1994; Doroshowi and Davies 1986). These free radicals or reactive oxygenic species stimulate lipid peroxidation and inhibit the mitochondrial function, causing cell damage which provokes cardiotoxicity, one of the side effects of the antibiotic (Lee et al., 1991). In this paper, the cytoprotective effect of melanoidins in isolated rat hepatocytes subjected to oxidative stress with adryamicin was established by studding lactate dehydrogenase (LDH), ATP levels, lipid and protein oxidation, in vitro assays

MATERIAL AND METHODS

Preparation of the melanoidin solutions in the reaction mixture of gluclose-glycine

The instruction for the separation of the melanoidin solutions were prepared according to the method previously published by Hofmann et. al (2001). 0.05 mol of both glucose and glycine were dissolved in 20 ml distilled water, frozen freeze dried over night and heated at 120ºC for 2h. This mixture was dialysed at 4 ºC with double-distilled deionized water with a dialysis tubing to obtained a high molecular weight fraction (molecular weight > 12.400 Da). This fraction were freeze-dried and used as solid fraction.

Isolation and treatment of hepatocytes

In order to carry out the study, we used isolated hepatic cells from male Wistar rats, aged between 3-4 months, and weighting between 250-300 g. They were fed with a standard IPM-20 diet. Hepatocytes were isolated by a liver perfusion with collagenase as described elsewhere (Berry and Friend, 1969) and cell viability tested with trypan blue was always greater than 90%. 2 ml of hepatocytes suspension containing approximately $2x10^6$ cells in Krebs-Henseleit saline equilibrated with O_2/CO_2 (95/5; v/v) were incubated in a shaking water bath at 37 ºC for 1 hour, in 25 ml conical flasks sealed with rubber stoppers. Control toxicity was carry out incubated isolated hepatocytes in presence of different concentration of melanoidins (10, 50 ?g). Incubation mixtures containing hepatocytes and adriamycin 50 ?M in absence/ presence of melanoidins were made up to 4 ml final volume with Krebs-Henseleit buffer solution (pH 7.4). Once the incubation time had elapsed, the cells were processed to carry out the relevant tests of oxidative stress.

Analytic Methods

LDH leakage measurement: Cell viability was determined by quantification of the lactate dehydrogenase released into the extracellular medium (Bergmeyer and Bernt, 1974).

Determination of thiobarbituric acid reactive substances (TBARS): Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the spectrophotometric method of Stacey and Priestly (1978) with 1,1,3,3-tetramethoxypropane used as standard.

Protein carbonyl measurement: Protein oxidation was analyzed by measuring protein carbonyl content after treatment of hepatocytes with 2,4-dinitrophenyl hydrazine (DNPH), determined spectrophotometrically at 360 nm and calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} (Levine et al. 1990).

Adenosine triphosphate (ATP): The ATP levels was determined on isolated rat hepatocytes by measuring spectrophotometrically at 340 nm NADPH oxidation in the presence of glucose by Lamprecht and Trautschold method (1974).

Protein measurement: Total protein concentration was evaluated using bovine albumin as a standard by Lowry method (Lowry et al., 1951).

Statistical analysis

Results are presented as the means ? SD for the indicated number of independent experiments. Statistical analysis was performed using Student´s *t*-test for unpaired data. Differences were assumed to be statistically significant at $P<0.05$.

RESULTS

The melanoidins effect was studied in hepatocytes incubated for 60 minutes in absence (melanoidins) and in presence of 50 adriamycin (ADR). The value corresponding to hepatocytes incubated for 60 minutes in absence of substrates, this is the control value (C).

The Fig. 1 shows the levels of LDH leakage measured as an index of cell toxicity. It was observed that adriamycin induces citotoxicity because to increase significantly the levels of LDH. In the figure, the last two columns refer to the hepatocytes incubated with only melanoidins at 10 and 50 ?g. We can observe that the values are equal or lower than the control, which verifies that melanoidins do not have any toxic effect. When incubated melanoidins with adriamycin, the levels of LDH decreased reaching control values at the two doses studied. Thus. These results reveal protective effect of melanoidins in hepatocytes viability treated with adriamycin.

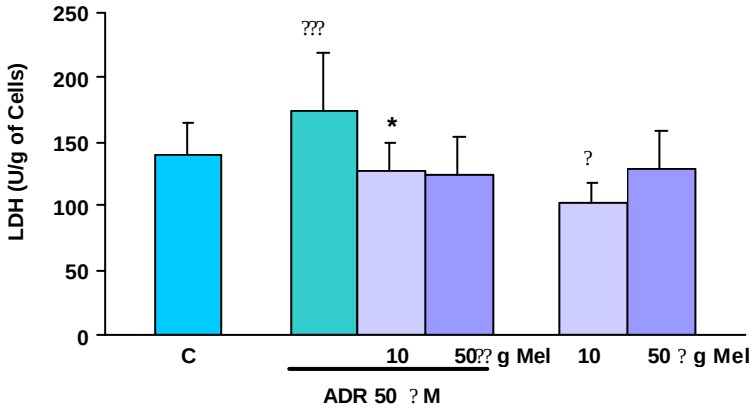


Fig.1.- Lactate dehydrogenase (LDH) levels in isolated rat hepatocytes. Results are expressed as mean ± SD of 8 separate experiments. C= control ADR= adriamycin and Mel= melanoidins. Statistical significance was evaluated by Student's *t*-test. ?P<0.05 ??P <0.005 ADR vs C and Melanoidins vs C. *P<0.05 **P<0.005 ADR+ Melanoidins vs ADR.

The damage to lipids was studied by quantification of the products formed in lipid peroxidation and which react with thiobarbituric acid (TBARS), table 1. When in the incubation medium there are melanoidins alone the values of TBARS are similar or decreasing below of the values control hepatocytes.

Table 1. Effect of melanoidins on the levels of thiobarbituric acid reactive substances (TBARS) in isolated rat hepatocytes treated with adriamycin

Concentrations	TBARS (nmol/g of Cells)
Control	280 ? 73,8
Melanoidins (Mel) 10 ? g	210.91 ? 66.9
Melanoidins (Mel) 50 ? g	125.27 ? 69.6??
Adriamycin (ADR) 50 ? M	410 ? 104.09?
ADR 50 ? M + Mel 10 ? g	243.46 ? 42.3*
ADR 50 ? M + Mel 50 ? g	112.4 ? 38.2**

Results are expressed as mean ± SD of 8 separate experiments.
Statistical significance was evaluated by Student's *t*-test.
?P<0.05 and ??P<0.005 adriamycin vs control and melanoidins vs control.
*P<0.05 **P<0.05 adriamycin + melanoidins vs adriamycin alone.

We can observe that when the adriamycin is in the incubation medium, the levels of TBARS increases significantly ($p<0.05$). When melanoidins are present in the medium, there is a considerable reduction of TBARS levels increased by the effect of adriamycin. These melanoidin changes were significant at adriamycin 50 ? M (decreased of 41% and 73% for 10 ? g and 50 ? g respectively).

The Fig. 2 shows the levels of protein carbonyl groups. We observed that there is an increase higher significative ($p<0.005$) in the levels of carbonyl groups in presence of adriamycin compared with the control hepatocytes. Decreased of carbonyl group levels were observed when incubated 50 ? g of melanoidins and adriamycin 50 ? M. However no significant differences were observed when 10 ? g of melanoidins were present in the incubation medium.

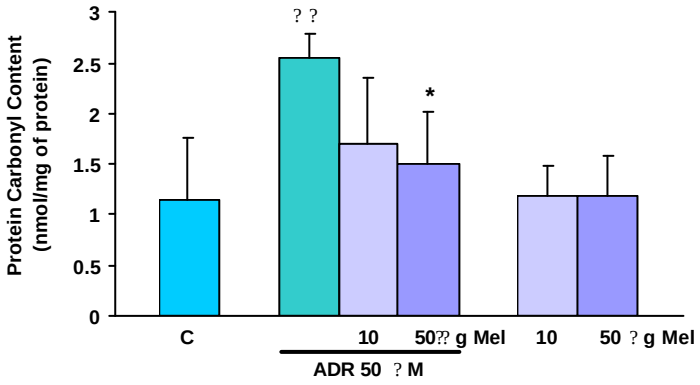


Fig.2.- Protein carbonyls content in isolated rat hepatocytes. Results are expressed as mean ± SD of 8 separate experiments. C=control ADR= adryamicin and Mel= melanoidins. Statistical significance was evaluated by Student's *t*-test. ??P<0.005 adriamycin vs control. *P<0.05 adriamycin + melanoidins vs adriamycin alone.

The main source of energy for cells is the adenosine triphosphate (ATP) and adriamycin interacts with the first complex of the electronic transfer chain submitting changes in ATP levels.

Table 2. Melanoidins effect on the levels of adenosine triphosphate (ATP) in isolated rat hepatocytes treated with adriamycin.

Concentrations	ATP (? mol/g of Cells)
Control	1.64 ? 0.29
Melanoidins (Mel) 10 ? g	1.20 ? 0.28
Melanoidins (Mel) 50 ? g	1.39 ? 0.22
Adriamycin (ADR) 50 ?M	0.97 ? 0.32??
ADR 50 ?M + Mel 10 ? g	1.52 ? 0.49*
ADR 50 ?M + Mel 50 ? g	1.35 ? 0.39*

Results are expressed as mean ±?SD of 8 separate experiments.
Statistical significance was evaluated by Student's *t*-test.
? P<0.05 and ?? P<0.005 adriamycin vs control.
*P<0.05 and **P<0.05 adriamycin + melanoidins vs adriamycin alone.

The table 2 shows the ATP levels. The incubation the isolated hepatocytes with melanoidins alone decrease no significantly the ATP values. In this table we observe that in presence of adriamycin the levels of ATP decreased significantly ($p<0.005$). The presence of melanoidins in adriamycin-hepatocytes resulted in a significant increase ($p<0.05$) in the ATP values in all cases studied to compared with it control group.

DISCUSSION

The precise molecular mechanism melanoidins antioxidant activity is still unknown although hydroxyl, superoxide scavenging activity and metal chelating ability had been suggested (Faist and Erbersdobler, 2001; Wagner et al. 2002). So, results of studies on the physiological benefits of food and beverages suggest that some of this effects can be attributed to melanoidins (Chuyen et al., 1990; Borrelli et al, 2002).

Recently, a standard melanoidins prepared from glucose and glycine has been established in order to have a material that can be used for the study of different aspect of melanoidins such as this antioxidant properties or protective effect to reactive oxygen species (ROS) (Ames et al., 2000).

The adriamycin is a useful compound for the study of oxidative stress because the antioxidant activity is thought to play an important role in the protection against adriamycin toxicity (van Acker, 2001). The exposure of hepatocytes to adriamycin, result in a increase in the formation of oxidative damage. For these, this system represent a good model for evaluate the effect of melanoidins on antibiotic toxicity mediated by ROS. Studies previous have reported the induction of citotoxicity using a drug concentration of 50 ?M (van Acker et al., 2001).

In order to predict toxicity cell, was studied the lost of viability in hepatocytes measuring the LDH leakage, and deleterious effects were determined by measuring the ATP levels. The results obtained on LDH leakage and ATP levels when hepatocytes were treated with

melanoidins alone showed no presence of citotoxicity, not there are harmful changes with respect to control values. Moreover, melanoidins presented protective effects of LDH and ATP levels against adriamycin citoxicity observed.

The presence of melanoidin in the incubation medium of isolated rat hepatocytes subject to a oxidative stress resulted in a decrease of lipid oxidation. These findings are similar to those reported by Chuyen (1998) who observed that liver TBARS values of rats fed with peptide-glucose reaction products showed much lower values than those of control rats fed with unheated peptide-glucose mixture.

The cytoprotector effect of the melanoidins using glucose-glycine model might to be due for their highest potential to inhibit the hydroxyl radical (Yoshimura et al, 1997). Other explication might to be the proposed by Wagner et al. (2002), were the Maillard reactions products contain at least one group hydroxyl which might act as hydrogen donator and therefore have an ability to scavenge free radicals.

In conclusion, melanoidins formed by a glucose-glycine model at 120 ºC were highly effective in the protection of hepatocytes from oxidative adryamicin toxicity by distinct mechanisms decreased the lipid and protein oxidation and increasing the levels of ATP.

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