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ANTIOXIDANT EFFECT OF MELANOIDINS AFTER INDUCTION OF OXIDATIVE STRESS IN ISOLATED RAT HEPATOCYTES

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Introduction

In this work we have studied the effect of melanoidins synthesised from glucose and glycine, after the induction of oxidative stress generated by the antitumoral antibiotic adriamycin in isolated rat hepatocytes. Adriamycin or doxorubicin (Fig,1) is a quinonic antibiotic belonging to the anthracyclines group. Clinically, it is a potent antitumoral antibiotic used for the treatment of a variety of human cancers including lymphomas, leukemias, and solid tumors. The citostatic effect of this drug implies the inhibition of topoisomerase II and RNA polymerase II, insertion into chromosomal DNA and formation of complexes with transsition metals, provoking erroneous transcription and replication, and generation of reactive oxygen species (ROS) (Valls *et al.*,1994).

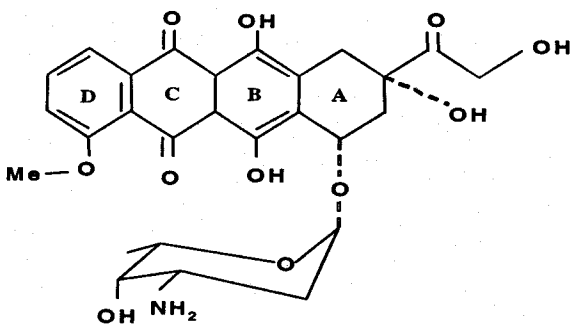


Figure 1.- Structural formula of adriamycin (doxorubicin)

The clinical use of the antitumoral agent, adriamycin, is largely limited, because it is a cumulative dose-related and this provokes cardiotoxicity. This toxicity is generally believed to be caused by the formation of oxygen free radicals.

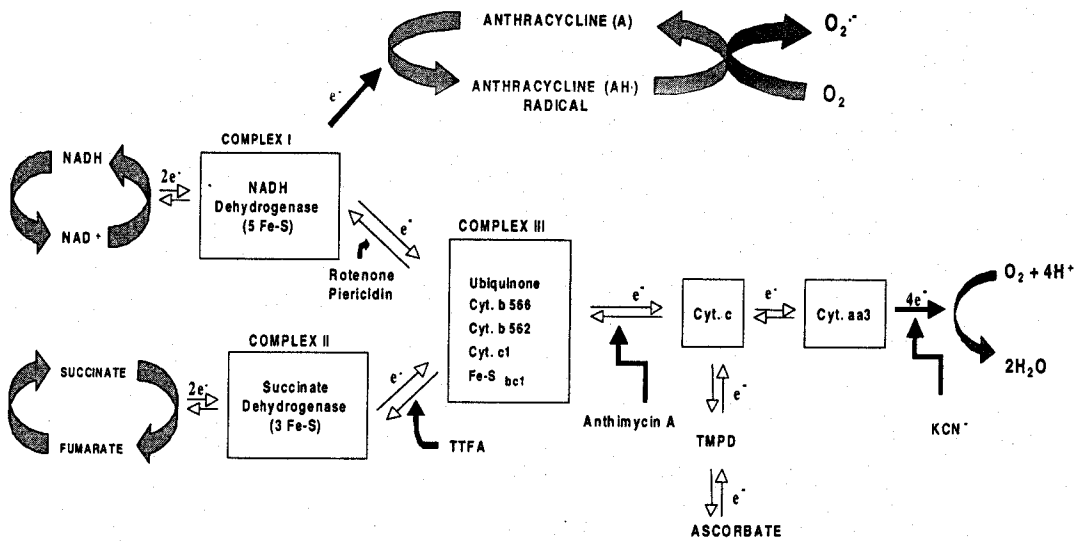
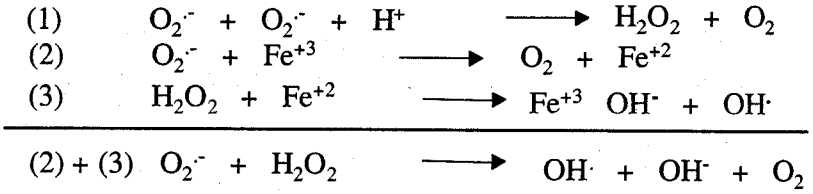


Figure 2.- Redox cycling of anthracyclins by mitochondria

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.

In this study our main objective is to reduce or eliminate ROS by the administration of melanoidins, in order to avoid the side effects of this antibiotic.

Materials and Methods

In order to carry out the study, we used isolated hepatic cells from male Wistar rats, aged between 3-4 months, and weighing between 250-300 g. They were fed a standard IPM-20 diet. Hepatocytes were isolated by a simplified version of the perfusion method of Berry and Friend (1969). Cell viability was tested with trypan blue. Approximately 95% of the isolated hepatocytes were found to exclude the dye. 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 °C (degrees celsius) for 1 hour, in 25 ml conical flasks sealed with rubber stoppers. Incubation mixtures containing cells and 50 µM of adriamycin in absence/presence of melanoidins (10, 50 µg), 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

- cell viability assessment, by quantification of the lactate dehydrogenase released into the extracellular medium (Bergmeyer and Bernt,1974).
- Damage caused to lipids, the extent lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the spectrofotometric method of Stacey and Priestly (1978).
- Damage caused to proteins, by quantification of the protein carbonyl groups (Levine *et al.*,1990)
- Adenosine triphosphate (ATP) levels was determined by Lamprecht and Trautschold method (1974).
- GSH content of isolated hepatic cells was determined using the assay of Brigelius, of the glutathione -S- transferase (Brigelius *et al.*,1983), and the protein content by Lowry method. (Lowry *et al.*,1951).

Results and Discussion

We first determined cellular viability (Fig. 3) by quantification of the lactate dehydrogenase (LDH) released into the extracellular medium. Being T0, the value corresponding to the cells not incubated, and T60, cells incubated for 60 minutes in absence of substrates, this is the control value. The first column of each block represents those cells incubated for 60 minutes with ADR (50 µM). We can observe that as the concentration of adriamycin rises, the release of LDH increases. The last two columns refer to the cells incubated with only melanoidins at 10 and 50 µg. We can observe that the values are equal to the control, which verifies that melanoidins do not have any toxic effect. When melanoidins are present in the incubation medium together with adriamycin, the levels of LDH decrease, reaching values even lower than control T60, being significant in the case studied. These results reveal the protective effect of melanoidins in cellular viability.

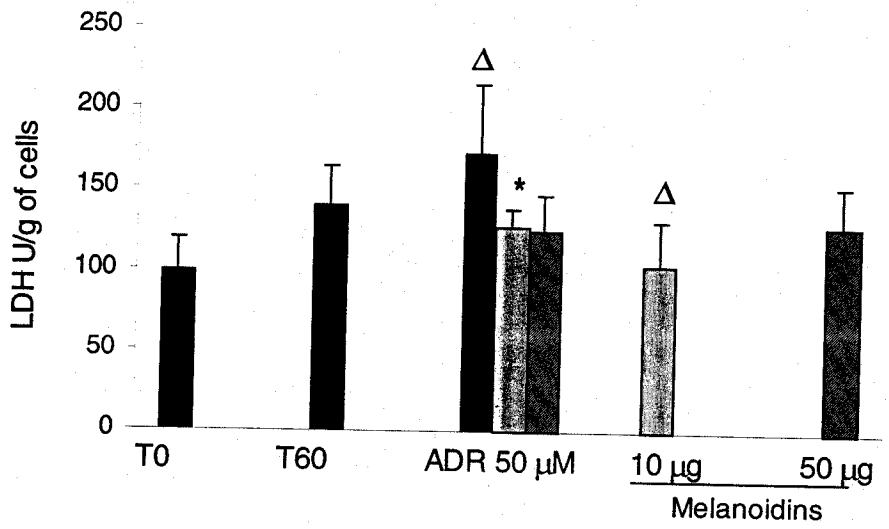


Figure 3 Lactate dehydrogenase (LDH) levels in isolated rat hepatocytes.
Results are expressed an mean ϒSD of 8 separate experiments. Statistical significance was evaluated by Student´s *t*-test.
$^{\Delta}P< 0.05$ and $^{\Delta\Delta}P<0.005$ - Compared with the control (T60)
*P<0.05 and **P<0.005 - Compared with corresponding adriamycin concentration

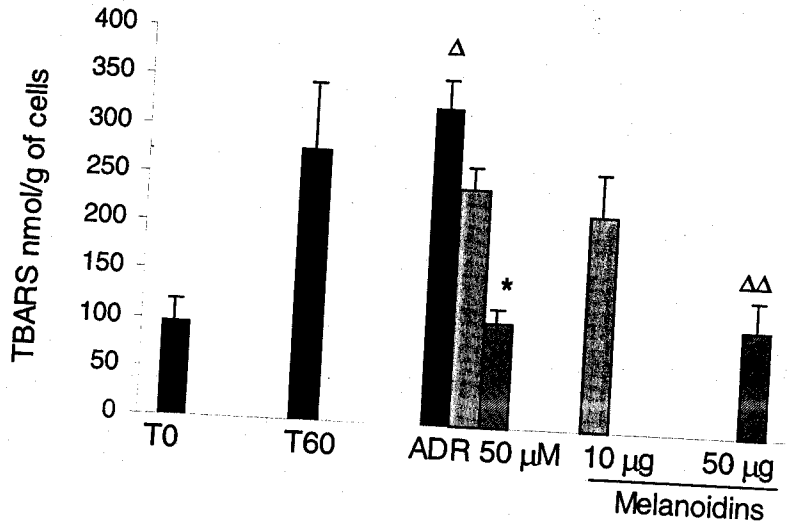


Figure 4 Levels of thiobarbituric acid reactive substances (TBARS) in isolated rat hepatocytes. Results are expressed an mean ϒSD of 8 separate experiments. Stadistical significance was evaluated by Student´s *t*-test.
$^{\Delta}P< 0.05$ and $^{\Delta\Delta}P<0.005$ - Compared with the control (T60)
*P<0.05 and **P<0.005 - Compared with corresponding adriamycin concentration

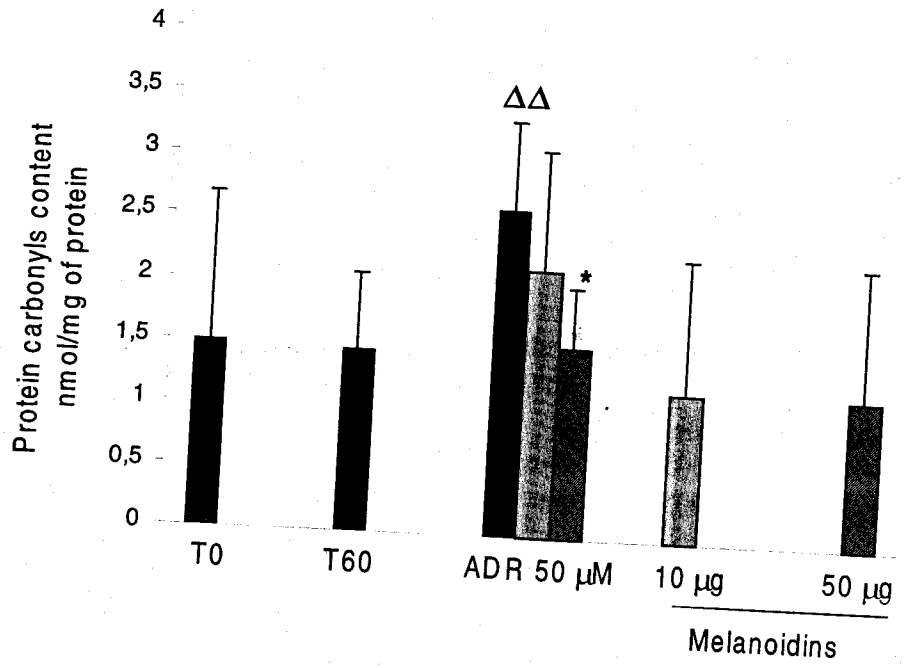


Figure 5 Protein carbonyls content in isolated rat hepatocytes.
Results are expressed an mean ϒSD of 8 separate experiments. Statistical significance was evaluated by Student´s *t*-test.
$^{\Delta}P< 0.05$ and $^{\Delta\Delta}P<0.005$ - Compared with the control (T60)
*P<0.05 and **P<0.005 - Compared with corresponding adriamycin concentration

Later we studied the damage caused to lipids and proteins. The damage to lipids was studied by quantification of the products formed in lipid peroxidation and which react with tiobarbituric acid (TBARS), figure 4. It was observed that adriamycin induces peroxidation, increasing levels of TBARS. When melanoidins are present in the medium, there is a considerable reduction until control values are reached. When the concentration of melanoidins is 50 μg, values are identical to control T0, being significant in the concentration of ADR studied. The higher the concentration of melanoidins is, the greater is the protective effect.

In figure 5 we study the damage caused to proteins, by quantification of the protein carbonyl groups. Here, there is also an increase in the presence of adriamycin compared with the control, and a reduction when there are melanoidins present in the medium, being equal to control T60.

These results highlight the antioxidant effect of melanoidins against macromolecules, such as lipids and proteins.

Since the main source of energy is the adenosine triphosphate (ATP) and adriamycin interacts with the first complex of the electronic transfer chain, we quantify the levels of ATP, figure 6. In this graph we observe that in presence of adriamycin the levels of ATP decrease, although it is not significant. When melanoidins are present in the medium they increase, reaching the control values.

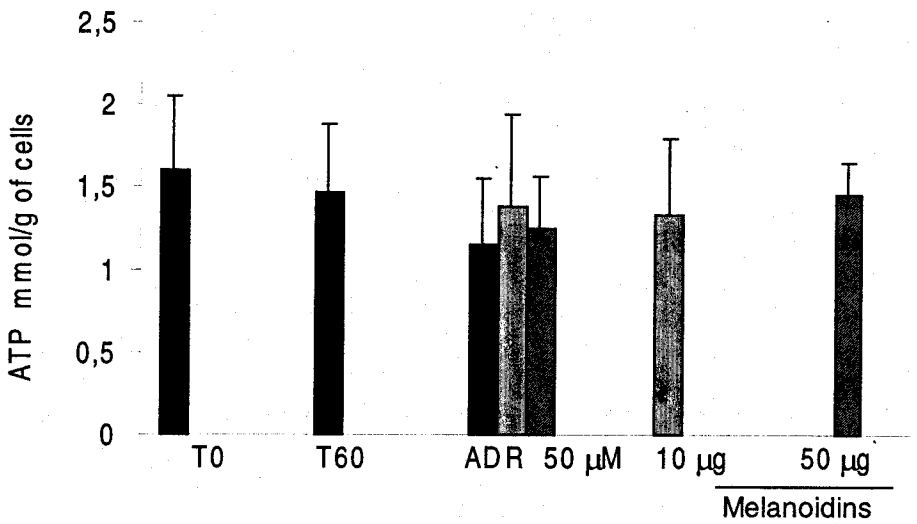


Fig.6.- Levels of adenosin triphosphate (ATP) in isolated rat hepatocytes.
Results are expressed an mean ϒSD of 8 separate experiments. Statistical significance was evaluated by Student´s *t*-test.
$^{\Delta}P< 0.05$ and $^{\Delta\Delta}P<0.005$ - Compared with the control (T60)
*P<0.05 and **P<0.005 - Compared with corresponding adriamycin concentration

However, when we study non enzymatic antioxidant defence, as in the case of glutathione (GSH), figure 7, we observed that in the presence of melanoidins the levels of GSH are equal to control T60, not having any effect. But when melanoidins are present together with adriamycin the values are lower. This would be on effect not desired in the antioxidant defence.

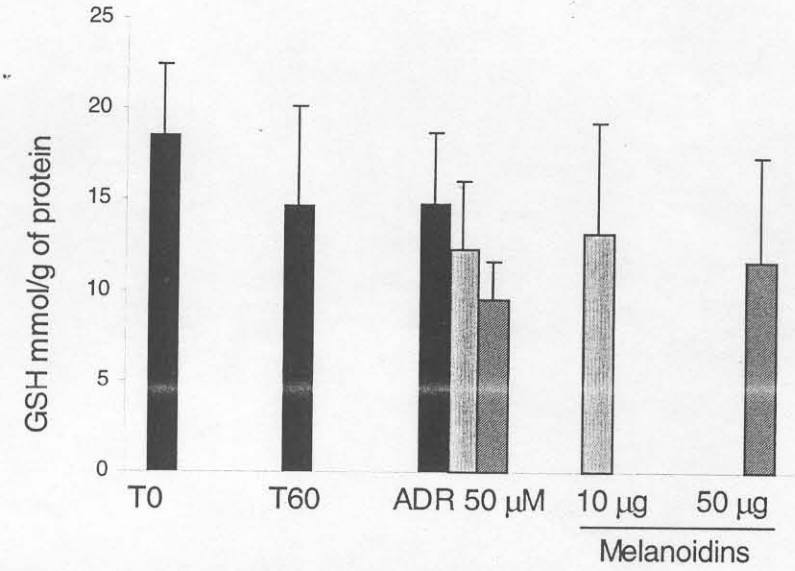


Figure 7 Glutathione (GSH) levels in isolated rat hepatocytes.
Results are expressed an mean ϒSD of 8 separate experiments. Stadistical significance was evaluated by Student´s *t*-test.
$^{\Delta}P< 0.05$ and $^{\Delta\Delta}P<0.005$ - Compared with the control (T60)
*P<0.05 and **P<0.005 - Compared with corresponding adriamycin concentration

In conclusion, melanoidins synthesized from glucose and glycine have a protective effect over macromolecules. They increase cell viability, protect lipids and proteins from the oxidative stress and induce ATP formation, therefore, they have an antioxidant effect. But, on the other hand, they maintain or decrease GSH levels, an adverse effect for antioxidant defence. At the time being we have only determined levels of GSH, but it would be interesting to determine other parameters involved in antioxidant defence.

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