Effect of Naringin on the DNA Damage Induced by Daunorubicin in Mouse Hepatocytes and Cardiocytes

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Naringin (Nar) is a flavonoid that has shown antigenotoxic effect against the chromosome damage induced by various compounds. The aims of the present investigation on Nar were threefold: a) to determine its DNA breaking potential in mouse hepatocytes and cardiocytes, b) to evaluate its capacity to inhibit the DNA damage induced by daunorubicin (Dau) in the same tissues, and c) to determine its capacity to trap free radicals *in vitro* using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method. For the two first purposes we applied the comet assay to three groups of animals administered with Nar by the oral route (50, 250, 500 mg/kg), and made the observations before the chemical administration and at 3, 12, and 21 h postadministration. Other three groups of mice were given equal doses of Nar, and 1 h later they were intraperitoneally injected with 1 mg/kg of Dau. The results showed that Nar did not induce DNA breakage in both types of studied cells, in contrast with the significant damage induced by Dau in hepatocytes and cardiocytes. Moreover, the administration of Nar protected the DNA damage produced by Dau, showing a maximum reduction of 71.3% and 51.1% in hepatocytes and cardiocytes, respectively. With respect to the antioxidant potential, 20 mM of Nar produced a free radical scavenging activity as high as 95%. Our study established a high DNA breaking potential of Dau, and a protective effect by Nar, probably related with its capacity to trap free radicals.

Key words antigenotoxic; naringin; antioxidant; daunorubicin; micronucleus; mouse

Naringin (Nar) (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) is a chemical found in different parts of the grapefruit (*Citrus paradisi* Macf.) (Rutaceae), including its flower, fruit and peel.[1] Genotoxic studies made on this compound have determined no effect using the Ames test, as well as no increase in the rate of micronuclei (MN) in mouse blood cells.[2,3] On the contrary, these studies found a 36% mean reduction in the damage induced by benzo(*a*)pyrene in the *Salmonella typhimurium* assay (strains TA 98 and TA 100, with and without metabolic activation), and an inhibition of 54.2% in the rate of MN formed by ifosfamide in mouse blood cells. Other studies in mouse bone marrow also reported a significant reduction produced by Nar in the frequency of chromosomal aberrations and MN caused by gamma-radiation.[4,5] Besides, an *in vitro* assay in cultured precision-cut human and rat liver slices that were analyzed with the unscheduled DNA synthesis test showed a protective effect of the flavanone against the damage induced by the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine; however, no effect was observed in connection with the damage induced by 2-acetylaminofluorene and aflatoxin B_1.[6]

Moreover, Nar has also shown other interesting properties, such as its effect as hypocholesteremic, and inducer of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase.[7,8] Therefore, further studies on the biological properties of Nar are advisable.

On the other hand, the antineoplasic agent daunorubicin (Dau) is known to produce genotoxic alterations in *in vitro* and *in vivo* models, where gene mutations and increases in the rate of sister chromatid exchanges and of MN have been detected.[9—11] The compound may also affect the normal structure of the DNA by intercalation, alkylation, cross-linking, and apoptosis *via* topoisomerase II alterations.[12,13] In particular, the production of reactive oxygen species (ROS) during the metabolism of Dau has been related with the development of severe cardiotoxicity and hepatotoxicity in experimental animals, suggesting the need to avoid or reduce this damage that limits the usefulness of the medicament.[14—16] Based on the indicated knowledge, we designed the present study to investigate the DNA breaking potential of Dau in mouse cardiac and hepatic cells, as well as to determine the protective role of Nar in this type of damage. In addition, we determined the *in vitro* free radical trapping capacity of Nar.

MATERIALS AND METHODS

Chemicals and Animals Dau was acquired from Lemery Laboratories (Mexico City, 97% pure); 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 4′,5,7-trihydroxyflavanone 7-rhamnoglucoside (Nar), ethidium bromide stain, trypan blue, low melting point agarose (LMPA) and normal melting point agarose (NMPA) were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). We used male mice (NIH) with 25 g of weight, obtained from the National Institute of Hygiene. The animals were maintained in metallic cages at 23 °C and a 12 h dark–light cycle, with free access to food (Purina) and tap water. The protocol was approved by the Committee of Ethics and Biosecurity of the National School of Biological Sciences.

Genotoxicity/Antigenotoxicity Protocol Initially, we confirmed that Nar is an agent with low toxicity in mouse. As previously determined,[3] no mortality was observed when doses up to 5000 mg/kg were tested. Then, three mice were assigned to each of eight groups treated by gavage with the

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compounds (except for Dau, which was intraperitoneally administered). One of these groups received 0.3 ml of distilled water, another group was treated with 1 mg/kg of Dau, three more groups were administered with 50, 250 and 500 mg/kg of Nar, respectively; the last three groups of the assay were also treated with 50, 250 and 500 mg/kg of Nar, but 1 h later they were administered 1 mg/kg of Dau. The selected doses of Nar and Dau had been used in our earlier report to evaluate their effect on MN.[3)]

The mice of each group were sacrificed at 3, 12, and 21 h after the chemical administration, and the liver and heart were dissected. Each organ was placed in cold phosphate buffered saline (PBS) and cut in fragments of approximately 3 mm to separate the cells. Then, the gross tissue was detached and the remaining cellular suspension of each organ (about 10000 cells/ml) was examined with the trypan blue exclusion technique to evaluate cellular viability, which was found higher than 85%. Next, the alkaline unicellular electrophoresis (comet) assay was made in two slides per animal as described earlier,[17)] with slight modifications. Seventy five microliters of 1% NMPA was layered on a cold fully frosted slide and coated with another layer formed by a 1 : 1 mixture made with 75 μl of the cellular suspension plus 2% LMPA at 45 °C. Finally, another layer of LMPA was put on top of the second agarose. The slides were then placed in a chilled lysis solution pH 10.5 for 2 h. The solution consisted of NaCl 2.5 M, ethylenediaminetetraacetic acid (EDTA) 100 mM, trizma base 10 mM (pH 10), 1% triton X-100, and 10% dimethyl sulfoxide (DMSO). The slides were immersed in a horizontal electrophoresis chamber containing chilled alkaline solution (NaOH 300 mM, and Na_2EDTA 1 mM, at a pH>13), and left in the solution for 20 min in the dark to allow DNA unwinding and the expression of alkali-labile sites. Afterwards, the electrophoresis was carried out for 20 min at 25 V and 300 mA. The slides were rinsed gently 5 times with 400 mM trizma (pH 7.5) to neutralize the excess alkali, and the nucleoids of each slide were stained with 50 μl of ethidium bromide to be examined at 400×magnification with an epifluorescent microscope (Axiophot-1, Zeiss) equipped with a digital camera (ZWS-47DE) and software for the capture, processing and image analysis (Zeiss KS400 version 3.01). We measured the total length containing the nucleus and the migrated DNA (T), and divided the result by the nucleus diameter (N) so as to obtain the T/N ratio. By this analysis cells exhibiting no damage show a ratio with a tendency to 1.[18)] Furthermore, four grades of damage were established in 100 nucleoids per dose/time for each organ. Grade 1 corresponded to round, compact nucleoids without comet tails, and grade 4 to those with the longer tails. Statistical analysis of the obtained data was made with the ANOVA and Student–Newman Keuls tests by using the Instat software, version 2.

Radical-Scavenging Activity of Naringin The method used for this purpose was described by Russo *et al.*,[19)] and is based on the color loss determination of the stable radical DPPH. In a final volume of 1 ml ethanol we mixed Nar (from 2 to 20 mM) and DPPH (86 mM), and incubated the mixture for 10 min at room temperature. The absorbance was spectrophotometrically assessed in triplicate at 517 nm against a blank (ethanol). The %DPPH radical scavenging activity was calculated according to the equation: %DPPH radical scavenging=[(mixture absorbance+DPPH absorbance)/DPPH absorbance)]×100.

RESULTS

Figure 1 shows the T/N index of each group of mouse hepatocytes exposed to Dau and protected with Nar. We found that the three doses of the studied flavonone had no genotoxic effect at 3, and 12 h; at 21 h, however, Nar (50, 250 mg/kg) showed statistical differences in comparison with the control value. It is probable that these statistical increases do not signify relevant cellular damage, in light of the higher, but not significant values determined for the two lower doses of Nar at 3 and 12 h. The cells treated with Dau showed a significant level of DNA deterioration all through the assay. At 12 h the damage was equivalent to almost four times the level found in the control cells; this value, however, showed a 43.8% decrease at the last data point of the study (21 h), suggesting the involvement of DNA repair during this period. The administration of Nar followed by the mutagen prevented the DNA damage induced by Dau in a variable way: the two high doses of Nar showed a mean protection of 61.1%, with a maximum effect of 71.3% with 500 mg/kg of

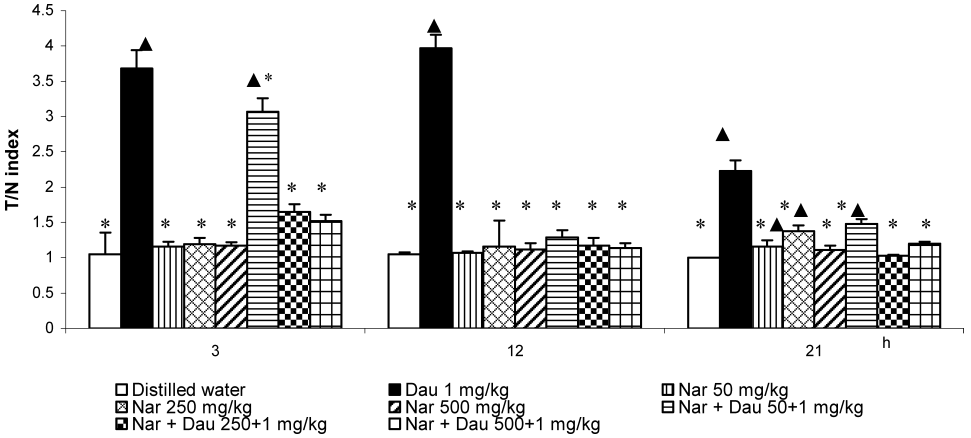

Fig. 1. T/N Index Determined in the DNA of Mouse Hepatocytes Exposed to Daunorubicin (Dau) and Treated with Naringin (Nar)

▲ Statistically significant difference with respect to the value obtained in the control cells, and * with respect to the value determined with Dau. ANOVA and Student–Newman Keuls tests (α=0.001).

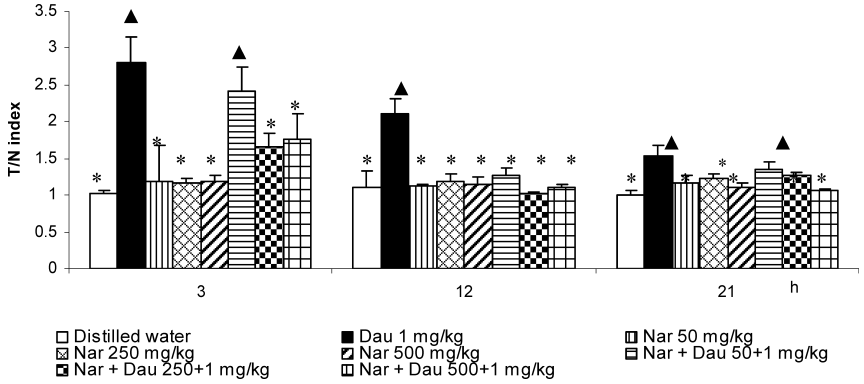

Fig. 2. T/N Index Determined in the DNA of Mouse Cardiocytes Exposed to Daunorubicin (Dau) and Treated with Naringin (Nar)

▲ Statistically significant difference with respect to the value obtained in the control cells, and ∗ with respect to the value determined with Dau. ANOVA and Student–Newman Keuls tests (α=0.001).

Table 1. Grades of Damage Observed in the DNA of Mouse Hepatocytes Exposed to Daunorubicin (Dau) and Treated with Naringin (Nar)

Agent	(mg/kg)	Time (h)	Grades of damage (%)			
			1	2	3	4
Distilled water		3	97.0	2.5	0.5	0
		12	96.3	2.7	0.3	0.7
		21	99.7	0.3	0	0
Dau	1.0	3	8.7	29.0	26.3	36.0
		12	3.3	22.7	28.6	45.4
		21	46.3	34.3	14.0	5.4
Nar	50	3	98.0	1.0	1.0	0
	250	3	97.4	1.6	1.0	0
	500	3	96.0	1.7	2.0	0.3
Nar	50	12	97.8	1.0	1.2	0
	250	12	95.3	2.1	2.6	0
	500	12	96.4	2.4	1.2	0
Nar	50	21	93.7	4.0	1.3	1.0
	250	21	94.5	2.5	2.5	0.5
	500	21	96.6	2.8	0.4	0.2
Nar+Dau	50+1	3	15.7	25.0	40.0	19.3
		12	94.0	3.3	1.3	1.4
		21	83.7	11.7	4.0	0.6
Nar+Dau	250+1	3	46.3	33.3	16.0	4.4
		12	97.0	1.0	0.7	1.3
		21	99.3	0.7	0	0
Nar+Dau	500+1	3	59.7	31.7	6.3	2.3
		12	96.0	3.0	0.7	0.3
		21	97.0	3.0	0	0

Table 2. Grades of Damage Observed in the DNA of Mouse Cardiocytes Exposed to Daunorubicin (Dau) and Treated with Naringin (Nar)

Agent	(mg/kg)	Time (h)	Grades of damage (%)			
			1	2	3	4
Distilled water		3	99.0	0	1.0	0
		12	95.5	3.0	1.5	0
		21	99.7	0.3	0	0
Dau	1.0	3	41.6	32.0	12.7	13.7
		12	44.3	26.7	21.0	8.0
		21	74.3	15.3	6.4	4.0
Nar	50	3	98.0	1.0	1.0	0
	250	3	93.5	5.3	1.2	0
	500	3	96.0	1.7	2.0	0.3
Nar	50	12	97.8	1.0	0.2	0
	250	12	94.9	3.1	1.0	1.0
	500	12	96.4	2.4	1.2	0
Nar	50	21	93.7	4.0	1.3	1.0
	250	21	93.7	2.1	3.6	0.6
	500	21	96.6	2.8	0.4	0.2
Nar+Dau	50+1	3	61.7	19.0	7.3	12.0
		12	95.0	1.3	1.3	1.4
		21	73.7	17.7	7.3	1.3
Nar+Dau	250+1	3	82.3	11.0	4.0	2.7
		12	99.0	1.0	0	0
		21	90.7	5.7	2.3	1.3
Nar+Dau	500+1	3	55.7	24.0	11.7	8.6
		12	97.3	1.7	0.3	0.7
		21	97.3	2.0	0.7	0

Nar at 12 h, and the least effect of the antimutagen corresponded to the low dose tested which only protected the DNA at 12 h of the evaluated schedule.

The genotoxicity produced by Dau in cardiocytes as well as the protective capacity of Nar is shown in Fig. 2. As observed in the hepatic cells, the administration of Nar produced no significant amount of DNA breakage all through the experiment, and Dau disrupted the integrity of the molecule. However, in this type of cells the highest T/N index increase was found at 3 h of treatment, with a decrease of 24.9 and 45.5% at 12 and 21 h, respectively. The administration of Nar before adding the mutagen produced significant prevention of the damage induced by Dau. The two high doses of Nar, 250 and 500 mg/kg, reduced the genotoxic effect of Dau a mean of 39% during the experiment. The greatest reduction was observed at 12 h, where 250 mg/kg of Nar induced an inhibition of 51.1%; likewise, as in the case of the liver, the least effect of the antimutagen corresponded to the low dose tested, which only protected the damage induced by Dau at 12 h postadministration.

Data obtained with the T/N index was congruent with the recorded grades of damage in hepatic and cardiac cells (Tables 1, 2). Most control animals as well as those administered with Nar presented mainly undamaged nuclei, while mice treated only with Dau had a significant increase in the rate of damaged nuclei. When Nar was added before administering Dau, the protective effect of the flavonone was manifested as a reduction in the number of grade 4 nuclei in comparison with the induced with Dau alone.

As regards the antioxidant potential of Nar, we found a positive response with all concentrations tested. The highest free radical scavenging potential was 95% when tested

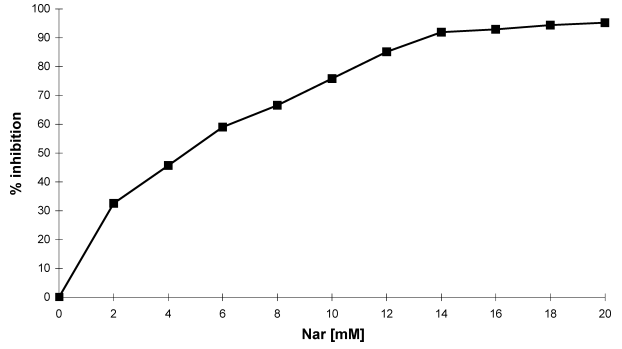

Fig. 3. Radical Scavenging Potential of Naringin (Nar)
Determination of color loss in the radical 1,1-diphenyl-2-pycril-hydrazyl (DPPH).

20 mM of Nar (Fig. 3).

DISCUSSION

The genotoxic effect observed in hepatocytes and cardiocytes resulting from the administration of Dau is important because of the severe collateral damage that could be produced by cumulative doses of the medicament in these organs, an effect which limits its clinical use, particularly in patients who have high risk factors for toxicity, for those who have received anthracycline therapy in the past, or for those who could receive other cardiotoxic agents.[13,20] Earlier studies have shown significant increases of sister chromatid exchanger (SCE) and MN in mice administered Dau,[10,11] as well as increases in the DNA breaking frequency in cancer patients treated with idarubicin, and in cultured cardiac myocytes derived from embryonic rat heart treated with doxorubicin.[21,22] These last authors also determined that DNA damage is an early event in cardiac toxicity. Overall, these data confirm the validity of using anthracyclins, and particularly Dau, as positive control agents in genotoxicity studies; besides, they also suggest the relevance of finding agents that can reduce the toxicity observed in patients treated with such medicaments.

The collateral cardiac toxicity which is generally described in regard to anthracyclin therapy has been attributed to the production of free radicals. This has been supported by studies in transgenic mice cell lines where the authors suggested mitochondria as the critical target, in cardiac myocytes isolated from treated rats that showed a persistent high rate of reactive oxygen species as well as depression in the activity of glutathione (GSH), or in the determination of free radical-induced DNA base modification in chromatin isolated from lymphocytes of cancer patients under chemotherapy.[21,23,24] Moreover, oxidation of DNA bases by anthracyclines has also been confirmed when incorporating enzymes such as endonuclease III and formamidopyrimidine-DNA glycosilase to the comet assay.[25] The above mentioned evidences are congruent with alterations found in cardiac biochemical markers, as well as in the morphology of the affected tissue, which may show loss and/or disassembly of myofibrils and mitochondrial anomalies.[14,26] Based on such knowledge, a number of studies have been made in search of antioxidants having the efficacy to avoid or ameliorate anthracyclin toxicity but without reducing antitumor capacity. With these two purposes in mind, several agents have been experimentally tested showing variable results; recently, administering vitamin E, melatonin, tomato-oleoresin, and rutoside-type flavonoids to mice or rats has given positive promising results.[27—30] A problem to solve in this type of studies is finding the appropriate dose and duration of exposure.

Furthermore, our results with Nar indicated that this chemical can be an appropriate antimutagen, and they agree with reports which show its inhibitory effect on the frequency of MN and chromosomal aberration in mice treated with lomefloxacin, a difluorinated quinolone antibacterial drug,[31] besides its protective action against the MN produced by ifosfamide or radiation in mouse.[4,5,11] In regard to its mechanism of action, besides producing a strong *in vitro* antioxidant effect, the chemical has also demonstrated a clear *in vivo* antioxidant potential, for example, by correcting the increase in lipid peroxidation and the reduction in glutathione level induced by lomefloxacin in mice,[31] by improving the activity of a number of antioxidant enzymes and non-enzymatic antioxidants in rats and rabbits as well as ameliorating the induced cardiac histological alterations.[32—34] Accordant with these considerations, the capacity of Nar to inhibit DNA damage produced by Dau found in our investigation could be related, at least partially, with an improved antioxidant condition induced by the flavonoid that could compensate for the oxidative stress caused by Dau. However, oxidative damage was not confirmed in the studied tissues; besides, a chemopreventive agent is also known to usually act under various mechanisms of action. Thus, explanations for the antigenotoxic effect of Nar may also be related with actions that modify the biotransformation of the antineoplastic, as well as the efficacy of its transportation or its effect on the cell cycle. Such explanations include the controversially described inhibitory effect on the CYP3A4 enzyme,[35—37] the modifying effect on other enzymes as CYP1A2, hepatic acyl-CoA:cholesterol acyltransferase, or esterase,[35,38,39] in addition to a pharmacokinetics intervention by altering the P-glycoprotein-mediated drug efflux,[40—43] or the induction of the G1 cell cycle arrest.[44] These proposals suggest that Nar may have more than one form of action, depending on the particular experimental conditions.

In our study, the lack of response observed with the low dose of Nar at 3 h of exposure may be related with its incapacity to cope with a high concentration of plasmatic Dau at that exposure time.[20] Besides, the effect of Dau on hepatocytes was higher than the level detected in cardiocytes, an effect probably associated with a larger amount of microsomal glycosidases and cytoplasmic aldoreductases,[45] which could have brought about an efficient biotransformation of Dau into its active metabolites. Our result may also be due to the short duration of the experiment, in light of the fact that cardiotoxicity is usually the main collateral effect in human treatments. With respect to hepatotoxicity, polymorphic mitochondria, cytoplasmic vacuolization and accumulation of lipid droplets have been observed in these cells, in addition to an increase in the activity of alanine aminotransferase.[15,20]

Interestingly, the damage decreased in both tissues at 21 h of treatment, suggesting the elimination of Dau and its metabolites, and/or the participation of DNA repair mechanisms. This is an expected process that is detected with the comet assay when a single dose is given to experimental models, as shown in repair studies on human lymphocytes,

and other animal-derived cells.[46)]

In summary, the findings obtained in the present study established that Dau is an inducer of DNA damage in hepatocytes and cardiocytes, cells which are also the most affected by the side effects observed in anthracyclin therapy. Moreover, we determined that Nar protects against the DNA deterioration induced by Dau in these target cells, which then suggests the pertinence of extending studies so as to define whether or not the tested flavonoid can be useful for ameliorating the collateral pathology found in Dau treatments.

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