Hesperidin Reduces Cisplatin-Induced DNA Damage in Bone Marrow Cells of Mice

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Abstract: Hesperidin is a bioflavonoid abundantly found in citrus fruits and displays chemoprotective effects against DNA (deoxyribonucleic acid) damage, however there are few reports about hesperidin effects against cisplatin—DNA damage induction. The aim of this work was to evaluate hesperidin antimutagenicity against cisplatin—DNA damage. (1) The antimutagenicity of hesperidin was assayed by bone marrow of mice *in vivo* using the micronucleus test. Hesperidin pre-treatment protocol reduced the frequency of MNPCE (micronucleated polychromatic erythrocytes) and the dose of 100 mg·kg^{-1} was highest efficiency, with 65.24% of damage reduction. In the simultaneous treatment protocol, the dose of 200 mg·kg^{-1} exhibited a more effective reduction of MNPCE, with 94.01% of damage reduction. (2) Hesperidin was also effective in reducing the MNPCE frequency in the post-treatment protocol for all doses, with 77.48%, 82.13% and 90.08% of damage reduction at the doses of 100, 200 and 400 mg·kg^{-1}, respectively. From the study, it can be concluded that hesperidin was able to promote the reduction of micronuclei frequency and DNA damage induced by cisplatin. Hesperidin is a powerful antioxidant compound and its chemoprotective effects on DNA may occur due to its association with the antioxidant cell system which is responsible for eliminate free radicals generated by chemical harmful to DNA.

Key words: Hesperidin, micronucleus test, DNA damage, chemoprotection, antimutagenicity.

1. Introduction

Flavonoids are a large group of phenolic compounds that are secondary metabolites of plants and fungi. More than 5,000 naturally occurring flavonoids have been reported in various plants and they exhibit beneficial medicinal effects due to the various biological properties. Hesperidin is a naturally occurring flavonoid found abundantly in citrus fruits and is also called bioflavonoid because of its wide range of pharmacological effects. The hesperidin molecule is composed of an aglycone unit, hesperetin and a disaccharide, rutinose [1, 2].

The biological activities of hesperidin have been extensively demonstrated in recent laboratory research. It has been observed that hesperidin displays chemoprotective effects against cancer and cardiovascular diseases by reducing oxidative stress, anti-inflammatory activities and also by decreasing the

expression of cell cycle proliferative and inflammatory markers [1, 3]. Furthermore, hesperidin reduces the functional and histological damage of kidney and liver induced by cisplatin without affecting their potential cytotoxic effect [4, 5].

Cisplatin is a very effective chemotherapeutic agent used in the treatment of a variety of tumors. This class of drugs reacts *in vivo*, binding to and causing cross linking of DNA (deoxyribonucleic acid), which ultimately triggers apoptosis. Despite its beneficial antineoplastic effects, cisplatin causes many undesirable side effects on different tissues including nephrotoxicity, hepatotoxicity and cardiotoxicity. These side effects limit its application in clinical oncology as a powerful chemotherapeutic agent [6, 7].

Mutagens such as cisplatin can promote toxicity, induce DNA damage and interference with the mitotic machinery, which can result in pre-mutagenic lesions and chromosome instability, mutations and other adverse outcomes [8]. Mutagenicity and

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antimutagenicity assays have been commonly used to identify substances with anticarcinogenic and antimutagenic potential and in the development of drugs used in the prevention and treatment of neoplasies [9]. Micronucleus test is a common assay used to assess DNA damage at chromosome level and the increased frequency of micronuclei expresses genome damage and instability [10]. In addition, mutagenicity and antimutagenicity assays have been used to identify potential antimutagens, anticancerigens and in the development of drugs that can be used to prevent and treat neoplasies [9].

The aim of the present study was to investigate the possible antimutagenic effects of hesperidin against the cisplatin-induced damage on bone marrow of mice *in vivo* using the micronucleus assay.

2. Material and Methods

2.1 Reagents

Hesperidin (Sigma-Aldrich, USA) dissolved in commercial corn oil at defined concentrations; Leishman's eosin methylene blue (Cromoline, Brazil) and Cisplatin (Fauldcispla®, Libbs), a cross-link agent used to induce damage to the genetic material, administered intraperitoneally (i.p.) at a dose of 5 mg·kg^{-1} of body weight (b.w.).

2.2 Selection of Doses

The dosages chosen for the experiments were based on the daily dosage of hesperidin suitable for humans, 200 mg·kg^{-1} per day. For the calculations a body weight of a human adult of 70 kg was assumed. The final dose used was 1/2 (100 mg·kg^{-1}), 1 (200 mg·kg^{-1}) and 2 (400 mg·kg^{-1}) times of the daily dosage recommended for humans.

2.3 Animals and Treatments

All animal studies were conducted in accordance with the ethical principles of animal experimentation stated by the Research Ethical Committee on Animal Use of UFES (Federal University of Espírito Santo).

110 Swiss albino mice (*Mus musculus*), 55 males and 55 females, with 6-8 weeks of age and about 30 g b.w were used. All animals were supplied by the biotery of UFES. The mice were housed in plastic cages under conditions of controlled light and temperature, with free access to water and food.

The potential of hesperidin as an agent to protect and/or repair genetic material was investigated using pre-treatment, simultaneous treatment and post-treatment protocols. For the proposed protocols, five experimental groups were used: the treated groups with hesperidin (100, 200 and 400 mg·kg^{-1} b.w.), the positive control (cisplatin) and the negative control (saline 0.9%). Each experimental group consisted of 10 animals (5 males and 5 females), randomly selected and separated by sex.

2.4 Control Groups

The negative control group received a single dose of saline solution (0.9%) by gavage intraperitoneally (i.p.). The positive control group received a single dose of saline (0.9%) by gavage and cisplatin intraperitoneally (5 mg·kg^{-1} b.w.). The animals were euthanized by cervical dislocation 24 h after the last treatment.

2.5 Treatment Groups

The pre-treatment groups received hesperidin (100, 200 and 400 mg·kg^{-1} b.w.) by gavage for 14 days and cisplatin (5 mg·kg^{-1} b.w., i.p.) on the 14th day. The simultaneous treatment groups received a single dose of hesperidin (100, 200 and 400 mg·kg^{-1} b.w.) and cisplatin (5 mg·kg^{-1} b.w., i.p.) simultaneously. The post-treatment groups received hesperidin (100, 200 and 400 mg·kg^{-1} b.w.) 24 h after application of the mutagenic agent (cisplatin, 5 mg·kg^{-1} b.w., i.p.). The animals were euthanized by cervical dislocation 24 h after the last treatment, for all protocols performed.

2.6 Micronucleus Assay in Mouse Bone Marrow

The micronucleus assay was performed as described by Schmid [11], with modifications. After the smear

drying, the cells were fixated with methanol (100%) and stained with Leishman for the differentiation of blood cells, especially PCE (polychromatic erythrocytes) and MNPCE (micronucleated polychromatic erythrocytes).

The antimutagenicity of hesperidin against the damage induced by cisplatin was determinated by analyzing 2000 PCEs per animal, taking into account the corresponding MNPCE frequency. Cytological analyses were performed using optical microscopy with a magnification of 1000 x.

2.7 Statistical Analysis

Statistical analysis was performed by the comparison between groups, separating and combining the genders, through ANOVA (analysis of variance) followed by Kruskall-Wallis test, a posteriori ($p < 0.05$). The statistical comparison between the responses to the treatments between the sexes was performed by Mann–Whitney test ($p < 0.05$). The relative damage reduction was calculated using Eq. (1) [12]:

%DR = (MNPCE (A) – MNPCE (B) / MNPCE (A) – MNPCE (C)) × 100

where "A" is the group treated with cisplatin, ''B'' is the group treated with hesperidin and cisplatin, and ''C'' is the negative control group (0.9% NaCl).

3. Results

In Tables 1 to 3, the results of the antimutagenic activity assay of hesperidin against DNA damage induced by cisplatin are summarized. For the pre-treatment protocol, all groups treated with hesperidin (n = 10) showed a significant diminution of MNPCEs frequency compared with the positive control group. Highest efficiency in reducing mutagenic damage in this protocol was found at the dose of 100 mg·kg^{-1}, with 65.24% of damage reduction (Table 1). When comparing the responses obtained between genders, male and female differed in the frequency of MNPCE only when were treated with hesperidin in the dose of 100 mg·kg^{-1}. For female groups, the dose of 400 mg·kg^{-1} was most effective in reducing mutagenic damages (Table 1).

Following the simultaneous treatment protocol, the group that received hesperidin at a dose of 200 mg·kg^{-1} (n = 10) exhibited a more effective reduction of the mutagenicity induced by cisplatin, with 94.01% of damage reduction (Table 2). Furthermore, it was observed that the dose of 400 mg·kg^{-1} was not able to reduce DNA damage (Table 2). The comparison of genders showed that there are no statistical differences in the mutagenicity in the simultaneous treatment (Table 2).

In the post-treatment protocol, all doses were effective in reducing the MNPCE frequency (n = 10) and a dose-dependent relationship between the doses was observed in micronucleus frequency and DNA damage reduction (Table 3). The relative reduction of damages in the groups treated with hesperidin at concentrations of 100, 200 and 400 mg·kg^{-1} b.w. were 77.48%, 82.13% and 90.08%, respectively. The

Table 1 Frequency of MNPCE and the percentage of reduction of the damages induced by the cisplatin, following the protocol of pre-treatment.

Treatment	MNPCE/1000 PCE ± SE			Reduction (%)		
	Per gender		Per group	Per gender		Per group
	Male	Female		Male	Female	
Negative control	2.60 ± 0.37^a	1.60 ± 0.27^a	2.10 ± 0.25^a	-	-	-
Positive control	57.30 ± 1.95^c	58.80 ± 2.45^c	58.05 ± 1.53^c	-	-	-
Hesperidin 100 mg·Kg^{-1}	14.50 ± 1.82^{ab}*	28.60 ± 1.34^{bc}*	21.55 ± 1.96^b	78.24	52.80	65.24
Hesperidin 200 mg·Kg^{-1}	29.30 ± 2.20^{bc}	26.90 ± 2.61^{bc}	28.10 ± 1.68^b	51.18	55.76	53.53
Hesperidin 400 mg·Kg^{-1}	24.00 ± 2.19^{bc}	23.50 ± 1.49^{ab}	23.75 ± 1.29^b	60.87	61.71	61.30

Values followed by different letters in the column differ statistically among themselves (Kruskall-Wallis test, $p < 0.05$); SE = standard error. * Statistically significant difference between genders (Mann-Whitney test, $p < 0.05$).

Table 2 Frequency of MNPCE and the percentage of reduction of the damages induced by the cisplatin, following the protocol of simultaneous treatment.

Treatment	MNPCE/1000 PCE ± SE			Reduction (%)		
	Per gender		Per group	Per gender		Per group
	Male	Female		Male	Female	
Negative control	2.60 ± 0.37^a	1.60 ± 0.27^a	2.1 ± 0.25^a	-	-	-
Positive control	57.30 ± 1.95^c	58.80 ± 2.45^c	58.05 ± 1.53^c	-	-	-
Hesperidin 100 mg·Kg^{-1}	13.20 ± 0.99^{bc}	12.60 ± 1.58^{bc}	12.9 ± 0.91^{bc}	80.76	80.76	80.70
Hesperidin 200 mg·Kg^{-1}	6.40 ± 0.58^{ab}	4.50 ± 0.81^{ab}	5.45 ± 0.53^{ab}	93.05	94.93	94.01
Hesperidin 400 mg·Kg^{-1}	57.60 ± 4.50^c	58.70 ± 5.04^c	58.15 ± 3.29^c	0	0	0

Values followed by different letters in the column differ statistically among themselves (Kruskall-Wallis test, $p < 0.05$); SE = standard error. * Statistically significant difference between genders (Mann-Whitney test, $p < 0.05$).

Table 3 Frequency of micronucleated polychromatic erythrocytes (MNPCE) and the percentage of reduction of the damages induced by the cisplatin, following the protocol of post-treatment.

Treatment	MNPCE/1000 PCE ± SE			Reduction (%)		
	Per gender		Per group	Per gender		Per group
	Male	Female		Male	Female	
Negative control	2.60 ± 0.37^a	1.60 ± 0.27^a	2.1 ± 0.25^a	-	-	-
Positive control	57.30 ± 1.95^c	58.80 ± 2.45^c	58.05 ± 1.53^c	-	-	-
Hesperidin 100 mg·Kg^{-1}	15.70 ± 2.32^{bc}	13.70 ± 1.83^{bc}	14.7 ± 1.46^{bc}	76.05	78.84	77.48
Hesperidin 200 mg·Kg^{-1}	13.22 ± 0.96^{bc}	10.10 ± 0.94^{bc}	11.58 ± 0.74^b	80.62	85.13	82.13
Hesperidin 400 mg·Kg^{-1}	9.33 ± 1.07^{ab}	7.20 ± 1.13^{ab}	8.21 ± 0.80^b	88.84	87.93	90.08

Values followed by different letters in the column differ statistically among themselves (Kruskall-Wallis test, $p < 0.05$); SE = standard error. * Statistically significant difference between genders (Mann-Whitney test, $p < 0.05$).

comparison between genders showed that there are no statistical differences between male and female in the mutagenicity (Table 3).

4. Discussion

Synthetic and natural substances can act as mutagenic, co-carcinogenic and/or carcinogenic agents, leading to several human diseases [13]. A variety of bioactive compounds has been demonstrated to be capable to act against these pro-mutagens and carcinogens, which might protect DNA against damage and its consequences for humans.

The genotoxicity of anticancer drugs, such as cisplatin, can result in secondary effects in non-tumor cells, mainly due to high levels of free radicals and ROS (reactive oxygen species) generated, resulting in oxidative stress [14]. The oxidative stress can alter lipids, proteins and nucleic acids by producing oxidized purines and pyrimidines, single strand breaks and alkali labile sites which may results in direct DNA damage [15, 16]. To avoid these toxic side effects, it is

common that the used of chemotherapeutic drugs in combination with various protective and detoxifying agents is to eliminate or reduce its adverse toxic effects. The use of antioxidants, such as flavonoids with the ability to reduce the reactivity of free radicals in chemically stable molecules, can decrease oxidative stress and consequently can avoid the oxidative damage to DNA and other molecules [15, 17].

Cisplatin interacts with DNA at the N7 position of purines forming adducts, which results in cross-links, a mechanism that induces mutagenicity. This might lead to the inhibition of the enzyme DNA polymerase and therefore might inhibit transcription, resulting in blockage of the cell cycle and apoptosis [18, 19]. Nucleotide excision is the main repair process by which DNA adducts formed by cisplatin are removed. This requires fully functional antioxidant and enzyme systems, which ensure the efficacy of the repair process [20].

An important characteristic of flavonoids, including hesperidin, is their antioxidant activity, which plays an

important role in the neutralization of free radicals such as hydroxyl radicals generated by chemical agents [21]. Consequently, the oxidative damage is lowered in the biological system exerting the chemoprotective effects by elimination of free radicals, inhibition of inflammation, induction of phase II enzymes, suppression of cell proliferation, induction of cell differentiation, inhibition of cell cycle and apoptosis [22, 23]. Previous studies with catechins, rutin, curcumin and daidzein have reported the antimutagenic activity of these compounds by *in vivo* and *in vitro* tests [24-26].

Hesperidin is a flavonoid considered as a powerful antioxidant agent in chemical and biological systems, with a strong cellular antioxidant protection. Recently, this substance gained special attention due to its chemoprotective and radioprotective actions [25, 27].

Our results demonstrated the antimutagenic activity of hesperidin against cisplatin-induced damage. Previous researches demonstrated its antimutagenic effect against the damage induced by cyclophosphamide [25], an alkylating drug that is activated by cytochrome P-450 in the liver [28, 29] and powerful protective effects on the radiation-induced DNA damage in mouse bone marrow cells [30]. Kalpana et al. [16] demonstrated its protective effect on H_2O_2 induced oxidative damage on pBR322 DNA and RBC (red ball cells) cellular membrane. Until today, there have been no studies reporting the antimutagenic potential of hesperidin against the damage induced by cisplatin.

The comparison of hesperidin effects against cisplatin damage induction showed some variation response between genders and suggested that in females the reduction of DNA damage is less effective (Table 1). Sex has been reported as a factor to be considered in mutagenesis studies because this factor has an influence on the basal production of micronuclei. As a consequence, women have been reported to be more pre-disposed to spontaneous formation of this structure [31].

Genetic and nongenetic factors can affect the mechanisms of absorption and metabolization of drugs and can change the efficacy and toxicity of drugs used simultaneously [32]. The pharmacokinetic and action of citric flavonoids in the human body have not been completely elucidated, but studies demonstrate that the absorption of these flavonoids occurs in the small intestine in the presence of intestinal enzymes and sugar molecule bound to the compound [33, 34].

After oral ingestion, hesperidin, a glycosylated flavone, is hydrolyzed by the intestinal microflora, converted to hesperetin by β-glucosidase, its aglyconated form, and is absorbed in the colon [33, 35-37]. Subsequently, in the intestine, hesperetin undergoes conjugation reactions, such as glucuronidation and methylation and is conducted by the system port of enterocytes to the liver. There is undergoing other reactions, such as methylation, sulfation and glucuronidation to form a variety of metabolites [38-41].

Some research has demonstrated the ability of hesperidin or its metabolites to inhibit some hepatic isoenzymes of the cytochrome P-450 [42, 43]. Such as cisplatin is a drug which has no known interactions with cytochrome P450 enzymes [44-46], it is supposed that hesperidin does not act at the hepatic level, under these experimental conditions. Some biochemical and molecular mechanisms of cisplatin resistance were identified, which include elevated cellular gluthathione levels [47] and increased DNA repair [48]. Additionally, lemon flavonoids, i.e. eriocitrin and hesperidin, can induce the increase of the concentration of enzymes like gluthathione as well as their antioxidative activity *in vivo* [49] that could be associated with the mechanisms used to promote the damages reduction as observed in this study (Tables 1-3).

5. Conclusion

In summary, in this study, it was shown that hesperidin was able to promote significant reduction of

DNA damage against cisplatin-damage induction, showing its chemoprotective effect on DNA molecule. Our results suggest that hesperidin can be associated with the antioxidant cell system and consequently enable it to eliminate free radicals generated by chemical harmful to DNA, acting in the preventing of damage formation (pre and simultaneous treatments) and as modulator in the repair of the damage (post-treatment). However, the exact mechanism of action of hesperidin against DNA damage induced by mutagenic agents as cisplatin is not fully understood yet.

Conflict of Interest

Authors declare no competing financial interests associated with the manuscript exist.

Acknowledgments

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