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The Impact of Morin, a Natural Flavonoid, on Cyclophosphamide-Induced Changes in the Oxidative Stress Parameters in Rat Livers*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Cyclophosphamide (CPX) has many adverse effects, partly due to oxidative stress induction in various tissues. Morin is one of the natural flavonoids with strong antioxidant properties.
Objectives. The aim of the current research was to estimate the influence of morin on changes in antioxidant parameters in rat livers after cyclophosphamide administration.
Material and Methods. The study was performed on Wistar rats. The rats in Group C received 0.9% saline; those in Group CX received cyclophosphamide (CPX); and those in Group M-CX received CPX with morin. Cyclophosphamide and morin were given by gastric gavage for 10 consecutive days at doses of 15 mg/kg and 100 mg/kg, respectively. Malondialdehyde (MDA) and glutathione (GSH) concentrations, superoxide dismutase (SOD) activity and catalase (CAT) activity were determined in liver tissue homogenates.
Results. CPX caused a significant decrease in SOD activity and GSH levels, but only the latter was fully restored by morin. There were no significant differences in CAT activity in the various groups. CPX also insignificantly decreased MDA levels, which was aggravated by co-administration of morin.
Conclusions. The results obtained indicate that morin may exert some protective action on CPX-induced changes in the antioxidant state in rat livers (**Adv Clin Exp Med 2014, 23, 4, 505–509**).

Key words: cyclophosphamide, morin, oxidative stress, liver, rat.

Cyclophosphamide (CPX) is widely used as an immunosuppressant and anticancer agent. After it is administered in a high single dose, it induces oxidative stress in tissues, which causes some adverse effects, especially hemorrhagic cystitis [1–3]. The action of CPX on the oxido-reduction state in tissues after repeated administration in small doses is not very clear and is probably more complex [4, 5].

Many studies focus on the possibilities of different natural and synthetic compounds decreasing CPX-induced toxicity. Different data suggest that substances belonging to the flavonoid group, e.g. morin, exhibit strong antioxidant properties and therefore may be useful in reversing CPX-induced changes in antioxidants state [6–9].

In a previous study the current authors found that a synthetic derivative of morin – water-soluble morin-5'-sulfonic acid sodium salt (NaMSA) – had antioxidative effects in rat kidneys and livers, reversing some CPX-induced changes in antioxidant level/activity [5]. The purpose of the current study was to assess the effect of morin, a natural flavonoid, on selected antioxidant parameters

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in the rat liver after 10 days of cyclophosphamide administration.

Material and Methods

Animals

The study was performed on male and female Wistar rats (203 g ± 18 g). The animals were purchased from the Department of Pathomorphology at Wroclaw Medical University (Wroclaw, Poland). The animals were kept in separate polypropylene cages. Conditions including a 12/12 h light/dark cycle, at temperature between 21 and 23°C, water and standard food *ad libitum* were maintained throughout the experimental procedures. The approval of the First Local Ethics Committee for Experiments on Animals in Wroclaw was obtained.

Chemicals

The chemicals used in the experiment were cyclophosphamide *in subst.* from Sigma (Germany); morin hydrate *in subst*. from Sigma (Germany), 0.5 g thiopental vials from Biochemie (Austria); and 0.9% NaCl solution from Polpharma S.A. (Poland).

Experiment

The experiment was performed according to methods described in earlier reports [5, 10]. Thirty-six rats of both sexes were divided randomly into 3 study groups (12 animals in each): The control group (Group C), receiving 0.9% NaCl solution at 9 am and at 2 pm; the cyclophosphamide group (Group CX), receiving CPX at a dose of 15 mg/kg at 9 am and 0.9% NaCl solution at 2 pm; and the cyclophosphamide-morin group (Group M-CX), receiving cyclophosphamide at a dose of 15 mg/kg at 9 am and morin at a dose of 100 mg/kg at 2 pm. All of the substances were dissolved in 0.9% NaCl solution (4 mL/kg volume) and were administered for 10 consecutive days by gastric gavage. The experiment was terminated on the 11th day. The animals were sacrificed by terminal anesthesia using intraperitoneal thiopenthal (70 mg/kg). Half of the liver was homogenized on ice using lysis buffer (pH 7.5) consisting of 1% NP40, 10 mM EDTA, 140 mM NaCl, 10% glycerol and 20 mM Tris base, and then centrifuged at 14000 rpm for 25 min at 4°C. In the supernatants obtained, the concentrations of malondialdehyde (MDA) and glutathione (GSH) as well as superoxide dismutase (SOD) and catalase (CAT) activities were determined.

Measurements of the Oxidative Stress Parameters

All parameters were assessed spectrophotometrically (MARCEL S350 PRO spectrophotometer).

MDA was assayed using a BIOXYTECH--MDA-586 kit (OxisResearch, USA) according to the manufacturer's instructions, and its level was expressed as μmol/mL.

GSH concentration was assayed using a BIOXYTECH GSH-400 kit (OxisResearch, USA) according to the manufacturer's instructions, and its level was expressed as μM.

SOD activity was assayed using a Ransod kit (Randox Laboratories, UK) according to the manufacturer's instructions, and its activity was expressed as U/mg of protein.

CAT activity was determined following decreases in the initial H_2O_2 concentration (30 mM used as the initial substrate) at 240 nm and 25°C, over a time frame of 60 s, following the procedure published by Johanson et al. [11]. Briefly, 100 μL of supernatant isolated from rat liver homogenates was placed in a cuvette and diluted to a final volume of 2 mL with phosphate-buffered saline (50 mM). The decrease in absorption at 240 nm 60 s after adding 1 mL H_2O_2 was observed. One unit of CAT was defined as the amount of enzyme that degraded 1 μL H_2O_2 per min. Values were expressed as U/mg of protein.

The total protein level in the homogenate supernatants was analyzed in a certified laboratory using a Flex kit and Dimension RxL-Max apparatus (Siemens). In brief, copper cations interact in alkaline solution with peptide bonds in proteins. Blue-colored copper (II) complexes were proportional to protein concentration and measured by the bichromatic technique.

Statistical Analysis

Data were expressed as means ± standard deviation (SD). Statistical analyses of the impact of the studied substances on CAT and SOD activities and on GSH and MDA levels were performed by ANOVA analysis of variance. Detailed comparisons between the study groups were evaluated by *post-hoc* Tukey's test. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using STATISTICA 9 PL software (StatSoft, Kraków, Poland).

Results

The mean values of the studied parameters, with corresponding SD values and levels of significance, are presented in Table 1.

When compared to the control group (Group C), CPX significantly decreased glutathione levels in rat livers, which was fully reversed by concomitant treatment with morin.

CPX evoked a significant decrease in SOD activity in rat livers in comparison to Group C, and this action was not reversed by the addition of morin to the CPX regimen. The difference was significant between Group M-CX and Group C, but not between Groups M-CX and CX.

No statistically significant differences were observed in CAT activity in rat livers among any of the groups.

Finally, CPX insignificantly decreased liver MDA concentration compared to the control group. This was aggravated by the co-administration of morin, and the differences between Group M-CX and Groups C and CX were statistically significant.

Discussion

This study assessed antioxidant parameters such as SOD, GSH and CAT in rat livers. CPX significantly decreased SOD activity and GSH levels, which was similar to many other reported findings [12–14]. In the current study, morin exerted a strong protective effect in the case of GSH liver concentrations, fully restoring CPX-induced GSH depletion, but failed to reverse decreased SOD activity, which is one of the most potent intracellular antioxidants [15]. Using the same experimental model, the current authors previously noted that CPX also significantly decreased GSH levels in rat kidneys, which was fully reversed by a water-soluble derivative of morin – NaMSA. NaMSA also fully protected rat kidneys, but not livers, from CPX-induced decreases in SOD activity [5]. Similarly, in the current study, the addition of morin, a natural compound, did not reverse decreased SOD activity in rat livers.

The authors did not observe any impact of CPX on CAT activity either in the current study or in the previous work using the same experimental model [5]. However, other authors documented decreased CAT activity in CPX-treated groups of rats or mice [12–14].

In the present study the mean value of the MDA concentrations in the CPX-receiving group was 5.0 μmol/mL. In the authors' previous work, following the same schedule of administration (15 mg/kg daily for 10 days), CPX significantly decreased malondialdehyde concentration in comparison to the control group, with a mean value of 4.86 μmol/mL [5]. In many studies, administering CPX caused an increase in MDA levels in various tissues as an effect of the prooxidative activity of this drug. Most of those studies were, however, performed on rats receiving high single dose of CPX [1, 16, 17]. In a study by Kim et al., CPX caused an increase in hepatic MDA levels, with decreased concentrations of reduced glutathione and CAT activity in female rats [12]. In other studies, CPX also increased MDA level in mouse livers as well as decreased SOD and CAT activity and GSH levels [13, 14]. Despite a similar cumulative dose of CPX to that used in our current study, different schedules of administration of the drug might be responsible for the different changes in MDA levels noted by other researchers. Askar et al. [4] also observed decreased MDA concentrations after administering CPX in small doses in an ischemia-reperfusion skin injury model. The results of the current study confirm that even small doses of CPX may deplete endogenous antioxidant storage, but still may not induce lipid peroxidation reflected as increased MDA concentration.

Table 1. Malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels in rat liver homogenates

Group / Parameter	C mean ± SD	CX mean ± SD	M-CX mean ± SD
MDA (μmol/mL)	5.49 ± 0.46	5.00 ± 1.12	3.83 ± 0.61[a, b]
GSH (μM)	100.59 ± 6.97	88.85 ± 3.20[a]	98.33 ± 6.42[b]
SOD (U/mg of protein)	1332.74 ± 86.03	1197.82 ± 107.23[c]	1228.21 ± 67.09[d]
CAT (U/mg of protein)	0.092 ± 0.021	0.097 ± 0.024	0.080 ± 0.029

[a] $p < 0.001$ *vs.* C; [b] $p < 0.005$ *vs.* CX; [c] $p < 0.005$ *vs.* C; [d] $p < 0.05$ *vs.* C. C – the control group; CX – the group receiving cyclophosphamide intragastrically at a 15 mg/kg dose at 9 am; M-CX – the group receiving cyclophosphamide intragastrically at a 15 mg/kg dose at 9 am and morin intragastrically at a 100 mg/kg dose at 2 pm.

The authors of the current study chose a naturally-occurring flavonoid – morin – to evaluate its potential protective action on CPX-induced oxidative stress. In the available literature, little has been reported about the activity of this substance and its effect on antioxidant parameter changes after CPX administration. Morin's antioxidant properties have been confirmed in various models [7–9]. Morin may also minimize the toxicity of antitumor agents such as doxorubicin or mitomycin C [18], or may influence the pharmacokinetics of anticancer/immunosuppressant drugs such as methotrexate [19] or cyclosporin A [20]. Unfortunately, in the earlier study the current authors did not observe that morin had any protective action against CPX-induced tissue toxicity such as changes in white blood cells, red blood cells or platelet level, nor on body weight loss. The weight loss parameter became even worse during simultaneous CPX and morin use [10].

The results of the present study with morin are very similar to the authors' previous work with NaMSA [5] despite the different physicochemical properties of the two substances (water-soluble vs. water-insoluble). The pharmacokinetic properties of substances are determined by their water/lipid solubility, among other factors. Water/lipid solubility may also have an impact on an agent's tissue and intracellular distribution. In *in vitro* models it has been observed that the water soluble sulfonic derivative of morin (NaMSA) was less potent than the lipid soluble original compound (morin) in cytotoxic and cytostatic activities, despite its better solubility in water and a higher concentration achieved in the culture medium [21].

Although flavonoids are considered antioxidants, it has also been demonstrated that under specific conditions (such as oxygen and transition metals) they may act as prooxidants and may induce unfavorable DNA damage. This mechanism is not fully clear, but autoxidation of polyphenols may play a role in this process. Sahu and Gray's study indicated that pro- or antioxidative activity may be dependent on the redox state of the cell environment [22], which may at least partly explain the dual effect of morin observed in the current study.

The results obtained in the current study suggest that morin has a potential to protect against some CPX-induced changes in the oxidative stress parameters, especially against depletion of GSH levels in rat livers. It is probable that the experiment was terminated at a point when the full prooxidative action of CPX had not yet been revealed, but when the antioxidant defense started to be impaired. Unfortunately, the positive changes evoked by morin in the level of some antioxidants were not accompanied by a decrease in CPX's tissue toxicity as observed in the authors' previous study.

References

[1] **Abraham P, Rabi S:** Protective effect of aminoguanidine against cyclophosphamide-induced oxidative stress and renal damage in rats. Redox Rep 2011, 16, 8–14.

[2] **Korkmaz A, Topal T, Oter S:** Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation. Cell Biol Toxicol 2007, 23, 303–312.

[3] **Topal T, Oztas Y, Korkmaz A, Sadir S, Oter S, Coskun O, Bilgic H:** Melatonin ameliorates bladder damage induced by cyclophosphamide in rats. J Pineal Res 2005, 38, 272–277.

[4] **Askar I, Oktay MF, Gurlek A, Bac B:** Protective effects of some antineoplastic agents on ischemia-reperfusion injury in epigastric island skin flaps. Microsurgery 2006, 26, 193–199.

[5] **Merwid-Ląd A, Trocha M, Chlebda E, Sozański T, Magdalan J, Ksiądzyna D, Kopacz M, Kuźniar A, Nowak D, Pieśniewska M, Fereniec-Gołębiewska L, Kwiatkowska J, Szeląg A:** Effects of morin-5'-sulfonic acid sodium salt (NaMSA) on cyclophosphamide-induced changes in oxido-redox state in rat liver and kidney. Hum Exp Toxicol 2012, 31, 812–819.

[6] **Ray S, Chowdhury P, Pandit B, Ray SD, Das S:** Exploring the antiperoxidative potential of morin on cyclophosphamide and flutamide-induced lipid peroxidation and changes in cholesterol profile in rabbit model. Acta Pol Pharm 2010, 67, 35–44.

[7] **Karthik Kumar V, Vennila S, Nalini N:** Modifying effects of morin on the development of aberrant crypt foci and bacterial enzymes in experimental colon cancer. Chem Toxicol 2009, 47, 309–315.

[8] **Sreedharan V, Venkatachalam KK, Namasivayam N:** Effect of morin on tissue lipid peroxidation and antioxidant status in 1, 2-dimethylhydrazine induced experimental colon carcinogenesis. Invest New Drugs 2009, 27, 21–30.

[9] **Zhang R, Kang KA, Piao MJ, Maeng YH, Lee KH, Chang WY, You HJ, Kim JS, Kang SS, Hyun JW:** Cellular protection of morin against the oxidative stress induced by hydrogen peroxide. Chem Biol Interact 2009, 177, 21–27.

[10] **Merwid-Ląd A, Trocha M, Chlebda E, Sozański T, Magdalan J, Ksiądzyna D, Pieśniewska M, Szeląg A:** The effects of morin, a naturally occurring flavonoid, on cyclophosphamide-induced toxicity in rats. Adv Clin Exp Med 2011, 20, 683–690.

[11] **Johanson LH, Borg HLA:** A spectrophotometric method for determination of catalase activity in small tissue sample. Anal Biochem 1988, 174, 331–336.

[12] **Kim SH, Lee IC, Lim JH, Moon C, Bae CS, Kim SH, Shin DH, Park SC, Kim HC, Kim JC:** Protective effects of pine bark extract on developmental toxicity of cyclophosphamide in rats. Food Chem Toxicol 2012, 50, 109–115.

[13] **Tripathi DN, Jena GB:** Intervention of astaxanthin against cyclophosphamide-induced oxidative stress and DNA damage: a study in mice. Chem Biol Interact 2009, 180, 398–406.
[14] **Tripathi P, Tripathi R, Patel RK, Pancholi SS:** Investigation of antimutagenic potential of Foeniculum vulgare essential oil on cyclophosphamide induced genotoxicity and oxidative stress in mice. Drug Chem Toxicol 2013, 36, 35–41.
[15] **Korkmaz A, Topal T, Oter S:** Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation. Cell Biol Toxicol 2007, 23, 303–312.
[16] **Oboh G, Akomolafe TL, Adefegha SA, Adetuyi AO:** Attenuation of cyclophosphamide-induced neurotoxicity in rat by yellow dye extract from root of Brimstone tree (Morinda lucida). Exp Toxicol Pathol 2012, 64, 591–596.
[17] **Al-Yahya AA, Al-Majed AA, Gado AM, Daba MH, Al-Shabanah OA, Abd-Allah AR:** Acacia Senegal gum exudate offers protection against cyclophosphamide-induced urinary bladder cytotoxicity. Oxid Med Cell Longev 2009, 2, 207–213.
[18] **Kok LD, Wong YP, Wu TW, Chan HC, Kwok TT, Fung KP:** Morin hydrate: a potential antioxidant in minimizing the free-radicals-mediated damage to cardiovascular cells by anti-tumor drugs. Life Sci 2000, 67, 91–99.
[19] **Hong SS, Jin MJ, Han HK:** Enhanced systemic availability of methotrexate in the presence of morin in rats. Biopharm Drug Dispos 2008, 29, 189–193.
[20] **Fang SH, Hou YC, Chao PD:** Pharmacokinetic and pharmacodynamic interactions of morin and cyclosporin. Toxicol Appl Pharmacol 2005, 205, 65–70.
[21] **Król W, Dworniczak S, Pietsz G, Czuba ZP, Kunicka M, Kopacz M, Nowak D:** Synthesis and tumoricidal activity evaluation of new morin and quercetin sulfonic derivatives. Acta Pol Pharm 2002, 59, 77–79.
[22] **Sahu SC, Gray GC:** Lipid peroxidation and DNA damage induced by morin and naringenin in isolated rat liver nuclei. Food Chem Toxicol 1997, 35, 443–447.

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