J. Pineal Res. 2003; 34:282–287

Journal of Pineal Research
ISSN 0742-3098

Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats

Abstract: Regarding the mechanisms of methotrexate (MTX) hepatotoxicity and nephrotoxicity, several hypotheses have been put forward, among which oxidative stress (including depletion of glutathione) is likely. This investigation elucidates the role of free radicals in MTX-induced toxicity and the protection by melatonin. Wistar albino rats were injected with MTX intraperitoneally. Following a single dose of MTX (20 mg/kg), either saline (MTX group) or melatonin (10 mg/kg, MTX + Mel group) was administered for 5 days. In other rats, physiologic saline (control group) or melatonin (10 mg/kg, Mel group) was injected for 5 days, following a single injection of saline. On the sixth day, rats were killed to obtain blood, liver, and kidney tissue samples. Malondialdehyde (MDA), an end product of lipid peroxidation, and glutathione (GSH), a key antioxidant, levels were evaluated in blood and tissue homogenates. Reactive oxygen metabolite-induced inflammatory changes in kidney and liver tissues were evaluated by measuring myeloperoxidase (MPO) activity, an index of neutrophil infiltration. MTX administration resulted in increased MDA levels and MPO activity and decreased GSH levels in the blood, liver, and kidney whereas melatonin reversed these effects. When melatonin was administered alone, no significant changes in biochemical parameters were noted. In conclusion, the present study suggests that melatonin may be of therapeutic benefit when used with MTX.

Nermina Jahovic[1], Hülya Çevik[1], A. Özer Şehirli[2], Berrak Ç. Yeğen[1] and Göksel Şener[2]

[1]Department of Physiology, School of Medicine; [2]Department of Pharmacology, School of Pharmacy, Marmara University, Istanbul, Turkey

Key words: glutathione, kidney, lipid peroxidation, liver, melatonin, methotrexate, myeloperoxidase activity

Address reprint requests to Göksel Şener, PhD, Marmara University, School of Pharmacy, Tbbiye Cad. 34668 İstanbul, Turkey. E-mail: gokselsener@hotmail.com

Received November 7, 2002; accepted January 21, 2003.

Introduction

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for leukemia and other malignancies. Over the past five decades, low-dose MTX has also been used for the treatment of various inflammatory diseases such as psoriatic and rheumatoid arthritis. Cyclic high doses of MTX, as used for acute leukemia [1, 2], or the relatively high doses of MTX used to treat severe psoriasis have been associated with liver hepatotoxicity, including progressive hepatic fibrosis and cirrhosis [3, 4]. Also, MTX can cause increased serum creatinine levels, uremia and hematuria, while its administration in high doses has been reported to cause acute renal failure [5]. In some cases, MTX-induced renal failure could be prevented by folinic acid supplementation [6] or leucovorin, a biochemical antidote of MTX [7]. Severe MTX poisoning with acute hepatorenal dysfunction has been treated with plasma exchange and hemodialysis [8].

MTX-induced toxicity appears to be a consequence of the interaction of many factors: dosing schedule and length of treatment, patients' risk factors, type of disease, and presence of genetic and molecular apoptotic factors [9]. The conversion of MTX to its major extracellular metabolite, 7-hydoxymethotrexate, takes place in the liver, where it is oxidized by a soluble enzymatic system [10]. Inside cells, MTX is stored in a polyglutamated form [11]. Long-term drug administration can cause accumulation of MTX polyglutamates and decreased folate levels [12]. The presence of higher levels of polyglutamates causes a longer intracellular presence of the drug, and this has been suggested as a mechanism for MTX hepatotoxicity [13]. Moreover, being a high affinity inhibitor of dihydrofolate reductase, MTX indirectly affects the synthesis of thymidilate, thereby suppressing DNA synthesis [14]. Additionally, it was demonstrated that the cytosolic nicotinamide adenosine diphosphate [NAD(P)]-dependent dehydrogenases [15] and NADP malic enzyme are inhibited by MTX, suggesting that the drug could decrease the availability of NADPH in cells [16]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of cellular glutathione, an important cytosolic antioxidant, which is protective against reactive oxygen species (ROS). Thus, the significant reduction in glutathione (GSH) levels promoted by MTX leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to ROS [17]. Considering the relationship between GSH and the deleterious effects of MTX, interest has been focused on compounds that act as antioxidants and are capable of stimulating GSH synthesis.

Melatonin, the chief indolamine produced by the pineal gland, has been shown to be an effective antioxidant and free-radical scavenger [18–20]. Melatonin, because of its small size and high lipophilicity, crosses biologic membranes

easily, thus reaching all compartments of the cell. There is a substantial body of evidence for a protective effect of melatonin against DNA, lipids, and proteins, which are the result of a number of endogenous and exogenous free-radical generating processes [21–23].

The purpose of this study was to establish the role of oxidative stress in MTX-induced hepatotoxicity and nephrotoxicity. Presuming that free radical scavengers are protective against drug-induced nephrotoxicity [24, 25] and hepatotoxicity [26, 27], the present study was also undertaken to investigate the putative protective effect of melatonin against MTX-induced hepatorenal oxidative injury.

Materials and methods

Experimental design

All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Both sexes of Wistar albino rats (200–250 g) were kept at a constant temperature (22 ± 1°C) with 12 h light and dark cycles.

Methotrexate (David Bull Laboratories, Mulgrave-Victorica, Australia) and melatonin (Sigma, St Louis, MO, USA) were injected intraperitoneally. Following a single dose of methotrexate (20 mg/kg), either saline (MTX group) or melatonin (10 mg/kg, MTX + Mel group) was administered for 5 days. In other rats, physiologic saline (control group) or melatonin (10 mg/kg, Mel group) was injected for 5 days, following a single injection of saline. On the sixth day, animals were killed by decapitation and trunk blood was collected, centrifuged (3000 g, 10 min, 4°C) and serum was stored at 80°C until the time of malondialdehyde (MDA) and GSH assays. The liver and kidney were excised and stored for later analysis of MDA and GSH levels, and myeloperoxidase (MPO) activity.

Malondialdehyde and glutathione assays

Tissue samples of liver and kidney were homogenized in ice cold 150 mM KCl for determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation [28]. Results are expressed as nmol MDA/g tissue. GSH was determined by the spectrophotometric method using the Ellman's reagent [29]. Results are expressed μg GSH/g tissue. Serum MDA and GSH levels were measured by the same methods.

Myeloperoxidase activity

Tissue associated MPO activity was measured using a procedure similar to that documented by Hillegas *et al*. [30]. Liver or kidney samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity is expressed as U/g tissue.

Statistics

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± S.E.M. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $P < 0.05$ were regarded as significant.

Results

Methotrexate treatment decreased the liver GSH levels significantly ($P < 0.01$) almost to 65% of the control, while melatonin treatment following MTX prevented this reduction in GSH ($P < 0.05$) (Fig. 1A). Similarly, kidney GSH levels were also depleted ($P < 0.001$) in MTX-treated rats. However, treatment with melatonin abolished the MTX-induced GSH reduction with the levels of the thiol being of the control levels (Fig. 1B).

The levels of liver MDA, as products of lipid peroxidation, were increased after treatment with MTX to levels that are found to be significantly higher than those in the control animals ($P < 0.001$). On the other hand, melatonin treatment following the MTX challenge reversed MDA levels back to the control values ($P < 0.01$) (Fig. 2A). Kidney MDA levels were also increased significantly ($P < 0.01$) following the administration of MTX, and this increase was prevented by melatonin treatment ($P < 0.01$) (Fig. 2B).

Tissue MPO activity was measured as an indirect evidence of neutrophil infiltration. MTX treatment increased both the liver ($P < 0.05$) and the kidney ($P < 0.01$) MPO activities, which were reversed back to control levels in MTX + Mel group ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3A,B).

MTX treatment caused a significant increase in serum MDA ($P < 0.01$) and a concomitant decrease in GSH ($P < 0.001$) levels. Melatonin treatment for 5 days reversed the elevation in MDA and maintained GSH at control levels (Fig. 4).

Discussion

The results of the present study demonstrate that MTX treatment causes oxidative tissue damage, as assessed by increased lipid peroxidation and decreased GSH levels in the liver and kidney, while melatonin treatment protects against the oxidative injury. In addition, MTX-induced elevation in MPO activity was inhibited by melatonin treatment, indicating that the antioxidative effect of melatonin may be neutrophil-dependent.

Antitumor drugs are being increasingly utilized as adjuvant therapy for patients at high risk for recurrent disease [31]. Recent advances showed that oxygen radicals and hydrogen peroxides are linked with the development of several pathological processes associated with chemotherapy,

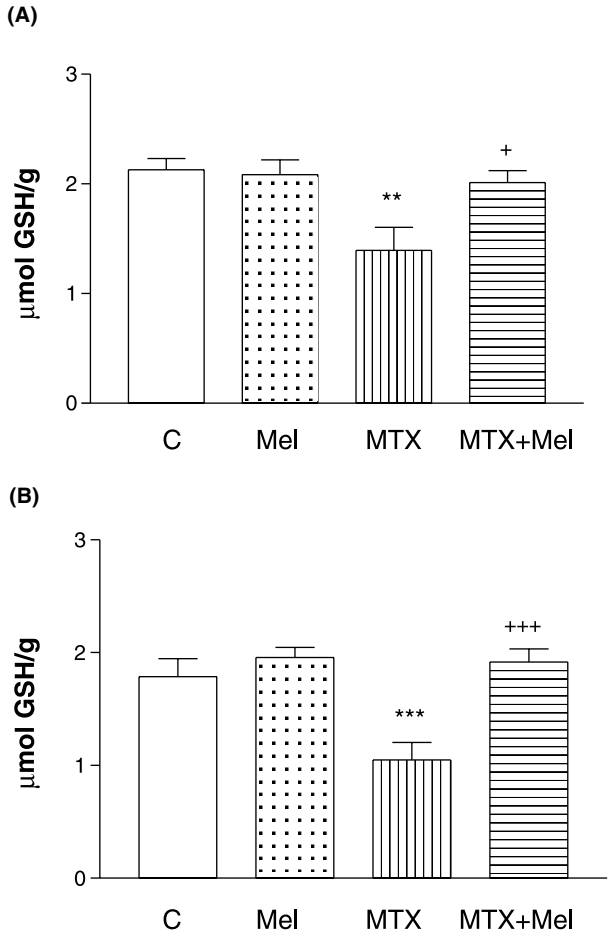

Fig. 1. Glutathione (GSH) levels in (A) liver and (B) kidney of rats after MTX and/or Mel treatment. (C, control; Mel, melatonin; MTX, methotrexate; MTX + Mel, methotrexate + melatonin groups.) $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control group; $^{+}P < 0.05$, $^{+++}P < 0.001$, MTX + Mel group compared with MTX group.

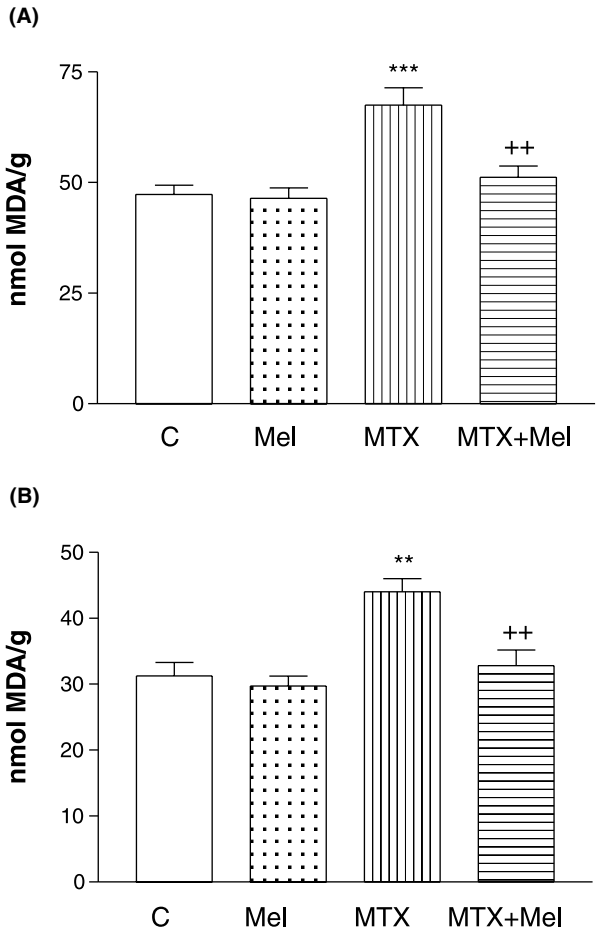

Fig. 2. Malondialdehyde (MDA) levels in (A) liver and (B) kidney of rats after MTX and/or Mel treatment. (C, control; Mel, melatonin; MTX, methotrexate; MTX + Mel, methotrexate + melatonin groups.) $^{**}P < 0.01$, compared with control group; $^{++}P < 0.01$, MTX + Mel group compared with MTX group.

including adverse effects of antitumor drugs [31, 32]. MTX, a widely used drug in antimetabolite cancer therapy or in various forms of arthritis, is known to have toxic effects due to oxidative reactions that take place during its metabolism in the liver [10]. MTX can cause liver hepatotoxicity, including steatosis, cholestasis, fibrosis, and cirrhosis [33]. Administration of a high-dose of MTX may also results in acute renal failure possibly due to precipitation of MTX and/or 7-OH-MTX in the renal tubules. This nephrotoxicity leads to delayed MTX elimination [34].

By binding to dihydrofolate reductase with greater affinity than folic acid, MTX limits the conversion of folic acid to tetrahydrofolate, a molecule necessary for the synthesis of DNA [35]. MTX's inhibition on the synthesis of purine and pyrimidine thymidilate results in improper DNA synthesis and subsequent apoptosis [36]. Owing to its ability to cross biological membranes easily and to its high concentrations in the nuclei of cells [37], melatonin has been shown to protect nuclear DNA from oxidative destruction in several experimental situations, all of which involve the generation of free-radical species [38–40]. For example, melatonin reduces hepatic DNA damage of rats exposed to the carcinogen, safrole, alleviates the free-radical-induced suppression of the Ca^{2+}-pump in cardiomyocytes, protects against paraquat-induced oxidative damage, and recently melatonin was also shown to prevent lipopolysaccharide-induced oxidative injury in phenobarbital-treated rats, as well as protect against kainic acid-induced neural damage [41–43]. In our study, melatonin brought about a reduction in lipid peroxidation products and increased the GSH levels in hepatic and renal tissues, as well as in plasma, suggesting the antioxidant and free-radical scavenging activity of melatonin may reduce the toxic effects of MTX. Previously, it was shown that when antioxidants are given as adjuvant therapy, a significant clinical improvement is demonstrated. When a high dose of vitamin E [44] or a combination of antioxidants [45] is given, the activity of glutathione peroxidase was restored with concomitant reduction in MDA levels and an increase in depleted of sulfhydryl groups caused by MTX treatment. Neuman *et al.* [9] have studied the role of silymarin, a natural antioxidant, in

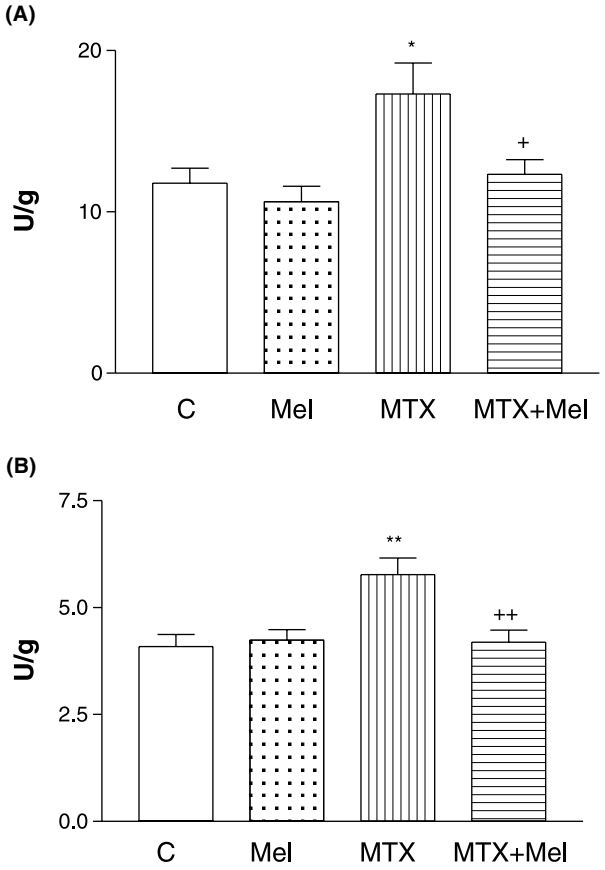

Fig. 3. Myeloperoxidase (MPO) activity in (A) liver and (B) kidney of rats after MTX and/or Mel treatment. (C, control; Mel, melatonin; MTX: methotrexate; MTX + Mel: methotrexate + melatonin groups.) $^{*}P < 0.05$ $^{**}P < 0.01$, compared with control group; $^{+}P < 0.05$, $^{++}P < 0.01$, MTX + Mel group compared with MTX group.

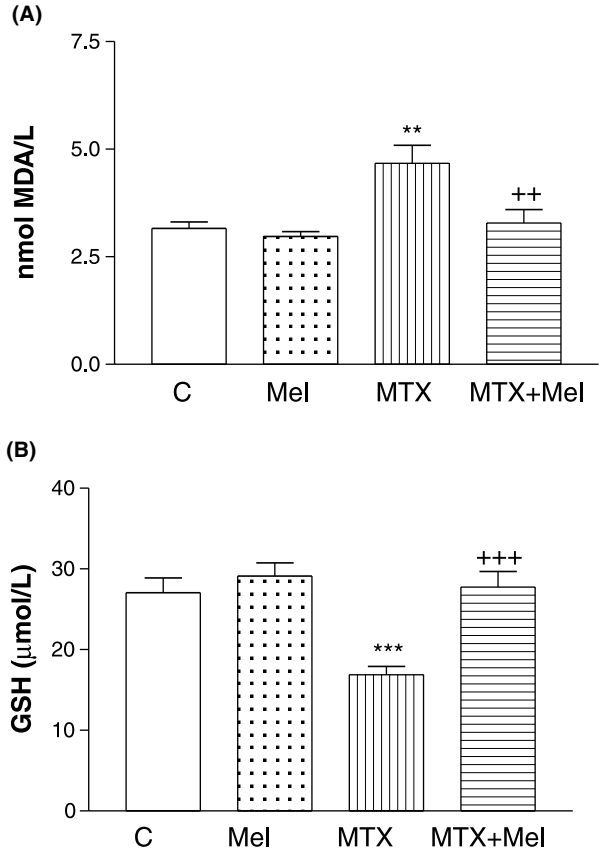

Fig. 4. The effects of MTX and/or Mel treatment on (A) serum malondialdehyde (MDA) and (B) glutathione (GSH) levels. Each group consists of eight rats. (C, control; Mel, melatonin; MTX, methotrexate; MTX + Mel, methotrexate + melatonin groups.) $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control group; $^{++}P < 0.01$, $^{+++}P < 0.001$, MTX group versus MTX + Mel group.

preventing MTX cytotoxicity and suggested that the mechanism of silymarin's cellular protection is the enhancement of intracellular GSH, a phenomenon which occurs in the mitochondria.

GSH is one of the most important molecules in the cellular defense against chemically reactive toxic compounds or oxidative stress. In its reduced form, GSH is necessary for the detoxification of xenobiotics. Decreased cellular GSH levels and capacity for GSH synthesis sensitize cells to radiation and to certain drugs [46]. It was reported that GSH could have a role in maintaining activity of the pentose phosphate cycle at a level which is appropriate for the severity of the oxidative challenge, as well as for the capacity of the cellular antioxidant defenses [47]. The significant reduction in GSH levels promoted by MTX, represents an alteration in the cellular redox state, suggesting that the cells could be more sensitive to reactive oxygen metabolites [48], and leads to a reduction of effectiveness of the antioxidant enzyme defense system [17]. The experimental data indicate that exaggerated inhibition of glucose-6-phosphate dehydrogenase by MTX contributes to a decrease of the availability of NADPH, an inhibition of glutathione reductase activity and finally an inhibition of GSH cycle. On the other hand, Rouse *et al.* [49] suggested that intravenous glutamine protects liver cells from MTX-induced oxidant injury by increasing intracellular GSH metabolism. In the present study, MTX administration caused significant decreases in GSH levels in both kidney and liver, which were restored with the antioxidant melatonin. It was demonstrated that melatonin, besides its detoxification of free-radicals, also stimulates the activity of several antioxidative enzymes, including the MTX-inhibited glucose-6-phosphate dehydrogenase [38].

There is substantial evidence for a role of reactive oxygen metabolites in mediating the renal and hepatic toxicity of some xenobiotics and the pathogenesis of organ failure [50, 51]. As oxidative stress has been implicated a common link between chronic liver damage and hepatic fibrosis, free-radicals may play a role in MTX-induced hepatic and renal toxicity. Lipid peroxidation, mediated by oxygen free-radicals, is believed to be an important cause of destruction and damage to cell membranes and has been suggested to be a contributing factor to the development of MTX-mediated tissue damage. Attention has been focused on the role of (ROS) in mediating the microvascular disturbances that precede tissue damage induced by various chemicals

[52]. Besides their direct damaging effects on tissues, free-radicals seem to trigger the accumulation of leukocytes in the tissues involved, and thus aggravate tissue injury indirectly through activated neutrophils. It has been shown that activated neutrophils secrete enzymes (e.g. MPO, elastase, proteases) and liberate oxygen radicals [53]. Herein, elevated MPO levels in both liver and kidney tissues indicate that neutrophil accumulation contributes to MTX-induced oxidative organ injury. Furthermore, the results also suggest that melatonin has a preventive effect through the inhibition of neutrophil infiltration.

The severity of MTX-associated liver injury is related to both the dose and duration of the treatment. In some experimental studies, liver injury was not observed with high dose MTX, presumably because the duration of exposure to the drug was limited by the systemic toxicity (e.g. bone marrow and gastrointestinal injury) that necessitated killing the animals [2]. In our study, a single dose of 20 mg/kg MTX caused toxic effects on the liver and kidney that was suitable for studying the oxidative injury without systemic toxicity, that would otherwise limit the survival of the animals. High doses of MTX that have been used to treat malignancies may result in acute renal failure possibly due to precipitation of MTX and 7-OH-MTX in the renal tubules, which consequently leads to toxicities including myelosuppression, gastrointestinal toxicity, hepatitis and mucositis [54]. Our results suggest that melatonin may be a choice of rescue therapy in preventing lethal MTX toxicity. In support of this suggestion, it was reported that plasma melatonin concentrations, which increase after the initial chemotherapy, could be an essential contribution to the success of chemotherapy [44]. As the total antioxidant capacity of human serum positively correlates with its melatonin concentration [55], supplementing patients with adjuvant therapy of melatonin may have some benefit for successful chemotherapy [56].

In conclusion, the findings of the current study illustrate that exogenously administered melatonin is capable of reversing the oxidative toxic effects of MTX. These data suggest that melatonin, by preventing hepatorenal toxicity, may enhance the selectivity of antitumor drugs in the patients who require high doses of MTX.

References

1. Hersh EM, Wong VG, Henderson ES, Freirich EJ. Hepatotoxic effects of methotrexate. Cancer 1966; **19**:600–606.
2. Hall PM, Jenner MA, Ahern MJ. Hepatotoxicity in a rat model caused by orally administered methotrexate. Hepatology 1991; **14**:906–910.
3. Tobias H, Auerbach R. Hepatotoxicity of long term methotrexate therapy for psoriasis. Arch Intern Med 1973; **132**:391–396.
4. Roenigk HH, Bergfield WF, St Jacques R, Owens FJ, Hawk WA. Hepatotoxicity of methotrexate in the treatment of psoriasis. Arch Dermatol 1971; **103**:250–261.
5. Kintzel PE. Anticancer drug-induced kidney disorders. Drug Safety 2001; **24**:19–38.
6. Iqbal MP, Sultana F, Mahboobali N, Pervez S. Folinic acid protects against suppression of Growth by methotrexate in mice. Biopharm Drug Dispos 2001; **22**:169–178.
7. Schiff MH, Whelton A. Renal toxicity associated with disease-modifying antirheumatic drugs used for the treatment of rheumatoid arthritis. Semin Arthritis Rheum 2000; **30**:196–208.
8. Goto E, Tomojiri S, Okamoto I, Tanaka K. Methotrexate poisoning with acute hepatorenal dysfunction. J Toxicol Clin Toxicol 2001; **39**:101–104.
9. Neuman MG, Cameron RG, Haber JA, Katz GG, Malkewcz IM, Shear NH. Inducers of cytochrome P450 2E1 enhance methotrexate-induced hepatotoxicity. Clin Biochem 1999; **32**:519–536.
10. Chladek J, Martinkova J, Sispera L. An in vitro study on methotraxate hydoxylation in rat and human liver. Physiol Res 1997; **46**:371–379.
11. Galivan J, Nimec Z, Balinska M. Regulation of methotrexate polyglutamate accumulation in vitro: effects of cellular folat content. Biochem Pharmacol 1983; **32**:3344–3347.
12. Kamen BA, Nylen PA, Camitta BM, Bertno JR. Methotrexate accumulation and folate depletion in cell as a possible mechanism of chronic toxicity to the drug. Br J Hematol 1981; **49**:355–360.
13. Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients. Arth Rheum 1986; **29**:832–835.
14. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med 1983; **309**:1094–1104.
15. Vogel WH, Snyder R, Schulman MP. The inhibition of dehydrogenases by folic acid and several of its analogs. Biochim Biophys Res Comm 1963; **10**:97–101.
16. Caetano NN, Campello AP, Carnieri EGS, Kluppel MLW, Oliveira MBM. Effects of methotrexate (MTX) on $NAD(P)^+$ dehydrogenases of HeLa cells: malic enzymes, 2-oxoluterate and isocitrate dehydrogenases. Cell Biochem Funct 1997; **15**:259–264.
17. Babiak RM, Campello AP, Carnieri EG, Oliveira MB. Methotrexate: pentose cycle and oxidative stress. Cell Biochem Funct 1998; **16**:283–293.
18. Reiter RJ, Maestroni GJM. Melatonin in relation to the antioxidative defense and immune systems: possible implications for cell and organ transplantation. J Mol Med 1999; **77**:36–39.
19. Cuzzocrea S, Costantino G, Mazzon E, Micali A, De Sarro A, Caputi AP. Beneficial effects of melatonin in a rat model of splanchnic artery occlusion and reperfusion. J Pineal Res 2000; **28**:52–63.
20. Reiter RJ, Acuna-Castroviejo D, Tan DX, Burkhart S. Free radical-mediated molecular damage. Ann NY Acad Sci 2001; **939**:200–215.
21. Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: a potent endogenous hydroxyl radical scavenger. Endocrine J 1993; **1**:57–60.
22. Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. J Biomed Sci 2000; **7**:444–458
23. Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. A review of the evidence. Cell Biochem Biophys 2001; **34**:237–256.
24. Sener G, Sehirli AO, Altunbas HZ, Paskaloglu K, Ayanoglu-dulger G. Melatonin protects against gentamicin-induced nephrotoxicity in rats. J Pineal Res 2002; **32**:231–236.

25. Mcginnes JE, Proctor PH, Demopoulos HB, Hokanson JA, Kirkpatrick DS. Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase). Physiol Chem Phys 1978; **10**:267–277.
26. Amimoto T, Matsura T, Koyama SY, Nakanishi T, Yamada K, Kajiyama G. Acetaminophen-induced hepatic injury in mice: the role of lipid peroxidation and effects of pretreatment with coenzyme Q10 and alpha-tocopherol. Free Radic Biol Med 1995; **19**:169–176.
27. Kourounakis AP, Rekka EA, Kourounakis PN. Antioxidant activity of guaiazulene and protection against paracetamol hepatotoxicity in rats. J Pharm Pharmacol 1997; **49**:938–942.
28. Beuge JA, Aust SD. Microsomal lipid peroxidation. Meth Enzymol 1978; **52**:302–311.
29. Beutler E. Glutathione in Red Blood Cell Metabolism. A Manual of Biochemical Methods. Grune & Stratton, New York, 1975; pp. 112–114.
30. Hillegas LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assesment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990; **24**:285–295.
31. Sugiyama S, Hayakawa M, Kato T, Hanaki Y, Shimizu K, Ozawa T. Adverse effects of anti-tumor drug, cisplatin, on rat kidney mitochondria: disturbances in glutathione peroxidase activity. Biochem Biophys Res Comm 1989; **159**: 1121–1127.
32. Zhang JG, Zhong LF, Zhang M, Xia YX. Protection effects of procaine on oxidative stress and toxicities of renal cortical slices from rats caused by cisplatin in vitro. Arch Toxicol 1992; **66**:354–358.
33. Vonen B, Morland J. Isolated rat hepatocytes in suspension: potential hepatotoxic effects of six different drugs. Arch Toxicol 1984; **56**:33–37.
34. Van den bongard HJGD, Manhot RAA, Beijnen JH *et al*. Successful rescue with leucovorin and thymidine in a patient with high-dose methotrexate induced acute renal failure. Cancer Chemother Pharmacol 2001; **47**:537–540.
35. Tsurusawa M, Saeki K, Fujimoto T. Differential induction of apoptosis on human lymphoblastic leukemia Nalm-6 and Molt-4 cells by various antitumor drugs. Int J Hematol 1997; **66**:79–88.
36. Heenen M, Laporte M, De Graef C. Methotrexate induces apoptotic cell death in human keratinocytes. Arch Dermatol Res 1998; **290**:240–245.
37. Acuna-castroviejo D, Martin M, Macias M *et al*. Melatonin, mitochondria and cellular bioenergetics. J Pineal Res 2001; **30**:65–74.
38. Karbownik M, Reiter RJ, Garcia JJ, Tan DX, Qi W, Manchester LC. Melatonin reduces rat hepatic macromolecular damage due to oxidative stress caused by δ-aminolevulinic acid. Biochim Biophys Acta 2000; **1523**:140–146.
39. Morioka N, Okatani Y, Wakatsuki A. Melatonin protects against age-related DNA damage in the brains of female senescence-accelerated mice. J Pineal Res 1999; **27**:202–209.
40. Vijayalaxmi, Meltz ML, Reiter RJ, Herman TS. Melatonin and protection from genetic damage in blood and bone marrow: whole-body irradiation studies in mice. J Pineal Res 1999; **27**:221–225.
41. Sewerynek E, Melchiorri D, Reiter RJ, Ortiz GG, Lewinski A. Lipopolysaccharide-induced hepatotoxicity is inhibited by the antioxidant melatonin. Eur J Pharmacol 1995; **293**:327–334.
42. Melchiorri D, Reiter RJ, Sewerynek E, Chen LD, Nistico G. Melatonin reduces kainate-induced lipid peroxidation in homogenates of different brain regions. FASEB J 1995; **9**:1205–1210.
43. Sewerynek E, Melchiorri D, Ortiz GG, Poeggeler B, Reiter RJ. Melatonin reduces H_2O_2 induced lipid peroxidation in homogenates of different rat brain regions. J Pineal Res 1995; **19**:51–56.
44. Subramaniam S, Shya mala Devi CS. Vitamin E protects intestinal basolateralmembrane from CMF-induced damages in rat. Ind J Physiol Pharmacol 1995; **39**:263–266.
45. Helmy M, Shohayeb M, Helmy MH, El-bassiouni EA. Antioxidants as adjuvant therapy in rheumatoid disease. A peliminary study. Arzneimittelforschung 2001; **51**:293–298.
46. Ross D. Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther 1988; **37**:231–249.
47. Przybtkowski E, Averill-bates DA. Correlation between glutathione and stimulation of the pentose phosphate cycle in situ in Chinese hamster ovary cells exposed to hydrogen peroxide. Arch Biochem Biophys 1996; **325**:91–98.
48. Goldman D. Analysis of the cytotoxic deteminants for methotrexate (NSC-740): a role for free intracellular drug. Cancer Chemother Rep 1975; **6**:51-61.
49. Rouse K, Nwokedi E, Woodliff JE, Epstein J, Klimberg VS. Glutamine enhances selectivity of chemotherapy through changes in glutathione metabolism. Ann Surg 1995; **221**:420–426.
50. Mitchell JR, Jallow DJ, Gillette JR, Brodie BB. Drug metabolism as a cause of drug toxicity. Drug Metab Disposition 1973; **1**:418–423.
51. Baliga R, Ueda N, Walker PD, Shah SV. Oxidant mechanisms in toxic acute renal failure. Drug Metab Rev 1999; **31**:971–997.
52. Parks DA, Granger DN. Ischemia-reperfusion injury: a radical view. Hepatology 1988; **8**:680–682.
53. Sullivan GW, Sarembock IJ, Linden J. The role of inflammation in vascular diseases. J Leukoc Biol 2000; **67**:591–602.
54. Schornagel JH, Mcvie JG. The clinical pharmacology of methotrexate. Cancer Treat Rev 1983; **10**:53–57.
55. Benot S, Goberna R, Reiter RJ, Garcia-maurino S, Osuna C, Guerrero JM. Physiological levels of melatonin contribute to the antioxidant capacity of human serum. J Pineal Res 1999; **27**:59–64.
56. Reiter JR, Tan DX, Sainz RM, Mayo JC, Lopez-Burillo S. Melatonin: In reducing the toxicity and increasing the efficacy of drugs. J Pharm Pharmacol 2002; **54**:1299–1321.