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Preventive role of mirtazapine in methotrexate induced nephrotoxicity in rats

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ABSTRACT: Methotrexate (MTX) is used in the treatment of rheumatic diseases, psoriasis, and cancer. Since more than 90% of MTX is excreted via the kidneys, nephrotoxicity is one of the most important reasons restricting the use of this drug. In our study, we aimed to evaluate biochemical and histopathological properties to determine whether mirtazapine has a protective effect on MTX-related nephrotoxicity in rats. A group of rats were given a dose of 30 mg/kg of mirtazapine orally through a tube and 1 h after a dose of 5 mg/kg of MTX intraperitoneally. The rats in the control group received MTX alone intraperitoneally. A group of healthy rats was given orally distilled water in equal volume as dissolvent, then distilled water was administered intraperitoneally 1 h later. These procedures were repeated for 7 days. At the end of this period, all animals were sacrificed by giving a high dose of sodium thiopental. The results of biochemical analysis revealed that in the kidney tissues of rats given MTX, malondialdehyde, myeloperoxidase, glutathione, glutathione S-transferase, and nitric oxide levels were 42.3 ± 2.4 μmol/g protein, 31.3 ± 1.9 U/g, 6.5 ± 1.0 nmol/g protein, 5.7 ± 0.8 U/g, and 14.7 ± 1.2 μmol/g, respectively; in the mirtazapine group, these values were found to be 24.2 ± 1.9 μmol/g protein, 19.7 ± 1.8 U/g, 11.5 ± 1.3 nmol/g protein, 10.5 ± 1.1 U/g, and 25.2 ± 2.7 μmol/g, respectively. No biomarkers for protein nitrosylation, nitration, or DNA damage were measured. In this study mirtazapine was found to be efficacious to prevent nephrotoxicity due to MTX, suggesting that MTX can be used safely in higher doses and for a longer course in combination with mirtazapine in cancer chemotherapy.

KEYWORDS: oxidative/nitrosative stress, depression, inflammation

INTRODUCTION

Methotrexate (MTX), a cytotoxic chemotherapeutic agent, is used in the treatment of acute lymphoblastic leukaemia, lymphoma, osteosarcoma, breast cancer, head and neck cancers and also in the therapy of non-oncologic disorders such as rheumatic diseases and psoriasis[1–4]. High-dose methotrexate interrupts synthesis and repair of DNA and cell division by inhibiting several enzymes of the folic acid cycle. However, beside the therapeutic effects, there are also toxic effects including nephrotoxicity as well as gastrointestinal, central nervous system, hepatic, and bone marrow toxicity. Since more than 90% of MTX is excreted via the kidneys, nephrotoxicity is one of the significant reasons for restricting its use[5–7]. The main damages caused by MTX in the kidneys occur in a wide clinical range, varying from subclinical tubulopathy to acute renal failure[8]. The increased production of reactive oxygen radicals is defined as one of the important causes of MTX-related renal toxicity. The use of MTX causes increased activities of malondialdehyde (MDA) and myeloperoxidase (MPO) and a decline in glutathione level in the kidneys and in other tissues[9]. Furthermore, it is suggested that excessive nitric oxide (NO) production has an impact on renal damage developed due to MTX administration[10]. These studies indicate that oxidative damage is an important mechanism involved in the pathogenesis of MTX-related nephrotoxicity. For this reason, it was thought that anti-oxidant therapy could be helpful to prevent or to ameliorate MTX nephrotoxicity. Studies have showed that several antioxidants are protective in MTX nephrotoxicity. However, MTX-related toxicity still remains one of the significant causes restricting its use in desired doses. Hence several trials have thus far been performed to ameliorate MTX toxicity.

An antidepressant is a psychiatric medication used to alleviate mood disorders, such as major de-

pression and dysthymia, and anxiety disorders such as social anxiety disorder. Drugs including the monoamine oxidase inhibitors, tricyclic antidepressants, tetracyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin-norepinephrine reuptake inhibitors are most commonly associated with the term. Mirtazapine is a tetracyclic antidepressant used primarily in the treatment of depression. Human studies and animal models of depression provide evidence that oxidative and nitrosative stress (O&NS) pathways are involved in treatment resistance [11]. So there is a relation between antidepressant agents and O&NS. Studies also suggest that major depressed patients could benefit from treatments with selected antioxidants [11].

In addition, mirtazapine prevents ischaemia-reperfusion induced oxidative stress in kidney tissue [12]. Mirtazapine inhibits the production of both enzymatic and non-enzymatic oxidant parameters in the stomach tissue but increases the anti-oxidants [13]. Thus mirtazapine could be a protective agent in MTX-induced nephrotoxicity. In a literature reviews, we did not find any information or innovation relating to protective effects of mirtazapine on MTX induced renal toxicity. For this reason, the goal of our study is to ascertain biochemically and histopathologically whether mirtazapine has any protective effect on MTX induced nephrotoxicity in rats.

MATERIALS AND METHODS

Animals

In this study, a total of 18 Wistar albino male rats with weights varying between 220–235 were used, which were supplied from Ataturk University Practice and Research Centre for Medical Experiments. Before the study, the animals were kept and fed in groups at room temperature (22 °C). Animal experiments were performed in accordance with national guidelines for the use and care of laboratory animals and approved by the local animal care committee of Ataturk University.

Chemical substances

Of the chemical substances used for the experiments, thiopental sodium was provided by IE Ulagay-Turkey and mirtazapine was obtained from Organon Pharmaceuticals-USA. MTX was provided by Kocak Farma-Turkey.

General procedure

Rats to be used in the study were divided in to three groups to undergo (i) methotrexate (MTX), (ii) methotrexate + mirtazapine (MTX+Mrtz), and (iii) intact.

Performing study

In this series of our trial, a group of rats were given a dose of 30 mg/kg of mirtazapine orally through a tube. A dose of 5 mg/kg of MTX was given via intraperitoneal route 1 h after mirtazapine administration. The rats in the control group were injected MTX alone using the same route. The group of healthy rats was given orally distilled water in equal volume as dissolvent, then distilled water was administered intraperitoneally 1 h later. These procedures were repeated for 7 days. At the end of this period, all animals were sacrificed by giving an overdose of sodium thiopental. Their kidneys were extracted to perform biochemical analysis and histopathological examination. The results obtained from mirtazapine group were compared with those achieved from MTX group and control group.

Biochemical analysis

Venous blood samples were collected into tubes without anticoagulant. Serum was separated by centrifugation after clotting and stored at -80 °C until assayed. Creatinine and urea levels were determined in Cobas 8000 (Roche) spectrophotometrical system with colorimetric method. BUN (blood urea nitrogen) levels were determined with formula ($\mathrm{BUN} = \mathrm{URE} \times 0.48$).

In alkaline solution, creatinine forms a yellow-orange complex with picrate. The colour intensity is directly proportional to the creatinine concentration and can be measured photometrically (505 nm). Assays using rate-blanking minimize interference by bilirubin. Serum and plasma samples contain proteins which react non-specifically in the Jaffe method. Serum and plasma results must be corrected by 0.3 mg/dl (26 μM) to obtain accurate values. This correction causes a measurement error of $\leqslant 1\%$ in urine specimens because these do not contain non-specific proteins.

Urea is hydrolysed by urease to form CO_2 and ammonia.

$$\mathrm{Urea} + \mathrm{H_2O} \xrightarrow{\text{urease}} 2\,\mathrm{NH_4^+} + \mathrm{CO_2}$$

The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of GLDH to yield glutamate and NAD^+.

$$\alpha\text{-ketoglutarate} + \mathrm{NH_4^+} + \mathrm{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + \mathrm{NAD^+} + \mathrm{H_2O}$$

The decrease in absorbance due to consumption of NADH is measured kinetically. The NADH complex to decrement is determined photometrically (340 nm).

Biochemical analysis of kidney tissue

After the macroscopic analyses, the malondialdehyde (MDA), myeloperoxidase (MPO), glutathione (GSH), glutathione S-transferase (GST), and nitric oxide (NO) levels and enzyme activities in rat kidney tissues were determined. For this purpose, rat kidneys were frozen at −80 °C before until biochemical investigations. To prepare the tissue homogenates, kidney tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 ml of an appropriate buffer, homogenized on ice using an Ultra-Turrax homogenizer for 15 min, filtered, and centrifuged using a refrigerated centrifuge at 4 °C. The supernatants were used to determine the enzymatic activities. All assays were carried out at room temperature in triplicate.

Determination of lipid peroxidation or MDA formation

The concentrations of gastric mucosal lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test[14]. Briefly, the rat stomachs were promptly excised and rinsed with cold saline. To minimize the possibility of haemoglobin interfering with free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed, and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium lauryl sulphate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate, and 0.3 ml distilled water. The mixture was incubated at 98 °C for 1 h. Upon cooling, 5 ml of n-butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 1280g. The absorbance of the supernatant was measured at 532 nm. A standard curve was generated using 1,1,3,3-tetramethoxypropane. The recovery was over 90%. The results were expressed as nanomoles MDA per gram wet tissue (nmol/mg tissue).

MPO activity

MPO activity was measured according to the modified method of Bradley et al[15]. The homogenized samples were frozen and thawed three times, and centrifuged at 1500g for 10 min at 4 °C. MPO activity in the supernatant was determined by adding 100 ml of the supernatant to 1.9 ml of 10 mmol/l phosphate buffer (pH 6.0) and 1 ml of 1.5 mM o-dianisidine hydrochloride containing 0.0005% (wt/vol) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a UV-vis spectrophotometer. MPO activity in renal tissues was expressed as millimoles per minute per milligram tissue (mmol/min/mg tissue).

GSH determination

The amount of GSH in the gastric mucosa was measured according to the method of Sedlak and Lindsay[16]. The mucosal surface of the stomach was collected by scraping, weighed, and then homogenized in 2 ml 50 mM Tris-HCl buffer containing 20 mM EDTA and 0.2 mM sucrose, pH 7.5. The homogenate was immediately precipitated with 0.1 ml of 25% trichloroacetic acid, and the precipitate was removed by centrifugation at 1380g for 40 min at 4 °C. The supernatant was used to determine GSH using 5,5′-dithiobis(2-nitrobenzoic acid). Absorbance was measured at 412 nm using a spectrophotometer. The results of the GSH level in the mucosa were expressed as nanomoles per milligram tissue (nmol/mg tissue).

GST activity

Total GST activity was determined as described by Habig and Jakoby[17]. Briefly, the enzyme activity was assayed spectrophotometrically at 340 nm in a 4 ml cuvette containing 0.1 M PBS (pH 6.5), 30 mM glutathione, 30 mM 1-chloro-2,6-dinitrobenzene and tissue homogenate.

Determination of NO

Determination of nitric oxide (NO): NO is readily oxidized to NO_2^- and NO_3^- since it is an extremely short-lived radical. For this reason, NO measurement was done after reduction of NO_3^- existing in the ambient to NO_2^- [18].

Histopathological analysis

The kidneys of rats were fixed using 10% formaldehyde solution. Paraffin sections in 5 μm thickness were cut from paraffin blocks prepared after tissue processing procedure, and the slides were stained with haematoxylin-eosin. The frequency and severity of renal lesions were evaluated on at least two tissue sections using the light microscope (Olympus CX51). The renal cortex and medulla were examined to determine the severity of renal damage.

Statistical analysis

All data were subjected to one-way ANOVA using SPSS 18.0 software. Differences among groups were attained using the least significant difference option and significance was declared at $p < 0.05$. Results are the means ± standard error of the mean.

Table 1 The effect of mirtazapine on oxidative stress parameters in MTX induced nephrotoxicity in MTX (methotrexate), Mrtz+MTX (methotrexate + mirtazapine), and intact groups. Results are the mean ± standard error of the mean. $N = 6$ (number of animals).

Drugs	Dose (mg/kg)	MDA	MPO	GSH	GST	NO
MTX	5	42.3 ± 2.4	31.3 ± 1.9	6.5 ± 1.0	5.7 ± 0.8	14.7 ± 1.2
p		–	–	–	–	–
Mrtz+MTX	30+5	24.2 ± 1.9	19.7 ± 1.8	11.5 ± 1.3	10.5 ± 1.1	25.2 ± 2.7
p		0.0001	0.0001	0.007	0.007	0.004
Intact	–	21.2 ± 1.6	17.2 ± 1.6	14.0 ± 1.1	16.8 ± 1.3	33.5 ± 2.3
p		0.0001	0.0001	0.0001	0.0001	0.0001

RESULTS AND DISCUSSION

The histopathologic and biochemical results of our study confirmed that MTX causes renal injury. When mirtazapine was given together with MTX to ascertain the effect of mirtazapine preventing from MTX-related nephrotoxicity, mirtazapine provided a protective effect on renal damage developed with MTX reversing biochemical parameters indicating renal damage to almost control levels. Moreover, histopathologic examinations also demonstrated that mirtazapine had a protective effect on renal injury developed with MTX in rats.

In our study, it was observed that the levels of MDA and MPO significantly increased in the kidney tissue of MTX group in comparison with the intact group, whilst GSH and GST levels declined (Table 1). In the group of rats that received mirtazapine plus MTX, the levels of MDA, MPO, and GSH were similar to that of the group of intact animals and the difference between the groups was statistically insignificant. An increased level of MDA, which is one of the end-products of lipid peroxidation, represents an increase in free-oxygen radicals in the tissues[19]. The free radicals generate harmful effects initiating lipid peroxidation in the cell. MDA causes further damage in the cells[20]. In some studies, it was demonstrated that MDA and MPO levels were high, but the amounts of GSH and GST were low in the damaged renal tissue. These data are consistent with our MDA, MPO, GSH, and GST results achieved from both healthy and damaged tissues.

MPO enzyme existing within phagocytic cells (PNL) catalyses the production of toxic hypochlorous acid (HOCl) from H_2O_2. Excessive production of MPO and other reactive radicals result in oxidative damage because of the production of abundant amounts of superoxide anion (O^{2-}) and hydroxyl radical (OH^-) by PNL cells. However, in the tissues antioxidant defensive mechanisms evolve against these toxic oxygen radicals. When these anti-oxidant defence mechanisms fail, severe damage occurs in the tissues[21]. The amount of MPO was found to be high in the kidney tissue of the rats given MTX in comparison with the group of intact rats. However, mirtazapine blocked significantly the elevation of MPO in the rat kidney tissue in comparison with MTX group.

GSH, an anti-oxidant, reacts with peroxides and free radicals and transforms them to non-toxic products. GSH keeps –SH groups of proteins in reduced forms hindering their oxidation. With this mechanism, GSH protects the cells against oxidative damage that can be caused by free radicals[22]. In the kidney tissue of the rats given MTX, GSH, and GST levels were found to be much lower in comparison with the group of intact rats. But, the declines of GSH and GST levels were significantly blocked in the kidney tissue of rats given mirtazapine in comparison with MTX group. NO, also known as endothelial-derived relaxing factor, is released into circulation during hypoxia, endotoxin, or cellular damage stress[23]. Over increase in the intracellular concentrations of NO, triggers toxic events result in cell death[24]. Occurrence of $O_2^{\cdot}$ is parallel with NO, as they affect each other and cause the formation of $OH^{\cdot}$ and $NO_2^{\cdot}$. During this reaction toxic intermediate products such as peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH) occur[25].

There are studies suggesting that increased production of NO plays a role in renal damage and support the claiming that NO improves the renal damage occurring in renal disorders. In our study, the amount of NO was found to be lower in rats given MTX in comparison with the control group. When mirtazapine was given together with MTX, the amount of NO increases. These results are supported by the studies claiming remedial effect of NO on renal damage.

Oxygen free radicals not only acts on lipids but also reacts with DNA generating the mutagenic 8-hydroxyguanine, which is considered as an indicator

Table 2 The effect of mirtazapine on renal function tests in MTX induced nephrotoxicity in MTX (methotrexate), Mrtz+MTX (methotrexate + mirtazapine), and intact groups. Results are the means ± standard error of the mean.

Drugs	BUN	p	Cre	p
MTX	140.3 ± 6.3	–	2.2 ± 0.1	–
Mrtz+MTX	70.0 ± 3.9	0.0001	1.3 ± 0.1	0.0001
Intact	38.7 ± 2.2	0.0001	0.70 ± 0.04	0.0001

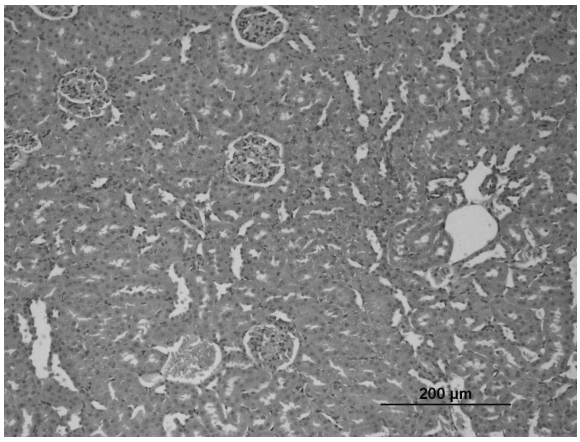

Fig. 1 Renal tissue (cortex) of intact group.

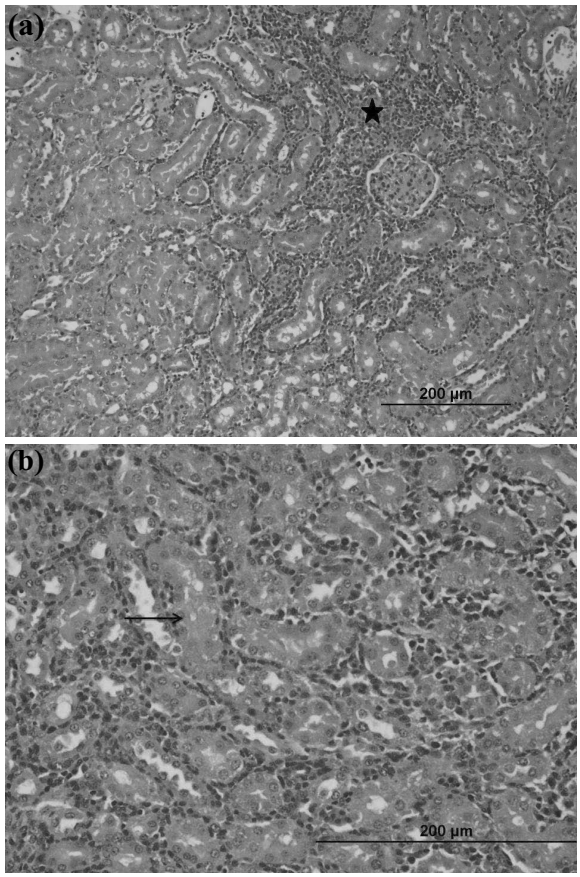

Fig. 2 Renal tissue (corticomedullary junction) of rats administered MTX.

of DNA damage in tissue[26]. In recent studies it has been reported that 8-hydroxyguanine levels increase in tissues with reduced GSH and increased MDA levels[27]. GSH play roles in the prevention of potential damage of free radicals, inactivation of toxic substances, DNA synthesis, repair of damaged DNA fragments, and other metabolic functions[28]. Our results, together with the information obtained from literature, demonstrate that oxidants play an important role in DNA damage.

In our study, it was found that mirtazapine significantly decreased serum BUN and creatinine levels, which were increased due to methotrexate (Table 2). Serum BUN and creatinine are important biochemical parameters for measuring renal functions. Increases in serum BUN and creatinine concentrations are the indicators of severe kidney damage such as loss of functional nephrons. One evidence for the preventive effect of mirtazapine on nephrotoxicity is that mirtazapine blocked the increase in BUN and creatinine levels in blood samples of rats given methotrexate (Table 2).

Histopathological analysis

In the intact group, rats revealed normal renal morphology in both cortex and medulla (Fig. 1). Normal histomorphologic tissue is observed in cortex, glomeruli, proximal, and distal convoluted tubules. However, there were histopathologic signs such as interstitial inflammation, swelling, and desquamation in rats given MTX (Fig. 2). Fig. 2a shows dense mixed type tubulointerstitial inflammation (★) that is more prominent at the corticomedullary junction in the MTX-given group. There is remarkable swelling in the tubular epithelial cells and desquamated cells within the tubule lumen in the corticomedullary junction (⟶) (Fig. 2b). No damage was observed in the glomeruli. Such pathological changes were absent in the kidney tissue of rats given mirtazapine (Fig. 3). Normal histomorphology was observed in the cortex (Fig. 3a) and medulla (Fig. 3b) in the group given mirtazapine + MTX. Tubulointerstitial inflammation and tubular damage was not observed in Fig. 3.

These results indicate that histopathological findings are consistent with the biochemical tests. Literature data and results from our study may suggest that the protective effect of mirtazapine on methotrexate nephrotoxicity results from its anti-oxidant activity.

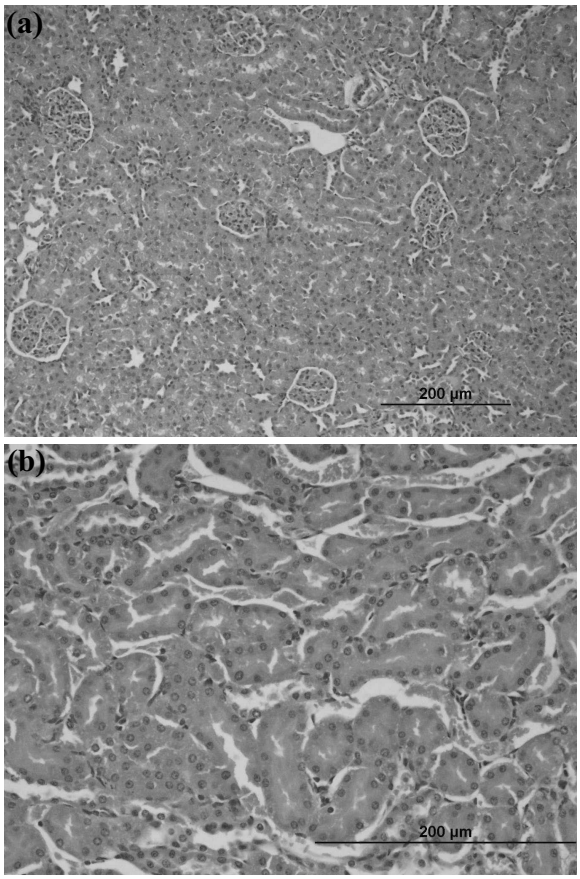

Fig. 3 Renal tissue (cortex and medulla) of rats given mirtazapine + methotrexate.

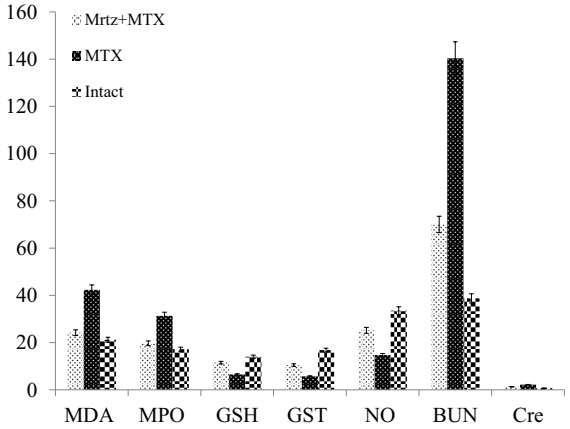

Fig. 4 The effect of mirtazapine on oxidative stress parameters in MTX induced nephrotoxicity in MTX (methotrexate), Mrtz+MTX (methotrexate + mirtazapine), and intact groups.

However, this does not mean that nephroprotective effect of mirtazapine is generated from its anti-oxidant property alone. Mirtazapine also blocks 5-HT_2 and 5-HT_3 receptors[29]. It is known that stimulation of 5-HT_2 and 5-HT_3 receptors is associated with toxic side effects. For this reason, the role of these receptors can also be regarded in nephroprotective effect of mirtazapine. However, there is a need for further comprehensive studies to determine whether these receptors play a role in the mechanism of act of nephroprotective effect of mirtazapine. Maes and his colleagues proposed the role of oxidative mechanisms in the development of depression[30]. However, Bilici and his colleagues demonstrated the importance of the relationship between major depression and oxidant/antioxidant activities[31]. Studies on the pathogenesis of depression have shown that, inflammation occurs concomitantly with depression; inflammation is thought to be due to depression or it can lead to depression in process of time[32]. Müller and his colleagues reported that inflammation plays a role in pathophysiology of depression and pro-inflammatory cytokines, and PGE_2 increased in inflammation[33]. Information from the literature shows that, the antidepressant activity of mirtazapine may result from the antioxidant ability and the inhibition of PG and proinflammatory cytokines.

In conclusion, that mirtazapine was found to be efficacious to prevent nephrotoxicity due to MTX shows that MTX can be used safely in higher doses and for a longer course in combination with mirtazapine in cancer chemotherapy. To clarify this issue, more detailed studies must be done for DNA damage, such as protein nitration/nitrosylation measurements in the future.

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