

The use of ^{99m}Tc -phytate for assessment the protective effect of vitamin E against hepatotoxicity induced by methotrexat in rat

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Abstract

Hepatotoxicity is one of the most common side effects of methotrexate (MTX) therapy in patients. The aim of this study was to evaluate the protective effect of vitamin E against MTX-induced hepatotoxicity using ^{99m}Tc -phytate as a radiopharmaceutical agent in animals. Rats were divided into five groups as follows: control, solvent, Vit E (100 mg/kg), MTX (20 mg/kg) and Vit E + MTX. Animals were intraperitoneally injected with Vit E for 17 days before MTX injection and continued for 4 days. ^{99m}Tc -phytate was injected into the tail of rats after the 21 days of Vit E administration. Percentage of the injected dose per gram of liver and spleen tissues (%ID/g) was calculated in treated rats. Liver imaging was obtained with gamma camera. In other experiment, liver of treated rats was assessed for histopathology. ^{99m}Tc -phytate uptake (%ID/g) of livers in control, solvent, Vit E, MTX and Vit E + MTX groups were $8.99\% \pm 1.37$, $8.53\% \pm 2.91$, $8.65\% \pm 3.84$, $3.22\% \pm 1.09$ and $8.38\% \pm 2.68$. Vit E administration resulted in a significant increase of the level of %ID/g in MTX-injected animal. Vit E pre-treatment improved the shape of liver in MTX-treated rat which was seen abnormal view in planar imaging. Histopathological examinations approved the protective effect of Vit E against MTX-induced hepatotoxicity in rats. The results of this study show that Vit E significantly attenuates the MTX-induced hepatotoxicity in rats, and ^{99m}Tc -phytate is an acceptable radiopharmaceutical agent for assessment of liver and spleen damages in the animal model.

KEY words: Vitamin E, liver, ^{99m}Tc -phytate, hepatotoxicity, hepatoprotective

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Introduction

Methotrexate (MTX) is a folic acid antagonist which has been widely used as an effective anticancer agent in chemotherapy regimen [1]. It has been prescribed extensively as a cytotoxic agent in the treatment of leukemia, breast cancer, testicular tumors and other malignancies. It is used in psoriasis and rheumatoid arthritis that are inflammatory related diseases [2–4]. MTX causes some toxic effects including nausea, vomiting, diarrhea, alopecia, stomatitis, bone marrow depression and hepatotoxicity in patients [5]. MTX treatment increases biomarkers associated with liver injury and are involving in hepatotoxicity [6]. MTX inhibits enzymes corresponding in production of the main sources of nicotinic adenine dinu-

cleotide (NADPH) resulting in intracellular decreases of NADPH [7]. Following NADPH decreasing, glutathione decreases and hepatocytes become vulnerable against reactive oxygen species (ROS). Enhancement of ROS level causes oxidative stress in cell [8]. Hepatotoxicity and production of ROS are main side effects induced by MTX [9]. Then it has been advised to prevent MTX-induced hepatotoxicity, it is using concomitant with antioxidants [10]. Several protective agents such as pentoxifyllin, alpha lipoic acid and beta carotenes have been investigated in different studies for prevention of MTX-caused hepatotoxicity [10, 11]. Vitamin E is considered a lipid soluble vitamin and an important antioxidant. This vitamin is able to neutralize peroxide and free radicals. Vitamin E protects cellular membrane against oxidative damages [12]. Vitamin E, as a free radical scavenger, protects cellular organelles against harmful effects of free radicals [13].

^{99m}Tc -phytate colloid is a radiopharmaceutical agent that has been used for functional imaging of liver and spleen in nuclear medicine. Since 1973, ^{99m}Tc -phytate has been used as an imaging

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radiotracer for liver and spleen assessments. Most of the colloid particles are captured by the Kupffer cells in the liver which is well imaged in nuclear medicine practice [14, 15]. Radionuclide distribution in the liver has a great relevance with chronic hepatic disease severity, histological fibrosis severity and hepatic functions. So, quantitative evaluation of ^{99m}Tc -phytate uptake in liver could be used as a hepatic function tool [16].

The purpose of this research was to evaluate the protective effect of vitamin E on hepatic and spleen injuries caused by MTX using quantitative ^{99m}Tc -phytate uptake measurements and planar imaging and then radiopharmaceutical findings were compared with pathological data in rat.

Material and methods

Animal

Wistar rats weighing 180 ± 220 g were purchased from the Pasteur Institute (Amol, Iran). Rats were housed in a good condition in the university animal house and given standard mouse pellet and water ad Libitum. Animals were kept under controlled lighting condition (light: dark, 12:12 h) and temperature ($22 \pm 1^\circ\text{C}$). Animal experimental and ethical issues were approved by Research Committee of Mazandaran University of Medical Sciences, Sari, Iran (ID# 1969).

Chemicals

MTX was purchased from Mylan (2 mg/2 mL vial, France). Vitamin E (Vit E), as injection form in olive oil, was from Osvaeh Pharmaceutical Co. (Iran). All other chemicals were obtained from Merck Company (Germany).

Experimental group and treatment schedules

Twenty animals were randomly divided into 5 groups (four rats each group):

- Group 1: control; the rats were administered daily intraperitoneal (ip) of normal saline for 21 days;
- Group 2: solvent; the rats were administered daily intraperitoneal (ip) of olive oil for 21 days;
- Groups 3: Vit E; the rats were treated with Vit E ($100 \text{ mg kg}^{-1} \text{ bw}$) for 21 days;
- Group 4: MTX; the rats were administered a single dose of MTX ip injections ($20 \text{ mg kg}^{-1} \text{ body weight (bw)}$) at 17 days after olive oil treatment and 4 days before killing;
- Groups 5: Vit E+ MTX; rats were injected with Vit E ($100 \text{ mg kg}^{-1} \text{ bw}$) at 21 days before single dose of MTX treatment and continued for 4 days.

Radiopharmaceutical assessment

^{99m}Tc , as sodium pertechnetate, was eluted from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (IEOI, Iran) just before the radiolabelling procedure. Phytate (IEOI, Iran) was used as a freeze-dried commercial kit. ^{99m}Tc -phytate was prepared by adding 740 MBq of ^{99m}Tc -pertechnetate to 3.5 mL of saline to the kit. Radiolabelling was performed according to manufacture guideline. After slowly shaking, it is kept at room temperature for 15 minute. ^{99m}Tc -phytate (0.1 mL) was injected through the tail vein. After deep anesthesia (by ketamine/xylazine), rats were killed and the livers and spleens were removed by dissection 2 hours after the radiopharmaceutical injection. Livers and

spleens were weighed in pre-weighed containers. Radioactivity in each organ samples was counted using a gamma counter (Delshid, Iran). The percentage of injected dose per gram of tissue (%ID/g) was calculated by dividing the radioactivity count per minute in each tissue by total count injected and the mass of the organ.

Scintigraphic imaging with ^{99m}Tc -phytate

Planar imaging studies were performed on anesthetized rats to obtain a visual confirmation of the liver uptakes and shapes. Images were acquired at 2 hours postinjection of ^{99m}Tc -phytate using an E-CAM dual head (Siemens Medical Solutions, Hoffman Estates, IL) equipped with a low energy high-resolution collimator.

Histopathological assessment

After fixation the liver tissue in 10% formalin, samples were processed according to standard histological techniques and embedded in paraffin blocks. Paraffin blocks were sectioned using a rotary microtome into $3 \mu\text{m}$ thick sections placed on glass slides and fixed for histological staining. Staining was performed using hematoxylin. Liver tissue was evaluated on hepatic paranchyme showing morphological changes. The tissue sections were assessed by blinded pathologist. The average of ten microscopic fields was recorded by him for each specimen. Histopathological criteria and the morphological parameters were defined semi-quantitatively according to modified scoring system [17, 18] which performed as follow:

(-), (+-), (+), (++) and (+++) is indicating: no or absent, few or < 1 single altered foci into microscopic HPF (High Power Field $\times 400$), mild or between $1 \leq 2$ altered foci in 10 microscopic HPF, moderate or between 2–3 altered foci in to microscopic HPF, and severe or > 3 altered foci in to microscopic HPF, respectively.

Statistical analysis

The data values are presented as means \pm standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), as well as post hoc Dunnett's Multiple Comparison Test. P value < 0.05 was considered as significant and highly significant, respectively (Prism Software, USA).

Results

Quantitation of liver and spleen uptakes of ^{99m}Tc -phytate

The percentage of the injected dose per gram of ^{99m}Tc -phytate in liver of control, solvent, Vit E, MTX, and Vit E + MTX groups were $8.99\% \pm 1.37$, $8.53\% \pm 2.91$, $8.65\% \pm 3.84$, $3.22\% \pm 1.09$ and $8.38\% \pm 2.68$ (Figure 1A). MTX injection reduced ^{99m}Tc -phytate uptake in liver as compared with other groups as control, solvent and Vit E. It was observed significant difference between MTX group and other groups in ID/g% values of livers ($P < 0.05$). Vit E pre-treatment significantly increased the ^{99m}Tc -phytate uptake in the livers of rats injected by MTX. The mean of ID/g% value in liver was significantly higher in Vit E + MTX group as compared with MTX treatment alone group ($P < 0.05$). The ID/g% of ^{99m}Tc -phytate in spleen of control, solvent, Vit E, MTX, and Vit E + MTX groups were $4.00\% \pm 1.07$, $5.19\% \pm 1.34$, $4.40\% \pm 2.11$, $1.86\% \pm 0.49$ and $2.94\% \pm 0.98$ (Figure 1B). It was observed a significant difference between MTX group with solvent and Vit E groups in ID/g% values ($P < 0.05$). The ^{99m}Tc -phytate spleen uptake was about 2.7

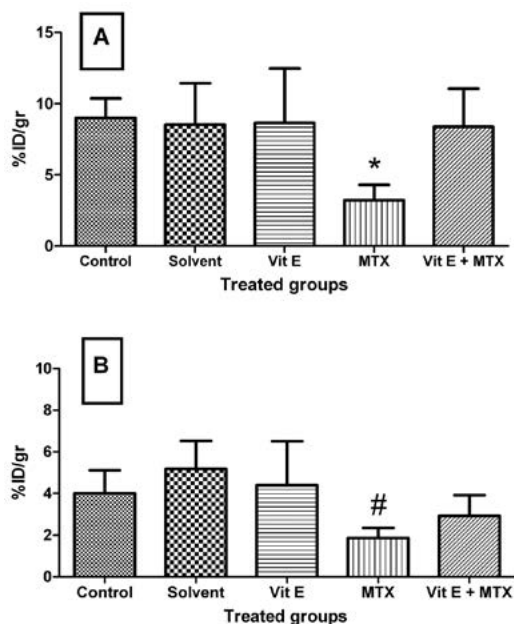


Figure 1. The percentage of the injected ^{99m}Tc -phytate dose per gram of liver (A) and spleen (B) tissues (% ID/g) in control, solvent (olive oil), vitamin E (100 mg/kg), methotrexate (MTX), vitamin E + methotrexate (Vit E + MTX) groups are shown (n = 4). *Comparison of all groups with MTX groups $P < 0.05$, non-significant comparison of control, solvent, Vit E and Vit E + MTX between groups. # Comparison of solvent and Vit E with MTX groups $P < 0.05$, non-significant comparison of control and Vit E + MTX with MTX

fold less in MTX treatment group as compared to solvent group ($P < 0.05$). However Vit E pre-treatment increased the ^{99m}Tc -phytate uptake in the spleens of rats injected by MTX, the mean of ID/g% value in spleen was statistically non significantly higher in Vit E + MTX group as compared with MTX treatment alone group.

Gamma camera imaging

The whole body images of treated rats are presented in Figure 2. The hepatic uptakes of ^{99m}Tc -phytate were homogeneous in control, solvent and Vit E treated rats. The shape of liver was abnormal in MTX-injected rat. The uptake of ^{99m}Tc -phytate was concentrated as a circle or round shape. It is interesting that the Vit E pre-treatment improved the shape of liver in planar image.

Histopathological assessment

The impaired hepatic caused by MTX was further confirmed by histological examination of liver (Fig. 3). As shown in Figure 3, liver sections from control, solvent and Vit E in rats showed normality whereas liver in animals treated with MTX revealed a marked liver necrosis and degeneration, extensive epithelial vacuolization, swelling and dilation (Fig. 3). The hepatic damage was less severe in the rat treated with Vit E + MTX when compared with MTX-treated alone rat (Fig. 3). The histopathological alteration semi-quantitative results of some injury related parameters are shown in Table 1. Histological evaluations of the liver sections of animals from control, solvent and Vit E groups showed normal structure, architectural integrity and regenerative nodules. The animals from MTX group

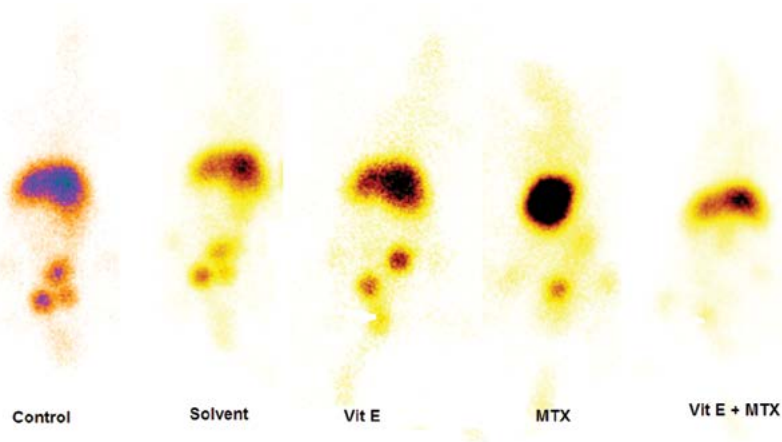


Figure 2. Whole body images of rats were injected ^{99m}Tc -phytate. Rats are as control, solvent, Vit E, methotrexate (MTX), Vit E + MTX. The uptakes of ^{99m}Tc -phytate in control, solvent and Vit E and also Vit E + MTX are homogenous while scintigraphic imaging shows an abnormal shape of liver in MTX-treated rat

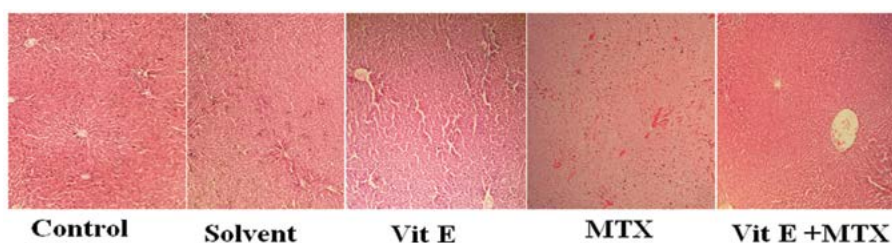


Figure 3. Histological examination of rats treated with methotrexate (MTX) and/or vitamin E (Vit E). The liver of control, solvent and Vit E treated rats show normal. Liver of rat administered with MTX at a single dose of 20 mg/Kg exhibited a distinct histological difference when compared with control, these liver shows large numbers of cells with degeneration. Improved cellular degeneration was seen in rat treated with Vit E at dose 100 mg/Kg and MTX

Table 1. The effect of vitamin E (Vit E) against hepatotoxicity induced by methotrexate (MTX)

Group	Criteria				
	Control	Solvent	Vit E	MTX	Vit E + MTX
Change in architecture	-	-	-	+	-
Congestion	-	+,-	+,-	+++	++
Dilation	-	+,-	+,-	+++	+
PMNS	-	+,-	+	+++	+
Mononuclears	-	-	+,-	++	+
Eosinophills	-	-	-	++	-
Necrosis	-	-	+,-	+	+,-
Apoptosis	-	-	-	++	+
Cytoplasmic	-	-	+,-	++	+
Hydropic degeneration	-	-	+,-	++	+
Activated kupffer cell	-	-	+,-	++	+
Fatty change macrovesicular steatosis	-	++	+,-	-	-
Uncertain cellular limit	-	+,-	+,-	++	+,-
Tronbosis	-	-	-	++	+
Endothelial cells injuries	-	-	-	++	+,-

(-), (+,-), (+), (++) and (+++) is indicating: no or absent, few or < 1 single altered foci in to microscopic HPF (High Power Field *400), mild or between 1 ≤ 2 altered foci in 10 microscopic HPF, moderate or between 2-3 altered foci in to 10 microscopic HPF, and severe or > 3 altered foci in to 10 microscopic HPF, respectively

exhibited histopathological alterations, with variations in the number of pathological criteria such as of congestion, dilatation, apoptotic and cytoplasmic and endothelial cell injury (Table 1). Vit E + MTX group showed preserved liver architecture with markedly reduction in number of pathological alterations.

Discussion

It is important to recognize and understand the side effect of chemotherapy on the liver, since this is a major organ which is responsible for drug, endogenous and exogenous substances clearance. Since some chemotherapeutic agents such as MTX may cause liver damage, the evaluation of possible hepatotoxicity is important in healthcare. In this study, hepatoprotective effect of Vit E after a single injection of MTX was evaluated in rats. MTX induced significantly live damage that was prevented by Vit E treatment. The hepatotoxicity was evaluated using ^{99m}Tc-phytate that is used as a radiopharmaceutical agent for assessment of hepatic and spleen diseases in patients. This protective effect was confirmed with histopathology. This is the first time study to investigate using MTX in treatment with Vit. E in animals that was verified with a radionuclide method using ^{99m}Tc-phytate as a radioactive colloid radiopharmaceutical. Hepatotoxicity is a common side effect caused by MTX in patients [19, 20]. Elevated ROS and induced pro-inflammatory process are main suggested mechanisms involved in hepatotoxicity induced by MTX [21]. Several compounds have been reported to have protective effect against hepatotoxicity induced by MTX. These compounds acted mainly through antioxidant and anti-inflammatory effects [22–24]. Our findings showed that MTX significantly reduced the liver uptake of ^{99m}Tc-phytate about 2.5-fold less level than control rats. The present results show that MTX causes Kupffer cell malfunction in the liver and these damaged cells unable to uptake normally this radiopharmaceutical agent. Also the reduction in uptake of

^{99m}Tc-phytate was observed in spleen of MTX-treated rats. The reticuloendothelial system (RES) plays an important role in the biological defense system. Kupffer cells are the main constituent of the RES in the liver and spleen, and when their function is impaired post drug treatment the complications may occur [25, 26]. ^{99m}Tc-phytate is used for imaging areas of functional reticuloendothelial cells in the liver, spleen and bone marrow. It is a useful radiopharmaceutical agent in the diagnosis, localization and evaluation of liver and spleen pathology. In the blood, phytic acid binds with calcium ions to form colloidal particles which are captured by the RES, principally by Kupffer cells in the liver. In imaging purpose, ^{99m}Tc-phytate reacts *in vivo* with calcium ions in the blood and forms ^{99m}Tc-Sn-Ca-phytate that is rapidly cleared by the RES from the blood. Uptake of the radioactive colloid by RES is dependent upon both the relative blood flow rates and the functional capacity of the phagocytic cells [27]. In a study, the side effect of ionizing radiation on the liver in rat was evaluated using gamma camera (^{99m}Tc-phytate) imaging and MRI [28]. Exposure to ionizing radiation (4 Gy) markedly induced liver injury that was visualized by gamma scintigraphy with changing in the shape of the liver and the relative proportions of the volumes of its right and left portions that these findings were confirmed by MRI and pathology. Both techniques proved that the reticuloendothelial cells were damaged by ionizing radiation, which might result in malfunction of the Kupffer cells and or a decrease in their number [28]. Histological findings of MTX-induced hepatotoxicity are including macrovesicular steatosis, hepatocellular necrosis and inflammation, edema, fibrosis, morphological abnormalities and development of cirrhosis [29–31]. Chemotherapeutic agents caused drug induced acute hepatitis and hepatomegaly, decreased liver enhancement and widening of periportal space due to edema which are nonspecific imaging findings of acute hepatitis [32]. In our study an abnormal shape of liver in MTX-treated animal that attributed liver pathologic abnormalities was observed.

In this study, Vit E administration increased the liver uptake of ^{99m}Tc -phytate in rats injected with MTX, and the level of radioactivity uptake was close to normal range. It was interesting that the shape of liver was changed in MTX-injected rat and liver was an abnormal shape. While the shape of liver in Vit E + MTX treated rat was similar to control rat. It was a confirmation in liver damages assessment between quantitation of liver uptake of ^{99m}Tc -phytate and liver image. Pathological examination confirmed that MTX significantly caused hepatotoxicity in rats that this liver damage was prevented by Vit E treatment. Vit E exhibited hepatoprotective effect in rats that co-injected with MTX. Vit E has free radical scavenging, increasing endogenous antioxidant and anti-inflammatory effects [33, 34]. These mechanisms are suggested to contribute in reduction of deleterious effects of MTX in liver and spleen by Vit E.

Conclusion

In this study, it was demonstrated the Vit E was effective in the prevention of hepatotoxicity induced by MTX in animal. Our results showed that quantization of injected dose per gram of ^{99m}Tc -phytate in liver and spleen as well as liver image to be acceptable techniques for assessment of liver and spleen damages and/or their tissues protective effects in animal model.

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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