

Full Length Research Paper

Effect of methotrexate combined with ginger, silymarin or propolis on the mRNA expression levels of cytochrome P450 oxidoreductase (POR), caspase 3 (CASP-3) and interleukin 6 (IL-6)

Amal A. M. Hassan^{1*}, Manal F. Ismail² and Hanaa M. Mohamed³

¹National Research Centre, Genetic Engineering and Biotechnology Division, Cell Biology Department, Cairo, Egypt.

²Cairo University, Faculty of Pharmacy, Biochemistry Department, Cairo, Egypt.

³Beni Suef University, Faculty of Science, Zoology Department, Beni Suef, Egypt.

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The present study was performed to evaluate the effect of three natural antioxidants on the adverse effect of methotrexate (MTX) in normal liver cells. TaqMan RT-PCR technology was used to estimate the mRNA expression levels for three genes after rats injection with a single dose of 20 mg/kg b.w MTX or the same MTX dose combined with ginger, silymarin or propolis oral administration. The doses of ginger, silymarin or propolis were similar (200 mg/kg b.w) and were daily administrated to rats for 21 days before MTX injection and four days after MTX injection. The three genes were: cytochrome P450 oxidoreductase (POR) that encodes POR enzyme, caspase 3 (CASP-3) that encodes CASP-3 enzyme and interleukin 6 (IL-6) that encodes IL-6 pro-inflammatory cytokine. Results indicate that the used MTX single dose of 20 mg/kg b.w did not significantly affect POR mRNA expression level in rat liver. Moreover the administration of ginger, silymarin or propolis with MTX did not significantly increase or decrease POR mRNA expression level. Results also reveal insignificant increase in CASP-3 mRNA expression level after MTX injection and also after administration of ginger or propolis with MTX. The administration of silymarin with MTX significantly increased the CASP-3 mRNA expression level. IL-6 mRNA expression level was insignificantly upregulated after injection with MTX and also after administration of silymarin with MTX whereas a significantly upregulation in the IL-6 mRNA expression level was reported after administration of ginger or propolis with MTX.

Key words: Methotrexate, ginger, silymarin, propolis, mRNA expression.

INTRODUCTION

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent that is widely preferred in the treatment of malignancies and some autoimmune diseases (Cetin et

al., 2011). MTX is a structural analogue of folic acid. It works as a dihydrofolate reductase (DHFR) inhibitor that blocks DNA synthesis through the depletion of the

*Corresponding author. E-mail: amal.a.hassan@hotmail.com.

intracellular-reduced folate pools required for the purines and thymidine biosynthesis, and leads to cell cycle arrest and apoptosis in different cell types. MTX promotes adenosine release and inhibits pro-inflammatory cytokines production. It also suppresses the lymphocytes proliferation and reduces serum immunoglobulin through the folic acid metabolism inhibition (Kobayashi et al., 2002).

While the cytotoxic effect of MTX is not selective for cancer cells, it also affects the normal tissues specially that have a high rate of proliferation, including the hematopoietic cells in the bone marrow and actively dividing cells of the intestinal mucosa (Jahovic et al., 2004). It was also reported that MTX induced damage to the normal liver cells that was well characterized by fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation (Hemeida and Mohafez, 2008).

Numerous studies showed that different antioxidants such as flavonoids reduce the adverse effects of chemotherapeutic agents on normal cells (Choi et al., 1999; Borek, 2004). In addition to that, other studies have suggested that antioxidants can directly induce apoptosis in tumor cells (Nomura et al., 2001).

Propolis (bee glue) is a natural antioxidant produced by the honeybee (*Apis mellifera ligustica*). It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids which have been reported to induce the immune system. Many of the physiological actions of these flavonoids have been attributed to their antioxidant properties (Bhadauria et al., 2008).

Silymarin is an active extract from milk thistle. It is a powerful antioxidant said to protect liver cells (and other cells in the body and brain) from toxins. Silymarin apparently promotes liver cell protein synthesis and decreases the oxidation of glutathione. Silymarin may potentially be beneficial in a number of diseases involving liver disease, if in the early stages. Early research indicates that silymarin may also have anti-cancer and pro-apoptotic properties (Yassin et al., 2010).

Ginger or ginger root is the rhizome of the plant *Zingiber officinale*. Ginger is known to possess antioxidant properties. 6-Gingerol, a natural product of ginger, has been known to possess anti-tumorigenic and pro-apoptotic activities (Oyagbemi et al., 2010).

Previous studies were performed to investigate the beneficial effect of propolis and ginger extracts against MTX-induced hepatotoxicity using histopathological and biochemical approaches (Cetin et al., 2011; Badr et al., 2011; Mansour et al., 2012). However, to the best of our knowledge, no studies were performed to estimate the mRNA expression levels of genes encoding for the liver enzymes or other genes that are involved in MTX mode of action after treatment with MTX combined with propolis, ginger or silymarin. In the present study three of these genes were chosen for investigation after MTX injection combined with propolis, silymarin and ginger.

The first gene is cytochrome P450 oxidoreductase (POR) that encodes the P450 oxidoreductase enzyme which is required for the normal functioning of more than 50 enzymes in the cytochrome P450 family. Cytochrome P450 enzymes are involved in the formation (synthesis) and breakdown (metabolism) of various molecules and chemicals within cells (Rhee and Galivan, 1986). The two other genes caspase 3 (CASP-3) and interleukin 6 (IL-6) are involved in the processes of apoptosis and inflammation. CASP-3 gene encodes for the enzyme caspase 3 which is required for DNA fragmentation and the morphological changes associated with apoptosis (Yang et al., 2004). Where, IL-6 gene encodes interleukin 6 cytokine that functions in inflammation and the maturation of B cells. The IL-6 pro-inflammatory cytokine was reported to have an important role in MTX uptake inside the cell (Yoshida et al., 2005; Hashizume et al., 2012).

The aim of this work was to study the effect of MTX alone and MTX in combination with propolis, silymarin or ginger on the mRNA expression levels of the three genes: POR, CASP-3, and IL-6 in rat liver cells using RT-PCR technology.

MATERIALS AND METHODS

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt, following the 18th World Medical Association (WMA) General Assembly, Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Methotrexate (MTX) was purchased from Ebewe Pharma, Austria. Silymarin and Gingerol were kind gift from Kahira Pharm. and Chem. IND. Co., Egypt and Mepaco Co., Egypt, respectively. Propolis was purchased from TWIN Laboratories Inc., N.Y. (USA).

Experiment strategy and treatments

Sixty adult male albino rats (*Rattus norvegicus*) were used in this study. The animals were allocated into five groups. Group 1 (G1) and group 2 (G2) were administered with saline; group 3 (G3) received a daily dose of 200 mg/kg b.w ginger extract; group 4 (G4) received a daily dose of 200 mg/kg b.w silymarin and group 5 (G5) received a daily dose of 200 mg/kg b.w propolis. The daily dose of 200 mg/kg b.w of ginger, silymarin and propolis was chosen in the present work according to the previous studies which reported that the daily administration of the above antioxidants is not toxic at this dose (El-Abhara et al., 2008; Eminzade et al., 2008; Bhadauria et al., 2007). All treatments were orally administered for 21 days. On day 21, animals in G2, G3, G4 and G5 were injected intraperitoneally with a single dose of 20 mg/kg b.w MTX. This dose of MTX was reported to cause injury in liver cells (Gulgun et al., 2010). The oral administration of saline, ginger, silymarin and propolis were continued for 4 consecutive days in all groups. All animals were sacrificed on day 25.

Extraction of RNA and complementary DNA (cDNA) synthesis

Liver tissue samples from all animal groups were obtained for RNA

Table 1. The mean relative quantity values (mean RQ values) for each target gene in the five rat groups.

Groups	Parameter		
	POR mRNA mean RQ values \pm standard deviation	CASP-3 mRNA mean RQ values \pm standard deviation	IL-6 mRNA mean RQ values \pm standard deviation
Control	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00
MTX	0.95 \pm 0.01	2.48 \pm 0.69	2.78 \pm 0.03
Ginger + MTX	0.77 \pm 0.04	2.15 \pm 0.67	71.28 \pm 9.12 ^{ab}
Silymarin + MTX	0.97 \pm 0.18	3.06 \pm 1.77 ^a	27.66 \pm 8.36
Propolis + MTX	1.07 \pm 0.30	1.89 \pm 0.83	113.75 \pm 10.03 ^{ab}

Significance level: at $P < 0.05$; a: significant increase compared to control group; b: significant increase compared to MTX group.

extraction. Total RNA was extracted from each sample using PeqGold TriFast™ (PEQLAB Biotechnologie GmbH) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on the extracted total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences) according to the manufacturer's instructions. The resulting cDNA from each sample was subjected to real time PCR amplification (RT-PCR) using TaqMan technology in order to quantify mRNA expression levels of three target genes; POR, CASP-3 and IL-6 in the different groups of the experiment.

Primers and fluorogenic probes design

Sequences from the cDNA of the three target genes in rat; POR, CASP-3, IL-6 and from the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) which was used as an internal control were amplified using TaqMan RT-PCR technology. The design and synthesis of the primers and probes of the three tested genes cDNA were performed using TaqMan® Gene Expression Assays-Applied Biosystem. TaqMan probe/primer sequences were designed to be specific for rat cDNA of POR (Assay ID: RN00580820), CASP-3 (Assay ID: RN00563902), IL-6 (Assay ID: RN01410330). The probe/primer set designed for GAPDH cDNA, although amplifying GAPDH cDNA in the rat was also capable of amplifying mouse and human GAPDH because of its conserved nature across species.

Rt-PCR amplification conditions and profile

Real time-PCR reaction of each target gene cDNA was performed with the housekeeping gene cDNA in the same tube (multiplex PCR) where the probe of the housekeeping gene cDNA was labeled by VIC fluorescent dye and the three target gene cDNA probes were labeled by FAM fluorescent dye. RT-PCR reactions were conducted in 25 μ L final volume. Reaction mixture consisted of 12.5 μ L of 2x master mix (TaqMan Universal PCR Master Mix, ABI), 1.25 μ L of the target gene primer/probe mix (20x), 1.25 μ L of the housekeeping gene primer/probe mix (20x), 5 μ L of cDNA (typically 25 g of total RNA) and 5 μ L of DEBC-treated water. The quantitative real-time PCR reactions were carried out in an RT-PCR Cycler-Rotor-Gene Q 2 Plex- with 2 channels (QIAGEN). RT-PCR amplification profile started by a hold step for 15 min at 95°C followed by 45 repeats of 15 sec at 94°C and 60 sec at 50°C.

Calculation of mRNA expression levels

Data were analyzed by the software version Rotor-Gene 2.0.2.4 as following: The delta ct (threshold cycle) was calculated for the target

gene in each sample of the different groups by subtracting the ct value of the housekeeping gene from the ct value of the target gene, where the ct value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold. The delta delta ct ($\Delta\Delta Ct$) of the target gene in each sample in all groups was calculated by subtracting the delta ct value of the target gene in the calibrator (The calibrator: is one of untreated control animals) from the delta ct value of the same gene in each sample. Finally, the mRNA expression level of the target gene in a given sample was calculated as a relative quantity (RQ) compared to its expression in the calibrator; where, $RQ = 2^{-\Delta\Delta Ct}$. RQ value < 1 means that the mRNA expression is down regulated whereas RQ value > 1 means an up regulation in the expression.

Statistical analysis

For the determination of significant inter-groups differences in the mRNA expression of the three genes under investigation, statistical analysis for the obtained RQ data was performed using one-way analysis of variance (ANOVA) included in SAS version 11 for windows. The ct values of the housekeeping gene in the different groups of each multiplex PCR were also analyzed by the ANOVA test to investigate the effect of the different treatments of the present study on its mRNA expression level. Significance values were determined at $P < 0.05$.

RESULTS

In the present study, quantitative gene expression data of POR, CASP-3 and IL-6 genes were normalized to the expression level of the housekeeping gene GAPDH. Aanalysis of the ct values of the GAPDH gene in the different tested groups of each multiplex PCR indicated that there were no significant differences in the expression levels of GAPDH mRNA due to the different treatments compared to the control and the methotrexate treated groups.

The mRNA expression levels of POR, CASP-3 and IL-6 genes are presented as the mean values of RQ \pm standard deviation for each target gene in five animal groups: control (G1), MTX (G2), ginger + MTX (G3), silymarin + MTX (G4) and propolis + MTX (G5) (Table 1). The mean RQ values of each gene in the different groups were statistically compared with the same gene mean RQ values of the control and MTX groups (at $P < 0.05$) and the

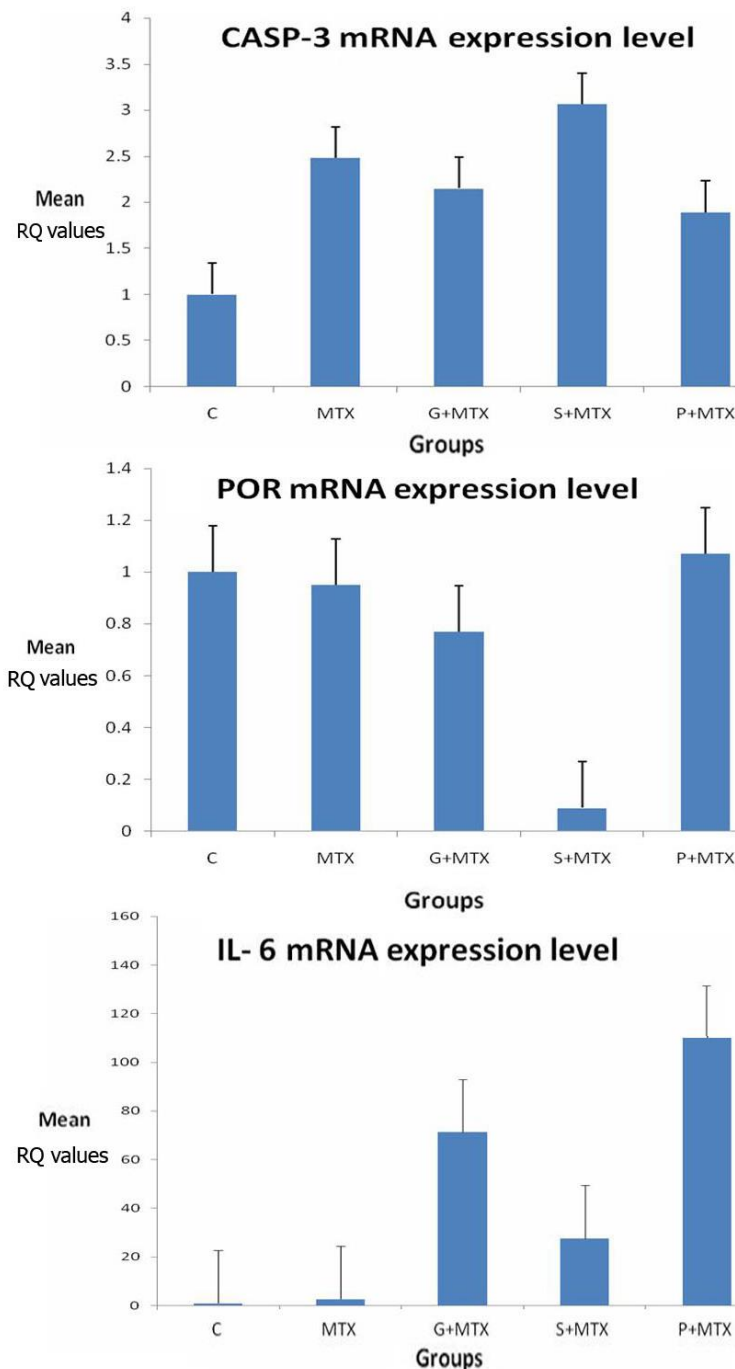


Figure 1. mRNA expression levels of each of the tested genes in the five rat groups. C= Control; MTX= Methotrexate; G = Ginger; S = Silymarin; P = Propolis.

significant differences in mRNA expression levels are illustrated (Table 1). Results of mRNA expression levels are also represented in a histogram (Figure 1) that was done using the mean RQ values of each gene in each of the five animal groups.

Results reveal that there was no statistical difference in the mRNA expression level of POR gene in the different

groups compared to control and to the MTX groups.

The mRNA expression level of CAPS-3 gene was elevated in the MTX group (mean RQ=2.48±0.69) compared to its expression level in the control group (mean RQ=1.00±0.00). It was also elevated in ginger + MTX, silymarin + MTX and propolis + MTX but the increase in the expression level was significant (at the

level 0.05) only in the silymarin + MTX group (mean RQ=3.06±1.77) compared to control group (mean RQ=1.00±0.00).

Interleukin 6 mRNA expression level was also elevated in all the treated groups compared to the control group. The increase in the mean RQ values was statistically significant (at the level 0.05) only in the ginger + MTX (mean RQ=71.28±9.12) and propolis + MTX (mean RQ=113.75±10.03) groups compared to both control (mean RQ=1.00±0.00) and MTX (mean RQ=2.78±0.03) groups.

DISCUSSION

In the present study TaqMan RT-PCR technology was used for the estimation of mRNA expression levels of POR, CASP-3 and IL-6 genes in rat liver cells after injection of methotrexate (MTX) alone or MTX with ginger, silymarin or propolis oral administration. TaqMan technology has a number of advantages over conventional methods for assessing the potential of a drug to cause a target gene induction. The key advantages of this method that makes it both precise and reproducible (Bustin, 2000; Ginzinger, 2002) are features, such as it is a completely homogenous assay with a specific target gene being detected. The TaqMan method is also exquisitely sensitive, being able to amplify small amounts of mRNA in contrast to commonly used methodologies, which typically require relatively large amounts of total RNA and are unsuitable for high throughput and usually only semi-quantitative in nature (Baldwin et al., 2006).

To evaluate the effect of MTX alone and MTX in combination with ginger, silymarin or propolis on the liver enzymatic system, the POR gene mRNA expression level was chosen for investigation. It is known that POR enzyme is required for the normal functioning of more than 50 enzymes in the cytochrome P450 family. Cytochrome P450 enzymes are involved in the synthesis and metabolism of various molecules and chemicals within cells (Ding et al., 2001; Rhee and Galivan, 1986). Results of the present study revealed that no significant difference were detected in the POR mRNA expression level after injection of MTX neither alone nor in combination with ginger, silymarin or propolis. These results indicate that the used MTX single dose of 20 mg/kg did not significantly affect POR mRNA expression level in rat liver. Moreover the administration of ginger, silymarin or propolis with MTX did not significantly increase or decrease POR mRNA expression level.

Results of the present study also indicate that the mRNA expression level of CAPS-3 gene was elevated in the group treated with MTX (mean RQ=2.48±0.69) compared to its expression in the control group (mean RQ=1.00±0.00) but the increase in the expression level was not significant. It is well known that apoptosis is the main mechanism of action of most cancer chemothera-

peutic agents including methotrexate (Kobayashi et al., 2002). The increase of the mRNA expression level of CAPS-3 gene due to MTX treatment was expected because caspase3 enzyme is required for DNA fragmentation and the morphological changes associated with apoptosis (Yang et al., 2004). Previous studies indicated that the response to methotrexate in tumor cells depends on caspase 3 function (Hattangadi et al., 2004). The administration of ginger, silymarin or propolis with MTX also elevated mRNA expression level of CASP-3 where the elevation was significant only in the silymarin + MTX group (mean RQ=3.06±1.77) compared to control group (mean RQ=1±0.00). This result indicate that administration of silymarin with MTX significantly increase the CASP-3 mRNA expression level. This may consequently leads to the increase of the CASP-3 enzyme activity and apoptosis tendency in liver cells. It was reported before that silymarin has pro-apoptotic activities and it has been shown to inhibit skin carcinogenesis in mice by the induction of apoptosis through caspase3 enzyme activation (Katiyar et al., 2005).

Interleukin 6 is an interleukin that acts as a pro-inflammatory cytokine. It is encoded by the IL-6 gene. Interleukin 6 secretion during infection leads to inflammation and stimulation of the immune response (Nishimoto, 2006). Although MTX is known to have anti-inflammatory actions and it was reported to inhibit pro-inflammatory cytokines production in a dose-dependent manner (Yoshida et al., 2005), results of the present study revealed that the single dose of MTX (20 mg/kg) insignificantly upregulated the IL-6 mRNA expression level (mean RQ=2.78±0.03) compared to its expression in the control group (mean RQ=1.00±0.00). It was reported that IL-6 cytokine has a role in the uptake of MTX inside the cell (Hashizume et al., 2012) as it will be explained in the following. Methotrexate enters cells via the reduced folate carrier SLC19A1, suggesting that SLC19A1 is associated with the efficacy of MTX. IL-6 cytokine is responsible for the regulation of SLC19A1 expression and it is able to reduce the efficacy of MTX by decreasing the expression of SLC19A1 (Hashizume et al., 2012).

The slight upregulation of the IL-6 mRNA expression level which reported in the present study against the single dose of 20 mg/kg MTX treatment may support the finding of Hemeida and Mohafez (2008) that the same single dose of MTX had caused mild inflammation in normal liver cells. The administration of ginger + MTX, silymarin + MTX or propolis + MTX over increased the mRNA expression of IL-6 compared to its expression in the control and MTX groups.

The increases were considered statistically significant only in the ginger + MTX (mean RQ=71.28±9.12) and propolis + MTX (mean RQ=113.75±10.03) groups compared to control (mean RQ=1.00±0.00) and methotrexate (mean RQ=2.78±0.03) groups. It was reported in a previous

study that the oral administration of squeezed ginger augmented the production of IL-6 in mouse leukemic monocytes (Ueda et al., 2010). In another study, it was reported that IL-6 expression increased by a dose-dependent manner due to silymarin administration (Johnson et al., 2003). Also, propolis oral dose of 200 mg/kg, for three consecutive days, was reported to upregulate IL-6 cytokine production in mice spleen cells (Orsatti et al., 2010). The increase of IL-6 mRNA expression level that reported in the present study after administration of silymarin, ginger or propolis with MTX perhaps will lead to the increase in the production of IL-6 pro-inflammatory cytokine, reduction of SLC19A1 expression and consequently reduction of the efficacy of MTX in rat liver cells.

Conclusion

Results of the present study indicated that: the upregulation of mRNA expression level of casp-3 gene after the administration of silymarin with MTX may add to the adverse effect of methotrexate in the normal rat liver cells.

Also, the upregulation of mRNA expression level of IL-6 gene after the administration of silymarin, ginger or propolis with MTX perhaps may decrease the efficacy of MTX in the normal rat liver cells.

The administration of silymarin, ginger or propolis with MTX may protect normal cells from MTX damage but it may also have an adverse effect by decreasing the efficacy of MTX in the diseased cells.

Conflict of interests

The author(s) did not declare any conflict of interest.

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