THE PROTECTIVE EFFECT OF DAFLON AGAINST THE RENAL-HEPATIC TOXICITY INDUCED BY METHOTREXATE IN RABBITS

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ABSTRACT
Methotrexate (MTX) is considered a folic acid antagonist, used frequently in the treatment of various types of diseases and has had a major role in the treatment of inflammation and suppressive material for immunity in Non-tumor diseases like acute arthritis and psoriasis. This study was designed to investigate the effect of methotrexate and pre-treatment with daflon in attenuation of its development for renal-hepatic toxicity. Twenty-four healthy, local, domestic rabbits were randomized divided into 4 groups: group 1 (control), group 2 (20mg/kg MTX group), group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX). At the end of experiment, blood samples were collected from the heart then the liver and kidney tissues were excised. Plasma level of S.ALT, S.AST, ALP, total protein, bilirubin, urea, creatinine and oxidation parameters (GSH, and MDA) were measured. The Results show that the compared with the control group, levels of S.ALT, S.AST, ALP, urea, creatinine and MDA were increased (p<0.05), while total protein and GSH were decreased (p<0.05) in MTX group with deterioration of liver and kidney tissues. While group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX) showed decreased levels of S.ALT, S.AST, ALP, urea, creatinine and MDA while total protein and GSH were increased (p<0.05) in as compared with MTX group with improvement of liver and kidney structures. Conclusions: The results of the present study reveal that daflon has been shown to attenuate MTX induced hepatorenal toxicity.

Key words: Methotrexate, hepatotoxicity, renal toxicity, daflon, oxidative stress.

INTRODUCTION
Methotrexate (MTX), a folic acid antagonist, is a cytotoxic drug which is commonly used in the cancer therapy (El-Sheikh et al., 2015) and as anti-inflammatory and immunosuppressive agent in other diseases such as Reiter's syndrome, arthritis, biliary cirrhosis and psoriasis (Weinblatt, 1985). It has many therapeutic uses and it is well tolerated at low doses, however, when it has been used in high doses or for long duration, many serious side effects are resulted, including hepatorenal toxicity (Cronstein et al., 1991) which may be resulted from increased oxidative stress both through increased activity of reactive oxygen species and impairment of anti-oxidative defense mechanism via depletion of intra hepatic glutathione store (Jahovic et al., 2003). Nowadays, it is usual to use supplements rich with polyphenols, particularly flavonoids which have protective effects against reactive oxygen species (ROS) and also enhanced antioxidant defense system in the body (Pandey and Rizvi, 2009). Flavonoids are natural polyphenols appear in many plants and have antioxidant, anticancer, anti-atherogenic activity and the ability to scavenge any radicals may cause damage in the body (Erdogan et al., 2015).

Daflon (Micronized purified flavonoid fraction) is a drug similar to synthetic treatment included 10% flavonoids expressed as hesperidin and 90% a flavone derivative (Katsenis, 2005). It is a phlebotonic drug commonly used in chronic venous or lymphatic insufficiency, and this effect of Daflon can be attributed to decrease the inflammatory and antioxidant effects of the flavonoid that contain (Delbarre et al., 1995). The antioxidant effect is attributed to its ability to decrease the proportion of hydroxyl radicals (Rapavi et al., 2007), with increasing in concentration of free SH-group, and the scavenging activity that give it's important role (Mcginness et al., 1977). The aim of this study was to investigate the protective effect of daflon against to hepatorenal oxidative injury induced by MTX, supposing that free radical scavenging activity may have a protective role against drug which lead to the toxicity in liver and kidney cells (Amimoto et al., 1995).

MATERIALS AND METHODS
1. Experimental Animals: A 24 local rabbits used in the experiment, ages 2-3 months and their weights (1300-1500 g) were involved in this study. Before the study, the animals were allowed for acclimatization for 3 days in the conditions of the experiment and were kept on controlled temperature and environment with a 12 h. light/ dark cycle. All rabbits have to eat and drink.
2. Preparation of drugs: Methotrexate (EBE-WE pharma, AUSTRIA) was used in 20mg/kg per day (Asvadi et al., 2011, Swayeh et al., 2014). Daflon® (Servier, Paris, France) consisting of 90% diosmin and 10% hesperidin, was putting in isotonic solution (0.9% NaCl) immediately before they began. Daflon® was used in a dose of (100 and 200) mg/kg/ per day orally (Delbarre et al., 1995).

Keywords: Methotrexate, hepatotoxicity, renal toxicity, daflon, oxidative stress.
3. Experimental design: Rabbits were divided into 4 groups randomly, each group including 6 animals and treated orally for 7 days: Group 1 (control): Rabbits were treated with normal saline only. Group 2 (group MTX): Animals were injected methotrexate in intra-peritoneal (ip) with (20 mg/kg) in the fourth day of the experiment period continued to four consecutive days. Group 3 (Daflon 100+MTX): Animals were treated 100 mg/kg of daflon by mouth once daily for 7 days, while MTX was injected in (20mg/kg) in the fourth day of the experiment continued for four successive days. Group 4 (Daflon 200 + MTX): Rabbits were given 200 mg/kg of daflon orally once per day(for 7 days) and MTX was injected 20 mg/kg daily in the fourth day from the experimental period (7 days) and continued to four days. At the end 8th day of the experiment, blood collection were done to all rabbits by inhalation of chloroform for anesthesia. The blood collected by direct puncture in the heart, than the blood centrifuged at 3000 rpm for 10 min. to get plasma which was stored at -20°C for determination of S.ALT, S.AST, ALP, total protein, TSB, urea, creatinine and oxidation parameters (GSH, and MDA). After scarification, prepared the sections by (Luna, 1978) then were examined by microscope.

4. Statistical analysis: All data were compared with analysis of variance (ANOVA). T-test was used to identify the difference between two groups, Use P < 0.05 as Probability level were regarded as statistically significant (Dawood and Ilias, 1990).

RESULTS AND DISCUSSION

This study performed a histological and biochemical studies into whether daflon has a preventive and improved characteristics on the pathological effects induced on the tissue by methotrexate in rabbits. Methotrexate (MTX) is one of the folic acid synthesis inhibitor, commonly used in the treatment of many effects of changes (Tawfeeq et al., 2014). It is used in high doses for malignancies like leukemia, lung and breast carcinomas, while low doses used as medicines for inflammation conditions and in the therapy of many autoimmune disorders including psoriasis, juvenile idiopathic arthritis and rheumatoid arthritis (Funk et al., 2013). Toxicity induced by MTX appears to be a result of the interaction of many factors that include the duration of treatment, dosing schedule, type of disease, patient risk factors and the presence of genetic disease or molecular apoptotic factors (Cetinkaya et al., 2006). In present study, histopathological changes were examined to support the results of the parameters of liver and renal functions.

1. Histology and physiology of liver: The examination of liver tissue was demonstrate that liver structure in the normal state of control group with lobular structure, Central vein, Sinusoids and Hepatic cords are consisted of hepatocytes arranged radially (Figure 9A). While the treatment with use MTX led to congestion and dilatation of central vein, necrosis of hepatocytes and accumulation of inflammatory cells, in addition to extension in sinusoids (Figure 9B). As far the combined treatment of MTX and daflon in group 3 showed normal hepatic structure with simple changes in the tissue as dilatation in hepatic sinusoids and cell death (Figure 9C), but the improvement was more significant in group 4 (Daflon200+MTX), where observed the tissue closer to the normal tissue in control group.
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The functions enzymes tests have supported what appeared in this study that the treatment with MTX in rabbits induce hepatotoxicity, which reflected in a significant increased\( (P <0.05) \) in the rates of ALT, AST and ALP as shown when compared with control group, while treatment with daflon and MTX in group 3 showed a significant decrease in the levels of ALT, AST and ALP when compared with MTX group, but the reduction was higher significant in group 4 (Daflon 200 + MTX) when compared with group 3 (Daflon 100 + MTX)(Fig.1,2,3). As for total protein (T. protein) was significantly decreased of the second group (MTX) through the compared with control group. In contrast, this test was significantly increased in groups that treated with daflon and MTX when compared with MTX group, and the increase in total protein was significant in group 4 (Daflon 200 + MTX) when compared with group 3 (Daflon 100 + MTX)(Fig.4).

Liver histopathological observation supported the results of liver function tests which showed that severe degree of damage in hepatic tissue. The present study agree with (Vardi et al., 2010), who observed that various of the side effects of methotrexate be associated with a presence of hydrogen peroxides and free oxygen radicals and these peroxides and radicals lead to cell death by binding between the large cellular molecules, especially the lipid molecules of cell membrane causing egression of AST and ALT from Inside the cell to serum which is the clear sign of hepatic injury, these results also are in agreement with the reports of other studies (Uraz et al., 2008, Walker et al., 2000) and this increased in ALT and AST enzymes are functional inefficiency of cell membranes and evidence of intracellular leakage in the liver (Drotman and Lawhorn,1978), that may be due to the pathological effects of methotrexate by the ability to discouragement of dihydrofolate reductase (DHFR) and Obstruction of thymidylate synthetase (which are important for DNA synthesis) and lead to restriction in nucleic acids industry (Al-khateeb et al., 2014). This disability in the formation of amino acids, DNA and RNA might lead to injury in the cell membranes and organelles of parenchymal cells in the liver and permitting to infiltration of enzymes (Hersh et al., 1966). Adding to this, may be occur changes in the structure and shape of nucleus by the increase in oxidative stress that a resulted from destruction and distortion of DNA, which have a key role in the development of apoptosis (Morey et al., 2013), so
MTX caused with increase the oxidative stress in the renal and hepatic cells. While daflon prevents induced oxidative stress by methotrexate, because of their ability to effect against methotrexate injury in kidney and liver tissue by sweeping the free radicals formed (Amimoto et al., 1995). In the present study too, we observed that adding daflon to MTX was found to have hepato protective effect as demonstrated by a significant decrease in liver enzymes activities with improvement in liver histology. This result in agree with (Paysant et al., 2008, Kose et al., 2012, Erdogan and Ilgaz, 2015, Kalantar et al., 2017).

2. Histology and physiology of Kidney: Regarding the histological changes in kidney, The tissue presented with acute congestion and retrogression in glomerulus, the area of the Bowman capsule was expanding, tubular degradation and leakage of inflammatory cells in group 2 (MTX group), when compared with control group (Figure F), which showed normal kidney histology, Glomerulus, Bowman capsule, Distal convoluted tubules and proximal convoluted tubules (Figure E). Treatment with daflon and MTX showed improved in the tissue of kidney, with some mild expansion of convoluted tubules and abnormal shape for tissue in group 3 (Figure G). While the improvement was more significant in group 4 (Daflon 200+ MTX) (Figure H) when compared with group 3 (Daflon 100+MTX).

Serum urea and creatinine levels were assessed as markers of renal functions which supported its results the histopathological effect in kidney of this study. Where this study showed the treatment with MTX alone in rabbits developed renal injury, that reflected by a significant increase (P<0.05) in the levels of urea and creatinine when the comparison with control group, while treatment with daflon and MTX revealed a significant decrease in the proportions of urea and creatinine in group 3 (Daflon 100 + MTX) and group 4 (Daflon 200 + MTX) when compared with control group, but the reduction was more significant in group 4 (Daflon 200 + MTX) when compared with group 3 (Daflon 100 + MTX) (Fig. 5, 6).
Renal failure (acute stage) is one of the common pathological changes in the kidneys that results from the use of high doses of methotrexate continuously and which is found at 3-10% of treatment courses (Widemann et al., 2006). The most common patho-physiological cases of acute renal failure (ARF) is appearance of methotrexate in the urine and deposition of its metabolic products in the acidic environment with renal outcomes (Jahovic et al., 2003). In this study, our result is agree with (Kolli et al., 2008, Tousson et al., 2014) who reported that MTX increase urea and creatinine activities, which stimulate of toxicity in the kidney, in addition they are supported by kidney histopathological examination which shows severe renal injury in MTX treated group. On the contrary, the results are disagreement with (Chan et al., 2006). This study also showed that daflon has renal protective effect for the first time through reduction in urea and creatinine with improvement in kidney histopathological changes and here the study agree with (Asvadi et al., 2011, Morsy et al., 2013, El-Sheikh et al., 2015).

According to the results of this study, daflon has a dose dependent hepatorenal protective effect (increasing with dose increment). And here the improvement by daflon may be attributed to his role in anti-inflammation and antioxidant, as well as the protection from nephrotoxicity and hepatotoxicity (Amimoto et al., 1995, Delbarre et al., 1995).

3. Oxidation parameters: Concerning oxidation parameters, the result demonstrated that treatment with MTX leading to increasing with MDA level, which is lipid peroxidation product, and decreasing with GSH level which is considered as antioxidant enzyme in MTX group. Adding daflon to MTX in group 3 and group 4 significantly attenuated lipid peroxidation as evident by significant decreases in MDA level which associated with a significant increase in GSH level as shown in (Fig. 7, 8).

Oxidative stress is a parameter used to denote the change in equilibrium between oxidizing agents and antioxidants which leads to injury or damage to a specific part of the body. If the equilibrium unable to continue in the tissues, there are many of histopathological and physiological changes will caused damage in cell (Jahovic et al., 2003). The damage that resulted by oxidative stress with the common effects of peroxidation and generation of radicals are fixed sign of methotrexate pathological effect (Jahovic and Sener, 2004). It is the metabolic product to metabolize free radicals resulting from peroxidation of lipid (Kose et al., 2012). Its usefulness as an indicator of lipid release and peroxidation in saturated layers such as cell membranes, in addition to being a final outputs of irregular oxidation (Sahna et al., 2006). The lipid peroxidation may cause by Methotrexate via the clear increases in MDA and may be mediated by oxygen-free radicals which be an important pathological factor of distortion and fragmentation to the cell membranes and was suggested to be a contributing factor of the effect of MTX- mediated the
changes in the tissue (Kose et al., 2012). GSH is very important part for defense as antioxidant agents in living cells (Iyyaswamy and Rathinasamy, 2012). In the present experiment, MDA in the second group (MTX) was observed in high level, while the glutathione showed decreased when compared with control group. Our results agree with Delbarre et al., (1995), Cirilik et al., (2006) and Fu et al., (2008) who found that, the treatment with MTX causes clear decrease in GSH with increase in the level of MDA. Regarding daflon is an effective through the reduction in lipid peroxidation products and graduated increase in GSH in blood, suggesting that the active effect as anti-oxidant and the scavenging activity of the radicals by daflon may reduce the pathological changes by MTX (Megenness et al., 1977, Rapavi et al., 2007).

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