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Possible Protective Effects of Two Different Doses of Cyanocobalamin Against Methotrexate Nephrotoxicity Model in Rats

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Abstract

Nephrotoxicity is defined as rapid deterioration in kidney functions. It arises from direct exposure to drugs or their metabolites. Methotrexate is a famous chemotherapeutic drug with anti-inflammatory and immunosuppressive properties. A high-dose methotrexate-induced renal dysfunction can be life threatening. Cyanocobalamin, one of the forms of vitamin B_{12}, acts as a coenzyme in the conversion of homocysteine to methionine in the cytosol, and the conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondrion. The study was designed to investigate the possible protective role of intraperitoneal cyanocobalamin administered in 2 different doses, against rat model of nephrotoxicity induced by methotrexate. Rats utilized in this study were randomized into 4 groups (ten rats per each group); **Group 1-** (Control) rats intraperitoneally injected with 0.5ml normal saline once daily for 7 consecutive days. **Group 2-** Rats intraperitoneally injected with 0.5ml normal saline once daily for 7 consecutive days; and at day 2, a single intraperitoneal dose of methotrexate (20mg/kg) is to be injected. **Group 3**- Rats intraperitoneally injected with a 0.5mg/kg cyanocobalamin once daily for 7 consecutive days, and at day 2, a single intraperitoneal dose of methotrexate (20mg/kg). **Group 4**- Rats intraperitoneally injected with a 2mg/kg cyanocobalamin once daily for 7 consecutive days, and at day 2, a single intraperitoneal dose of methotrexate (20mg/kg). Co-administration of cyanocobalamin at doses cyanocobalamin 0.5mg/kg and 2mg/kg with methotrexate showed significant-reduction- ($P<0.05$) in malondialdehyde, significant elevation ($P<0.05$)-in the glutathione level, significant upregulation in renal Nrf2 expression and significant down regulation in renal keap1 expression each compared to corresponding levels in methotrexate-only treated group. In conclusion this study demonstrated that co-administration of cyanocobalamin at two different doses with MTX resulted in attenuation of its nephrotoxicity by the utilization of selected parameters.

Keywords: Nephrotoxicity, methotrexate, cyanocobalamin, malondialdehyde, glutathione, Nrf2, Keap1.

التأثيرات الوقائية المحتملة لجرعتين مختلفتين من السيانوكوبالامين تجاه نموذج السمية الكلوية المستحثة بالميثوتريكسيت في الجرذان

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الخلاصة

تعرف السمية الكلوية بأنها تدهور سريع في وظائف الكلى. ينشأ من التعرض المباشر للأدوية أو مستقلباتها. الميثوتريكسيت هو دواء علاجي كيميائي مشهور له خصائص مضادة للالتهابات ومثبطة للمناعة. ان الجرعات العالية من الميثوتركيست تسبب الخلل الكلوي وممكن ان تكون مهددة للحياة. يعمل السيانوكوبالامين ، وهو أحد أشكال فيتامين ب ١٢ ، بمثابة أنزيم مساعد في تحويل الهوموسيستين إلى ميثيونين في العصارة الخلوية، وتحويل ميثيل مالونيل- CoA إلى سكسينيل- CoA في الميتوكوندريون. صُممت الدراسة للتحقيق في الدور الوقائي المحتمل للسيانوكوبالامين داخل الصفاق الذي يتم تناوله في جرعتين مختلفتين ، تجاه نموذج الجرذان للسمية الكلوية التي يسببها الميثوتريكسات. تم تقسيم الجرذان المستخدمة في هذه الدراسة بصورة عشوائية إلى ٤ مجموعات (عشرة فئران لكل مجموعة). المجموعة الأولى (مجموعة السيطرة)- حقنت الجرذان بمحلول ملحي عادي ٠,٥ مل داخل الصفاق مرة واحدة يومياً لمدة ٧ أيام متتالية. المجموعة الثانية- حقنت الجرذان بمحلول ملحي عادي ٠,٥ مل داخل الصفاق مرة واحدة يومياً لمدة ٧ أيام متتالية. وفي اليوم الثاني ، تم حقن جرعة واحدة داخل الصفاق من الميثوتريكسيت (٢٠ ملغم/كغم). المجموعة الثالثة- تم حقن الجرذان ب ٠,٥ ملغم/كغم من السيانوكوبالامين داخل الصفاق مرة واحدة يومياً لمدة ٧ أيام متتالية ، وفى اليوم الثاني تم حقن جرعة واحدة من الميثوتريكسيت (٢٠ملغم/كغم) داخل الصفاق. المجموعة الرابعة: تم حقن الجرذان داخل الصفاق بجرعة ٢ ملغم/كغم من السيانوكوبالامين مرة واحدة يومياً لمدة ٧ أيام متتالية ، وفي اليوم الثاني ، تم حقن جرعة واحدة داخل الصفاق من الميثوتريكسيت (٢٠ ملغم/كغم). سبب الاعطاء المشترك للسيانوكوبالامين بجرعات ٠,٥ ملغم/كغم و ٢ ملغم/كغم كل مع الميثوتريكسيت انخفاضًا معنويا ($P<٠,٠٥$) في مستوى المالونديالديهايد ، ارتفاعًا معنويا ($P<٠,٠٥$) في مستوى الجلوتاثيون، زيادة معنوية في التعبيرالكلوي Nrf2 وانخفاض معنوي التنظيم في تعبير ال keap1 الكلوي مقارنة بالمستويات المقابلة في المجموعة المعالجة بالميثوتريكسيت فقط. يمكن الاستنتاج بان هذه الدراسة أوضحت أن الإعطاء المشترك للسيانوكوبالامين بجرعتين مختلفتين مع الميثوتريكسيت أدى إلى توهين السمية الكلوية عن طريق استخدام عوامل مختارة.

الكلمات المفتاحية: سمية كلوية ، ميثوتريكسيت ، سيانوكوبالامين ، مالونديالديهيد ، جلوتاثيون، Nrf2، Keap1

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Introduction

Nephrotoxicity can be defined as any renal damage caused directly or indirectly by drugs or their metabolites, with acute renal failure, tubulopathies, and glomerulopathies as prominent clinical presentations [1].

Methotrexate (MTX) is a famous chemotherapeutic drug with anti-inflammatory and immunosuppressive properties that is used to treat autoimmune disorders. Despite its wide range of clinical use, MTX's efficacy is often limited by severe adverse effects, primarily nephrotoxicity and hepatotoxicity; it also has other side effects such as intestinal damage, myelosuppression [2-4].

The pathogenesis of MTX nephrotoxicity involves several pathways, including oxidative stress (OS) and inflammation [3].

A characteristic hallmark of high dose-MTX-induced acute kidney injury (AKI) is a rapid increase in serum creatinine after injection of high dose-methotrexate (HD-MTX) [5].

Two major pathways are thought to be involved in MTX-induced nephrotoxicity. The first is MTX-induced crystal nephropathy, which is caused by the precipitation of MTX and its metabolites within the renal tubules. The second mode is direct tubular toxicity; where, MTX can cause overproduction of the reactive oxygen species (ROS) in the kidney [6].

Furthermore, researchers revealed that acidic urine can increase the risk of MTX-induced nephrotoxicity because MTX is poorly soluble at low pH, resulting in intratubular MTX crystallization and obstruction of urine flow [7].

Increased hydration, high-dose leucovorin, and glucarpidase (if needed) effectively lower serum MTX concentrations and protect cells from such drug, but these treatments must be started as soon as possible to -avoid further toxicity, -facilitate renal recovery, and -allow patients to resume HDMTX therapy once renal function has returned to normal [8].

Cyanocobalamin is the public name of the B_{12}-active corrinoid (also known as cobalamin) with a cyanide ion (CN^-) at the β-position of the cobalt atom [9]. Intracellular vitamin B_{12} is stored as methylcobalamin and deoxyadenosylcobalamin, two active coenzymes. Methylcobalamin is a cofactor for cytoplasmic methionine synthase, which catalyzes homocysteine methylation to methionine. This transmethylation reaction also includes folate (vitamin B9), which is necessary for nucleic acid synthesis. The second active coenzyme (deoxyadenosylcobalamin) is a cofactor for methylmalonyl-CoA mutase enzyme, which catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondria. Succinyl-CoA then enters the Krebs cycle where it is used to synthesize lipids and carbohydrates [10-12].

Vitamin B_{12} has several antioxidant properties including: (a) direct scavenging of reactive oxygen radicals (RORs), mainly superoxide anion ($O_2^{\bullet-}$); (b) indirect activation of ROS scavenging by restoration of reduced glutathione (GSH); (c) modulation of cytokine and growth factor (GF) production to offer protection from immune response-induced OS; (d) lowering of homocysteine-induced OS; and (e) reduction of OS caused by advanced glycation end products [13].

The Nrf2 (Nuclear factor-erythroid 2-related factor 2) is the key transcription factor that regulates the antioxidant response. Under physiological condition, Kelch-like ECH-associated protein 1 (Keap1) sequesters Nrf2 in the cytosol. Upon activation by ROS, Nrf2 dissociates from Keap1, translocates into the nucleus, forms a dimer with a small musculoaponeurotic fibrosarcoma (MAF) protein, binds to the antioxidant response element (ARE) and promote the transcription of several antioxidant and cytoprotective genes [14, 15].

This study is designed to investigate the protective effect of cyanocobalamin (0.5mg/kg and 2mg/kg) each intraperitoneally (IP)-injected in combination on methotrexate (MTX)-induced nephrotoxicity in rats.

Materials and Methods

Animals

Forty (40) white Albino rats of both sexes, weighing 180-200g were used in this study. Rats were obtained from the Animal House of the College of Pharmacy-University of Baghdad; and maintained under controlled conditions of temperature, humidity and light/dark cycle. The animals were fed commercial pellets and tap water *ad libitum*.

Chemicals and kits

Methotrexate (MTX) vial (50mg/2ml) Mylan, France; Cyanocobalamin (vitamin B_{12} ampule 1mg/2ml) Kontam, Hong Kong; Rat GSH ELISA kit Mybiosource, USA; Rat MDA ELISA kit Mybiosource, USA. In addition, TRIzol reagent Thermo Fisher Scientific (USA). GoTaq® 1-Step RT-qPCR System. Promega, USA. PCR primers for Nrf2, keap 1, and β–actin genes were synthesized and purchased from Macrogen, Korea.

Experimental design

Wistar rats utilized in this study were randomly-divided into 4 groups (10 rats each) and received their treatment by IP route as follows:

Group 1- Control/Experimental healthy rats IP injected with 0.5ml normal saline (0.9% NaCl) once daily for 7 consecutive days.

Group 2- Experimental healthy rats IP injected with 0.5ml normal saline (0.9% NaCl) once daily for 7 consecutive days; and at day 2, a single dose of MTX (20mg/kg) is to be I.P injected [16].

Group 3- Experimental healthy rats I.P injected with a 0.5mg/kg cyanocobalamin [17] once daily for

7 consecutive days, and at day 2, a single IP dose of MTX (20mg/kg) to be injected.

Group 4- Experimental healthy rats I.P injected with a 2mg/kg cyanocobalamin [17] once daily for 7 consecutive days, and at day 2, a single IP dose of MTX (20mg/kg) to be injected.

The animals in each group were euthanized by anesthetic ether 24 hour after the end of the treatment.

Preparation of kidney tissue homogenate

After the rat euthanized by anesthetic ether, the kidney was quickly excised, rinsed in ice-cold buffer phosphate saline pH 7.4 to remove excess thorough blood and weighed before homogenization, then minced tissue and homogenized with the aid of homogenizer after putting the tube in a beaker containing ice. After that the homogenate was then centrifuged for 20 min at 3000 rpm using cold centrifuge and the supernatant was utilized for the estimation of GSH, and MDA levels.

Determination of mRNA expression of renal Nrf2 and keap 1 genes

Total RNA was extracted from kidney tissues depending on the steps of TRIzol™ Reagent protocol. Quantus fluorometer was applied for the detection of the extracted RNA concentration. The isolated RNA was ready for use in cDNA synthesis. One step RT-qPCR protocol used to investigate quantitatively mRNA of the transcription factor Nrf2 and keap 1. All real-time RT-PCR reactions were conducted in a total volume of 10 µl. The thermal profile used was at 37 °C for 15 min for RT. Enzyme activation at 95 °C for 5min for initial activation denaturation, followed by 40 cycles of 95 °C for 20s denaturation, 56 °C for 20 s annealing and 72 °C for 20 s extension. As a reference gene, the β-actin is used. After the PCR amplification, the ΔΔ Ct was used for calculating by subtraction of the β-actin Ct from each sample Ct, qRT-PCR primers Nrf2, keap 1 and B-actin sequences [18, 19] were shown in table 1.

Statistical analysis

Data were analyzed as mean±standard error of the mean (SEM); and performed by using the Graphpad Prism, version 8. The analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test was done. Data differences were considered significant at $P<0.05$.

Table 1. Primers

Primer Name	Sequence
B actin- F	5`-CCACCATGTACCCAGGCATT-3`
B actin- R	5`-ACGCAGCTCAGTAACAGTCC-3`
NRF2-F	5`-TTGTAGATGACCATGAGTCGC-3`
NRF2-R	5`-TGTCCTGCTGTATGCTGCTT-3
Keap1-F	5`-GGACGGCAACACTGATTC-3`
Keap1-R	5`-TCGTCTCGATCTGGCTCATA-3`

Results

Effect on reduced glutathione (GSH) level

Rats IP injected with MTX at day 2 at a dose of 20mg/kg (**Group 2**) showed significant reduction ($P<0.05$) in the level of GSH compared to the corresponding level in control (**Group 1**) rats. Mean±SEM of GSH levels were respectively 58.21± 4.254 and 77.85± 4.585. Figure 1

Furthermore, in figure 1 there were significant elevation ($P<0.05$) in GSH level in (**Group 3 and 4)** rat treated with cyanocobalamin dose 0.5mg/kg and 2mg/kg for 1 week in combination with MTX each compared to corresponding level in (**Group 2**) rats; where, mean±SEM of GSH levels were respectively 76.57± 3.445, 78.05± 5.753 and 58.21± 4.254.

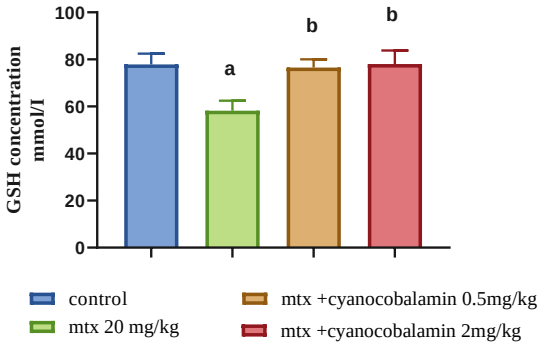

Figure 1. Bar chart showing levels of GSH in kidney tissue homogenate (mmol/l) in different experimental groups

Each value represents mean±standard error of means (SEM) a l atter , is significantly different (*P*<0.05) compared to control group; b latter, is significantly different (*P*<0.05) compared to MTX group N=10.

Effect on Malondialdehyde (MDA) levels

Rats IP injected with MTX at day 2 at a dose of 20mg/kg (**Group 2**) showed significant elevation (*P*<0.05) in the level of MDA compared to that level in control (**Group 1**) rats. The mean±SEM of MDA levels were respectively 2.882± 0.3345 and 1.838± 0.2085. Figure 2
Furthermore in figure 2 there were significant reduction (*P*<0.05) in MDA level in (**Group 3 and 4)** rat treated with cyanocobalamin dose 0.5mg/kg and 2mg/kg for 1 week each in combination with MTX at day 2 as compared to corresponding level in (**Group 2**); where, mean±SEM of MDA levels were respectively 1.856± 0.08614, 1.881± 0.1498, and 2.882± 0.3345.

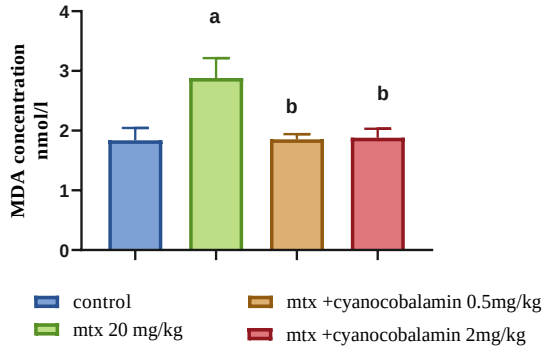

Figure 2. Bar chart showing levels of MDA in kidney tissue homogenate (nmol/l) in different experimental groups
Each value represents mean±standard error of means (SEM) .- a latter, is significantly different (P<0.05) compared to control group; b latter, is significantly different (*P*<0.05) compared to MTX group. N=10.

Effect on Nrf2 gene expression

Rats IP injected with MTX at day 2 at a dose of 20mg/kg (**Group 2**) significantly (*P*<0.05) downregulated renal Nrf2 mRNA expression compared to control group **(Group 1)**; where, mean±SEM of Nrf2 level 0.3529±0.05385 and 1.982± 0.1495. Figure 3.Furthermore, figure 3 showed that there were significant (*P*<0.05) up-regulation in renal Nrf2 mRNA expression in (**Group 3 and 4**) rats treated with cyanocobalamin dose 0.5 mg/kg and 2mg/kg for 1 week in combination with MTX at day 2 ,respectively compared to rats treated with MTX (**Group 2**); where, mean±SEM of Nrf2 level are 2.000±0.4949, 2.258±0.6545 and 0.3529±0.05385, respectively.

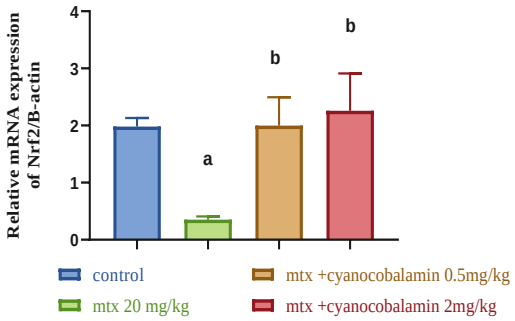

Figure 3. Bar chart showing levels of relative mRNA expression of Nrf2/B actin in kidney tissue in different experimental groups
Each value represents mean±standard error of means (SEM) - a latter,- is significantly different (P<0.05) compared to control group; b latter, is significantly different (*P*<0.05) compared to MTX group. N=10.

Effect on Keap1 gene expression

Rats IP injected with MTX at day 2 at a dose of 20mg/kg (**Group 2**) upregulated renal keap1 mRNA expression significantly (*P*<0.05) compared to that level in control (**Group 1**) rats; where, mean±SEM of keap 1 level 1.370±0.2115 and 0.7660± 0.0895. Figure 4.Furthermore, figure 4 showed that there were significant dawn-regulation (*P*<0.05) in renal keap1 mRNA expression in (**Group 3 and 4**) rats treated with cyanocobalamin dose 0.5 mg/kg and 2mg/kg for 1 week in combination with MTX at day 2, respectively compared to rats treated with MTX (**Group 2**); where, mean±SEM of keap1 level 0.6361±0.1280, 0.7690±0.04357 and 1.370±0.2115, respectively.

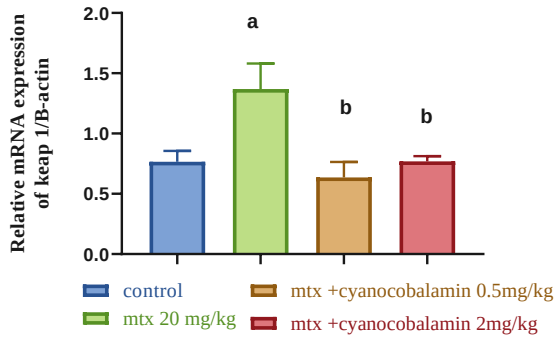

Figure 4. Bar chart showing levels of relative mRNA expression of Keap1/B actin in kidney tissue in different experimental groups

Each value represents mean ± standard error of means (SEM) - a latter, is significantly different (P<0.05) compared to control group; b latter, is significantly different (*P*<0.05) compared to MTX group. N=10.

Discussion

Methotrexate (MTX), a chemotherapeutic agent that is therapeutically-used for the treatment of various cancers. High doses of such drug can cause acute renal failure with an increase in the serum creatinine (SCr) levels, uremia, and hematuria [20].

One of the key mechanisms through which MTX can cause tissue damage is the OS. Since, it can increase ROS production by suppressing homocysteine remethylation, depletion of the reduced form nicotinamide dinucleotide phosphate (NADPH), stimulation of neutrophils, activation of NADPH oxidase enzyme activity, and mitochondrial dysfunction; moreover, ROS then can cause cell damage through oxidizing lipid and proteins, inactivating antioxidant enzymes, and causing DNA damage, leading to a dysfunctional cellular protective response [18].

Furthermore, researchers reported that administration of high dose of MTX resulted in -elevated MDA level, -depletion of glutathione reservoirs, and -reduction in tissue antioxidant capacity [21].

These ROS negatively affect nephrons, resulting in structural and functional changes of glomeruli and renal tubules [22].

The result of this study revealed that rats treated with 20mg/kg MTX IP, showed significant reduction in the level of GSH and significant elevation in the level of MDA as compared to control group.

Additionally, MTX reduced the efficiency of the antioxidant enzyme defense mechanism, and making the cells more vulnerable to ROS; furthermore, GSH is a sensitive marker of OS and it plays an essential role in preserving the integrity of the cell; since such marker involved in several detoxification reactions in the organism and it is one of the most important non-enzymatic antioxidants [23].

Furthermore, ROS-induced oxidation of polyunsaturated fatty acids (PUFAs) in biological systems resulting in the generation of LP products such as MDA [24].

Although the endogenous antioxidant response system in human can strongly-regulate the level of ROS and reduce correlated cellular damage, the exogenous antioxidants can play a significant role; since exogenous antioxidants were discovered to have a priming impact on the antioxidant response system; furthermore, both exogenous and endogenous antioxidant response systems work together to provide a more effective and efficient defense against harmful redox modulations [25].

Several molecular pathways play a remarkable role in kidney pathophysiology among them; Kelch-like ECH-associated protein1 (Keap1)/ nuclear factor erythroid 2–related factor2 (Nrf2). Since, the Keap1/Nrf2 pathway is one of the most significant OS cytoprotective pathways, and Nrf2 plays a critical physiological role in protecting the kidney against a variety of illnesses [26].

Moreover, researchers mentioned that the increased in Nrf2 expression was linked to lower OS and enhanced antioxidant defenses [27]. Additionally, Hassanein, E. H. M.*et al* (2018) reported that MTX significantly down-regulate renal Nrf-2, while up-regulate renal Keap-1 [28].

Rats injected with a single dose 20mg/kg MTX downregulated renal Nrf2 mRNA expression and upregulated renal Keap1 mRNA expression significantly (*P*<0.05); while there were significant (*P*<0.05) -up-regulation in renal Nrf2 mRNA expression in rats treated with cyanocobalamin doses [0.5mg/kg and 2mg/kg] for 1 week in combination with MTX and -down-regulation in renal Keap1 mRNA expression in rats treated with cyanocobalamin doses [0.5 mg/kg and 2mg/kg] for 1 week in combination with MTX (figure 3 and 4). Therefore, activation of Nrf2/antioxidant signaling by cyanocobalamin can attenuate MTX-induced OS, inflammation and kidney injury.

Researchers mentioned that the Nrf2 signaling pathway played a vital role in the protection against cell injury induced by OS and electrophiles; where, excessive production of ROS and oxidative injury are associated with the pathogenesis of several diseases and disorders; and there is crosstalk between the Nrf2/ARE signaling and other signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which represented the main underlying mechanism by which Nrf2 exerts its anti-inflammatory activities; furthermore, given the dual role of Nrf2 activation in the prevention of OS and inflammation, pharmacological activation of this signaling pathway could represent a powerful strategy for treating diseases associated with excessive release of ROS and proinflammatory mediators [29].

In agreement with the perception that the activation of the Nrf2 pathway is coupled with antioxidant effects and was linked to the decreased levels of NF-κB and inducible nitric oxide synthase (iNOS) expression in experimental rats, the Nrf2 is important for the expression of antioxidant genes like superoxide dismutase (SOD), catalase (CAT) and heme oxygenase-1 (HO-1), researchers postulated that the improved efficacy of antioxidant nephroprotective defense may be explained by their ability to regulate the Nrf2 signaling pathway positively [30].

Additionally it has been demonstrated that, MTX diminished Nrf2/ARE/HO-1 signaling in the liver and kidney of rats [14].

Although exposure to moderate OS can lead to Nrf2 activation, the excessive and sustained ROS generation can diminish Nrf2 signaling in the kidney and liver of rats challenged with MTX; thus, the diminished Nrf2/HO-1 pathway is a direct consequence of the sustained ROS generation induced by MTX [31].

Conclusion

According to the results obtained from this study, it could be concluded that the protective effect of cyanocobalamin at a dose of 0.5mg/kg and 2mg/kg were observed when co-administered with 20mg/kg MTX. Notably, co-administration of cyanocobalamin at 2 different doses with 20mg/kg MTX resulted in attenuation of MTX induced nephrotoxicity.

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