Allopurinol and Febuxostat Equally Attenuate Methotrexate-Induced Testicular Injury In Rats Via Activation Of EGF/ ERK_{1/2}/HO-1 Signaling Pathway.

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Research Article

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Abstract

Methotrexate (MTX) is commonly used in the management of several malignancies and autoimmune disorders; however, testicular damage is one of the most detrimental effects of MTX administration. In the current study, we evaluated the possible protective effect of xanthine oxidase inhibitors either purine analogue; allopurinol (ALL) or non-purine analogue; febuxostat (FEB) in testicular injury induced by MTX in rats. Gonadotoxicity was induced by a single dose of MTX (20 mg/kg, i.p.). ALL and FEB were administered orally in the following daily doses (100, 10 mg/kg, respectively) for 15 days. Total and free testosterone were measured in serum. In addition, total antioxidant capacity (TAC), epidermal growth factor (EGF), malondialdehyde (MDA), tumor necrosis factor- α (TNF- α), extracellular signal-regulating kinase1/2 (ERK1/2), and nitric oxide (NO) end products were measured in testicular tissues. At the same time, immunoexpression of HO-1 in testicular tissue was measured. Histopathological examination was done. ALL and FEB increased total and free serum testosterone. Both drugs showed a significant reduction in testicular MDA, NO, TNF- α levels with an increase in TAC, EGF, and ERK1/2 levels in testicular tissue. Furthermore, both drugs enhanced HO-1 immunoexpression in testicular tissue. All these findings were parallel to the preservation of normal testicular architecture in rats treated with ALL and FEB. In conclusion, All and FEB were protective against testicular damage induced by MTX through anti-inflammatory, anti-apoptotic, and antioxidant actions which might be through activation of the EGF/ERK1/2/HO-1 pathway. At the same time, no significant difference between ALL and FEB was noticed in this protective effect.

Introduction

Methotrexate, a folic acid antagonist, is a commonly prescribed medication with diverse indications including treatment of several types of cancer and autoimmune diseases. According to the most recent WHO's list of essential medicines, methotrexate (MTX) was included [1]. [2].

However, the unwanted effect of methotrexate on high proliferating organs represents a major concern. Testicular injury is among the limiting adverse effects of methotrexate ([2]). Exposure to MTX causes testicular damage. Such effect is mediated through oxidative stress, apoptotic changes, inflammation and disturbance in blood supply [3].

Allopurinol (ALL) and febuxostat (FEB), xanthine oxidase (XO) inhibitors, have been shown to have protective effects in various tissue-injuries. ALL inhibits synthesis of uric acid, reduces production of reactive oxygen species, downregulates some chemokines, cytokines and reduces tumor necrosis factor- α (TNF- α) and nuclear factor- κ B (NF- κ B) [4].

Febuxostat (FEB) is a nonpurine XO inhibitor, is a more potent serum uric acid-lowering action compared with ALL [5]. FEB also antagonizes oxidative stress and inflammation in several models of cell injury. Furthermore, It was reported that FEB reduces cardiotoxicity, nephrotoxicity, hepatotoxicity and pulmonary inflammation that are induced by certain cancer chemotherapeutic agents [6].

The present study evaluated and compared the expected protective effects of ALL and FEB on testicular damage induced by MTX in rats. The mechanisms of their effects were explored.

Materials And Methods

2.1. Ethics

Animal handling, medications, and scarification were carried out following the guidelines for the care of experimental animals indicated by Institutional Ethical Committee (Faculty of Medicine, Minia University, Egypt) and according to the NIH Guide for taking care and use of laboratory animals (Approval number 325:11/2019).

2.2. Chemicals

Chemicals and kits were obtained from the indicated companies; Carboxy methylcellulose (CMC) (El-Nasr Pharmaceutical Co., Egypt), Epidermal growth factor (EGF) ELISA kit (Cusabio, USA), total antioxidant capacity (TAC) colorimetric kit (Biodiagnostic, Egypt), Testosterone ELISA kit (Elabscience Biotechnology Co. Ltd), malondialdehyde (MDA) colorimetric kit (BioMed Diagnostic, Egypt), extracellular signal-regulated kinases 1/2 (ERK1/2) ELISA kit (Cusabio, USA), Nitric oxide (NO) colorimetric Kit (Biodiagnostic, Egypt), TNF- α ELISA Kit (Elabscience Biotechnology Co. Ltd., USA), Caspase-3 and hemeoxygenase-1 (HO-1) antibodies (Lab Vision Corporation, USA), FEB powder (Global Napi Co., Egypt), ALL powder (GalaxoSmithKline Co., Egypt) and MTX powder (Sreeven Pharma Pvt Ltd., Egypt).

2.3. Animals and experimental design

Adult male albino rats (200-250g, age 8-10 weeks) were purchased from the National Research Centre, Giza, Egypt. Rats housed in laboratory cages and feed a standard diet of rat chaw and tap water. Rats allowed 7 days to acclimate to the surroundings before beginning any experimentation. The temperature was maintained at 22 ± 2 °C. A 12/12 h light/dark cycle was maintained.

Experimental design

Rats were allocated to four groups (n=8) as follows:

Group 1: Negative control group received carboxy methyl cellulose (CMC) for 14 days and i.p. saline at 11th day.

Group 2: Received vehicle for 14 days and at 11th day rats received a single injection of MTX (20 mg/kg, i.p.) [7].

Group 3: Received oral allopurinol (100 mg/kg/d) for 14 day and at 11th day received a single i.p. injection of MTX (20 mg/kg) [8].

Group 4: Received oral febuxostat (10 mg/kg/d) for 14 day and at 11th day received a single i.p. injection of MTX (20 mg/kg) [9].

After completing the experiment, blood gathered from the anesthetized rats and sera stored at -80 °C for determination of testosterone. Testis were dissected and washed with phosphate buffer solution and stored at -80°C for measuring TAC, MDA, NO, EGF and TNF- α . The other half of testis immersed in Bouin's fluid for histological study.

2.4. Biochemical measurements in testicular tissue

Levels of MDA, TAC and NO_x measured calorimetrically using kit according to the manufacturer's instructions. TNF- α , ERK_{1/2}, and EGF levels in testicular tissue homogenate was assessed by using ELISA kits according to manufacturer's instructions.

2.5. Determination of serum testosterone

Serum testosterone measured by ELISA kit guided by the manufacturer's instructions.

2.6. Histological study

2.6.1. Histopathological examination

Hematoxylin-eosin staining was used and examined by optical microscope. Tissue damage in testis was graded and by multiplying each area grade by the percentage of totally occupied surface, the final result for each testis was calculated. It was scored as the following: grade 1; normal testicular structure with intact germinal epithelium, grade 2; testicular Injury with germinal cells (less orderly and non-cohesive) together with closely packed seminiferous tubules, grade 3; testicular Injury with disorganized germinal cells with nuclear pyknotic and less distinct seminiferous tubule borders and grade 4; closely packed seminiferous tubules with coagulative necrosis of the germinal cells. The pathologist who examined and graded the histological changes in the testes was blinded to the type of the treatment of each animal. Grade 1 is defined by normal tissue [10].

2.6.2. Immunohistochemical study of caspase-3 and Heme oxygenase-1 in testicular tissue

Immunohistochemical examination of caspase-3 and HO-1 were performed using monoclonal mouse antibodies. Guided by anti-caspase-positive cells percentage in the field, immunoexpression was semi-quantitatively scored as follows: 0, when absent; 1, positive cells were less than 10%; 2, when positive cells were from 10% to 50%; 3, when positive cells were from 51% to 90% and 4, when positive cells were more than 91%. HO-1 immunoreactivity was evaluated as positive cells number (based on positive staining percentage) and staining intensity. Extent was scored as: 0, less than 5%; 1, from 6% to 25%; 2, from 26% to 50% and 3, more than 50%. Intensity of staining was scored as: 0, weak; 1, moderate; 2, strong. The scoring was calculated by multiplying extent score by intensity [11, 12].

2.7. Statistical analysis

Mean $\pm$ SEM is used to express results. One-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test for multiple comparisons were used. P value < 0.05 is considered significant. Graph Pad Prism7 program was used to analyze data.

Results

3.1. Effect on serum testosterone.

Levels of free and total serum testosterone were decreased in MTX group when compared to control group. However, when compared to MTX group, the serum levels of free and total testosterone were increased in MTX+ALL and MTX+FEB groups [Table 1]. No significant difference was noticed between MTX+ALL and MTX+FEB groups.

Table 1
effect of allopurinol and febuxostat on free and total serum testosterone.

	Free testosterone (pg/ml)		Total testosterone (ng/ml)	
	Level	p values	Level	p values
Control	3.53 $\pm$ 0.09		3.14 $\pm$ 0.13	
MTX	2.32 $\pm$ 0.13 ^a	$p_1 < 0.0001$	1.68 $\pm$ 0.25 ^a	$p_1 < 0.0001$
MTX+ALL	2.98 $\pm$ 0.18 ^b	$p_2 = 0.0385$	2.79 $\pm$ 0.17 ^b	$p_2 = 0.0011$
MTX+FEB	3.12 $\pm$ 0.22 ^b	$p_2 = 0.0096$	3.24 $\pm$ 0.17 ^b	$p_2 < 0.0001$
Data represent the mean $\pm$ SEM (n=8). ^a statistically significant from control group; ^b statistically significant from MTX group; P_1 , p value from control group and P_2 , p value from MTX group. Significance is at $p < 0.05$.				

3.2. Effect on oxidative stress parameters in testicular tissue.

As compared to control group, testicular TAC was reduced in MTX group. Meanwhile, in MTX+ALL and MTX+FEB groups, TAC in testicular tissue was increased, as compared to MTX group. Testicular MDA and NO was increased in MTX group, as compared to control group. In groups treated with ALL and FEB, a decrease occurred in testicular MDA and NO, as compared to MTX-treated group [Table 2]. No significant changes in MDA, NO and TAC were noticed between MTX+ALL and MTX+FEB groups.

Table 2
Effect of allopurinol and febuxostat on total antioxidant capacity, malondialdehyde and nitric oxide in rat testes.

	TAC		MDA		NO	
	(mmol.g tissue ⁻¹)		(mmol.g tissue ⁻¹)		(mmol.g tissue ⁻¹)	
	Level	p values	Level	p values	Level	p values
Control	1.62±0.04		40.2±3.2		28.7±1.00	
MTX	0.99±0.06 ^a	$p_1 < 0.0001$	88.9±2.49 ^a	$p_1 < 0.0001$	73.5±5.12 ^a	$p_1 < 0.0001$
MTX+ALL	1.48±0.09 ^b	$p_2 = 0.0017$	70.8±1.34 ^{ab}	$p_1 < 0.0001$ $p_2 = 0.0005$	54.8±1.71 ^{ab}	$p_1 = 0.0003$ $p_2 = 0.0097$
MTX+FEB	1.51±0.12 ^b	$p_2 = 0.0008$	69.1±3.62 ^{ab}	$p_1 < 0.0001$ $p_2 = 0.0002$	48.8±5.43 ^{ab}	$p_1 < 0.005$ $p_2 = 0.0006$
Data represent the mean ± SEM (n=8). TAC, Total antioxidant capacity; MDA, Malondialdehyde and NO, nitric oxide. ^a statistically significant from control group; ^b statistically significant from MTX group; P_1 , p value from control group and P_2 , p value from MTX group. Significance is at $p < 0.05$.						

3.3. Effect on testicular TNF-α, EGF and ERK1/2.

As compared to control group, testicular TNF-α was elevated in MTX group. In groups treated with ALL and FEB, testicular TNF-α was decreased, as compared to MTX group. The level of testicular TNF-α in MTX+FEB was higher than its level in MTX+ALL. Levels of EGF in testicular tissue were reduced in MTX group, as compared to control group. However, in MTX+ALL and MTX+FEB groups, levels of EGF in testicular tissue were increased, as compared to MTX group. Levels of ERK1/2 in testicular tissue were reduced in MTX group, as compared to control group. However, in MTX+ALL and MTX+FEB groups, levels of EGF in testicular tissue were increased, as compared to MTX group [Table 3]. The changes in TNF-α, EGF and ERK1/2 were insignificant in MTX+FEB group, as compared to MTX+ALL group.

Table 3
effect of allopurinol and febuxostat on tumor necrosis factor- α , epidermal growth factor and extracellular signal-regulated kinases 1/2 in rat testes.

	TNF- α (pg/g tissue ⁻¹)		EGF (pg/g tissue ⁻¹)		ERK1/2 (pg/g tissue ⁻¹)	
	Level	p values	Level	p values	Level	p values
Control	32.8 $\pm$ 1.95		28.4 $\pm$ 1.68		10.96 $\pm$ 0.76	
MTX	68.34 $\pm$ 2.11 ^a	$p_1 < 0.0001$	15.2 $\pm$ 0.89 ^a	$p_1 < 0.0001$	3.96 $\pm$ 0.26 ^a	$p_1 < 0.0001$
MTX+ALL	55.9 $\pm$ 5.43 ^{ab}	$p_1 = 0.0001$ $p_2 = 0.0471$	20.1 $\pm$ 0.57 ^b	$p_1 = 0.0003$ $p_2 = 0.0415$	10.69 $\pm$ 0.53 ^b	$p_2 < 0.0001$
MTX+FEB	36.7 $\pm$ 1.71 ^{bc}	$p_2 < 0.0001$ $p_3 = 0.0011$	22.6 $\pm$ 1.49 ^b	$p_1 = 0.0118$ $p_2 = 0.0013$	11.51 $\pm$ 0.72 ^b	$P_2 < 0.0001$
data represent the mean $\pm$ SEM (n=8). TNF- α , Tumor necrosis factor- α , EGF, Epidermal growth factor and ERK1/2, extracellular signal-regulated kinases 1/2 in rat testes. ^a statistically significant from control group; ^b statistically significant from MTX group; ^c statistically significant from MTX+ALL group; P_1 , p value from control group; P_2 , p value from MTX group and P_3 , p value from MTX+ALL group. Significance is at $p < 0.05$.						

3.4. Histological study

3.4.1. Effect on histopathological picture of testicular tissue

In control group, there was no histopathological changes with preserved normal histological structure of seminiferous tubules, complete luminal spermatogenic series and the interstitial leydig cells in between. In MTX group, necrobiosis and nuclear pyknosis were detected in some of spermatogonial cells of the seminiferous tubules. Treatment with ALL or FEB restored germinal epithelium cells with no histopathological alteration [figure 1].

3.4.2. Immunohistochemical results for caspase 3

Examination of wide fields of control showed faint caspase-3 cytoplasmic expression in the testicular tissue. In MTX group a deeply stained cytoplasmic expression in most cells was noticed. Furthermore, MTX+ALL and MTX+FEB groups exhibited less cytoplasmic expression in the previously mentioned cells [figure 2].

3.4.3. Immunohistochemical results for hemeoxygenase-1

Examination of wide fields of control showed strong caspase cytoplasmic expression in the testicular tissue. In contrast, MTX group showed weak cytoplasmic expression in most cells. Furthermore,

MTX+ALL and MTX+FEB groups showed a strong cytoplasmic expression in testicular tissue [figure 3].

3.4.4. Morphometric Results

The results of grading of testicular damage are shown in Table 4. An increase in total score of testicular tissue damage in MTX group when compared to control group. In contrast, reduction in total scoring of tissue damage in MTX+ALL and MTX+FEB groups, as compared to MTX group.

Table 4
Histopathological scoring of testicular damage.

	Score	P Values
Control	1.00 ± 0.00	
MTX	3.52 ± 0.14 ^a	<i>p1< 0.0001</i>
MTX + ALL	1.14 ± 0.04 ^b	<i>p2< 0.0001</i>
MTX + FEB	1.09 ± 0.04 ^b	<i>p2< 0.0001</i>
Data represent the mean ± SEM (n=8).C, control group; MTX, methotrexate group; ALL, allopurinol group; FEB, febuxostate group. ^a statistically significant from control group; ^b statistically significant from MTX group; <i>P1</i> , <i>p</i> value from control group and <i>P2</i> , <i>p</i> value from MTX group. Significance is at <i>p</i> < 0.05.		

There was an increase in semiquantitative scoring of immunoreactive cells for anti-caspase-3 in MTX-treated group when compared to control group. Semiquantitative scoring of immunoreactive cells for anti-caspase-3 was reduced significantly in MTX+ALL and MTX+FEB, as compared to MTX group. on the other hand, semiquantitative scoring of immunoreactive cells for HO-1 in MTX group was significantly reduced, as compared to control group. Meanwhile, it was increased significantly in MTX+ALL and MTX+FEB when compared to MTX group [figure 4]. At the same time, no significant difference was noticed in MTX+FEB group, as compared to MTX+ALL group.

Discussion

Methotrexate is used as a cancer treatment of vast types of oncological disorders and also used in other disorders as psoriasis, rheumatic diseases and inflammatory bowel diseases [13].

Methotrexate Testicular toxicity has robust side effect causing subsequent infertility. Data from previous studies stated a disorganization in the seminiferous tubules of the testis, a reduction in sperm number, and sperm DNA destruction after MTX administration [14]. In current work, we evaluated the contribution of xanthine oxidase inhibitors in abrogation acute chemotherapy testicular cytotoxic effect. To the best of our knowledge, this is the first in vivo study to observe a protective effect of purine analogue XO inhibitor; febuxostat *versus* non- purine analogue XO inhibitor; allopurinol, in testicular damage induced by MTX in rats.

MTX toxicity accumulates ROS and depletes antioxidants and hence damaging cellular macromolecules [15]. lipid peroxidation with consequent cell damage results from reaction of polyunsaturated fatty acids and membrane lipids with reactive oxygen species [16]. Also, NO is increased as a result of inducible nitric oxide synthase activation [17]. The reaction of ROS and NO produces the versatile oxidant peroxynitrite that can damage DNA and other cellular molecules [18]. Similarly, in the current work, MTX administration caused reduction in TAC and increase in MDA and NO in testes.

Inflammation has a role in MTX-induced testicular cytotoxicity. previous studies report an increase in circulating pro-inflammatory cytokines with MTX administration [19]. The former action caused by activation of the transcription factor NF- κ B through exaggerated production of ROS and the activated NF- κ B has a direct relation to enhancement of the pro-inflammatory cytokines such as TNF- α [20].

In current study, after administration of MTX, cellular TNF- α activity significantly increased. Many studies illustrate that, TNF- α has a role in the pathogenesis of MTX-induced testicular injury [21].

In current study rats received ALL and FEB showed a significant decrease in MDA, NO and TNF- α levels and increased in TAC in testis. These findings are in accordance with previous studies demonstrated that ALL possess antioxidant properties, which may be attributable to inhibition of xanthine oxidase which results in attenuation of oxidative stress, enhanced the antioxidant defenses and amelioration of inflammatory status in different tissues liver [22], [23], [24] [8]. In consistence with our results, **Amirshahrokhi** reported the antioxidant and anti-inflammatory effects of FEB [9]. These effects result from FEB ability to antagonize oxidative stress and inflammation induced by XO. FEB also antagonized cardiotoxicity induced by doxorubicin, cisplatin-induced nephrotoxicity, nephritis and lung inflammation [25, 26].

Caspase 3, is a crucial enzyme in programmed cell death pathway is implicated in germ cell apoptosis with MTX administration [27]. In current work, MTX- induced caspase-3 expression in testis, which agrees with [21] who reported that MTX-induced elevation in caspase 3 immunostaining. Induction of oxidative stress and inflammation are two supposed mechanisms induce apoptosis of testicular cells [28]. At the same time, activation of proapoptotic proteins may have a role in gonadotoxicity induced by MTX [14, 29].

Interestingly, ALL or FEB significantly decreased testicular caspase-3 when supplemented with MTX. Other reports showed that XO- inhibitors are able to antagonize apoptosis [30].

The well-acknowledged participation of oxidative stress induction and inflammation aggravation in stimulating apoptosis emphasizes that the anti-apoptotic effect of ALL and FEB might be related to amelioration of oxidative stress and inflammation. Our results are supported by previous findings about the antagonism of germ cell apoptosis and improvement of spermatogenesis with ALL treatment [31].

One of the key growth factors regulating cell survival is EGF that has been shown in Leydig cells, Sertoli and peritubular cells. By binding to EFG receptors, IT activates an extensive network of signal

transduction pathways that regulate expression of both pro- and anti-apoptotic proteins that was effective in blocking the apoptosis [32]. Also, withdrawal of EGF from the extracellular environment increased the sensitivity to apoptotic stimuli [32]. Furthermore, EGF antagonized inflammation, ROS generation in many models of cell damage [33], [34], [35], [36], [37].

In an attempt to explore the contribution of EGF pathway in testicular damage, we evaluate EGF in testes which showed that, MTX reduced testicular EGF level, as compared to control group. In contrast, ALL and FEB induced expression of EGF in testicular tissue. These findings run parallel to improvement of oxidative stress, inflammation, and apoptosis status noticed with ALL and FEB.

In order to go deeper in understanding the mechanistic pathway underlying the protective effect of EGF which increased in treated groups we examined the status of downstream tyrosine kinase ERK_{1/2}, one of MAPKs, and has a pivotal role in the cellular death and survival [38]. Moreover, ERK_{1/2} signaling inhibition caused subsequent induction of apoptosis-associated proteins [39]. In current work, p-ERK_{1/2} increased in the testicular tissue exposed to MTX + ALL and increased in MTX + FEB groups when compared with MTX intoxicated rats. Interestingly, recent studies demonstrated that several drugs provide protective effect through activating ERK signaling cascade [40–42]. Surprisingly, the current results are supported by reports exhibited the link between activation of EGF and induction of ERK_{1/2} [43].

Heme oxygenase (HO)-1, member of the family of heat shock proteins (HSP-32), plays critical roles in physiological iron homeostasis, antioxidant defense and antiapoptotic effects [44]. Furthermore, induction of HO-1 results in reduction in infiltration of inflammatory cell and exudates, whereas HO-1 downregulation enhanced inflammatory exudates, illustrating the role of HO-1 in modulating inflammatory response [45].

Interestingly, MTX in the current study reduced HO-1 expression as evidenced by the undeniable elevation in semiquantitative scoring of immunoreactive cells for HO-1. Running in the same stream, Mahmoud and co-workers reported that MTX reduced expression of HO-1 [46]. In contrast, ALL and FEB upregulated HO-1 in testicular tissue which agrees with previous numerous studies stated the enhancing effect of xanthine oxidase inhibitors on HO-1 expression and linked that to the cytoprotective effects of ALL and FEB in different models of tissue injury. In these models of tissue injury, the amelioration of HO-1 expression was directly related inflammation, oxidative stress and apoptosis inductions. On the other hand, ALL and FEB reverted the reduction of HO-1 expression which was restorative to tissue integrity and functions [47],[48], [49]. The current induction of HO-1 is supported with data revealed that activation of HO-1 gene occurred via regulation of ERK1/2 and this enhances cell survival [50]. Furthermore, it was stated that protection against cell damage attained by HO-1 signaling induction involved ERK_{1/2} pathway modulation [40].

These findings are shedding light on the contribution of EGF, ERK_{1/2} and HO-1 in oxidative stress, apoptosis and inflammatory cascade induced during testicular cytotoxicity. Surprisingly, EGF receptor-mediated signaling pathways affected ERK_{1/2} that inturn induced HO-1 [44]. This finding can support the

assumption that inhibition EGF, ERK_{1/2} and HO-1 signaling pathway can provoke apoptosis, oxidative stress and inflammation and vice versa.

At the end of the day, the undeniable increase of serum testosterone and normalization of histopathological picture in ALL and FEB groups, as compared to MTX group, confirmed the protection afforded by ALL and FEB in testis damage. To the best of our knowledge this is the first time to study the comparative effect of purine analogue *versus* non-purine analogue xanthine oxidase inhibitors. On the light of the previous data, we found that ALL and FEB are equipotent in their cytoprotective effect in testicular damage.

In conclusion, EGF through subcellular activation of ERK1/2/HO-1 pathway might have a crucial role in the protective effect of ALL and FEB in MTX induced testicular damage as shown through antioxidant, anti-inflammatory, and anti-apoptotic actions. Furthermore, no significant difference was noticed between ALL and FEB in a such valuable effect.

Declarations

Ethical Approval

Study was approved by Institutional Ethical Committee (Faculty of Medicine, Minia University, Egypt) (Approval number 325:11/2019).

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors' contributions

RR and RM designed and performed the experiment, data analysis and supervision. MI and WA cooperated in performing the experiment, revised manuscript and wrote final draft. The authors declare that all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors declare that they have no conflicts of interest.

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Availability of data and materials

The original source data was submitted to the journal and is available.

References

1. Organization WH (2019) World Health Organization model list of essential medicines: 21st list 2019. World Health Organization
2. Çakır T et al (2011) Caffeic acid phenethyl ester (CAPE) prevents methotrexate-induced hepatorenal oxidative injury in rats. *J Pharm Pharmacol* 63(12):1566–1571
3. Abdelzaher WY et al (2020) Role of nitric oxide donor in methotrexate-induced testicular injury via modulation of pro-inflammatory mediators, eNOS and P-glycoprotein. *Hum Exp Toxicol* 39(12):1700–1709
4. Prieto-Moure B et al (2017) Allopurinol Protective Effect of Renal Ischemia by Downregulating TNF- α , IL-1 β , and IL-6 Response. *J Invest Surg* 30(3):143–151
5. Kojima S et al (2019) Febuxostat for Cerebral and CaRdiorenovascular Events PrEvEntion StuDY. *Eur Heart J* 40(22):1778–1786
6. Namai-Takahashi A et al (2019) Xanthine Oxidase Inhibitor, Febuxostat Ameliorates the High Salt Intake–Induced Cardiac Hypertrophy and Fibrosis in Dahl Salt-Sensitive Rats. *Am J Hypertens* 32(1):26–33
7. Rjiba-Touati K et al (2018) Recombinant human erythropoietin prevents etoposide-and methotrexate-induced toxicity in kidney and liver tissues via the regulation of oxidative damage and genotoxicity in Wistar rats, vol 37. *Human & experimental toxicology*, pp 848–8588
8. Aldaba-Muruato LR *Protective effects of allopurinol against acute liver damage and cirrhosis induced by carbon tetrachloride: Modulation of NF- κ B, cytokine production and oxidative stress.* *Biochimica et Biophysica Acta (BBA) - General Subjects* et al (2012) **1820**(2): p. 65-75
9. Amirshahrokhi K (2019) Febuxostat attenuates ulcerative colitis by the inhibition of NF- κ B, proinflammatory cytokines, and oxidative stress in mice. *Int Immunopharmacol* 76:105884
10. Cosentino MJ et al (1986) Histopathology of prepubertal rat testes subjected to various durations of spermatic cord torsion. *J Androl* 7(1):23–31
11. Pescarmona E et al (1999) Expression of p53 and retinoblastoma gene in high-grade nodal peripheral T-cell lymphomas: immunohistochemical and molecular findings suggesting different pathogenetic pathways and possible clinical implications. *J Pathol* 188(4):400–406

12. Lundvig DM et al (2014) Delayed cutaneous wound closure in HO-2 deficient mice despite normal HO-1 expression. *J Cell Mol Med* 18(12):2488–2498
13. Abdel-Raheem IT, Khedr NF (2014) Renoprotective effects of montelukast, a cysteinyl leukotriene receptor antagonist, against methotrexate-induced kidney damage in rats. *Naunyn Schmiedebergs Arch Pharmacol* 387(4):341–353
14. Daggulli M et al (2014) Protective effects of carvacrol against methotrexate-induced testicular toxicity in rats. *Int J Clin Exp Med* 7(12):5511–5516
15. Mahmoud AM et al (2017) *Methotrexate hepatotoxicity is associated with oxidative stress, and down-regulation of PPAR γ and Nrf2: Protective effect of 18 β -Glycyrrhetic acid*. *Chemico-biological interactions*. 270:: p. 59–72
16. Naik SR, Panda VS (2007) Antioxidant and hepatoprotective effects of Ginkgo biloba phytosomes in carbon tetrachloride-induced liver injury in rodents, vol 27. *Liver international*, pp 393–3993
17. Ali N et al (2017) Protective effect of Chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. *Chemico-Biol Interact* 272:80–91
18. McKim SE et al (2003) Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. *Gastroenterology* 125(6):1834–1844
19. Wang Y et al (2018) Melatonin protects methotrexate-induced testicular injury in rats. *Eur Rev Med Pharmacol Sci* 22(21):7517–7525
20. Mahmoud AM et al (2017) Possible involvement of Nrf2 and PPAR γ up-regulation in the protective effect of umbelliferone against cyclophosphamide-induced hepatotoxicity. *Biomed Pharmacother* 86:297–306
21. Morsy MA et al (2020) The Possible Contribution of P-Glycoprotein in the Protective Effect of Paeonol against Methotrexate-Induced Testicular Injury in Rats. *Pharmaceuticals* 13(9):223
22. Mahmoud AM, Germoush MO, Soliman AS (2014) Berberine attenuates isoniazid-induced hepatotoxicity by modulating peroxisome proliferator-activated receptor gamma, oxidative stress and inflammation. *International Journal of Pharmacology* 10(8):451–460
23. Germoush MO, Mahmoud AM (2014) Berberine mitigates cyclophosphamide-induced hepatotoxicity by modulating antioxidant status and inflammatory cytokines. *J Cancer Res Clin Oncol* 140(7):1103–1109
24. Mahmoud AM et al (2017) Modulatory effect of berberine on adipose tissue PPAR γ , adipocytokines and oxidative stress in high fat diet/streptozotocin-induced diabetic rats. *J Appl Pharm Sci* 7(4):1–10
25. Krishnamurthy B et al (2015) Febuxostat ameliorates doxorubicin-induced cardiotoxicity in rats. *Chemico-Biol Interact* 237:96–103
26. Kataoka H, Yang K, Rock KL (2015) The xanthine oxidase inhibitor Febuxostat reduces tissue uric acid content and inhibits injury-induced inflammation in the liver and lung. *Eur J Pharmacol* 746:174–179

27. Savitskaya M, Onishchenko G (2015) *Mechanisms of apoptosis*. Biochemistry (Moscow), **80**(11): p. 1393-1405
28. Sheikhbahaei F et al (2016) Protective effects of thymoquinone against methotrexate-induced germ cell apoptosis in male mice. International journal of fertility & sterility 9(4):541
29. Nouri HS, Azarmi Y, Movahedin M (2009) Effect of growth hormone on testicular dysfunction induced by methotrexate in rats. Andrologia 41(2):105–110
30. Wang S et al (2015) Febuxostat pretreatment attenuates myocardial ischemia/reperfusion injury via mitochondrial apoptosis. Journal of Translational Medicine 13(1):209
31. Sukhotnik I et al (2008) Effect of allopurinol on germ cell apoptosis following testicular ischemia–reperfusion injury in a rat, vol 24. Pediatric surgery international, pp 61–661
32. Henson ES, Gibson SB (2006) Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cell Signal 18(12):2089–2097
33. Uguralp S et al (2004) The effect of sustained and local administration of epidermal growth factor on improving bilateral testicular tissue after torsion. Urol Res 32(5):323–331
34. Sen S et al (2020) Anti-inflammatory effect of epidermal growth factor conjugated silk fibroin immobilized polyurethane ameliorates diabetic burn wound healing. Int J Biol Macromol 143:1009–1032
35. Kuhn MA et al (2002) Heparin-binding EGF-like growth factor (HB-EGF) decreases oxygen free radical production in vitro and in vivo. Antioxidants and Redox Signaling 4(4):639–646
36. Tomaszewska R et al (2002) The influence of epidermal growth factor on the course of ischemia-reperfusion induced pancreatitis in rats. J Physiol Pharmacol 53(2):183–198
37. Taira T et al (1996) Expression of epidermal growth factor and its receptor in rabbits with ischaemic acute renal failure. Virchows Arch 427(6):583–588
38. Zhu C-H et al (2020) Dexmedetomidine reduces ventilator-induced lung injury via ERK1/2 pathway activation. Mol Med Rep 22(6):5378–5384
39. Zhang L et al (2020) Mitotic checkpoint kinase Mps1/TTK predicts prognosis of colon cancer patients and regulates tumor proliferation and differentiation via PKC α /ERK1/2 and PI3K/Akt pathway. Med Oncol 37(1):1–10
40. Bucolo C et al (2019) Curcumin prevents high glucose damage in retinal pigment epithelial cells through ERK1/2-mediated activation of the Nrf2/HO-1 pathway. J Cell Physiol 234(10):17295–17304
41. Shi Y et al (2018) Neuroprotective role of dexmedetomidine pretreatment in cerebral ischemia injury via ADRA2A–mediated phosphorylation of ERK1/2 in adult rats. Experimental and therapeutic medicine 16(6):5201–5209
42. Qiu Z et al (2020) Dexmedetomidine inhibits neuroinflammation by altering microglial M1/M2 polarization through MAPK/ERK pathway. Neurochem Res 45(2):345–353

43. Yamamoto T, Cui X-M, Shuler CF (2003) Role of ERK1/2 signaling during EGF-induced inhibition of palatal fusion. *Dev Biol* 260(2):512–521
44. Kuroda H et al (2010) Inhibition of heme oxygenase-1 with an epidermal growth factor receptor inhibitor and cisplatin decreases proliferation of lung cancer A549 cells. *Lung Cancer* 67(1):31–36
45. Willis D et al (1996) Heme oxygenase: a novel target for the modulation of inflammatory response. *Nat Med* 2(1):87–90
46. Mahmoud AM, Hozayen WG, Ramadan SM (2017) Berberine ameliorates methotrexate-induced liver injury by activating Nrf2/HO-1 pathway and PPAR γ , and suppressing oxidative stress and apoptosis in rats, vol 94. *Biomedicine & Pharmacotherapy*, pp 280–291
47. Luo J et al (2020) Allopurinol reduces oxidative stress and activates Nrf2/p62 to attenuate diabetic cardiomyopathy in rats. *J Cell Mol Med* 24(2):1760–1773
48. Mao X et al (2013) N-acetylcysteine and allopurinol confer synergy in attenuating myocardial ischemia injury via restoring HIF-1 α /HO-1 signaling in diabetic rats. *PLoS ONE* 8(7):e68949
49. Kim H et al (2020) Febuxostat, a novel inhibitor of xanthine oxidase, reduces ER stress through upregulation of SIRT1-AMPK-HO-1/thioredoxin expression. *Clin Exp Nephrol* 24(3):205–215
50. Mathew T, Sarada S (2018) Intonation of Nrf2 and Hif1- α pathway by curcumin prophylaxis: A potential strategy to augment survival signaling under hypoxia, vol 258. *Respiratory physiology & neurobiology*, pp 12–24

Figures

Figure 1

Effect on testis histopathological picture. A representative photomicrograph of a section in testis tissue (20x). A) control group, B) MTX group, necrobiosis and nuclear pyknosis were detected in some of spermatogonial cells of the seminiferous tubules, C) MTX+ALL group; well-organized normal germinal epithelium, D) MTX+FEB group; a well-preserved tubular structures and normal germ cells.

Figure 2

Effect on caspase-3 immunoreactivity in testicular tissue (40x). A; control group, B; MTX group, C; MTX+ALL group and D; MTX+FEB group.

Figure 3

Effect on hemoxygenase-1 immunoreactivity in testicular tissue (40x). A; control group, B; MTX group, C; MTX+ALL group and D; MTX+FEB group.

Figure 4

Effect of allopurinol and febuxostat on semiquantitative scoring of immunoreactivities of caspase 3 (A) and hemeoxygenase-1 (B) in rat testes. Data represent the mean $\pm$ SEM (n=8). a statistically significant from control group; b statistically significant from MTX group. Significance is at $p < 0.05$.