ijlpr 2021; doi 10.22376/ijpbs/lpr.2021.11.2.P167-173

International Journal of Life science and Pharma Research

Research Article

Pharmacy for better health

Punicalagin Reverses Methotrexate-Induced Nephrotoxicity by Attenuating Oxidative Stress, Inflammation and Apoptosis

Saleem H. Aladaileh[1,2*], Farhan K. Al-Swailmi[1], Mohammad H. Abukhalil[2,3*], And Mohammed H. Shalayel[1]

[1] *Department of Pharmacy Practice, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin 31991, Saudi Arabia*

[2] *Department of Medical Analysis, Princess Aisha Bint Al-Hussein Faculty of Nursing and Health Sciences, Al-Hussein Bin Talal University, Ma'an 71111, Jordan*

[3] *Department of Biology, Faculty of Science, Al-Hussein Bin Talal University, Ma'an 71111, Jordan*

Abstract: Methotrexate (MTX) is a trusted anticancer agent; however, its multi-organ toxicity limits its clinical application, including nephrotoxicity. Punicalagin (PUN) is an ellagitannin found in the fruit peel of *Punicagranatum* with anti-inflammatory and antioxidant properties. The present study aimed to investigate the renoprotective effects of PUN in a well-established rat model of MTX-induced nephrotoxicity. Rats were divided randomly into four groups (six animals per group) as follows: the first group (control) was a normal control group. The second group (PUN) received PUN (30 mg kg^{-1} day^{-1}) daily through oral gavage for 10 consecutive days. The third group (MTX) a single i.p. injection of MTX (20 mg/kg) on day 7. The fourth group (PUN + MTX) received PUN (30 mg kg^{-1} day^{-1}) via oral gavage for 10 consecutive days and a single i.p. injection of MTX (20 mg/kg) on day 7. MTX-induced nephrotoxicity was associated with increased creatinine and urea in the serum. MTX also resulted in increased oxidative stress as evidenced by elevated malondialdehyde and protein carbonyl levels along with decreased glutathione content and superoxide dismutase and catalase activities in the kidney. Moreover, renal tissues were characterized by increased proinflammatory cytokines, cytochrome c and caspases-3, indicating increased inflammation and apoptosis in the kidney. PUN pretreatment prevented kidney injury, oxidative stress, inflammation and apoptosis, and enhanced antioxidant defenses in the kidney. Collectively, these findings indicate that PUN may represent a novel renoprotective strategy against MTX-induced oxidative stress, inflammation and apoptosis in the kidney, which deserves further exploration in future studies.

Keywords: Methotrexate; Oxidative stress; Apoptosis; Nephrotoxicity; Polyphenols; Punicalagin

***Corresponding Author**

Saleem H. Aladaileh, PhD , Department of Pharmacy Practice, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin 31991, Saudi Arabia, Department of Medical Analysis, Princess Aisha Bint Al-Hussein Faculty of Nursing and Health Sciences, Ma'an 71111, Jordan

Recieved On 15 February 2021
Revised On 27 February 2021
Accepted On 03 March 2021
Published On 05 March 2021

Funding This work is supported by Deanship of Scientific Research, University of Hafr Al-Batin, [Grant No. G-115-2020].

Citation Saleem H. Aladaileh, Farhan K. Al-Swailmi, Mohammad H. Abukhalil, And Mohammed H. Shalayel , Punicalagin Reverses Methotrexate-Induced Nephrotoxicity by Attenuating Oxidative Stress, Inflammation and Apoptosis.(2021).Int. J. Life Sci. Pharma Res.11(2), L167-173 http://dx.doi.org/10.22376/ijpbs/lpr.2021.11.2.P167-173

1. INTRODUCTION

Methotrexate (MTX), an alkylating agent, is used for the treatment of an array of malignancies; however, its clinical application is limited due to its clinical side effects, including nephrotoxicity.[1,2] MTX-induced renal dysfunction is believed to be mediated by the precipitation of MTX and its metabolites, [7-hydroxy-methotrexate (7-OH-MTX)] and [2,4-diamino-N10-methylpteroic acid] (DAMPA) in the renal tubules, leading to the loss of tubular cells by necrosis and apoptosis and consequent infiltration of inflammatory cells.[1,3] The mechanism of MTX nephrotoxicity is poorly understood; however, many studies have been conducted to understand this mechanism. These studies showed that MTX nephrotoxicity might be caused by the elevation of reactive oxygen species (ROS), inflammation, mitochondrial dysfunction, caspase activation and DNA damage, which ultimately lead to renal dysfunction.[4-9]It is well-known that oxidative stress and inflammation play key roles in the activation of apoptotic signaling pathways in the kidney, which include the mitochondrial-dependent caspase pathway.[10] Therefore, mitigating oxidative stress and inflammation may protect against MTX-induced nephrotoxicity. Indeed, extensive evidence shows that natural plant products may offer new perspectives for novel therapies aimed to preventing MTX-induced nephrotoxicity.[5,11,12]Punicalagin (PUN) is the most abundant ellagitannin found in pomegranate which has a wide range of biological activities.[13,14] In multiple preclinical disease models, PUN has been shown to have strong antioxidant, anti-inflammatory and anti-apoptotic effects.[13,15,16] PUN was shown to prevent endotoxemic acute kidney injury in rats by reducing inflammation and counteracting oxidative/nitrative stress and apoptosis.[17] In addition, PUN prevents high glucose-induced cellular stress and neural tube defects through inhibition of lipid peroxidation and protein nitration.[16] PUN was also found to protect against myocardial ischemia-reperfusion-induced myocardial injury in rats by restoring antioxidant defenses and mitigating lipid peroxidation. Another study showed that PUN has been proven to attenuate streptozotocin (STZ)-induced cardiac injury in rats by increasing the redox state and Bcl-2 expression and suppressing caspases and p53.[15] Importantly, PUN was proven to be harmless and well-tolerated without adverse biological effects in rats.[18]Several pharmacological actions of PUN have been investigated; however, to the best of our knowledge, none is known about its protective effects against MTX-induced nephrotoxicity. As a result, our study was conducted to evaluate the protective effect of PUN against MTX nephrotoxicity in rats. Our findings may have a novel mechanistic insight into the protective effects of PUN in nephrotoxicity and a promising tool for the prevention of multi-organ toxicity of MTX chemotherapy.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

Male albino Wistar rats, weighing 200–220 g, were used in this experiment. All animals were acclimatized for one week before experimentation. Rats were housed in an air-conditioned atmosphere at standard temperature (23 ± 2 °C) on a 12 h light/dark cycle. They received a standard diet and water *ad libitum*. All the animal protocols conformed to the guidelines of the National Institutes of Health (NIH publication No. 85-23, revised 2011) and were approved by the local authorities (G-115-2020). Rats were randomly allocated into four groups (six animals per group) as follows: the first group (control) was a normal control group. The second group (PUN) received PUN (30 mg kg^{-1} day^{-1}) daily through oral gavage for 10 consecutive days. The third group (MTX) a single i.p. injection of MTX (20 mg/kg) on day 7 [5]. The fourth group (PUN + MTX) received PUN (30 mg kg^{-1} day^{-1}) via oral gavage for 10 consecutive days and a single i.p. injection of MTX (20 mg/kg) on day 7. The dose of PUN (Santa Cruz Biotechnology, Texas, USA) was determined based on previous studies demonstrating the antioxidant and anti-inflammatory actions of PUN *in vivo*.[19,20] PUN was dissolved in 1% Tween 80 and MTX was dissolved in saline. On the 11th day, all rats were anesthetized and sacrificed and blood and kidney samples were collected for further investigations. Serum was prepared by centrifugation of the blood and was used to measure creatinine and urea. The kidneys were cleaned in cold phosphate buffered saline (PBS). Kidney samples were homogenized (10% w/v) in cold PBS, and centrifuged. The resulting clear homogenate was used for evaluation of different biochemical parameters.

2.2 Biochemical assays

Urea and creatinine levels in serum were determined following the reagent kits instructions provided by Spinreact (Girona, Spain). Kidney homogenate was used to assess malondialdehyde (MDA), protein carbonyl and antioxidants. The MDA levels were assessed by coupling MDA with thiobarbituric acid as previously described by Ohkawa et al.[21] Protein carbonyl contents were estimated as described by Levine et al.[22] The superoxide dismutase (SOD)23 and catalase (CAT)[24] activities and reduced glutathione (GSH) [25] contents were determined in renal tissues. Levels of interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α) and IL-6 levels in the kidney were measured using ELISA kits obtained from R&D Systems (MN, USA). Cytochrome c and caspase-9 levels in the kidney were determined using ELISA kits obtained from MyBioSource (CA, USA) and Cusabio (Wuhan, China), respectively. All assays were performed according to the manufacturer's instructions.

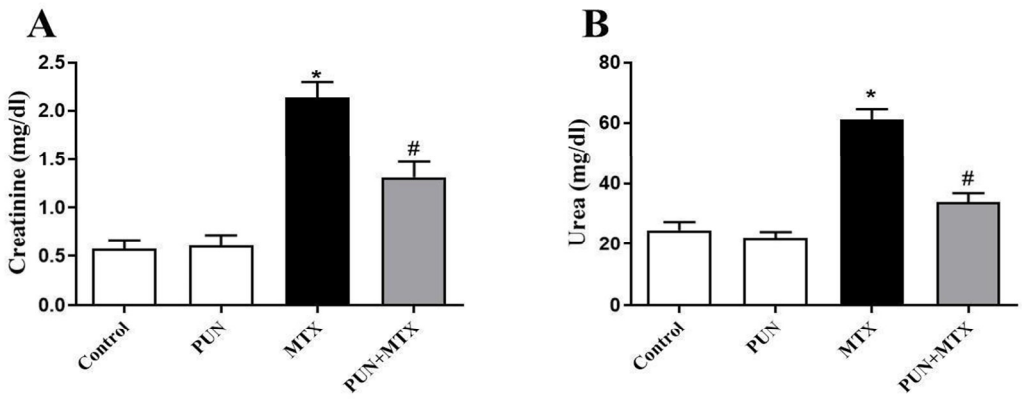
3 STATISTICAL ANALYSIS

The results significance was determined by one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism 7 software (San Diego, CA, USA). All results are presented as the mean ± standard error of the mean (SEM) and a P value <0.05 was considered significant.

4 RESULTS

4.1 PUN attenuates serum creatinine and urea levels in MTX-induced rats

For assessment of the effect of PUN on MTX-induced renal dysfunction, serum urea and creatinine levels in serum were determined. There was a significant increase in the measured serum creatinine and urea levels in MTX-treated rats compared to the control group (Figure1A and B). In contrast, pre-treatment with PUN decreased the serum creatinine and urea levels compared to those in MTX-treated rats and prevented MTX-induced kidney dysfunction, with no effect on the kidney of normal animals (Figure 1A and B).

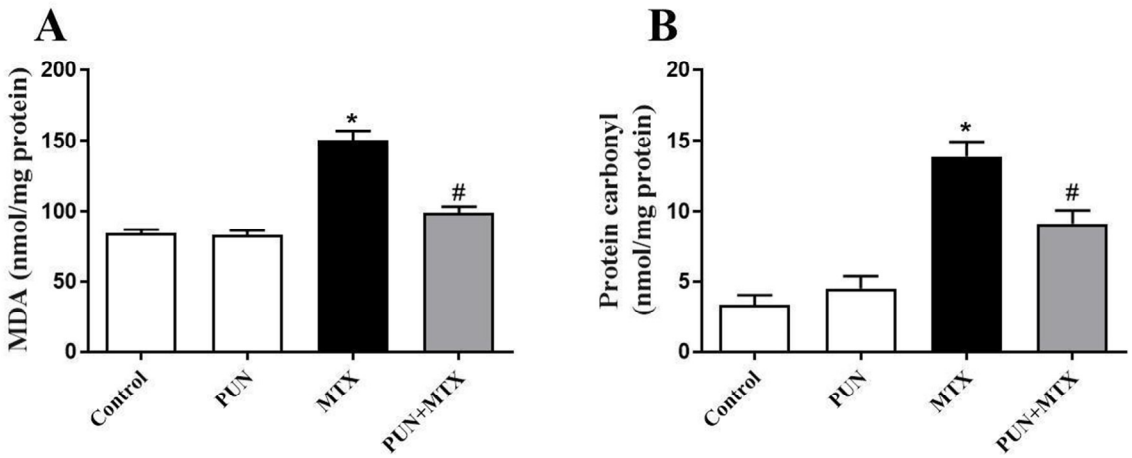


Results are represented as Mean ± SEM, n=6. *P< 0.05 versus control group. # P< 0.05 versus MTX group.

Fig 1: ***The effect of pretreatment of MTX-intoxicated rats with PUN on (A) creatinine and (B) urea levels in serum.***

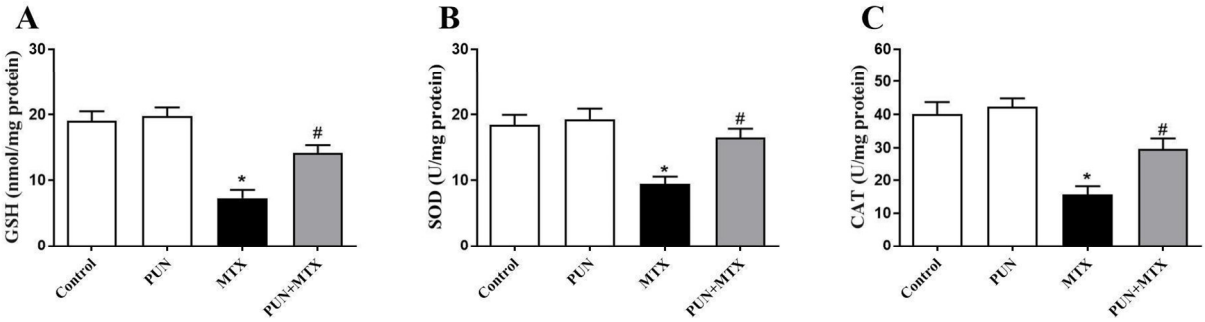
4.2 PUN prevents MTX-induced increased renal oxidative stress and enhances antioxidant defenses

Because MTX nephrotoxicity is known to be associated with increased oxidative stress, this study further evaluated the oxidative stress markers and antioxidants in the kidney. Kidneys of MTX-intoxicated rats showed a significant increase in MDA (Figure 2A) and protein carbonyl (Figure 2B), along with decreased GSH contents and SOD and CAT activities (Graph 3A-C). All these changes were significantly attenuated when MTX-intoxicated rats were pretreated with PUN. PUN alone had no effect on the above-measured variables.



Results are represented as Mean ± SEM, n=6. * P < 0.05 versus control group. # P < 0.05 versus MTX group.

Fig 2: ***The effect of pretreatment of MTX-intoxicated rats with PUN on levels of (A) MDA and (B) protein carbonyl in the kidney.***

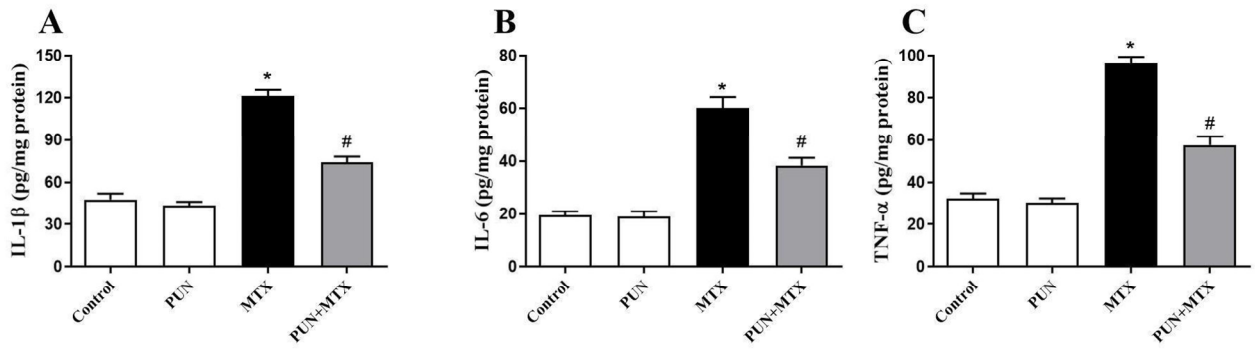


Results are represented as Mean ± SEM, n=6. * P < 0.05 versus control group. # P < 0.05 versus MTX group.

Fig 3: ***The effect of pretreatment of MTX-intoxicated rats with PUN on (A) GSH content and (B) SOD and (C) CAT activities in the kidney.***

4.3 *PUN pretreatment attenuates MTX-induced renal inflammation*

The effect of PUN on inflammation was investigated by determining pro-inflammatory cytokine levels in the kidney. The inflammatory response following MTX administration was shown by the elevated TNF-α, IL-1β and IL-6 levels in renal tissues (Figure 4A-C). Remarkably, PUN attenuated MTX-induced increase levels of the assayed inflammatory mediators. All of the measured proinflammatory cytokines were not affected in PUN-treated rats (30 mg/kg).

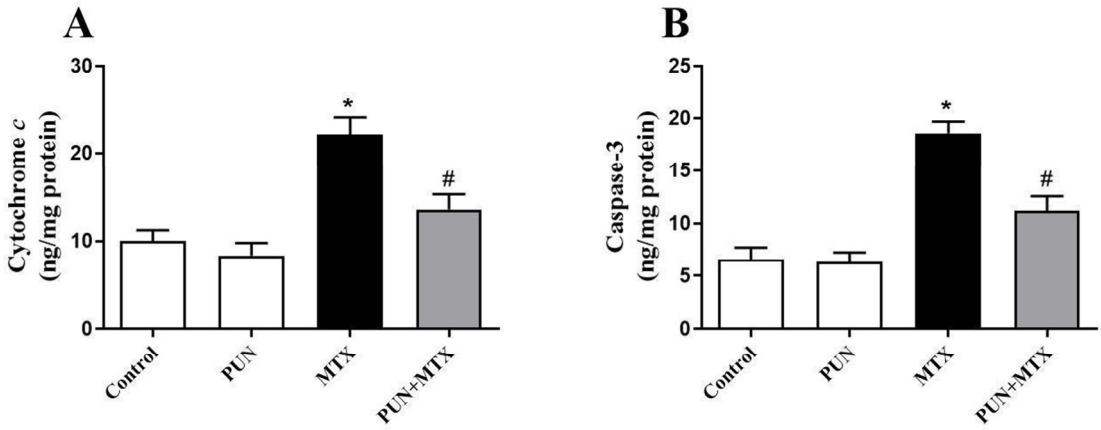


Results are represented as Mean ± SEM, n=6. * P < 0.05 versus control group. # P < 0.05 versus MTX group.

Fig 4: ***The effect of pretreatment of MTX-intoxicated rats with PUN on (A) IL-1β (B) IL-6 and (C) TNF-α levels in rats kidney.***

4.4 *PUN protects against MTX-induced renal apoptosis*

Increased oxidative and inflammation play a major role in apoptosis induction. Therefore, we next evaluated apoptosis markers in the kidney. MTX-induced apoptosis in rats was evidenced by increased cytochrome c and caspase-3 levels in the kidney (Figure 5A and B). Interestingly, pretreatment of MTX-injected rats with PUN significantly prevented apoptosis in the kidney by attenuating cytochrome c and caspase-3 levels. PUN alone had no effect on cytochrome *c* and caspase-3 levels in the kidney.



Results are represented as Mean ± SEM, n=6. * P < 0.05 versus control group. # P < 0.05 versus MTX group.

Fig 5: ***The effect of pretreatment of MTX-intoxicated rats with PUN on (A) cytochrome c and (B) caspase-3 levels in the kidney.***

5 DISCUSSION

MTX, a widely used chemotherapeutic agent, is well-known to produce significant oxidative damage in multiple organs, which restricts its clinical use.[5,8,11,26] Therefore, there is an urgent need for the development of novel approaches to prevent MTX chemotherapy-induced organ injury. PUN is a bioactive ellagitannin constituent of pomegranate with strong antioxidant and anti-inflammatory effects in various preclinical disease models.[13,16,17,19,20,27] In the present study, we showed that PUN exerts protective effects against MTX-induced kidney injury by (i) attenuating oxidative stress, (ii) decreasing renal inflammation, (iii) preventing apoptosis. Consistent with several previous studies [7,11], MTX-induced nephrotoxicity was shown by increased serum levels of creatinine and urea. Creatinine is commonly measured as an index of glomerular function.[28] Urea is a result of protein breakdown, and most of it is excreted through the kidney.[29] High levels of these kidney injury biomarkers indicate deleterious changes in the kidney.[28,30] Herein, pre-treatment of MTX-intoxicated rats with PUN attenuated the circulating levels of creatinine and urea, indicating that PUN maintained normal structural and architectural integrity of the kidney. In accordance, PUN ameliorated endotoxemic acute kidney injury in rats.[17] Likewise, PUN prevented renal dysfunction in a mouse model of diabetes.[31] One of the important molecular

mechanisms through which MTX injures the kidney is oxidative stress.[5,8,11,26] It has been reported that MTX induces ROS production by inhibiting the remethylation of homocysteine[32], depletion of cellular nicotinamide adenine dinucleotide phosphate (NADPH)[33], and stimulation of neutrophils and associated increased superoxide-generating NADPH oxidase.[34,35] In turn, ROS can cause cell injury through lipid peroxidation, protein oxidation, inactivation of antioxidant enzymes and DNA damage, culminating in dysfunctional cellular protective responses.[36] Lipid peroxidation can interrupt the membrane fluidity and permeability and also inactivate the membrane-bound proteins, which eventually lead to destruction of the membrane.[37] Moreover, oxidative modification of proteins may cause damage to the active sites of enzymes, including antioxidant enzymes, and disrupt the conformation of structural proteins, raising havoc throughout the cell. Therefore, maintenance of the cellular redox balance is considered an effective strategy to protect against MTX-induced kidney injury. Herein, PUN supplementation effectively prevented MTX-induced renal lipid peroxidation and protein carbonylation and decreased the GSH level and SOD and CAT activities. In accordance with these observations, PUN attenuated oxidative stress and enhanced the antioxidant defenses in rodent models of endotoxemia-induced acute kidney injury[17], cyclophosphamide-induced hepatotoxicity [38] and STZ-induced cardiac injury.[15] Importantly, activation of Nrf2, a central regulator of an array of detoxifying and antioxidant defense gene expression[39], may have a role in mediating the antioxidant action of PUN. In the same context, it has been reported that PUN inhibited lipopolysaccharide (LPS)-induced ROS and NO generation in RAW264.[7] macrophages *via* activation of Nrf2/HO-1 *in vitro*.[27] These results demonstrate that PUN can protect against MTX-induced kidney injury *via* attenuation of oxidative stress and restoration of antioxidant defenses. Inflammation is believed to be one of the main causes of MTX-induced nephrotoxicity. Indeed, excessive generation of ROS can activate important stress signaling and pro-inflammatory pathways in the kidney after MTX administration.[4,6,7] In experimental renal disease, podocytes and mesangial cells activation has been reported during glomerular injury, along with tubular cells during the course of proteinuria or primary tubulointerstitial diseases, such as ischemia reperfusion, obstruction, and septic or toxic acute kidney injury.[40]- [44] Herein, MTX-intoxicated rats showed elevated levels of proinflammatory cytokines in the kidney. In fact, multiple lines of evidence indicate that inflammation and oxidative stress work in league to generate cellular damage and apoptosis.[4,6,7] Consistent with several previous studies [4,26,45,46], the kidneys of MTX-injected rats exhibited increased apoptosis, as evidenced by the elevated levels of cytochrome c and caspase-3. In fact, MTX-mediated apoptosis is believed to be induced by generation of an excessive amount of ROS, which in turn induces DNA damage, eventually culminating in the activation of the mitochondrial apoptotic pathway by increasing the expression of pro-apoptotic proteins and reducing the expression of anti-apoptotic proteins.[7,26,46]Therefore, targeting oxidative stress and consequent proinflammatory signaling activation is therapeutically important for MTX nephrotoxicity. Interestingly, pretreatment of MTX-injected rats with PUN significantly reduced TNF-α, IL1β, IL-6, cytochrome c and caspase-3 levels in the kidney. In accordance, a previous report demonstrated the inhibitory effect of PUN on proinflammatory production in LPS-induced nephrotoxicity in rats.[19] Recently, PUN has been reported to mitigate pyroptosis-related inflammatory factors IL-1β, NLRP3, GSDMD, and caspase-1 in kidney tissue of diabetic mice.[31] In addition, PUN prevented apoptosis in the kidney by modulating of caspases 3, 8 and 9 activities and theBax/Bcl2 ratio in LPS-induced endotoxemic acute kidney injury.[17] Another study showed that PUN attenuated STZ-induced cardiac apoptosis in rats by increasing Bcl-2 and decreasing Bax, Caspase-3 and -9 levels.[15] The anti-apoptotic potential of PUN could be attributed to its inhibitory effect against free radicals and pro-inflammatory cytokines overproduction.

6 CONCLUSION

In conclusion, these findings demonstrate some mechanistic insights into how PUN confers protection against MTX-induced nephrotoxicity in rats. PUN prevents MTX-induced oxidative, inflammation and apoptosis and significantly improves GSH content and SOD and CAT activities in the kidney. Therefore, PUN may represent a promising approach for the prevention of nephrotoxicity caused by MTX treatment and perhaps other forms of MTX toxicity.

7 FUNDING ACKNOWLEDGEMENT

This work was supported by the Deanship of Scientific Research, University of Hafr Al-Batin, [Grant No. G-115-2020].

8 AUTHORS CONTRIBUTION STATEMENT

Mohammad H. Abukhalilcarried out the experiment. Saleem H. Aladaileh and Mohammad H. Abukhalil and Farhan K. Al-Swailmi wrote the manuscript with support from Mohammed H. Shalayel.

9 ACKNOWLEDGMENTS

The authors would like to thank the Deanship of Scientific Research, University of Hafr Al-Batin for funding this study.

10 CONFLICT OF INTEREST

Conflict of interest declared none.

11 REFERENCES

1. Malyszko J, Kozlowska K, Kozlowski L, Malyszko J. Nephrotoxicity of anticancer treatment. Nephrol Dial Transplant. 2017;32(6):924-36. doi: 10.1093/ndt/gfw338, PMID 28339935.
2. Kremer JM. Toward a better understanding of methotrexate.Arthritis Rheum. 2004;50(5):1370-82. doi: 10.1002/art.20278, PMID 15146406.
3. Widemann BC, Adamson PC. Understanding and managing methotrexate nephrotoxicity.Oncologist.

2006;11(6):694-703. doi: 10.1634/theoncologist.11-6-694, PMID 16794248.
4. Abd El-Twab SM, Hozayen WG, Hussein OE, Mahmoud AM. 18 β-glycyrrhetinicacid protects against methotrexate-induced kidney injury by up-regulating the Nrf_2/ARE/HO-1 pathway and endogenous antioxidants. Ren Fail. 2016;38(9):1516-27. doi: 10.1080/0886022X.2016.1216722, PMID 27499091.
5. Abdel-Daim MM, Khalifa HA, Abushouk AI, Dkhil MA, Al-Quraishy SA. Diosmin attenuates methotrexate-induced hepatic, renal, and cardiac injury: a biochemical and histopathological study in mice. Oxid Med Cell Longev. 2017;2017:3281670. doi: 10.1155/2017/3281670, PMID 28819543.
6. Arab HH, Salama SA, Maghrabi IA. Camel milk attenuates methotrexate-induced kidney injury via activation of PI3K/Akt/eNOS signaling and intervention with oxidative aberrations. Food Funct. 2018;9(5):2661-72. doi: 10.1039/c8fo00131f, PMID 29667662.
7. Mahmoud AM, Germoush MO, Al-Anazi KM, Mahmoud AH, Farah MA, Allam AA. Commiphoramolmol protects against methotrexate-induced nephrotoxicity by up-regulating Nrf_2/ARE/HO-1 signaling. Biomed Pharmacother. 2018;106:499-509. doi: 10.1016/j.biopha.2018.06.171, PMID 29990838.
8. Mahmoud AM, Hozayen WG, Ramadan SM. Berberine ameliorates methotrexate-induced liver injury by activating Nrf_2/HO-1 pathway and PPARγ, and suppressing oxidative stress and apoptosis in rats. Biomed Pharmacother. 2017;94:280-91. doi: 10.1016/j.biopha.2017.07.101, PMID 28763751.
9. Erboga M, Aktas C, Erboga ZF, Donmez YB, Gurel A. Quercetin ameliorates methotrexate-induced renal damage, apoptosis and oxidative stress in rats. Ren Fail. 2015;37(9):1492-7. doi: 10.3109/0886022X.2015.1074521, PMID 26338102.
10. Hamzeh M, Hosseinimehr SJ, Khalatbary AR, Mohammadi HR, Dashti A, Amiri FT. Atorvastatin mitigates cyclophosphamide-induced hepatotoxicity via suppression of oxidative stress and apoptosis in rat model. Res Pharm Sci. 2018;13(5):440-9. doi: 10.4103/1735-5362.236837, PMID 30271446.
11. Morsy MA, Ibrahim SA, Amin EF, Kamel MY, Rifaai RA, Hassan MK. Curcumin ameliorates methotrexate-induced nephrotoxicity in rats. AdvPharmacol Sci. 2013;2013:387071. doi: 10.1155/2013/387071, PMID 24381587.
12. Elmansy RA, Seleem HS, Mahmoud AR, Hassanein EHM, Ali FEM. Rebamipide potentially mitigates methotrexate-induced nephrotoxicity via inhibition of oxidative stress and inflammation: A molecular and histochemical study. Anat Rec. 2021;304(3):647-61. doi: 10.1002/ar.24482, PMID 32589351.
13. Cao K, Xu J, Pu W, Dong Z, Sun L, Zang W, Gao F, Zhang Y, Feng Z, Liu J. Punicalagin, an active component in pomegranate, ameliorates cardiac mitochondrial impairment in obese rats via AMPK activation [Sci Rep:14014]. Sci Rep. 2015;5:14014. doi: 10.1038/srep14014, PMID 26369619.
14. Li J, Wang G, Hou C, Li J, Luo Y, Li B. Punicalagin and ellagic acid from pomegranate peel induce apoptosis and inhibits proliferation in human HepG2 hepatoma cells through targeting mitochondria. Food AgricImmunol. 2019;30(1):897-912. doi: 10.1080/09540105.2019.1642857.
15. El-Missiry MA, Amer MA, Hemieda FAE, Othman AI, Sakr DA, Abdulhadi HL. Cardioameliorative effect of punicalagin against streptozotocin-induced apoptosis, redox imbalance, metabolic changes and inflammation. Egypt J Basic Appl Sci. 2015;2(4):247-60. doi: 10.1016/j.ejbas.2015.09.004.
16. Zhong J, Reece EA, Yang P. Punicalagin exerts protective effect against high glucose-induced cellular stress and neural tube defects. BiochemBiophys Res Commun. 2015;467(2):179-84. doi: 10.1016/j.bbrc.2015.10.024, PMID 26453010.
17. Fouad AA, Qutub HO, Al-Melhim WN. Nephroprotection of punicalagin in rat model of endotoxemic acute kidney injury.ToxicolMech Methods. 2016;26(7):538-43. doi: 10.1080/15376516.2016.1211207, PMID 27464552.
18. Cerdá B, Cerón JJ, Tomás-Barberán FA, Espín JC. Repeated oral administration of high doses of the pomegranate ellagitanninpunicalagin to rats for 37 days is not toxic. J Agric Food Chem. 2003;51(11):3493-501. doi: 10.1021/jf020842c, PMID 12744688.
19. Fouad AA, Qutub HO, Al-Melhim WN. Punicalagin alleviates hepatotoxicity in rats challenged with cyclophosphamide. Environ ToxicolPharmacol. 2016;45:158-62. doi: 10.1016/j.etap.2016.05.031, PMID 27310207.
20. Yaidikar L, Byna B, Thakur SR. Neuroprotective effect of punicalagin against cerebral ischemia reperfusion-induced oxidative brain injury in rats. J Stroke Cerebrovasc Dis. 2014;23(10):2869-78. doi: 10.1016/j.jstrokecerebrovasdis.2014.07.020, PMID 25282190.
21. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351-8. doi: 10.1016/0003-2697(79)90738-3, PMID 36810.
22. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 1990;186:464-78. doi: 10.1016/0076-6879(90)86141-h, PMID 1978225.
23. Nishikimi M, Appaji NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazinemethosulfate and molecular oxygen.BiochemBiophys Res Commun. 1972;46(2):849-54. doi: 10.1016/s0006-291x(72)80218-3, PMID 4400444.
24. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121-6. doi: 10.1016/s0076-6879(84)05016-3, PMID 6727660.
25. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine.Anal Biochem. 1980;106(1):207-12. doi: 10.1016/0003-2697(80)90139-6, PMID 7416462.
26. Aladaileh SH, Hussein OE, Abukhalil MH, Saghir SAM, Bin-Jumah M, Alfwuaires MA, Germoush MO, Almaiman AA, Mahmoud AM. Formononetinupregulates Nrf_2/HO-1 signaling and prevents oxidative stress, inflammation, and kidney injury in methotrexate-induced rats. Antioxidants. 2019;8(10):430. doi: 10.3390/antiox8100430, PMID 31561418.

27. Xu X, Li H, Hou X, Li D, He S, Wan C, Yin P, Liu M, Liu F, Xu J. Punicalagin induces Nrf_2/HO-1 expression via upregulation of PI3K/AKT pathway and inhibits LPS-induced oxidative stress in RAW264. 7 Macrophages.Mediators Inflam. 2015;2015:380218. doi: 10.1155/2015/380218, PMID 25969626.
28. Gowda S, Desai PB, Kulkarni SS, Hull VV, Math AA, Vernekar SN. Markers of renal function tests. N Am J Med Sci. 2010;2(4):170-3. PMID 22624135.
29. Salazar JH. Overview of urea and creatinine. Lab Med. 2014;45(1):e19-20. doi: 10.1309/LM920SBNZPJRJGUT.
30. Ferguson MA, Vaidya VS, Bonventre JV. Biomarkers of nephrotoxic acute kidney injury.Toxicology. 2008;245(3):182-93. doi: 10.1016/j.tox.2007.12.024, PMID 18294749.
31. An X, Zhang Y, Cao Y, Chen J, Qin H, Yang L. Punicalagin protects diabetic nephropathy by inhibiting pyroptosis based on TXNIP/NLRP3 pathway. Nutrients. 2020;12(5):1516. doi: 10.3390/nu12051516, PMID 32456088.
32. Abdel-Raheem IT, Khedr NF. Renoprotective effects of montelukast, a cysteinyl leukotriene receptor antagonist, against methotrexate-induced kidney damage in rats. NauSchm Arch Pharm. 2014;387(4):341-53. doi: 10.1007/s00210-013-0949-x, PMID 24363042.
33. Caetano NN, Campello AP, Carnieri EG, Kluppel MLW, Oliveira MBM. Effect of methotrexate (MTX) on NAD (P)+ dehydrogenases of HeLa cells: malic enzyme, 2-oxoglutarate and isocitrate dehydrogenases. Cell Biochemistry and Function: cellular biochemistry and its modulation by active agents or disease 1997;15:259-64.
34. Kılıc S, Emre S, MetinA, Isıkoglu S, Erel O. Effect of the systemic use of methotrexate on the oxidative stress and paraoxonase enzyme in psoriasis patients. Arch Dermatol Res. 2013;305(6):495-500. doi: 10.1007/s00403-013-1366-1, PMID 23660995.
35. Chan ES, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis Res Ther. 2002;4(4):266-73. doi: 10.1186/ar419, PMID 12106498.
36. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol. 2002;192(1):1-15. doi: 10.1002/jcp.10119, PMID 12115731.
37. Smathers RL, Galligan JJ, Stewart BJ, Petersen DR. Overview of lipid peroxidation products and hepatic protein modification in alcoholic liver disease. ChemBiol Interact. 2011;192(1-2):107-12. doi: 10.1016/j.cbi.2011.02.021, PMID 21354120.
38. Tsai MS, Chien CC, Lin TH, Liu CC, Liu RH, Su HL, Chiu YT, Wang SH. Galangin prevents acute hepatorenal toxicity in novel propacetamol-induced acetaminophen-overdosed mice. J Med Food.2015;18(11):1187-97. doi: 10.1089/jmf.2014.3328, PMID 26501381.
39. Satta S, Mahmoud AM, Wilkinson FL, Yvonne Alexander M, White SJ. The role of Nrf_2 in cardiovascular function and disease.Oxid Med Cell Longev. 2017;2017:9237263. doi: 10.1155/2017/9237263, PMID 29104732.
40. Sanz AB, Sanchez-Niño MD, Ramos AM, Moreno JA, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A. NF-kappaB in renal inflammation. J Am SocNephrol. 2010;21(8):1254-62. doi: 10.1681/ASN.2010020218, PMID 20651166.
41. Guijarro C, Egido J. Transcription factor-κB (NF-κB) and renal disease. Kidney Int. 2001;59(2):415-24. doi: 10.1046/j.1523-1755.2001.059002415.x, PMID 11168923.
42. Sanz AB, Sanchez-Niño MD, Izquierdo MC, Jakubowski A, Justo P, Blanco-Colio LM, Ruiz-Ortega M, Selgas R, Egido J, Ortiz A. TWEAK activates the non-canonical NFkappaB pathway in murine renal tubular cells: modulation of CCL21. PLOS ONE. 2010;5(1):e8955. doi: 10.1371/journal.pone.0008955, PMID 20126461.
43. Ruiz-Ortega M, Bustos C, Hernández-Presa MA, Lorenzo O, Plaza JJ, Egido J. Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-κB activation and monocyte chemoattractant protein-1 synthesis. J Immunol. 1998;161(1):430-9. PMID 9647253.
44. López-Franco O, Suzuki Y, Sanjuán G, Blanco J, Hernández-Vargas P, Yo Y, Kopp J, Egido J, Gómez-Guerrero C. Nuclear factor-κB inhibitors as potential novel anti-inflammatory agents for the treatment of immune glomerulonephritis. Am J Pathol. 2002;161(4):1497-505. doi: 10.1016/s0002-9440(10)64425-2, PMID 12368222.
45. Dalaklioglu S, Sahin P, Ordueri EG, Celik-Ozenci C, Tasatargil A. Potential role of poly (ADP-ribose) polymerase (PARP) activation in methotrexate-induced nephrotoxicity and tubular apoptosis. Int J Toxicol. 2012;31(5):430-40. doi: 10.1177/1091581812457430, PMID 22914891.
46. Helal MG, Said E. Tranilast attenuates methotrexate-induced renal and hepatic toxicities: role of apoptosis-induced tissue proliferation. J BiochemMolToxicol. 2020;34(5):e22466. doi: 10.1002/jbt.22466, PMID 32045101.