Ameliorative effect of metformin on methotrexate-induced genotoxicity: An *in vitro* study in human cultured lymphocytes

ABEER M. RABABA'H[1], KAREM H. ALZOUBI[1], OMAR F. KHABOUR[2] and MERA ABABNEH[1]

[1]Department of Clinical Pharmacy, College of Pharmacy; [2]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan

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Abstract. Methotrexate is a folic acid antagonist that has been shown to be genotoxic to normal healthy cells. Metformin is an insulin-sensitizing agent, with multiple potential pharmacodynamic profiles. The aim of the present study was to evaluate the genotoxic effect of methotrexate on DNA and the potential ameliorative effect of metformin on chromosomal damage induced by methotrexate. The present study was performed *in vitro*, and the frequency of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in human cultured lymphocytes were measured. Blood samples from five non-smoking healthy men aged 20-35 years were donated and used in the present study. Treatment of cultured blood cells with methotrexate significantly increased the number of cells with CAs (P<0.0001) and the frequency of SCEs (P<0.0001). The chromosomal injury induced by methotrexate was significantly reduced by pretreatment of the samples with metformin (P<0.0001). Importantly, the treatment of the cells with metformin alone did not affect the frequency of SCEs compared with the control group (P>0.05). Additionally, methotrexate and metformin alone, and combined, induced significant decreases in the proliferative index compared with the control group (P<0.05). In conclusion, metformin ameliorated the genotoxicity induced by methotrexate in cultured human lymphocytes.

Introduction

Methotrexate (MTX), a folic acid antagonist, exhibits anti-proliferative activity, and immune- regulatory and anti-inflammatory properties (1-3). The chemical structure of MTX is: (2~)-2-[[4-[(2,4-diaminopteridin-6-yl) methyl methylamino] benzoyl]amino] pentanedioic acid (4). MTX competitively inhibits the activity of dihydrofolate (DHF) reductase enzyme (DHFR), which is a small protein (~19 kDa, 186 amino acids) that catalyzes the reduction of DHF into tertahydrofolate (THF) (1,5). Moreover, THF is essential for the synthesis of purines and several amino acids, as well as for DNA synthesis (5).

The clinical use of MTX as an antimetabolite in cancer management is associated with dose-dependent toxic adverse effects, such as alopecia, ulcerations, pulmonary toxicity, abdominal discomfort, hepatotoxicity, myelosuppression and nephrotoxicity (6,7). Moreover, the administration of MTX is associated with neurotoxicity that is reported along with neurological complications, delays in treatment and prolonged hospitalization (8,9). MTX administration also causes genetic alterations and DNA damage by enhancing the accumulation of oxidative DNA lesions (10,11). Furthermore, MTX has been reported to cause double-stranded breaks, which can result in chromosomal relocations, and is extremely harmful to dividing cells (12,13). Previous studies have revealed the significant role of oxidative stress as a participating factor in neurotoxicity and hepatotoxicity (14-16). Hence, the excessive production of reactive oxygen species (ROS) contributes to the incidence and advancement of MTX-induced toxicity (17). The redox-state altering properties of MTX have been suggested as an essential immunosuppressive mechanism and found to induce apoptosis via ROS production (1). The produced reactive species react with different biological macromolecules, thereby generating lipid peroxides that are capable of producing additional ROS or converting them into reactive compounds that are able to crosslinks within DNA and proteins, resulting in cellular toxicity (14,18). Thus, it is hypothesized that cellular oxidative damage with lipid peroxidation is a characteristic of MTX-induced toxicity. Moreover, a decrease in tetrahydrobiopterin levels (an important cofactor for nitric oxide synthesis that is produced by the DHFR) potentiates MTX-induced ROS production (1). Antioxidants function to reverse the increase in oxidative stress induced by MTX. Currently, to the best of our knowledge, there are no studies aiming to antagonize the genotoxicity induced by MTX. Thus, identifying an agent that can ameliorate the MTX-induced genotoxicity may significantly improve the outcomes of patients who are administered MTX.

Metformin, a biguanide anti-hyperglycemic drug, is the first-line agent used in the management of type 2 diabetes mellitus. It reduces hepatic glucose synthesis, decreases glucose

Correspondence to: Dr Abeer M. Rababa'h, Department of Clinical Pharmacy, College of Pharmacy, Jordan University of Science and Technology, PO Box 3030, Irbid 22110, Jordan
E-mail: amrababah@just.edu.jo

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intestinal absorption and increases insulin sensitivity by elevating peripheral glucose uptake and consumption (19,20). Additionally, metformin is widely used in the treatment of polycystic ovary syndrome and as an adjunct treatment for cancer (19-22). The molecular mechanisms responsible for the effects of metformin may include, but are not limited to: The reduction of cellular oxidative stress, the suppression of inflammation and the reduction of levels of inflammatory biomarkers through 5' AMP-activated protein kinase-dependent and independent pathways (23). Cheki *et al* (24) reported that metformin has potential protective effects against cisplatin-induced genotoxicity in rat bone marrow cells due to its antioxidant properties. Moreover, another study demonstrated that metformin exerted protective effects against acetaminophen-induced liver toxicity by reducing overall hepatic oxidative stress (25). Thus, the present study aimed to assess the potential ameliorative effects of metformin against MTX-induced genotoxicity in cultured human lymphocytes.

Materials and methods

Participants. In the present study, peripheral venous blood was collected from five male subjects (median age 30 years old). The participants did not consume alcohol or drugs, did not smoke cigarettes/or waterpipe tobacco and were not on any medications. The blood samples were freshly collected from donors (pre-dietary intake) on the same day of the experiments to diminish any possible dietary effects. Written informed consent was obtained from all participants who decided to participate in the present study after providing them with an explanation of the aims and the objectives of the present study, and the present study was approved by the Institutional Review Board of Jordan University of Science and Technology (approval no. 27/132/2020). Additionally, the present study was performed in accordance with guidelines described in the Declaration of Helsinki (26) for research involving humans.

Human blood cell culture and drug treatment. The blood samples were cultured within 1 h of collection. Each sample of freshly drained blood (1 ml) was mixed with 9 ml culture medium (PB-MAX™ Karyotyping Medium; Gibco; Thermo Fisher Scientific, Inc.). PB-Max (Gibco; Thermo Fisher Scientific, Inc.) is an optimized fully supplemented RPMI-1640 medium that includes 15% FBS, L-glutamine and phytohaemagglutinin (a mitogen that stimulates the division of blood lymphocytes) (10,27-29).

A stock solution of 2.2 mM MTX (≥98% purity, Sigma-Aldrich; Merck KGaA) was prepared using sodium hydroxide as a solvent. The final concentration of MTX in the culture medium was 0.5 μM, and it was added to the cell culture 24 h before harvesting. Human cultured lymphocytes require ~24 h to complete one cycle of cell division (30). Therefore, the 24 h exposure window was selected to make sure that cultured cells were exposed to drugs in all phases of the cell cycle. The MTX concentration used was based on previously published literature which showed that MTX-induced chromosomal damage (5,10). Metformin (kindly provided by MS Pharma-United Pharmaceutical Manufacturing Company®) was added to the corresponding flasks. The final metformin concentration in the culture medium was 12 μM and it was added at the beginning of culture. The metformin concentration used in the present study was based on previously published literature (31). To assess the effects of MTX and/or metformin on DNA, four different groups were used: Control, metformin, MTX and metformin + MTX groups. A total of 2 h prior to harvesting, 100 μl 10 μg/ml Colcemid (a spindle inhibitor; cat. no. 477-30-5; Sigma-Aldrich; Merck KGaA) was added to arrest mitosis at the metaphase stage. The procedure of human lymphocyte cell culture preparation was performed according to previously published protocols (10,29,32).

Chromosomal aberration (CA) assay. The cultured cells were transferred into screw capped 15-ml tubes and centrifuged at 134 x g, 4˚C for 10 min. The supernatant was then removed and the pellet was resuspended using pre-warmed KCL solution (0.56% KCL) followed by incubation at 37˚C for 30 min. Subsequently, the lymphocytes were centrifuged for 10 min at 134 x g, 4˚C to collect swollen lymphocytes. The supernatant was then removed and the pellets were fixed by the addition of the freshly prepared fixative, absolute methanol and glacial acetic acid [3:1 (v/v)] in a drop-wise manner, followed by incubation in the dark for 20 min at room temperature. The pellet was then washed three times using the aforementioned fixative solution and suspended in ~1 ml fixative solution. Then, the suspended solution was dropped on pre-chilled slides to disperse the metaphases. Finally, the slides were allowed to dry and were then dyed with 5% Giemsa (Sigma-Aldrich; Merck KGaA) for 4 min at room temperature, rinsed with distilled water and then air-dried for the CA test. CAs were evaluated in 100 well-spread metaphases for each group/donor (two repeats per donor were used) using a high-resolution light microscope (Nikon Corporation) with immersion oil at x1,000 magnification. The CAs were classed as gaps or breaks/exchanges.

Sister chromatid exchange (SCE) assay. The blood cultures were treated with 25 μl 5-bromo-2-deoxyuridine (BrdU 0.01 g/ml; cat. no. 72218-68-9; Gibco; Thermo Fisher Scientific, Inc.) prior to the incubation period. As BrdU is highly susceptible to light, the procedures were performed in the dark to prevent photolysis. The protocols of cell culture preparation, cell harvesting/dropping and slide preparation were comparable to those used in the CA assay. The prepared slides for SCE were stained at room temperature for 22 min with 10 μg/ml Hoechst 32285 dye, then rinsed with distilled water and mounted in McIlvaine buffer (0.18 g citric acid and 2 g sodium phosphate dissolved in 100 ml distilled water; pH 8.0). Subsequently, the prepared slides were exposed to two UV lamps (350 nm) at a distance of 7 cm at 40˚C for 30 min. Subsequently, the slides were washed carefully with distilled water and dried at room temperature. The slides were then stained at room temperature for 4 min using 5% Giemsa stain in Gurr buffer (pH 6.8; Gibco; Thermo Fisher Scientific, Inc.), washed with distilled water and air-dried at room temperature. For SCE scoring, ~50 well-spread second-division metaphases (M2) per donor that contained 42-46 chromosomes were scored using a high-resolution light microscope (Nikon Corporation) with immersion oil at x1,000 magnification as described above. M2 chromosomes were distinguished based on the presence of two differentially stained chromatids (one

Table I. Frequency of chromosomal aberrations per donor induced by the different treatments[a].

Donor/treatment	Frequency of chromatid/ chromosome exchanges	Frequency of chromatid/ chromosome breaks	Frequency of chromatid /chromosome gaps
Donor 1			
Control	0	0.03	0.12
Metformin	0.01	0.03	0.06
Methotrexate	0.06	0.16	0.25
Methotrexate + Metformin	0.05	0.06	0.09
Donor 2			
Control	0.01	0.04	0.1
Metformin	0	0.02	0.05
Methotrexate	0.08	0.17	0.28
Methotrexate + Metformin	0.04	0.07	0.08
Donor 3			
Control	0	0.05	0.13
Metformin	0.01	0.02	0.08
Methotrexate	0.07	0.16	0.25
Methotrexate + Metformin	0.05	0.09	0.07
Donor 4			
Control	0	0.03	0.1
Metformin	0	0.04	0.06
Methotrexate	0.07	0.19	0.26
Methotrexate + Metformin	0.04	0.06	0.09
Donor 5			
Control	0.01	0.04	0.12
Metformin	0.01	0.04	0.05
Methotrexate	0.06	0.22	0.27
Methotrexate + Metformin	0.04	0.08	0.1
Total mean			
Control	0.004	0.038	0.114
Metformin	0.006[c,d]	0.030[c,d]	0.060[b-d]
Methotrexate	0.068[b,d]	0.180[b,d]	0.262[b]
Methotrexate + Metformin	0.044[a,b]	0.072[a]	0.086[a,b]
P-value	P<0.0001	P<0.0001	P<0.0001

[a]Frequency was calculated based on scoring of 100 cells per each treatment/donor. [b]Significantly different from the control group. [c]Significantly different from the methotrexate group. [d]Significantly different from the methotrexate + metformin group.

lightly and the other darkly stained). Conversely, chromosomes in the M1 phase were distinguished by having both chromatids darkly stained. Chromosomes in the M3 phase were differentiated by exhibiting a combination of differentially stained chromatids, whereas chromosomes in the M4 phase had both chromatids lightly stained (10). To perform the cell kinetics analysis, the proliferative index (PI) was calculated via scoring 100 metaphases from each donor using the following formula: (1 x M1 + 2 x M2 + 3 x ≥M3)/100; where M1, M2 and M3 represent the number of cells at the first, second and third metaphases, respectively (29).

Statistical analysis. Data analysis was performed using GraphPad Prism version 7 (GraphPad Software, Inc.). Multiple comparisons were performed using a one-way ANOVA followed by a Tukey's post hoc test. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

The effect of metformin (12 μM) on MTX (0.5 μM)-induced genotoxicity in cultured human lymphocytes was examined using CA assays. The frequency of CAs induced by the indicated drugs is presented in Table I. Treatment of the cultured cells with MTX significantly increased the number of CAs. This included aberration exchanges (P<0.0001), chromatid/chromosome breaks (P<0.0001) and gap aberrations (P<0.0001). With respect to metformin, no significant changes in the frequency of all examined CAs were observed (P>0.05). However,

co-treatment of the cultures with MTX and metformin induced significantly fewer CAs compared with the group treated with MTX alone (P<0.0001). The reduction in the frequency of CAs ranged between 35% of aberrations exchanges to 67% in gap aberrations. The trend in changes in the frequency of CAs induced by MTX and metformin was observed in cells obtained from different blood donors (Table I).

The SCE frequencies were increased in the MTX-treated human lymphocytes compared with other treatment groups (P<0.0001; Fig. 1). When the cells were treated with metformin, lymphocytes were protected against the genotoxic effects of MTX (P<0.0001). The results of the present study revealed that MTX (0.5 μM) led to a statistically significant increase in the frequency of SCEs in normal healthy lymphocytes (P<0.0001). However, pre-treatment of the cells with metformin (12 μM) induced a significant decrease in the SCE frequency; it should be noted that the SCE frequency did not return to normal levels. Furthermore, treatment of the cells with metformin alone did not affect the frequency of SCE compared when compared with the control group (P=0.4278).

The effects of MTX and metformin on the PI are shown in Fig. 2. MTX and metformin, when used alone and when used in combination, induced a significant decrease in the PI compared with the control group (P<0.0001; Fig. 2). In addition, a significant difference in the PI was observed between the group treated with metformin alone and the MTX + metformin-treated group (P<0.0001). Fig. 3 provides representative images and examples of the chromosomal damage (ring and gap aberrations, SCEs and chromosomal exchange) observed in the present study.

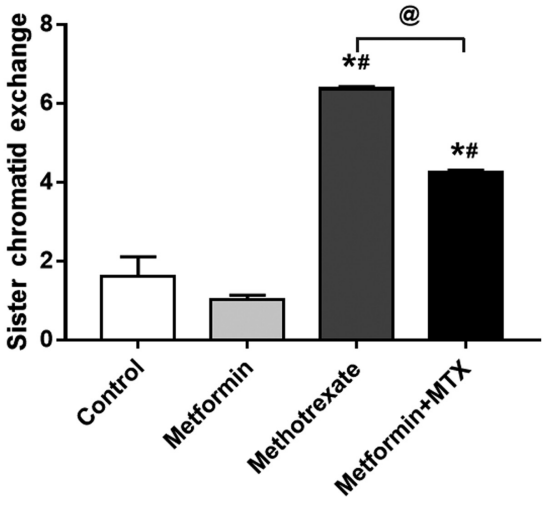

Figure 1. Average frequency of SCEs in the control, metformin, methotrexate, and the metformin + MTX groups. There was an increase in the SCE frequencies in MTX-treated human lymphocytes compared with the other treatment groups (P<0.0001), whereas treatment with metformin alone did not affect the frequency of SCE compared with the control (P=0.4278). Data are presented as the mean ± the standard error of the mean. *P<0.05 vs. control group; #P<0.05 vs. metformin group; @P<0.05 vs. metformin + methotrexate combination group. SCE, sister chromatid exchange; MTX, methotrexate.

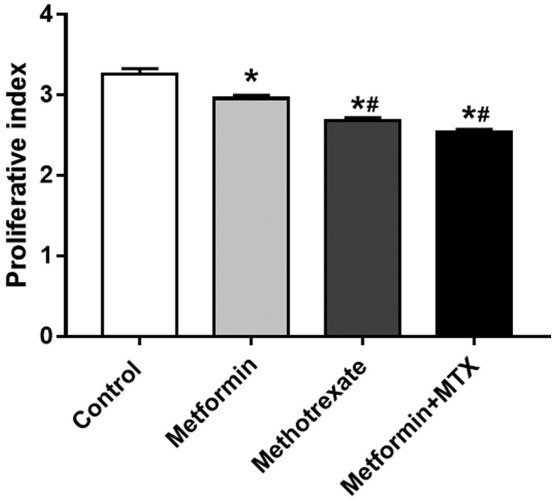

Figure 2. PI of the human cultured blood cells treated with metformin, MTX, and metformin + MTX combination. The figure shows a significant decrease in the PI of MTX and metformin, either alone or in combination, when compared with the control group (P<0.0001). The PI was calculated as follows: (1 x M1 + 2 x M2 + 3 x ≥M3)/100; where M1, M2 and M3 represent the number of cells at the first, second and third metaphases, respectively. *P<0.05 vs. control group; #P<0.05 vs. metformin group. Data are presented as the mean ± the standard error of the mean. PI, proliferative index.

Discussion

Previous studies have shown that treatment with several of the most effective anticancer agents causes direct cellular toxicity (1,16,28). Furthermore, treatment with various anticancer agents has been demonstrated to exert carcinogenic, teratogenic and mutagenic effects in experimental environments. For example, MTX administration has been shown to enhance the accumulation of oxidative DNA injuries, which, in turn, have been shown to induce DNA damage (11,33). Hence, protecting normal cells from conditions that may cause malignancy is an essential means to inhibit long-term impairments or damage due to the administration of chemotherapeutic agents.

Metformin remains the first-line medication used for the treatment of patients with type 2 diabetes mellitus. Several studies have demonstrated that metformin exhibits antioxidant, anti-apoptotic and anti-inflammatory properties (34-37). The aim of the present study was to investigate the potential ameliorative effects of metformin on the genotoxicity induced by MTX in human cultured lymphocytes. The cytogenetic effects were examined by performing CA and SCE assays *in vitro*. Both CAs and SCEs are commonly utilized as determining factors of DNA damage and genotoxicity, and both assays are widely used for examining the genotoxicity of therapeutic drugs and environmental agents (38-41).

The primary results of the present study indicated the ability of metformin to decrease the genotoxicity induced by MTX, as shown by the reduction in the frequencies of CAs and SCEs in the cells pre-treated with metformin. The results obtained in the present study revealed that the SCE and CA frequencies in the MTX treated cells were significantly higher compared with those in the control group, showing the genotoxic effects of MTX on normal healthy cells. These findings are in agreement with those of a previous study, where the genotoxicity of MTX was also shown (10). Moreover, it has been shown that MTX substantially increases the frequency of CAs in cultured human lymphocytes (42). Similarly, Atteritano *et al* (43) demonstrated a marked increase in the SCE frequency in the MTX group in patients with rheumatoid arthritis. Another

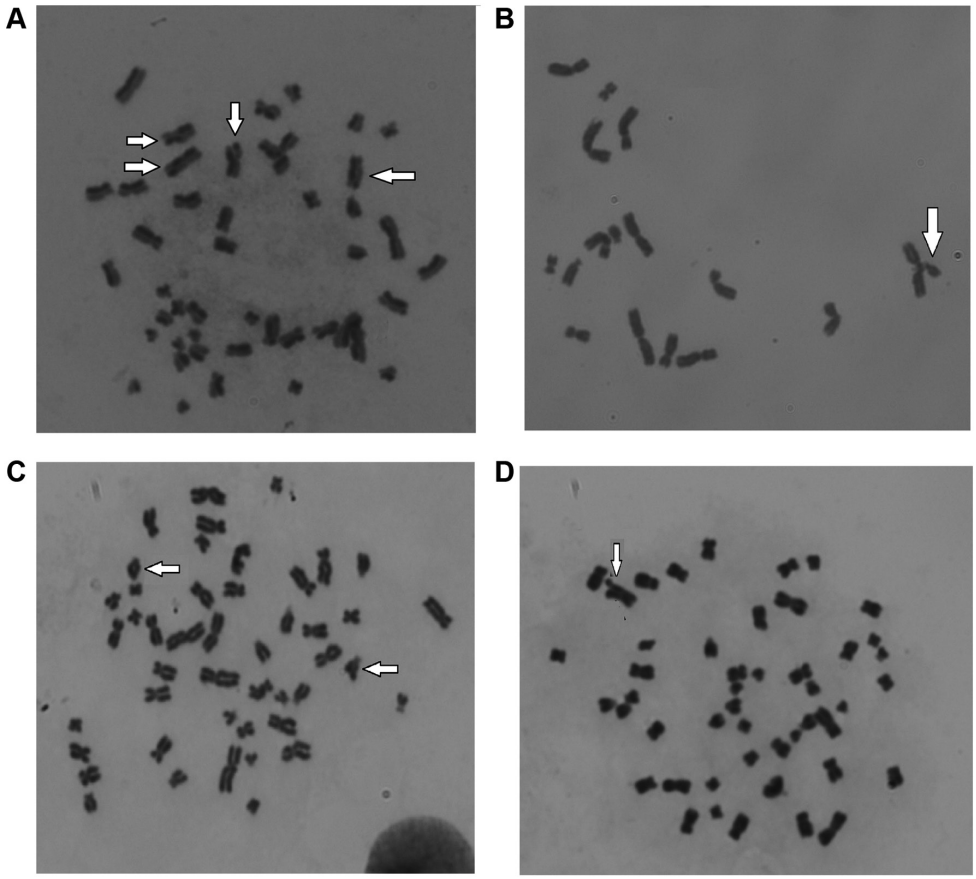

Figure 3. Representative images of chromosomal damage. (A) Several SCEs indicated by different arrows. (B) A chromosomal exchange involving 3 chromosomes. (C) A ring aberration (left arrow) and a terminal deletion (right arrow), and (D) a gap aberration. SCE, sister chromatid exchange.

study by Said Salem *et al* (44) reported a significant increase in the number of micronucleated polychromatic erythrocytes in mouse bone marrow cells treated with MTX compared with the corresponding controls.

A suggested mechanism underlying the increase in CA and SCE frequencies in cultured lymphocytes treated with MTX may involve the generation of ROS induced by MTX, which potentiate cellular damage (25). Several anticancer agents, including MTX, induce cellular genotoxicity via DNA oxidation, ROS production and reducing the total antioxidant capacity (17,44). Several studies have demonstrated that metformin exerts antioxidant activities by reducing the malondialdehyde serum concentrations and increasing the activities of superoxide dismutase, catalase and glutathione reductase (45-47). The results of the present study revealed that the administration of metformin decreased the chromosomal damage induced by MTX, as evidenced by a prominent decrease in the CA and SCE frequencies. Collectively, it was suggested that metformin can attenuate the genotoxic effects on normal cells induced by MTX by attenuating oxidative stress through reducing ROS generation. This finding is in agreement with the results reported in an animal study by Ashoka and Mustak (48), which revealed the protective effect of rutin, a potent antioxidant flavonoid composite, in preventing MTX-induced genotoxicity. However, further human *in vivo* studies are required to confirm these results.

The results of the present study demonstrated that metformin reduced the incidence of spontaneous SCE, break and gap frequencies compared with the control. Hence, metformin appears to decrease the spontaneous levels of SCEs and gap aberrations, possibly via the reduction of the basal oxidative stress level. However, the detailed mechanisms responsible for this suppressive effect requires further investigation.

The protective effects of metformin on DNA damaging agents was documented in several studies. For example, using HepG2 cells, metformin has been shown to protect against DNA damage induced by formaldehyde (49). In a study that was performed using cells derived from elderly subjects, metformin has been shown to protect against pro-oxidant stimulus-induced DNA damage (50). Finally, using human A549 cells, metformin conferred protection against UVC-induced DNA damage (51). Thus, using different models, metformin seems to be a potent option for use with agents that induce cellular DNA damage.

In the present study, to assess the cytotoxicity of MTX and metformin, cell kinetic analysis was performed, which involves the determination of the PI. Following treatment of the cultured cells with MTX for 24 h, it was found that MTX was cytotoxic to healthy normal human lymphocytes, as evidenced by the significant reduction in the calculated PI. However, metformin was not capable of attenuating the cytotoxic effects of MTX, and a slight further decrease in the PI was observed with the use of metformin. The results of the present study are consistent with those of previous research, where it has been demonstrated that MTX reduced the PI of cultured human lymphocytes (10) and other cell types, such as neurons and

cancer cells. However, in a study that was conducted on rats, treatment of animals with metformin ameliorated the reduction in the number of proliferating cells, and the survival of the cells and immature neurons induced by MTX in the brain (52). Thus, the combined effects of metformin and MTX could differ according to model and or tissues used. The mechanisms by which MTX induced reduction in cellular proliferation include the induction of the intracellular ROS, interference with pyrimidine metabolism, activation of cellular apoptosis, reduction of methyltransferase activity and the reduction in cellular utilization of folates (53,54). Therefore, the observed weak impact of metformin on MTX-induced inhibition of cellular proliferation could be due to the multiple mechanisms utilized by this drug to mediate its effects. Collectively, both metformin and MTX appeared to modulate changes in the PI in cultured human lymphocytes; pre-treatment of the cells with metformin did not lead to any marked alterations in the MTX-mediated reduction in PI. The PI was evaluated to examine the influence of metformin on the cytotoxicity of MTX, and was not predicted to imitate the genotoxicity findings.

The present study has some limitations, including the subjectivity in the genotoxicity parameter scoring (CAs, SCEs and PI), and the lack of *in vivo* experiments. Hence, further *in vitro* and *in vivo* studies are required to confirm the findings presented herein, and to extrapolate a small *in vitro* study to a large comprehensive analysis using larger sample sizes. Moreover, MTX genotoxicity was detected at 24 h following treatment with a single dose. Thus, further studies investigating the dose-response effects with longer treatment durations are required. This could provide a basis for future experimental study to improve our understanding of the molecular mechanism underlying the actions of MTX. Finally, in the present study, oxidative stress biomarkers and how they are modulated by the treatment were not investigated. Such experiments are recommended in future investigations.

In conclusion, the present study demonstrated that MTX was genotoxic to normal human cultured cells, as illustrated by the results of the CA and SCE assays. Conversely, metformin exerted an ameliorative effect against MTX-induced chromosomal injury, as it significantly reduced the CA and SCE frequencies. As the present study was conducted using an *in vitro* lymphocyte cell culture model, the results are not generalizable to other cell types, and thus additional studies are required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AMR, MA and KHA conceived the study. AMR, KHA, OFK and MA designed the study. AMR, KHA and OFK performed the experiments. AMR and OFK analyzed the data. AMR, OFK and MA wrote the manuscript. AMR, KHA, OFK and MA edited the manuscript. OFK and AMR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Jordan University of Science and Technology (approval no. 27/132/2020). Written informed consent was obtained from all study participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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