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The ameliorative effect of methotrexate on the biochemical changes in rats' experimentally-induced hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a deadly and intricate cancer primarily linked to oxidative stress and inflammation in the hepatic tissue. This study attempted to look into the impact of methotrexate (MTX) on HCC. Three groups of rats were categorized. Group I (a normal control group). Group II (Diethylnitroseamine (DENA)/Carbon tetrachloride (CCL_4) group): HCC group; 200 mg of DENA per kg b.w. i.p. was administered to rats once. 3 ml of CCl_4 per kg b.w. s.c. per week for six weeks, following two weeks of DENA application to enhance the carcinogenic impact. Group III (treated): methotrexate (MTX) treated; rats were given MTX at a dose of 0.1 µM/week/ i.p. for 4 weeks after receiving injections of DENA/CCL_4 at doses similar to those in group 2. DENA/CCL_4 injection resulted in a substantial increase in serum AST, ALT, ALP activities, hepatic inflammatory markers (IL-6, TNF-α), and hepatic MDA levels, in addition to a non-significant decrease in hepatic TAC level. Treatment with methotrexate in HCC rats caused marked improvement effects in all biochemical parameters. According to these findings, methotrexate might be considered an effective option in hepatic cancer treatment.

1. INTRODUCTION

Currently, cancer remains the deadliest disease affecting humans, even with significant improvements in treatment (Saad et al., 2017). Death rates from cancer are highest in the developing nations (El-Aassar et al., 2019). The liver cancer ranks third overall in terms of mortality worldwide (Aboseada et al., 2021). In 2020, 7.69% of fatalities attributable to cancer worldwide were caused by liver cancer (Hassona et al., 2022).

An N-nitroso alkyl molecule known to be a strong hepatotoxin is diethylnitrosamine (DENA). Preclinical research has often employed DENA-induced HCC models to investigate HCC pathogenesis and to test new medications. Following diethylnitrosamine administration, DENA is converted in hepatocytes into intermediary metabolites that interact with DNA to produce an intricate structure that has a higher chance of undergoing carcinogenic alterations. Highly reactive and unstable molecules, which are produced by the metabolic processes involved in the transformation of DENA, cause hepatic inflammation, apoptosis, and necrosis. DENA hepatotoxic effects could potentially participate in the induction of hepatic cancer (Santos et al., 2017). By ethylating different nucleophilic sites in deoxyribonucleic acid, DENA generates tiny foci of dysplastic hepatocytes (Newell et al., 2008). DENA injections combined with other non-genotoxic carcinogens such as carbon tetrachloride (CCl_4) have produced HCC development in rats. According to some research, the only way to successfully induce HCC tumors in rats is DENA administration (Vedarethinam et al., 2016).

Among the many chemotherapeutic medications, methotrexate (MTX) is a significant one that has been used for many years to treat cancer, including non-Hodgkin lymphomas, acute lymphoblastic leukemia, osteosarcoma (Chen et al., 2020). According to Zhao et al. (2022), MTX is an antimetabolite of folate with remarkable counter-inflammatory properties. Zheng and Cantley (2019) stated that owing to their structure resemblance, MTX inhibits folic acid effects. Folic acid is a crucial helper molecule for several enzymes involved in the manufacture of purines, thymidine, and methionine as well as the translation of mitochondrial proteins. This prevents the cell from dividing, inhibits DNA synthesis and repair, and ultimately causes the cell to die (Wojtuszkiewicz et al., 2015).

The current work was performed to evaluate methotrexate's therapeutic benefits on liver cancer caused by diethylnitrosamine/CCl_4.

2. MATERIAL AND METHODS

2.1. Experimental animals:

Forty-five male albino rats weighing 120-170 grams were purchased from the Faculty of Veterinary Medicine, Benha University. Throughout the experiment, the rats were kept in special boxes with optimal environmental, and nutritional circumstances. The rats were allowed to acclimate for 2 weeks earlier than when the experiment started. The experiment methods were authorized by

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Benha University's ethical animal committee (BUFVTM 11-12-22).

2.2. Chemicals

Diethylnitroseamine carbon tetrachloride and methotrexate were bought from Sigma-Aldrich, St. Louis, MO, USA.

2.3. Experimental design

Following the adaption period, rats were handled as follows and equally split into three groups:

*G*roup I (a normal control group): Every day of the trial, rats received an oral gavage of 1 mL of physiological saline.

Group II (HCC group): Rats received 200 mg per kg b.w. once intraperitoneally injection of DENA. Following two weeks of DENA treatment, rats were weekly given 3 ml of CCl_4/kg b.w. s.c. for six weeks to enhance the carcinogenic impact (Sundaresan and Subramanian, 2003).

Group III (Methotrexate treated group): Rats were treated with DENA and CCL_4 injection as in group 2 (Sundaresan and Subramanian, 2003), and then subsequently treated with methotrexate (0.1 µM/week/i.p.) for four weeks (Yiang et al., 2014).

2.4. Sampling

2.4.1. Blood Samples

Samples of blood taken from the eye's retro-orbital plexus were centrifuged at 3,000 rpm for 15 minutes and sera were preserved at -20 °C to assess the biochemical parameters.

2.4.2. Liver tissues for biochemical analysis

To create 10% homogenate, minced liver tissue (one gram) was homogenized in nine-volume ice-cold potassium phosphate buffer **(**0.05 mM, pH 7.4**)**, and centrifuged for 15 minutes at 4°C and 6000 rpm. The biochemical parameters were measured in the supernatant.

2.5. Analysis

ELISA kits were used to measure activities of serum aspartate aminotransferase (AST) (ab263883), alanine transaminase (ALT) (ab285264), and alkaline phosphatase (ALP) (ab287823), hepatic interleukin-6 (IL-6) (CSB-E04640r) and tumor necrosis factor-alpha (TNF-α) (ab46070) levels, hepatic malondialdehyde (MDA) (CSB-E08558r) and total antioxidant capacity (TAC) (ZX-44109-96) levels. The supplier's protocols were followed during all the assays.

2.6. Statistical Analysis

The data were statistically analyzed using SPSS version 25 (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) with Duncan's post-hoc test was used to determine the significant differences between the groups. The statistical significance was set at $P \leq 0.05$.

3. RESULTS

3.1.Assessment of hepatic tissue injury markers

When compared to a normal control group, rats administered with DENA/CCL_4 to cause liver damage showed a substantial rise in AST, ALT, and ALP activities; in contrast, the MTX-treated group when compared to the HCC group, showed a notable decline in AST, ALT and ALP activities (Table 1).

3.2.Assessment of hepatic inflammatory markers

When comparing the HCC group to the control group, inflammatory markers like hepatic IL-6 and TNF-α levels were noteworthy elevated; however, in the group that received MTX, these elevations of IL-6 and TNF-α levels were significantly decreased in comparison to the HCC group (Table 2).

3.3.Assessment of hepatic oxidative stress and anti-oxidant markers

Rats administered DENA/CCL_4 had a notable rise in liver MDA level and a non-notable decline in liver TAC level in comparison to a control group. Fortunately, rats treated with MTX revealed both a notable rise in liver TAC level and a notable decline in liver MDA level (Table 3).

Table 1 Methotrexate's impact on some serum parameters in rats' experimentally-induced hepatocellular carcinoma (Mean ± SEM)

Animal Groups	Normal control Group	DENA + CCL4 (HCC) group	MTX treated group
AST (U/L)	65.08 ± 3.36^{c}	225.93 ± 8.19^{a}	163.00 ± 6.58^{b}
ALT (U/L)	44.24 ± 6.83^{c}	202.56 ± 28.02^{a}	133.43 ± 3.27^{b}
ALP (U/L)	77.44 ± 4.17^{c}	134.48 ± 3.75^{a}	104.21 ± 3.99^{b}

Values with different superscripts within the same row differed significantly at P<0.05.

Table 2 Methotrexate's impact on hepatic inflammatory markers in rats' experimentally-induced hepatocellular carcinoma (Mean ± SEM)

Animal Groups	Normal control Group	DENA + CCL4 (HCC) group	MTX treated group
IL-6 (ng/g tissue)	1.26 ± 0.14^{b}	4.20 ± 0.68^{a}	1.76 ± 0.35^{b}
TNF-α (ng/g tissue)	10.71 ± 0.83^{c}	53.28 ± 5.12^{a}	33.80 ± 2.89^{b}

Values with different superscripts within the same row differed significantly at P<0.05.

Table 3 Methotrexate's impact on hepatic oxidative stress and anti-oxidant markers in rats' experimentally-induced hepatocellular carcinoma (Mean ± SEM)**.**

Animal Groups	Normal control Group	DENA + CCL4 (HCC) group	MTX treated group
MDA (nmol/g)	22.95 ± 3.06^{c}	81.93 ± 1.30^{a}	39.72 ± 3.57^{b}
TAC (µM/100mg tissue)	2.51 ± 0.31^{b}	1.22 ± 0.08^{b}	5.89 ± 1.17^{a}

Values with different superscripts within the same row differed significantly at P<0.05.

4- DISCUSSION

The majority of occurrences of liver cancer are hepatocellular carcinoma (HCC), which is the third most common cause of cancer-related deaths globally (Kinsey and Lee, 2024). One of the most popular and successful medications for treating severe and resistant forms of immune-mediated diseases or various types of cancer is methotrexate, a structural analogue of folic acid (Koźmiński et al., 2020). The present study aimed to investigate the efficacy of methotrexate against diethylnitrosamine/CCl4-induced HCC in a rat animal model.

Highly reactive and unstable molecules produced by DENA/CCL_4 further interact with biomolecules (lipids and proteins) that interfere with organelles within cells, particularly the nucleus, resulting in damage to DNA and RNA, consequently leading to the formation of HCC (Bashandy et al., 2023). Diethylnitrosamine (DENA) is a nitrosamine type that is known to create DNA adducts, which in turn induce liver cancer (Somade et al., 2021). DENA's production of oxidative stress further complicates the underlying causes of hepatic cellular carcinoma (Kabil et al., 2021). DENA and other recognized cancer causative agents promote cell proliferation, which leads to hepatic cells necrosis and damage (Sulaimon et al., 2021). Laboratory animals are an important research way to

studying hepatic cellular carcinoma and are widely used in cancer studies. In rat models, DENA has been demonstrated to cause early deteriorating assault, inflammatory response, and proliferation (Shetty et al., 2021). Rupturing the integrity of cell membranes through lipid peroxidation caused by the highly reactive free radical metabolites of CCL_4 can damage liver tissue. This can result in compensatory proliferation of cells and an increase in the frequency of DNA mutations, which can ultimately lead to cancer (Manibusan et al., 2007). Inducing HCC is often effective when DENA is used as an initiator and CCL_4 is used as a promoting agent (Hui et al., 2019).

Methotrexate functions as an antifolate antimetabolite in cancer. Methotrexate-polyglutamate is formed when methotrexate is absorbed into the cell via carriers known as human reduced folate carriers. The enzyme dihydrofolate reductase, which catalyzes the conversion of dihydrofolate into tetrahydrofolate, the active form of folic acid, is inhibited by both methotrexate and methotrexate-polyglutamate (Mikhaylov et al., 2019). The synthesis of the nucleotides in both DNA and RNA requires tetrahydrofolate. DNA synthesis is further inhibited by methotrexate-polyglutamate, which also prevents thymidylate synthase and purine from synthesizing de novo. Because of its cytotoxic effect, this mechanism is used in the treatment of cancer (Singh et al., 2019).

When compared to the normal control group, serum liver enzymes like ALT, AST, and ALP were found to be significantly elevated in the DENA/CCL_4 group. This indicates that the chemical agents such as DENA used for the induction of hepatocarcinogenesis caused liver damage, as reported by multiple studies using animal models of HCC (You et al., 2021; Yassin et al., 2022). The increase in serum transaminase activity is a defining feature of liver damage caused by CCl_4. The extracellular fluids contain the ALT, AST, and ALP enzymes, which are released by injured hepatocytes and exhibit elevated activity (Alimullah et al., 2024). However, as a result of MTX treatment, these parameters significantly decreased in the MTX-treated group at the end of the current investigation. These findings were consistent to the research published by Saleem et al. (2020). According to Talwar and Srivastava (2003), these outcomes might be because the numbers of damaged liver cells were lowered in the MTX-treated group.

Tumor-associated macrophages and neoplastic cells both release TNF-α and IL-6 (Dranoff et al., 2004). According to Nadia et al. (2023), cytokines including TNF-α and IL-6, which are mostly produced in kuffer cells, have been shown to cause hepatic oxidative stress, which could lead to inflammation. As expected, we found that the HCC group had higher levels of TNF-α and IL-6 than the normal control group. These results were in line with the findings published by Hamid et al. (2017), who reported that CCl_4 increased the proinflammatory cytokines TNF-α and IL-6. Also these results in line with the findings published by Sánchez-Meza et al. (2023), who found that diethylnitrosamine+2-acetylaminofluorene raised the mRNA expression level of IL-6 and TNF-α. The pro-inflammatory effects of DENA/CCl_4 might be the reason of our results of elevated IL-6 and TNF-α level. These findings matched the results of a research published by Uehara et al. (2014). On the other hand, MTX therapy decreased TNF-α and IL-6 levels. Thus, the current study suggests that MTX treatment lowered inflammation via lowering levels of TNF-α and IL-6. These findings were close to the findings of study published by Zălar et al., (2021). Our findings would suggest that, in cases of hepatocarcingenesis, a low dose of MTX may be advantageous for liver health.

Malondialdehyde (MDA) measured as a lipid peroxidation marker is a commonly used as an indicator of oxidative stress to determine the degree of oxidative injury in patients with hepatic impairment (Baltacıo˘glu et al., 2014). When compared to the normal control group, our findings showed a significant increase in MDA level and a concurrent decrease in total antioxidant capacity, indicating the oxidative toxic effect of DENA/CCl_4. These outcomes were in line with the findings reported by Abouzed et al. (2022), who reported that rats treated with DENA/CCl_4 showed decreased liver antioxidant enzyme activity and a significant increase in MDA level. According to earlier study on DENA-induced hepatocellular carcinoma, oxidative damage and ROS formations led to the development of carcinogenesis, which was characterized by inflammation, decreased antioxidant enzyme activity, and antioxidant depletion (Abdu et al., 2022). Rats given DENA showed decreased enzymatic antioxidant activity and elevated MDA formations (Zhang et al., 2023). Chronic CCl_4 treatment has been shown to be characterized by lipid peroxidation and endogenous antioxidant exhaustion (Chang et al., 2021). These outcomes were similarly to the results published by Ogaly et al. (2022), who found that intrahepatic MDA generation was greatly elevated and liver TAC was dramatically decreased following an 8-week chronic treatment of CCl_4. Furthermore, it is well known that trichloromethyl free radical (CCl_3*) and trichloromethyl proxy free radical (CCl_3OO*) are the two metabolites that are usually produced when cytochrome P-450 facilitates the biotransformation of CCl_4. According to Khan et al. (2016), these metabolites induced lipid peroxidation, highly reactive and unstable molecule formation, and a decrease in the activity of the liver's antioxidant enzymes.

In comparison to a normal control group, the current outcomes demonstrated a notable rise in the hepatic MDA level in the HCC group. These results could be attributed to DENA, a strong carcinogenic compound that causes deteriorating assault by releasing highly reactive and unstable molecules, that in turn damages cells when it is metabolized. This result is in line with the findings published by Imosemi and Owumi (2022). The current investigation revealed that in the HCC group, total antioxidant capacity was lower than that of a normal control group. This outcome was aligned with a study conducted by Mohseni et al. (2019). Our findings demonstrated that methotrexate treatment raised TAC and decreased MDA levels, indicating that MTX can both lower oxidative stress and boost antioxidant capability. The results of studies reported by Dogru et al. (2019) and Zălar et al. (2021) support our findings. The stable adduct malondialdehyde-acetaldehyde (MAA) is formed when proteins and lipoproteins react with MDA and AA (acetaldehyde) produced by lipid peroxidation (Kim et al., 2016). Studies have demonstrated that MTX blocked the creation of MAA adducts, and thus lowered ROS levels. Also, MTX exhibits inherent antioxidant qualities that allow it to scavenge free radicals, particularly superoxide (O^{2-}). This reduces intracellular oxidative stress (Zalewska et al., 2019).

5. CONCLUSIONS

In conclusion, methotrexate, by preserving hepatic functions, reducing inflammation, and increasing

antioxidant activity, may help to improve liver status hepatocarcinogenesis.

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