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The Effect of Curcumin on the Activity of MMP-17 and MMP-24 in Hepatocytes of Mice Exposed to Thioacetamide

Sahar Farzaneh[1], Masoud Salehipour*[1], Farzaneh Tafvizi[1], Vahid Naseh[1]

Abstract

Background: Hepatocellular carcinoma is the most primitive form of liver cancer, which is related to chemo carcinogens such as thioacetamide (TAA) and tissue remodeling molecules such as Matrix metalloproteinases (MMPs). Antioxidants, like curcumin (Cur), can inhibit these factors. In this research, the effect of curcumin on the expression and activity of two MMP enzymes, MMP-14 and MMP-17, which are involved in the carcinogenesis of mice after chronic exposure to thioacetamide, is investigated.

Methods: In this study, 30 mice were divided into six groups and studied for 4 months. The first group, control; the second group, curcumin; the third group, TAA; the fourth group, TAA and curcumin simultaneously; the fifth group, first treated with TAA for 2 months and then curcumin; and finally, the sixth group, first treated with curcumin for 2 months and then TAA. Afterward, the mice were euthanized, and their liver tissues were transferred to the laboratory for analysis of gene and protein expression.

Results: The averages of gene expression were calculated using SigmaPlot software and showed that the expression of MMP-17 and MMP-24 genes and the levels of their proteins were significantly increased by thioacetamide (****$p<$ 0001) compared to the control group. Pathological observations indicated necrosis and dysplastic foci in the TAA group.

Conclusion: Considering the crucial roles of MMPs in various diseases, including hepatocellular carcinoma, the regulation of their gene expression and enzymatic activity is significant in preventing tumor progression. Compounds such as thioacetamide and polyphenols like curcumin can modulate the activity of MMP-17 and MMP-24.

Keywords: Carcinoma, Curcumin, Hepatocellular, Matrix Metalloproteinases, Thioacetamide.

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, which occurs after long-term exposure of liver cells, hepatocytes, to various agents such as chemicals, radiation, alcohol and hereditary factors, including abnormal activation of signaling pathways and disruption of the balance between activation and deactivation of proto-oncogenes and anti-oncogene (1,2).

Matrix metalloproteinases (MMPs) are the key mediators of tumor progression. These are the large group of proteases that play an important role in tissue remodeling, breaking down the extracellular matrix, collagens and gelatins through various physiological processes. The activity of MMPs increases under pathological conditions such as diseases or after exposure to mechanical stimuli, due to the release of pro-inflammatory cytokines. Matrix metalloproteinases are secreted by various types of connective tissue and pro-inflammatory cells such as fibroblasts,

1: Department of Biology, Islamic Azad university of Parand Branch, Parand, Iran.
***Corresponding author: Masoud Salehipour; Tel: +98 21 56733158; E-mail: m.salehipour@piau.ac.ir.**
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osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes (3, 4).

The MMP-17 and MMP-24 are membrane MMPs, known as MT4-MMP and MT5-MMP, respectively (5-7). It seems that MMP-17 is involved in the process of tumorigeneses, metastasis, and other diseases like arthritis (8, 9). MMP-24 is mainly expressed in the brain, kidney, pancreas, and lung (7). Studies have shown that MMP-24 has proteolytic activity against pregelatinize A, which leads to creation of the active form of this enzyme and promotes tumorigenesis (9). Therefore, like many other cancers, an increase in the expression of these two MMPs is expected in liver cancer.

Thioacetamide used in the industry, is a liver carcinogen toxin causing hepatocellular carcinoma through various pathways. This compound induces malignant neoplasia by damaging cell signaling pathways, increasing the risk of genetic errors, and stimulating cell development by affecting proliferation, differentiation, and apoptosis mechanisms (10-14). Long-term exposure to thioacetamide can also cause biliary dysplasia and cholangiocarcinoma (15).

Curcumin (Cur) is the main active polyphenol derived from turmeric, (Curcuma longa), and possesses antioxidant activity by removing chemicals through enzyme activity. Curcumin is used as a food supplement and is beneficial to the recovery of liver and cardiovascular disorders, diabetes, infertility, and various cancers. Many studies have revealed the positive molecular effects of this substance in cancer treatment (16-18).

This study aims to investigate the effect of curcumin on gene expression, protein level, and activity of MMP-17 and MMP-24 enzymes in mice after chronic exposure to thioacetamide.

Materials and Methods

Animal model

In this study, 30 NMRI male mice (6–8 weeks) were obtained from Razi Institute and kept at the animal house of Azad University of Parand under controlled conditions. The temperature was maintained at 22±1 °C, humidity at approximately 60±10%, and a 12:12-hour light-dark cycle was followed. This study adheres to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health, as well as the Animal Care and Utilization Committee of the University of Parand (NO: IR.IAU.VARAMIN.REC.1399.003), Iran.

Experimental Design

The study involved six groups of mice, each consisting of five individuals:

1. Control Group: Received a normal diet.
2. Curcumin Group: Received daily oral administration of curcumin (Sigma, Germany) at a dose of 15 mg/kg body weight via gastric gavage.
3. TAA Group: Treated with thioacetamide (Sigma, Germany) to induce liver cancer.
4. Simultaneous Treatment Group: Administered both thioacetamide and curcumin simultaneously.
5. TAA-Then-Curcumin Group: Treated with thioacetamide for two months, followed by curcumin treatment.
6. Curcumin-Then-TAA Group: Administered curcumin for two months, followed by thioacetamide treatment.

To induce liver cancer, a concentration of 200 mg/L thioacetamide (equivalent to 33 mg/L per day for each mouse, based on an average body weight of 40±540 \pm 540±5 g) was dissolved in drinking water. This solution was provided to the mice for 14 weeks. After 14 weeks, the mice were anesthetized using a combination of ketamine and xylazine (BREMER, Germany) at a 1:3 dose ratio. The liver tissues were collected and divided into two parts. One part was preserved in a 10% formalin solution (Merck, Germany) for histopathological analysis, while the other part was stored in microtubes for laboratory analysis of gene expression and protein levels.

Real-Time PCR

Liver tissue RNA was extracted by Trizol (Invitrogen, USA). The purification was determined by measuring the absorbance at 260

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nm/280 nm (Nanodrop, Biotek, USA) and agarose gel electrophoresis (Razitajhiz, Iran). The extracted RNA was converted to complementary DNA (cDNA) using the Takara kit (Japan). The expression levels of mRNA transcripts were quantified by real-time PCR. The real-time PCR was performed using SYBER Green PCR on Rotor Gene 6000 (Corbett, Australia). The PCR product was detected by monitoring the fluorescence increase due to the binding of SYBR Green to double-stranded DNA.

Specific primers (Takapou zist, Iran) were designed for the MMP-17 and MMP-24 genes by Allel ID (V6) software (Table 1).

Table 1. Primers used in this study.

Primer	Forward	Reverse
MMP-17	CACCCACTTTGATGACGATG	CCCTGGTAGTACGTTGCAT
MMP-24	TATCATGCTCCCTTCTACAATA	CTGCGGACCGGGAGTGT

The cycling parameters were as follows: 94 °C for 15 minutes for one cycle, followed by 45 cycles of 94 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. The efficiency of the reaction was checked using LinRegPCR and delta-delta CT (ΔΔCT). The housekeeping GAPDH transcript was used to normalize the amount and quality of the RNAs.

Western Blotting

The liver cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Abcam, Cambridge, MA, USA) and quantified by the Bradford method. 40 µg of protein per sample was separated onto 10% SDS-PAGE gels and transferred onto polyvinyl difluoride (PVDF) filter membrane (Bio-Rad, Hercules, CA, USA). Then, PVDF membranes were blocked with 5% skim milk in PBS for 2 h and incubated with rabbit anti-MMP-17 and anti-MMP-24 (1:1000, ABcam Corp (ab51075), USA), at 4 °C overnight. Finally, membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit) (1:10000, ABcam Corp (ab205718), USA). The immuno-reactive bands were visualized with an infrared image processing system to analyze the molecular weight and optical density (19).

Zymography

Samples from the concentrated conditioned media were added to the electrophoresis sample buffer. Twenty microliters of each sample was loaded into each lane of a 10% sodium dodecyl sulfate polyacrylamide gel containing 0.1% gelatin and fibrinogen as a specific substrate of MMP-24 and MMP-17 enzymes, then electrophoresed at 25 mA and 4 °C for 1.5 h. Page Ruler pre-stained protein ladder plus (Fermentas) was used as the molecular weight marker standard. After electrophoresis, the gels were gently soaked in 2.5% Triton X-100 at 37 °C twice for 30 min each. The gels were then incubated in metalloproteinase activation buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$, and 0.01% NaN_3) overnight at 37 °C. The gels were rinsed in distilled water, stained with 0.5% Coomassie blue R-250 for 2 h, and destained with 40% methanol and 10% acetic acid until appropriate color contrast was achieved. The clear bands on the zymogram were indicative of the enzyme activity (20-22).

Histopathology

Liver tissue samples were fixed in 10% formalin (Merck, Germany), embedded in paraffin, and sectioned with a microtome (Poya Ahraz, Iran) to obtain 5 µm thick slides. The slides were then stained with hematoxylin/eosin (SIGMA), and the morphology of the hepatocytes wall, nuclei, as well as the number and degree of damage, were evaluated using an optical microscope (OLYMPUS).

Statistical analysis

All results are presented as means±S.D. Differences between the groups were

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determined using one-way ANOVA and Tukey post-hoc multiple comparison tests. The level of significance for all statistical analyses was set at $p< 0.05$. Analysis was performed using SigmaPlot (V12.2) software.

Results

The effect of Cur and TAA on the expression of MMP-17 and MMP-24 genes in different groups

The expression of the MMP-17 gene in mice exposed to TAA significantly increased compared to the control group and the groups which were first treated with thioacetamide and then curcumin (green column) (****p= 0.0001). The level of MMP-17 in the group that was first treated with Cur and then TAA showed a slight increase (light blue) (*p= 0.05). Additionally, in the group treated with TAA and Cur simultaneously, the level of MMP-17 increased (p= 0.9571), but it was not statistically significant (Fig. 1).

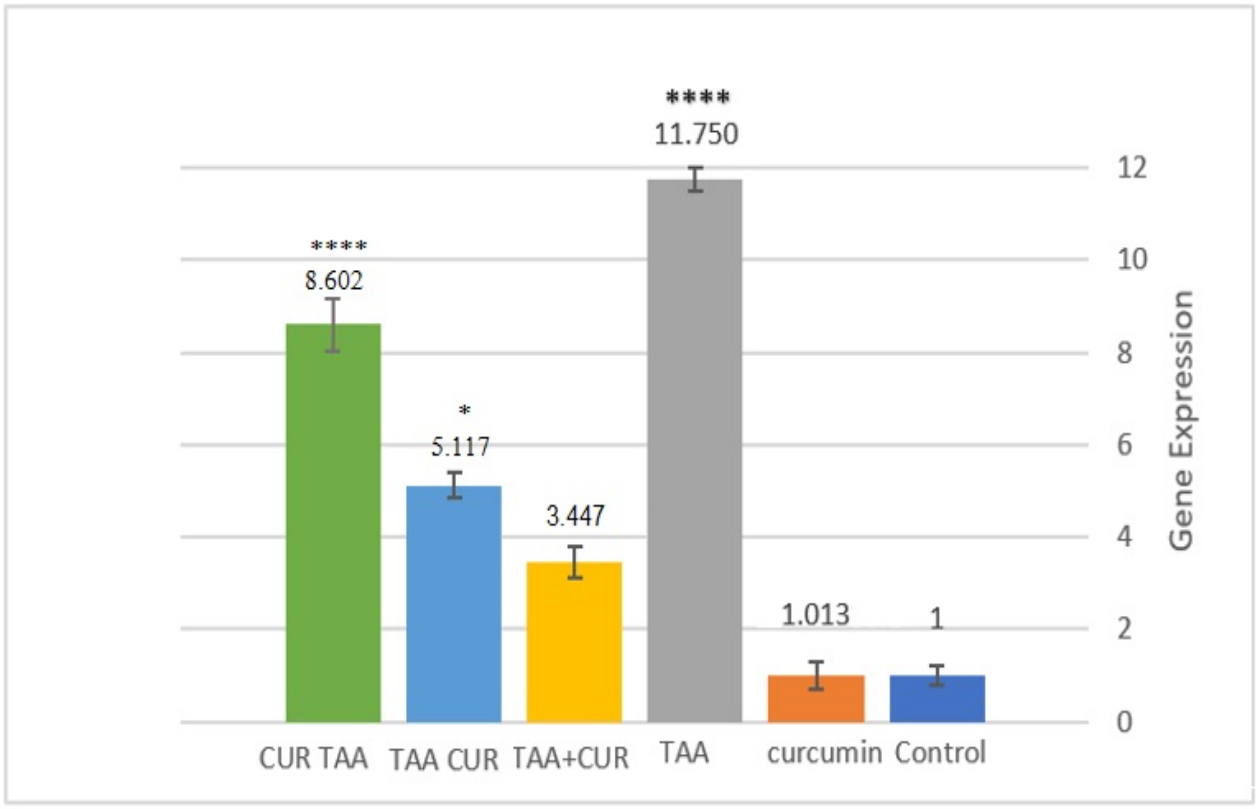

Fig. 1. The expression of the MMP-17 gene in different groups. In the group receiving TAA (thioacetamide), the level of MMP-17 expression was significantly higher compared to other groups receiving TAA. The mRNA levels were analyzed using the Cyber green method and normalized with GAPDH gene expression.

The expression of MMP-24 mRNA in the group treated with TAA significantly increased compared to the other groups (****$p< 0.0001$) (gray graph). In other groups, such as the group that was first treated with thioacetamide for 2 months and then curcumin, the gene expression was increased, but it was not significant (light blue column) (p= 0.5217). Similarly, in the group that received TAA and Cur simultaneously, the level of MMP-24 gene expression was slightly increased (p= 0.3674), but it is not statistically significant (Fig. 2).

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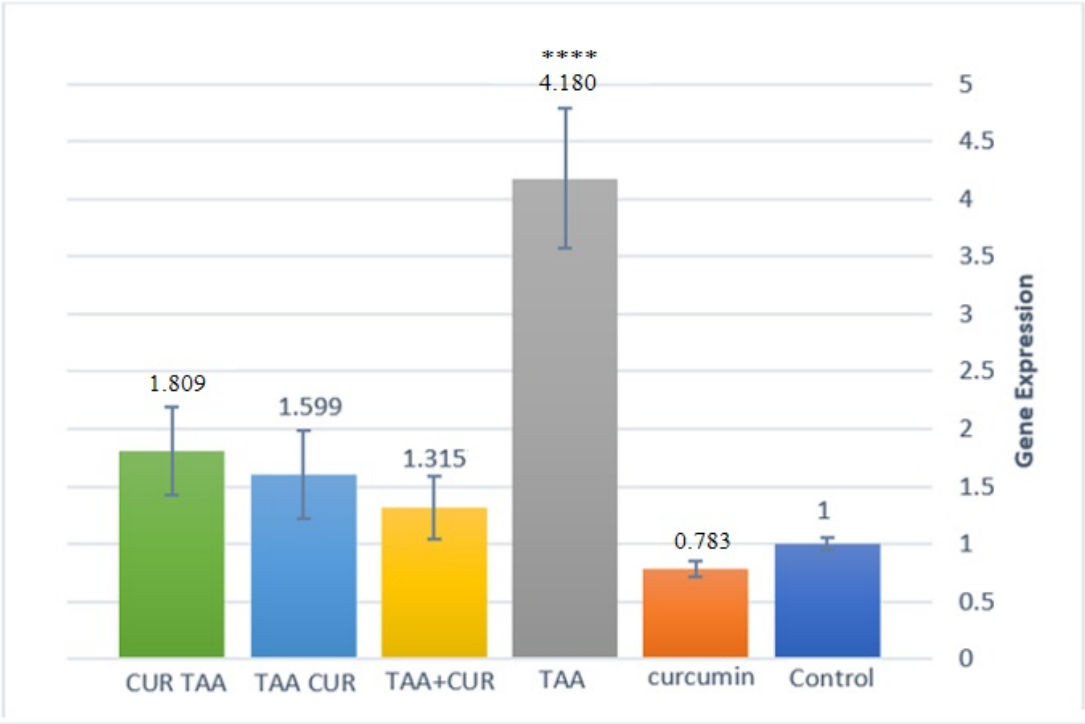

Fig. 2. The expression of MMP-24 gene in different groups compared to the control group. The group treated with TAA for 4 months showed a significant increase in MMP-24 mRNA expression. In the other groups, a slight increase in expression was observed; however, it was not statistically significant. The mRNA levels were analyzed using the Cybergreen method and normalized to GAPDH gene expression.

The effect of Cur and TAA on MMP-17 and MMP-24 proteins using western blotting method in the treated groups

The amount of MMP-17 and MMP-24 proteins in the liver tissue of mice treated with thioacetamide, especially in the group treated with TAA for 4 months, was increased compared to the control group. However, in the groups treated with curcumin, the level of MMPs protein was decreased (Fig. 3).

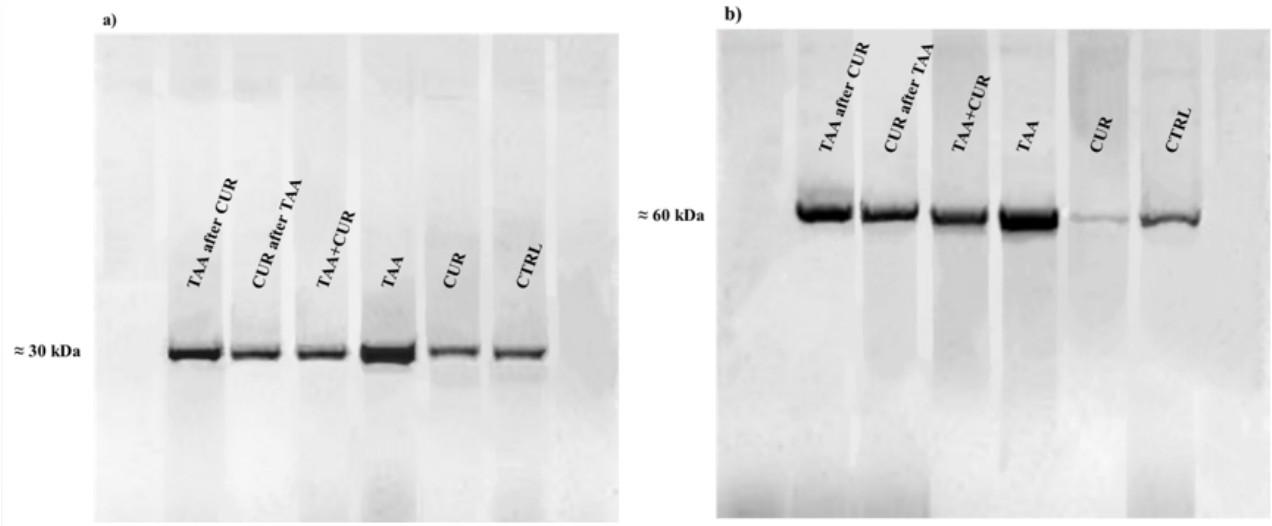

Fig. 3. The effects of TAA and curcumin on MMP-17 and MMP-24 protein expression in liver cells. MMP-17 protein expression increased in the TAA-treated group compared to the control group. Densitometric analysis of MMP-24 protein expression revealed increased levels in the TAA-treated group compared to both the control and curcumin-treated groups. Molecular weight markers and bands of MMP-17 and MMP-24 proteins were observed on the gel.

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The effect of Cur and TAA on the activity of MMP-17 and MMP-24 enzymes using zymography

The enzymatic activity of MMPs was determined by densitometry after electrophoresis and gel washing with Triton and zymography buffer. The gel was stained with Coomassie blue and then destined by methanol and acetic acid. The colorless areas corresponding to the bands indicated the activity of the enzyme (Fig. 4).

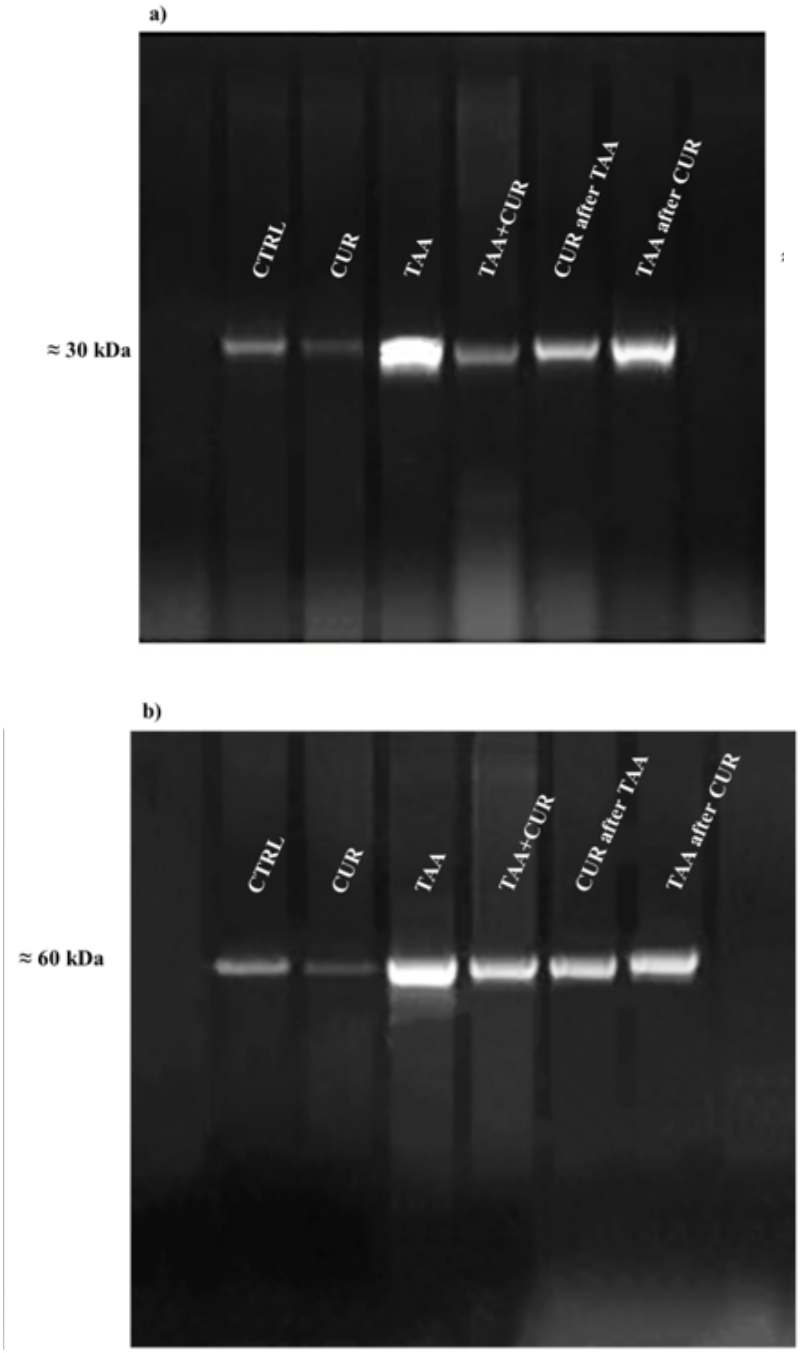

Fig. 4. The effects of TAA and curcumin on MMP-17 and MMP-24 enzyme activity in liver tissue cells. MMP-17 enzyme activity was significantly higher in the TAA-treated group compared to the control group, especially when compared to the curcumin-treated group. MMP-24 enzyme activity also increased greatly in the TAA-treated group compared to the control group. Bands indicating enzyme activity were observed in the decolorized gel.

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Histopathology

Liver samples were examined for morphology, cytoplasmic staining, nuclear size, and cellular atypia (abnormality and malformation). The liver tissue of mice exposed to TAA for 4 months showed fibrosis and necrosis in some areas. No histopathological damage was observed in the control and curcumin groups, and the liver cells exhibited normal structure. In the group initially treated with TAA followed by curcumin, moderate dysplasia was observed. This was characterized by hypertrophy of liver cell nuclei, irregular nuclear borders, and degeneration along with swelling of the cell walls in some cells.In some areas, the space of Disse and sinusoid was expanded. No significant changes were observed in the other groups. In the liver of some mice in the simultaneous treatment groups and the group that first received curcumin and then thioacetamide, slight changes in cell size were observed (Fig. 5).

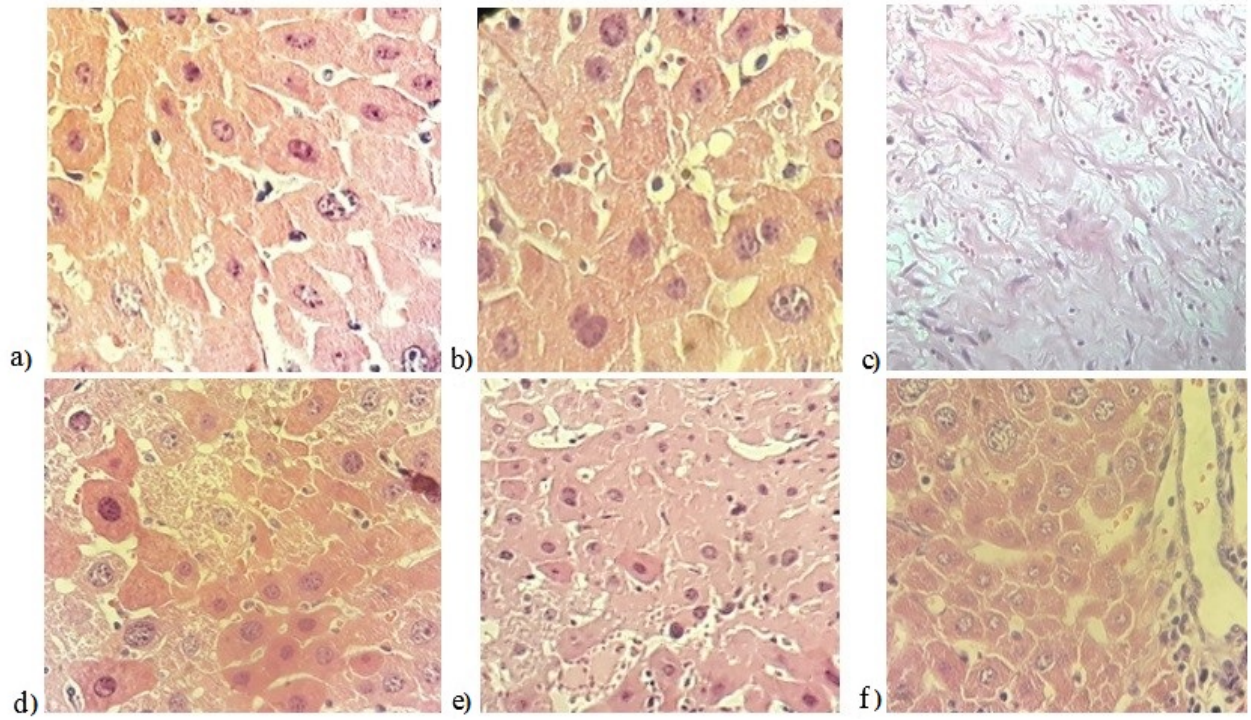

Fig. 5. Microscopic comparison of liver tissue in different groups. a) The control group showed normal hepatocytes. b) The curcumin-treated group did not show pathological lesions in hepatocytes. c) The TAA-treated group showed increased liver fibroblasts and macrophages, along with complete cell structure destruction and necrosis. d) The simultaneous group showed an increase in hepatocyte size in some areas. e) that first received group, TAA and then curcumin showed enlarged hepatocyte nuclei and hypertrophy in some areas. f) The group that first received curcumin and then TAA showed a slight increase in hepatocyte size (100x).

Discussion

Liver cancer is the fifth most common disease in the world, comprising 83% of liver diseases. The liver is a vital organ in the body that regulates physiological and metabolic processes. Liver cancer can be caused by various factors such as viral infections, alcohol consumption, fungal toxins, and exposure to chemicals. The environment contains over 2,000 different human-made chemicals that undergo metabolism in the body, with the liver being one of the main organs involved in this process (23). One of these toxic compounds is thioacetamide, which is widely used in industry and was used as a cancer inducer in this study. The obtained results revealed a significant change in the expression of MMP-17 and MMP-24 genes in the experimental groups. Mice exposed to thioacetamide for 4 months showed a significant increase in the expression of

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MMP-17 and MMP-24 genes. Interestingly, even in the group treated with curcumin antioxidants before thioacetamide exposure, the expression of MMP-17 increased. This suggests a reduction in the defence power of liver cells, possibly due to decreased intracellular glutathione and altered redox potential, leading to an imbalance between the production and collection of oxidants and disruption of membrane system balance. This, in turn, results in changes in cell permeability and damage.

In similar studies conducted by other scientists on the signaling pathways that cause liver fibrosis and inflammation in liver stellate cells induced by thioacetamide, it was found that this substance can increase the expression of microRNAs such as miRNA-17, which have a potential role in modulating the expression of precancerous genes (24). These studies also observed increased levels of ALT (1.7 times) and AST (2.4 times), as well as increased expression of genes involved in liver fibrogenesis, such as α-SMA (25). Other studies using TAA as a liver damage inducer reported changes in blood factors related to liver damage, such as albumin, GSH, and IL-6, as well as alterations in genes involved in fibrosis, such as TIMP-1 and MMP-2, cyclin D, and inflammatory factors like TNF-α (26).

Today, there is an expanding body of research on the protective effects of various antioxidants against diseases like cancer and liver diseases, allowing for further investigation of the molecular mechanisms of their effects. However, the properties of these compounds often focus on preventing damage caused by free radicals. Curcumin, for instance, has been shown to regulate the expression of apoptotic proteins, such as P53 and Bax messenger RNA, and reduce the expression of Bcl2, thereby reducing the sensitivity of liver cells to cytotoxicity caused by thioacetamide. Additionally, curcumin can induce apoptosis in damaged cells, which serves as a defence mechanism against liver inflammation and fibrosis (27). Recent research on the semi-structural analogue of curcumin, Dehydrozingerone (DHZ), has shown that it effectively reduces oxidative stress caused by TAA, modifies the MAPK pathway, and reduces fibrosis in liver tissue by increasing catalase enzyme activity (28).

The investigation of MMP-17 and MMP-24 protein expression levels in this study demonstrated a significant increase in the groups induced by thioacetamide. Similarly, studies on colorectal cancer patients have shown significant upregulations of MMP3, MMP-1, MMP-12, MMP9, MMP7, and MMP-14 at the genome and protein levels. Moreover, MMP-17, MMP-14, MMP-19, and MMP-11 showed a significant increase in expression at higher tumor stages (29). Comparing these findings with the present study indicates that thioacetamide can alter gene expression levels and the activity of MMP-17 and MMP-24 enzymes at the protein level, which are critical contributors to the development of liver cancer. Notably, the protein expression levels of these MMPs in the curcumin-treated groups were even lower than in the control group, indicating the wide range of pleiotropic effects of curcumin. It can directly or indirectly affect various genes, proteins, molecules involved in inflammatory pathways, growth factors, enzymes, and adhesion molecules through numerous undefined mechanisms (30,31). Previous studies have demonstrated that curcumin can suppress tumors by reducing inflammatory factors such as TNF-α, ILs, and IFN-γ, increasing P53 activity, reducing collagen degeneration, and decreasing MMP9 expression (32). Furthermore, curcumin has been found to reduce liver metabolic activity by decreasing cytochrome p450 activity, increasing glutathione-S transferase, inhibiting angiogenesis and cell proliferation, and ultimately reducing cancer incidence (33-37). It has been shown to inhibit the activation of liver stellate cells (HSCs), which play a role in liver fibrosis, and induce apoptosis in response to chemical-induced liver damage such as TAA. Curcumin treatment increases the expression of P53 protein and Bax mRNA, while decreasing the expression of

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Bcl-2, thus increasing hepatocyte sensitivity to TAA and inducing apoptosis as a protective mechanism for the liver tissue (38). Curcumin can react with active oxygen species by forming stable rings and prevent Reactive Oxygen Species (ROS) activities in hepatotoxins like alcohol, CCl4, and TAA. Moreover, it indirectly protects the liver by increasing the Nrf2 (erythroid-derived nuclear factor 2) pathway (39).

Various studies have reported different results regarding the role of MMP-17 in carcinogenesis. For example, Chabottaux et al. showed that the expression of MT4-MMP gene and protein in breast cancer significantly increases, promoting angiogenesis, blood vessel growth, and metastasis (40). On the other hand, Nuttall et al. found that MT4-MMP mRNA expression decreases in advanced tumor grades and demonstrated differential regulation of GPI-MT-MMPs in brain cancer (41). However, most studies indicate an increase in MMP-17 expression in advanced stages of certain cancers, including digestive cancer (42). MMP-17 in breast cancer has been found to play a regulatory role in tumor progression by affecting cell signaling pathways involved in angiogenesis, which is a crucial process in tumorigenesis. Angiogenesis allows solid cancer cells to receive nutrients and oxygen through the formation of blood vessels, facilitating their proliferation (43). This study's molecular and pathological findings also indicate an increase in MMP-17 gene and protein expression, accompanied by extensive tissue changes at the cellular level. By expressing MT4-MMP, undifferentiated cells are induced and interact with other MMPs, such as MT1-MMP/MMP-14, through signaling molecules like growth factors, receptors, extracellular matrix (ECM), and adhesion molecules. MT1-MMP is overexpressed in cancer cells and upregulates VEGF, stimulating angiogenesis by regulating the Src, Akt, and mTOR signaling pathways (43).

MMP-24 is one of the MMPs activated during inflammation and the carcinogenic process of hepatocytes. Research has shown that the metastatic activity of MMP-24 is associated with the absence of Capicoa receptors (CIC) or increased expression of its effector (ETV4), which phosphorylates and changes the conformation of MAPK receptors, activating them in response to cellular stress. This degenerates CIC and initiates the cancerous process of the cells (44). Studies by Benson et al. in 2013 demonstrated significantly upregulated MMP-24 mRNA expression in breast cancer cells compared to normal cells (45). In this study, when liver tissue was exposed to toxic chemicals for an extended period, the levels of MMP-24 gene and protein expression increased.

Activated Kupffer cells also increase the production of growth factors such as TGF-β, OSM, and activate the JAG1 pathway, which in turn activates quiescent liver stem cells (Stella cells) and induces their differentiation into hepatocytes. This process involves increased expression of collagens, α-SMA, TIMP-1, and MMPs (23), providing a conducive environment for cellular carcinogenesis.

In conclusion, although the exact mechanism of action of MMPs in cancers remains poorly defined, the present research demonstrates strong upregulation of MMP-17 and MMP-24 gene and protein expression in the livers of mice exposed to the hepatocarcinogen thioacetamide. Notably, these expressions were significantly reduced or partially inhibited in groups treated with curcumin simultaneously, or before and after thioacetamide induction. Given the role of MMPs in the breakdown and rearrangement of cellular matrix and basement membrane during cancerous tissue development and the increasing prevalence of cancer-causing chemicals like thioacetamide, the need for the use of antioxidants such as curcumin becomes increasingly evident. Pharmacological evidence confirms curcumin as a non-toxic food additive with numerous benefits.

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Further investigations should evaluate the expression of other MMPs in human samples and different cell lines. A simultaneous examination of other genes and factors, such as Scr and ETV4, is necessary to gain a comprehensive understanding of curcumin's effects on thioacetamide-induced homeostasis in liver cells, alongside the expression of MMP-17 and MMP-24 genes.Measuring the levels of other markers such as tumor markers CEA (Carcino Embryonic Antigen) and AFP (Alpha Feto protein), liver damage detection tests like GGT (Gamma glutamyl transferase), and less invasive methods like CT scan and ultrasound can provide a more accurate understanding of the effects of curcumin.

Conflict of interest

The authors have no conflicts of interest to declare.

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