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Original Article

Investigation of TNFα Level and *Metallothionein* Gene Expression in Livers of Rats Exposed to Dietary Aluminum

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Abstract

Aluminum chloride is a chemical compound widely used in both pharmaceutical and industrial sectors. The present study aimed to assess the effect of aluminum chloride on TNF levels and *metallothionein* gene expression in rat livers. A total of 16 Wistar rats were used as an experimental model and assigned to four groups (n=4). The treated groups received aluminum chloride (Sigma/USA) at a dose of 25g/kg body weight via a feeding tube as follows: group 1: Non-treated rats as the control group, group 2 were treated with aluminum chloride for 8 weeks, group 3 were treated with aluminum chloride for 12 weeks, and group 4 received aluminum chloride for 16 weeks. The TNF-α was measured in liver tissue using an enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry and real-time polymerase chain reaction (RT-PCR) were used to analyze metallothionein gene expression in rat liver. To estimate TNF levels, the results revealed that levels were considerably higher ($P<0.01$) in all experimental groups, especially in group 4 which underwent treatment for 16 weeks (401±22.1 ng/ml), as compared to that in the control group. For the immunohistochemistry assay, a gradient intensity of staining for liver tissue was observed, ranging from zero staining in the control group to moderate, medium, and high staining in the experimental groups after 8, 12, and 16 weeks of aluminum chloride treatment, respectively. The greatest amount of methylothionine expression was observed in the livers of group 4 which received aluminum chloride for 16 weeks (15.5-fold), with a significant difference ($P<0.01$) from the other experimental groups. In both immunohistochemical and RT-PCR experiments, aluminum administration had a substantial influence on TNFα levels and metallothionein expression in rat livers.

Keywords: Aluminum chloride, Immunohistochemistry, Liver, Metallothionein, RT-PCR, TNF

1. Introduction

Aluminum chloride has industrial and biological applications, and toxicity research on this compound is limited to the cases of acute exposure. Aluminum chloride may infiltrate the food chain, causing animal and human poisoning (1). Oral absorption of aluminum chloride is thought to cause genotoxic damage (2). Prabhakar, Reddy (3) have elucidated the possible involvement of oxidative stress and altered antioxidant status in the induction of aluminum toxicity after acute oral treatment. Several studies have indicated that the toxicity of aluminum chloride *in vitro* and *in vivo* has a severe impact on cellular shape and components, leading to apoptosis, as well as DNA and protein damage (4).

In addition, aluminum chloride exposure can result in genetic damage, inflammatory response, carcinogenicity, cytotoxicity, the generation of reactive oxygen species (ROS), and mitochondrial dysfunction (5). The effect is determined by the amount consumed, the velocity of the entrance, tissue distribution, concentration reached, and excretion rate (6). Aluminum toxicity is caused by the reduction of enzyme activity and protein synthesis, as

well as alterations in DNA composition and cell membrane permeability.

Aluminum chloride toxicity alters cellular components and structure (7), protein structure and function (via activation or inhibition), as well as gene expression which encodes these proteins (8). The recognition of the impact of aluminum chloride on gene coding is a key regulator of its cellular metabolism (9). TNF-αTNF is an anti-inflammatory and proinflammatory cytokine involved in apoptosis, proliferation, inflammation, immunology, and cirrhosis (10). The MT-2 gene encodes a low molecular weight protein that binds to divalent heavy metal ions.

The conserved cysteine residues coordinate metal ions through mercaptide linkages (11). These proteins are antioxidants that aid with heavy metal detoxification and metal homeostasis in the cell (12). Since MT2A is expressed in a variety of organs, tissues, and cultured cells, it has received assiduous attention in recent years due to its important pathophysiological function in detoxification, antioxidation, and inflammation (13). The MT2A upregulates mineral homeostasis, oxidative stress for detoxification, immune defense, cell cycle progression, angiogenesis, cell proliferation, and differentiation; moreover, it has a specific function in regulating autophagy and apoptosis (14).

The disruption of the metallothionein genes causes problems with heavy metal protection, oxidative stress, immunological responses, and carcinogens (15). Mammalian metallothionein-2A is present in the nucleus during cell proliferation and regeneration. Given the importance of the topic, the current study sought to examine the effect of aluminum chloride treatment on TNFα level and MT2A expression in the liver by immunohistochemistry and real-time polymerase chain reaction (RT-PCR) in rats as an experimental model.

2. Materials and Methods

A total of 17 male albino rats (230-310 g) were placed in cages with floors covered with fine sawdust. The animals received a standard rodent diet and sanitary water at 20°C-25°C according to Mohammed (16) and the guidelines approved by the Animal Ethics Committee of the University of Baghdad. The animals were assigned to four groups (n=4 in each group) and were treated with aluminum chloride (Sigma/USA), 25g/kg weight according to Sanai, Okuda (17) via a feeding tube as follows:

1. Rats in group 1 received no treatment (Control group)
2. Rats in group 2 were treated with aluminum chloride for 8 weeks.
3. Rats in group 3 were treated with aluminum chloride for 12 weeks
4. Rats in group 4 were treated with aluminum chloride for 16 weeks.

The rats were thoroughly sedated with an intramuscular injection of 80 mg/kg ketamine (Sigma/USA) one week following the final day of the experiment. The rats were then sacrificed, and the livers were separated into three sections: the first was used to estimate TNF levels, the second was used to determine MT2A expression via RT-PCR, and the third was fixed in 10% formaldehyde (Sigma/USA) for 24 h prior to being used in Lynch (18) 's immunohistochemistry assay.

2.1. Estimation of TNFα Level in Rat Liver

A total of 50 mg of liver tissue was dissolved in 450 mg lysis buffer (Sigma, USA) by blender, and the mixture was centrifuged for 15 min (15,000×g at 4°C). A TNFα kit (Thermo Fisher/USA) was used for the detection of TNF-α in the liver according to Mohammed (19) and the manufacturer's instructions at 450 nm absorption.

2.2. Immunohistochemistry Detection

Paraffin blocks were prepared according to Lillie (20). Immunohistochemistry assay was performed using antibody and DAB chromogen substrate kit (Abcam/USA), as well as hematoxylin as nuclear antibody contrastain, based on Mohammed (21) to detect MT2A expression in liver tissues.

2.3. Reverse Transcription Polymerase Chain Reaction Assay

Total RNA was extracted from liver tissue using the TRIzol reagent (Thermo Fisher/USA) in accordance with the manufacturer's instructions. Using an RNA purification kit (Bioneer/Koria), the RNA pellet was dissolved in 25 µ molecular biology grade water (Sigma), and the tubes were then frozen.

Sambrook and Russell (22) used Nanodrop (BioNeer/Korea) to measure RNA concentration and purity. According to Due to Wang and Seed (23) and Mohammed, AL-Thwani (24), the RNA-primer mixture (Thermo Fisher/USA) was prepared by the addition of RNA to hexamer dNTP and DEPC H_2O random hexamers. Thereafter, it was incubated at 65°C for 5 min and on ice for 1 min, then briefly mixed with a reaction mixture (Thermo Fisher/USA) containing MgCl2 DTT, RNAase, as well as RT, and kept according to Zhang, Volkmann (25). The RT-PCR was performed employing SYBR green Mix (2X) (Thermo Fisher/USA) and primers provided by Alpha DNA/Canada (Table 1). Table 2 illustrates the 25 microliters of real-time mixed reactions incorporating components. According to Pfaffl, Horgan (26), the rotation program was followed. Tables 2, 3 and 4 represent the usage of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

Table 1. Primer sequence

Primer		5-'3 'forward
MT2	F Primer	TCGCTCGATTTCTACCG
	R Primer	TGTCGGAAGCCCTTTGC
GAPDH	F Primer	GTCTTCACTACCATGGAGAAGG
	R Primer	TCATGATGACCTTGCCAG

Table 2. Polymerase chain reaction components

Ingredient	Quantity (µl)
cDNA	0.2
H_2O	11.3
Primer pair mix (5 pm/ µl)	1
SYBR Green Mix (2x)	12.5
Final volume	25

Table 3. Polymerase chain reaction amplification program for MT2 gene

Step	Tm (°C)	Time	Cycles
Initial denaturation	95°C	10 min	1x
Denaturation	94°C	15 sec	
Annealing	60°C	30 sec	35X
extension	72°C	20 sec	
Final extension	86°C	5 sec	1X
Melting Curve	60-99°C	40 min	1X
Cooling	40°C	30 sec	1X

Table 4. Polymerase chain reaction amplification program for the *GAPDH* gene

Step	Tm (°C)	Time	Cycles
Initial denaturation	95°C	10 min	1x
Denaturation	94°C	15 sec	
Annealing	58 °C	30 sec	35X
Extend	72°C	20 sec	
Final extension	84°C	5 sec	1X
Melting curve	60°C-99°C	40 min	1X
Cooling	40°C	30 sec	1X

2.4. Statistical Analysis

The impact of variations on research parameters was assessed using SAS statistical analysis system (27). The one-way analysis of variance (ANOVA) test of least significant difference was employed to compare the mean.

3. Results

The TNF-α has a major role to play as an antioxidant, anti-apoptotic, and anti-inflammatory agent (28). Many pathologic processes, such as gene expression, aging, apoptosis, and necrosis, are caused by DNA damage (29). The MT2A, as a free radical scavenger, has been also shown to protect cells and tissues against oxidative stress in studies. Heavy metal exposure is known to induce MT2A production, which is a sensitive biomarker (30). Inflammatory mediators, such as metals, medicines, and metals, promote MT2 expression in the liver. The MT2 is hypothesized to be involved in metal metabolism, transport, homeostasis, and toxicity or detoxification (31).

The MT2 is a direct promoter of tumor formation and progression by increasing DNA damage and genomic

instability. Moreover, it interacts with heavy metals, influencing the intracellular and extracellular metal distribution and donation to numerous critical factors and enzymes (32). Regarding the examination of TNF-α expression in rat liver tissues, the findings revealed statistically significant (*P<0.01*) differences in TNF-expression across the whole groups of aluminum-treated rats, with the levels varying depending on the dose of aluminum administered.

The results pointed out that the rats in group 4 which were treated with aluminum chloride (25 g/kg body weight/day) for 16 weeks had the highest level of TNF (401±22.1 ng/ml) with a significant difference (*P≤0.01*), compared to other groups. On the contrary, as compared to the control group, all experimental groups demonstrated a statistically significant increase, as displayed in table 5.

Table 5. Effect of aluminum chloride on the level of tumor necrosis factor in rat liver

Groups	TNFα (ng/ml) mean±SD
1	65.11±4.3
2	101±4.6
3	198.1±1.51
4	401±22.1
LSD value	49.652**
P-value	*0.0001*

**(*P≤0.01*), LSD: least significant difference

The MT2 expression levels were found to be significantly higher in all groups of rats that received aluminum chloride treatment, as compared to the control group, and these levels varied depending on how long the rats had been exposed to aluminum chloride. The MT2 protein was discovered in the cytoplasm of hepatocytes and inflammatory cells, and the accumulation of MT2 protein was evaluated as follows: The score is 1 for MT2. According to Zhang, Liu (33), the conditions were classified as negative, weak, moderate, and strong staining in the presence of only a small amount of staining, as well as 2%-33%, 33%-79%, and 80% staining, respectively. The current findings revealed that MT2 expression was observed in all groups of rats treated with aluminum chloride. Moreover, it was indicated that there was a definite relationship between MT2 protein accumulation and the length of time the rats were exposed to aluminum chloride. Figure 1 displays the microscopic appearance of liver tissue sections obtained from rats in group 1 (controls), which contained normal hepatocytes, central veins, Kupffer cells, and sinuses, as well as negative staining with DAB against the anti-H&E stain, implying that there was no changes detected in MT2 protein expression.

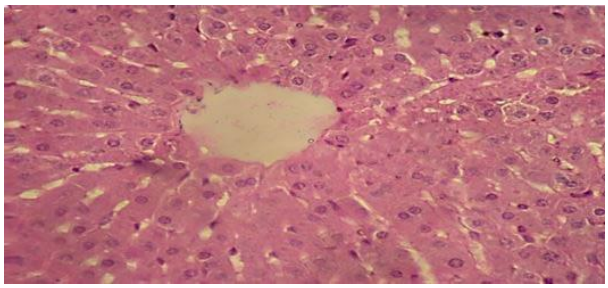

Figure 1. Normal central vein (CV), hepatocyte (HC), Kupffer cell (KC), sinus (S), and negative immunohistochemistry localization of MT2 staining with DAB against the H&E staining in liver tissues of Group 1 rats (control group) (X400)

The MT2 expression was found to be low in liver tissues taken from rats in group 2 that were treated with aluminum chloride (25g/Kg of body weight a day for 8 weeks), as shown in figure 2. The faint staining indicates a poor expression of MT2 in liver tissues collected from rats.

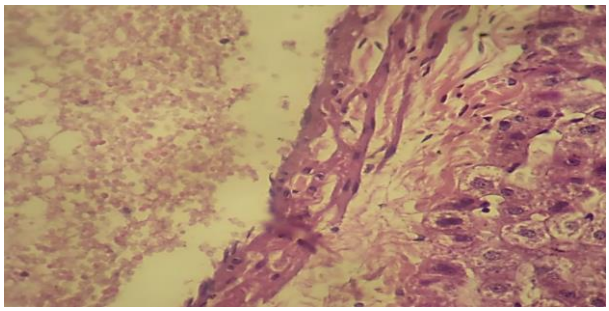

Figure 2. Liver tissues of group (2) rats (Treatment with aluminum chloride 25 g/kg body weight/day) for 8 weeks, showing weak immunohistochemistry localization for MT2 staining with DAB against counterstain H&E dye (X400)

As depicted in figure 3, moderate staining in liver sections from rats treated with aluminum chloride (25

g/kg body weight/day) for 12 weeks demonstrated a moderate expression of MT2.

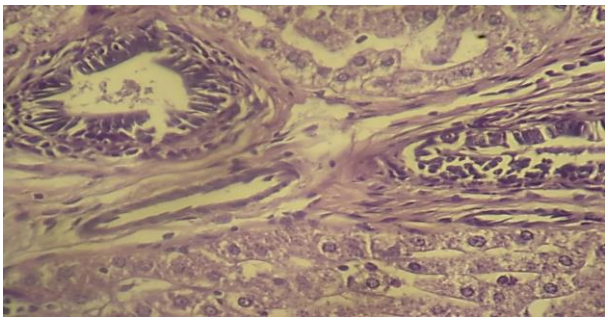

Figure 3. Liver tissues from rats in group 3, demonstrating considerable immunohistochemical localization of MT2 staining with DAB against the counterstain H&E dye after a 12-week treatment with aluminum hydroxide (25 g/kg body weight/day) (X400)

Strong staining was observed in liver sections of rats in group 4 treated with aluminum chloride (25 g/kg body weight) for 16 weeks, represented by MT2 overexpression (Figure 4).

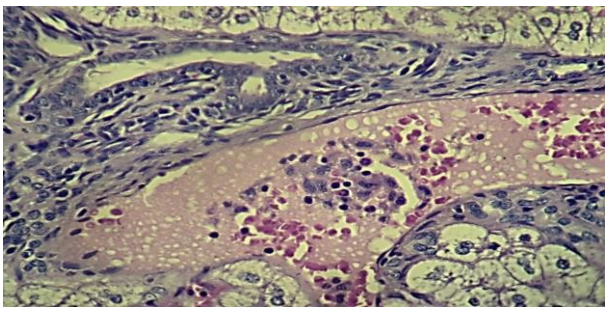

Figure 4. Rat liver tissues of group 4 (Treated with aluminum chloride 25g/kg body weight/day) for 16 weeks), illustrating a strong immunohistochemical localization for MT2 staining with DAB against counter stain H&E (X400)

When rats were treated with aluminum chloride (25 g kg body weight/day) for 8 and 12 weeks, the results of RT-PCR displayed fundamental and time-dependent elevations in MT2 mRNA expression in the livers of rats in the experimental groups, even in the lowest exposure period (9.3-fold) and medium exposure period (11.2-fold) with a significant difference ($P \leq 0.005$). Moreover, the rats in group 4 which were treated with aluminum chloride (25 g/kg body weight/day) for 16 weeks showed the highest MT2 expression (15.5-fold) with a highly significant difference ($P \leq 0.01$), when compared to the other experimental groups. Furthermore, all experimental groups displayed a significant increase, in comparison with the control group (0.8-fold), as illustrated in figure 5.

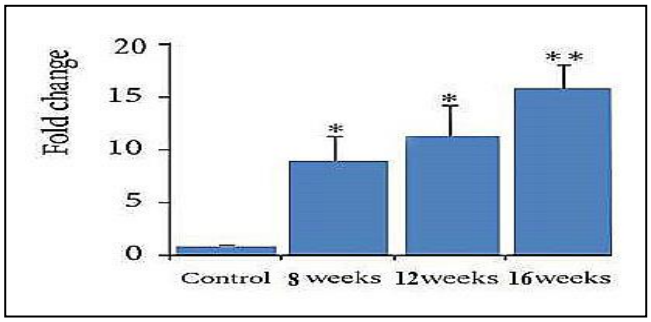

Figure 5. Effect of aluminum chloride treated with 25g/kg body weight/day) for 0,8,12, and 16 weeks) on MT2 expression in rat liver measured in the real-time polymerase chain reaction

4. Discussion

As evidenced by the result of this study, aluminum treatment increased the levels of TNF and MT2 expression in either immunohistochemistry or RT-PCR, which resulted in liver damage defined by apoptosis. The first hepatocyte damage triggers a greater inflammatory response, resulting in increased MT2 expression in liver tissue (34). In fact, aluminum chloride treatment elicits a wide range of immune responses (35). Moreover, it was found that aluminum chloride treatment caused a significant ($P \leq 0.01$) increase in TNFα level, especially in rats in group 4 which were treated with aluminum chloride for 16 weeks, as compared to controls. It can be attributed to the fact that TNFα is considered a major inflammatory factor in broad hepatotoxicity, which is produced by macrophages in inflammatory or damaged tissues (35).

The results also pointed out that aluminum chloride caused a significant increase in MT2 gene expression in all experimental groups, particularly group 4 which revised aluminum chloride (25 g/kg body weight/day) for 16 weeks, as compared to the control group. The rats in the experimental groups had elevated MT2 expression and strong staining in liver tissues, as well as increased expression in RT-PCR. Moreover, a significant time-dependent increase with grading was

detected in MT2 expression in both RT-PCR and immunohistochemistry, as compared to control.

It has been demonstrated that greater bodily damage is directly related to increased MT2 expression. The MT2 expression was detected after 8 weeks of aluminum chloride treatment, whereas overexpression was detected after 16 weeks, indicating enhanced hepatotoxicity caused by aluminum chloride (36). The MT2 may be involved in several liver infections, toxins, and injuries (31). It is produced when a DNA injury marker associated with chronic hepatitis damages tissue (37), is required for normal hepatocyte propagation during liver regeneration (38), and is a mediator of hepatotoxicity in several animal models (39).

To summarize, the results of the current study pointed out that aluminum chloride treatment had a dramatic impact on TNF level and MT2 expression in rat livers in both immunohistochemistry and real-time RT-PCR assays, necessitating the development of strategies to protect individuals in order to maintain overall health.

Authors' Contribution

The author read and approved the final version of the paper. B. J. M. came up with the idea for the study and did the lab work, animal handling, and data analysis.

Ethics

The animals received a standard rodent diet and sanitary water at 20°C-25°C according to Mohammed (16) and the guidelines approved by the Animal Ethics Committee of the University of Baghdad.

Conflict of Interest

The authors declare that they have no conflict of interest.

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