

Scientific paper

Protective Effects of 3-benzoyl-7-hydroxy Coumarin on Liver of Adult Rat Exposed to Aluminium Chloride

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Abstract

In this study, the effects of 3-benzoyl-7-hydroxy coumarin molecule on mineral and antioxidant enzymes were investigated in rat liver exposed to oxidative stress with aluminium chloride (AlCl_3). Adult male Wistar albino rats were divided into four groups as Control, Coumarin, AlCl_3 , and Coumarin + AlCl_3 . Coumarin at the dose of 10 mg/kg and AlCl_3 at the dose of 8.3 mg/kg were administered for 30 days every other day. In AlCl_3 group, malondialdehyde (MDA), iron (Fe), aluminium (Al) and copper (Cu) levels increased compared to the control group, while glutathione (GSH) level, glutathione S-transferase (GST), and carboxylesterase (Ces) enzyme activity levels decreased. In Coumarin + AlCl_3 group, MDA, Fe, Al and Cu levels decreased with the effect of coumarin compared to AlCl_3 group, while GSH level, and GST enzyme activity levels increased. According to our results, AlCl_3 generates oxidative stress in rat livers, and we believe that 3-benzoyl-7-hydroxy coumarin has an ameliorative effect on antioxidant enzyme system, Al, Fe and Cu levels.

Keywords: Aluminium; coumarin; rat; liver; carboxylesterase; mineral

1. Introduction

Aluminium (Al) is a metal commonly found in soil. People are exposed to Al throughout life in many ways such as through cooking utensils, drinking water, antacids, deodorants, food additives, spices, teas and dialysis processes. Al accumulates in many organs such as liver, brain, heart and lung. As a result of its toxic effects, Al causes many disorders such as anaemia, brain diseases, muscle weakness and lung problems.^{1,2} In particular, in Alzheimer's Disease, it has been suggested to play a role in the progression of the disease.³ Liver is one of the most important organs of metabolism, regulating detoxification and secretory functions. Studies have reported that Al accumulates in liver and causes harmful effects.^{4,5} Significant changes in haemato-biochemical parameters of rats and rabbits exposed to Al were detected. Al causes an increase in hepatic MDA levels and a decrease in antioxidant enzymes.^{6,7}

Coumarins are defined as "2H-1-benzopyran-2-on". These chemicals are one of the important members of the natural class of polyphenolic compounds.⁸ In addition to being isolated from plants, coumarins were also synthesized synthetically. Coumarins have many biochemical effects such as anticancer,⁹ antiinflammatory,¹⁰ and antihyperlipidemic.¹¹ In our study, 3-benzoyl-7-hydroxy cou-

marin was used. Hydroxycoumarins are a phenolic compound. The free radical scavenger and metal chelator properties of these substances are known.¹²

There are many esterase enzymes such as acetylcholine esterase, buthylcholin esterase, carboxylesterase, cholesterol esterase, which differ in biological functions in our body. Increases in metabolites produced by these enzymes can lead to pathological conditions, such as Alzheimer's Disease, hypercholesterolemia, brain cancer, and Coronary Artery Disease; and therefore, researchers have focused on various esterase inhibitors.¹³ Among these enzymes, carboxylesterase is an important detoxification enzyme. Carboxylesterase (Ces, EC 3.1.1.1) is a member of the superfamily of esterase that catalyses the hydrolysis of esters, amides and thioesters.¹⁴ These enzymes are expressed mainly in the liver of mammals.¹⁵ Many drugs and xenobiotics have effects on the influence expression of Ces enzymes.¹⁶ Another important detoxification enzyme is Glutathione S-transferase (GST). The most important task of this enzyme is to scavenge reactive oxygen species (ROS).¹⁷ Reduced glutathione (GSH) is a non-enzymatic antioxidant molecule, and is essential for antioxidant functions.¹⁸ Macro and trace elements have many functions in human metabolism, such as cofactors and antiox-

idants. Lack and excess of elements in metabolism cause serious damage. In some studies, it has been reported that the levels of other elements change as a result of Al exposure.¹⁹

There is information in the literature about the protective role of various coumarin derivatives (fraxin, esculetin, grandi vittin, agosyllin, aegolinol benzoate, osthol etc.) against oxidative stress⁸, but there is no information about the effect of the 3-benzoyl-7-hydroxy coumarin we used in our study against oxidative stress. Therefore, in the present study, the effects of 3-benzoyl-7-hydroxy coumarin on Fe, Al, Cu, zinc (Zn), magnesium (Mg), manganese (Mn), CES, GST, GSH and MDA were investigated in the liver of rats exposed to AlCl₃.

2. Materials and Methods

2.1. Animals and Experimental Procedure

All experimental protocols were approved by the Local Ethics Committee of Adiyaman University (Number of the permission: 2019/27). Rats used in the experiment were treated in accordance with national and international laws and policies regarding the care and use of experimental animals. Twenty-eight adult (aged 2.5 months) male *Wistar albino* rats (260 ± 10 g body weight) were obtained from the Department of Animal Experiments Local Ethics Committee of Adiyaman University (Adiyaman, Turkey). Rats were randomly divided into four groups as Control (C), 3-benzoyl-7-hydroxy coumarin (CM), AlCl₃ (A), and 3-benzoyl-7-hydroxy coumarin+AlCl₃ (CM+A) (n = 7 each group). The C group rats received vehicle solutions with only distilled water. AlCl₃ was dissolved in distilled water, and the animals were given 8.3 mg / kg intraperitoneally (IP).²⁰ 3-benzoyl-7-hydroxy coumarin was administered to the animals in distilled water at a dose of 10 mg / kg orogastrically.²¹ During the application, in order to prevent possible complex formation between aluminium and coumarin, AlCl₃ and coumarin exposures were applied at different time intervals. The exposure of AlCl₃ was made between 09:00 am and 10:00 am every other day for 30 days. Coumarin administration was performed between 04:00 pm and 05:00 pm every other day for 30 days. The rats were housed under a standard light/dark schedule (12-h light/12-h dark cycle) at constant temperature (21 ± 1°C) and humidity (55 ± 5%) with free access to pelleted food and fresh tap water. The rats were sacrificed at the end of day 30. For biochemical analyses, liver samples were stored at -50°C until the assays were performed.

2.2. Homogenization and Centrifugation of Liver Tissue

Ice-cooled 0.1 M K-phosphate buffer containing 0.15 M KCl, 1 mM EDTA and 1 mM DTT in a 1 : 4 ratio of total tissue weight (w/v) was used to homogenize liver samples.

Homogenization was performed with a Heidolph RZ 2021 brand homogenizer. The homogenates were centrifuged at 15000 g for 30 minutes at 4 degrees Celsius using the Hettich ROTINA 420 R centrifuge. The supernatant obtained after centrifugation was used for testing enzyme activity. The levels of MDA, reduced GSH, and activities of Ces and GST were determined spectrophotometrically at appropriate wavelengths using a microplate reader system (ThermoTM Varioskan Flash, Thermo Scientific).

2.3. Measurement of Glutathione S-transferase Activities

The GST activity was measured using a modified version of the method developed by Habig et al. (1974), and adapted to the microplate reader system.²² The mixture consisted of 100 mM K-phosphate buffer (pH 6.5), 1 mM GSH (used as cofactor), 10 mM CDNB (used as a substrate) and supernatant. During the measurement, 10 microliter supernatant, 100 microliters phosphate buffer + 100 µl GSH mixture and finally CDNB were pipetted into microplate wells, respectively. Absorbance changes expressing the decrease in CDNB amount depending on the reduced GSH consumed during the reaction were measured at 344 nm for 3 min at 25 °C, and the specific GST activity was calculated as nmol min⁻¹g protein⁻¹.

2.4. Measurement of Ces Activities

The Ces activity was determined using the modified version of the procedure described by Santhoshkumar and Shivanandappa (1999) for the microplate reader.²³ The reaction solution consisted of 5 µL of supernatant and 0.1 mM 250 µL pH 7.4 trizma buffer. The reaction mixture pipetted into microplate wells was incubated for 3 min at 25 °C. The reaction was initiated by the addition of 5 µL of the 0.5 mM p-nitrophenol acetate (PNPA), into the reaction solution in wells. The p-nitrophenol released during the reaction due to the use of p-nitrophenol acetate as a substrate by Ces was monitored at 405 nm for 2 min at 25 °C. The specific Ces activity was calculated as nmol min⁻¹g protein⁻¹.

2.5. Determination of Malondialdehyde and Reduced GSH Levels in Tissues

The analysis of MDA, a secondary product of lipid peroxidation was carried out as described by Placer et al. (1966).²⁴ The reaction mixture was freshly prepared by adding 500 µL homogenate to 1.5 mL reaction solution by mixing equal volume of 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 N HCl (1:1:1, w/v). The reaction mixture was heated for 30 min at 100 °C in a water bath. After the mixture was cooled to room temperature, the centrifugation was performed at 15000 g for 15 min. Subsequently, by transferring supernatant samples into mi-

croplate wells, absorbance changes depending on MDA formed during the reaction were recorded at 532 nm. MDA levels were expressed as (nmol/g wet weight tissue). The reduced GSH level was determined by measuring the absorbance value at 412 nm of a compound formed by the reaction of reduced GSH with 5,5-dithiobis 2-nitro-benzoic acid (DTNB).²⁵ The level of reduced GSH was expressed as nmol mg⁻¹ protein.

2. 6. Determination of Total Protein in Samples

The total protein content in the supernatant samples was determined using the colourimetric method of Bradford 1976, and with Bovine Serum Albumin (BSA) as the standard.²⁶ Results were expressed as milligram protein. All analyses were performed in triplicates. Total protein level was used to determine for GSH level and CES, GST enzyme activities in the liver.

2. 7. Liver Mineral Analyses

Liver Al, Fe, Cu, Zn, Mg, and Mn concentrations were measured using a NexION 350 inductively-coupled plasma mass spectrometer (ICP-MS, Perkin Elmer, MA, USA) at the Central Research Laboratory of Adiyaman University. The livers (250 mg) were digested in an acid solution (5 ml of nitric acid (HNO₃, 65%) using a microwave digestion system.²⁷ The digestion solutions were diluted and analysed by using the ICP-MS. For quality control, duplicate samples were used to measure the precision of the analysis. Detection limits were calculated as three times the standard deviation for the reagent blanks.²⁸ The results of the liver mineral concentrations are presented as ppm for Al, Fe, Cu, Zn, Mg, and Mn.

2. 8. Synthesis of 3-benzoyl-7-hydroxy Coumarin Molecule

The synthesis of 3-benzoyl-7-hydroxy coumarin compound was carried out according to reference²⁹ briefly as follows: 2,4-dihydroxybenzaldehyde (2.762 g), ethyl benzoyl acetate (3.844 g), piperidine (three drops) and acetone (50 mL) were added into a three-necked reaction flask and then, it was refluxed for 2 hours on a magnetic stirrer. At the end of the process, the reaction mixture was transferred into excess methanol to precipitate the 3-benzoyl-7-hydroxy coumarin compound (Figure 1). Finally, it was recrystallized in ethanol and dried under vacuum.

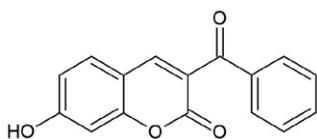


Figure 1: Schematic representation of 3-benzoyl-7-hydroxy coumarin

2. 9. Statistical Analyses

Data were expressed as mean ± SEM (standard error of mean). Statistical comparison of biochemical parameters was done using One-Way Analysis of Variance followed by the Tukey-HSD test. P < 0.05 was considered to be statistically significant.

3. Results

Liver mineral levels are presented in Table 1. Fe and Cu levels of group A increased compared to all other groups (p < 0.05). There was no statistical difference between the Zn levels of all groups (p > 0.05). While the Al level of group A increased compared to all other groups (p < 0.001, p < 0.05), Al level of CM+A group decreased compared to A group (p 0.05). Mn levels of A and CM+A groups decreased compared to C and CM groups.

Table 1: Liver mineral levels (ppm)

Minerals	C	CM	A	CM+A
Fe	103.20±3.49	100.55±3.00 ^x	116.84±2.38 ^a	102.25±2.2 ^x
Zn	25.79±1.66	25.95±0.66	24.43±1.26	29.16±1.22
Mg	238.06±6.09	230.34±5.11	232.74±9.28	240.56±1.93
Al	29.98±3.64	28.75±3.08 ^z	94.14±5.44 ^c	69.07±6.33 ^{cx}
Mn	2.90±0.07	2.94±0.06 ^x	2.55±0.04 ^a	2.65±0.09 ^a
Cu	5.37±0.76	4.69±0.23 ^x	7.32±0.53 ^a	5.29±0.40 ^x

Comparison with group C. a: p < 0.05, b: p < 0.01, c: p < 0.001

Comparison with group A. x: p < 0.05, y: p < 0.01, z: p < 0.001

Liver MDA, GSH and enzyme activity levels are shown in Table 2. Group A's MDA level was found to be increased compared to other groups (p < 0.001; p < 0.05). GSH level of group A decreased compared to other groups (p < 0.05; p < 0.001). Ces enzyme activity levels of A and CM + A groups decreased compared to C and CM groups (p < 0.05). GST enzyme activity level of A group decreased compared to other groups (p < 0.05).

Table 2: MDA, GSH and enzyme activity levels of the liver. GST and Ces activities are expressed as nmol/min/mg protein ± mean standard error. GSH and MDA levels are expressed as nmol GSH/mg protein ± mean standard error, and nmol MDA/g wet weight tissue ± mean standard error, respectively.

Parameters	C	CM	A	CM+A
MDA	45.02±2.54	48.05±3.41 ^z	78.75±5.85 ^c	58.56±3.65 ^{ax}
GSH	88.45±3.02	96.41±4.23 ^z	73.75±4.05 ^a	86.32±5.45 ^x
Ces	1.78±0.06	1.81±0.05 ^x	1.47±0.02 ^a	1.48±0.09 ^a
GST	31.41±0.76	33.80±1.67 ^x	24.34±1.04 ^a	32.20±1.90 ^x

Comparison with group C. a: p < 0.05, b: p < 0.01, c: p < 0.001

Comparison with group A. x: p < 0.05, y: p < 0.01, z: p < 0.001

4. Discussion

Al is a metal commonly found in air, soil and water. This metal is widely used in industry, agriculture, transportation and daily lives of people. Al, absorbed by the gastrointestinal tract, accumulates in the liver and other organs. In many studies, Al has been reported to cause hepatotoxicity, nephrotoxicity and cardiotoxicity.^{30,31}

In the present study, we observed that Al, Fe and Cu concentrations increased significantly in the liver of rats that were given AlCl₃ intraperitoneally. In many experimental studies, it has been reported that the concentration of Al increases in rat liver exposed to Al.^{32,33} Al is a non-redox metal, and many studies have reported that Al affects concentrations of other metal ions.^{34,35} Yang et al. reported that the Al, Fe, Cu concentrations were increased in rat liver overloaded with Al.¹⁹ In addition, it has been reported that Fe levels increase in rat liver exposed to Al.³⁶ Al is known to be linked to transfer protein carrying Fe³⁺, thereby reducing the binding of Fe²⁺. The increase in free intracellular Fe²⁺ causes peroxidation of membrane lipids, thereby causing membrane damage.³⁷ Chronic Cu²⁺ and Fe³⁺ overload causes accumulation of Cu²⁺ and Fe³⁺ in the liver and kidney, which causes Fe²⁺ overload. Al has been reported to increase Fe-induced oxidative stress injuries.^{38–40}

In this study, liver Mn concentration of the A and combination groups decreased compared to the C group. In some studies, it has been reported that the Mn concentration decreases in rat liver exposed to Al.^{41,42} In our study, we observed that the Zn and Mg concentration levels of group A decreased relatively compared to group C. Trace elements have important physiological functions in metabolism, and their abnormal usability causes serious negative effects. Trace elements, such as Cu, Zn, Fe, Mn, and Mg are essential co-factors for antioxidant enzymes. These enzymes are very important in preventing the oxidation of nucleic acids, lipids or proteins.⁴³

In our study, the level of MDA increased in rat liver treated with AlCl₃. Also, GSH levels and GST and Ces enzyme activity levels decreased in antioxidant defence system. Based on these results, we believe that increased oxidative stress with AlCl₃ and reduction of enzymes involved in antioxidant defence could be caused by peroxidation damages. The glutamyl-cysteine-synthetase enzyme carries out the synthesis of the GSH molecule in the liver. In addition, it was found that enzymes, such as glucose-6-phosphate dehydrogenase and NADP-isocitrate dehydrogenase control the synthesis of the GSH molecule in the liver. In many studies, the inhibition effect of AlCl₃ on enzymes controlling GSH biosynthesis has been reported. AlCl₃ has pro-oxidant activity, and causes changes in the activity of antioxidant enzymes. In similar studies, while AlCl₃ increased the level of MDA in rat liver, it decreased GSH level and the activity of Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase.^{44–46} In another study, it was reported that activities of

catalase, GPx, SOD, and GST enzyme decreased in rat liver tissues with the toxic effects of Al.⁴⁷

In the present study, it was observed that the Al, Fe and Cu levels increased in the liver of the rats exposed to oxidative stress with AlCl₃, and the 3-benzoyl-7-hydroxy coumarin substance decreased the levels of these metals. In addition, it was determined that the level of MDA, which is the high lipid peroxidation product of AlCl₃, was reduced by 3-benzoyl-7-hydroxy coumarin. It was observed that 3-benzoyl-7-hydroxy coumarin substance improved the GSH level and GST enzyme activity, which are effective in the antioxidant system. In this study, Ces enzyme activity decreased due to the toxic effect of AlCl₃. Also, in the combination group, the activity of Ces enzyme decreased with the effect of 3-benzoyl-7-hydroxy coumarin.

The results of the present study show that 3-benzoyl-7-hydroxy coumarin exerted antioxidant properties against the pro-oxidant effects of AlCl₃. We found that 3-benzoyl-7-hydroxy coumarin improves both Al, Fe and Cu concentrations in the liver, and positively affects the antioxidant system. The antioxidant activity of coumarin is due to its ability to scavenge free radicals and chelate metal ions. The antioxidant capacity of many coumarin substances has been reported to be due to its molecular structure.⁴⁸ The number and location of hydroxy, acetoxy and methoxy groups in the structure of coumarin affect the antioxidant capacity. These functional groups are very important for detoxification functions of coumarins.⁴⁸ In a similar study, the hepatoprotective effect of four different coumarins (coumarin (1,2-benzopyran), esculetin (6,7-dihydroxycoumarin), scoparone (6,7-dimethoxycoumarin), and 4-methylumbelliferone (7-hydroxy-4-methyl)) were investigated against hepatic damage caused by CCl₄ in rat liver, and it was reported that two coumarins (esculetin and scoparone) had hepatoprotective effects. It has been explained that antioxidant properties of coumarins are attributed to their chemical structure.⁴⁹ In another study, the ameliorative effect of coumarin (1,2-benzopyron) was investigated in rats with ferric nitrilotriacetate (Fe-NTA)-induced renal oxidative stress. Fe-NTA increases the level of MDA with the effect of oxidative stress while decreasing the GSH level and GST enzyme activity. However, the ameliorative effect of the coumarin (1,2-benzopyrone) molecule has also been reported.⁴⁸ Many studies have shown that coumarin has an antioxidative effect against various oxidative stress agents.^{50–52} Atmaca et al. (2011) suggested that coumarin and its derivatives showed antioxidative effects against rat liver damage induced by carbon tetrachloride by testing various antioxidant biomarkers.⁴⁹ Various studies have shown that coumarin derivatives, such as fraxin, esculetin, grandivittin, agacyllin, aegolinol benzoate and osthol have free radical scavenging effects.⁸

In our study, we revealed the relationship between the toxic effect of Al on the liver and the detoxification enzymes such as Ces and GST. Al reduced GST and Ces en-

zyme activities in rat liver. We could not find studies related to Ces enzyme activity to compare our results; however, there are reports indicating that Al decrease GST enzyme activity. In our previous study, we had found that Ces and GST enzyme activities decreased in liver of rats given lead acetate.⁵³ In this study, although we saw the ameliorative effect of 3-benzoyl-7-hydroxy coumarin in GST enzyme activity, we also found that this had no ameliorative effect on Ces enzyme activity in the combination group. It may be considered that the ineffectiveness of coumarin on Ces activity depends on the dose applied. Perhaps Ces activity would have been affected if different doses of coumarin were tried. We believe that more in vitro and in vivo studies are needed regarding the effect of coumarin on Ces activity. In the study conducted by Khan et al., it was reported that coumarin applied in increasing doses against the oxidative stress induced by Fe-NTA has an ameliorative effect on GST enzyme activity at high dose.⁴⁸

In advanced pharmacological studies, we believe that this study is important for the effects of coumarin-derived substances on Ces enzyme activities.

5. Conclusion

In conclusion, we observed that AlCl₃ induces oxidative stress in rat liver. We have determined ameliorative effects of 3-benzoyl-7-hydroxy coumarin on deleterious effects of aluminium on the antioxidant enzymes, Fe, Cu and Al levels. However, we observed that the 3-benzoyl-7-hydroxy coumarin did not ameliorate the effect of Ces enzyme activity. In future studies, we believe that this study is important for investigating the effects of toxic substances and coumarin derivatives on Ces enzyme activity. As a result, we believe that 3-benzoyl-7-hydroxy coumarin may have an ameliorative effect against oxidative stress induced by Al.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Povzetek

V tej raziskavi so bili proučeni učinki molekule 3-benzoil-7-hidroksi kumarina na minerale in antioksidativne encime v jetrih podgan, izpostavljenih oksidativnemu stresu z aluminijevim kloridom (AlCl_3). Odrasli samci podgan Wistar albino so bili razdeljeni v štiri skupine, poimenovane kontrola, kumarin, AlCl_3 in kumarin + AlCl_3 . Kumarin v odmerku 10 mg/kg in AlCl_3 v odmerku 8,3 mg/kg sta bila aplicirana 30 dni vsak drugi dan. V skupini AlCl_3 so se v primerjavi s kontrolno skupino zvišale koncentracije malondialdehida (MDA), železa (Fe), aluminija (Al) in bakra (Cu), medtem ko so se ravni glutaciona (GSH) in encimske aktivnosti glutation S-transferaze (GST) in karboksilesteraze (Ces) zmanjšala. V skupini kumarin + AlCl_3 so se ravni MDA, Fe, Al in Cu z delovanjem kumarina zmanjšale v primerjavi s skupino AlCl_3 , medtem ko so se ravni GSH in aktivnost encima GST povečale. Glede na naše rezultate, AlCl_3 povzroča oksidativni stres v jetrih podgan in verjamemo, da ima 3-benzoil-7-hidroksi kumarin blažilni učinek na antioksidativni encimski sistem in ravni Al, Fe ter Cu.



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