Applied Biochemistry and Biotechnology
https://doi.org/10.1007/s12010-019-03177-w

The Protective Effects of Silymarin on Thioacetamide-Induced Liver Damage: Measurement of miR-122, miR-192, and miR-194 Levels

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Received: 26 September 2019 / Accepted: 11 November 2019/Published online: 06 December 2019
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Abstract
This study aims to investigate the protective effects of silymarin (Sm) in thioacetamide (TAA)-related liver damage. What makes this study special is that it attempts to determine the expression of changes in the liver at the level of gene expression. Routine liver damage markers were compared with changes in the levels of microRNA (miRNA) known as new biomarkers. With this in mind, we divided the rats into four groups including control, TAA, Sm + TAA (50 + 50 mg/kg), and Sm + TAA (100 + 50 mg/kg). Blood and tissue samples belonging to the rats were collected in consideration of morphological, immunohistochemistry, miRNAs levels, and biochemical evaluations. Our study results showed that miR-122, miR-192, and miR-194 levels had decreased in the experimental groups given TAA, whereas miR-122, miR-192, and miR-194 levels had increased in the doses of Sm + TAA-given group. Therefore, Sm treatment undertaken before exposure to the toxin successfully altered its effects upon the study animals.

Keywords Thioacetamide · Silymarin · miRNA · Immunohistochemistry · Rat

Introduction

The liver is a critical organ with many functions including regulation of essential hormones, elimination of many substances, bile secretion, blood detoxification,

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metabolism, and synthesizes. Liver disease is still a serious, worldwide problem correlated with morbidity and mortality. The chief causes of liver diseases are known to be excessive alcohol consumption, viruses, toxins, parasitic diseases, hepatitis, hepatotoxins, antibiotics, and chemotherapeutic agents including paracetamol, carbon tetrachloride (CCI4), and TAA [1]. TAA is a hepatotoxin that is frequently used to generate experimental liver damage [2]. Ledda-Columbano et al. (1991) reported that TAA causes apoptosis in hepatocytes [3]. In addition, it has been emphasized which TAA causes the increase in the levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) known as liver damage markers significantly [4]. TAA is bioactivated by CYP2E1 [5–8] to TA sulfoxide (TASO) and to the highly reactive sulfdioxide ($TASO_2$), which initiates hepatic necrosis by covalent binding [5].

miRNAs are a molecule of about 20–22 nucleotides in length, involved in the regulation of genes epigenetically [9]. Many studies show that miRNAs play a significant part in some types of tissue damage and cancer, as well as regulating important mechanisms in the cell. Furthermore, miRNAs can function as a new biomarker in the detection of tissue damage in many metabolic diseases [10, 11]. Recent studies have showed that some miRNAs including miR-122 and miR-192 are powerfully and specifically expressed in hepatic dysfunction in experimental animals and in humans [12]. Liver damage also significantly changes the expression levels of miRNAs. In fact, it was emphasized that miR-122 and miR-194 levels increased as a result of liver damage in experimental studies. miR-122 that is a 22-nucleotide is expressed by gene *hcr* found in the liver cells. About 70% of the total miRNA in the body is found in the liver. Also, it is expressed as 66,000 copies per cell [10]. miR-122 is the richest miRNA in hepatic tissue, and it constitutes majority of total miRNAs (about 70%) [13]. miR-122 plays a role in many of cell division factors, histone regulation genes, and pathways [14]. Recent studies have shown that miRNA can be a more sensitive marker [15, 16]. miR-192 is expressed by the liver, colon, kidney, and small intestine [16], and upregulation of miR-192 examined many cancer such as gastric, pancreatic, and esophageal [13, 17]. It has been demonstrated that serum levels of miR-192 is elevated in various liver diseases, and it related metastasis and survival in cancer patients. Besides, miR-192 could be useful as a toxicological biomarker for the identification of chemical hazards [18]. miR-194 is exceedingly expressed in liver cells, but two types of mesenchymal cells in the liver, not in Kupffer cells or hepatic stellate cells. miR-194 expression was reduced in liver cell in vitro. Besides, the expression of miR-194 was low in liver cancer cell lines. The overexpression of miR-194 in liver mesenchymal-like cancer cells decreased the expression of the mesenchymal cell marker N-cadherin and suppressed migration and invasion of the mesenchymal-like cancer cells in vitro and in vivo [19].

Silybum marianum L. (milk thistle) belongs to the family Asteraceae extensively used as far as traditional medicine is concerned for many years in treating various diseases [20, 21]. Sm is a purified mixture of four isomeric flavonoids derived not only from the fruit but also from the seeds of the *Silybum marianum* [L.] *Gaertner* (milk thistle) plant. Sm has also been used in treating various liver disorders (jaundice, hepatitis, cirrhosis, etc.) and gallbladder disease in protecting the liver against poisoning from chemical and toxins, thanks to its antioxidative and antihepatotoxic features [22, 23]. All these taken into account, we aim to determine the protective effects of Sm upon rats on TAA-related liver injury and to investigate expression levels of miR-122, miR-192, and miR-194.

Material and Method

Chemicals

TAA, DMSO, and Sm were bought from Sigma Chemical Co. (St. Louis, MO, USA), while immunohistochemistry (IHC) antibodies were bought from Santa Cruz Biotech (Santa Cruz, USA). Malondialdehyde (MDA), glutathione peroxidase (GPx), ALT, AST, ALP, and LDH ELISA kits were bought from YL-BIONT, Korea. miRNeasy Mini Kit 50 test was purchased from Macherey Nagel, Germany. The rest of the chemicals we used for this study were of analytical grade.

Animals

Adult healthy male Sprague–Dawley rats, aged 8 weeks plus weighing 180 ± 20 g, were kept at a controlled temperature of 25 °C on a 12-h light–dark cycle so that they would have free access to standard diet and water ad libitum. Animals were adapted to the ambient conditions for as long as 1 week prior to the onset of the experiment. We attended to the rats in accordance with the regulations issued by the Institute Animal Ethics Committee, with the protocol approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg. No. KU/IAEC/PhD/100 dated 26.07.2012).

The Experimental Design

Rats were separated into four groups (n = 7 each group) randomly.

Group (I), vehicle control: each animal was given 0.5 mL of 0.2% DMSO, the solvent of Sm, by gavage for the first 14 years, and then 0.5 mL of saline, the solvent of TAA, was applied for the second 14 days.

Group (II), TAA group: each animal was injected with 50-mg/kg TAA for the second 14 days. No application was made in the first 14 days.

Group (III), treatment group: each animal received 50-mg/kg Sm for the first 14 days and for the second 14 days 50 mg/kg TAA injection.

Group (IV): each animal received 100-mg/kg Sm for the first 14 days and for the second 14 days 50-mg/kg TAA injection.

During the experiment, all of the rats were fed 5% dextrose containing 0.9% saline and potassium (20 mEq/L) to prevent weight loss, hypoglycemia, and renal failure [24].

Finally, 24 hours after the TAA treatment had been launched, the animals were given ketamine/xylazine in 5 is to 1 ratio. Their blood samples were obtained from their left ventricule. Plus, we obtained their serum by means of blood centrifugation at 3000 rpm (956 g-Eppendorf 5804 R) for 10 min at.

Biochemical Determinations

In a private medical laboratory, we achieved serum analysis such as ALT, AST, ALP, and LDH. Also, we used serum in determining GPx and MDA with the help of ELISA kits.

Histopathology Analysis

The liver samples of not only normal but also of treated animals underwent fixation with 10% formalin in phosphate-buffered saline for 24 hours before being embedded in paraffin. The sections with a thickness of 5–6 μm were cultivated through a microtome and stained with hematoxylin–eosin prior to being examined under the microscope to observe possible histopathological changes of the liver.

Immunohistochemistry

Immunostaining was achieved in accordance with the standard methods with only minor modifications. Once the liver sections had been fixed overnight at 56 °C, they were deparaffinized in fresh xylene and rehydrated with the help of graded ethanol solutions. We dipped the specimens in a freshly prepared solution of 1% H2O2 in ice-cold methanol for 20 minutes to quench endogenous peroxidase. Once these specimens could be rinsed in phosphate-buffered saline (PBS), they were incubated for 1 h in blocking solution (3% BSA, 0.1% Tween-20 in PBS) at room temperature (RT). The incubation process was achieved with anti-Bcl-2, anti-Bax, and anti-caspase-3 antibodies (goat polyclonal) (1:500) in blocking solution for 12 hours at 4 °C, re-equilibrated to RT and cleansed with PBS, incubated with horse radish peroxidase (HRP) antibody conjugates (1:2500) in blocking solution without Tween-20 for 2 hours at RT. The specimens were cleansed using PBS before being incubated with 0.2% solution of 3,3′-diaminobenzidine (DAB) up until the desired stain intensity was achieved at RT, which was then exposed to washing in distilled water. These sections were counterstained using hematoxylin and mounted with di-n-butylphthalate-polystyrenexylene (DPX). Immunoreactivity was measured blindly by three free observers with the total number of positively stained cells being measured.

miRNA Analysis by qRT-PCR

miRNA was isolated from tissue using a miRNeasy kit (Macherey Nagel, Duren, Germany) with minor modifications. Briefly, 40-μg livers were homogenized with liquid nitrogen and were added 200-μL nuclease-free water, and then, it was mixed with 700 μL of QIAzol. It waited for 5 minutes before adding140-μL chloroform. Samples were then mixed gently for 15 seconds and centrifuged at 12,000 g for 15 minutes at 4 °C. 350 μL was taken from the upper aqueous phase; it was mixed with 70% methanol in a fresh microtube and added the total volume to miRNa syminisp in column. The column was centrifuged at 8000 g for 15 seconds at 25 °C. The quantity which contains the small RNA fraction including the miRNA complement was mixed with 450-μL pure ethanol. Data was subjected to normalization to common reference samples and to the average of two endogenous control genes (G6PDH and β-catenin) and auto scaling (Table 1). Kolmogorov-Smirnov test was performed for normality and t-test for statistical significance of the data.

miRNA Purification

The small RNA elution mixture was applied to a MinElute column, 700 μL at a time. The immobilized RNA was then washed with various buffers before a final 80% ethanol wash. The

Table 1 miRNA sequences

Oligo name	5'-3' sequence
Stemloop – miR-122-5p	GAAAGAAGGCGAGGAGCAGATCGAGGAAGAA GACGGAAGAATGTGCGTCTCGCCTTCTTTCCC TTTCGC
Forward- miR-122-5p	AGGAGAGAGACAAAGGAGAAAG
Stemloop- miR-192-5p	GAAAGAAGGCGAGGAGCAGATCGAGGAAGAA GACGGAAGAATGTGCGTCTCGCCTTCTTTCCG GCTGGC
Forward- miR-192-5p	CAGACCAAAGAAAAGACAGCC
Stemloop- miR-194-5p	GAAAGAAGGCGAGGAGCAGATCGAGGAAGAA GACGGAAGAATGTGCGTCTCGCCTTCTTTCCT CCTCGC
Forward miR-194-5p	AGAAACAGCAACACCAAGAGGA
Reverse primer	CGAGGAAGAAGACGGAAGAAT

column was then dried by centrifugation. The small RNA fraction was eluted in 14 μL of nuclease-free water and stored at 80 °C.

Statistical Analysis

Using one-way analysis of variance (ANOVA), all the experimental data were compared for multiple groups and then analyzed using SPSS 18.0 software. Data of statistical significance were further analyzed, so that their mean levels were compared with the aid of the Duncan's multiple range test. The data were expressed as mean ± SEM, plus, $p<0.05$.

Results

Hepatoprotective Effects of Sm on Serum Enzymes

Injection of TAA raised serum biochemical parameters like AST, ALT (Table 1), ALP, and LDH (Table 2). Our results revealed that the TAA-given group had showed a considerable increase ($p < 0.001$) in enzymatic activity levels when compared to the control group. By contrast, TAA and Sm given together had notably decreased ($p < 0.001$) AST, ALT, ALP, and LDH levels in TAA-intoxicated animals.

Effect of Sm on Liver Antioxidant States

The TAA-intoxicated animals showed a remarkable decrease ($p <$ 0,001) in GPx, MDA, and uric acid levels (Table 2), whereas the levels of malondialdehyde (MDA) (Fig. 2) had control group. These changes were determined to have significantly restored ($p < 0.001$) in the 50 Sm

Table 2 Effect of Sm on liver function test in rat (median ± standard deviation, $n = 7$) (*$p < 0.05$; ***$p < 0.001$)

	CONTROL	TAA	50Sm + TAA	100Sm + TAA
GPx	0.892 ± 0.030	0.809 ± 0.015*	0.849 ± 0.009	0.872 ± 0.029*
MDA	0.131 ± 0.012	0.224 ± 0.024***	0.176 ± 0.020***	0.117 ± 0.026***

+ TAA and 100 Sm + TAA-treated animals. As for the oral administration of vehicle control (%0.02 DMSO+%0.9 SF), it showed no changes in nonenzymatic antioxidant levels. Levels of specific liver marker enzymes in the serum and plasma were measured with a view of determining the extent of liver damage in all the experiment groups. Levels of marker enzymes in the Sm and TAA-given groups returned to normal when compared with those of the normal vehicle control (Fig. 1).

Effect of Sm miR-122 on miR-192 and miR-194 Expression Known as New Liver Markers

As shown in Fig. 3, the levels of miR-122 on miR-192 and miR-194 were significantly lower in the TAA-treated group than in the control group ($p < 0.001$). In the experimental groups given Sm + TAA, there was a significant increase in the levels of miR-122 on miR-192 and miR-194. However, it is seen that 100-mg/kg Sm provides better protection than 50-mg/kg Sm. The mechanisms leading to a decrease in miR-122, miR-192, and miR-194 levels after TAA are unclear, but the most likely explanation is that hepatocyte injury can spread these miRNAs into the circulation.

Histopathological Observations

In hematoxylin–eosin staining (Fig. 4), several degenerations including nuclear vacuolization, hemorrhage, edematous, and inflammatory areas were observed in thioacetamide-treated group, while control group was normal with typical nucleus and cytoplasm. In 50-mg/kg Sm co-treated group, still the similar degenerations were observed. However, in 100-mg/kg Sm co-treated group, liver histology was similar to that of control group.

Immunohistochemical Results

TAA led to an increase in staining of TNF-α, TUNEL (Fig. 4), caspase-3 (Fig. 4), Bax (Fig. 5), and a decrease in staining of Bcl-2 (Fig. 5) when compared to the control group. 50-mg/kg Sm

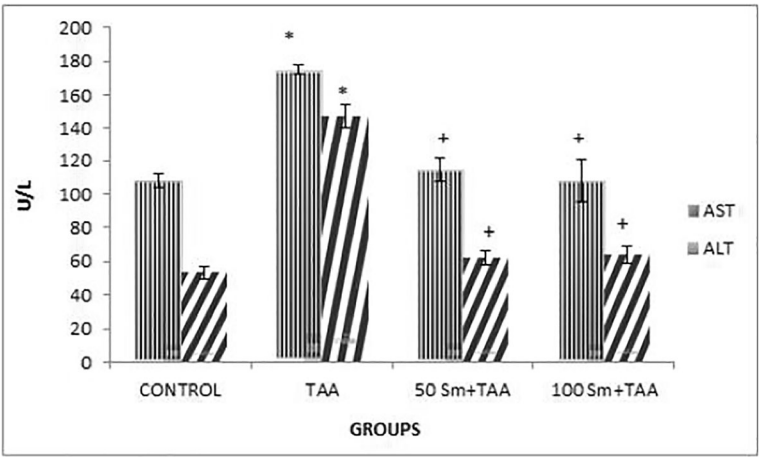

Fig. 1 Effect of Sm on AST and ALT level in groups (*compared with control group $p < 0.001$; +compared with TAA $p < 0.001$)

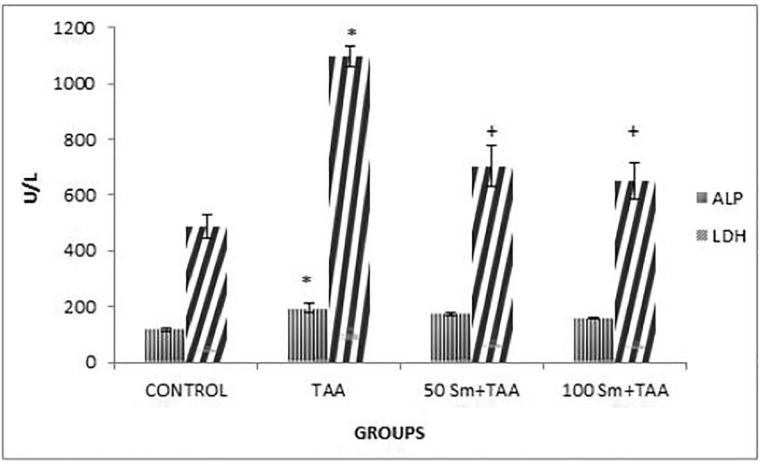

Fig 2. Effect of Sm on ALP and LDH level in groups (*compared with control group $p < 0.001$; +compared with TAA $p < 0.001$)

co-treatment did not alter the staining of Bax, caspase-3, TUNEL, and Bcl-2, whereas 100-mg/kg Sm co-treatment changed the staining markedly when compared to TAA alone group. In conclusion, high-dose Sm was effective in protecting against TAA-induced liver damage, while low-dose Sm was not.

Discussion

The current study revealed that pre-treatment with silymarin resulted in the protection against TAA-induced liver dysfunction by reduction of apoptosis and oxidative stress.

Liver enzymes (ALT, AST, ALP, and LDH) in serum are marker of damage in cells or inflammation in liver. Liver enzymes and some chemicals have leaked into the blood from the

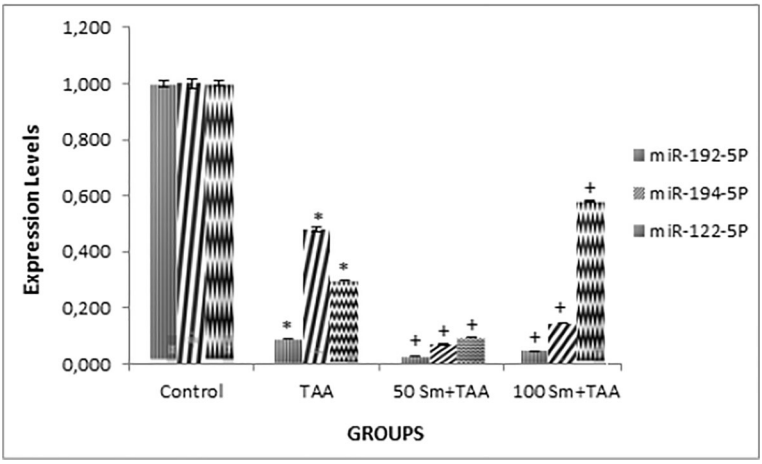

Fig. 3 Effect of Sm on liver miRNAs expression levels in rat. (*compared with control group $p < 0.001$; +compared with TAA $p < 0.001$)

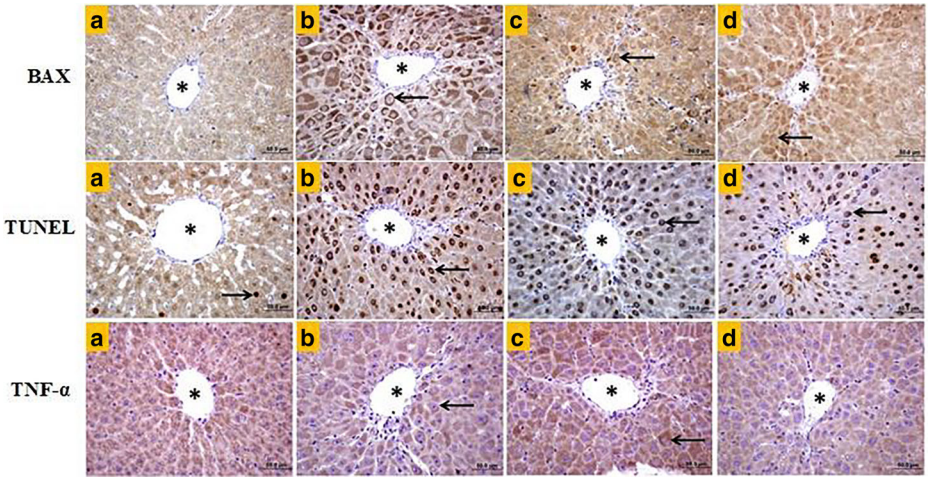

Fig. 4 The rat liver sections of experimental groups. (A**)** In the control group, **(**B**)** in the TAA alone group, (C) in the 50 Sm + TAA group, and (D) in the 100 Sm + TAA group. BAX, TUNEL, and TNF-α, all bars indicate 50 μm

damaged liver tissue higher amount than normal, and this can also be demonstrated by blood tests (Anbarasu et al., 2012). In a study, 400-mg/kg TAA was injected over 10 days intraperitoneally, and AST, ALT, ALP, and LDH enzymes and bilirubin level increased significantly. Also, histopathological results have confirmed liver necrosis. In another study, it was reported that 50-mg/kg TAA causes the increase in AST, ALT, ALP, LDH, and bilirubin (Somaia et al., 2015) and stated that it causes severe liver damage. Anbarasu et al. demonstrated that TAA induced a significant rise in AST, ALT, ALP, total bilirubin with a reduction of total protein, superoxide dismutase (SOD), catalase, and glutathione S-transferase (GST) [25]. Similarly, Somaia et al. indicated that TAA caused significant elevation of hydroxyproline (Hyp) and nitric oxide (NO) contents in liver homogenate and increased serum levels of AST, ALT, ALP,

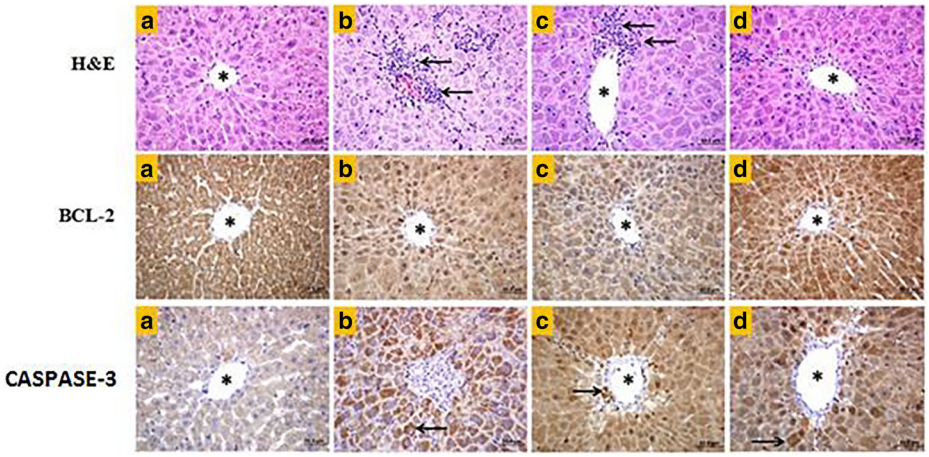

Fig. 5 The rat liver sections of experimental groups. (A) In the control group, (B) in the TAA alone group, (C) in the 50 Sm + TAA group, and (D) in the 100 Sm + TAA group. H&E, BCL-2, and Caspase-3, all bars indicate 50 μm

and total bilirubin [26]. In our study, as shown in the above studies, liver enzymes increased TAA-induced group compared to the control.

Generation of a great amount of reactive oxygen species (ROS) owing to TAA can overcome the antioxidant defense mechanism and apoptosis; this in turn can harm cellular function and structure. GPx is an enzyme family that has peroxidase activity and maintains an organism from free radical damage. GPx decreases lipid hydroperoxides and diminishes free hydrogen peroxide to the water. A study on the role of the antioxidants enzymes in TAA damage has shown that GPx and SOD decreased markedly TAA group compared to the control group in Wistar rats [24]. Anbarasu et al. demonstrated that TAA induced a significant reduce in GPx [25]. In our study, it was seen that the GPx level increased TAA-induced group compared to the control group.

An end product generated as a result of metabolizing arachidonic acid and polyunsaturated fatty acids (PUFAs) is MDA as enzymatic or nonenzymatic [27]. MDA has been used for many years because of reaction with thiobarbituric acid (TBARS) [28], and so MDA is one of the most popular and reliable markers that determine oxidative stress in clinical situations [29]. Somaia et al. indicated that TAA caused significant elevation of MDA [26]. MDA results of our study were similar to those of the literature.

It has been showed that TAA caused hepatotoxicity and enhanced the expression of Bax and degradation in the Bcl-2 expression considerably [30]. TAA (300 mg/kg) caused apoptosis. This was expressed by a decrease in Bax expression and an increase in Bcl-2 expression [31]. Lim et al. showed that 200-mg/kg TAA causes apoptosis in hepatocytes. They observed a decrease in levels of anti-apoptotic Bcl-2 and Bcl-XL mRNA and an increase in levels of proapoptotic Bax following TAA application of liver cells [32]. Our apoptosis evaluation result is in accordance with literature.

microRNAs (miRNA) which are noncoding RNAs and composed of about 20 nucleotides act as gene expression regulators posttranscriptionally. 2588 in human, 1915 in mouse, and 765 miRNA in rat are discovered, and they have been found in the miRBase database [33]. It is recognized that miRNAs can be biomarkers in disease diagnosis due to both tissue and cell-specific behavior [34]. Wang et al. (2009) showed which acute acetaminophen (APAP) poisoning increased miR-122 and miR-192 levels in sera of mice. Besides, serum miR-122 and miR-192 quantities have both a dose- and duration-dependent increase, and they can be determined earlier compared to serum aminotransferases [6]. Continuously, miR-122 and miR-192 quantities increased in patients with ALF (acute liver failure) caused by APAP compared to healthy people [14]. In another study, it has been showed that miR-122, miR-192, miR-483, miR-194, and miR-210 together with 31 miRNAs levels elevated in sera of patients with APAP compared to healthy controls [13]. Antoine et al. (2013) indicated that serum miR-122 quantity can be an important biomarker for ALF patients caused by APAP even if ALT levels do not increase serum. Besides, thanks to miR-122, liver failure can be detectable with high precision [33]. In our results, it was observed that TAA caused a decrease in miR-122, miR-192, and miR-194 levels. The mechanisms leading to a decrease in miR-122, miR-192, and miR-194 quantities after TAA are unclear, but the most likely explanation is that hepatocyte injury can spread these miRNAs into the circulation. Although there were some changes in miR-122 and miR-192 levels in liver damage caused by drug, acetaminophen, lipopolysaccharide and D-galactosamine, there are no studies in the literature on how miRNA levels of TAA and Sm were affected.

Sm is an antihepatotoxic agent in liver failure, and at the same time, it has effect of antioxidative, anti-inflammatory, cell protective, and anticarcinogenic [35]. There are many

studies showing that Sm is anti-apoptotic [36]. For example, Madani et al. (2008) stated that apoptosis caused by TAA (50 mg/kg b.w.) was reduced in the Sm group (25 mg/kg b.w.) [37]. In a similar study, it was shown by Patel et al. (2000) which Sm (750 mg/kg b.w.) protect apoptosis and liver damage induced by Fumonisin B1 (FB1) toxin (2.25 mg/kg b.w.). Also, they indicated that Sm has increased the regeneration potential of damaged liver tissues [38]. In another study, it showed that reactive oxygen species, apoptotic proteins (Bd-xL and p53), and liver failure caused by doxorubicin (60 mg/kg b.w.) were diminished by Sm (16 mg/kg b.w.) [38]. A study led by Santosh revealed that Sm increased anti-apoptotic proteins (Bcl-2 and Bcl-xL), whereas it decreased proapoptotic proteins (Bax) in JB6 C141 cancer cells [39]. Our study showed similar results to the above-mentioned studies, we have found that liver damage induced by 50 mg/kg TAA, was alleviated in groups that were treated Sm, there was deduction in the levels of miR-122, miR-192, miR-194, AST, ALT, ALP and LDH. Sm reduced the number of activated TNF-a, TUNEL, Bax and caspase-3 cells and also increased the number of Bcl-2 positive cells. Histopathological observations further confirm the membrane stabilizing effect of Sm in TAA challenged rats. Morever, 100 mg/kg Sm was more successful in maintaining TAA-induced damage than 50 mg/kg Sm.

Conclusion

In our study, it was determined that Sm is known to protect the liver, can be used in the treatment of liver as well as liver diseases, and can be added to the drug protocols. However, we think that the active substances of Sm should be investigated more extensively. On the other hand, it is thought that miRNA analysis can be extended and epigenetic drugs may be involved in the treatment of HE. miR-122 level in 100 mg/kg Sm + TAA group should be investigated why it is higher than TAA group and which genes are cleaved by miR-122 when miR-192 and miR-194 are increased.

Funding Information Project with coded 2017-1621 was supported by the scientific research projects of Eskisehir Osmangazi University. This study was made from PhD thesis by Ozgun Teksoy at Eskisehir Osmangazi University.

Compliance with Ethical Standard

Conflict of Interest The authors declare that they have no conflict of interest.

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