Original Article

Aloe Vera Attenuates Oxidative Stress in Rats with Non-alcoholic Steatohepatitis

Wongphoom J[1]
Klaikeaw N[1]
Werawatganon D[2]
Siriviriyakul P[2]

ABSTRACT

AIM: To examine the effects of *Aloe vera* on antioxidant level in rat with non-alcoholic steatohepatitis (NASH) rats.

Materials and Methods: Rats were randomly divided into three groups. Group 1 (control group, *n = 6*), rats fed ad libitum with standard diet containing 35% of energy from fat, 47% from carbohydrate, and 18% from protein for 8 weeks. Group 2 (NASH group, *n = 6*), rats fed ad libitum with high-fat high-fructose diet (HFHFD) containing 55% of energy from fat, 35% from carbohydrate, and 10% from protein for 8 weeks. Group 3 (*Aloe vera* group, *n = 6*), rats fed ad libitum with high-fat high-fructose diet (HFHFD) plus *Aloe vera* in DMSO (50 mg/kg) by gavage feeding daily for 8 weeks. The concentration of *Aloe vera* was 50 mg/kg. All rats were sacrificed to collect blood and liver samples at the end of treatment period.

Results: Hepatic malondialhyde (MDA) was increased significantly in the NASH group as compared with the control group (MDA; 377 ± 77 *vs.* 129 ± 51 nmol/mg protein, *p*=0.000). The glutathione (GSH) in the NASH group was also significantly decreased as compared with the control group (GSH; 9 ± 2 *vs.* 24 ± 8 nmol/mg protein, *p*=0.001).

The NASH group showed macrovesicular steatosis, microvesicular steatosis, cellular ballooning, and lobular inflammation in microscopic study of paraffin section. On the other hand, the *Aloe vera* group showed significant decrease in the levels of MDA compared with the NASH group (MDA; 199 ± 35 *vs.* 377±77 nmol/mg protein, *p*=0.000), as well as improved GSH level (GSH; 18 ± 4 *vs.* 9 ± 2 nmol/mg protein, *p*=0.039). *Aloe vera* treatment could alter MDA and GSH levels, and also improve the liver histopathology.

Conclusion: *Aloe vera* may attenuate the oxidative stress. It can increase GSH and improve liver histopathology in rats with NASH.

Key words : NASH, *Aloe vera*, Oxidative stress, MDA, GSH

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[1]*Department of Pathology,* [2]*Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand*

Address for Correspondence: *Prof. Dr. Duangporn Werawatganon, MD,, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. dr.duangporn@gmail.com*
Telephone: +662-256-4267 Fax: +662-256-4267

Introduction

Non-alcoholic steatohepatitis (NASH) is the most common liver disease worldwide[1]. Histologically, it may present as macrovesicular -microvesicular steatosis, inflammation, fibrosis and finally cirrhosis[2]. The starting point for and NASH is unclear for starting point. The "two-hit" hypothesis (the "two-hit" theory) has been proposed, with NASH undergoing two steps of liver injury. In the first hit, fat accumulates in the liver causing steatosis in 5-10% of the liver cells. Consequently to the second hit, the liver cells progress to inflammatoin, steatosis and fibrosis[3], leading to NASH.

NASH is associated with the metabolic syndrome and, insulin resistance. Popular "fastfood" diet comprises fat, oil and fructose[4]. Fructose is hepatotoxic and is readily transferred to the liver cells[5]. Fat accumulation in the liver cells appears as macrovesicular as well as microvesicular fat in the hepatic cytoplasm. Oxidative stress is the major factor that induces liver cell inflammation at the second stages. This is promoted by lipid peroxidation products or MDA[6,7]. Reactive oxygen species (ROS) including oxidant reactives such as peroxide, superoxide and nitric oxide activate the progression of NAFLD[8]. This results in the imbalance of the oxidant-antioxidant system in the liver[9]. In addition, ROS events further promote liver injury, necroinflammation and apoptosis of the liver cells. Consequent on the increase reactive oxygen species production, proinflammatory cytokines such as tumor necrosis factor (TNF) and iron overload appear in NASH[10]. Glutathione is the most studied ROS that comprises a tri-peptide of the amino acids glutamine, cysteine, and glycine, and co-factor enzymes for cytoplasmic in the body[11]. GSH plays a role in the detoxifi cation and controls immuno-modulatory mechanisms, and protects cells from damage and aging[12]. NASH patients have decreased GSH levels[13] and increased oxidative stress levels represented by MDA contents[14].Therapy is not absolutely essential, but prevention and retarding the progression of NASH is needed.

Aloe vera (*Aloe barbadensis miller*) is one of approximately 420 species of plants in the same the genus[15]. Originated in dry areas of Africa, Asia, and Southern Europe, *Aloe vera* for several decades has been widely used worldwide[16]. The plant consists of two parts, the outer green rind including the vascular bundles and the parenchyma containing *Aloe vera* clear gel. The major component is formed in the aloe parenchyma tissue or pulp. The plant contains many important antioxidants such as vitamins A, C and F, Vitamins B (thiamine), niacin, vitamin B_2 (riboflavin), vitamin B_{12}[16-18] minerals, saccharides, amino acids, anthraquinones and enzymes[19]. *Aloe vera* has been used in various cosmetics, food supplements, pharmaceutical products and medicines. It is thus known as the healing plant[20]. Medically, *Aloe vera* is used as laxative, anti-inflammatory[20-23], immunostimulant, antiseptic, anti-tumorous, anti-ageing and anti-diabetic agents[21]. In addition, *Aloe vera* can be used as a biochemical liver marker[24-26], as when the liver cells are damaged, *Aloe vera* can block certain hepatic enzyme systems, such as cytochrome P450 and other enzymes involving in ethanol metabolism[25]. *Aloe vera* can lower blood glucose,serum AST, serum ALT, and alkaline phosphatase[20,22]. Recently, a study by Ayse C. *et al.* showed that Aloe vera increased GSH levels and decreased serum ALP and ALT activities in the liver of type-II diabetic rat models[20]. Another recent study by Werawatganon D, *et al.* revealed a correlation between liver MDA level (a marker of peroxidation) and IL-12, IL-18, (makers of inflammation), which were all decreased when *Aloe vera* was given. That study confirmed that *Aloe vera* was protective against hepatocyte damage[27]. In our study, we studied the about effects of *Aloe vera* on oxidative stress in an experimental rat model.

Materials and Methods

Animal preparation

Male Sprague-Dawley rats weighing 220-260 grams from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, were chosen. The animals were allowed to rest for one week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University. They were kept at a controlled temperature of 25 ± 1˚C under standard conditions (12 hour dark: 12 light cycle) and fed with regular dry rat chow ad libitum, with free access to drinking water.

Experimental protocol

Rats were randomly divided into 3 groups of 6 rats each, as follows.

Group 1 (Control group): rats were fed ad libitum for 8 weeks with standard diet containing 35% of energy from fat, 47% from carbohydrate, and 18% from protein.

Group 2 (NASH group): rats were fed ad libitum for 8 weeks with high-fat high-fructose diet (HFHFD) containing 55% of energy from fat, 35% from carbohydrate, and 10% from protein to induce NASH.

Group 3 (*Aloe vera* group): rats were fed ad libitum for 8 weeks with HFHFD plus *Aloe vera* in DMSO (50 mg/kg) by gavage feeding daily.

Aloe vera preparation

Aloe vera powder was supplied by Lipo Chemical Co, United States. The powder was dissolved in DW before use. The concentration of Aloe vera is 50 mg/kg. *Aloe vera* was suspended in DW 1.75 mL/rat by gavage once a day for eight weeks.

At the end of the study, all rats were sacrificed using an intraperitoneal injection of an overdose of thiopental sodium (45 mg/kg). The rat abdominal wall was opened, the liver excised quickly and cleansed in ice-cold normal saliva (NSS). One lobe of the liver was frozen in liquid nitrogen and stored at -80˚C for MDA and GSH analysis. The remaining pant of the liver was fixed in 10% formaldehyde for histological examination.

Hepatic malondialdehyde determination

MDA is an end-product of lipid peroxidation in cells. MDA was measured using the rate of production of thiobarbituric acid-reactive substances (TBARS) assay kit (Cayman, USA), as per the principle of the MDA method. One gram of liver tissue was homogenized in buffer on ice. An aliquot of 0.2 mL was mixed with a solution containing 20% acetic acid, 0.8% thiobarbituric acid, and 8.1% sodium dodecyl sulfate under high temperature, and was heated in water bath at 95˚C for 60 minutes. After centrifuging for 10 minutes at 1000 g, the MDA was quantitated using a spectrophotometer at 532 nm. The content of MDA was expressed in terms of nmol/mg protein.

Hepatic GSH

Hepatic GSH was assessed using a commercially kit based enzymetic method as per the instruction manual (Cayman, USA). The GSH produced in each sample was measured by the absorbance of TNB at 405 or 412 nm, and the result was expressed as as molar extinction coefficient (nmol/mg protein).

Histopathological analysis

Liver tissue was processed by standard technique. The collected tissue was fixed in 10% formalin at room temperature for 24-48 hrs, embedded in parafin and sectioned at 3 μm using a microtome machine. Each liver paraffin-embedded section was stained with hematoxylin and eosin (H&E) for histopathological characterization. Steatosis and necroinflammation in each specimen was determined by the pathologist and graded with semiquantitative scores according to the criteria described by Brunt *et al*.[28].

The severity of steatosis was graded as the percentage of parenchymal cells containing fat as follows: 0 = less than 5% of hepatocytes containing fat, 1 = less than 33% of hepatocytes containing fat, 2 = 33% - 66% of hepatocytes containing fat, and 3 = more than 66% of hepatocytes containing fat.

Hepatic inflammation was scored from 0 to 3: 0 = no inflammation, 1 = mild focal zone 3 hepatocyte inflammation, 2 = moderate zone 3 hepatocyte inflammation, and 3 = severe zone 3 hepatocyte inflammation.

The presence of ballooning degeneration was graded from 0 to 2: 0 = no ballooning cell, 1 = few balloon hepatocytes, and 2 = many balloon hepatocytes.

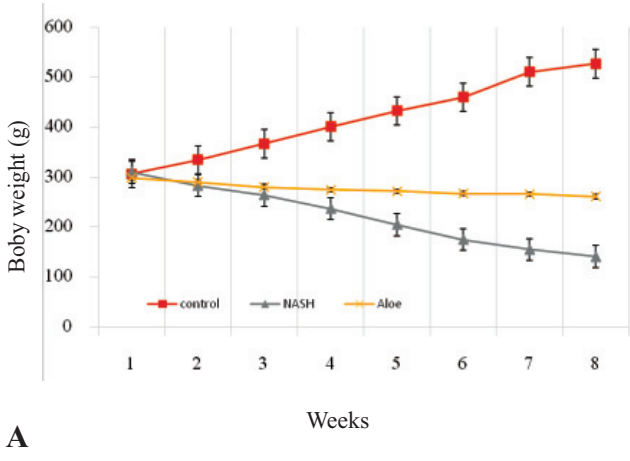
Statistical analysis

All data were presented as mean and standard deviation (SD). One-way ANOVA and Tukey post-hoc was used to compare the results. Descriptive statistics was used for presenting histological examination of liver tissue. The value of $p<0.05$ was considered statistically significant.

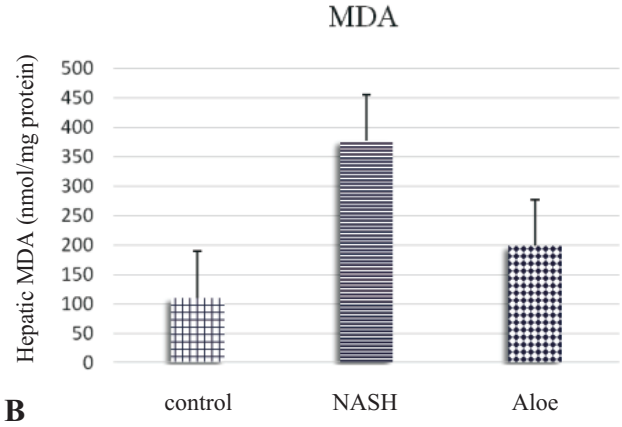
RESULTS

The change of body weight in each group of rat

No rats died before the end of the study. The average initial body weight and after 8 weeks of the study were as following; rats feed with HFHFD (NASH group) were noted for a lower weight than the group feed with standard diet (417±11.2 g *vs.* 223±14.0 g, respectively; $p<0.05$). After 8 weeks, there was a significantly increased body weight in the *Aloe vera* group, comparing with the NASH group (276±3.6 g *vs.* 223±14.0 g, $p<0.05$) (Figure 1A).



A



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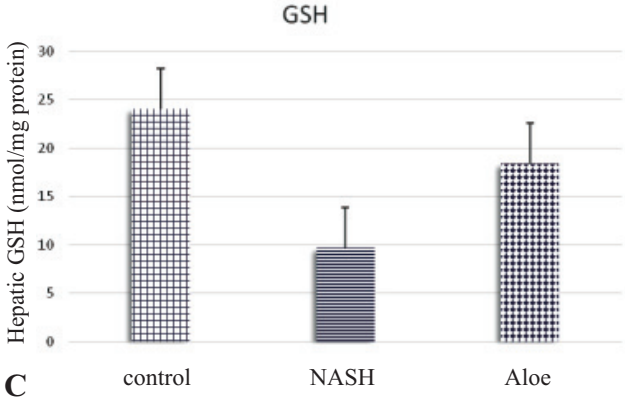

C

Figure 1. The mean ± SD of *Aloe vera* on body weight, hepatic malondialdehyde, and hepatic GSH in rats with non-alcoholic steatohepatitis.
A: body weight;
B: Hepatic malondialdehyde (MDA);
C: Hepatic GSH.
$p = 0.001$ NASH vs. control
$p < 0.05$ NASH vs. *Aloe*

Histopathology of liver tissue

H&E staining was employed to display the histological score (Table 1). Steatosis, hepatocyte ballooning or lobular inflammation was not found in the liver tissue of the control group. However, the liver tissue of rats in the NASH group showed steatosis (2+) with macrovesicular and microvesicular fat, cellular swelling in hepatocytes (2+) and lobular inflammation (2+) compared with the control group. After treatment with Aloe vera, liver pathology was significantly improved compared with the NASH group. The liver tissue in the *Aloe vera* group exhibited mild steatosis (1+), cellular swelling (1+) and mild lobular inflammation (1+). The histological scores of steatosis and necroinflammation are summarized in Table 1.

Hepatic MDA level

The MDA level in the NASH group was significantly increased compared with the control group (377 ± 77 *vs.* 129 ± 51 nmol/mg protein, $p<0.05$). After treatment with *Aloe vera*, the MDA level was significantly decreased in the *Aloe vera* group compared with the NASH group (199 ± 35 *vs.* 377±77 nmol/mg protein, $p <0.05$) (Figure 1B).

Hepatic GSH level

Glutathione (GSH) in the NASH group was significantly decreased compared with the control group (9 ± 2 *vs.* 24 ± 8 nmol/mg protein, $p<0.05$). After treatment with *Aloe vera,* the liver GSH was significantly increased in the *Aloe vera* group compared with the NASH group (18 ± 4 *vs.* 9 ± 2 nmol/mg protein, $p=0.039$) (Figure 1C).

DISCUSSION

There are other models for studying NASH, such as the model of high-fat diet, and the model of methionine and choline deficient diet. In our study, we chose high-fat and high-fructose diet to induce NASH in Sprague-Dawley rats, which accordance to the pathogenesis of stations and inflammation in the liver. Fructose is primarily metabolized in the liver, unlike glucose which requires insulin[29]. In a previous animal

Table 1. Summary of scores of steatohepatitis and necroinflammation levels in all experimental groups graded by Brunt *et al* [28].

Group	n	Steatosis				Inflammation				Ballooning		
		0	1	2	3	0	1	2	3	0	1	2
Control	6	6	-	-	-	6	-	-	-	6	-	-
NASH	6	-	-	3	3	-	5	1	-	-	4	2
NASH + *Aloe vera*	6	3	3	-	-	2	4	-	-	3	3	-

Data are expressed as the number of rats presenting each score of histopathology. Levels of steatosis: 0 = < 5%; 1 = < 33%; 2 = 33-66%; 3 = > 66%. Levels of inflammation: 0 = normal; 1 = mild; 2 = moderate; 3 = severe. Levels of ballooning degeneration: 0 = no ballooning; 1 = few balloon cells; 2 = many balloon cells.

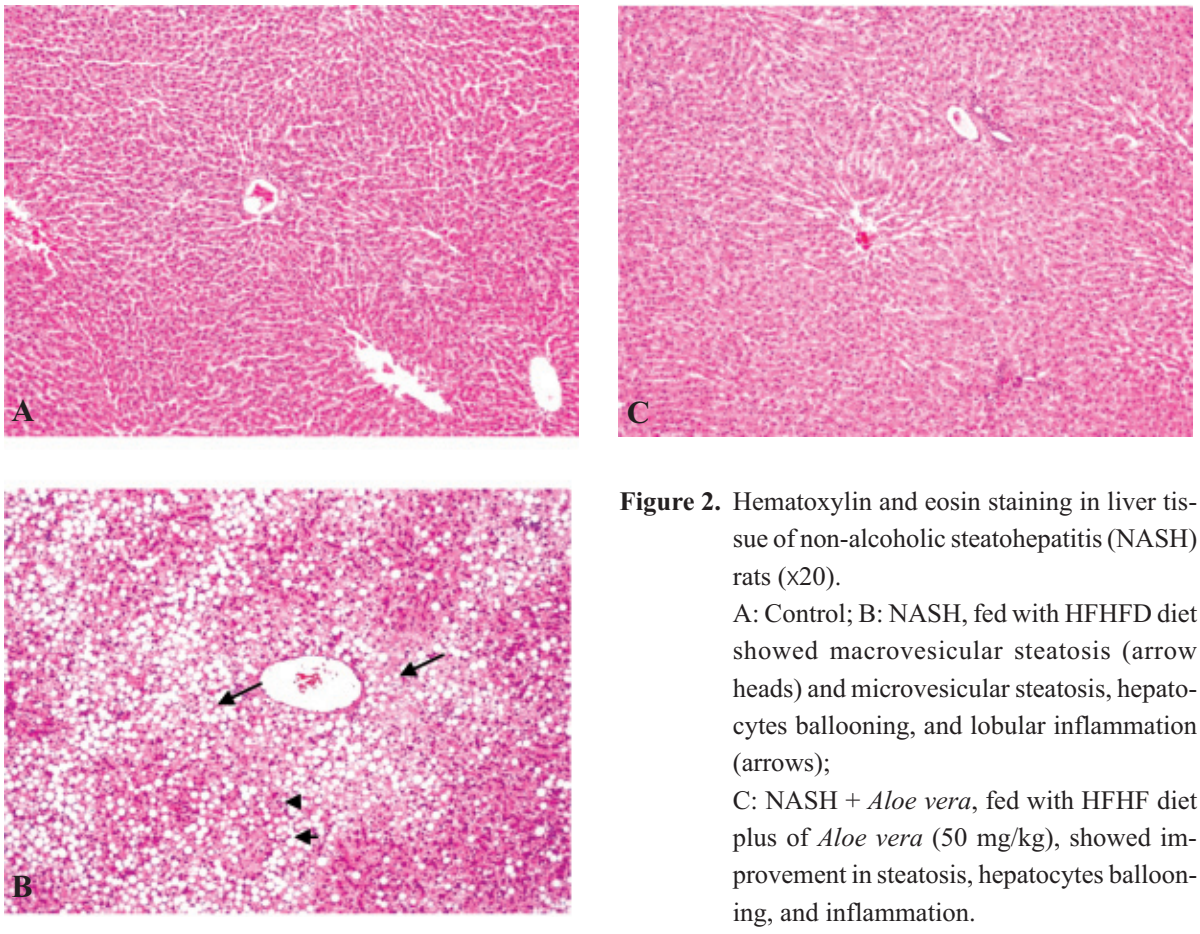

Figure 2. Hematoxylin and eosin staining in liver tissue of non-alcoholic steatohepatitis (NASH) rats (x20).
A: Control; B: NASH, fed with HFHFD diet showed macrovesicular steatosis (arrow heads) and microvesicular steatosis, hepatocytes ballooning, and lobular inflammation (arrows);
C: NASH + *Aloe vera*, fed with HFHF diet plus of *Aloe vera* (50 mg/kg), showed improvement in steatosis, hepatocytes ballooning, and inflammation.

study, fructose was said to increase lipid peroxidation and to activate of inflammation[30] of the liver cells by reducing intracellular ATP as well as oxidative responses. Therefore, high fructose consumption may contribute to NAFLD pathogenesis because fructose induces ATP depletion and promotes hepatic necroinflammation[33-35]. A high fructose intake may play a role in the development of fibrosis and liver failure[4,5].

Elevation of free fatty acid (FFA) from lipolysis is due to reduction of liver oxidation and an increase of adipose tissue[38]. FFA metabolism increases the production of ROS, which is important to activate inflammation to second hit in NASH[7]. High levels of ROS can activate lipid peroxidation in the liver and release metabolite products such as thiobarbituric acid, a markers of lipid oxidation[6]. MDA reacts with thiobarbituric acid[6,7,38,39] and an elevated level of MDA was found

in NASH[40]. In our study, high levels of liver MDA were noted in rats fed with high-fat high-fructose diet[41-45]. Although high level of MDA was found in liver tissue, reduction of antioxidant substances[46-48] such as glutathione (GSH)[49-51] were reported. Glutathione is a tri-peptide considered metabolic toxicity[51] to the liver and the kidneys, and plays an important role in the antioxidant system[49-48]. We found that *Aloe vera* contributed to antioxidant and improved liver lipid peroxidation as well as liver histopathology in *Aloe vera* feeding rats (50 mL/kg)[52]. *Aloe vera* retarded the second stages of NASH, thus improving the liver histology and reducing the remembers of inflammatory cells, as evident by histopathological findings. The bioactive component of *Aloe vera* is aloe-emodin or 1,8 dihydroxyanthraquinone[53], which has an anti-inflammatory property that mitigates inflammatory cytokines such as nitric oxide synthase (iNOS) and interleukin-6 (IL-6). The *Aloe vera* NF-ĸB, MAPK, and PI3K pathways[54,55], contributing to the inhibition of TNF alpha synthesis. Moreover, the aloe-emodin component decreases the number of neutrophils in the liver tissue[56]. This has led to the use of *Aloe vera* in skin burns and inflammatory bowel diseases. Overall, it can be said that *Aloe vera* has an antioxidant protection property[57-58]. In addition, *Aloe vera* can decrease MDA level[5]. This was evident from previous studies showing that *Aloe vera* induced antioxidant production and improve MDA in rats[59-62]. In our study, GSH-antioxidant probably was protective for liver tissue, playing a role in the intercellular interaction of free radicals and decreasing oxidative damage caused by escalating glutathione. This process could result in deleterious effects due to accumulation of ROS. Our findings correlated with other studies asserting the effect of *Aloe vera* and GSH-in NASH[63,64].

In conclusion, the results of this study suggested that *Aloe vera* could restore GSH, improve body weight, decreasing MDA levels as well as ameliorating the liver histopathology.

Our findings also indicate that *Aloe vera* can reduce inflammation and oxidative stress on the liver, thus making it a potential therapeutic agent for the treatment of NASH.

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