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Research Article

HISTOPATHOLOGICAL STUDY OF THE PROTECTIVE EFFECT OF SPIRULINA PLATENSIS ON FETAL LIVER OF MICE WITH ACUTE FATTY LIVER OF PREGNANCY

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ABSTRACT

Objective: The protective effect of Spirulina platensis on the fetal liver of mice with acute fatty liver of pregnancy was investigated.

Methods: Small female mice were divided into four groups: A control group with a standard diet, a high-fat diet to induce liver steatosis, a high-fat diet plus Spirulina, and a high-fat diet plus Simvastatin given through gavage to protect the liver against steatosis. After 2 months, the female mice became pregnant by polygamy method at the same time they were treated by different diets. On day 17, the fetuses were removed by C-section, and histological studies were carried out on their livers.

Results: The results showed a significant decrease in liver steatosis in the group treated by Spirulina compared with the other groups ($p<0.05$). The fatty liver of pregnancy could lead to liver failure and death in both mother and fetus, and medications like Simvastatin that is used for the treatment of fatty liver are harmful to the fetus. However, Spirulina shows a positive effect on the treatment of both fetus and mother.

Conclusions: The results of this study show that Spirulina is an effective medical supplement in the treatment of fatty liver of pregnancy.

Keywords: Acute fatty liver of pregnancy, high-fat diet, Spirulina, liver steatosis, fatty liver of pregnancy, fetal liver.

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INTRODUCTION

The liver is one of the most important organs in the body with an important role in detoxification, storage of glycogen, and metabolism of fats and carbohydrates. One of the most important and common diseases of the liver are the fatty liver which is divided into three types: Alcoholic fatty liver, non-alcoholic fatty liver, and fatty liver of pregnancy. What makes this condition worse is the irreversible destruction of liver cells if the disease is not diagnosed in the early stages. The fatty change could be subdivided into microvesicular and macrovesicular steatosis. In macrovesicular steatosis, the hepatocytes contain one bulky vacuole that displaces the nucleus. This type is mostly caused by obesity, diabetes, or alcohol abuse. In microvesicular steatosis, the cytoplasm is occupied by several small fat vesicles, and the nucleus remains centrally placed. It usually results from mitochondrial β-oxidation defects, which are usually associated with toxins or metabolic disorders [1].

Acute fatty liver of pregnancy (AFLP) is a rare but important disease that occurs in the third trimester of pregnancy. AFLP is one of the factors causing the microvesicular fatty liver. From the histological point of view, in microvesicular fatty liver, cell necrosis is minor and variable; the hepatocytes become swollen and have central nuclei with prominent nucleoli. In some examinations, centrizonal cholestasis is seen, and examinations by electron microscopes show that the mitochondria are swollen and pleomorphic, which would also decrease the oxidation of fatty acids, and the ribosomal patterns which are on the rough endoplasmic reticulum are seen abnormal [2]. Abdominal pain, nausea, vomiting, and fatigue are common symptoms of AFLP. Sometimes the mothers have been reported to experience hypoglycemia, hepatic encephalopathy, and moderate to severe coagulopathy, and preeclampsia [3]. Laboratory experiments show an increase in bilirubin and aminotransferase levels, and sometimes it involves neutrophilic leukocytosis, and with the progression of the disease, thrombocytopenia and hypoalbuminemia may also be seen [3,4]. In diagnosing this disease, examining the white blood cells shows a decrease in platelets and normoblasts [5]. Disseminated intravascular coagulation is also common in this disease [6]. Fibrinogen, thromboplastin, and prothrombin are usually abnormal in this disease, and the blood uric acid level increases. As the disease has drastic effects on both mother and child, diagnosis must be made early on. AFLP diagnosis is usually made based on laboratory findings and imaging appearances. In general, if AFLP is not diagnosed early on, it will cause significant maternal and fetal mortality. The main cause of the disease remains unknown; however, molecular studies show that the leading cause of AFLP is mitochondrial dysfunction [7-9]. AFLP usually occurs after unsuccessful or successive pregnancies for the mother. Pathophysiological studies of AFLP show defects in mitochondrial fatty acid beta-oxidation. In general, in normal conditions, no abnormal fatty oxidation is seen in a person who is heterozygous for enzymatic mutations in fatty acid oxidation. However, when a heterozygous mother carries a fetus that is homozygous for such mutations, a lot of fetal fatty acids return to the mother's circulation. This extra burden of long-chain fatty acids, and accumulation of triglyceride causes hepatic fat deposition and impaired hepatic function [3]. Natarajan *et al.* [10] showed that one of the key factors causing liver diseases is oxidative stress, and, in fact, microvesicular liver steatosis leads to mitochondrial dysfunction and oxidative stress. Oxidative stress was also found in preeclampsia patients, a condition which is found in 40% of the patients with AFLP. It is well-known that the most important cellular source of free radicals in preeclampsia patients is mitochondria; additionally, peroxisomes play a role in fatty acids oxidation [11].

Most patients, especially pregnant women, cannot bear the adverse effects of chemical medicines. However, biological substances, which are derived from plants and constitute an important part of modern pharmacotherapy, have few side effects on the patients. Some medications that are used for the treatment of chronic liver diseases are probably used during pregnancy, too; however, some medications

are toxic for the fetus and therefore are not prescribed. As maternal nutrition is an important factor during pregnancy, proper nutrition could help the fetus develop well and ensures the health of the fetus; however, incorrect or unbalanced maternal nutrition causes fetal disorders [12].

Spirulina is a blue-green alga from the Oscillatoriaceae family, which is typically found in tropical and subtropical areas. The nutritional value of Spirulina is high. Spirulina contains a great amount of protein (60–70% of its dry weight), a lot of anti-oxidants such as β-carotene, phycocyanin, and elements such as K, Na, Ca, Mg, Fe, Zn, vitamins (tocopherols), essential amino acids, and polyunsaturated fatty acids, especially γ-linolenic acid, phenolic compounds amino acids, gamma-Linolenic acid, and minerals [13-15]. Thanks to being rich in nutrients, and having therapeutic effects and non-toxicity, Spirulina has been known as one of the most important dietary supplements and useful in the prevention of some diseases in the 21st century [16,17]. Consuming Spirulina supplements are suggested for the prevention and control of hypocholesterolemia [18], hyperglycerolemia [19], galactosamine-induced hepatotoxicity [20] obesity, inflammation [21], cancer [22], cardiovascular diseases [23], insulin resistance, diabetes, nonalcoholic fatty liver, malnutrition, anemia, allergic rhinitis, toxicity of substances [14,16,19], and also for its analgesic effect [24]. Due to its antioxidant properties, Spirulina Platensis has drawn a lot of attention for removing hydroxyl radicals and preventing lipid peroxidation by lowering liver lipid profiles and lipoperoxide products [25].

This study aims to investigate whether a high-fat diet during pregnancy could cause AFLP in both mother and fetus. The protective property of Spirulina against AFLP in mother was also investigated. Although it has been proofed that Spirulina has a protective effect on the liver, there is no report on the effect of Spirulina on the fetal liver during pregnancy. Therefore, the objective of this research is to evaluate the prevention effect of Spirulina on the fetal liver of mice fed with a high-fat diet during pregnancy and study this organ histomorphometrically.

METHODS

Preparing Spirulina pellets and fat emulsion

Powdered Spirulina platensis used in the experimental diet was purchased from Riz Jolbak Parsian Co. The daily needed pellet of each mouse was ground into powder, and then Spirulina was added to it at the ratio of 1:10, and it was mixed with warm water so it would turn into a paste. Then, we turned the paste into pellets suitable for the animals to consume and kept them at room temperature until they dried.

The definition of high-fat-diet is different from humans to the mouse. Standard mouse diets contain lower amounts of fat than the recommended human diet. In contrast to approximately 30% of total energy intake from fats in humans, the standard mouse diets contain less than 10% of kcal fat, whereas high-fat diets and very high-fat diets contain 30%–50% and more than 50% of kcal fat, respectively [26,27]. In this project, the method proposed by Zou *et al*. [28] was used to induce steatosis in the experimental groups. The high-fat emulsion diet contained 77% of its energy from fat, 14% from total milk powder, and 9% from carbohydrates. The composition of macronutrients in this emulsion is shown in Table 1. In the prepared emulsion, proteins were provided by total milk powder, carbohydrates by saccharose, and fat by corn oil. Each diet was supplemented with a vitamin and mineral mixture. The emulsion was stored at 4°C, heated in a 42°C water bath, and fully mixed before use.

Table 1: Composition of the high-fat emulsion diet ingested through gavage to mice

Component	Dosage
Corn oil	400 g
Sucrose	150 g
Whole milk powder	80 g
Cholesterol	100 g
Sodium deoxycholate	10 g
Tween 80	36.4 g
Propylene glycol	31.1 g
Multi vitamin	2.5 g
Salt	10 g
Mixed minerals	1.5 g
Distilled water	300 ml
Total energy	43–42

Experimental animals

Inducing fatty liver

Sixty small female NMRI mice weighing 28±3 g were supplied by the Department of Experimental Animals of Pasteur Institute. To acclimate them to the new environment, the mice were kept in special cages for 1 week before the beginning of the experiment on a 12-h light/dark cycle and temperature of 2±23°C with sufficient supply of water and standard food, produced by Behparvar Co. Experimental procedures were approved by the Ethics Committees for Animal Experiments of Parand Azad University. The mice were randomly assigned to four groups as follows:

- Group (C): The healthy control group
- Group (C-): The group fed with high-fat diet (as the negative control)
- Group (C+): The group fed with high-fat diet + Simvastatin (as the positive control)
- Group (SP): The group fed with high-fat diet + Spirulina.

To get liver steatosis, the negative control group received 10 ml/kg body weight of lipid emulsion every day for 56 days through gavage. Simultaneously, the positive control group received a daily dose of a suspension of 75 mg/kg body weight Simvastatin in 0.1 % methylcellulose through gavage [29]. The Simvastatin was given through gavage to the group (C+) to protect the liver against steatosis, and they were examined for histopathological changes of the liver and the results compared with the Group (SP). The SP group received Spirulina in their diet in addition to gavage of fat emulsion, and finally, 10 ml/kg body weight of normal saline was given through gavage to the mice in the control group.

Pregnancy

The mice become pregnant after 56 days (8 weeks) of diets through polygamy. The two female mice were mated with one male mouse. Dams failing to become pregnant were allowed to remate. Day zero of pregnancy was determined by the formation of a vaginal plug. Dams were maintained on their diets through pregnancy. On the 17th day, the mice were anesthetized using ketamine and xylazine, and the cesarean section was performed and the fetuses and placenta were removed from uterine horns. Five fetuses were selected randomly from each pregnant mouse, and they were dissected using stereomicroscope and their livers were removed for pathological examinations.

Morphological evaluation

For histopathological analysis, liver specimens fixed in 10% neutral-buffered formalin for 24 h and were then embedded in paraffin, sliced at 5-μm thickness and stained with hematoxylin and eosin (H&E) staining for detection the degree of hepatic steatosis [30]. The pathological changes were assessed and photographed under a Nikon optical microscope (ECLIPSE E200). Liver biopsy for liver steatosis based on lipid changes in hepatocytes was scored according to Takahashi and Fukusato [31] as follow zero: No steatosis, normal liver; I: <25% of hepatocytes affected; II: 26–50% of hepatocytes affected; III: 51–75% of hepatocytes affected; and IV: More than 76% of hepatocytes affected. The cells were counted using a lens with a rectangular framework measuring 83 μm×62 μm on an area of 5146 μm^2. Grading was done at ×40 in ten microscopic fields.

Total bilirubin measurement

To test the changes in total bilirubin, which is one of the symptoms of AFLP, blood samples were taken from the heart before and during

pregnancy. The serum total bilirubin was assayed according to Gaber [32].

Statistical analysis

SPSS statistical software was used to analyze the data. Quantitative data were expressed as mean ± standard deviation, and Scheffe *post hoc* test was used to examine differences between groups and one-way ANOVA was used to compare liver fat levels across all groups. The significance level was set at $p<0.05$.

RESULTS

The effect of Spirulina platensis on maternal blood bilirubin

The effect of Spirulina platensis on maternal blood bilirubin in mothers with AFLP before and during pregnancy is shown in Table 2. According to Table 2, before pregnancy, serum bilirubin levels increased significantly in the negative group compared with all other groups. Serum bilirubin levels increased significantly in the Group (C+) and (SP) compared with the control group. However, it decreased compared with the Group (C-). Compared with Group (SP), serum bilirubin levels decreased significantly in the Group (C+) ($p<0.05$). The results also showed that serum bilirubin levels during pregnancy increased significantly in the Group (C−) compared with the other groups. Serum bilirubin level increased significantly in the Group (C+) and (SP) compared with the Group (C); however, compared with the Group (C+), it decreased significantly in the Group (SP) ($p<0.05$).

Histopathology of the effect of Spirulina platensis on fetal liver damage

In microscopic examinations, there was no abnormal condition such as necrosis and infiltration of inflammatory cells in the fetal livers tissue of the control healthy group (Fig. 1a); however, in the liver of fetuses whom mothers fed with a high-fat diet for 8 weeks, Group (C−), lipid changes, and fatty liver in mothers caused heavy damages to the fetal livers as well, and a big part of hepatocytes was destroyed, and hyperemia and infiltration of inflammatory cells into hepatocytes can be seen. In addition, *sinusoidal spaces* are tighter and the concentration of blood islands is high (Fig. 1b). In the Group (C+), which had received a high-fat diet plus Simvastatin, more inflammation and necrosis were seen than those in the Group (SP). In fact, in Group (C+) hyperemia and infiltration of inflammatory cells accompanied liver damage and necrosis of hepatocytes. In comparison, Group (C+) shows more hepatocyte concentrations, wider sinusoidal spaces, and fewer blood islands than Group (C−) (Fig. 1c). In the fetal livers of the Group (SP), hepatic hyperemia and inflammatory cells were seen but liver damage and concentration of inflammatory cells were less than those in the Group (C+). In addition, compared with the negative control group, there is a high concentration of hepatocytes, small narrow sinusoidal spaces, and few blood islands, signifying the effectiveness of Spirulina (Fig. 1d).

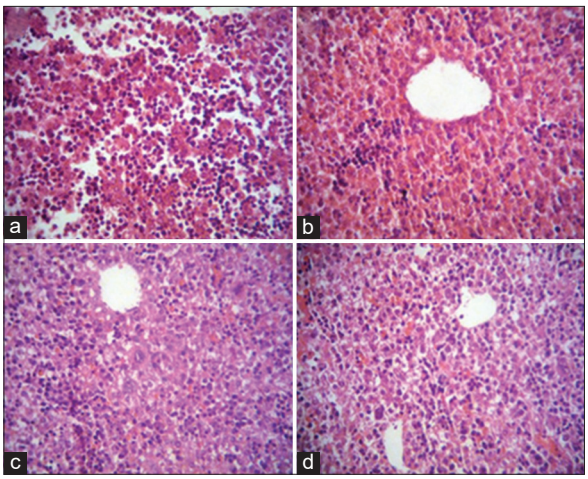

Fig. 1: Histological changes in the liver of 17-day fetuses from dams with the following diets: (a) Group (C), (b) Group (C-), (c) Group (C+), and (d) Group (SP), (Staining by haematoxylin-eosin and magnification of ×40)

Table 2: Bilirubin level (TB) before pregnancy and during pregnancy

Treatment	TB (mg/dl) before pregnancy	TB (mg/dl) during pregnancy
Group (C)	0.33±0.01	0.39±0.02
Group (C-)	0.90±0.04	0.85±0.04
Group (C+)	0.35±0.04	0.44±0.03
Group (SP)	0.31±0.04	0.58±0.03

Data expressed as mean±standard deviation for each group ($p<0.005$)

Cell count

To examine the disease more closely, the liver fat content of the fetus livers was measured, and hepatocytes, macrophages, blood islands, bile ducts, and megakaryocytes were counted and results are furnished in Table 3. The results showed that fetus liver fat content in Group C− increased significantly compared with the Group C while the fat content of the fetus from the Group SP is in the range of the fat content of the Group C+. Moreover, no histomorphometric change was seen in the liver tissue of the fetuses in the Group C. Hepatocyte changes in the fetal livers in Spirulina and positive groups decreased compared with the control group, but they increased compared with the negative group. There was no significant difference in the macrophage variable between the control and Spirulina groups; however, it decreased significantly in the control group compared with the positive and negative groups ($p<0.001$). In addition, it decreased significantly in the positive group compared with the negative group. The blood islands variable increased significantly in the negative group compared with the other groups, and it increased significantly in Spirulina and positive groups compared with the control group, but it decreased significantly compared with the negative group. The bile duct variable increased significantly in Spirulina and positive groups compared with the control group, but it decreased significantly compared with the negative group. The megakaryocytes variable increased significantly in the negative group compared with the positive and control groups, but it was not significantly different from the Spirulina group, and it decreased significantly in the positive group compared with the other groups. The liver fat was significantly lower in the control group compared with the Spirulina and negative groups. In addition, although the liver fat was lower in the control group than in the positive group, the difference was not significant. Finally, the liver fat in the Spirulina and positive groups was significantly lower than that in the negative group.

Table 4 shows the result of one-way ANOVA of the liver fat scores and cell variables in the liver of the fetus in the four groups of C, C−, C+, and SP. A significant difference across all four groups in the liver fat ($p<0.001$), hepatocytes ($p<0.001$), and macrophages ($p<0.001$) can be observed. In addition, no significant difference was seen across the four groups in blood islands ($p=0.615$), bile ducts ($p=0.611$), and megakaryocytes ($p=0.973$). Scheffe *post hoc* test was used to examine the differences in liver fat, the result of which is shown in Table 5.

As shown in Table 5, liver fat is significantly lower in the control group than in Spirulina ($p<0.003$) and negative ($p<0.001$) groups. In addition, although there was a difference in liver fat between the control group and the positive group, the difference was not statistically significant ($p=0.168$). The liver fat in the Spirulina group was more than that in the positive group, but the difference was not significant ($p=0.424$). On the other hand, liver fat in the Spirulina group was significantly lower than that in the negative group ($P= 0.001$). Finally, liver fat in the positive group was significantly lower than that in the negative group ($p\leq0.001$).

Table 3: Mean and standard deviation of liver fat content and cell variables in the liver in the four groups of C, C−, C+, and SP

Variable	Control	Spirulina	Positive	Negative
Liver fat	1.60±0.70	2.90±0.56	2.30±0.48	4.20±0.78
Hepatocytes	106.8±10.51	101.50±24.71	84.90±20.04	70.40±14.09
Macrophages	2.60±1.07	2.60±0.84	6.0±1.76	8.70±1.70
Blood islands	0.90±0.73	1.20±1.03	1.30±1.15	1.50±1.08
Bile ducts	0.30±0.48	0.40±0.51	0.40±0.51	0.60±0.51
Megakaryocytes	2.10±0.73	2.20±1.39	2.0±0.94	2.20±1.22

Table 4: One-way ANOVA for comparing the liver fat scores and cell variables in the fetus liver in the four groups of (C), (C−), (C+), and (SP)

Variable	F	p
Liver fat	27.72	p<0.001
Hepatocytes	8.28	p<0.001
Macrophages	44.35	p<0.001
Blood islands	0.606	0.615
Bile ducts	0.613	0.611
Megakaryocytes	0.075	0.973

Table 5: Scheffe *post hoc* test to examine pairwise differences in liver fat

Variable	Group	Group	Mean differences	p
Liver fat	Control	Spirulina	−1.20	0.003
		Positive control	−0.70	0.168
		Negative control	−2.60	<0.001
	Spirulina	Positive control	0.50	0.424
		Negative control	−1.40	0.001
	Positive control	Negative control	−1.90	<0.001

DISCUSSION

This study was aimed at investigating the protective effect of Spirulina platensis on the fetal liver of mice with AFLP. The results showed that there was a significant difference in maternal total serum bilirubin between the experimental groups and the control group before and during pregnancy. In fact, quantification of serum bilirubin is evidence for the assessment of liver function and an unusual increase in the levels of bilirubin in the serum indicates severe perturbation of hepatocellular function. In this research, the bilirubin level in the Spirulina group before pregnancy increased significantly compared with the positive and control groups, but it decreased significantly compared with the negative group ($p<0.05$). Then, after pregnancy, the bilirubin level in the Spirulina group showed a significant decrease compared with all the other three groups ($p<0.05$). This finding shows that Spirulina could probably treat bile duct damage in the liver (Table 3) and could be able to strengthen biliary dysfunction. Similar protective effects were observed for the *Spirulina fusiformis* [20]. A lot of studies have investigated the effect of Spirulina on different blood parameters and even on liver enzymes, all of which have pointed out the beneficial effects of this cyanobacterium. Therefore, we can consider Spirulina as an effective dietary supplement in reducing enzyme disorders, bile duct disorders, and other complications. Studies have shown that this alga is a probiotic organism that enhances health [33]. This alga is rich in unsaturated fatty acids, phycocyanin [34], and phenolic compounds [35]. Different researchers have studied the effect of Spirulina on proteins and blood lipids [36,37], anti-oxidant properties especially phycocyanin, its antiviral, and immunomodulatory properties in animals [38]. Studies have found that oxidative stress is involved in many liver diseases, and it leads to liver damage [20,39]. Therefore, antioxidants play an important role in scavenging free radicals and thereby protecting the liver. In addition, Spirulina contains plenty of superoxide dismutase (SOD) (an antioxidant enzyme), which removes free radicals [40]. Many algae and cyanobacteria are rich in phycocyanobilin (PCB) and are considered rich sources of the chromophore, which helps absorb light as a component of holoprotein phycocyanin [41]. PCB is one of the derivatives of biliverdin that, in mammals, is expressed by biliverdin reductase to phycocyanobilin, which is quite similar to bilirubin in structure. Studies have shown that about 0.66% of the dry mass of Spirulina is PCB (phycocyanin comprises around 14% of the total dry weight of Spirulina and PCB constitutes 4.7% of the mass of phycocyanin), making it easy to be absorbed by the body and to have a high antioxidant activity (15–29). Considering the findings of studies on the effect of Spirulina on blood factors, we could probably conclude that the existence of the abovementioned compounds in this alga reduced the bilirubin level during pregnancy in Spirulina group compared with the other groups in the present study. However, studies show that this is a pathologic condition and requires more examinations. In general, the serum bilirubin level is an indicator of the liver function and abnormal increase in bilirubin level indicates a severe disorder of hepatocellular function [42]. Unlike HELLP syndrome and liver complication in pre-eclampsia and eclampsia, AFLP is a disease with real liver dysfunction. In AFLP, hyperbilirubinemia is seen without hemolysis; however, in pre-eclampsia, hyperbilirubinemia is rare and is in interaction with hemolysis [4]. In severe cases of pre-eclampsia, elevated bilirubin level to less than 6 mg/dL has been reported [2]. On the other hand, elevated bilirubin level (usually above 5 mg/dc) indicates AFLP [43]. The findings of the present study about bilirubin levels support the findings of studies by other researchers [4,10,43]. Studies have also found that bilirubin or its precursor (biliverdin) could prevent a large range of disorders as an oral antioxidant. Recent studies have found that bilirubin in nanomolar concentrations acts as a powerful inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Expression of heme oxygenase-1 is induced by intracellular oxidant stress, which produces bilirubin from heme through biliverdin; this mechanism provides feedback control of the oxidant stress-mediated by NADPH oxidase. Bilirubin's suppressive impact on NADPH oxidase activity likely explains the growing epidemiological literature that associates increased serum bilirubin, or high-expression polymorphisms of heme oxygenase-1, with diminished risk for vascular disorders, certain cancers, and various other diseases. Recent molecular studies have pointed out that the cause of AFLP is probably mitochondrial dysfunction. Some studies have shown that AFLP is like autosomal diseases that result from fatty acids oxidation defects. The least likely cause of AFLP is beta-oxidation defect. The pathogenesis of AFLP still seems to be elusive. A lot of studies have pointed out that this disease is directly and indirectly associated with the deficiency of the long-chain 3-hydroxyacyl-CoA dehydrogenase in the fetus, which is a fatty acid beta-oxidation defect [9]. The fetal liver tissue was investigated in mice with AFLP in the present study.

At the beginning of the study, examining the liver tissue showed that lipid affected liver structure and caused liver steatosis. Then, semi-thin sections of the livers were prepared to be investigated by a microscope. Histological studies showed that the increase in the intake of fat by the mother affected both maternal and fetal liver. These examinations also showed that due to the high intake of fat, lipid vesicles increased in all experimental groups (negative, Spirulina, and positive) compared with the control group, which showed deposition of lipid in fetal liver cells. It was also showed that in the Spirulina group, lipid vesicles, hepatic

hyperemia, and inflammatory cells were seen, but they decreased significantly compared with the positive and negative groups and increased significantly compared with the control group (Fig. 1d) ($p<0.05$). In other words, Spirulina had a positive effect on both maternal and fetal livers. Considering the findings of the present study, we can say that Spirulina decreases deficiencies in lipid metabolism resulting from a high-fat diet. In addition, the findings showed that in Spirulina and positive groups, forming lipid vesicles in liver parenchymatous cells decreased significantly compared with the negative group, signifying the effectiveness of Spirulina and Simvastatin in decreasing lipid deposition in liver structure (Fig. 1c and d). This finding is supported by the findings of the study by Blé-Castillo *et al*. [29]. In fact, the findings showed that high-fat diet affects lipid metabolism in fetal liver, and the existence of lipid vesicles in fetal livers is a sign of lipid concentration and increased lipid deposition [44,45] stated that factors such as hepatocyte dysfunction (especially mitochondria), deficiency in prolactin proliferator receptors, and receptor inflammation are involved in causing NASH. In a similar study, Pruis *et al*. [44] stated that regardless of electron transport, signs of change in mitochondrial function, microvesicular fat accumulation, decreased plasma b-hydroxybutyrate, and downregulation of PPARA mRNA are seen. We can assume that peroxisomal and mitochondrial b-oxidation systems are not properly regulated so that they can adapt to the increase in fatty acids production. The hepatic triglyceride accumulation prevents regulation of fatty acid synthesis, mitochondrial B-oxidation, and fatty acid export. These factors could play an important role in causing AFLP in both mother and fetus.

The liver tissue was then examined and it was observed that the number of hepatocytes increased significantly in the Spirulina group compared with the positive and negative groups; however, it decreased significantly compared with the control group ($p<0.001$). This finding shows that Spirulina could prevent hepatocyte apoptosis. Examining hepatocytes showed a significant increase in the number of hepatocytes in the control group compared with the other groups. In fact, this finding shows that hepatocyte apoptosis is an important pathologic feature of NAFLD [46,47]. In addition, hepatocyte apoptosis and inflammation have been seen as directly associated with fibrosis. Different studies on NAFLD in humans and animals and during *in vitro* hepatocyte steatosis have shown that the accumulation of lipid vesicles in liver cells increases apoptosis [47]. This fact shows an association between hepatocyte apoptosis and liver fibrogenesis [48]. Fibrogenic activity is started by engulfment of apoptotic bodies by Ito cells in these cells, and it may be how hepatocyte apoptosis promotes fibrosis [49]. In the present study, we showed that hepatocyte apoptosis predicts NASH accurately [46]. Then, further examinations showed a significant increase in the number of macrophages in this tissue in the negative and positive groups compared with Spirulina and control groups ($p<0.001$). We know that chronic inflammation and macrophage infiltration into adipose tissue are found in AFLP [50]. In this study, in addition to changes in sinusoidal blood, considerable changes were seen in the phagocytic activity of liver macrophages. Similar results were also reported by McCurdy *et al*. [51] and Frazier *et al*. [52]. As macrophages release reactive oxygen species, nitro radicals, cytokines, and vasoactive prostanoids when they are in the activated phase, they may cause parenchymal injury, the microvascular inflammatory response, and activation of the neighboring Ito cells.

Blood islands decreased significantly in the control group compared with the other groups; in addition, they decreased significantly in the Spirulina group compared with the positive and negative groups ($p=0.1$). Bile ducts in the control and Spirulina groups decreased significantly compared with the positive and negative groups, and compared with the control group, it increased significantly in the Spirulina group ($p=0.1$).

Histological studies of the experimental groups (positive, negative, and Spirulina) are showed widespread changes in sinusoidal spaces. The sinusoidal spaces were dilated in the control group compared with the other groups (Fig. 1a), but in the other groups, these spaces were reduced depending on the type of treatment (Fig. 1b-d). Studies have shown that enlarged hepatic parenchymal cells, which are swollen with lipid, cause reductions in sinusoidal perfusion [53].

These structural changes in sinusoids were seen in the negative group (Fig. 1b), and they make them inefficient conduits, which finally causes damage to liver tissue [54]. Like the present study, other studies have also found considerable reductions in the number of sinusoids [54-57]. These alterations are accompanied by progressive damages to the sinusoidal endothelial cells (SEC), as well as the development of a basal lamina and deposition of collagen in Disse space [54]. In addition, adhesion of leukocytes to the sinusoidal endothelium may exist, followed by leukocyte infiltration into the hepatic parenchyma to form inflammatory foci [58]. On the other hand, microvascular blood flow is further restricted due to the deposition of collagen in Disse space as well as the narrowing and distortion of the sinusoidal lumen. This situation gets worse when leukocytes are trapped in the narrowed sinusoids or adhere to the endothelium following a hepatic microvascular inflammatory response. Furthermore, lipid accumulation in hepatic parenchymal cells and oxidative stress seem to be more important factors than the microvascular changes in steatosis. Studies have shown that interrupted sinusoidal blood flow because of lipid accumulation in parenchymal cells and collagen deposition in Disse space seems to be linked with hepatocellular injury in AFLP [53].

One of the most important findings of the present study was the fact that a persistent high-fat diet caused more lipid transfer to the fetus. Lipolysis from maternal adipose tissue increases plasma glycerol concentration and fatty acids [51]. This situation along with dietary fatty acids and hepatic triglycerides (TGs) results in an increase in TG-rich lipoproteins in the mother's circulation and consequently increases the transfer of fatty acids to the fetus [51,59-61]. Lipotoxicity is a major concern when lipid is accumulated within the liver. It could result in insulin resistance, oxidative stress, activation of pro-inflammatory cytokines, and liver fibrosis in the end [51,62-65]. The obtained results show that the chance of lipid accumulation in the fetal liver soars when there is exposure to maternal lipid-derived fuels during early pregnancy. This lipid accumulation is linked with lipotoxicity, which may result in macrophage infiltration and elevated production of inflammatory cytokine. The oxidative stress pathway is activated due to this inflammation, and it can activate the transcriptional regulators of hepatic gluconeogenesis [51].

In summary, the findings of the present study show that Spirulina has a more beneficial effect on the fetal liver than Simvastatin as a chemical medicine. Spirulina proved effective in minimizing high-fat-diet-induced damage to lipid metabolism. It consists of protein (55%–70%), carbohydrates (15%–25%), minerals, essential fatty acids (18%), vitamins especially B and E, high levels of beta carotene, and zeaxanthin, pigments such as phycocyanin, carotenoid, Chlorophyll, and other substances. In addition, Spirulina has high levels of Gamma-linolenic acid and it can have a protective effect on liver against toxins as the main causes of free radicals, cirrhosis, steatosis, and necrosis in liver cells [40]. In general, the body is endowed with systems of enzymatic and non-enzymatic antioxidants that scavenge and neutralize free radicals and prevent damage to other tissues. The antioxidant systems include SOD, glutathione peroxidase, catalase, and macromolecules such as albumin, ferritin, ceruloplasmin, and also molecules such as uric acid, bilirubin, ascorbic acid, and alpha-tocopherol [66,67]. Studies have shown that antioxidants contain elements that control and scavenge free radicals produced during the natural metabolism of the body. Blé-Castillo *et al*. [29] observed the protective effect of Spirulina against free radicals as well as oxidative stress, toxins, and medications due to the presence of antioxidants, tocopherols, phenolic acids, phycocyanin, selenium, beta-carotene, and flavonoid compounds. We know that scavenging free radicals are one of the main mechanisms that prevent free radical chain reactions. Studies have shown that the liver and kidney are the first targets of free radicals as they are exposed

to toxic and xenobiotic substances more the other tissues [68]. Having antioxidant properties and being rich in phycocyanin C, Spirulina could reduce free radicals and consequently protect the maternal and fetal liver. In addition, phycocyanin C plays a key role in scavenging lipid and peroxidation chain reactions. SODs, members of the metalloenzymes family, have also been known as antioxidants that scavenge free radicals. In fact, when free radicals increase in the body and the existing antioxidants do not suffice to combat them, phycocyanin in Spirulina regulates the enzymatic activity and restores the body's capability to fight free radicals. In addition to phycocyanin C, beta-carotene and tocopherols are other antioxidants found in Spirulina [38]. In additionally, Spirulina has Vitamin E, which is an antioxidant against lipid peroxidation chain. Vitamin E is a lipid-soluble antioxidant that prevents the peroxidation of unsaturated lipids [67]. Studies on the protective effect of Vitamin E suggest that this vitamin removes free radicals from hydrophobic environments [42,69]. In fact, the radical (R) is deactivated by a molecule of Vitamin E (α-Tocopherol) [69,70]. It seems after absorption of *in vivo* Vitamin E, it reacts with free radicals and probably with oxidizing intermediates. Therefore, Vitamin E prevents free radical chain reaction and protects cell membranes [69,71]. Finally, the therapeutic effect of Spirulina in treating liver diseases found in this study is supported by the findings of other studies done on Spirulina.

CONCLUSIONS

The findings of the present study showed that Spirulina has a protective and beneficial effect on the fetal liver in AFLP in mice. The protective efficiency of Spirulina is very promising as evidenced by histopathological studies. The AFLP protective property of the Spirulina is attributed to the presence of various constitutions which are present in Spirulina. Some other medications used for the treatment of fatty liver could probably be used during pregnancy too; however, some of them are dangerous for the fetus and cannot be used during pregnancy. However, such an effect did not observe in the case of Spirulina. As maternal nutrition is important during pregnancy, proper nutrition could ensure the health and development of the fetus; however, incorrect or unbalanced maternal nutrition causes fetal disorders. In addition, it is well-known that the liver detoxifies the body, and during the prenatal period, all substances that pass through the placenta enter the fetal liver directly. If these substances are toxic, they affect liver development and function adversely. Therefore, considering the findings of the present study, Spirulina having all the aforementioned nutrition could be considered as a green, non-toxic, and beneficial dietary supplement during pregnancy and reduce the risk of fetal disorders. It is worth noting that this study has been done on animals and whether it has similar effects on humans requires further studies.

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AUTHORS' CONTRIBUTIONS

The author declares that all the named authors have contributed equally to this article.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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