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ORIGINAL ARTICLE

Effect of folate supplementation on immunological and autophagy markers in experimental nonalcoholic fatty liver disease

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ABSTRACT. *Background and aims*: Chronic hepatic inflammation is an important pathogenic mediator of nonalcoholic fatty liver disease (NAFLD) that contributes to disease severity. It is commonly suggested that autophagy dysfunction may be an underlying cause of nonalcoholic fatty liver disease. However, the exact role of autophagy in lipid metabolism remains controversial. There has been a growing interest in the role of folate supplementation for the treatment and/or prevention of NAFLD. We aimed in this study to investigate the effects of different doses of folate supplementation on several immune markers and autophagy trying to explore the complex role of IL-22 and autophagy in NAFLD. *Methods*: Fifty Wistar rats were randomly separated into experimental (n = 40) and control groups (n = 10), which were fed for eight weeks with a high-fat diet (HFD) containing 40% fats or a standard diet, respectively. The experimental group was further subdivided into four subgroups where the first subgroup was left untreated while the other three were treated with different doses of folate (50, 100, and 150 μg/kg of body weight, respectively). At the end of the experimental period, animals from each group were sacrificed for blood and tissue analyses. *Results*: NAFLD rats showed decreased IL-22 serum levels and increased LC3B expression as compared to controls. Folate treatment was significantly associated with improvement in disease parameters, reduced presence of the pro-inflammatory cytokines TNF-α and CXCL8 and LC3B expression, and increased IL-22 levels in a dose-dependent manner. *Conclusion*: These results highlight the capacity of folate to modulate the production of several pro-inflammatory cytokines and autophagy thereby having a favorable impact disease progression.

Key words: Nonalcoholic Fatty Liver Disease, Folate, IL-22, TNF-α, CXCL8, LC3B

INTRODUCTION

Nonalcoholic Fatty Liver Disease (NAFLD) is a spectrum of liver disorders associated with accumulation of too much fat inside liver cells (exceeding 5% of liver volume) that is not due to excessive alcohol consumption or secondary causes of steatosis [1]. At least 20%–30% of patients with NAFLD may develop progressive liver disease called nonalcoholic steatohepatitis (NASH), which can result in cirrhosis and related complications (as liver failure or liver cancer) [2].

NAFLD is closely associated with insulin resistance, obesity, and metabolic syndrome, thus the prevalence of NAFLD is estimated to be 25% worldwide [3]. It is well accepted that inflammation is a central component of NAFLD pathogenesis and that mediators of immunity, especially pro-inflammatory cytokines, are able to control many key features of liver diseases. Various cytokines and chemokines, such as tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10) and chemokine ligand 8 (CXCL8/IL-8), are associated with hepatic inflammation and fibrosis [4]. Interleukin-22 (IL-22) is a member of the IL-10 family of cytokines that is produced by many immune cell types, such as helper T (Th)17, Th22, natural killer (NK) cells, and natural killer T (NKT) cells [5]. It binds to a heterodimeric receptor (R) complex composed of IL-10R2 and IL-22R1 that is expressed in hepatocytes, liver progenitor cells (LPCs), and hepatic stellate cells (HSCs) [6]. It has been suggested that IL-22 has a protective role in the liver by reducing fibrosis in some pathological conditions; however, the results are contradictory [7].

Folate is a water-soluble vitamin B that plays an essential role in one-carbon transfer reactions involved in nucleic acid biosynthesis, methylation reactions, and sulfur-containing amino acid metabolism. Vitamin B group has eight types of compounds but only vitamins B3 and B12 have been associated with NAFLD, whereas the evidence for the involvement of other vitamin B compounds is lacking [8]. The liver is the primary organ responsible for storage and metabolism of folates. It has been reported that a low level of endogenous folates in rodents perturbs folate-dependent one-carbon metabolism, and may be associated

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with development of metabolic diseases such as NAFLD.

Autophagy is physiologically a homeostatic and survival-promoting pathway that captures, degrades, and recycles intracellular proteins and organelles in lysosomes [9]. It is also considered a second type of programmed cell death when over-activated under certain circumstances. LC3B has an important role in the formation of autophagosome and its amount is correlated with the autophagy level and, therefore, used as a marker of autophagy [10].

Hepatic autophagy occurs at the basal level and may be elevated under stress conditions, thus contributing to the maintenance of normal hepatocyte functions and responding to pathogenic changes in the liver [11]. It has been shown that autophagy can be involved in the pathogenesis of NAFLD that can also affect autophagy. However, the exact role of autophagy in the lipid metabolism is controversial where some publications report a lipolytic function of autophagy, while others claim a lipogenic function [12, 13].

The effects of folate on immune mediators and hepatic autophagy in *in vivo* NAFLD models are unclear. In addition, whether autophagy is a friend or foe in the pathogenesis of NAFLD still needs to be clarified.

MATERIALS AND METHODS

Experimental Animals

Fifty (8 weeks old) healthy Wistar male rats, weighing an average of 150 g, were obtained from the Medical Technology Center, Medical Research Institute, Alexandria University, Egypt. The rats were housed 4 per cage at an ambient temperature of 23 ± 1°C in a 12-h light/dark cycle and 45 ± 5% humidity. Rats had free access to chow diet and water for a week prior to the experiment.

Ethical Statement

All the experiments fulfil the guidelines of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and recommendations of Egypt's guide for the care and use of laboratory animals [14]. The current study follows the ARRIVE Guidelines for reporting animal research and a completed ARRIVE checklist is included. All efforts were made to curb the distress of rats during the experimental period.

Experimental Design.

Rats were randomly divided into five groups each group consisting of ten rats as follows. Group (1) served as the normal control rats and were fed a standard diet, while the rest of the animals received a high-fat diet (HFD) containing 40% fats for 8 weeks to develop NAFLD. Group (2) was left untreated to serve as untreated NAFLD rats whereas Groups (3), (4), and (5) were treated with different doses of folate (50, 100, and 150 μg/kg of body weight, respectively). Treatments were gavaged orally for 4 weeks, during which rats were maintained on HFD to mimic the situation in humans. Food intake was monitored daily and body weight was measured weekly during the experiment.

Serum parameters

At the end of 4-week treatment, overnight fasted animals were anesthetized with ketamine 100 mg/kg and xylazine 10 mg/kg, and blood was then collected *via* cardiac puncture. Serum was separated by centrifugation (800 × g, 4 °C, 20 min), and used to assess fasting blood sugar (FBS), Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (AST) using colorimetric kits (Spectrum Diagnostics, Cairo, Egypt) and adiponectin as well as insulin by a commercially available ELISA kits (Abnova, Jhongli, Taiwan).

The homeostasis model assessment index for insulin resistance (HOMA-IR) was then calculated using the following formula [15]:

$$HOMA-IR = \frac{[\,fasting\,glucose(mmol/L) \times\ fasting\,insulin(\mu IU/ml)]}{22.5}$$

In addition, triglycerides (TG), total cholesterol (TC), and HDL-C were determined using Boehringer Mannheim colorimetric kits (Mannheim, Germany). LDL-C was estimated according to the Friedewald equation [16]:

$$\mathrm{LDL-C} = \mathrm{TC} - \left(\mathrm{HDL-C} + \frac{1}{5}\mathrm{TG}\right)$$

Tissue parameters

Immediately after blood collection, livers were excised, washed in ice-cold saline, and divided into aliquots and preserved at -80 °C. One aliquot was used to extract hepatic lipids according to the method modified by Bligh and Dyer [17], where the chloroformic layer, containing all lipids, was utilized to assay TGs using the previously mentioned kit.

Another aliquot of 30 mg weight was used for total RNA extraction using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. An aliquot was used for the ELISA determination of TNF-α, CXCL8 (IL-8), and IL-22.

Reverse transcription of total RNA

MiScript II RT Kit (Qiagen, Germany) was used for the reverse transcription of the extracted RNA into cDNA according to the manufacturer's instructions.

Assessment of hepatic expression of LC3B at the mRNA level

The cDNA was used to quantify the gene expression of LC3B in liver by Rotor-Gene Q qPCR (Qiagen, USA) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany). Quantitative PCR amplification conditions began with an initial denaturation at 95 °C

for 10 minutes and then the amplification phase by 40 cycles of PCR as follows: Denaturation at 95 °C for 5 seconds, annealing at 55 °C for 15 seconds, and extension at 60 °C for 15 seconds. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for normalization. The primers used for the determination of rat genes are as follows: LC3B; F 5'-CCCACCAAGATCCCAGTGAT-3' and R 5'-CCAGGAACTTGGTCTTGTCCA-3' [18], and GAPDH: F 5'-GGGTGTGAACCACGAGAAATA-3' and R 5'-AGTTGTCATGGATGACCTTGG-3' [19].
Rotor-Gene Q-Pure Detection version 2.1.0 (build 9) (Qiagen, Valencia, CA, USA) was used to determine the values of threshold cycle (C_t). For each gene, the relative change in mRNA in samples was determined using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH.

Assessment of hepatic TNF-α, CXCL8 (IL-8), and IL-22 by ELISA

A 0.5 g aliquot of liver tissues was homogenized in phosphate-buffered saline in a ratio of 1:9 and centrifuged at 4 °C for 10 minutes at 10000 × g. The supernatant was used for the ELISA determination of the hepatic content of TNF-α, CXCL8 (IL-8), and IL-22 (CUSABIO, Wuhan, China), according to the manufacturer's instructions. The protein concentration was determined using the modified Lowry's method [20].

Statistical analysis

The values are expressed as mean ± SD of 10 animals and were analyzed using SPSS statistical software version 18 (SPSS, Chicago, IL). Multiple comparisons were performed using one-way ANOVA, followed by the Tukey post-hoc test. The correlation coefficients (r) between different assayed parameters were evaluated using Pearson correlation coefficient; $P \leq 0.05$ was considered the significance limit for all comparisons.

RESULTS

Effect of folate on liver function tests

The means of serum aminotransferase and bilirubin were significantly higher in the untreated NAFLD group compared to control. The treatment with folate led to significant dose-dependent decline in liver function tests compared to the untreated group with complete normalization of ALT and AST activities in the NAFLD rats treated with the higher folate doses (100, 150 μg/kg). Although the bilirubin level showed significant dose-dependent decline with the folate treatment, its level was still significantly higher than the control rats even with the highest dose used (*table 1*).

Effect of folate on lipid profile

As summarized in *table 2*, there was significant increase in serum TG, cholesterol, LDL-C, and hepatic TG, together with significant HDL-C decrease in untreated NAFLD compared to control. The treatment of NAFLD rats with folate leads to a significant dose-dependent improvement in the lipid profile. The serum levels of total cholesterol, LDL-C, and HDL-C were completely normalized with the high doses (100, 150 μg/kg). However, the hepatic and serum TG levels were still higher than the control values (*table 2*).

Effect of folate on glucose homeostasis

table 3 summarized the changes in the glucose homeostasis parameters, the untreated NAFLD group showed significantly higher levels of fasting blood sugar (FBS), insulin as well as HOMA-IR. NAFLD rats treated with folate showed dose-dependent ameliorative effects on these parameters in spite of significant higher values compared to the healthy control rats (*table 3*).

Effect of folate on adiponectin level

The untreated NAFLD rats had significantly lower adiponectin level compared to healthy control rats. The NAFLD rats treated with folate showed a significant dose-dependent improvement in the adiponectin level compared to the untreated NAFLD rats (*figure 1*).

Effect of folate on immunological markers

As shown in *figures 2A and 2B*, there was a marked elevation level of TNF-α and CXCL8 (IL-8) in the untreated NAFLD group compared to the control

Table 1
Comparison between the different studied groups according to liver function tests.

Liver function tests	Control	NAFLD			
		Untreated	Folat 50 μg/kg	Folat 100 μg/kg	Folat 150 μg/kg
ALT (U/L)	32.3 ± 4.1	63.1^{*} ± 11.4	$51.7^{*\#}$ ± 5.3	$36.8^{\#@}$ ± 4.9	$32.4^{\#@}$ ± 2.9
AST (U/L)	115.6 ± 17.9	171.3^{*} ± 21.1	$146.5^{*\#}$ ± 17.8	$124.2^{\#@}$ ± 7.8	$117.4^{\#@}$ ± 9.7
Bilirubin (mg/dl)	0.3 ± 0.1	1.1^{*} ± 0.1	$1.0^{*\#}$ ± 0.1	$0.8^{*\#@}$ ± 0.1	$0.7^{*\#@\&}$ ± 0.04

Data was expressed using Mean ± SD.
*:p ≤ 0.05 *vs.* Control
#:p ≤ 0.05 *vs.* Untreated
@: p ≤ 0.05 *vs.* Folat 50 μg/kg
&: p ≤ 0.05 *vs.* Folat 100 μg/kg

Table 2
Comparison between the different studied groups according to lipid profile.

Lipid profile	Control	NAFLD			
		Untreated	Folat 50 μg/kg	Folat 100 μg/kg	Folat 150 μg/kg
TG (mg/dl)	36.3 ± 7.2	62^{*} ± 9.8	$50.8^{*\#}$ ± 3.9	$43.7^{\#}$ ± 8.7	$47.9^{*\#}$ ± 9.3
Cholesterol (mg/dl)	131.9 ± 13.3	175.9^{*} ± 18.3	$153.4^{*\#}$ ± 7.3	$142.4^{\#}$ ± 4.5	$141.1^{\#}$ ± 4.3
HDL-C (mg/dl)	49.6 ± 4.2	31.5^{*} ± 1.8	$37.9^{*\#}$ ± 4.5	$47.5^{\#@}$ ± 5.9	$48.3^{\#@}$ ± 2.1
LDL-C (mg/dl)	75.6 ± 11.4	133.4^{*} ± 19.1	$105.8^{*\#}$ ± 9.6	$87.8^{\#@}$ ± 3.2	$84.04^{\#@}$ ± 3.4
Hepatic TG (mg/g tissue)	30.6 ± 4.5	108.6^{*} ± 3.3	$92.4^{*\#}$ ± 12.9	$63.2^{*\#@}$ ± 8.9	$57.1^{*\#@}$ ± 7.03

Data was expressed using Mean ± SD.
*:p ≤ 0.05 *vs.* Control
#:p ≤ 0.05 *vs.* Untreated
@: p ≤ 0.05 *vs.* Folat 50 μg/kg
&: p ≤ 0.05 *vs.* Folat 100 μg/kg

Table 3
Comparison between the different studied groups according to glucose homeostasis.

Glucose homeostasis	Control	NAFLD			
		Untreated	Folat 50 μg/kg	Folat 100 μg/kg	Folat 150 μg/kg
FBS (mg/dl)	77.4 ± 4.7	161.8^{*} ± 6.1	$141.2^{*\#}$ ± 5.8	$122.8^{*\#@}$ ± 9	$118^{*\#@}$ ± 6.4
Insulin (mIU/ml)	6.2 ± 1.1	14.5^{*} ± 1.6	14.2^{*} ± 1	$12.1^{*\#@}$ ± 1.3	$10.1^{*\#@\&}$ ± 0.8
HOMA-IR	1.2 ± 0.2	5.7^{*} ± 0.9	$4.9^{*\#}$ ± 0.4	$3.7^{*\#@}$ ± 0.3	$3^{*\#@\&}$ ± 0.2

Data was expressed using Mean ± SD.
*:p ≤ 0.05 *vs.* Control
#:p ≤ 0.05 *vs.* Untreated
@: p ≤ 0.05 *vs.* Folat 50 μg/kg
&: p ≤ 0.05 *vs.* Folat 100 μg/kg

group; on the other hand, the level of IL-22 was significantly decreased (*figure 2C*). The treatment of NAFLD rats with folate led to dose-dependent decline in the levels of CXCL8 (IL-8) and TNF-α and dose-dependent increase in the IL-22 level. In spite of these ameliorative effects of folate treatment on the immunological parameters, their levels were still significantly higher than the control group.

Effect of folate on autophagy marker; LC3B

Regarding LC3B expression at mRNA level, there was significant upregulation in hepatic expression of LC3B in untreated NAFLD group compared to control. The treatment of these rats with folate led to significant dose-dependent suppression in LC3B expression compared to the untreated rats, but the

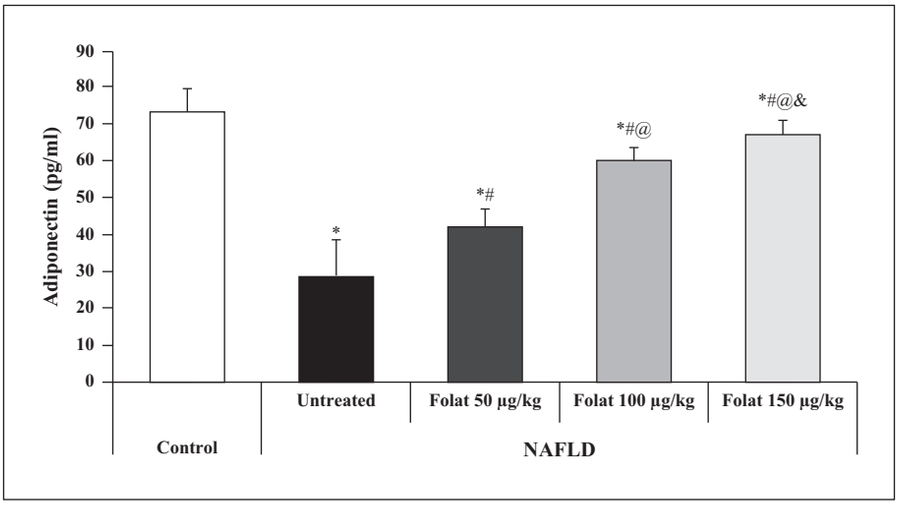

Figure 1
Effect of treatment with different doses of folate (50, 100 or 150 μg/kg) on the adiponectin level (pg/ml) in NAFLD rats. Data represented means ± S.E.M as compared with normal control group (*), untreated NAFLD group (#), NAFLD group treated with Folat 50 μg/kg (@), NAFLD group treated with Folat 100 μg/kg (&) (one-way ANOVA followed by Tukey post hoc test) at P≤0.05.

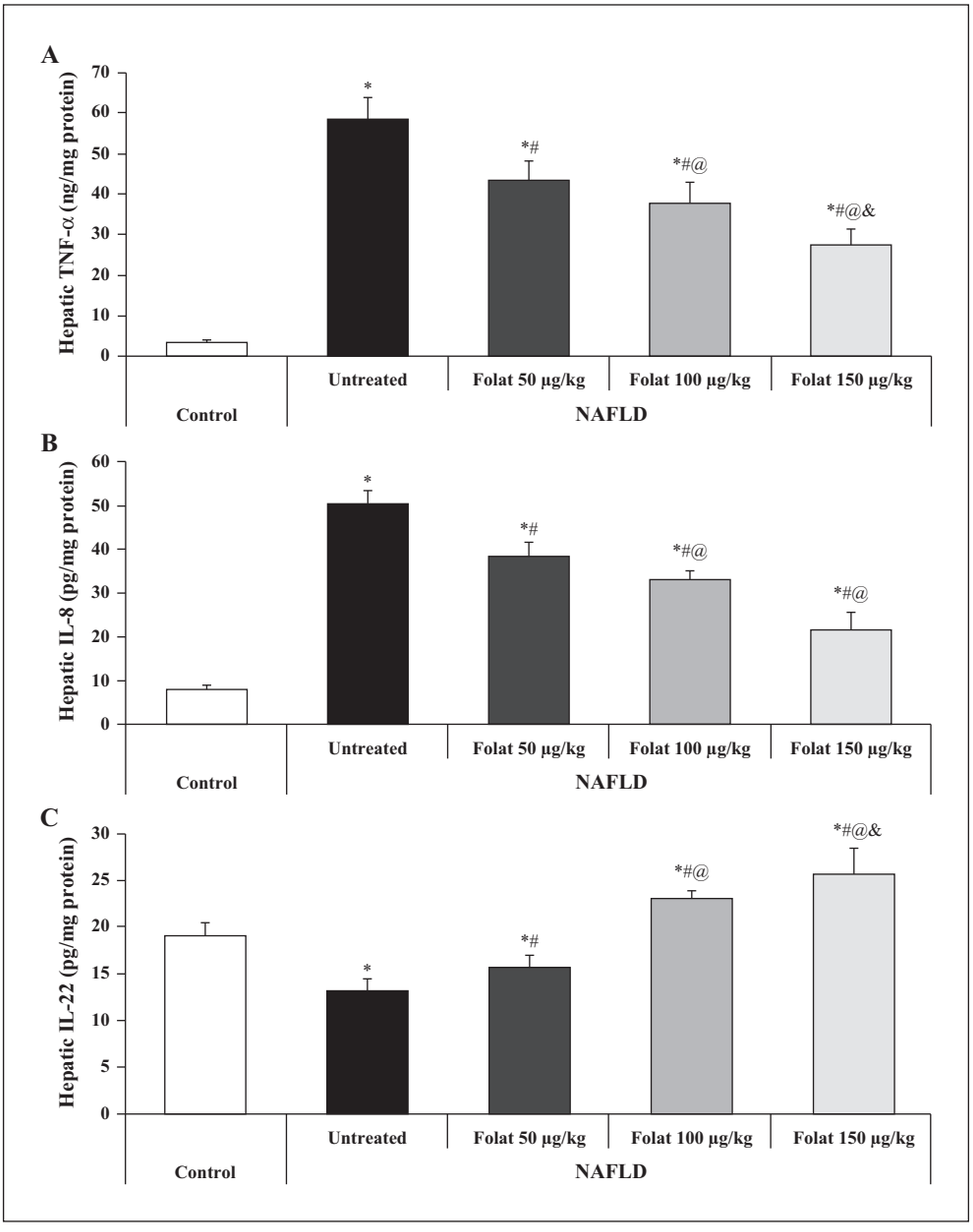

Figure 2

Effect of treatment with different doses of folate (50, 100 or 150 µg/kg) on hepatic immunological markers in NAFLD rats. A) hepatic TNF-α level (ng/mg protein), B) hepatic IL-8 level (pg/mg protein) and C) hepatic IL-22 level (pg/mg protein). Data represented means ± S.E.M as compared with normal control group (*), untreated NAFLD group (#), NAFLD group treated with Folat 50 µg/kg (@), NAFLD group treated with Folat 100 µg/kg (&) (one-way ANOVA followed by Tukey post hoc test) at $P \leq 0.05$.

expression was still higher than control group even with the highest dose used (*figure 3*).

Association between LC3B expression and immunological markers with different disease parameters

The correlation studies in NAFLD rats showed that LC3B expression, CXCL8 (IL-8), and TNF-α were positively correlated with liver function tests, serum TG, cholesterol, LDL, hepatic TG, and glucose homeostasis markers, whereas they were negatively correlated with HDL-C and adiponectin levels. On the other hand, the IL-22 level showed opposite pattern of correlations (*table 4*).

Association between LC3B expression and immunological markers

As shown in *table 5*, LC3B expression showed a positive correlation with both TNF-α and CXCL8 (IL-8) and a negative correlation with IL-22.

DISCUSSION

NAFLD is a complex chronic liver condition, being considered the most common liver problem which may progress to other liver diseases. It is often regarded as the hepatic manifestation of the metabolic syndrome since it has a strong association with obesity, insulin resistance, and multiple factors that influence its pathogenesis. The underlying mechanism involves

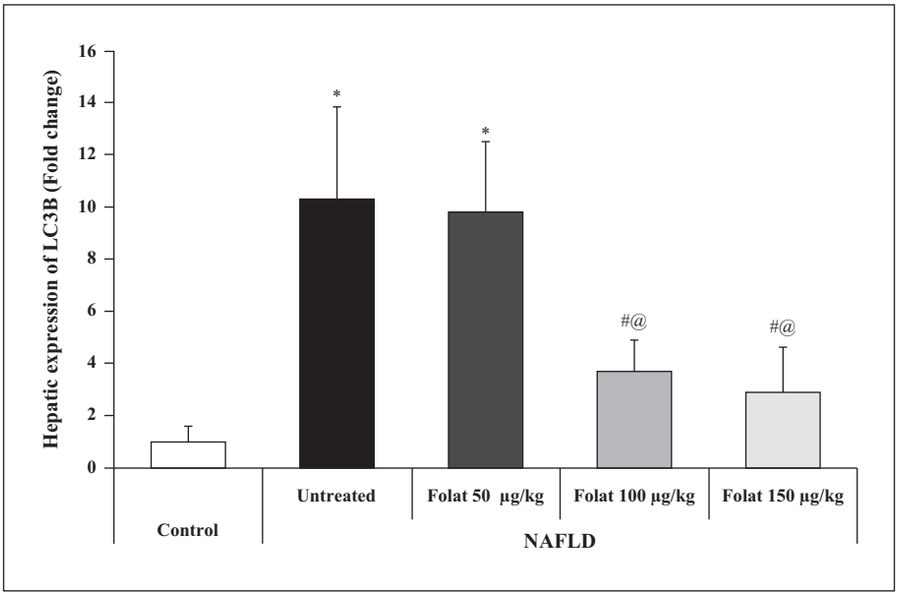

Figure 3
Effect of treatment with different doses of folate (50, 100 or 150 µg/kg) on hepatic expression of LC3B expression (fold change) in NAFLD rats. Data represented means ± S.E.M as compared with normal control group (*), untreated NAFLD group (#), NAFLD group treated with Folat 50 µg/kg (@) (one-way ANOVA followed by Tukey post hoc test) at $P \leq 0.05$.

Table 4
Correlation between LC3B expression and immunological markers with different disease parameters in NAFLD groups (n = 40).

		CXCL8 (IL-8)	TNF-α	IL-22	LC3B Expression
ALT	**r**	0.805*	0.817*	-0.830*	0.790*
	p	0.000	0.000	0.000	0.000
AST	**r**	0.727*	0.765*	-0.772*	0.752*
	p	0.000	0.000	0.000	0.000
Bilirubin	**r**	0.907*	0.851*	-0.925*	0.722*
	p	0.000	0.000	0.000	0.000
TG	**r**	0.514*	0.555*	-0.426*	0.405*
	p	0.001	0.000	0.006	0.010
Cholesterol	**r**	0.737*	0.614*	-0.674*	0.423*
	p	0.000	0.000	0.000	0.007
HDL-C	**r**	-0.807*	-0.805*	0.798*	-0.649*
	p	0.000	0.000	0.000	0.000
LDL-C	**r**	0.812*	0.707*	-0.770*	0.506*
	p	0.000	0.000	0.000	0.001
Hepatic TG	**r**	0.843*	0.806*	-0.867*	0.780*
	p	0.000	0.000	0.000	0.000
FBS	**r**	0.866*	0.799*	-0.845*	0.680*
	p	0.000	0.000	0.000	0.000
Insulin	**r**	0.755*	0.668*	-0.841*	0.652*
	p	0.000	0.000	0.000	0.000
HOMA-IR	**r**	0.857*	0.762*	-0.888*	0.666*
	p	0.000	0.000	0.000	0.000
Adiponectin	**r**	-0.869*	-0.813*	0.902*	-0.684*
	p	0.000	0.000	0.000	0.000

r: Pearson coefficient
*: Statistically significant at $p \leq 0.05$

the modification and interaction of immunity, metabolism, and other factors where micronutrients such as vitamins may also play a key role. However, no pharmacologic treatment has yet been proven for this disease.

Various studies have demonstrated that obese subjects have lower folate serum levels in comparison with normal-range BMI subjects [21, 22], suggesting that folate deficiency may have a role in obesity and associated NAFLD. Moreover, Sid et al. [23] highlighted the importance of folate in the progression of NAFLD, therefore raising the possibility that supplementary folate may be a treatment option. Furthermore, it was demonstrated that inclusion of serum folate levels in the current NAFLD prediction score has led to a significant improvement in NAFLD prediction [24]. These findings are consistent with our results, in which folate treatment led to a significant dose-dependent improvement in liver function tests and lipid profile in NAFLD rats. Although the mechanism of action of folic acid is not fully understood, its beneficial effects might be attributed to homocysteine reduction and its ability to antagonize oxidative stress [25].

It was documented that impaired methylation capacity promotes hepatic fat accumulation through an impairment of phosphatidylcholine (PC) synthesis which is associated with accumulation of hepatic TG due to a reduction in VLDL secretion [26]. In addition, folate deficiency induces the expression of genes involved in hepatic lipid synthesis that may contribute to hepatic steatosis [27]. However, in a study by Polyzos et al. [28], no correlation was found between the level of folate and the severity of liver disease.

Adiponectin is a specific secretory adipokine that regulates fatty acid oxidation and inhibits lipid accumulation and maintains whole-body glucose homeostasis, including hepatic insulin sensitivity [29]. It has been shown that serum adiponectin levels were lower in NAFLD patients compared to healthy individuals [30]. However, a direct association between folate status and both adiponectin and insulin resistance in NAFLD has not been well established. It was apparent from our results that the treatment of NAFLD rats with folate was associated with lower insulin resistance and higher adiponectin level. In agreement with these results, it has been observed that folic acid supplementation improved insulin resistance, and induced DNA methylation and gene expression changes in genes associated with obesity [31]. On the other hand, it has been observed recently that high-dose folic acid supplementation may promote insulin resistance and disrupt glucose metabolism possibly by depressing adiponectin expression [32].

Table 5
Correlation between LC3B expression and immunological markers in NAFLD groups (n = 40).

Immunological parameters	LC3B Expression	
	r	p
CXCL8 (IL-8)	0.664*	<0.001*
TNF-α	0.750*	<0.001*
IL-22	-0.821*	<0.001*

r: Pearson coefficient
*: Statistically significant at $p \leq 0.05$

Cytokines and chemokines might play an active role in the development and progression of NAFLD through stimulation of hepatic inflammation, cell necrosis and apoptosis, and induction of fibrosis [33]. In this study, we evaluated some inflammatory cytokines as TNF- α, CXCL8 (IL-8), and IL-22 that are modulated by hepatic cells upon lipid accumulation and involved in pathogenesis of liver diseases. In line with the present data, it was reported that TNF-α and IL-8 cytokines were increased in the NAFLD group and were positively correlated with disease severity [34, 35]. TNF-α is one of the most important mediators of NAFLD development that upregulates key molecules associated with lipid metabolism, inflammatory cytokines, and fibrosis in the liver [36].

CXCL8 (IL-8) is a CXC chemokine ligand that orchestrates neutrophil recruitment within inflamed tissues. It has been observed that serum IL-8 levels were significantly higher in NASH patients compared with those with hepatic steatosis or healthy controls [37] where lipid accumulation in hepatocytes may induce production of IL-8.

Another important cytokine is IL-22 that is secreted by different immune cells and may play a hepatoprotective role in liver inflammation/fibrosis, being supported by our results that showed that IL-22 was significantly lower in the NAFLD group compared to normal with negative association with the disease parameters. It has been shown that IL-22 administration partially inhibited HFD-induced upregulation of lipogenesis-related genes that are involved in lipid synthesis and reduced the expression of TNF-α in the liver [35]. On the contrary, Su S-B et al. [38] reported elevated IL-22 expression in peripheral blood of NAFLD patients that may play an inflammatory and immune response role in progression from NAFL to NASH or fibrosis.

The current results showed that folate treatment of NAFLD rats was associated with dose-dependent decline in TNF-α and CXCL8 levels together with increase in IL-22 level, suggesting that the hepatoprotective effect of folate in NAFLD may be attributed, in part, to its anti-inflammatory action.

In agreement with our results, it has been reported that folic acid and the methyl donor mixture reduce proinflammatory gene expression in monocytes and decrease the secretion of TNF-α and IL-1β cytokines from differentiated macrophages by inhibiting the NF-κB pathway [39]. The specific mechanisms for the beneficial effects of folic acid or methyl donors on inflammation have not been clearly elucidated; however, one of the possible explanations is epigenetics, via DNA and histone methylation [40]. The increased IL22 level with increased folate may be explained on the protective role of IL-22 that promotes tissue repair by directly activating anti-apoptotic, proliferative programs, and antioxidants in various types of cells, including hepatocytes [35].

Autophagy is a critical intracellular pathway that targets defective organelles to the lysosomes for

degradation. It influences lipid metabolism in a number of ways, from lipogenesis to lipolysis [41]. The two mechanisms are not contradictory, but coexist and coact on lipid regulation, where the imbalance between lipogenesis and lipolysis may result in steatosis. The current results revealed upregulation of hepatic LC3B expression in the NAFLD group, whereas folate treatment was associated with decreased LC3B expression with increasing the dose. The increased autophagy level in the untreated group may reflect its compensatory role as long-term lipid load may change membrane lipid composition and reduce autophagosome/lysosome fusion both *in vitro* and *in vivo*, which may partly impair lipophagy and accelerate lipid accumulation [42]. This may explain why increased LC3B expression was associated with disease progression in the form of increased inflammatory markers and disease markers as shown in the results. The decreased autophagy level with increased folate may be explained on the protective role of folate that may decrease the need for autophagy. In concordance, it has been demonstrated that folate deficiency in pregnant mice caused a marked inhibition of mTORC1 and mTORC2 signaling in multiple tissues [43].
Although autophagy ensures a well-balanced inflammatory response that is accompanied by the restoration of homeostasis, the current results revealed a negative correlation between LC3B expression and both IL-22 and adiponectin that may be context dependent.

CONCLUSIONS

The results of our study suggest a therapeutic role of folate in improving liver enzyme function and the production of immune mediators in NAFLD. In addition, the correlation between serum levels of TNF-α, CXCL8 (IL-8), and IL-22 and clinical parameters of the disease may indicate their value in the diagnosis of NAFDL.

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