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Glycine alleviates liver injury induced by deficiency in methionine and or choline in rats

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Abstract. – *Objectives:* **Nonalcoholic steatohepatitis (NASH) is an advanced stage of non-alcoholic fatty liver disease (NAFLD) from steatosis. Methionine and choline are important amino acids play a key role in many cellular functions. Glycine is a non-essential amino acid having multiple roles in many reactions. This study aimed to investigate liver damage induced by feeding male albino rats either methionine deficient (MD), choline deficient (CD), or MCD diets. And to clarify the alleviatory effect of dietary glycine supplementation (5%) on reduced complications caused by feeding each of the deficient diets.**

Material and Methods: **Nutritional status, liver functions, lipids profile, hepatic oxidative stress, hepatic antioxidant enzymes, tumor markers and hepatic fatty acid transport protein gene were assessed.**

Results: **Rats fed with either MD or MCD diet had less body weight gain unlike rats fed the CD diet. Liver injury was detected in deficient groups by elevating plasma ALT, AST, ALP, total and direct bilirubin, albumin and protein levels. Lipid accumulation was more prominent in rats fed the MCD or CD diet than in those fed the MD diet. Fatty acid transport protein (FATP) was significantly elevated in the different glycine supplemented groups.**

Conclusion: **Oral administration of glycine confers a significant protective effect by optimizing all the assessed parameters and gene expression.**

Key Words:

Methionine, Choline, Glycine, Non-alchohlic fatty liver, Fatty acid transport protein.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in the liver (conventionally set as more than 5% by weight) in the absence of secondary causes such as increased alcohol consumption and other forms of chronic liver disease[1]. NAFLD is the most common chronic liver disorder in the world, with a prevalence of approximately 20% in the general population and up to 95% among those with obesity. NAFLD represents a broad spectrum of liver disease ranging from mild steatosis to steatohepatitis featuring severe steatosis (> 60% hepatocytes affected) hepatocellular injury, progressive chronic inflammation and fibrosis[2].

Liver plays a central role as both victim and culprit in obesity-related disorders and obesity. Insulin resistance (i.r.) of the white adipose tissue results in an increased fatty acid flux to the liver with a subsequent ectopic fat deposition in the hepatocytes. Fatty liver disease has been linked to insulin resistance and progression of atherosclerosis and, thus, by itself may be a major aggravating factor in the pathogenesis and progression of the metabolic syndrome and its associated disorders[1].

Lipid abnormalities can also affect glucose homeostasis. This is usually explained by the "lipotoxicity" hypothesis. According to this hypothesis, abnormal accumulations of triglycerides and fatty acyl-CoA in muscle and liver can result in i.r. Because insulin resistance induces peripheral lipolysis and the delivery of free fatty acids to the liver, levels of potentially hepatotoxic free fatty acids are increased. Hepatocytes protect themselves by binding, transforming, catabolizing, and exporting excess free fatty acids[3].

Nutritional models based on methionine choline deficient (MCD) diet leads to the impaired formation of very-low-density-lipoproteins (VLDL), contributing to the development of steatosis, hepatic inflammation, and fibrosis[4]. Rodents fed on MCD diet have been shown to have higher levels of serum tumor necrosis factor-alpha (TNF-α) and are more sensitive to en-

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dotoxin exposure. Additionally, they had increased levels of oxidative stress, which may be important in the progression of steatosis to non-alcoholic steatohepatitis (NASH)[5]. However, choline can be biosynthesized via methionine and choline deficiency alone does not seem to impair the VLDL excretion. Therefore, the clinically relevant pathological features such as increased oxidative stress and the Kupffer cell-mediated inflammatory response contributing to the development of steatohepatitis, as described with the MCD diet, are not necessarily similarly induced by the choline deficient (CD) diet[2].

Glycine is a non-essential amino acid, having multiple roles in many reactions, such as gluconeogenesis, purine, haem synthesis and bile acid conjunction. Glycine is said to activate chloride channels in Kupffer cells, which hyperpolarizes the cell membrane and blunts intracellular Ca^{2+}concentrations similar to its action in neurons, and also decreases the levels of superoxide ions from neutrophils via glycine-gated chloride channels. Glycine has been reported to inhibit the activation of macrophages and TNF-α release. Glycine reduces reperfusion injury, prevents liver damage after chronic exposure to alcohol, and attenuates lipid peroxidation and glutathione depletion induced by different hepatotoxins[6]. A diet supplemented with glycine minimizes injury by endotoxin shock induced by D-galactosamine[7] or cyclosporine[8]. Moreover, glycine prevents hepatic cancer by inhibiting angiogenesis and endothelial cell proliferation[9].

The present study was designed to explore a possible new strategy to improve recovery from NAFLD in rat model. Glycine, a non toxic amino acid, may be a useful tool during the prognosis of liver diseases. Hence, the effect of oral administration of glycine on liver function, lipids profile, tumor initiation and the expression of main hepatic lipid transporter gene are evaluated in rats with NAFLD induced by methionine and choline either alone or in combination.

Material and Methods

Diets and Animals

Adult male albino rats "Sprague-Dawely strain" weighing 90-105 g were obtained from Research of Bilharzias Institute. Academic of Scientific Research and Technology. Cairo, Egypt. Rats were acclimatized to laboratory conditions for 3 days, maintained at constant 24 C° with 12 h light-dark cycle and fed a standard control diet and water *ad libitum*. After acclimatization, the rats were randomized into seven experimental groups (n = 10/group) and fed on a different purified diets prepared for 4 weeks. Groups were designed as follows[10].

Control group; rats received standard control diet.

MD group: Rats received standard control diet deficient in methionine.
CD group: Rats received standard control diet deficient in choline.
MCD group: Rats received standard control diet deficient in methionine and choline.
MD+G group: Rats received standard control diet deficient in methionine and supplemented with 5% glycine.
CD+G group: Rats received standard control diet deficient in choline and supplemented with 5% glycine.
MCD+G group: Rats received standard control diet deficient in methionine and choline and supplemented with 5% glycine.

Glycine supplementation was equivalent to 5 g/100 g diet according to Stachlewitz et al[7].

The daily food consumption and body weight were measured and after the designed period rats were sacrificed under ether anesthesia. Blood was collected from the hepatic portal vein, centrifuged (10 min, 3000 rpm, 4° C) and plasma was stored at -80 C°. Livers were removed, weighed and stored at –80° C.

Biochemical Analysis

Liver Function Tests

Plasma samples were analyzed colorimetrically for the assessment of the activities of alanine and asparate aminotransferases (ALT and AST) and alkaline phosphatase (ALP) by using (QCA) kits (Quimica Clinica Aplicada S.A. kits. Aspartado, Amposta, Spain). Also, the concentration of each of the total and direct bilirubin, protein and albumin were determined colorimetrically using (QCA) kits (Quimica Clinica Aplicada S.A. kits. Aspartado, Amposta, Spain).

Assessment of Lipids Profile

Hepatic lipids were extracted by the chloroform: methanol extraction method. Total lipids, total cholesterol (TC) and triacylglycerols (TAG) were de-

termined colorimetrically in both liver extract and plasma using (QCA) kits (Quimica Clinica Aplicada S.A. kits. Aspartado, Amposta, Spain).

Assessment of Hepatic Lipid Peroxidation

The hepatic oxidative stress was assessed as malondialdeyde (MDA) in liver supernatant of a phosphate buffered saline (PBS) solution, pH 7.4 containing 0.16 mg/ml heparin. Both of the hepatic antioxidant activities of SOD and GSH were determined. SOD activity was assessed in liver supernatant of ice-cold 0.25 M sucrose solution. GSH activity was assessed in liver supernatant of 3% sulfosalicylic acid (5% homogenate) using (QCA) kits (Quimica Clinica Aplicada S.A. kits. Aspartado, Amposta, Spain).

Assessment of tumor markers

The plasma activities of both α-L-Fucosidase and arginase were measured colorimetrically using kits (QCA). (Quimica Clinica Aplicada S.A. kits. Aspartado, Amposta, Spain).

Expression of Hepatic Fatty Acid Transport Protein (FATP)

Total RNA extraction was performed from frozen liver specimens with high pure RNA isolation kit (Roche) (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Transcription was performed according to the manufacturer's instructions. Conditions for PCR amplifications were as follows: initial denaturation at 95° C for 10 min; followed by 40 cycles of 60 s denaturation at 95° C; 30 s annealing at 60° C; and 30 s extension at 72° C. The primer set used for amplification of FATP were as follows; 5'-TCAAGGTGTGCTCAACAGCC-3` and 5'-AGGATAAAACACACCAACTGT-3`

The signal intensities of PCR products were separated on agarose gel and were visualized by fam staining. The products' signal intensities were determined by comparative delta CT method according to (Vandesompele et al)[11].

Statistical Analysis

The data were statistically analyzed by SPSS version 9.0 statistical packages (SPSS Inc, Chicago, IL, USA). Data were presented as a mean ± S.D.; statistical differences between groups were performed using Student's t-test. Differences considered significant when $p < 0.05$ and $p < 0.01$.

Results

In all of the experimental groups, physical activity was similar and appearance of animals remained healthy.

The mean body weight gain of rats fed the MCD diet was significantly less than the rats fed either CD, MD or control diets ($p < 0.01$). Rats administered either of the deficient diets had a highly significant elevation ($p < 0.01$) in both absolute and relative liver weights comparing with control group. Whereas, rats fed on the different glycine supplemented diets had a significant decrease ($p < 0.01$) in each of the body weight gain and the absolute liver weights (Table I).

The plasma and hepatic lipid profiles were significantly elevated ($p < 0.01$) in all of the groups fed the deficient diets compared with those fed the control diet, which has the significantly lowest concentration. Supplementation of glycine to the different deficient diets decreases significantly both of the plasma and hepatic lipids by com-

Table I. Body weight gain, absolute liver and relative weight values of adult male albino rats fed on different experimental diets.

Relative liver weight (g %)	Absolute liver weight (g)	Body weight gain (g)	Group
3.42 ± 0.77	6.91 ± 1.41	106.54 ± 11.19	Control
4.42 ± 0.52	8.15 ± 0.78	94.44 ± 15.85	MD
3.94 ± 0.35	8.90 ± 0.96	134.0 ± 11.08	CD
4.32 ± 0.26	7.59 ± 0.88	80.64 ± 15.97	MCD
5.09 ± 0.27	7.74 ± 0.73	61.66 ± 13.48	MD + G
5.83 ± 0.40	6.76 ± 0.67	25.56 ± 9.65	CD + G
4.00 ± 0.31	4.56 ± 0.64	32.40 ± 7.72	MCD + G

Values are the mean ± S.D.

Table II. Hepatic total lipids, total cholesterol (TC), and triacylglycerol (TAG) values of adult male albino rats fed on different experimental diets.

Hepatic TAG (mg/g)	Hepatic TC (mg/g)	Hepatic total lipids (mg/g)	Group
55.81 ± 7.30	39.91 ± 6.90	57.91 ± 12.67	Control
79.27 ± 8.15	53.70 ± 11.04	129.23 ± 11.78	MD
105.19 ± 11.98	57.70 ± 14.05	153.24 ± 12.33	CD
116.68 ± 12.19	58.50 ± 10.49	169.66 ± 14.23	MCD
61.92 ± 5.97	41.98 ± 10.49	91.08 ± 14.28	MD+G
95.64 ± 9.31	42.58 ± 8.88	114.20 ± 13.65	CD+G
77.69 ± 8.38	50.23 ± 10.05	84.19 ± 11.11	MCD+G

Values are the mean ± S.

paring the supplemented groups with its respective deficient groups. Although hepatic lipid accumulation was more prominent in rats fed the MCD or CD diets than those fed the MD diet, that this feature is *vice versa* in plasma values (Tables II and III).

All of the estimated liver function tests shown in Table IV of rats fed the different deficient diets were significantly elevated ($p < 0.01$) comparing with both of the control and the glycine supplemented diets. However, there was no significant difference among the values of the plasma direct bilirubin of the different experimental diets, except between the groups of rats fed the MCD diet and the control diets ($p < 0.01$). Supplementation of glycine to the different deficient diets improves the liver function by comparing between that of the deficient groups with its respective supplemented ones.

Each of the plasma α-L-fucosidase and arginase activities of all the experimental groups were higher significantly ($p < 0.01$) compared with those of rats fed the control diet. Otherwise, the added glycine could significantly lower both of the activities of rats fed the glycine supplemented diets (Table V).

Table V showed the ameliorative effect of glycine in lipid peroxidation in all of the rats fed the supplemented diets with a highly significant difference ($p < 0.01$). The lipid peroxidation is expressed as hepatic malondialdehyde (MDA) and hepatic antioxidant enzyme activities of superoxide dismutase (SOD) and reduced glutathione (GSH).

Figure 1 and Table VI show quantitatively a clear significant increment in the expression of the hepatic FATP gene of rats fed each of the experimentally glycine supplemented diets compared with the other groups of rats fed the glycine unsupplemented diets. Moreover, there was a significant difference between the hepatic gene expression of rats fed the control diet and all of the other groups of rats fed the different experimental diets except those fed the MCD + G diet.

Table III. Plasma total lipids, total cholesterol (TC), and triacylglycerol (TAG) concentrations of adult male albino rats fed on different experimental diets.

Plasma TAG (mg/ dL)	Plasma TC (mg/ dL)	Plasma total lipids (mg/dL)	Group
52.70 ± 10.70	40.70 ± 10.93	260.15 ± 10.21	Control
75.77 ± 10.65	76.00 ± 7.04	686.77 ± 14.25	MD
78.10 ± 8.23	95.13 ± 13.74	707.41 ± 7.13	CD
71.83 ± 10.28	71.13 ± 6.58	498.60 ± 12.06	MCD
55.40 ± 9.20	58.85 ± 11.22	451.20 ± 10.60	MD+G
57.39 ± 8.30	68.43 ± 10.30	456.59 ± 12.71	CD+G
57.20 ± 15.20	50.90 ± 13.28	266.30 ± 8.09	MCD+G

Values are the mean ± S.D.

Table IV. Plasma total and direct bilirubin, albumin and protein concentrations and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities of adult male albino rats fed on different experimental diets.

Plasma ALP (U/L)	Plasma AST (U/L)	Plasma ALT (U/L)	Plasma albumin (mg/dL)	Plasma protein (g/dL)	Plasma direct bilirubin (mg/dL)	Plasma total bilirubin (mg/dL)	Group
1.16 ± 0.42	8.25 ± 1.04	6.31 ± 0.93	3.08 ± 0.82	7.59 ± 0.92	$0.21 \pm 8.0 \times 10^{-2}$	0.40 ± 0.13	Control
2.62 ± 0.65	9.05 ± 1.62	7.03 ± 1.04	3.71 ± 0.49	8.97 ± 1.04	$0.28 \pm 9.9 \times 10^{-2}$	$0.70 \pm 8.5 \times 10^{-2}$	MD
2.35 ± 0.94	8.75 ± 0.63	7.33 ± 0.83	3.98 ± 0.83	7.77 ± 1.01	$0.22 \pm 9.2 \times 10^{-2}$	$0.49 \pm 7.2 \times 10^{-2}$	CD
2.54 ± 1.08	9.69 ± 1.23	8.07 ± 0.54	3.42 ± 0.56	8.08 ± 0.92	$0.32 \pm 7.3 \times 10^{-2}$	0.70 ± 0.13	MCD
1.32 ± 0.92	8.92 ± 1.08	7.53 ± 1.20	2.97 ± 0.77	7.59 ± 1.26	$0.21 \pm 8.4 \times 10^{-2}$	0.54 ± 0.12	MD + G
2.24 ± 0.86	8.10 ± 1.49	6.95 ± 0.64	3.27 ± 0.73	6.05 ± 1.04	$0.17 \pm 6.0 \times 10^{-2}$	$0.44 \pm 8.2 \times 10^{-2}$	CD + G
1.80 ± 1.03	8.24 ± 0.88	6.55 ± 1.17	3.04 ± 0.9	6.36 ± 1.0	$0.17 \pm 8.6 \times 10^{-2}$	0.47 ± 0.17	MCD + G

Values are the mean ± S.D.

Discussion

The present study elucidated three different nutritional models as a precursors for the NAFLD in an animal model, based on deficiencies in methionine and/or choline. Also, there is a trail to evaluate the role of glycine in the prevention of the fatty liver complications. Our data showed that the highest accumulation of fats was recorded in the groups of rats fed the CD diet followed by those fed the MD diet, then finally the rats fed the MCD diet. These findings were closely related to those of Vetelainen et al[2] and Picard et al[12]. Since overweight and obesity are closely associated with NAFLD, with the likelihood of the appearance of the NASH increasing the degree of obesity[13]. Glycine administration recorded an ameliorative effect on the degree of fat accumulation. This feature is emphasized by numerous reports which have documented the resolution of a fatty liver following a gradual weight loss[14]. However, van der Poorten et al[15] showed a closely linkage between the visceral fast assessed using magnetic resonance imaging and the severity of NAFLD. The visceral fat is not only a storage organ for free fatty acids (FFA), but also seems to participate directly in NAFLD pathogenesis in different ways, therefore interfering with both liver fat accumulation and progression from fatty liver to NASH. Furthermore, visceral fat acts as an endocrine organ able to interfere with cytokine/adipokine network, secreting different molecular mediators, such as FFA, adiponectin, leptin, TNF, IL-6, monocyte chemoattractant protein 1 (MCP-1), angiotensinogen, etc[16].

Feeding of animals a CD-high fat diet showed an accumulation of fat in liver with an improvement in both insulin sensitivity and glucose tolerance. These data suggested that the hepatic fat

Table V. Plasma α-L-Fucosidase and arginase activities and hepatic lipid peroxidation status of adult male albino rats fed on different experimental diets.

Hepatic SOD (U/g)	Hepatic GSH (mg/g)	Hepatic MDA (nmol/g)	Plasma arginase α-L-Fucosidase (U/L)	Plasma (U/L)	Group
9.99 ± 1.85	24.87 ± 1.58	5.29 ± 0.058	0.80 ± 0.37	0.65 ± 0.45	Control
12.96 ± 2.55	33.96 ± 1.42	10.89 ± 1.25	49.21 ± 6.36	3.42 ± 0.98	MD
14.25 ± 0.68	36.48 ± 1.78	11.36 ± 0.59	40.12 ± 13.1	3.46 ± 0.90	CD
17.98 ± 0.68	40.95 ± 0.99	13.89 ± 1.75	38.28 ± 11.9	4.35 ± 1.36	MCD
10.55 ± 1.47	29.48 ± 0.68	6.89 ± 1.11	33.52 ± 8.82	2.98 ± 0.65	MD + G
11.39 ± 1.55	27.55 ± 1.25	8.63 ± 0.89	32.29 ± 10.2	2.39 ± 0.90	CD + G
11.38 ± 2.89	31.65 ± 0.96	9.58 ± 0.68	31.15 ± 9.95	2.03 ± 1.20	MCD + G

Values are the mean ± S.D.

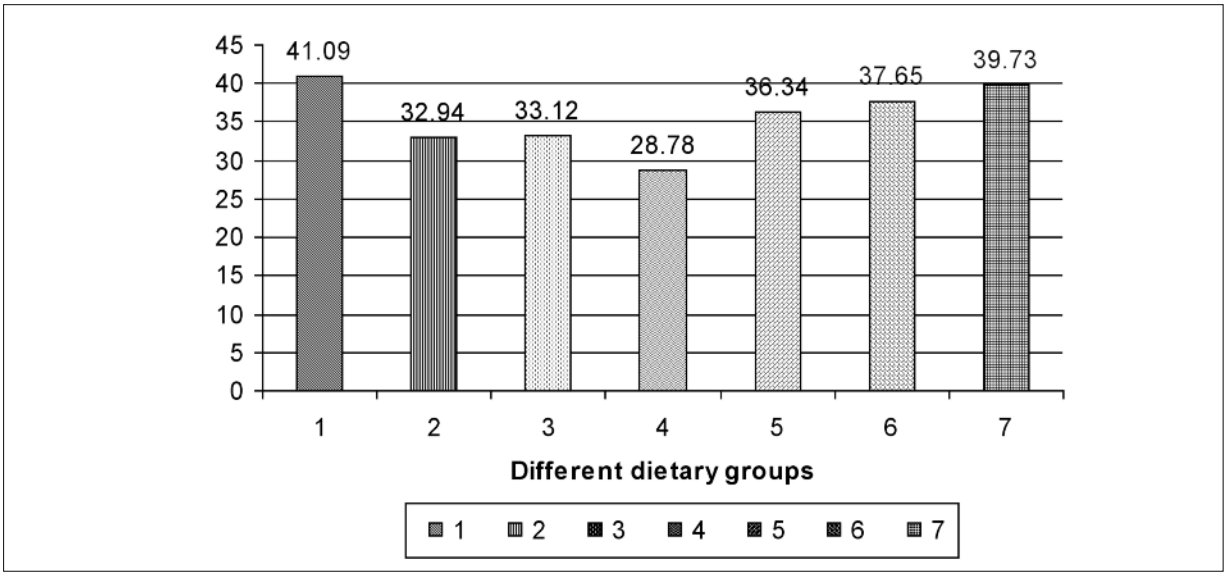

Figure 1. Shows quantitatively a clear increment in the expression of the hepatic FATP gene of rats fed each of the experimentally supplemented diets.

accumulation does not cause insulin resistance in diet-induced obesity. Thus, unlike the MCD diet, the insulin-sensitizing effect of CD diet could not attributed to weight loss[17].

The present study showed the greater hepatic fat accumulation in rats fed the CD or MCD diets than those fed the MD diet. Whereas plasma fats levels were elevated in the rats receiving the CD diet than that of the other two deficient groups. In addition, the hepatic FATP gene expression showed a clear increment in all of the three different deficient groups (MD, CD and MCD).

The proposed biochemical basis of fatty accumulation in choline and/or methionine deficiency is attributed to the impaired phosphatidyl choline (PC) synthesis, which is essential for the hepatic VLDL secretion. PC synthesis occurs through a direct incorporation of the performed choline into the phosphatidyl compounds (CDP pathway) or through the stepwise methylation of the adenosylmethionine[18]. The unimpaired PC synthesis via the CDP pathway was detected in the choline-deficient mice[19]. The results of this study were related with the previous above investigations demonstrating that the deprivation of a dietary choline and or methionine to animals induces a fatty liver. However, the mechanism operating in each case may differ. MCD diet-fed animals accumulate fat in the central perivenous zone of the liver, whereas animals on CD diet first accumulate fat in the periportal zone before it spills over into the other areas. Deficiency of both methionine and choline impairs the hepatocyte secretion of VLDL both *in vivo* and *in vitro*[17].

Table VI. Hepatic expression of the FATP gene of adult male albino rats fed on different experimental diets.

Hepatic FATP expression	Group
41.09 ± 0.91	Control
32.94 ± 1.94	MD
33.12 ± 1.88	CD
28.78 ± 0.22	MCD
36.34 ± 0.34	MD +G
37.65 ± 2.35	CD + G
39.73 ± 2.27	MCD + G

Values are the mean ± S.D.

The hepatocyte accumulation of TAG in the NAFLD may be due to insulin resistance which has a central role both in the disease development and progression[20]. The impaired peripheral insulin action leads to an uninhibited white adipose tissue lipolysis resulting in an increased flux of fatty acids to the liver and to a compensatory hyperinsulinemia which in turn determines an enhanced *de novo* hepatic lipogenesis. These two factors are central in leading to the hepatic accumulation of TAG[21]. Hepatic *de novo* lipogenesis accounts for 25% and is driven by an increased hepatic activity of critical transcription factors such as sterol regulatory element-binding pro-

tein-1c, carbohydrate response element-binding protein and peroxisome proliferator-activated receptor. Other pathways such as impaired hepatic fatty acid oxidation and/or impaired synthesis or secretion of VLDL seem to be less important[1].

The chronic exposure of non-adipose cells and tissues to elevated concentrations of fatty acids, TAG or cholesterol may trigger toxic effects. Thus, hepatic lipotoxicity may ensure when the hepatic capacity to utilize (oxidize) store and export fatty acids as TAG is overwhelmed by fatty acid flux from the periphery (usually visceral white adipose tissue) or hepatic *de novo* lipogenesis. Another factor may be the breakdown of hepatocellular TAG (stored in lipid droplets) by intracellular lipases, which may also contribute to (increased) intracellular fatty acid load. In addition, alterations in the expression of fatty acid transporter on the plasma membrane (FATP) and fatty acid binding protein may also critically determine the flux and concentration of fatty acid in the liver and thereby promote the lipotoxicity[22]. This explanation elucidated the increase in the hepatic expression of FATP and plasma activities of arginase and α-L-fucosidase in all of the three deficient diets (MD, CD and MCD).

FATP is a fatty-acid transporter on the plasma membrane. To verify the mechanism of glycine in improvement the status of NAFLD, the expression of FATP was compared among the different dietary groups either glycine supplemented or not. This study revealed that the expression of FATP was significantly elevated in all of the glycine supplemented groups (MD + G, CD + G and MCD + G) compared with their respective non supplemented groups. Similarly, the expression of FATP m-RNA was elevated in the PPAR-α and -δ treatment NAFLD rats[3].

All of the estimated hepatic function tests were highly elevated in the rats administered any of the deficient diets, indicating a symptom of the liver injury induced by the the accumulation of lipid according to NAFLD and its complications. These results were closely correlated with the feature of Cave et al[23] who reported that, although NAFLD may present at any stage, including cirrhosis with hepatocellular carcinoma, the most common presentation was in the asymptomatic, non-drinking patients where we may observe mildly elevated aminotransferases.

In the present study glycine, the non-essential amino acid, addition (5 g/100 g diet) to each of the methionine and/or choline deficient diets reverted the increase in body weight gain, the elevation in plasma and hepatic lipids profile, liver function tests, tumor markers and the disturbance in the expression of hepatic FATP gene.

Liver is the most common site of damage in laboratory animals administered drugs and other chemicals. The extent of hepatic damage in the glycine treated rats was reduced than non-treated animals. These findings agree with Senthilkumar and Nalini[6], who found that, glycine supplementation (0.6 g/kg body weight) significantly lowered the activities of serum AST, ALT, ALP and GGT. In addition, Yin et al[24] showed a reduced hepatic damage following the administration of glycine to rats with alcohol induced liver injury.

It was reported that, glycine administration (1% in drinking water) to male Wistar rats reduced the adipose tissue accumulation by stimulating the hepatic fatty acid metabolism (transport, activation or β-oxidation). This in turn decreased the non-esterified fatty acids concentration, which had been postulated to be a link with obesity and reducing TAG25.

The hypocholesterolemic effect of glycine in humans was firstly believed to be due to the alteration in insulin/glucagon ratios. Hence, the increased postprandial plasma glycine concentration, following the consumption of a casein meal supplemented with it, resulted in an elevated plasma glucagon level with a decreased insulin/glucagon ratio[26]. Lower insulin/glucagon ratios are associated with enhanced hormone-sensitive lipase in adipose tissue and decreased activities of hepatic lipogenic substrates[27].

There is evidence that glycine action is exerted through the glycine receptors[28]. The glycine receptor is a pentameric chloride channel, expressed in neurons, Kupffer cells, neutrophils, pancreas, and other cells in the body. After glycine-receptor binding, ion chloride influx promotes membrane hyperpolarization, inhibiting calcium influx into the cell[29]. Since cytokine production is dependent on influx of calcium into the cell, the same effect may occur in fibroblast and adipose cells. Hence, when type 2 diabetes patients were treated with glycine, the pro-inflammatory cytokines diminished after 3 months of treatment[30].

There are interrelations among the adipokines, each having an impact on the expression of the other. Adiponectin, for example, reduces LPS-mediated increase in m RNA IL-6 expression in pig adipocytes, by attenuating NF-$_{K}$B activation, and increases PPAR-γ2 expression in adipocytes[31]. In another study, adiponectin showed similar effects on TNF-α

and IL-6 expression in porcine macrophages. Besides, extra cellular IL-6 counter-regulated adiponectin gene expression and secretion in 3T3-L1 adipocytes[32].

The most dramatic effect of glycine in 3T3-L1 cells was the increment in PPAR-γ expression, without any apparent adipocyte structural modification or lipid accumulation[30]. Hence, there is evidence that PPAR-γ expression is controlled by adiponectin and *vice versa*[31]. Thus, the experimental finding in 3T3-L1 cells demonstrated clearly the effect of glycine on the expression of the anti-inflammatory molecules. The regulation of adipokine expression has important consequences in fat tissue, and other insulin-target organs due to the TNF-α and IL-6. These pro-inflammatory adipokines stimulate the serine phosphorylation of the insulin receptor substrate-1, diminish the insulin-induced tyrosine phosphorylation, and subsequently block the next steps of insulin signaling, where IRS-1 is associated with phosphatidyl inositol 3 kinase and glucose transporter type 4 (GLUT4) translocation, with the appearance, therefore, of the i.r.[33].

Conversely, adiponectin acts as an anti-inflammatory protein and counteracts the effects of the chronic inflammation. Hence, adiponectin and in consequence glycine exerts a beneficial effect in type 2 diabetes, obesity and atherosclerosis in human or in animal models[34]. These findings supported our results of an effective addition of glycine in alleviating the NAFLD complications, since it has an inflammatory component.

In this study, the ameliorating mechanism of glycine in NAFLD may be explained also through its antioxidant properties. It is well known that reactive oxygen species (ROS) play an important role in the etiology of i.r. and hyperglycemia, and can activate the transcription nuclear factor $_{K}$B (NF-$_{K}$B) pathway[35]. NF-$_{K}$B is the main nuclear transcription factor that modulates the pro-inflammatory cytokines production. Thus, our findings showed an amelioration in the hepatic oxidative stress (MDA), modulated the hepatic antioxidant enzymes activities (SOD and GSH), and blocked the production of the tumor markers (α-L fucosidase and arginase). So, the adipokine expression must be modulated in consequence in the glycine supplemented groups. In conclusion, glycine showed a great benefit in the improvement of the NAFLD status either by its indirect effect on adipokine via its antioxidant properties or by the enhancement of the hepatic FATP expression[35].

Conclusions

The completed model of non-alcoholic steatohepatitis (NASH) was found in rats fed the standard control diet deficient in both methionine and choline (MCD). However, methionine or choline deficiency from diets did not exert such severity.

Glycine supplementation had a significant protective effect against complications from different models of steatosis induced by methionine and/or choline deficiency at different levels.

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