Induction of Non-Alcoholic Fatty Liver Disease Associated with Metabolic Syndrome in Rats and the Amelioration Effects of some Promising Antioxidants

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) represents one of the most common liver diseases. It is strongly associated with obesity and insulin resistance and is thought to be parameters of the metabolic syndrome. NAFLD can progress to non-alcoholic steatohepatitis then to cirrhosis and liver failure. This study aimed to investigate whether L-carnitine or/Salvia officinalis (Sage) can improve non-alcoholic fatty liver in an animal model and whether this therapeutic approach resulted in amelioration in carbohydrate profile (Glucose, insulin, insulin resistance index, hepatic glycogen and glyoxalase I & II activities) and lipids (cholesterol, triglycerides, free fatty acids, leptin, resistin, adiponectin and hepatic total lipids & cholesterol). The obtained results revealed a significant (p<0.05) increase in carbohydrate profile (glucose, insulin, insulin resistance index, hepatic glycogen & glyoxalase system) in NAFLD rats than those in their control ones. Lipid parameters (cholesterol, triglycerides, free fatty acids, leptin, resistin and hepatic total lipids & cholesterol) were significantly (p<0.05) elevated in NAFLD rats compared with their corresponding control group. On the other hand, induction of NAFLD to rats caused a significant (p<0.05) decrease in adiponectin level. When, NAFLD rats group was treated with L-carnitine or/Sage, a considerable amelioration effects in all previous studied parameters were pronounced dependent on certain mechanisms. These results suggested that mitochondrial membrane stabilization, improved insulin resistance as well as oxidative stress inhibition may be the essential mechanisms for the hepatoprotective effect of L-carnitine or/Salvia officinalis (Sage) on non-alcoholic fatty liver disease in rats.

Key Words: Non-Alcoholic Fatty Liver Disease / Metabolic Syndrome / Antioxidants / Rats.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common type of chronic liver injury in many countries. NAFLD includes a spectrum of syndromes ranging from simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (1). The overall prevalence of NAFLD is 15%-40% in the world. It has dramatically increased over the past 15 years mainly as a consequence of its close association with two major worldwide epidemics: obesity and type II diabetes mellitus (T2DM) (2).

The increase in consumption of high calorie diets, specifically through refined carbohydrates and/or fructose, positively correlates with an alarming increase in obesity, type 2 diabetes and NAFLD (3). Moreover, NAFLD has been convincingly associated with the metabolic syndrome and insulin
resistance (4). Insulin resistance, through inhibition of lipid oxidation and increased fatty acid and triglycerides synthesis, is believed to be a key factor in the development of fatty liver. Moreover, insulin resistance is also characterized by altered production of adipokines variables such as adiponectin, leptin, resistin and tumor necrosis factor-α (TNF-α) (5).

L-Carnitine (CA) (β-hydroxy-γ-trimethyl ammonium butyric acid) is a vitamin like compound obtained from the diet; it is also synthesized in the body from the essential amino acids lysine and methionine. It is essential for the transport of long chain fatty acids into the mitochondrial matrix through the action of specialized acyl transferases. CA is also reported to possess antioxidant properties (6) and have effects on insulin action. Clinically, CA has been shown to improve insulin sensitivity in uremic and diabetic patients (7,8). The earliest study of the effect of CA on steatohepatitis was performed by Bowyer et al. (9). They claimed that CA deficiency was not the major cause of liver steatosis. However, a recent study showed that L-carnitine could improve the outcome of NASH, because it reduces lipid levels, limits oxidative stress and modulates inflammatory responses. It performs a number of essential intracellular and metabolic functions such as fatty acid transport, detoxification of potentially toxic metabolites, regulation of the mitochondrial acyl-CoA/CoA ratio and stabilization of cell membranes (10).

Salvia officinalis (Sage) is an aromatic and medicinal plant of Mediterranean origin well known for its antioxidant properties mainly due to its composition in phenolic compounds (11). Sage extracts revealed strong antioxidant activity by increasing the stability of food oils, by the ability to scavenge free radicals as well as by having oxygen radical absorbance capacity (12). In addition, sage has the protective effects against enzyme-dependent and enzyme-independent lipid peroxidation (13). Recently, it has shown that treatment with sage tea for 14 days lowered fasting plasma glyceroxidation but had no effects on glucose clearance in response to an intraperitoneal glucose tolerance test in rats (14). Using hepatocyte primary cultures a decreased gluconeogenic response to glucagon and a higher responsiveness to insulin were found after treatment with sage tea in vivo (14).

The objective of the present study was to investigate the development of fatty liver associated with metabolic syndrome in response to a high carbohydrate high fat diet and to determine whether L-carnitine and Salvia officinalis (Sage) can ameliorate the hepatic metabolic derangements in male albino rats.

MATERIAL AND METHODS

Seventy adult male albino rats (Rattus rattus) with ages 12 ± 1 week old and their weight 200±10g were employed in this study. They were housed in a well ventilated vivarium at Biological Applications Department, Nuclear Research Center, Atomic Energy Authority, Egypt. The animals were caged in wire bottom galvanized metal wall boxes under controlled environmental and nutritional conditions (25°C and 55-60 % relative humidity). The animals fed on a standard diet. Food and tap water were served ad libitum with fresh supplies presented daily.

Aqueous extraction of sage (Salvia officinalis):

The tested plant was sage (Salvia officinalis). The tested plant was used in aqueous extracts at concentration of 0.2 g/ml by soaking these plant in boiling water, after 5 min each extract was filtered and the filtrate was fed intragastrically to rats at a temperature of 37±1°C. Control rats were administered an equivalent volume of distilled water intragastrically at 37±1°C.
Experimental design:

This study was included two experiments: In the first experiment, the rats were randomly divided into two main groups according to the type of diet. In the first group (normal control rats group), twenty rats were fed on a control diet, containing 60% corn starch, 20% casein, 0.7% methionine, 5% corn oil, 10.6% wheat bran, 3.5% salt mixture and 0.2% vitamin mixture and served as control. While, the second group [non-alcoholic fatty liver (NAFL) rats group], fifty rats were fed on the same previous diet except that corn starch was replaced with an equal amount of fructose (30%) and lard (30%) according to Du Vigneaud & Karr (15) and approved by Pooranaperundevi et al. (16).

After one month, ten rats from each previous group were taken to compare the alterations in the profiles of serum lipid, hormones and carbohydrate associated with changes in both liver lipid and carbohydrate profiles due to induction of non-alcoholic fatty liver disease (NAFLD) with pronouncing of metabolic syndrome in rats.

In the second experiment (50 rats), five comparisons were made between normal control rats (10 rats) and four equal subgroups of rats with experimentally non-alcoholic fatty liver disease (NAFLD) (40 rats). The first experimentally non-alcoholic fatty liver disease (NAFLD) subgroup was served as recovery group. The second non-alcoholic fatty liver disease (NAFLD) subgroup rats were injected intraproteinally with L-carnitine (Sigma Chem. Co., St Louis, Mo. U.S.A) at a dose of 300 mg /kg body weight for 2 months (17) as served as L-carnitine subgroup. The third non-alcoholic fatty liver disease (NAFLD) subgroup rats were treated intragastrically with aqueous extracts of sage (Salvia officinalis) at concentration of 0.2 g/ml for 2 months according to Wang et al. (18). The fourth non-alcoholic fatty liver disease (NAFLD) subgroup rat was received both L-carnitine and aqueous extracts of sage for the same previous period. All animal subgroups were divided into two intervals (One and two months; five rats in each interval).

At the end of each experimental period, fasting blood samples were collected from each group by decapitation. Serum glucose, total cholesterol, triglycerides and free fatty acids concentrations were estimated colorimetrically using commercial kits from Randox, Ltd., Co. (UK). Serum resistin and adiponectin levels were assayed using commercial ELISA (Sandwich Immunoassay Technique) specific kit for rats (Diagnostic Automation, INC. USA). However, serum insulin and leptin levels were determined by radioimmunoassay (RIA) specific kit for rats using solid phase component system purchased from ICN Pharmaceuticals Inc, USA.

The homeostatic model assessment (HOMA) value as a measure of IR was calculated using the following formula: fasting insulin (µU/L) x fasting glucose (mmol/L)/22.5 (19).

After sacrifice, livers were removed aseptically at the end of each experimental period. For estimation of total cholesterol and triglycerides 200mg liver tissues wash with an ice-cold sterile 0.15M KCl with an ice-cold 0.05% butylhydroxytoluene solution (W/3V) and then homogenized in a Potter – Elvehjem homogenizer using a Teflon pestle. Each sample was then centrifuged for 20 min at 20,000 g and 4°C. The supernatant was aspirated for measuring the content of total cholesterol and triglycerides using commercial kits from Randox, Ltd., Co. (UK).

The glycogen kit is a convenient assay to measure glycogen levels in biological samples. In the assay, glucoamylase hydrolyzes the glycogen to glucose which is then specifically oxidized to produce a product that reacts with OxiRed probe to generate colour (λmax = 570 nm) according to Dalrymple & Hamm (20). So, glycogen estimation was done in terms of glucose equivalent to glycogen in mg/gm of tissue unit. This assay can detect glycogen 0.0004 to 2 mg/gm of tissue unit. This kit was purchased from BioVision Research Products Co. (USA).
Glyoxalase I was assayed by measuring the rate of formation of S-D-lactoylglutathione. The assay mixture contained 7.9mM MG, 1mM GSH and 14.6mM magnesium sulfate, and 182mM imidazole HCl, pH 7.0. After 5 minutes, 0.1ml of sample (50µg protein) was added and increase in absorption at 240 nm was measured and the activity was calculated using the co-efficient 2.86/mM/cm (21). The enzyme activity is calculated as µmole/g/min of the product formed. One unit of the enzyme is defined as the amount of enzyme catalyzing the formation of 1µmol of S-D-lactoylglutathione/min/mg protein under the assay conditions. Glyoxalase II was assayed by measuring formation of GSH from S-D-lactoylglutathione (22). The reaction was started by the addition 0.5 ml of 1.5mM S-D-lactoylglutathione to 0.1ml of sample and GSH formation was measured after 15 min by reaction with 0.75mM DTNB. The activity was expressed as mmol GSH formed/min/mg protein. The kits of glyoxalase I & II were purchased from amsbio, Life Science Int. Co. USA.

Data were statistically analyzed using Student “t” test in the first experimental. Moreover, two way analysis of variance (ANOVA) followed by Duncan’s multiple range test in the second experimental according to Snedecor & Cochran (23).

RESULTS AND DISCUSSION

Insulin resistance is a key event in the pathophysiology of the metabolic syndrome. Hyperinsulinemia caused by an increased insulin secretion by the pancreatic beta cells and decreased insulin degradation by the liver (a compensatory phenomenon to insulin resistance). Hyperinsulinemia leads to an increase in fat mass and lipogenesis associated with increased concentrations of free fatty acids (FFA). These changes are linked with further reduction in insulin signaling and an increase in both hepatic glucose and lipid production (24). In the current study, NAFLD rats group recorded a significant (p<0.001) elevation in the levels of serum glucose and insulin associated with a significant increment in the value of insulin resistance (HOMA-IR) as compared to their corresponding control group (Table 1). These results may be due to a defect in insulin binding caused by decreasing receptor number or their affinity or/and defects at the level of effect molecules such as glucose transporters and activities of their enzymes involved in glucose metabolism (25). Moreover, Ackerman et al. (26) reported that insulin resistance leads to defective insulin-mediated inhibition of lipolysis while hyperinsulinemia enhances the hepatic synthesis of free fatty acids and inhibits the synthesis of apolipoprotein. Furthermore, Shang et al. (27) reported that fat accumulation in the liver and insulin resistance cause and potentiate each other creating a vicious cycle of metabolic dysfunction and resulting in the development and progression of NAFLD.

Table (1): Comparison between normal and non-alcoholic fatty liver disease (NAFLD) rat groups on serum carbohydrate profile related to metabolic syndrome (Mean).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control rats n = 10</th>
<th>Non-alcoholic fatty liver disease n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose</td>
<td>4.592 ± 0.067</td>
<td>7.853 ± 0.102*</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>40.796 ± 1.316</td>
<td>79.748 ± 1.928*</td>
</tr>
<tr>
<td>(µU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>8.326 ± 0.217</td>
<td>27.834 ± 0.731*</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE - n= number of rats. - * significant at (P< 0.001).

Non-alcoholic fatty liver disease (NAFLD) represents a wide spectrum of disorders, the hallmark of which is hepatic steatosis. NAFLD was considered a benign condition, but is now
increasingly recognized as a major cause of liver-related morbidity and mortality. Insulin resistance is the basis for accumulation of free fatty acids and triglyceride storage in hepatocytes or steatosis \(^{(28)}\). The pronouncing of NAFLD may be attributed to the fructose supplementation which led to excess formation of malonyl-CoA which inhibits \( \beta \)-oxidation, increase hepatic lipid droplet formation due to the activation of mitogen-activated protein kinase 8 (MAPK8) and phosphofructokinase (PKC) which contribute to serine phosphorylation of IRS-1 (pSer-IRS-1) associated with hepatic insulin resistance and increase synthesis of forkhead box protein O1 (FOXO1) which promotes gluconeogenesis and hyperglycemia \(^{(20)}\).

In this study, administration of high carbohydrate (Fructose)-high fat diet for one month to induce NAFLD in rats caused a significant increment in serum total cholesterol, triglyceride and free fatty acids as compared to their corresponding normal fed rats (Table 2). These results may be due to the elevation in the lipid oxidation, the disturbance in the hypothalamus-pituitary-thyroid axis, the elevation of ceramides levels or/and the decrease of \( \beta \)-oxidation of lipid in the matrix of mitochondria. These results are in agreement with the viewpoint that mitochondrial dysfunction participates in the pathogenesis of NAFLD at different levels, mainly including lipid oxidation impairment and the induction of peroxidative production \(^{(30)}\). In parallel study, the increases in intracellular triglycerides content in tissues of humans with insulin resistance and metabolic syndrome can be related to increases in the uptake of FFA from plasma, an enhanced rate of de novo fatty acids synthesis and a deregulation of intracellular lipid partitioning in which fatty acid oxidation is impaired and its esterification enhanced \(^{(31)}\).

### Table (2): Comparison between normal and non-alcoholic fatty liver disease (NAFLD) rat groups on serum lipid profile related to metabolic syndrome.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control rats ( n = 10 )</th>
<th>Non-alcoholic fatty liver disease ( n = 10 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>55.873 ± 1.039</td>
<td>112.707 ± 2.478 *</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>64.136 ± 1.528</td>
<td>137.841 ± 2.749 *</td>
</tr>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>0.623 ± 0.071</td>
<td>1.718 ± 0.106 *</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.791 ± 0.032</td>
<td>6.036 ± 0.078 *</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>3.387 ± 0.051</td>
<td>5.681 ± 0.089 *</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>8.695 ± 0.141</td>
<td>4.927± 0.087 *</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE  
- \( n = \) number of rats.  
- * significant at \( P< 0.001 \).

Setshedi et al. \(^{(32)}\) reported that deregulated lipid metabolism promoted by insulin resistance leads to increase generation of ceramides that exacerbate insulin resistance, inflammation, tissue injury and cell death. Ceramides are a lipid species that exert biological effects through cellular proliferation, differentiation and cell death, and interact with several pathways involved in insulin resistance, oxidative stress, inflammation and apoptosis; all of which are linked to NAFLD. Moreover, ceramides are generated via 3 main pathways: (1) \textit{de novo} synthesis by coupling sphinganine to a long-chain fatty acid, yielding dihydroceramide (2) hydrolysis of complex sphingolipids such as sphingomyelin or glycosphingolipids and (3) recycling after acylation of sphingosine, utilizing the “salvage pathway”. Also, ceramides cause insulin resistance by activating proinflammatory cytokines, inhibiting transmission of signals through phosphatidyl-inositol-3 kinase (PI3K) and Akt and activating protein...
phosphatase 2A (PP2A). Furthermore, ceramides can promote apoptosis by activating protein kinase C, caspases and cathepsin D (32).

Leptin is the main regulator of fat in the organism. It is released from the fat tissue into blood then dispensed into the tissues (adipose tissue, peripheral lymphoid tissue, central nervous system, gastrointestinal tract and liver) over the body by circulation and combines with its receptors, which then interact with Janus family protein tyrosine kinase/signal transduction and activates transcription factors (JAK/STAT), mainly JAK2/STAT3, to cause the related biological effects (33) and to exhibit the function of diet control and energy metabolism regulation (34). In the current work, serum leptin level was significantly increased in NAFLD rats group as compared to their corresponding control group (Table 2). These results are in agreement with that obtained by Chitturi et al. (33) and Angulo et al. (35).

Another interesting issue observed that insulin has a direct influence on the synthesis and secretion of leptin (36). It is thought that leptin participating in glycol-metabolism and fat-metabolism in liver mainly depends on the regulation of gene expression of phosphoenolpyruvic acid and efficiency of glyconeogenesis which stimulates liver intake of lactic acid and genesis of hepatic glycogen. Simultaneously, it activates the carnitine acyl transferase through protein kinase A, then regulates fat metabolism. Furthermore, leptin interferes with the role of insulin in the liver, on one hand, it counteracts the downregulation of phosphoenolpyruvate carboxykinase (PEPCK) induced by insulin, restrains the phosphorylation of insulin receptor substrate-1 to be involved in the signal transduction; on the other hand, leptin restricts triglyceride synthesis and elevates the insulin sensitivity of liver and peripheral tissues. Leptin can also decrease triglyceride by reducing diet intake (about 50%) and enhance energy consumption (37).

The role of resistin in the pathogenesis of NAFLD is not clear. In this work, NAFLD rats group showed significant (P<0.001) increment in the resistin level (Table 2). This increment is correlated to insulin resistance and body mass index. The results obtained are confirmed by the results obtained by Pagano et al. (38). The authors reported that increase resistin levels in NAFLD patients were related to the histological severity of the disease. On the other hand, Jarrar et al. (39) did not find statistically significant correlation between serum resistin in patients with NASH, simple steatosis and obese controls.

Resistin is up-regulated by proinflammatory cytokines including TNF-α, IL-6 and IL-1β. It promotes the synthesis of these cytokines by NF-kB activation (40). Moreover, Yagmur et al. (41) reported the correlation between serum TNF-α level (which was one of the parameters indicating chronic inflammatory condition) and serum resistin in patients with chronic liver diseases (chronic hepatitis B or C virus infection, alcohol, autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis).

A number of studies have demonstrated the association between hypoadiponectinemia and NAFLD (42). The low adiponectin levels in NAFLD rats group reported in this study may represent a pathogenic mechanism leading to altered hepatocyte lipid metabolism and fat accumulation (Table 2). In fact, high adiponectin levels have been reported to protect against both alcoholic and nonalcoholic fatty liver disease in mice by reducing fatty acid synthesis through inhibition of acyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression and activity (39). The reduction of ACC activity reduces the malonyl-CoA level, which is known to inhibit carnitine palmitoyltransferase I (CPT-I) activity and fatty acid oxidation. Therefore, the reduction of adiponectin in NAFLD could be due to increase in fatty acid synthesis, accumulation of triglycerides and decrease fatty acid oxidation (43).
However, adiponectin may protect against steatohepatitis also through its anti-inflammatory action. It is well known that inflammation is a key mechanism in the progression of fatty liver to hepatitis and cirrhosis\(^{(43)}\). Adiponectin level is correlating inversely with circulating levels of TNF-\(\alpha\) and IL-6 because both these cytokines inhibit adiponectin messenger RNA in adipose tissue (Angulo, 2006). On the other hand, adiponectin induces its anti-inflammatory properties by suppression of IL-6\(^{(44)}\). Studies of Ota \textit{et al.}\(^{(45)}\) supported the idea that excessive production of IL-6 versus the defective production of adiponectin may provide a link between insulin resistance and inflammation in NASH. Moreover, Hui \textit{et al.}\(^{(46)}\) observed that lower serum adiponectin level in NASH patients was associated with more extensive necroinflammation. On the other hand, Wong \textit{et al.}\(^{(47)}\) did not find the correlation between serum adiponectin concentration and the disease severity.

Hepatic total lipids and cholesterol levels were significantly (\(p<0.001\)) increased in NAFLD rats group compared to their values in the corresponding normal fed rats group (Table 3). These results seemed to be in complete accordance with studies made by Westerbacka \textit{et al.}\(^{(48)}\). They reported that high fat diets have been shown to increase \textit{de novo} lipogenesis. This would lead to altering the relative sources of liver triacylglycerol. Fatty acids in the liver come from several different sources: derived from dietary fat, released from adipocytes \textit{via} lipolysis and from \textit{de novo} hepatic lipogenesis. An imbalance of any of the pathways involved in triacylglycerol delivery, synthesis, export or oxidation could contribute to its accumulation in the liver\(^{(49)}\).

In the current study, the level of glycogen was increased in the liver of NAFLD rats group as compared to normal fed rats group (Table 3). Free fatty acids inhibit carbohydrate oxidation, insulin-stimulated glucose uptake and its incorporation into glycogen due to a decrease in the activity of glycogen synthase\(^{(50)}\). Also, a disturbance in fatty acid partitioning could contribute to insulin resistance due to the inhibition of cytosolic long-chain fatty (LCFA) acyl CoA oxidation\(^{(51)}\). The mechanism involves malonyl-CoA, which inhibits carnitine palmitoyltransferase (CPT). CPT controls the transfer of LCFA acyl-CoA from the cytosol into the mitochondria where they are oxidized. There is an association between elevations of malonyl-Co-A and insulin resistance. An increase in its concentration leads to an increase in cytosolic LCFA acyl Coas, its esterification, formation of diacylglycerol, triglycerides, ceramide and reactive oxygen species, all linked to insulin resistance\(^{(51)}\).

Table (3): Comparison between normal and non-alcoholic fatty liver disease (NAFLD) rat groups on liver lipids and carbohydrate profile related to metabolic syndrome.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control rats (n = 10)</th>
<th>Non-alcoholic fatty liver disease (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids (mg/g tissue)</td>
<td>40.683 ± 0.771</td>
<td>78.125 ± 0.934*</td>
</tr>
<tr>
<td>Total cholesterol (mg/g tissue)</td>
<td>2.352 ± 0.041</td>
<td>4.424 ± 0.079*</td>
</tr>
<tr>
<td>Glycogen (mg/g tissue)</td>
<td>7.127 ± 0.084</td>
<td>10.816 ± 0.112*</td>
</tr>
<tr>
<td>Glyoxalase-I (µmol/min/mg protein)</td>
<td>13.619 ± 0.137</td>
<td>7.174 ± 0.089*</td>
</tr>
<tr>
<td>Glyoxalase-II (µg of GSH consumed/min/mg protein)</td>
<td>4.902 ± 0.053</td>
<td>2.871 ± 0.041*</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE -  \(n=\) number of rats. - * significant at (\(P<0.001\)).

The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II. Glyoxalase I catalyses the formation of SD-lactoylglutathione by the reaction between methylglyoxal and glutathione while, glyoxalase II catalyses the hydrolysis of SD-lactoylglutathione to D-lactic acid and...
GSH. Glyoxal may be formed as degradation products of autoxidation of glucose or from glucose adducts to proteins (52). Glyoxal is more reactive than glucose and can react non-enzymatically with proteins forming cross links and adducts, the degradation of which can be associated with oxidative stress. Thus, the glyoxalase system converts the levels of toxic α-oxoaldehydes to nontoxic R-2-hydroxy acids. The physiological substrate of the glyoxalase system and methylglyoxal could be formed non-enzymatically from dihydroxyacetone phosphate and glyceraldehydes-3-phosphate. When fructose is consumed, as the sole source of carbohydrate, there is increased flux through the glycolytic pathway and increased formation of intermediates of glycolysis and production of oxoaldehydes could be expected upon fructose feeding (53).

Judging from the data in table (3), NAFLD rats group showed a significant (p<0.001) decline in activities of the glyoxalases I and II than those of their corresponding ones. Decreased glyoxalase I and II activities represents a harmful situation, as the disposal of methylglyoxal as well as products of lipid peroxidation is less efficient (16). Lapolla et al. (54) showed that patients with diabetic complications had a propensity to maintain relatively high levels of plasma glyoxal and S-D-lactoylglutathione as a result of a significant decrease in the activities of glyoxalase I and II.

Disorders such as liver disease, defects in fatty acid metabolism, and the administration of pharmacological agents can cause secondary L-carnitine deficiency (55). L-carnitine is associated with hypoglycemia, it promotes insulin sensitivity, thus lowering insulin resistance, possibly by increase the peripheral glucose utilization and regulating the cell energy metabolism or reducing free fatty acids as shown by González-Ortiz et al. (56). In the current work, NAFLD rats treated by L-carnitine recorded significant (p<0.05) decrease in the levels of serum glucose, insulin and insulin resistance (HOMA) (Table 4). The mechanism underlying this is not clear but it can assume that L-carnitine can interfere with processes involved in β-oxidation and accumulation of lipotoxic metabolites that might contribute to mitochondrial dysfunction and insulin resistance. Also, L-carnitine could act through mechanisms that are independent of the putative detoxifying role (57).

Table (4) : Amelioration effects of the administration of L-carnitine, sage and/or their mixture on serum carbohydrate profile in NAFLD rats (Mean ± SE).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>NAFLD</th>
<th>NAFLD + L-carnitine</th>
<th>NAFLD + Sage</th>
<th>NAFLD + L-carnitine + Sage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Groups (n=5))</td>
<td>(1 month)</td>
<td>(2 months)</td>
<td>(1 month)</td>
<td>(2 months)</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>4.588±0.065 a</td>
<td>7.106±0.094 b</td>
<td>6.197±0.089 c</td>
<td>6.201±0.092 d</td>
<td>5.491±0.079 e</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>40.829±1.358 a</td>
<td>70.328±1.776 b</td>
<td>65.748±1.518 c</td>
<td>68.028±1.624 d</td>
<td>57.902±1.479 e</td>
</tr>
<tr>
<td>(μU/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>8.326±0.221 a</td>
<td>22.211±0.652 b</td>
<td>18.108±0.589 c</td>
<td>18.749±0.601 d</td>
<td>11.064±0.435 e</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- A, B, C, D, E Means with a common superscript within a row are significantly different at (P<0.05).
- a, b Means with a common subscript within a column are significantly different at (P<0.05).

Supplementation of L-carnitine after induction of NAFLD to rats showed a significant (p<0.05) decrease in the serum lipid profile (cholesterol, triglycerides, free fatty acids, leptin & resistin) as well as hepatic total lipids and cholesterol associated with a significant increase occurred in the serum adiponectin levels through the whole experiment periods (1 & 2 months) (Tables 5 & 6). These results are in agreement with those of El-Metwally et al. (58) who reported that oral L-carnitine increases plasma free carnitine levels, improves dyslipidemia and decreases oxidative stress. Also, L-carnitine administration to obese rats reduces significantly serum hypertriglyceridemia via decreased synthesis of triglycerides by the liver or by inhibition of triglyceride release from the liver (56).
L-carnitine is necessary for mitochondrial transport metabolism of long-chain fatty acids, thus for myocardial energetic metabolism. Fatty acids cross mitochondrial membranes as acyl-carnitine derivatives to enter pathways for oxidation, acylation, chain shortening or chain elongation-desaturation. Therefore, L-carnitine-dependent fatty acid transfer is central to lipid metabolism; dietary supplementation of L-carnitine improves the utilization of fat providing marked reduction in serum levels of TG (60).

Furthermore, L-carnitine is an essential factor in the production of acetyl-CoA. It regulates the turnover of the fatty acids into phospholipids membranes, a process known as the deacylation – reacylation cycle of phospholipids membranes. L-carnitine is suggested to act as CoA buffer, maintaining the acyl CoA/CoA ratio in cells and exerts a function in several metabolic processes. The transport of acyl-CoA across the inner mitochondrial membrane to the matrix determine a reduced availability of CoA in the matrix and a decrease of CoASH. It determines a parallel increase in the acyl CoA/CoASH ratio, which inhibits the mitochondrial dehydrogenases; consequently, not only the oxidation of fatty acids but also the utilization of carbohydrates becomes impaired (60).

Moreover, L-carnitine could have beneficial effects on the mitochondrial respiratory chain. Several studies on aging showed that L-carnitine increases the mitochondrial content of cardiolipin reducing the mitochondrial impairment of electron transfer in liver. In addition, L-carnitine has some antioxidant and antiapoptotic properties (61). The mechanism whereby L-carnitine could mediate its antioxidant action is still unclear, but several studies pointed to increased levels of different antioxidant enzymes (e.g., SOD, catalase, glutathione peroxidase) and vitamins (e.g., vitamins C and E) (57). Finally, L-carnitine could exert its antiapoptotic effects by decreasing ROS production, removing toxic fatty acid derivatives and reducing generation of ceramides (62).

Table (5) : Amelioration effects of the administration of L-carnitine, sage and/or their mixture on serum lipid profile in NAFLD rats (Mean ± SE).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups (n=5)</th>
<th>Control</th>
<th>NAFLD + L-carnitine</th>
<th>NAFLD + Sage</th>
<th>NAFLD + L-carnitine + Sage</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. cholesterol (mg/dL)</td>
<td>1 month</td>
<td>55.72±0.38 A, a</td>
<td>101.58±1.93 B, b</td>
<td>85.19±1.23 C, c</td>
<td>90.97±1.74 D, a</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>56.17±0.39 A, a</td>
<td>92.01±1.62 B, b</td>
<td>70.28±0.80 C, c</td>
<td>79.36±0.95 D, b</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>1 month</td>
<td>64.58±1.54 A, a</td>
<td>129.21±2.39 B, b</td>
<td>96.45±1.60 C, c</td>
<td>108.83±1.80 D, b</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>65.02±1.59 A, a</td>
<td>111.31±1.95 B, b</td>
<td>84.11±1.23 C, c</td>
<td>92.19±1.52 D, b</td>
</tr>
<tr>
<td>Free fatty acids (mmol/L)</td>
<td>1 month</td>
<td>0.63±0.03 B, c</td>
<td>1.44±0.09 B, a</td>
<td>1.02±0.07 C, a</td>
<td>1.29±0.09 D, a</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>0.63±0.07 B, c</td>
<td>1.12±0.08 B, b</td>
<td>0.80±0.07 C, a</td>
<td>0.92±0.07 D, b</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>1 month</td>
<td>2.77±0.03 A, a</td>
<td>5.58±0.08 B, a</td>
<td>4.10±0.06 C, a</td>
<td>4.84±0.07 B, a</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>2.80±0.03 A, a</td>
<td>4.74±0.07 B, b</td>
<td>3.00±0.05 C, b</td>
<td>3.94±0.06 B, a</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>1 month</td>
<td>3.39±0.04 A, a</td>
<td>5.12±0.07 B, b</td>
<td>4.41±0.06 C, a</td>
<td>4.79±0.07 D, a</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>3.39±0.05 A, a</td>
<td>4.40±0.06 B, b</td>
<td>3.63±0.05 C, b</td>
<td>4.10±0.06 D, b</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>1 month</td>
<td>8.69±0.14 A, a</td>
<td>5.52±0.09 B, a</td>
<td>6.50±0.10 C, a</td>
<td>5.97±0.09 D, a</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>8.69±0.14 A, a</td>
<td>6.59±0.10 B, b</td>
<td>7.87±0.12 C, b</td>
<td>7.02±0.11 D, b</td>
</tr>
</tbody>
</table>

Means with a common subscript within a row are significantly different at (P<0.05).

Means with a common superscript within a column are significantly different at (P<0.05).
By reviewing tables (4-6), the administration of aqueous extracts of sage (*Salvia officinalis*) to the non-alcoholic fatty liver disease rats group led to a considerable correction in all studied parameters dependent on the time of administration. These results were confirmed by Ninomiya et al. (63) and Lima et al. (64). They concluded that sage tea drinking positively affected the antioxidant status of the liver, mainly the GST and GR activities of the mice livers and GST activity in rats.

The current work showed that sage-tea-drinking significantly reduced serum glucose level in NAFLD rats. This suggested an inhibition of gluconeogenesis and/or glycogenolysis in the liver. In addition, the response of the hepatocytes to insulin was significantly increased by sage. Alarcon-Aguilar et al. (65) showed that 4 h after an intraperitoneal injection of a sage water ethanolic extract blood glucose decreased significantly in fasted normal mice and in fasted mildly alloxan-diabetic mice but not in fasted severely alloxan-diabetic mice. Although, the authors stated that insulin might have mediated the hypoglycaemic effect of the extract, once the animals were tested in the fasted condition, it seems likely that an inhibition of gluconeogenesis was the cause of the effects observed in their study, as indeed suggested by the present results. Additionally, Eidi et al. (66) showed that 3 h after an intraperitoneal injection of a sage methanolic extract, blood glucose decreased significantly in fasted STZ-diabetic rats but not in fasted normal rats. This effect was not accompanied by the acceleration of insulin release (66). Generally, sage-tea-drinking increased rat hepatocyte glucose consumption, decreased fasting gluconeogenesis and inhibited the stimulation of hepatic glucose production by glucagon.

Table (6) : Amelioration effects of the administration of L-carnitine, sage and/or their mixture on liver lipid and carbohydrate profile in NAFLD rats (Mean ± SE).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups (n=5)</th>
<th>Control</th>
<th>NAFLD</th>
<th>NAFLD + L-carnitine</th>
<th>NAFLD + Sage</th>
<th>NAFLD + L-carnitine + Sage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids (mg/g tissue)</td>
<td>1 month</td>
<td>40.78±0.759&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.21±0.872&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.09±0.836&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68.43±0.852&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.22±0.784&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>40.75±0.763&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.39±0.824&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.12±0.781&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.12±0.811&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.87±0.761&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mg/g tissue)</td>
<td>1 month</td>
<td>2.36±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.896±0.066&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.22±0.054&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.23±0.056&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.87±0.047&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>2.35±0.042&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.03±0.052&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.70±0.048&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.71±0.046&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.35±0.043&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycogen (mg/g tissue)</td>
<td>1 month</td>
<td>7.16±0.087&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.02±0.098&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.55±0.086&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.56±0.088&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.01±0.112&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>7.16±0.089&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.11±0.092&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.76±0.112&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.76±0.112&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.87±0.112&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glyoxalase-I (µmol/min/mg protein)</td>
<td>1 month</td>
<td>13.58±0.132&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.23±0.096&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.02±0.113&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.55±0.109&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.72±0.128&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>13.59±0.135&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.67±0.102&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.68±0.119&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.63±0.124&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.99±0.133&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glyoxalase-II (µg of GSH consumed/min/mg protein)</td>
<td>1 month</td>
<td>4.89±0.051&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.22±0.041&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.88±0.041&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.87±0.041&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.33±0.041&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
<td>4.90±0.054&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.897±0.041&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.23±0.041&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.22±0.041&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.90±0.041&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c, d</sup> Means with a common superscript within a row are significantly different at (P<0.05).

In addition, Milagro et al. (67) reported that there was a link among increased fat depots, insulin resistance and liver oxidative stress. This effect was explained by Planas et al. (68) and Elsayed (69) who stated that several natural compounds in sage have been shown to act on cholesterol metabolism (by reducing its absorption or its synthesis) such as phytosterols and catechins as well as substances called sasoid-sasoid-b. This finding was in coincidence with the observation of Kamal and Mohamed (70) who illustrated that sennosides in senna improve gastrointestinal motility and influence colonic motility thereby reducing fluid absorption and facilitates weight loss.
In the current investigation, treatment of NAFLD rats group with sage decreased serum lipid profile and hepatic total lipids and total cholesterol compared to NAFLD rats group (Tables 5&6). These results are in agreement with Ninomiya et al. (63) and Carla et al. (71). The authors found that oral administration of sage significantly lowered total cholesterol, triglycerides in serum of rats and elevated serum levels of HDL-C. Also, Akram and Maryam (72) showed that oral administration of sage water extract led to a significant decrease in the serum cholesterol and triglyceride levels. These results suggested that S. officinalis tea consumption is accountable for the improvement of the lipid profile inducing an increase in the HDL-C particles, contributing therefore positively to the control of the dyslipidaemia observed in Type 2 diabetes but also related to other diseases (73). However, sage modulating results may attributed to several sage natural components have been shown to act on cholesterol metabolism by reducing its absorption or its synthesis, such as catechins (68). This is in addition to the polyphenols, especially, phenolic rosmarinic acid in sage which has potent antioxidant effects and protecting membrane lipids of fatty acids and phospholipids from oxidative stress (64).

Co-administration of L-carnitine and sage to the NAFLD rats caused the maximum correction effects on all studied parameters. These results may be attributed to the synergistic effects of both L-carnitine and sage. Interestingly, sage administration to NAFLD rats may lead to improvement of the physiological, biochemical and phar-malogical properties of L-carnitine by increasing the activity of lipooxygenase acting together as free radical scavengers, cell membrane stabilizers, hypolipidemic agents and improvements in the auto-immune system.

From the data of the current study, it could be concluded that the relation between adipose tissue and liver is emerging and may act as a major player in the link between metabolic syndrome and fatty liver disease. Thus, the changes of adipohormones levels provide the additional tool for NAFLD detection. Furthermore, the administration of antioxidants (L-carnitine or/and sage) to NAFLD rats proved to have better protective action against non-alcoholic fatty liver disease. L-carnitine has an important mitochondrial detoxification effect on hepatocytes and other cells. It improved symptoms of NAFLD and might improve metabolic conditions. Sage, on the other hand, acts as free radical scavengers, cell membrane stabilizer and lipid peroxidation reduction dependent on the time of duration.

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