Role of Melatonin, Glutamine and L-arginine in Prevention of Non-alcoholic Fatty Liver Disease in Rats


From
Department of Medical Physiology / Faculty of Medicine/Mansoura University, Mansoura, Egypt
MERC / Faculty of Medicine/Mansoura University, Mansoura, Egypt

Abstract
Over the next years, non-alcoholic fatty liver disease will represent the main cause of chronic liver disease with the reduction of hepatitis C burden. Recent researches are directed towards prevention. Prevention of NAFLD can be achieved by attenuation of oxidative stress. The aim of this work is to study the possible role of melatonin, glutamine and L-arginine in prevention of non-alcoholic fatty liver disease in rats induced by high fat, and high carbohydrate diet. The study included control, NAFLD, melatonin, glutamine and arginine groups. For all groups we have measured the serum concentration of glucose, lipid profile, liver enzymes, the concentration of glutathione (GSH) and malonyl aldehyde (MDA) in liver tissues. Then we performed histopathological study of liver tissue. There was significant increase in blood glucose level, triglycerides, Cholesterol and LDL in NAFLD, significant increase in liver enzymes (AST, ALT) and MDA, and significant decrease in GSH in NAFLD group as compared with control group. The use of melatonin, glutamine and L-arginine improved all the parameters as compared with NAFLD group. By histopathological study, marked improvement with slight fatty infiltration and near normal hepatocytes in melatonin group, moderate improvement with mild steatosis in glutamine group while mild improvement with L-arginine group. From this work we can conclude that: Early intervention with melatonin, glutamine or L-arginine has a protective effect in NAFLD.

Keywords
- NAFLD
- Melatonin
- L-arginine
- glutamine
- oxidative stress
- glucose
- lipid profile

Corresponding author: Gehan Abdel Hamid Hassan Elwakil (PhD), Lecturer of Medical Physiology, Physiology Department, Faculty of Medicine Mansoura University, Mansoura, Egypt. E-mail: gehanelwakeel19@gmail.com, Mob.: +01005583338, Short title: Antioxidants and non alcoholic fatty liver disease
**Introduction**

The global incidence of non-alcoholic fatty liver disease continues to increase worldwide because of increasing obesity (1). Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic fatty liver (NAFL) with simple steatosis without inflammation (2) and its progressive form; non-alcoholic steatohepatitis (NASH) with inflammation and hepatocellular injury with or without fibrosis (3). NAFLD is one of the manifestations of the metabolic syndrome, which includes obesity, type 2 diabetes mellitus, dyslipidemia and hypertension (4).

Non-alcoholic fatty liver disease is a disease characterized by the presence of intracytoplasmic fat of more than 5% of the liver weight in non-alcoholic patients (steatosis). It may be classified to macrovesicular, microvesicular or mixed, according to the size of fat droplets inside hepatocytes. Also, it may be accompanied by hepatocellular injury and inflammation (non-alcoholic steatohepatitis) (5). Fibrosis may also develop in the context of NASH which may eventually lead to liver failure (2). Hepatocellular carcinoma (HCC) can also occur in the setting of NAFLD (6). The pathogenesis of NAFLD has yet to be illuminated, although the 'two-hit' hypothesis is well recognized. The first hit refers to lipid metabolism and insulin resistance, which cause fat accumulation and simple fatty liver. The second hit includes oxidative stress, inflammation and other factors (7). Oxidative stress is thought to be one of the key factors in the pathogenesis of NAFLD.

It is predicted that over the next years, NAFLD will represent the main cause of chronic liver disease in adults and children with the reduction of hepatitis C burden (8). As there are no established treatment lines (9), recent researches are directed towards prevention. Prevention or amelioration of NAFLD can be achieved by prevention or attenuation of oxidative stress (4).

Melatonin, known chemically as N-acetyl-5-methoxytryptamine, is a hormone found in animals, plants and microbes (10) formed in the brain by pineal gland. Also, it is produced in other sites other than pineal gland as lymphocytes. Its synthesis and release are stimulated nocturnally, playing a role in the circadian rhythm (11). Melatonin has a well-established antioxidant effect (12), acting directly as a scavenger for free radicals as hydroxyl (16), peroxyl radicals (13) and peroxynitrite (14).

Diabetic rat models treated with melatonin showed improvement in their lipid profile and decreased TNF alpha to 50% (15), while removal of the pineal gland caused increased insulin resistance and progression of the disease (16).

Glutamine is a free amino acid, representing about ~60% of free amino acid pool in the body (17). The product of glutamine, glutathione, GSH (composed of glutamic acid, cysteine, and glycine), is considered as an essential antioxidant, being capable of neutralizing oxidative damage. Glutamine lowers the release of proinflammatory factors (18) by decreasing nuclear factor kappa ,NF-κB, (a transcription factor for proinflammatory genes) activation, thus reducing the release of reactive oxygen species (ROS),
which eventually contributes to alleviation of oxidative stress (19).

L-arginine (L-Arg), is considered as a conditionally essential amino acid, which through various metabolic pathways can generate nitric oxide (NO), polyamine and L-proline and contributes to cellular growth regulation (20). NO is generated from L-Arg by the inducible nitric oxide synthase (iNOS) enzyme which is activated with the increase in L-Arg concentration, through the L-Arg-NO pathway (21). Owing to its well known therapeutic qualities, L-Arg has been used in many basic and clinical research settings (22).

The aim of this work is to study the possible role of melatonin, glutamine and L-arginine in prevention of non-alcoholic fatty liver disease in rats induced by high fat, high carbohydrate diet.

**Materials and Methods**

**Aim of the study:**

The present study was designed to study the possible role of melatonin, glutamine and L-arginine in prevention or attenuation of non-alcoholic fatty liver diseases in rats fed high fat, high carbohydrate diet.

**Experimental animals:**

This study was conducted on about 40 adult male albino rats weighing 100-200 grams. Animals were bred and housed in the animal house of Medical Experimental Research Center (MERC), Mansoura University. All experimental protocols were approved by our local ethics committee in May, 2015.

**Experimental design:**

Rats were divided randomly into 5 groups (8 rats each) and for six weeks:

- **Control group (I):** Rats fed on an ordinary standard diet (SD; 80 % carbohydrates, 18 % proteins and 2 % fats) (23).
- **NAFLD group (II):** Rats fed on a high fat, high carbohydrate diet (55 % ordinary chow diet, 15 % beef tallow, 10 % sucrose, 5 % roasted peanuts, 5 % milk powder, 5 % egg, 3 % sesame oil and 2 % NaCl) (23).
- **Melatonin Group (III):** Rats fed on a high fat, high carbohydrate diet + melatonin in a dose of 5 mg/kg/day by intraperitoneal injection (23).
- **Glutamine Group (IV):** Rats fed on a high fat, high carbohydrate diet + glutamine in a dose of 1 gm/kg/day by gastric gavage (18).
- **L-arginine Group (V):** Rats fed on a high fat, high carbohydrate diet + L-arginine in a dose of 1 gm/kg/day by gastric gavage (21).

**Chemical agents**

Melatonin was obtained from Sigma Chemical Co., Egypt in the form of white to off-white powder in a 250 mg package. Glutamine was purchased from EL-Goumhouria Co., Cairo, Egypt in the form of white powder in a 25 gm package. L-arginine was purchased from Beta Co., Egypt in the form of white powder in a 100 gm package.

**Collection of samples:**

After six weeks, sacrifice of rats was done under thiopental anesthesia (30-40 mg/kg I.P injection). Then by cardiac puncture, blood samples were collected from the heart. After collection of the blood in test tubes, blood samples were left for 2 hours at room temperature to clot before being centrifuged at 1000 r.p.m for 20 minutes to obtain
serum samples, which were frozen and stored at -20°C till chemical analysis. Insertion of a needle in the inferior vena cava, after exposure of the abdominal cavity, was performed. In which, we perfused an amount of 5 ml saline into the hepatic circulation, then we dissected a small portion of the liver and preserved it in liquid nitrogen, but after weighing it first, to be used later for determining oxidative stress markers. As for the rest of the liver tissue, it was preserved in 10% formalin later for the histopathology.

Biochemical analysis

Glucose estimation:
Serum glucose concentration was determined by an enzymatic kit (bio-merieu, USA).

Lipid Profile:
a) Determination of Triglyceride Concentration:
Serum triglycerides concentration was determined by Triglycerides GPO−POD Enzymatic colorimetric Kit (Sigma-Aldrich Co. Egypt).

b) Determination of Serum Total Cholesterol
Serum cholesterol was estimated by CHOD-POD Enzymatic Colorimetric Kit (Sigma-Aldrich Co. Egypt).

c) Determination of high density lipoprotein (HDL) and low density lipoprotein (LDL) Concentrations:
Serum cholesterol was estimated by HDL−cholesterol Phosphotungstic Precipitation (Sigma-Aldrich Co. Egypt).

Principle:
Phosphotungstic acid, magnesium ions precipitate low density lipoproteins (LDL−VLDL). Then by centrifugation, HDL-cholesterol concentration is determined in the supernatant.

Calculation of LDL: by the Friedewald formula (24):

(Total cholesterol) minus (TAG/5) minus (HDL-cholesterol) = LDL-cholesterol (mg/dl).

Oxidative stress markers:

Tissue homogenization:
After separation of a small part of the liver, and by means of smooth glass homogenizer (Ultra-Turrax) with a motor driven Teflon pestle, the tissue was homogenized in 0.02 M buffer of sodium phosphate, of PH (7.4), in a ratio of 1:4 wt/vol, then at 3000 rpm at 4 °C, centrifugation was performed for 20 min. The resulting supernatant was used for determining (GSH) and (MDA) concentrations.

Determination of reduced glutathione (GSH) (25):
GSH in the liver tissue was determined using kits purchased from Biodiagnostic, Egypt.

Histopathology:
At the end of this study, we dissected the liver. Serial sections were cut (5 μ) after being placed in 10% neutral buffered formalin, processed by standard procedure for paraffin embedding and
then, these serial sections were stained with hematoxylin and eosin.

**Statistical analysis:**

By usage of the statistical package for social science (SPSS) version 17.0, statistical analysis of our data was achieved. Expression of the data was as Mean ± SD. Data were compared by ANalysis Of VAriance (ANOVA) with post hoc Tukey test, and by means of microsoft®Excel® for windows ®(Microsoft Inc.,USA).

**Results**

The table (1) shows a significant increase in blood glucose level, triglycerides, cholesterol, LDL, AST (aspartate aminotransferase), ALT (alanine aminotransferase) and MDA as well as a significant decrease in HDL and GSH levels in NAFLD induction group. This is in comparison with the control group.

The groups of the three tested substances (melatonin, glutamine and L-arginine) showed a significant improvement in the biochemical parameters, with the exception of L-arginine, which had no significant lowering effect on glucose level, and although HDL levels were increased in glutamine and L-arginine groups but these results hadn’t reached the significance value (table 1).

There was also an improvement (↓ fat infiltration) in the pathological view of the melatonin, glutamine and L-arginine groups (figure 3C, 3D and 3E respectively as compared to the pathological view of NAFLD group (figure 3B).

**Table 1 : Comparison of the biochemical parameters in all studied groups.**

<table>
<thead>
<tr>
<th></th>
<th>I (control)</th>
<th>II (NAFLD)</th>
<th>III (melatonin)</th>
<th>IV (glutamine)</th>
<th>V (L-arginine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>91.53 ± 16.401</td>
<td>173.95 ± 17.796 *</td>
<td>137.50 ± 10.672</td>
<td>133.61 ± 10.134</td>
<td>157.96 ± 6.682</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>76.83 ± 8.588</td>
<td>121.83 ± 9.641 *</td>
<td>89.85 ± 3.588</td>
<td>110.15 ± 5.732</td>
<td>97.91 ± 5.043</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>52.83 ± 6.337</td>
<td>99.83 ± 9.621 *</td>
<td>86.46 ± 3.239</td>
<td>76.95 ± 3.284</td>
<td>66.78 ± 7.080</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>15.30 ± 5.310</td>
<td>57.46 ± 11.234 *</td>
<td>26.38 ± 2.863</td>
<td>35.46 ± 4.069</td>
<td>41.21 ± 6.027</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>22.16 ± 2.926</td>
<td>17.66 ± 2.581 *</td>
<td>23.00 ± 2.190</td>
<td>19.66 ± 1.032</td>
<td>19.00 ± 1.414</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>54.20 ± 3.736</td>
<td>107.91 ± 8.041 *</td>
<td>71.95 ± 3.150</td>
<td>92.85 ± 6.876</td>
<td>62.93 ± 3.271</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>23.81 ± 1.746</td>
<td>83.18 ± 4.475 *</td>
<td>64.43 ± 3.978</td>
<td>72.38 ± 2.428</td>
<td>49.08 ± 3.262</td>
</tr>
</tbody>
</table>

* Significant as regard to control group, # Significant as regard to NAFLD group. (Significance at P ≤ 0.05),
Discussion

NAFLD group showed significant increase in blood glucose level as compared with control group and this can explained by that NAFLD is closely associated with insulin resistance (26), which is presented by hyperinsulinemia and increased hepatic glucose production (27). Physiologically, when insulin binds to its receptors, insulin signal is transmitted through phosphorylation of insulin receptor substrates, which then activates several metabolic pathways as phosphoinositide 3 kinase (PI3K) and protein kinase B (PKB) pathways. Eventually, these events lead to increased glucose uptake through glucose transporters GLUT 4 (28). Our findings were explained by Postic & Girard (28), who illucidated increased lipid metabolites as DAG.
Fig. 3A: Liver specimens showing A) normal liver architecture (Control group), B) severe fatty infiltration (NAFLD group), C) marked improvement with slight fatty infiltration and near normal hepatocytes (melatonin group), D) moderate improvement with mild steatosis (glutamine group) and E) mild improvement (L-arginine group)
interferes with insulin signaling cascade. This happens through inhibition of insulin receptor activity resulting in insulin resistance state. Also, FFA, NF-kB, TNF alpha, which are reported in association with NAFLD, have been found to interfere with insulin signaling pathways (29).

It has been shown by Yamashita et al. (30) that carbohydrate regulatory element binding protein (ChREBP) is the transcription factor that mediates glucose stimulatory action on lipogenesis. Glucose promotes the entry of ChREBP into the nucleus and activates its binding to DNA, which in turn helps its activation (31). ChREBP then activates liver pyruvate kinase (L-PK) enzyme that converts phosphoenol pyruvate into pyruvate, which then generates citrate through entering Krebs cycle. Citrate is the main substrate of acetyl CoA, which is utilized for the synthesis of fatty acids.

The second transcription factor involved in lipogenesis is sterol regulatory element binding protein (SREBP), which is activated by insulin (32). SREBP increases the transcription of all lipogenic genes (33). Even in cases with insulin resistance, it has been found that insulin still activates SREBP, which eventually increases de novo fatty acid synthesis (34). SREBP also stimulates acetyl CoA caboxyylase (ACC) enzyme, which forms malonyl CoA. Malonyl CoA is found to inhibit carnitine palmitoyl transerfase (CPT) enzyme, which transports fatty acids into the mitochondria for oxidation. Thus, it eventually reduces β oxidation and favors triglycerides formation.

Another transcription factor involved in the development of steatosis is PPAR-γ. Its expression has been found to increase markedly in animal models with fatty liver and insulin resistance (35). On the other hand, its deletion genetically has been found to attenuate the steatosis despite of the concomitant hyperglycemia and hyperinsulinemia (36).

AMP activated protein kinase (AMPK) is a sensor of cellular energy levels, activated by increased cellular AMP levels, which reflects a reduction in energy stores in the cell. Activated AMPK stimulates pathways to increase ATP production, such as fatty acid β oxidation. Also it inhibits processes which consume ATP, such as lipogenesis. It acts directly through phosphorylation of regulatory proteins and indirectly through regulation of gene expression in these pathways (37). By alteration of AMPK activity, triglycerides accumulation in the liver could be influenced.

The increase in liver enzymes (AST, ALT) and parameters of oxidative stress (↓GSH, ↑ MDA) in the group of NAFLD as compared with control group could be explained by:

Oxidative stress is thought to be the key factor in the pathogenesis of NAFLD through mitochondrial dysfunction. It impairs fat homeostasis in the liver and increases the production of reactive oxygen species as well, which induce hepatocellular injury (38). Moreover, Oxidative stress has a damaging effect at the cellular level (as membrane lipid peroxidation, cellular degeneration and apoptosis. It enhances the expression of proinflammatory cytokines and activates hepatic stellate cells, which induce fibrosis (39).

Mitochondrial β oxidation is the dominant oxidative pathway for the disposition of fatty acids under normal physiologic conditions, but can also be a major source of ROS (40).
Reduced glutathione (GSH), being one of the most important antioxidants, its depletion plays an important part in the context of NAFLD. The depletion could be due to; decreased uptake by the mitochondria because of increased cholesterol within mitochondrial membrane (41), decreased synthesis of S-adenosyl methionine, the precursor of GSH (42), or diminished expression of some GSH related enzymes (43).

By using melatonin for six weeks in a dose of 5mg/kg/day in group (III), there was a significant decrease in blood glucose level agreed with Hussain et al. (44). Also, as evidenced in multiple animal models such as Ha et al. (17), who found that melatonin, through insulin receptor substrate 1-phosphatidylinositol 3 pathway, stimulates glucose transport, which may explain its hypoglycemic effect. More evidence on the relationship between melatonin and glucose homeostasis was presented by: two epidemiological studies conducted by McMullan et al. (45), But our findings were in contrast to Greico et al. (46), who found no significant decrease in blood glucose level in type 2 diabetic patients receiving melatonin, although it improved their glycemic control (HbA1c).

Melatonin produced significant reduction in triglycerides, total cholesterol and LDL in comparison with NAFLD group (II), HDL was increased significantly in melatonin group (III) as compared to NAFLD group (II). These results agreed with the findings of Goyal et al. (47).

The hypolipidemic effect of melatonin is related probably to activation of (AMPK), as suggested by Rui et al. (48) in their model of alcoholic fatty liver disease. It may also be explained by its role in prevention of (lipopolysaccharide) LPS-induced hepatic steatosis, by lowering the activation of sterol regulatory element-binding protein (SREBP) induced by LPS and expression of SREBP target genes (49).

There was a significant decrease in liver enzymes (AST, ALT) level in melatonin group (III) as compared to NAFLD group (II). GSH was increased significantly and MDA was decreased significantly in melatonin group (III) as compared to NAFLD group (II). These results agreed with Hatzis et al. (23), and Greico et al. (46). The biochemical findings were supported by the improvement in the pathological view of liver specimens of this group as compared with NAFLD group.

These findings could be explained by the antioxidative properties of melatonin, directly as a free radical scavenger, and indirectly through increasing antioxidant defense mechanisms (50) through activation of antioxidant enzymes (51) (as superoxide dismutase (SOD) and glutathione peroxidase), and regulation of the transcription of their genes (52). It increases the synthesis of glutathione (53), helps the activity of other antioxidants (54), and protects the antioxidative enzymes from damage by oxidative stress (55). It lowers the production of free radicals and electron leakage in the respiratory chain of the mitochondria (56). Also it inhibits lipid peroxidation (57), along with inhibition of (nuclear factor kappa) NF-κB, a vital protein in the process of inflammation, thus playing a role in overcoming inflammation (58). Also, Zhang et al. (59) discussed the contribution of melatonin in cellular function preservation and protection against apoptosis by oxidative stress, which may explain
the reduction in liver enzymes in our study as compared to NAFLD group.

When we used glutamine in group (IV), blood glucose level was significantly decreased as compared to NAFLD group (II) agreed with the findings of Mansour et al. (60).

Reimann et al. (61), in their in vitro study, concluded a possible mechanism of the hypoglycemic effect of glutamine through glucagon like peptide (GLP-1) secretion, which may stimulate insulin secretion. Another in vivo study supported this explanation conducted by Green Field et al. (62), which showed that glutamine caused an increase in the circulating level of GLP-1 in human subjects in a dose dependent manner. However, Samocha-Bonet et al. (63) in their study ruled out this insulin secretagogue mechanism and suggested that the hypoglycemic effect of glutamine may be attributed to its gastric emptying slowing effect through GLP-1. Modi et al. (64) discussed the role of glutamine in the adaptive response of beta cells of the pancreas to insulin resistance states, as glutamine increases the secretion of insulin growth factor 2 (IGF2). Also glutamine decreases NF-kB expression (18), which is thought to interfere with insulin signaling pathway (65).

There was a significant decrease in triglycerides, cholesterol and LDL in glutamine group (IV) as compared to NAFLD group (II). These decreasing effects agreed with da Rosa et al. (66) findings, which reported a recovery in lipid profile parameters after glutamine supplementation. Dechelolte et al. (67) had found that glutamine blocks peripheral lipolysis and reduces hepatic uptake of fatty acids. But our results were in contrast to Mansour et al. (60), who found no hypolipidemic effects of glutamine. On the other hand, there was no significant change in HDL level in glutamine group (IV) as compared to NAFLD group (II) agreed with Mansour et al. (60) and in contrast to da Rosa et al. (66).

There was a significant decrease in liver enzymes (AST, ALT) levels in glutamine group (IV) as compared to NAFLD group (II). This agreed with; Hartmann et al. (68) in their study about the effect of glutamine on liver injury caused by ischemia reperfusion in rat’s intestines and with da Rosa et al. (66) in their model of type 2 diabetic rats. Also, GSH was increased significantly and MDA was decreased significantly in glutamine group (IV) as compared to NAFLD group (II). These results were in agreement with Lin et al. (18).

These previous findings could be explained by the anti-inflammatory and antioxidant properties of glutamine. The product of glutamine (GSH) is considered as an essential antioxidant, being capable of neutralizing oxidative damage. Glutamine itself lowers the release of proinflammatory factors as TNF alpha (65) by decreasing NF-κB activation. Also it stimulates (HSP), a protein group important for survival of cells under different stresses (69), thus reducing the release of ROS. This eventually contributes to alleviation of oxidative stress (18). Therefore, glutamine is involved in maintaining a stable environment and protects the organs from deleterious effects of oxidative stress. These results were supported by the improvement in the pathological view of this group.

By using L-arginine, there was no significant change in blood glucose level agreed with Jablecka et al. (70), But in contrast to Alam et al. (71), El Missiry et al. (72), in alloxan induced
Melatonin, glutamine & L-arginine in fatty liver

Diabetic rats, noticed hypoglycemic effects of L-arginine. These previous studies attributed the hypoglycemic effect of L-arginine to its insulin secretagogue effect besides, L-arginine role in promoting glucose oxidation and limiting denovo glucose synthesis. These conflicting results may be related to different doses, method or duration of administration of L-arginine.

On the other hand, there was a significant decrease in triglycerides, cholesterol and LDL levels in L-arginine group (V) as compared to NAFLD group (II) agreed with El Missiry et al. (72), Alam et al. (71) and Suliburska et al. (73) who discussed L-arginine’s beneficial effect on dyslipidemia. Gergel et al. (74) explained the hypolipidemic effect of L-arginine possibly by stimulating fatty acid oxidation to supply energy needed for protein synthesis in rabbits, also NO (L-Arg product) has been found to block peripheral lipolysis (75). As regard HDL, there was an increase in its level in L-arginine group (v) as compared to NAFLD (II) but this change was not statistically significant.

There was a significant decrease in liver enzymes (AST, ALT) level in L-arginine group (V) as compared to NAFLD group (II) agreed with Alam et al. (71). GSH was increased significantly and MDA was decreased significantly in L-arginine group (V) as compared to NAFLD group (II) agreed with Suliburska et al. (73) study. They discussed the anti-inflammatory and antioxidant properties of L-arginine in rats fed high fat diet, which could be due to the effect of L-arginine on lowering reactive oxygen species, scavenging superoxide anion by NO action and also inducing antioxidant enzymes in the body. NO was also found to inhibit the activity of cytochrome P4502E1 enzyme, which is involved in ROS production and lipid peroxidation, thus it eventually leads to combating oxidative stress (74). Also our results agreed with the findings of Alam et al. (71) and El Missiry et al. (72), who reported in their studies the beneficial effect of L-arginine in reducing oxidative stress.

The anti-inflammatory effect of L-arginine was recently investigated in an in vitro study on intestinal cells conducted by Meng et al. (76). They concluded that L-arginine, through NO production, inhibits interleukin 1 beta (IL-1B) mediated nuclear factor kappa activation, which results in down regulation of proinflammatory cytokines as TNF alpha. In agreement with these results, there was a degree of improvement in the pathological view of this group as compared to the NAFLD group.

In conclusion, each of melatonin, glutamine and L-arginine (to a lesser extent) are effective in prevention or attenuation of NAFLD in rat model fed high fat, high carbohydrate diet. Further studies in humans are required to support our results about the promising role of these three substances in NAFLD.

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