INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), most common chronic hepatic pathology, that occurs in the developed countries is estimated at 1/3 of the population. Amongst the numerous pathogenetic factors, oxidative stress and apoptosis of hepatocytes initiate many inflammatory processes and are involved in the progression of disease, particularly in transformation of non-alcoholic steatohepatitis (NASH) to cirrhosis. The aim of our study was to determine the effects of tryptophan and melatonin on the selected biochemical parameters in patients with NAFLD, and additionally, to evaluate the effects of tryptophan and melatonin in improvement of liver tissue in selected NAFLD patients. Seventy four patients with NAFLD confirmed by histopathological examination of liver biopsy samples, were admitted to the study. They were randomly assigned to three groups. Group I received the preparation Essentiale forte in the dose of 3 × 1 tablet per day and tryptophan 2 × 500 mg/day over the period of 14 months, group II received Essentiale forte and melatonin 2 × 5 mg/day over 14 months and group III received only Essentiale over the period of 14 months. In nine patients of groups I, II, and III, the liver biopsy was performed after 14-months of treatment period. Out of nine patients whom biopsy was performed, three of them were from group I, four from group II and two of them were from group III, respectively. After the 14-month treatment period, gamma-glutamyl transferase (GGPT) activity and levels of triglycerides and LDL-cholesterol were found to be significantly reduced in group I and II. The level of melatonin after the therapy was significantly elevated in group I and II and did not change in group III. Statistically significantly lower levels of IL-1, IL-6 and TNF-α were observed in patients receiving melatonin and tryptophan, comparing with group III treated with Essentiale forte only. These study findings demonstrate that melatonin and tryptophan substantially reduce the levels of pro-inflammatory cytokines and improve some parameters of fat metabolism in patients with NAFLD. In few patients with NASH melatonin and tryptophan reduced the inflammation in liver. We conclude that melatonin is worth considering for the therapy of NAFLD, particularly in patients with impaired fat metabolism accompanied by hypertriglyceridemia and hyper-LDL cholesterolemia.

Key words: liver, non-alcoholic fatty liver disease, melatonin, tryptophan, proinflammatory cytokines, triglycerides
pharmacological treatment have not been established. Some new effective treatment methods for NAFLD are still being searched for, including those that could effectively reduce both, the oxidative stress, apoptosis of hepatocytes and finally inhibiting progression of steatosis into fibrosis.

Melatonin (N-acetyl-5-methoxytryptamine) produced by the pineal gland is considered an endogenous substance exerting the antioxidative, anti-inflammatory and anti-apoptotic activity (15, 16). Melatonin is a scavenger of reactive oxygen metabolites (17); therefore, its use in NAFLD could be recommended, considering the pathogenetic mechanisms involved in the development of NAFLD, especially NASH. Melatonin is produced from its precursor L-tryptophan in human body. L-tryptophan cannot be synthesized by the organism, and therefore received cholesterol-lowering agents before and during the study.

Procedures

On inclusion, the following biochemical parameters were determined in all patients: alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGTP), bilirubin, lipid profile (total cholesterol, LDL and HDL, cholesterol, triglycerides), IL-1, IL-6 cytokines, TNF-α and melatonin.

For routine laboratory assays standard automated techniques were used (Alab Sp. z o.o. Lublin, Poland). Cytokines and TNF-α were examined by ELISA test (Cytokine Immunoassays, R&D System, USA). Plasma melatonin concentration was determined using RIA-technique as described in details previously (21). Blood samples were collected in EDTA-coated polypropylene tubes and centrifugated at 1700 g for 20 minutes at 4°C. Than plasma was stored at –60°C. Melatonin was determined using Human MT RIA kit (DRG Diagnostics GmbH, Marburg, Germany).

The patients were randomly assigned to three groups. The characteristics of patients are presented in Table 1. Group I received 300 mg of phospholipids, 3 times a day - glycerol esters of cholinephosphoric acid and unsaturated fatty acids (linolic, linoleic, oleic) in the form of preparation Essentiale (Rhone-Poulenc Rorer GmBH, Germany) in the dose of 3 × 1 tablet/day and Tryptophan (Ardeydorm, Ardeypharm, Germany) 2 × 500 mg/day over the period of 14 months. Group II received 300 mg of phospholipids, 3 times a day - glycerol esters of cholinephosphoric acid and unsaturated fatty acids (linolic, linoleic, oleic) in the form of Essentiale (Rhone-Poulenc Rorer GmBH, Germany) and Melatonin (Lekam, Zakroczym, Poland) 2 × 5 mg/day over 14 months. Group III received 300 mg of phospholipids, 3 times a day - glycerol esters of cholinephosphoric acid and unsaturated fatty acids (linolic, linoleic, oleic) in the form of Essentiale (Rhone-Poulenc Rorer GmBH, Germany) done over the period of 14 months.

Table 1. Characteristics of patients and study groups: I (tryptophan), II (melatonin), III (placebo).

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>28</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>F/M (n)</td>
<td>25/3</td>
<td>15/8</td>
<td>11/12</td>
</tr>
<tr>
<td>Mean age</td>
<td>34.2 ± 7.56</td>
<td>36.16 ± 5.77</td>
<td>29.33 ± 9.58</td>
</tr>
<tr>
<td>Simple hepatic steatosis /NASH (n)</td>
<td>22/6</td>
<td>17/6</td>
<td>17/6</td>
</tr>
<tr>
<td>Diabetes with insulin therapy (n)</td>
<td>5</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Diabetes treated with oral drugs (n)</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Glucose intolerance</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Hypercholesterolemia (n)</td>
<td>11</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Hypertriglyceridemia (n)</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Elevated LDL cholesterol (n)</td>
<td>6</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

n=number of patients
None of the patients enrolled for the study was treated with any preparations containing antioxidative compounds, vitamins or diet supplements before and during the study. After 14 months, the above-mentioned biochemical parameters were re-determined in each group of patients.

After 14 months treatment period the liver biopsy which was optional was performed in patients who agreed for this procedure. Percutaneous liver biopsy was performed by Tru-Cut automatic needle 16 G (COOK Quick-Core® Biopsy-Needle, Cook Medical Inc., USA) with specimen size obtained at least 2 cm long with presence of no fewer than 11 complete portal tracts. Liver biopsy specimens were fixed in 10% neutral buffered formalin and were processed to paraffin blocks. Four µm thick slides were cut on the microtome and stained with haematoxylin and eosin (H+E), Masson Trichrome, silver stain and PAS. Slides were then examined by one pathologist under the light microscope OLYMPUS CX 45.

All patients gave their informed written consent to be recruited for the study.

**Statistical analysis**

All results were expressed as a mean ± standard deviation. Numeric data were analyzed by the t-student test. Statistical significance was assumed at p<0.05. All calculations were performed using STATISTICA PL software.

**RESULTS**

No side effects of melatonin and tryptophan were observed; for instance none of patients complained on excessive sleepiness and/or dizzines.

As shown in Table 2 after the 14-month treatment period, GGPT activity and the plasma levels of triglycerides and LDL-cholesterol were significantly reduced in group I and II. The remaining lipogand parameters did not differ between these groups. The level of melatonin after the therapy with melatonin and tryptophan was significantly elevated in group I and II.

### Table 2. Comparison of plasma levels of liver enzymes and biochemical metabolic parameters in the study groups I, II and III after 14 months of treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline level</td>
<td>After 14 months of treatment</td>
<td>Baseline level</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>84±21</td>
<td>81±23</td>
<td>NS</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>64±29</td>
<td>71±15</td>
<td>NS</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>124±37</td>
<td>131±42</td>
<td>NS</td>
</tr>
<tr>
<td>GGTP (U/L)</td>
<td>193±50</td>
<td>149±41</td>
<td>0.005</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.8±0.02</td>
<td>0.8±0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>211±42</td>
<td>242±71</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>298±39</td>
<td>232±62</td>
<td>0.0008</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>195±57</td>
<td>156±45</td>
<td>0.004</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>48±17</td>
<td>51±21</td>
<td>NS</td>
</tr>
</tbody>
</table>

The values are given as mean ± standard deviation. NS- differences not statistically significant. Asterisk (*) indicates statistically significant (p<0.05) decrease in plasma levels of GGTP, triglycerides and LDL-cholesterol measured after 14 month of treatment with tryptophan or melatonin compared to the baseline level (recorded at the start of the study).

**Table 3. Comparison of plasma levels of pro-inflammatory cytokines and melatonin in the study groups I, II and III after 14 months of treatment.**

<table>
<thead>
<tr>
<th>Cytokines &amp; Melatonin</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At the start of the study</td>
<td>After 14 months of treatment</td>
<td>At the start of the study</td>
</tr>
<tr>
<td>IL-1 (pg/mL)</td>
<td>11.1±2.9</td>
<td>6.2±2.3</td>
<td>9.02±1.23</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>27.17±0.67</td>
<td>13.23±5.6</td>
<td>23.41±6.38</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>2.08±0.72</td>
<td>1.24±0.67</td>
<td>2.56±0.43</td>
</tr>
<tr>
<td>Melatonin (pg/mL)</td>
<td>21.7±5.8</td>
<td>58.71±6.81</td>
<td>23.96±4.93</td>
</tr>
</tbody>
</table>

The values are given as mean ± standard deviation. Asterisk (*) indicates statistically significant decrease (p<0.05) below the values recorded at the start of the treatment. Cross (+) indicates statistically significant (p<0.05) increase above the values recorded at the start of the treatment.
compared with that recorded in group III. Moreover, statistically significantly lower plasma levels of IL-1, IL-6 and TNF-α were observed in patients receiving melatonin and tryptophan after 14-months therapy (Table 3). The remaining biochemical parameters did not show significant differences after the 14-month treatment period compared with respective values observed at the initiation of the study.

In group III receiving Essentiale, the differences in all the biochemical parameters studied were not statistically significant at the start of study and at follow up 14 months later.

In the nine patients of all groups the liver biopsy was performed after the end of 14-months treatment period. Three of the patients were from group I, 4 out of 9 from group II and remaining 2 were from group III, respectively.

In patients from group I and II the histological examination of liver showed NASH, (Figs. 1 and 2) After treatment in all of them, the features of steatosis were observed (Figs. 3 and 4). NASH was not found in any of them. Results of liver biopsy were not changed in group III. Figs. 1-4 present microscopic examples of NASH. Macrovesicular steatosis and lobular inflammation are most prominent on Fig. 1, while Fig. 2 shows scattered fatty change in hepatocytes associated with ballooning of hepatocytes and focal lobular inflammation.

Oedema within the cytoplasm of hepatocytes forming ballooning change are also well seen on the Fig. 2 and Fig. 4. The first one shows also foci of lobular inflammation. Minor microscopic changes are shown on Fig. 3. Fig. 4 shows also some microvesicular steatosis.

**Fig. 1.** Liver biopsy. NASH (non-alcoholic steatohepatitis). Macrovesicular steatosis, ballooned hepatocytes, focal intralobular inflammation and scattered necrotic hepatocytes. H+E × 400.

**Fig. 2.** Liver biopsy NASH (non-alcoholic steatohepatitis). Macrovesicular steatosis, ballooned hepatocytes, focal intralobular inflammation and scattered necrotic hepatocytes. Single apoptotic hepatocyte present. H+E × 400.
DISCUSSION

Our present study which follows up of short term observation published before (18), demonstrated the decreased plasma levels of GGTP, triglycerides and LDL cholesterol after the 14-month therapy with melatonin and its precursor tryptophan. Moreover, the plasma levels of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α, were found to be attenuated in patients treated with melatonin and tryptophan. The level of total cholesterol, HDL-cholesterol and activity of aminotransferases, ALT, AST and ALP and GGTP did not show statistically significant differences after 14 months of treatment with melatonin or tryptophan.

The literature data on this issue are scarce. The similar research was conducted by Gonciarz et al., yet their observation period was shorter, i.e. 4, 8, 12 and 24 weeks of treatment with melatonin (21, 22). According to their study, the mean levels of aminotransferases ALT and AST after the 24-week treatment with melatonin were 42% and 33%, respectively (p<0.05), compared with the baseline levels in patients with NAFLD (22). These findings differ from our results, as our study did not show statistically significant differences in the activity of aminotransferases throughout the period of treatment with melatonin. As far as triglycerides are concerned, our data reported reduced levels of triglycerides whereas the results published by Gonciarz et al. (22) showed no changes in their levels. This discrepancy is likely to result from the longer period of treatment with melatonin in our study, namely 14 months versus 24 weeks, respectively. On the other hand, the results concerning GGTP activity (22) are comparable in both studies. Both, our results and those reported by Gonciarz et al.

![Fig. 3. Liver biopsy. NASH (non-alcoholic steatohepatitis). Focal macrovesicular steatosis. Liver cells show mild oedema of their cytoplasm and glycogenated nuclei. H+E × 400.](image3)

![Fig. 4. Liver biopsy. NASH (non-alcoholic steatohepatitis). Focal macrovesicular steatosis. Liver cells show mild edema of their cytoplasm and enlarged nuclei. Very focal mononuclear inflammatory cells present within the lobule. H+E × 400.](image4)
demonstrated reduced activity of GGTP in patients who underwent the therapy with melatonin and tryptophan (22).

GGTP was considered as surrogate marker of NAFLD and NASH and is positively associated with cardiovascular events (23). Some researches believe that triglycerides can play the same role. Cardiovascular disease is increased in NAFLD and represents the main cause of death in these patients (24). It is found that NAFLD is a predictor of cardiovascular events (25). Longitudinal increase of GGTP level is observed in patients with cardiovascular disease and NAFLD (25). The oxidative stress and the dysfunction of mitochondria are linked with disorders in β-oxidation of free fatty acids. According to the literature data, melatonin can improve these processes and restore the proper balance in mitochondrial metabolism. Therefore, the decrease of GGTP and triglycerides levels observed after long term treatment with melatonin and tryptophan can reflect the beneficial effect of this indoleamine and its precursor on the elements of metabolic syndrome and particularly cardiovascular events (23-25).

Melatonin and its precursor tryptophan could be useful in NAFLD because melatonin is an antioxidant agent and the oxidative stress is considered as one of the factors involved in the pathogenesis of NAFLD, especially NASH (9-11, 26, 27). Recent studies showed promising results e.g. vitamin C, E and betadine in prevention of NASH (28-31).

Some animal studies revealed the hypocholesterolemic and lipid peroxidation inhibitory effect of melatonin (32). The serum levels of cholesterol and triglycerides were reduced in mice with experimental diet-induced hypercholesterolemia in mice (33). This hypocholesterolemic effect of melatonin may be associated with enhanced catabolism of cholesterol to bile acids, inhibition of cholesterol synthesis and activity of LDL receptors as well as direct effects on the adipose tissue exerted via the specific MT1 and MT2 receptors (34, 35). It is worth to underline that LDL can support the atherogenic effect of NAFLD. According to Pan et al., (36) the effect of melatonin on liver lipometabolism in rats was dose-dependent. Melatonin in the doses of 5 and 10 mg/kg reduced high cholesterol levels in rats fed with high-fat diet whereas only the high dose, i.e. 10 mg/kg, additionally decreased the liver triglyceride content (36). This dose reduced hepatic steatosis, serum levels of ALT, AST and oxidative stress parameters. The protective effect of melatonin in hepatic steatosis in rats could be attributed to its antioxidative action. In the other animal study melatonin alone or administered in combination with pioglitazone or pentoxifylline reduced the insulin resistance index, total cholesterol and triglycerides, activities of liver enzymes and the increased hepatic reduced glutathione level in rats with NAFLD. Data in this study indicate that melatonin can be used as promising adjunct therapy in the clinical management of NAFLD (37).

According the available knowledge regarding the pathogenesis of NAFLD, melatonin is considered as antiinflammatory factor. Our findings demonstrated reduced levels of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α after the use of melatonin and tryptophan in patients with NAFLD. TNF-α is considered an important pro-inflammatory cytokine produced predominantly by the immune cells of the liver in subjects with NASH. IL-6, a multifunctional cytokine promotes insulin resistance (38). These cytokines are involved in the transformation of hepatic stellate cells (HSCs) into myofibroblasts, which contribute to the progression of liver fibrosis. Serum levels of IL-6 in patients with NASH is associated with liver fibrosis (39). These data suggest that cytokines may play an important role in liver fibrosis in NAFLD patients, and the inhibition of proinflammatory cytokines appears as a primary target for the treatment of liver fibrosis.

The role of oxidative and nitrosative stress in the pathogenesis of NAFLD has been implicated (14, 40). Furthermore, the involvement of increased levels of TNF-α and of cytokines in induction of oxidative and nitrosative stress in obese patients was emphasized. Obesity may lead to impaired mitochondrial respiratory chain (MRC) activity and dysfunction of mitochondria (41-43). The protective impact of melatonin on MRC activity was demonstrated in in vitro and in vivo studies (44, 45).

Likewise, we confirmed that melatonin improved the metabolic parameters of NAFLD and attenuated the plasma levels of TNF-α and other proinflammatory cytokines IL-1, IL-6, which indicates the antioxidant and anti-inflammatory properties of melatonin in patients with NAFLD. Our present study confirms the original data of Gonciarz et al. (22) who demonstrated the beneficial effect of 12-24 weeks course of melatonin on the liver enzymes, namely AST and GGT in NASH patients. In their study (22), amelioration of liver enzymes was accompanied by the 6-7 folds increase in plasma melatonin levels at 24th week of treatment with melatonin. It is of interest that plasma cholesterol, triglycerides and glucose concentrations as well as plasma alkaline phosphatase were unchanged as compared to control group plasma levels during this prolonged study period (22).

In another study, Grigorescu et al., (46) proposed the non-invasive biochemical markers that might be useful in distinguishing between NASH and simple steatosis. According to this analysis (46) the novel pathophysiological-based panel of biomarkers combining total cytokerin-18 (M65 antigen), IL-6 and adiponectin could efficiently predict NASH, being useful in differentiation between NASH and simple steatosis. This is of great importance since the incidence of NAFLD has increased in recent years and in some patients an unexpected evolution starting from fatty liver towards cirrhosis and hepatocellular carcinoma (HCC) has been observed (47).

For the first time we observed that melatonin and tryptophan reduced inflammation in liver tissue in patients with NASH who underwent the liver biopsy, since there is lack of a similar evidence in the literature. Undoubtedly, a certain limitation of the present study is the lack of histopathological evaluation of the liver after 14 months of melatonin and tryptophan use in all examined patients. To assess in detail the beneficial effect of melatonin in the development of NAFLD, the results of morphological parameters in liver biopsies determined in large cohort of patients would be helpful, but these studies await further research.

Our study demonstrates that melatonin and tryptophan substantially attenuate the levels of pro-inflammatory cytokines and improve some parameters of fat metabolism in patients with NAFLD. Thus, melatonin seems to be worth considering for the therapy of NAFLD, particularly in patients with impaired fat metabolism accompanied by hypertriglyceridemia and hyper-LDL cholesterolemia. The mechanism of this beneficial effect of melatonin and tryptophan in NASH patients may depend on amelioration of the inflammation in liver and antioxidative properties of this indoleamine.

Conflict of interests: None declared.

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