Original paper

Green tea extract prevents the development of nonalcoholic liver steatosis in rats fed a high-fat diet

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Green tea contains many polyphenolic constitutes, which might prevent non-alcoholic fatty liver disease (NAFLD). We aimed to investigate whether green tea extract (GTE) given at doses reflecting habitual consumption of green tea beverages prevents development of NAFLD in rats fed a high-fat diet (HFD).
Twenty-four male Wistar rats were randomly divided into four equal groups (two study and two control groups). The study groups received a HFD (approximately 50% energy from fat), enriched with 1.1% and 2.0% GTE, respectively, for a total of 56 days. The control groups were fed a HFD alone and normal standardised diet (low-fat diet), respectively, for the same period of time.
The percentage of hepatocytes affected by steatosis in the HFD group (median [1st-3rd quartile]: 25% [12-34%]) was higher ($p < 0.033$ and $p < 0.050$, respectively) than in the HFD-2.0%GTE group (9% [3-18%]) and normal diet group (10% [5-18%]). No significant differences were observed for the group consuming HFD-1.1%GTE, in which intermediate results were observed (15% [4-30%]).
This finding points towards the hepatoprotective potential of GTE in preventing dietary-induced liver steatosis. In view of the increasing incidence of overweight and obesity a simple and cheap dietary modification, such as GTE supplementation, could prove to be useful clinically.

Key words: catechins, epigallocatechin-3-gallate, EGCG, obesity, fatty liver.

Introduction

Obesity is one of the most widespread chronic diseases in the world. In the United States of America about one-third of the adult population is suffering from obesity [1]. The main cause of this disease is the Western diet, which is high in fat, especially animal fat [1, 2, 3]. Because of these disturbances excess fat mass is formed [4]. There is a statistically proven-connection between obesity, type 2 diabetes, cardiovascular diseases, and hypertension, which all constitute metabolic syndrome. Metabolic syndrome is often characterised by chronic inflammation and hepatic steatosis, which leads to non-alcoholic fatty

liver disease (NAFLD). NAFLD develops from a relatively mild form of steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [5]. NAFLD is the most common chronic liver disease in developed countries. The estimated prevalence of NAFLD ranges from 10% to 25% [6]. Until now, there has been no effective treatment of NAFLD. The only way to deal with this disease is weight loss and treatment of comorbid conditions [6]. Thus, dietary strategies that prevent the development of liver steatosis or its progression to NASH are critically needed [7]. One of them is drinking green tea (GT). Green tea is made from unfermented dried leaves of the plant *Camellia sinensis*, so its chemical composition is different than in black or oolong tea, in which the tea leaves are allowed to oxidise [8]. Green tea is widely consumed in many regions of the world, with the majority consuming 5-8 cups of green tea beverage per day [9]. Moreover, in was proven that drinking at least five cups of GT per day may have a prophylactic effect on cardiovascular diseases [10]. Green tea contains caffeine and polyphenols commonly known as catechins. The most popular catechin found in GT is (-)-epigallocatechin-3-gallate (EGCG). Other catechins are epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), and catechin [11] (Fig. 1). Experimental data suggest that GT and its catechins play a significant role in protecting against NAFLD by regulating energy homeostasis and lowering oxidative stress and inflammatory response [12]. However, the majority of presented studies (including controlled human trials) have been conducted to evaluate the effect of green tea supplementation on metabolic outcomes in patients with existing NAFLD [13]. Moreover, in some studies the protective effect of green tea or its constitutes (EGCG) against NAFLD was studied with use of relative high doses of green tea extract (Bose, Kasu, Nakamoto), which did not reflect habitual consumption of this beverage. Therefore, in our study we decided to test the hypothesis that supplementation of green tea extract (GTE) reflecting the usual consumption of green tea beverage (5-8 cups/day) may protect against development of non-alcoholic fatty liver in rats fed a high-fat diet (HF).

Material and methods

High-fat diet enriched with green tea extract GTE – animal study

Twenty-four 11-week-old male Wistar rats with initial body weights ranging from 266 g to 292 g were obtained from the Department of Toxicology of Poznan University of Medical Sciences, Poland. The animals were kept in individual cages in a temperature-controlled room at 22 ±2°C, with 55% to 60% humidity and a 12-h dark/12-h light cycle. The rats were fed a chow diet (Labofeed B) purchased from Labofeed (Feed Production Plant, Kcynia near Bydgoszcz, Poland) and with tap water *ad libitum*

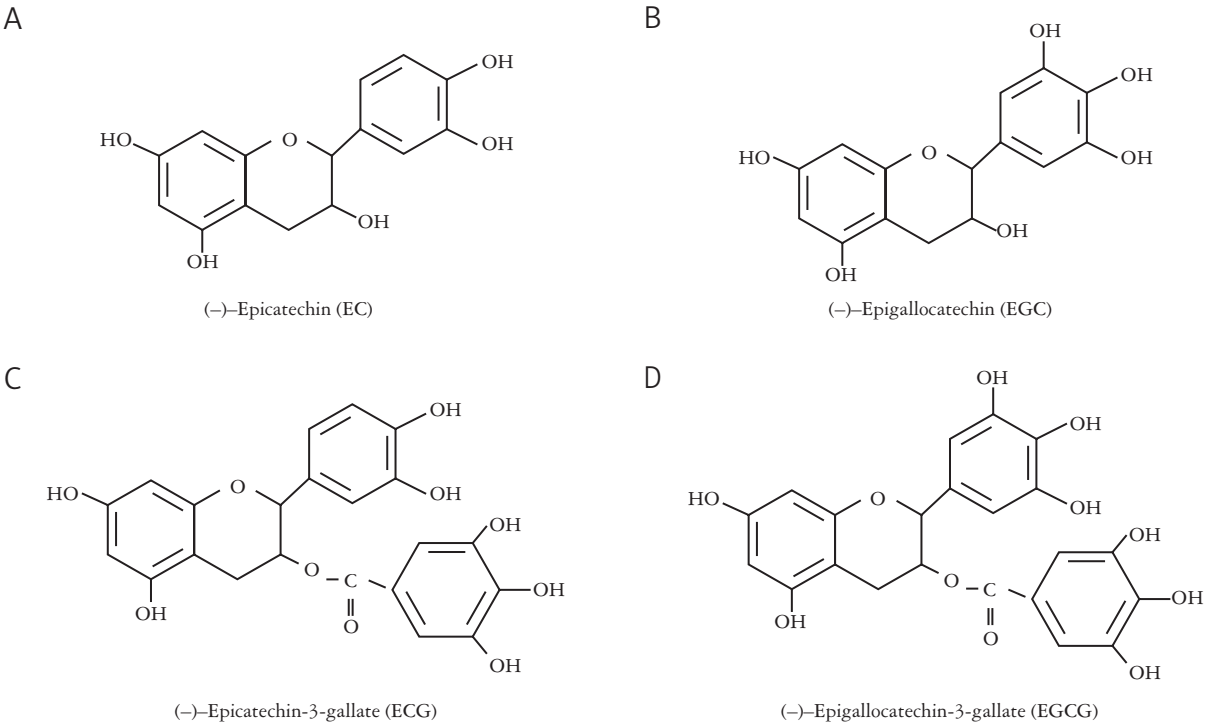

Fig. 1. The structure of green tea catechins

for one week before the experiment. All animals were treated in accordance with the World Health Organisation guidelines for animal care. The design of the animal study was approved by the Local Bioethical Committee on Animal Research at the Department of Animal Physiology and Biochemistry, Poznan University of Life Sciences (July 2008). After seven days of adaptation to laboratory conditions, the experimental animals were randomly divided into four groups of six animals each (two study groups and two control groups). There was no difference in body weight among the groups at the beginning of the experiment. The study groups received a HF diet (modified AIN-93G diet with added lard and sunflower oil, 30 g fat/100 g diet), enriched with 1.1% and 2.0% GTE, respectively, for a total of 56 days. The control groups were fed a HF diet alone and normal standardised diet (low-fat diet – AIN-93G), respectively, for the same period of time. The composition of the animals' diet is described in Table I.

Each ingredient in the diet was weighed (within ±0.5% of the amount required) and mixed. The mixture was then formed into equally sized granulate and placed in a temperature and humidity-controlled room to remove moisture. The rats received water and pellets *ad libitum*.

The food consumption (of individual rats) was calculated each day from the difference between the daily amount of food supplied and the amount of unconsumed food. The rats' body weights were measured every seven days using electronic scales.

At the end of the experiment (day 56), and after 16 hours of starvation, the animals were weighed and euthanised by intraperitoneal lethal dose injection of thiopental (40 mg/kg body weight).

Table I. Composition of experimental diets

Macronutrient composition	HF	HF GTE 1.1%	HF GTE 2.0%
Protein (% of energy)	15.3	15.4	15.5
Carbohydrate (% of energy)	34.0	33.4	33.0
Fat (% of energy)	50.8	51.2	51.5
Energy (MJ/100 g diet)	22.3	22.1	21.9
Diet composition [%]			
Casein	20.0	20.0	20.0
Sucrose	10.0	10.0	10.0
Sunflower oil	10.0	10.0	10.0
Lard	20.0	20.0	20.0
Potato starch	5.0	5.0	5.0
Wheat starch	30.2	29.1	28.2
AIN 93 Vitamin mix	1.0	1.0	1.0
AIN 93 Mineral mix	3.5	3.5	3.5
L-cystine	0.3	0.3	0.3
Aqueous green tea extracts	0	1.1	2.0

HF – high fat diet, HF GTE 1.1% – high fat diet enriched with 1.1% of green tea extracts, HF GTE 2.0% – high fat diet enriched with 2.0% of green tea extracts

Table II. Composition of green tea extract (GTE) used in animal diet (kg)

GTE components per kg of diet	HF GTE 1.1%	HF GTE 2.0%
Total polyphenols (mg of catechin equivalent)	5812.4	10568.0
(-)-EGCG (mg)	767.8	1396.0
(-)-EGC (mg)	454.4	826.3
(-)-ECG (mg)	199.1	362.1
(-)-EC (mg)	78.5	142.6
(-)-GC (mg)	52.0	94.6
(-)-CG (mg)	46.1	83.8
(-)-C (mg)	37.3	67.7
(-)-GCG (mg)	4.6	8.4
Caffeine (mg)	407.6	741.1

HF GTE 1.1% – high fat diet enriched with 1.1% of green tea extracts, HF GTE 2.0% – high fat diet enriched with 2.0% of green tea extracts, ECGG – (-)-epigallocatechin-3-gallate, EGC – (-)-epigallocatechin, ECG – (-)-epicatechin gallate, EC – (-)-epicatechin, GC – (-)-gallocatechin, CG – (-)-catechin gallate, C – (-)-catechin, GCG – (-)-gallocatechin gallate

Preparation of GTE

The GTE was prepared according to the method presented by Gramza and Regula [14] with the minor modification [15] from Japanese Sencha Fukuju Green Tea, which was available commercially (House of Tea).

The tea leaves (100 g) were ground and then boiled in double-distilled water (1000 ml), followed by stirring for 15 minutes at 70°C (the procedure was repeated three times). Collected extracts were filtered through filter paper, centrifuged (at 2700 g, for 15 min), frozen and lyophilised under a vacuum (Multi Branch Trade & Manufacturing Company "Elena", Zelazkow, Poland). HPLC analyses of the green tea catechin content were performed on a Waters Alliance HPLC System 2695 (Milford, Mass, USA) equipped with an X-Terra RP18 5 μm column (Milford). The composition of GTE is shown in Table II.

Based on the information that the mean EGCG content of a single cup (200 ml) of tea prepared with 2 g of green tea leaves ranges between 4.62 mg and 406.4 mg, the two presently employed doses of GTE, 1.1% and 2.0%, were equivalent to human intake of five and eight cups of green tea, respectively. Moreover, GTE at these doses delivers 407.6 and 741.1 mg caffeine per kilogram of animal diet (Table II).

Liver histology

Pieces of liver were fixed in 3.6% glutaraldehyde fixative in 0.1 M Na-cacodylate buffer, pH 7.4, followed by three washes in the buffer, then post fixed for one hour in 1% OsO_4, dehydrated, cleared, and infiltrated within EPON Resin. Semithin sections were stained with toluidine blue and examined with an Olympus XC50 light microscope. Ultrathin sections were stained with 2% uranyl acetate, followed by lead citrate, and examined with a JEM1011 electron microscope. Hepatic steatosis was graded as follows: 0 – no steatosis; 1 – mild (< 33% of hepatocytes affected); 2 – moderate (33-66% of hepatocytes affected); and 3 – severe (more than 66% of hepatocytes affected).

Statistical analysis

All statistical analyses were performed using Statistica 12.0 software (StatSoft Inc., Tulsa, USA). The Mann-Whitney test was used to assess differences between the studied groups. The level of significance was set at $p < 0.05$.

Results

In the majority of animals studied the liver steatosis was considered as low. The greatest percentage of steatosis found during examination of sections was 40%. The percentage of hepatocytes affected by steatosis in the control group consuming a HF diet (median [1st-3rd quartile]: 25% [12-34%]) was significantly higher

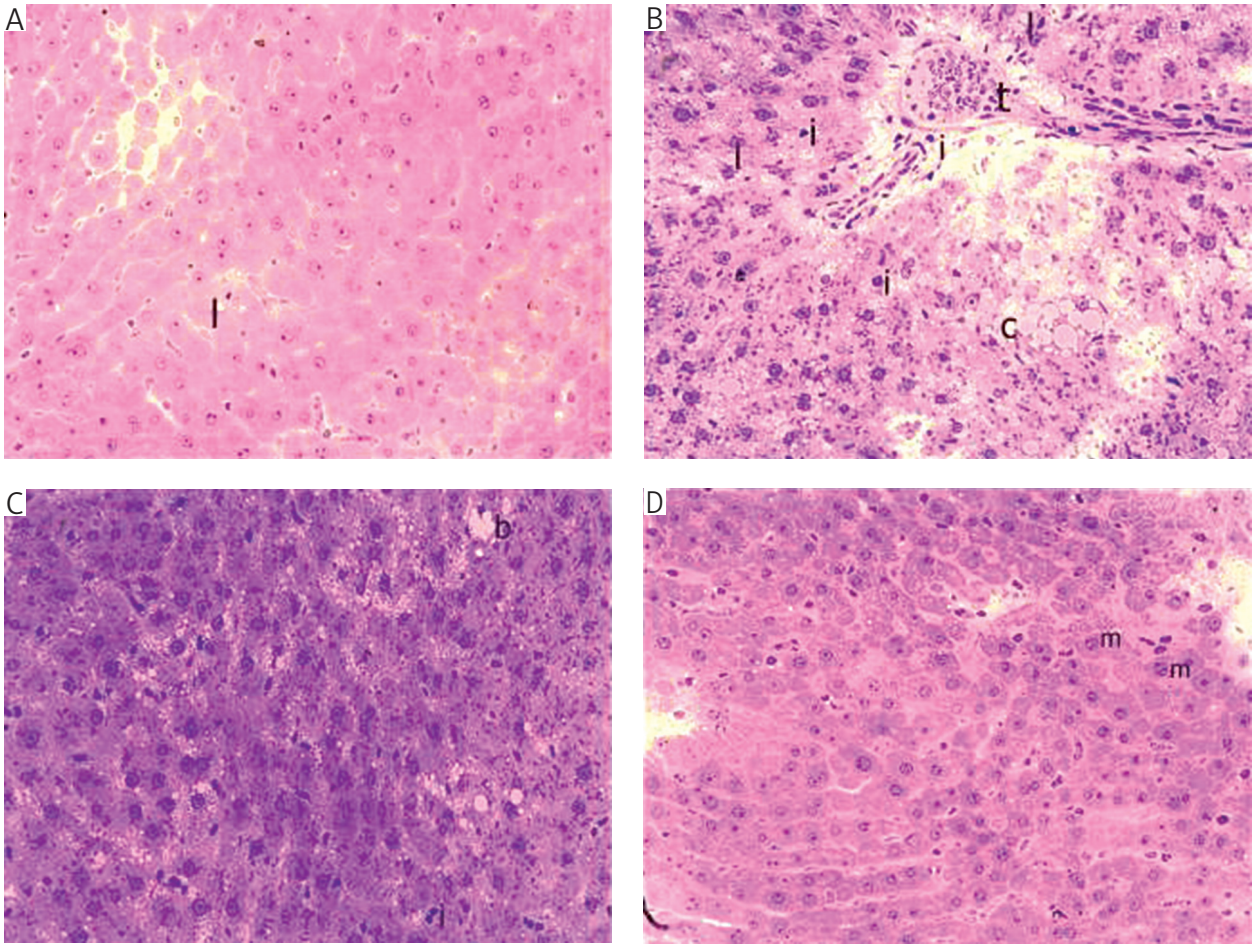

Fig. 2. Semithin sections of rats' livers examined by light microscope showing changes in fat accumulation in hepatocytes according to diet and GTE level. **A**) Liver of a rat fed with a low-fat diet. There are almost no lipid droplets in cells. There are only single, small droplets (l), which do not change the liver architecture. No sign of inflammation is seen. **B**) Liver of a rat fed with a high-fat diet. Presence of liver triad (t). Numerous hepatocytes with lipid droplets are found (l) with different sizes, but the predominance of macrovesicular steatosis is seen. Some huge lipid cysts in parenchyma merge with each other (c), which disrupts the liver histology. Few inflammatory cells are seen around the triad (i). **C**) Liver of a rat fed with a high-fat diet with 1.1% GTE. Predominance of microvesicular steatosis – numerous small lipid droplets in cytoplasm of hepatocytes. Large lipid droplets and ballooning (b) are seen rarely, so the structure of liver is not changed. There are sporadic inflammatory cells (i). **2**) Liver of a rat fed with a high-fat diet with 2.0% GTE. Only small, single lipid droplets are seen in hepatocytes. They do not change the liver structure. There are no damaged cells or ballooning. Mitotic activity (m) – numerous nucleoli in nuclei. Toluidine blue staining, objective magnification 20×

($p < 0.033$ and $p < 0.050$, respectively) than in the group consuming a HF diet enriched with 2.0% of GTE (9% [3-18%]) and the control group consuming the AIN-93G diet (10% [5-18%]). No significant differences were observed for the group consuming a HF diet enriched with 1.1% of GTE, in which intermediate results were observed (15% [4-30%]).

The livers of control rats (low-fat diet) had normal structure and its parenchyma had no signs of destruction or injury. Hepatic tissue consists of hepatic cords of hepatocytes. Hepatocytes had one or two nuclei with one or more nucleoli. There was no significant steatosis in control livers. There were no signs of ballooning, which would have led to cell degradation. Only single lipid droplets were observed in the cytoplasm of a few hepatocytes (Fig. 2A).

The hepatic tissue from rats fed a high-fat diet differed significantly from control livers (rats fed AIN-93G diet). There were definitely more lipid droplets in hepatocytes that had accumulated in the cells. Lipid droplets filled most of the cytoplasm, pushing the nucleus to the cell periphery. The next step was the collapse of cells and the release of their residues outside the cell. Lipid droplets in the majority

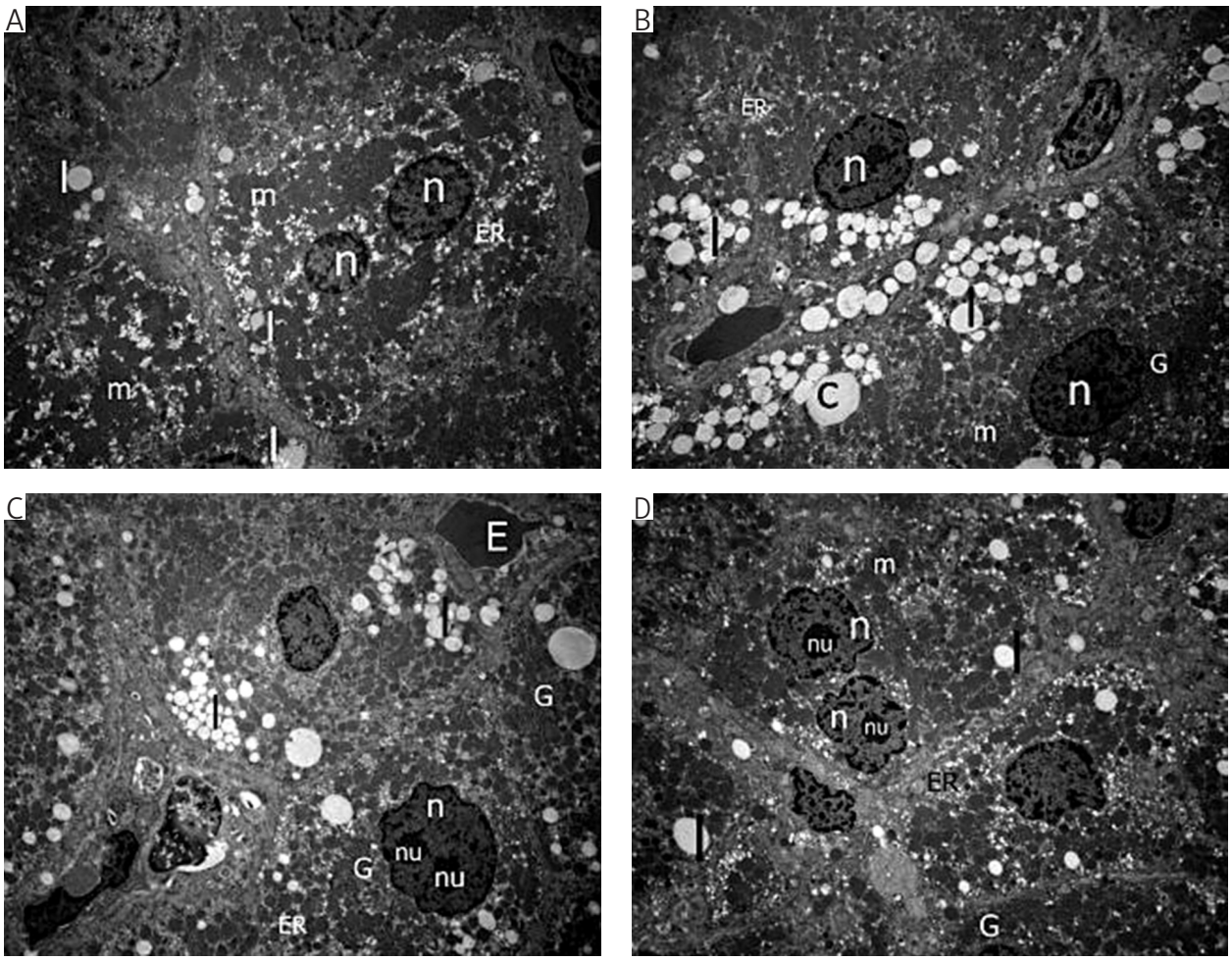

Fig. 3. Electron micrographs of a rat's liver with HF diet and different GTE level. **A**) Electron micrograph for a section in the liver in a rat fed with a low-fat diet, showing fragments of a few hepatocytes. One of the hepatocytes contains two nucleoli (n). Only a few small lipid droplets (l) are seen. All hepatocytes have numerous mitochondria(m) and endoplasmic reticulum (ER). **B**) Liver of a rat fed with a high-fat diet. Fragments of a few hepatocytes are seen. In cytoplasm of hepatocytes numerous lipid droplets (l) are present. Some of them are larger and merge with each other (c). Single nuclei (n) in hepatocytes are seen, numerous mitochondria (m), endoplasmic reticulum (ER), and Golgi apparatus (G). **C**) Electron micrograph of a section in the liver of a rat fed with a high-fat diet with 1.1% GTE. All hepatocytes contain medium sized lipid droplets (l); few of them are quite big, but they are found alone and do not merge with each other. One of the hepatocytes has nuclei (n) with two active nucleoli (nu). Mitochondria with medium polymorphism are seen. Golgi apparatus (G) and endoplasmic reticulum (ER) are also found. Also, erythrocyte (E) is found near one of the hepatocytes. **D**) Liver of a rat fed with a high-fat diet with 2.0% GTE. All hepatocytes contain a few medium sized lipid droplets (l). In one of the hepatocytes two nuclei with irregular nuclear envelope (n) are seen with active nucleoli (nu). In all hepatocytes numerous mitochondria (m), endoplasmic reticulum (ER), and Golgi apparatus (G) are found. TEM, magnification 7500×

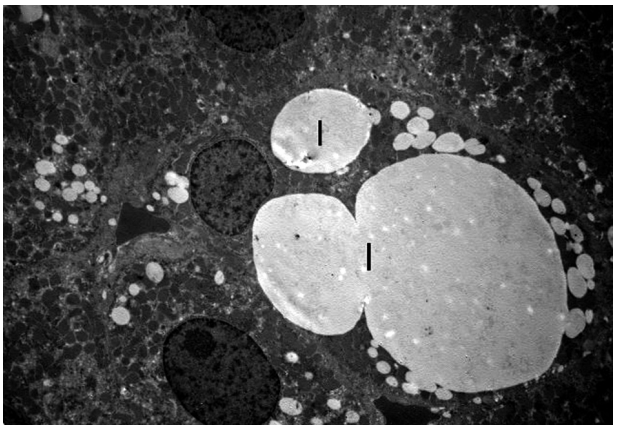

Fig. 4. Electron micrograph of a section of liver of a rat fed with a high-fat diet. In one hepatocyte large lipid droplets (l) are seen that occupy almost all the cells' area, and they push the nucleus to the cell periphery. TEM, magnification 7500×

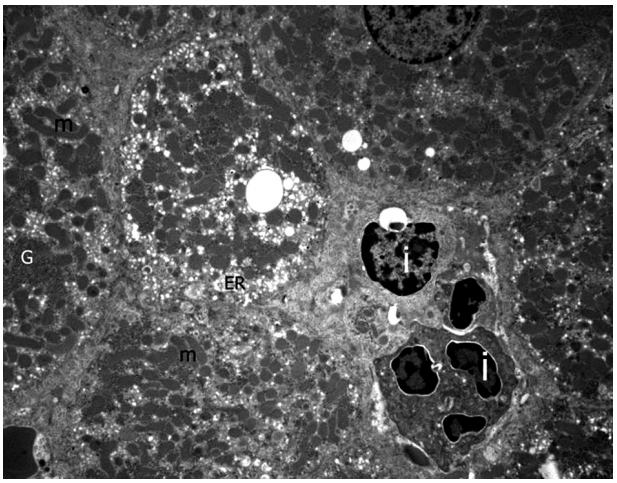

Fig. 5. Electron micrograph of a section of the liver of a rat fed with a high-fat diet. All hepatocytes contain mitochondria with average polymorphism (m), Golgi apparatus (G), and endoplasmic reticulum (ER). Inflammatory cell between hepatocytes is seen (i). TEM, magnification 7500×

of the tissue were large and created macrovesicular steatosis (Fig. 2B); when released by the collapse of cells occupying spaces between hepatocytes that impaired the structure of liver parenchyma, there were no longer hepatic cords.

Adding 1.1% GTE to the diet caused a reduction in liver steatosis (Fig. 2C). This reduction was significant, and steatosis changed from macrovesicular to microvesicular. Only in a few hepatocytes did we find ballooning and larger lipid droplets between cells generated by disintegration of hepatocytes. The addition of 2.0% GTE into the HF diet caused even more reduction in liver steatosis (Fig. 2D). The lipid content was definitely low; if any lipid droplets existed, they were small and did not change the structure of the hepatocytes. The liver parenchyma appeared similarly to that seen in a low-fat diet, without changes or interruptions in the hepatic cord system.

The same changes were observed during examination of tissues in a transmission electron microscope (Fig. 3). There were no changes in parenchyma in rats' livers fed with a low-fat diet (Fig. 3A). Only small droplets of lipids were found in hepatocytes. These droplets did not change the cell structure, and the hepatocytes contained all organelles. We observed a significant increase in the amount of fat droplets in hepatocyte rats fed a high-fat diet (Fig. 3B). In cytoplasm we observed numerous lipid droplets; some of them were large and merged into each other, creating cysts (Fig. 4). Inflammatory cells were found near hepatocytes (Fig. 5). Adding 1.1% GTE to the rats' diet caused a decrease in fat content (Fig. 3C). Small lipid droplets were still seen, but they were not as large as in the case of rats fed a HF diet. Adding 2.0% GTE to the diet caused an even greater decrease in the amount of lipid droplets in cytoplasm of hepatocytes (Fig. 3D). Only sporadic droplets were found, and they did not change the distribution of cell organelles.

Discussion

Hepatic steatosis evolves as a result of disorders of metabolic pathways in an organism. An increase in the amount of circulating free fatty acids appears to be the main stimulus in the pathogenesis of hepatic steatosis. In this process a very important role of various transcription factors, the effect of adipokines, disorganisation of hepatic fat oxidation, and low-density lipoprotein (VLDL) emission was found [15]. Non-treated hepatic steatosis develops into NASH, which is a chronic liver disease with high inflammatory activity, hepatocyte ballooning, the presence of Mallory's bodies, and sometimes fibrosis [16, 17]. The progression of NASH is best described using the "two-hit theory". According to this theory the first hit in the development of NASH is accumulation in liver excessive fat. The second hit is connected with cytokine and oxidative stress (OS), which leads to liver inflammation and fibrosis, which is the starting point in the development of NAFLD [18]. There is no efficient pharmacological therapy for NASH, so only physical activity and diet can help to reduce excessive fat accumulation in the liver.

Green tea is made from leaves of *Camilia sinensis* (family Theaceae). It contains polyphenols, the most important of which are the catechins: EGCG, EGC, ECG, and EC [11]. Many of the beneficial effects of green tea are assigned to EGCG, which has antioxidant, antihypertensive, anticarcinogenic, and hypercholesterolaemic effects [19, 20].

Our results indicate that supplementation of GTE reflecting habitual consumption of 5-8 cups of this beverage per day successfully reduced the amount of fat in the livers of rats during high-fat feeding. A high-fat diet triggers macrovesicular steatosis; however, adding GTE at 1.1% and 2.0% to the diet protects the liver against excessive fat accumulation in hepatocytes, development of hepatic steatosis, and further diseases connected with unnecessary lipid content in liver cells. A higher percentage of GTE in the diet caused an even more effective decrease in lipid droplets in hepatocytes. This is in agreement with studies by Nakamoto *et al*. [18]. They observed severe liver atrophy in NASH livers of rats. Histopathological examinations of livers showed hepatic macrovesicular steatosis, which was observed also in our semithin sections. In studies by Nakamoto *et al*. NASH was attenuated in all rats treated with fermented GTE [18], which was also noticed in our study after administration of 1.1% and 2.0% of regular GTE to all groups of rats fed the HF diet. However, one of the striking differences between the Nakamoto study and our present study was the content of EGCG. Nakamoto explained that only small amounts of EGCG were detected by HPLC analysis because of their decrease during the fermentation process. Therefore, it was suggested that fermented GTE prevents NASH progression through mechanism(s) different to those of EGCG. Due to abundant content, it seems that in our study EGCG in particular prevents steatohepatitis induced by a HF diet. Also, Kuzu *et al*. observed that EGCG in a high dose (1 g/l EGCG in drinking water) reduces the development of experimental NASH induced by a high-fat diet [21].

Other studies [22] demonstrated that obese (*ob*/*ob*) mice had severe macrovesicular steatosis and moderate inflammation. In contrast, very little steatosis and inflammation were seen in lean mice. Obese mice fed 1% GTE had reduced liver steatosis (0% GTE and 0.5% GTE in the diet had no effect on reduction of fat content in hepatocytes). Similar studies showed that GTE has a beneficial effect on leptin-deficient spontaneously obese mice [23]; it provided evidence that dietary supplementation of GTE protects against the development of hepatic steatosis and injury in obese (*ob*/*ob*) mice. Feeding with GTE at 1% and 2% reduced macrovesicular hepatic steatosis. GTE and EGCG have also been shown to inhibit fatty liver disease in mice fed a high-fat diet [24]. Our results, as well as the data in literature, suggest that only a level of GTE greater than 1% can attenuate hepatic steatosis. Lower levels of GTE (0.5%) do not prevent hepatic steatosis but can reduce the risk of hepatic injury and increase antioxidant activity in the liver [12]. Indeed, given the generally low oral bioavailability and rapid elimination of catechins, the benefits of green tea may only occur when consumed frequently (5-10 cups/day), as suggested by epidemiological studies [7].

Green tea activity consisting of lowering excessive fat accumulation in the liver is the starting point to reduce weight loss and obesity in experimental animals and also in humans. It is well known that GTE has an anti-obesity effect in humans [25, 26]. Drinking GT caused a decrease in levels of total plasma triglycerides and cholesterol in rats, it also reduced fat and starch absorption [27, 28, 29]. It was shown that consumption of water with extract of GT caused decreased body fat accumulation [30]. Studies by Murase *et al*. [31] showed that supplementation with GTE dose-dependently reduced body weight, adipose tissue mass, and accumulation of fat in the liver.

The effect of GTE on liver steatosis and body fat accumulation, as well as its anti-obesity action, is connected with the action of green tea catechins on lipid metabolism [32]. GTE reduced accumulation lipids in the liver by decreasing expression of adipose lipogenic genes and serum-free fatty acids. It also led to improved antioxidant activity in the liver and decreased levels of expression of tumour necrosis factor α. GTE can also downregulate proinflammatory events, which unimpeded can trigger oxidative and nitritative damage and cause NASH. GTE can reduce NADPH oxidase activity and expression of ROS. Thereby, GTE suppresses oxidative and nitritative damage that causes liver injury and NASH [33].

Although a lot of examples of the beneficial action of green tea exist in the literature, there are also reports about hepatotoxicity of GTE. If EGCG is administered at excessively high levels (750 mg/kg body weight of mice; more than 2% GTE), it can cause liver injury because of supraphysiological intake of green tea catechins [34]. Inoue *et al*. [35] have indicated that 1.0% green tea polyphenols disrupts kidney function through increased thiobarbituric acid-reactive substances (a reliable marker of oxidative damage) as well as down-regulation of antioxidant enzymes and heat-shock proteins in mice with dextran sodium sulphate-induced colitis, but also in normal mice, so it seems that a limitation of our study was that we failed to take into consideration a group of rats fed a normal (low-fat) diet enriched with GTE.

In the case of humans, the incidence of sporadic adverse events after consumption of large amounts of highly concentrated GTE are also available. Among such risks are the possibility of liver damage, the potential to interact with prescription drugs to alter their therapeutic efficacy, and the chance to cause harm when combined with other highly popular herbal remedies [36]. However, Donovan *et al*. reported that administration of decaffeinated green tea extract at a daily dose of 504 mg EGCG for

14 days did not alter CYP2D6 or 3A4 activity in healthy individuals [37]. According to Imai *et al*. this amount of EGCG may be anticipated with consumption of 10 or more cups of green tea per day [38].

Supplements of green tea contain much higher catechin and caffeine doses than green tea beverages. One capsule has been reported to be equal to 5-7 cups of tea. Theses capsules are usually taken more than once a day [39]. These data suggest that even when using herbs in the diet, it is recommended that the safe upper limit of its doses be identified.

Conclusions

The enrichment of a high-fat diet (representing a Western diet rich in animal fat) with GTE may prevent the development of dietary induced liver steatosis. Although our study was conducted on an animal model, these findings could be extrapolated to be directly applied to the human diet because the doses of GTE used in this study were equivalent to human intake of five and eight cups of green tea daily, which is approximately the amount of green tea consumed in many regions of the world [9]. In view of the increasing incidence of overweight and obesity, metabolic syndrome, and excessive energy and fat consumption, simple and cheap dietary modifications potentially influencing pathological processes may gain significance.

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The authors declare no conflict of interest.

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