Rats on a high energy diet showing no weight gain present with ultrastructural changes associated with liver fibrosis

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Abstract

Sibutramine is widely used as a weight-loss substance in the treatment of obesity and is a selective inhibitor of the neuronal reuptake of serotonin and noradrenaline. Although banned it is often a hidden ingredient in herbal and dietary supplements which are widely used by the general public. Various weight loss products, including sibutramine, have successfully been tested in animal models of diet-induced obesity. In the female Sprague-Dawley rat model, fed a high-energy diet that did not produce a significant increase in BMI, the cellular structure of the liver was evaluated using transmission electron microscopy. Compared to controls showing no damage, the livers of rats fed a high-energy diet were found to have increased fibrosis without steatosis, while for rats fed high-energy diet with sibutramine, fibrosis was increased and steatosis had developed. In conclusion, in female rats fed a high-energy diet that does not result in weight gain hepatic fibrosis occurs without steatosis. In these rats the co-administration of sibutramine increases the degree of fibrosis and steatosis develops. Although it has been widely believed that sibutramine is not hepatotoxic, this study clearly shows that at an ultrastructural level, rats fed a high-energy diet treated with sibutramine show signs of hepatotoxicity.

Key words: High energy diet; Sibutramine, ultrastructure, fibrosis

Introduction

Sibutramine is a selective inhibitor of the neuronal reuptake of serotonin and noradrenaline and is widely used as a weight-loss substance in the treatment of obesity. Inhibition of these neurotransmitters in the brain causes an increase in the synaptic concentration and thereby an increase in satiety and resultant reduced food intake [1,2]. The drug undergoes extensive first-pass metabolism, mainly by hepatic cytochrome p450 3A4 enzymes, to active primary (M1) and secondary (M2) amine metabolites through which it exerts its pharmacological actions and which are more potent than the parent compound. Most of the drug and its active metabolites are renally excreted [3]. The efficacy of sibutramine is greatly enhanced when used with intensive lifestyle modification. However, long-term data on the effects of sibutramine on major obesity-related morbidity and mortality are lacking [3]. Side effects of sibutramine that have been reported include insomnia, dry mouth, constipation, nausea and headache and it has also been associated with hypertension, increased pulse rate, tachyarrythmias and angina pectoris [3].

Although withdrawn from most markets worldwide due to the associated increased risk for cardiovascular and stroke sibutramine is often a hidden ingredient of herbal and dietary supplements [4]. Sibutramine is believed not to be hepatotoxic although increased liver function tests have been reported in sibutramine-treated obese patients, 1.6% compared to 0.8% for the placebo group. It is believed that sibutramine induced weight loss is beneficial in patients with non-alcoholic liver disease (NALD) but should not be used in patients with severe liver dysfunction [5]. Diet modification and increase in exercise is recommended for patients using weight loss drugs, but often these drugs are used with very little lifestyle modification, i.e. taken concurrently with a high energy diet (HED).

Animal models of diet-induced obesity have provided important information on food intake, changes in weight and effects of weight-loss promoting substances such as sibutramine, and have successfully been used as a model to study the pathophysiology of human obesity. Some animal species, such as the C57BL/6 mouse have been classified as diet-induced obese (DIO) rodents [6] whereas other models showed resistance to weight-gain under the conditions of a HED diet. Histological evaluation of the tissue structure of the liver of HED rats with weight gain reveals mild steatosis with fat droplet accumulation, ballooning degeneration of hepatocytes and inflammatory cell infiltration [7]. This effect on liver morphology in rats fed a HED without weight gain will be investigated, simulating patients that consume a HED but present with normal BMI. The hypothesis that will be tested is that sibutramine will show no hepatotoxicity in individuals with a normal BMI who consume a HED.

Materials and Methods

Animal model and experimental design

A diet-induced obese (DIO) Spraque Dawley rat model was used in this study. Ethical clearance was obtained from the University of Pretoria Animal Use and Care committee (ethical clearance number: h015-11). Twenty female Spraque Dawley rats, each of average weight 200-250g were used in this study and were maintained at the University of Pretoria Biomedical Research Centre (UPBRC). The animals were housed conventionally in cages complying with the sizes laid down in the SANS 10386:2008 recommendations. A room temperature of 22°C (+-2); relative humidity of 50% (+-20) and a 12hr light/dark cycle was maintained. Enrichment was provided according to standard procedures at the UPBRC. This experiment was conducted over a period of 88 days.

The animals were randomly divided into three groups with ten animals per group; NC (Normal chow), HED (high energy diet) and HED-S (high energy diet plus sibutramine treatment).

Animals in the NC group were on the normal diet [Epol mice cubes; a division of Rainbow farms (Pty) Ltd, Westville, Johannesburg, South Africa] for the first four days. For statistical purposes, day 0 was taken on the first day the animals in the HE group received the high energy diet. Animals in the NC group remained on the normal chow diet throughout the duration of the study. Animals in the HED group were on the HED from day 5 until day of termination. Animals in HED-S group were on the HE diet from day 5 until day of termination, but were treated with sibutramine for the last 28 days before termination. During the last 28 days, animals in the NC and HED groups were not treated with the solvent of sibutramine as it was dissolved in sterile water and water was provided ad libitum to all animals.

High energy diet (HED)

The HED diet consisted of 12% corn oil, 43% condensed milk and 45% Epol 4.4Kcal/g with 13% protein, 18% fat and 69% carbohydrates. The normal chow diet was replaced with the HE diet and was available to the animals *ad libitum*.

Sibutramine administration

Animals in the HED-S were treated for 28 days with physiological comparable levels of sibutramine hydrochloride monohydrate (BIOCOM biotech; Clubview, South Africa) dissolved in sterile water (1.32mg/kg body weight). Each of the animals in this group was orally gavaged daily with this product.

Statistical analysis of Weights and BMI

All animals were weighed daily to monitor any increase or decrease in weight over the experimental period. At weekly intervals the data was analysed. On the day of termination, the heart, lung, liver, kidney and visceral fat was removed and weighed. Statistical analysis

was performed with the aid of NCSS by using One-way Analysis of variance (ANOVA). The length of each animal from tail to nose tip was measured and the BMI using the mass on the date of termination was calculated.

Tissue for transmission electron microscopy

Liver samples were fixed in 2.5% glutaraldehyde / formaldehyde for 1 hour, rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) for 15 minutes before being placed in secondary fixative, 1% osmium tetraoxide solution, for 1 hour. Following fixation, the tissues were rinsed again as described above. The tissues were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. The samples were then embedded in resin and ultra-thin sections (80-100nm), cut with a diamond knife using an ultramicrotome, were contrasted with uranyl acetate for 5 minutes followed by 2 minutes of contrasting with lead citrate, after which samples were allowed to dry for a few minutes before examination with the JEOL Transmission Electron microscope (JEM 2100F).

Results

Table 1 shows the results obtained from the one-way ANOVA analysis of the weight, length and BMI of the animals in the different experimental groups on the day of termination. In Table 2 the average weight of the heart, lung, liver, kidney, duodenum and visceral fat of animals in the experimental groups are presented. Figure 1 shows the ultrastructural arrangement of hepatocytes and its organelles in the samples obtained from the NC (Figure 1 A and B) and HE (Figure 1 C and D) group. In Figure 2 the micropgraphs obtained from liver samples of the HED-S group are shown.

Table 1: Analysis of weight, length and BMI at termination. N=10 (Significance was set at a level of 0.05)

Group	**Weight (g)**	**Length (cm)**	**BMI (g/cm^2)**
NC	262.3 ± 5.700	40.8 ± 0.368	0.157 ± 0.003
HED	271.3 ± 5.465	40.96 ± 0.204	0.145 ± 0.002
HED-S	276.6 ± 5.346	41.65 ± 0.236	0.159 ± 0.002

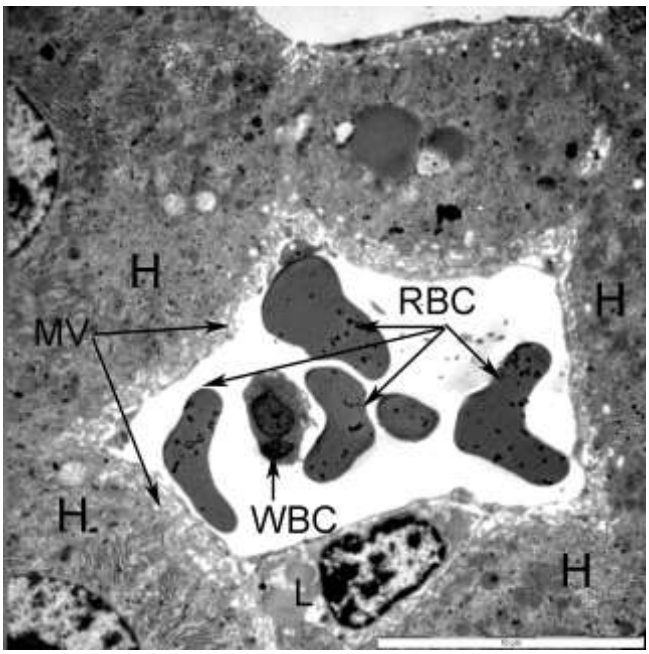
* Values are presented as Mean ± SD

Table 2: Average weight of selected organs on the day of termination

Group	Heart	Lung	Liver	Kidney	Duodenum	Visceral fat
NC	1.02±0.09	0.65±0.06	8.4±0.90	0.91±0.08	0.53±0.06	21.29±2.41
HED	1.02±0.01	0.67±0.06	8.13±0.72	0.87±0.09	0.50±0.06	24.20±3.80
HED-S	0.997±0.095	0.706±0.03	8.058±0.613	0.938±0.087	0.526±0.065	24.228±2.43

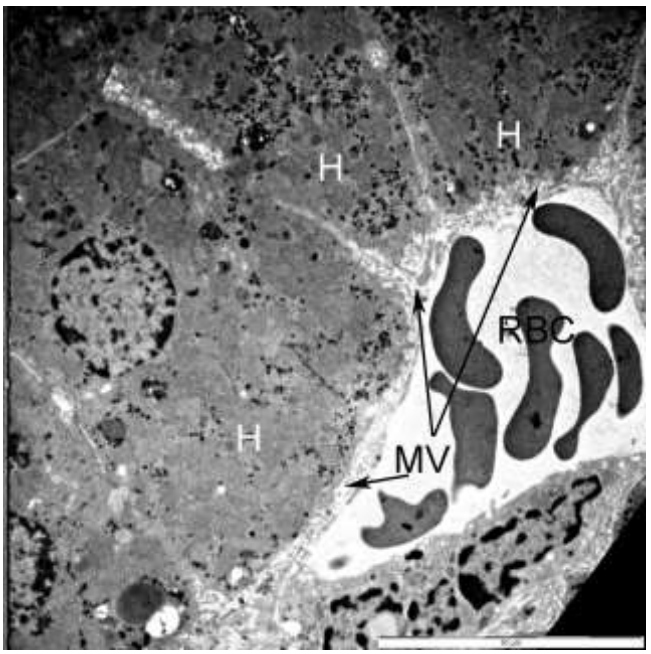
* Values are presented as Mean ± SD

A



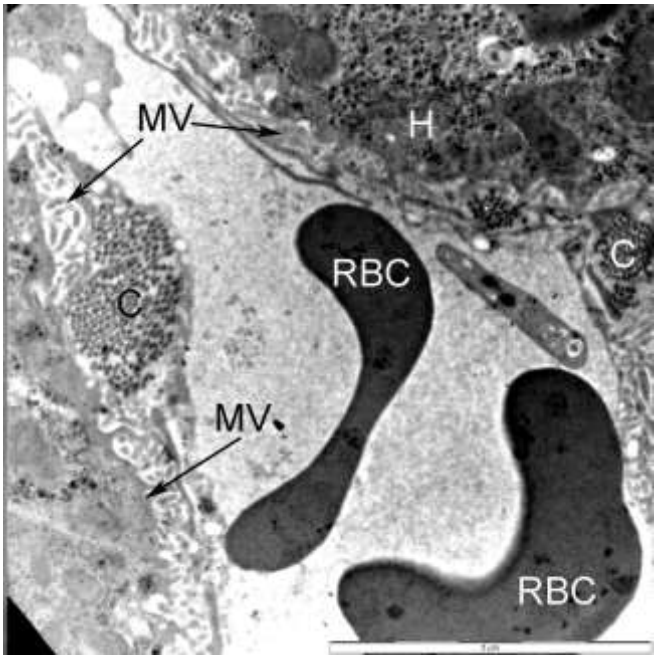
H = Hepatocyte; MV=Microvilli; RBC = Red Blood Cells; L=sinusoidal lining cell; WBC=White Blood Cells

C



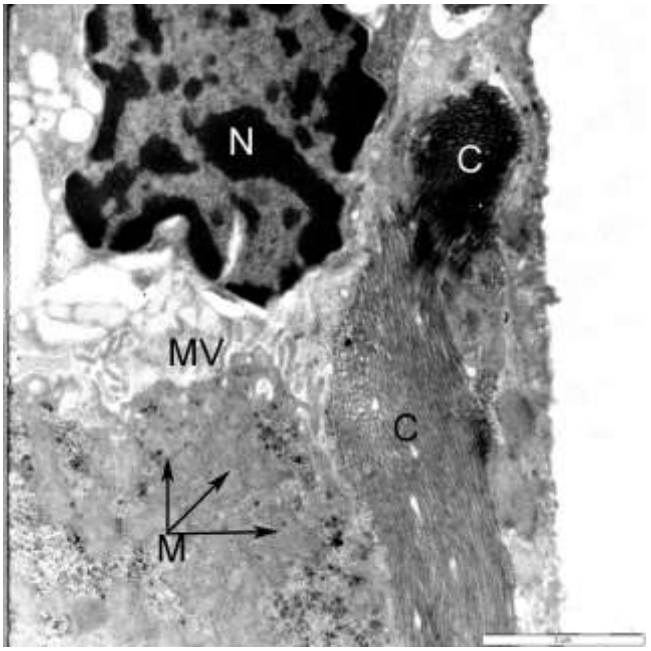
H = Hepatocyte; MV=Microvilli; RBC = Red Blood Cells

B



H = Hepatocyte; MV=Microvilli; RBC = Red Blood Cells; C=collagen fibers

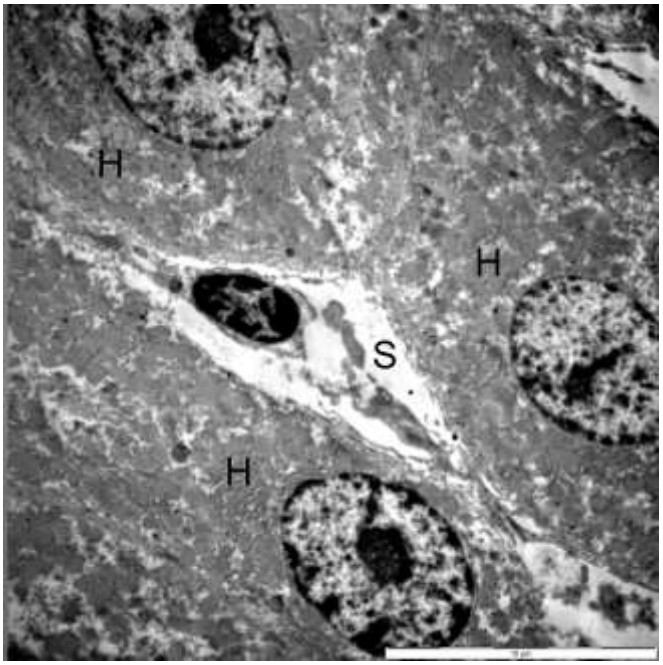
D



H=Hepatocyte; MV=Microvilli; M=mitochondria of hepatocyte; C=Collagen fibers; N = Nucleus

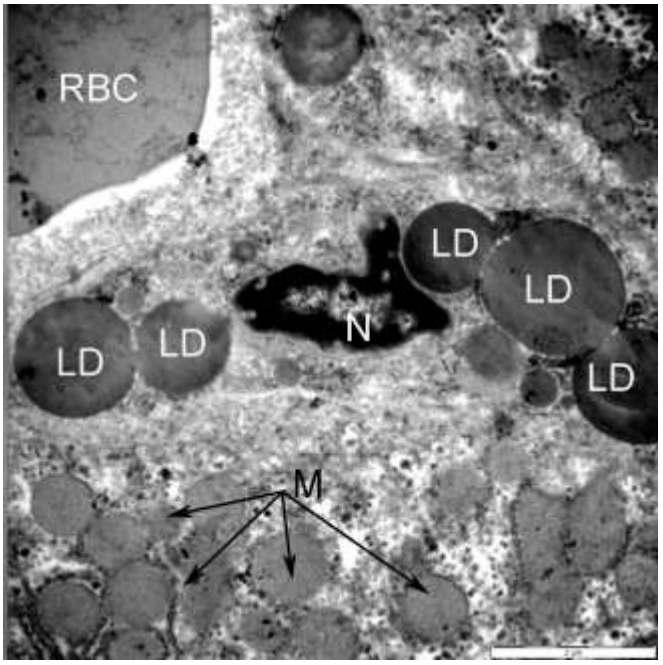
Figure 1. TEM micrographs of liver samples from NC (A and B) and HED (C and D).

A



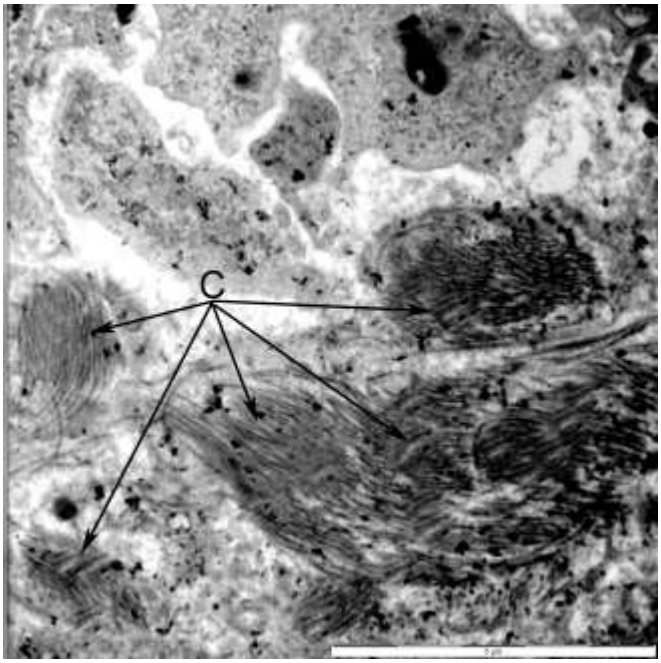
H=Hepatocyte; S=Sinusoid

C



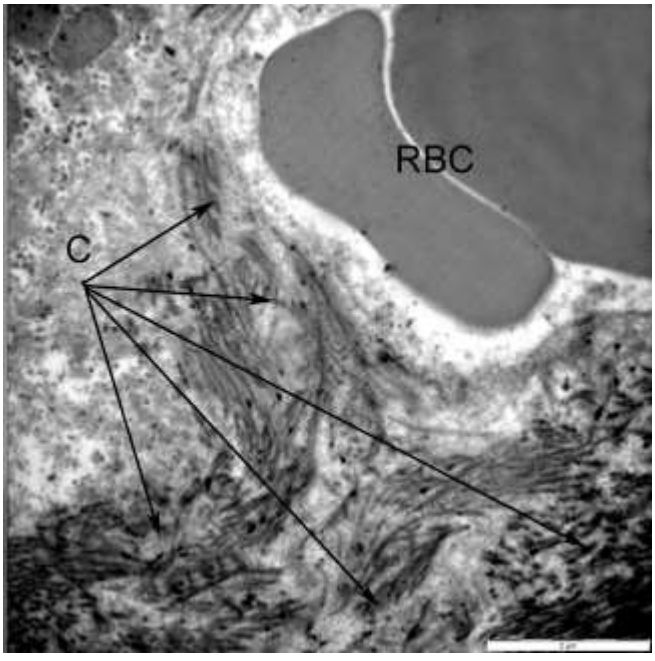
M=Mitochondria of hepatocyte; N = Nucleus of hepatic stellate cell; LD=Lipid Droplets

B



C=Collagen fibers

D



C=Collagen fibers; RBC=Red Blood Cell

Figure 2. TEM micrographs of liver samples from HED-S.

Discussion

Results obtained from the One-way ANOVA analysis of weight, length and BMI on the day of termination is indicated in Table 1. No significant differences were obtained between any of the experimental groups. No statistically significant difference in weight was obtained from analysis of the heart, lung, liver, kidney and duodenum of animals in the three groups. Also, no differences were obtained in the analysis of the weight of the visceral fat of animals in the experimental groups as indicated in Table 2. Although slight changes could be observed, these changes were not statistically significant.

The liver consists of parenchymal cells or hepatocytes that are closely associated with the sinusoids, a network of blood vessels [8]. Other cells present are non-parenchymal cells and include endothelial cells, Kupffer cells, dendritic cells and stellate cells. The endothelial cells form the largest portion of cells lining the sinusoids and Kupffer cells are macrophage-like cells that play a role in phagocytosis [8]. Dendritic cells are involved in the initiation of the immune response, and are responsible for antigen processing and secretion of cytokines. Hepatic stellate cells (HSC) are mesenchymal in origin and are found between the hepatocytes and the sinusoidal endothelial cells. These cells play an important role in retinoid homeostasis, and store 80% retinoids within the lipid droplets [9, 10].

Figure 1 A and C shows the normal ultrastructural arrangement of hepatocytes (H) with microvilli (MV) protruding into the space of Disse and blood cells in the sinusoidal space with a sinusoidal lining cell (L). Reticular fibers forms the scaffold network throughout the liver and are found in the space of Disse between the hepatocyte surface and the endothelial cells lining the sinusoid. Electron microscopy reveals that reticular fibers are small bundles of collagen fibrils consisting mainly of collagen type III with a diameter of 20-40 nm. In Figure 1 B several such bundles can be seen within the space of Disse.

The effect of a HED without weight gain on liver structure was also evaluated. It was found that the cellular arrangement of the NC (Figure 1 A) and HED (Figure 1 C) groups was

similar. In contrast to Figure 1 B, where small bundles of collagen was present in HED fed rats, the collagen bundles were thickened and tightly arranged masses of collagen fibers with some banding was observed (Figure 1 D).

Liver fibrosis develops as a result of chronic damage to the liver which is associated with the accumulation of extracellular matrix protein. Causes of liver fibrosis include chronic Hepatitis C Viral (HCV) infection, alcohol abuse and non-alcoholic steatohepatitis (NASH). In rat models for NASH, histological evaluation of the liver reveals steatosis with fat droplet accumulation, degeneration of hepatocytes via apoptosis [7] and inflammatory cell infiltration [7] and later liver fibrosis. In this study rats fed a HED, without any significant weight loss, did not develop steatosis with lipid accumulation but did develop fibrosis.

In chronic hepatic injury the development of fibrosis/cirrhosis is dependent on the efficacy of hepatocyte regeneration. If injury persists the hepatocytes are substituted with extracellular matrix (ECM) including fibrillar collagen. HSC are the main ECM producing cells and in normal liver tissue are resident in the Space of Disse. Following injury the HSC differentiate into myofibroblast-like cells which have contractile, proinflammatory and fibrogenic properties.

In these rats that showed liver fibrosis, a feature of NALD for which sibutramine is recommended; the effect of sibutramine at physiological dosages was evaluated. The micrographs of the liver in Figure 2 are representative of the animals in the HED-S group. Figure 2 A shows the normal arrangement of the hepatocytes (H) and sinusoid but the space of Disse compared to the NC group appears to be poorly defined, few microvilli are identifiable. In Figure 2 B and D there is evidence of fibrosis where the dense arrangement of collagen fibres is very clear. This is more prominent compared to the NC group (Figure 1 B). In Figure 1 C a HSC can be seen with its nucleus (N) and lipid droplets (LD) being prominent features. Rector et al., (2010) identified that mitochondrial dysfunction precedes hepatic steatosis [11]. The traditional two hit model hypothesis is used to explain the

development of NALD, which progresses from hepatic steatosis, inflammation, fibrosis and cirrhosis. However, recently Rector et al., (2010) proposed that mitochondrial damage due to oxidative stress occurs prior to hepatic steatosis [11]. Decreased mitochondrial functioning limits mitochondrial mediated fatty acid oxidation and as a consequence lipid droplet accumulation occurs. Using Swiss mice Da Silva (2010) found that sibutramine at 10, 20 and 40 mg/kg bw caused genotoxic effects in male Swiss mice 48 hours after administration measured with the comet assay and micronucleus test [12]. The dosage used was 1.5 to 6.5 times the highest dose prescribed for humans [12]. In our study, physiological dosages of sibutramine were administered over a period of 28 days. Accumulated dosage would be within the range of concentrations used by Da Silva et al. (2010). Many mitochondria are present in the hepatocyte and are in close proximity to the plasma membrane and this makes these organelles easy targets for genotoxicity caused by sibutramine and its metabolites. In this study, sibutramine and/or its metabolites may cause mitochondrial dysfunction resulting in impaired fatty acid oxidation and consequently the accumulation of lipid droplets in the HED-S group. Guzman et al. (2012) have found that in female Wistar rats that received 10mg/kg bw sibutramine for 15 days with subsequent weight loss, markers for oxidative stress were reduced in the cortex, hemispheres and medulla oblongata [13]. Da Silva et al (2010) also reported lack of sibutramine cytotoxicity determined by comparing the relationship between polychromatic erythrocytes and normochromatic erythrocytes in the bone marrow of mice [12]. Toxicity of sibutramine may be a function of rat models, depending on whether or not weight loss was achieved, tissue type as well as methods used for measurement.

In conclusion, in female rats on a HED that does not result in weight gain, hepatic fibrosis without steatosis develops. In these rats the co-administration of sibutramine increases the degree of fibrosis and also causes steatosis.

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