Pathological and biochemical investigation of the effects of L-carnitine and gemfibrozil on peroxisome proliferator activated receptors (PPARS) and lipidosis in rabbits on a high-fat diet

ABSTRACT

Obesity and fatty liver is a worldwide health problem in human with detrimental consequences where many investigations are undertaken to overcome this problem. In this study, gemfibrozil and L-carnitine were evaluated in prevention of obesity and lipidosis. The study involved 56 New-Zealand Albino rabbits, divided into 8 equal groups (n=7). The groups were as follow; group I (normal diet), II (normal diet +gemfibrozil), III (normal diet+L-carnitine) and IV (normal diet+gemfibrozil+L-carnitine), V (high fat diet), VI (high fat diet+gemfibrozil), VII (high fat diet+L-carnitine) and VIII (high fat diet+gemfibrozil+L-carnitine). Animals were blood sampled and wieght weekly during the experiment and at the end of the experiment for determination of biochemical parameters (glucose, total lipid). All rabbits were euthanised for histopathological examination and for distrubition of peroxisome proliferator activated receptors (PPARs) in tissues by immunohystochemistry. Gemfibrozil and L-carnitin treatment in rabbits given high fat diet resulted in statistically significant decrease in total lipid when compared to those only received high fat diet. Beta oxidation of high fat diet group was significantly higher than that of groups additionally received gemfibrozil and L-carnitine. Immunohistochemistry revealed an increase in PPAR, PPAR-α and β but not PPAR-γ expression in high fat diet group. On the contrary, L-carnitin administration had no effect on tissue PPAR expression. PPAR-α expression differed between groups received gemfibrozil and high fat diet and those did not. The most marked macroscopy finding was abdominal fat increase in high fat diet group (group V). On the other hand gemfibrozil administration resulted in significant abdominal fat decrease. Furthermore decreased abdominal fat was marked in gemfibrozil and L-carnitine given animals (group VIII) when compared to other groups. In conclusion, gemfibrozil and L-carnitine administration alleviated abdominal and hepatic fattening. Gemfibrozil also caused a significant increase in PPAR-α expression in the liver. It may be of use in avoiding abdominal fat (obesity) due to high fat diet by use of gemfibrozil, a synthetic PPAR-a ligand, and L-carnitine.

Keywords: Gemfibrozil, L-carnitine, Obesity, PPARs, Rabbits

INTRODUCTION

Obesity is a condition that results from the disruption of the balance between the energy intake through feed and consumption. The excess consumption of energy is mainly stored in the form of triglycerides in adipocytes. However, in the development of obesity namely morphological changes such as adipocyte hypertrophy and adipogenesis in adipose tissue, are under the control of numerous mechanisms. Fat deposition in the liver and obesity are inter related as obesity is the most important risk factor for the development of fatty liver (Selek et al, 2011).

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Research Article

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The severity of liver fat deposition is determined by the balance between fatty acid uptake, endogenous fatty acid synthesis, triglyceride synthesis, oxidation of fatty acids, and triglyceride exports (Stienstra et al., 2007). Activated receptors with peroxisome proliferators (PPARs) are nuclear receptors and have three different isoforms called PPAR-α, -β, and -γ (Wahli, 2002). Peroxisome proliferator activated receptors (PPARs) are nuclear receptors involved in the transcriptional control of genes involved in inflammation, lipid and carbohydrate metabolism, leading to the formation of obesity, hypercholesterolemia, insulin resistance and atherosclerosis (Brown and Plutzky 2007). PPAR-α is excreted from liver, heart and kidneys, PPAR-β is mainly from adipose tissue, skin and brain, and PPAR-γ is from adipose tissue, large intestine, heart, kidney, pancreas and the spleen (Akbiyik et al., 2004; Jeffrey et al., 2000; Tenenbaum et al., 2005; Tunca and Devrim, 2007). In obese animal models, fibrates have been shown to improve insulin sensitivity in addition to beneficial lipid modulation (Ogawa et all.,2000). They are activated by fatty acids and their derivatives. Although PPAR-β is activated by prostaglandin J2, PPAR-α is activated by leukotriene B4 (Xin et al., 1999). This enzyme affects body weight and energy balance as well as regulation of glucose metabolism (Hashimoto et al., 1999). These effects are partly due to the beta oxidation of hepatic fatty acids and lead to the degradation of fatty acids in the liver (Dreyer et al., 1992). PPAR-α fibrate derivatives play an important role in the effects of antilipidemic agents (Akbiyik et al., 2004; Hasimato et al., 1999; Tunca and Devrim, 2007). The PPARγ isoform, which is regarded as the main regulator of glucose homeostasis, is also highly expressed in various cell types and organs including adipocytes, muscle cells, liver and kidney. PPARγ agonists such as thiazolidinediones, pioglitazone, and rosiglitazone, which are currently prescribed as anti-diabetic drugs, act as insulin sensitisers (DeFronzo, 1999).

Fibrates (fenofibrate, gemfibrozil, etc.) are hypolipidemic drugs that stimulate PPARs in the cell (Akbiyik et al., 2004; Hashimoto et al., 1999; Jeffrey et al., 2000). For this reason, they regulate a series of gene expression that is important for lipid and lipoprotein mechanisms. Activated PPAR acts by increasing the expression of genes with PPRE by forming a heterodimer with the retinoid X receptor (Jeffrey et al., 2000). Fibrates also have activity in regulating energy hemostasis. These drugs can suppress obesity by reducing plasma triglycerides and fatty acid concentrations (Kraja et al., 2010; Moutzouri et al., 2010; Schoonjans et al., 1996; Tunca and Devrim, 2007; Yoon et al., 2003). Synthetic agonists of PPARα, such as fibrates, are used clinically to lower plasma triglycerides and increase high-density lipoprotein cholesterol (Tenenbaum et al., 2005). Gemfibrozil is used as a lipid regulator in human health as it lowers cholesterol, triglyceride, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) levels and increase high-density lipoproteins (HDL) and HDL/cholesterol ratio in the blood. For this reason, it is a main drug of choice against hyperlipidemia, coronary heart failure and myocardial infarction in diabetic patients (Costet et al., 1998; Schoonjans et al., 1996; Tunca and Devrim, 2007;).

L-Carnitine is a biological substance that plays a crucial role in energy production in mitochondria, and is a vital element synthesized in liver and kidneys from amino acids such as lysine and methionine. Oxidation of long chain fatty acids is not possible without L-carnitine. Free fatty acids can pass through the mitochondrial inner membrane by forming an ester with L-carnitine, so that the free fatty acids become ready for β-oxidation (Fritz, 1995; Kopec and Fritz, 1973, Longo et al., 2016) and energy is provided from the burning of fatty acids in mitochondria (Lohninger et al.,

1987). L-carnitine has also been shown to have modulatory and protective effects against metabolic intermediates that cause pathological disorders in organs and tissues in recent studies. These effects are; preparation of active fatty acids necessary for the synthesis of membranes, protection of membrane lipids against peroxidation, modification of membrane structure, transport of long chain fatty acids to mitochondria and oxidation, increasing of ATP production to optimum level, activation and support of immunity system and positive effects on receptor and transport proteins (Binienda and Ali, 2001; Brown, 1999; Kremser et al., 1995; Selek et al., 2011, Longo et al., 2016; Sung et al., 2016; Uhlenbruck, 1996;). Studies in food animals such as cattle and pig, also reported that the administration of exogenous L-carnitine increased digestibility of feed and fat (LaCount et al., 1995), and significantly reduced the amount of fat in the gluteal region resulting in an increase in meat/fat ratio (Owen et al., 1996). L-carnitine supplementation had positive effects in the reduction of serum triglyceride, cholesterol and free fatty acid levels in rats (Mondola et al., 1992), prevention of diabetes in carnivores, treatment of heart diseases such as cardiomyopathy and cardiac insufficiency, obesity and prevention of fatty liver (Keene, 1991).

This study is aimed to investigate pathological (macroscopic, microscopic and histochemical staining methods) and biochemical effects of Gemfibrozil, an antihyperlipoproteinemic fibrate, PPARs and L-carnitine in an experimental model in rabbits on high fed diet.

MATERIAL and METHOD

The study design, management of animals and experimental procedures are given elsewhere (Selek et al, 2011) Briefly a total of 56 New Zealand Albino rabbits, 6 months old, were assigned to 8 equal groups (n = 7). All animals were kept at 12-h light/dark in a ventilated unit with 55% humidity throughout the the experiment. Rabbits were provided normal ad libitum dieat containing 2500 Kcal metabolic energy and 4.5% crude fat (groups I, II, III and IV) and high fat diet containing 3200 Kcal metabolic energy and 15% crude fat (goups V, VI, VII and VIII) and drinking water. Peletted rabbit feed was purchased from Bayramoglu Yem Inc., Erzurum, Türkiye. The animals were medicated daily for nine weeks. Groups were as follow;

Group I: received normal diet only,

Group II; received normal diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW),

Group III: received normal diet+L-carnitine (200 mg/kg/day, BW),

Group IV: received normal diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW)+L-carnitine (200 mg/kg/day, BW)

Group V: received high fat diet only,

Group VI: received high fat diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW),

Group VII: received high fat det+L-carnitin (200 mg/kg/day, BW),

Group VIII: received high fat diet+Gemfibrozil (0.05%, 100 mg/kg/day, BW)+L-carnitin (200 mg/kg/day, BW).

Clinical Examination and Sample Collection

All rabbits were subjected to daily clinical examination following drug administration. The body weights of all rabbits were measured and recorded at a week interval during the study period. Blood samples were collected from vena auricularis into plain tubes at the start of the study (day 0) and every three weeks (week 3, 6 and 9) after a off feed period of 10 h with free access to water. Blood samples were centrifuged for 10 minutes at 3000 rpm and obtained sera was stored at -25ºC until analysis.

Sample Analysis

Glucose was spectrophotometrically determined using a commercial kit (IBL®, Germany) as instructed by manufacturer. Total lipid levels were measured according to the Kunkel phenol method. Peroxisomal β-oxidation analysis of liver tissue was performed spectrophotometrically at 340 nm wavelength according to the method reported by Lazarow (Lazarow, 1981).

Histopathological Examination

At the end of the study, animals were euthanized under general anesthesia and systemic necropsy was performed. Macroscopic changes noted were recorded for each animal. All abdominal organs and abdominal fat tissue (periovarian, perirenal, inguinal, mesenteric and omental adipose tissue) were weighed; tissue samples were taken for histo-pathological examinations. All tissues were fixed in 10% buffered formaldehyde solution. After routine procedures paraffin blocks were prepared and were cut at 4-6 μm thickness and stained with Hematoxylin Eosin (HE) for histopathological examination under light microscope. Histopathological changes were recorded.

For electron microscopic examinations, 1 mm^3 fragments from the liver were washed with 0.1 M cacodylate buffer (Ph 7.4) after 24 h of initial fixation in glutaraldehyde paraformaldehyde solution. It was then subjected to 2 hours fixation with 2% osmic acid. The samples were treated with known methods and blocked with araldite. Semi-thin sections and ultra-thin sections were stained with toluidine blue and uranyl acetate and citrate. Sections were examined on a Zeiss-9S electron microscope. In addition, the expression of PPARs in tissues was determined by immunohistochemical methods.

Immunohistochemical Examination

In this study, avidin-biotin peroxidase method was used as immunohistochemical method. For immunohistochemical staining, 4-6 μm thick sections from paraffin blocks were placed on the slide coated with 3-aminopropyltriethoxysilane (APES, Sigma-Aldrich, St. Louis, Montana, USA). After deparafinization and dehydration, the sections of the citrate buffer (pH 6.0) solution were subjected to microwave treatment at 800 watts and for 10 minutes of treatment with the aim of revealing the antigenic receptors. The sections, spontaneously cooled to room temperature, were incubated for 15 minutes in methanol (3%) solution of hydrogen peroxide to prevent endogenous peroxide activity in the tissues. This was followed by washing three times in PBS (Phosphate Buffer Solution). Primer antibodies used in the study (Table 1) were diluted in PBS. The tissues treated with polyclonal rabbit anti PPAR-α and goat PPAR-β antibodies were incubated with goat anti rabbit and rabbit anti goat immunoglobulin G for 30 minutes, respectively, in biotinylated order. The tissues incubated with monoclonal mouse anti PPAR-γ antibody were incubated with biotinylated rabbit anti mouse immunglobulin G for 30 minutes at room temperature. Negative controls were generated using goat or rabbit serum instead of the primer antibody. Subsequently, all sections were washed three times with PBS and incubated with peroxidase-conjugated streptavidin for an hour. The chromogenic solution of 3.3'-diaminobenzidine tetrahydrochloride (0.5 mg DAB/mL; Dako Corporation, Carpinteria, USA) dissolved in phosphate buffer solution was applied to the tissue sections as a color-evident substrate for 3 minutes. Background painting was done with Mayer Haematoxylin. For this purpose, background dyeing was carried out by staining for 5 seconds and then washing in tap water for 5 minutes. Subsequently, the sections were dehydrated and covered with immunomount. Finally, sections were examined by light microscopy.

Table 1. Specificity, clone, origin, firm and dilutions of primer antibodies used in immunohistochemical staining.

Specificity	Clone	Origin	Firm	Dilution
PPAR-α	Rabbit PAb IgG, H98, sc-9000	Human	SANTA CRUZ	1:100
PPAR-β	Goat PAb IgG, K20, sc-1987	Mouse	SANTA CRUZ	1:100
PPAR-γ	Mouse MAb IgG, E8, sc-7273	Human	SANTA CRUZ	1:100

Analysis of Immunohistochemical Staining Results

PPAR-α, PPAR-β and PPAR-γ immunoreactivity were examined using a grading system based on the number of positive cells in the areas that best reflected the staining character. The analysis for the quantification of immunological staining in the tissue was started based on the high intensity reaction areas. For each sample, a total of 400 magnifications were examined with 10 separate areas, each with a total of 100 grids (10x10) and a total area of 0.025 mm^2. Percentages of positive stained cells in each area were recorded and the average of these 10 sites was taken as the data of that animal. PPAR-α, PPAR-β and PPAR-γ staining intensities seen in the cytoplasm and nuclei of cells were made semiquantitatively for cytoplasm and nuclei separately.

Statistical Analysis

SPSS for Windows 10.0 program was used for the statistical analysis of the data obtained in the study. The data are listed as mean ± standard error on the tables. The comparisons of groups were performed using repeated measure of ANOVA. P-values <0.05 was accepted as significant.

RESULTS

Clinical Findings

Clinical examinations revealed no abnormal signs throughout the experiment.

Biochemical Analysis Findings

Biochemical parameters with regard to liver and oxidative stress parameters are given previously (Selek et al., 2011). Total lipid and glucose levels of the rabbits evaluated in the study are given in Table 2. The results of β-oxidation of fatty acids in the liver are shown in Table 3.

Table 2. Total Lipid and Glucose levels in rabbits fed on Normal and high fat diet (mean ± standard error).

Parameters	Group	Weeks				P
		0.	3.	6.	9.	
Total lipid	GrI	520.4±10.9	522.9±18.7 **B**	530.2±8.3 **BC**	528.7±13.2 **BC**	
	GrII	526.7±8.0	519.3±12.6 **B**	516.3±7.1 **C**	518.8±6.2 **C**	
	GrIII	522.9±8.9	517.8±13.5 **B**	523.3±12.6 **BC**	519.7±8.9 **C**	
	GrIV	521.8.±12.2	515.9±18.0 **B**	519.7±15.8 **BC**	514.7±17.0 **C**	
	GrV	534.1±12.0 **c**	577.1±5.9 **A,b**	607,4±7,3 **A,a**	632.2±7.7 **A,a**	**P<0.001**
	GrVI	538.9±12.6	551.2±12.6 **AB**	557.1±8.4 **B**	560.2±13.1 **B**	
	GrVII	535.4±10.6	548.4±15.2 **AB**	551.1±14.6 **BC**	563.7±12.8 **B**	
	GrVIII	537.2±14.7	547.2±20.2 **AB**	554.2±15.9 **B**	559.4±16.3 **B**	
	P		**P=0.064**	**P<0.001**	**P<0.001**	
Glucose	GrI	118.3±3.3	118.3±2.9	120.9±2.8	120.9±2.8 **BC**	
	GrII	119.4±1.8	122.1±2.8	123.4±2.9	123.1±3.2	

				ABC	
	GrIII	120.3±2.6	124.6±1.9 **ab**	127.7±1.7 **a**	129.4±1.7 **AB,a**	**P<0.05**
	GrIV	118.1±2.9	124.4±3.7	125.7±3.7	128.8±3.5 **AB**	
	GrV	118.6±3.4	118.7±3.2	116.3±2.8	117.4±3.1 **C**	
	GrVI	117.4±3.2	120.9±3.6	122.7±3.9	123.4±3.3 **ABC**	
	GrVII	118.9±3.2	124.1±2.4	126.6±3.7	131.4±3.9 **A**	
	GrVIII	119.4±2.9	123.7±3.2	126.9±3.0	130.6±3.9 **AB**	
P					**P<0.05**	

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **ABCDE:** Shows statistical significance according to week and intra-group on column base **abcde:** Shows statistical significance according to week and intra-group on line base

Table 3. The levels of peroxisomal β-oxidation in the liver tissue of rabbits fed a normal and high fat diet (µmol/minute/gr liver) (mean ± standard error).

Parameter	**Groups**				
	Group I	Group II	Group III	Group IV	
β- oxidation (µmol/min/gr liver)	0.91±0.24 **b**	0.99±0.11 **b**	1.14±0.13 **b**	1.39±0.32 **a**	**P<0.01**
	Group V	Group VI	Group VII	Group VIII	
	0.89±0.08 b	**1.04±0.08 b**	**1.48±0.27 a**	**1.51±0.33 a**	**P<0.001**

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base

Histopathological Examination Findings

Macroscopic Findings

On gross examination, the most striking finding was a significant increase in abdominal fat tissue observed only in the group treated with high fat diet (Group V). Gemfibrozil administration resulted in a significant decrease in abdominal fat tissue in all groups but the decrease was much more pronounced in gemfibrozil and L-carnitine treated group (Group VIII). The liver was pale and fragile in only animals fed high fat diet (Group V). Gemfibrozil and high fat diet also caused an increase in liver weight (Table 4, 5). The proportion of fat weight to body weight (tissue or organ weight x 100/live weight) is summarized in Table 4.

Histopathological Findings

Steatosis was noted in liver of only rabbits fed high fat diet and the details are given elsewhere (Selek et al 2011). A marked hypertrophy was observed in the adipocytes in omentum, inguinal fat and fat around kidney of rabbits fed with high fat diet. No significant histopathologic changes were observed in the kidneys except for mild glomerular changes in the rabbits fed with high fat diet. In the rabbits treated with gemfibrozil, degenerative and necrotic changes were observed mildly in the proximal tubulus epithelium, but no degenerative changes were observed in any of the rabbits treated with L-carnitine. In group V, mild hyperplasia in the pancreas Langerhans islets on rabbits was noted. In group V and VI vacuolization in the acinar cells of pancreas was

evident whereas no vacuoles found in the acinic cells in the other groups.

Table 4. Weekly animal weight change (gr) and carcass parameters in normal and high fat diet groups (gr) (mean ± standard error).

Parameter	Groups				P
	Group I	**Group II**	**Group III**	**Group IV**	
AW increase (gr)	1042.9±102.0 **a**	442.9±102.0 **c**	757.1±108.8 **ab**	628.6±89.2 **bc**	**P<0.01**
AW increase %	46.1±5.0 **a**	17.9±4.9 b	31.9±5.9 ab	27.7±4.8 b	**P<0.01**
Liver (gr)	103.8±2.9	100.3±5.0	98.9±6.6	104.3±5.9	
Brain (gr)	9.23±0.09 **b**	9.61±0.14 **ab**	9.71±0.17 **a**	9.42±0.14 **ab**	**P<0.05**
Omentum fat (gr)	23.8±3.7	22.7±4.8	20.9±2.3	16.8±3.2	
Upper renal fat (gr)	54.4±7.7	48.7±8.8	41.3±5.3	36.1±5.1	
Total abdominal fat (gr)	78.2±11.0	71.4±13.2	62.2±6.9	52.8±7.9	
Abdominal fat/AW ratio (%)	2.33±0.30	2.32±0.44	1.91±0.21	1.77±0.24	
Abdominal fat/carcas ratio (%)	4.46±0.54	4.45±0.76	3.69±0.38	3.29±0.45	
	Group V	**Group VI**	**Group VII**	**Group VIII**	
AW increase (gr)	771.4±101.7 **ab**	900.0±37.8 **a**	714.3±114.3 **ab**	514.3±96.2 **b**	**P<0.05**
AW increase %	33.8±4.8 **ab**	41.6±2.0 **a**	32.5±5.3 **ab**	23.1±4.9 **b**	**P<0.05**
Liver (gr)	92.9±5.5 **a**	93.7±3.9 **a**	86.8±2.9 **ab**	76.9±3.9 **b**	**P<0.05**
Brain (gr)	9. 57±0.33 **ab**	9.12±0.16 **b**	9.60±0.17 **ab**	9.90±0.12 **a**	**P<0.05**
Omentum fat (gr)	51.7±10.6 **a**	28.7±5.9 **b**	21.6±3.5 **b**	16.9±2.1 **b**	**P<0.01**
Upper renal fat (gr)	134.9±14.9 **a**	88.0±11.6 **b**	65.8±9.9 **bc**	48.1±8.5 **c**	**P<0.001**
Total abdominal fat (gr)	186.6±21.3 **a**	116.8±16.4 **b**	87.4±13.3 **bc**	65.0±10.0 **c**	**P<0.001**
Abdominal fat/AW ratio (%)	6.00±0.60 **a**	3.75±0.44 **b**	2.93±0.39 **bc**	2.27±0.30 **c**	**P<0.001**
Abdominal fat/carcas ratio (%)	**10.36±0.94 a**	**6.83±0.86 b**	**5.29±0.68 bc**	**4.21±0.62 c**	**P<0.001**

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group **abc:** Shows statistical significance according to week and intra-group on line base

Immunohistochemical Examination Findings

PPAR-α expression was significant especially in the liver, kidney and heart. High fat diet and gemfibrozil administration caused a significant increase in PPAR-α expression. On the contrary, L-carnitine administration was found to have no positive or negative effect on PPAR-α expression in tissues.

PPAR-α expression was significant especially in the liver, kidney and heart. The PPAR-α immunoreactive reaction in the liver, especially in hepatocytes, was determined both

cytoplasmic and nuclear. The number of cells stained with PPAR-α primer antibody and the staining intensity were higher in rabbits fed high fat diet+gemfibrozil (Group VI) and high fat diet gemfibrozil + L-carnitine (Group VIII) than rabbits in all groups and high fat diet group were higher compared to rabbits fed normal diet and normal diet+L-carnitine (Figure 1).

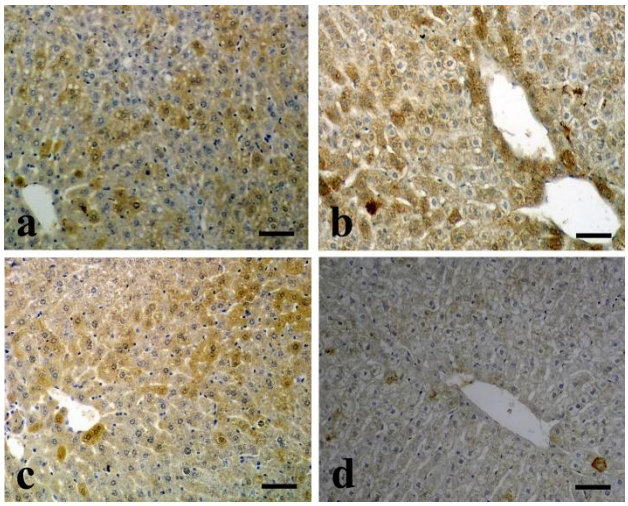

Figure 1. PPAR-α expression in the liver. Avidin Biotin Peroxidase Complex (ABC). High fat diet (a), high fat diet+ gemfibrozil (b), high fat diet+ gemfibrozil+ L-carnitine (d) fed rabbits showed less number of stained cells and staining intensity with PPAR-α in the liver than normal diet (d) fed rabbits. (Bar =50 µm).

There was a difference in PPAR-α expression between gemfibrozil and high fat diet groups and those received normal diet only. However, L-carnitine administration did not affect PPAR-α expression. The PPAR-α positive reaction in liver was more pronounced in periaciner (centrilobular) hepatocytes in rabbits fed normal diet and normal diet+L-carnitine. On the other hand, positive reactions occurred in hepatocytes located in periasiner and midzonal regions and a little less in centriaciner areas in rabbits treated with high fat diet and high fat diet+L-carnitine. Almost all areas of hepatocytes were stained positively in high fat diet+gemfibrozil (Group VI) and high fat diet +gemfibrozil+L-carnitine (Group VIII).

Table 5. Comparison of abdominal fat weight, abdominal fat weight/ animal weight, abdominal fat weight/ carcas ratio between groups with similar nutrition and medication by week in rabbits fed on normal and high fat diet (mean ± standard error).

Groups	Omentum fat (gr)	Upper renal fat (gr)	Total abdominal fat (gr)	Abdominal fat/AW (%)	Abdominal fat/carcas (%)
Group I	23.8±3.7	54.4±7.7	78.2±11.0	2.3±0.3	**4.46±0.55**
Group V	51.7±10.6	134.9±14.9	186.6±21.3	6.0±0.6	**10.36±0.94**
P	**P<0.05**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**
Group II	22.7±4.8	48.7±8.8	71.4±13.2	2.3±0.4	**4.45±0.76**
Group VI	28.7±5.9	88.0±11.6	116.8±16.4	3.8±0.4	**6.83±0.86**
P		**P<0.05**	**P<0.05**	**P<0.05**	**P=0.062**
Grup III	20.9±2.3	41.3±5.3	62.2±6.9	1.9±0.2	**3.69±0.38**
Grup VII	21.6±3.5	65.8±9.9	87.4±13.3	2.9±0.4	**5.29±0.68**
P		**P<0.05**		**P<0.05**	**P=0.064**
Grup IV	16.8±3.2	36.1±5.1	52.8±7.9	1.8±0.2	**3.29±0.45**
Grup VIII	16.9±2.1	48.1±8.5	65.0±10.0	2.3±0.3	**4.21±0.62**
P					

GrI: Normal diet group; **GrII:** Normal diet + Gemfibrozil group; **GrIII:** Normal diet + L-carnitin group; **GrIV:** Normal diet + L-carnitin + Gemfibrozil group **GrV:** High fat diet group; **GrVI:** High fat diet + Gemfibrozil group; **GrVII:** High fat diet + L-carnitin group; **GrVIII:** High fat diet + L-carnitin + Gemfibrozil group

The PPAR-a positive reaction in the kidneys was most prominently found in the proximal tubulus epithelium. In all groups, almost all of the proximal tubular epithelium was stained positively, but the density of staining was more prominent especially in rabbits treated with high fat diet and high fat+gemfibrozil diet than the other groups. Moreover, a positive reaction was found in distal tubular epithelium in these groups. There was no positive reaction in the glomerulus.

PPAR-α expression was also detected in epithelium cells in the stomach and intestines, cortical cells in the adrenal gland, cardiac

muscle and smooth muscle cells, adipocytes and macrophages (Figure 2).

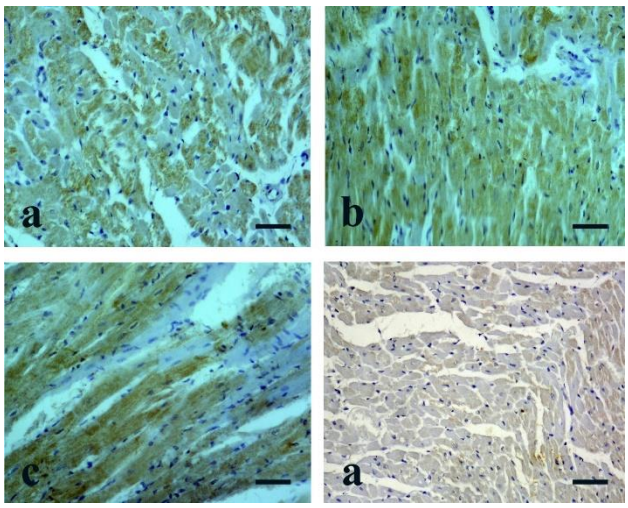

Figure 2. PPAR-α expression in the heart muscle (ABC). High fat diet (a), high fat diet+ gemfibrozil (b), high fat diet+ gemfibrozil+ L-carnitine (d) fed rabbits showed more number of stained cells and staining intensity with PPAR-α in the liver than normal diet (d) fed rabbits. (Bar a, b,c ve d =100 µm, e = 50 µm).

The staining density in the liver was more prominent in the rabbits fed the high fat diet, while no significant difference was found between the groups in terms of number of cells stained positive with PPAR-β primer antibody in tissues (Figure 3). PPAR-β immunoreactivity was present in almost all tissues. The positive reaction in liver tissue was mostly nuclear and cytoplasmic localization, usually granular. However, diffuse staining was also observed in groups that were given high fat diet. Positive immunological reactions in the liver, which were granular in almost all hepatocytes, showed diffuse staining especially in centri-acinar areas in high fat fed animals.

No positive staining was observed in immunohistochemical staining with PPAR-γ primer antibody. Positive reaction to tissue staining with PPAR-γ in mouse tissues was detected to remove doubts about whether the primer antibody is working.

The positive reaction in rabbits treated with normal diet and normal diet+L-carnitine in the kidneys was particularly severe in the tubulus epithelia in the cortical region, and mostly nuclear localization however very dense cytoplasmic staining was observed in the high fat diet animals (Figure 4 a-c). As for the adrenal gland, in almost all groups, PPAR-β expression was observed especially in cortical cells, both cytoplasmic and nuclear localization.

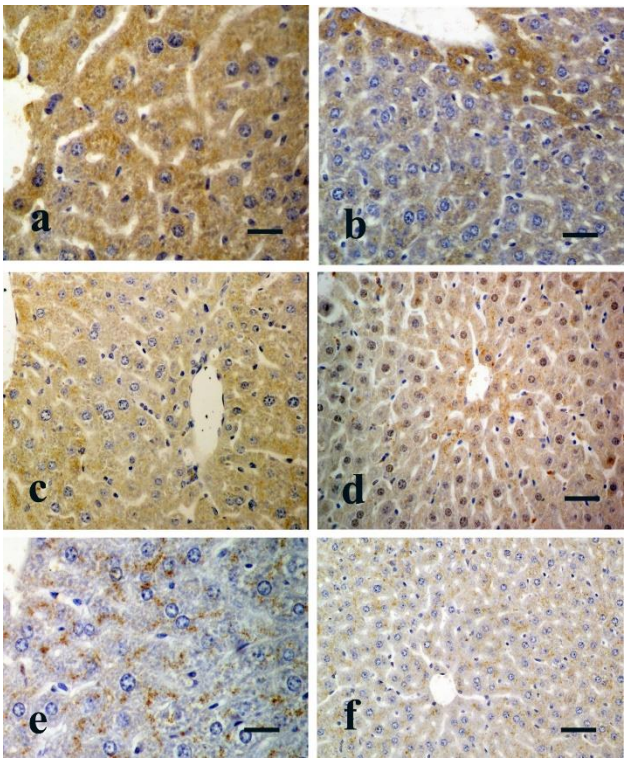

Figure 3. PPAR-β expression in the liver and kidneys. Avidin Biotin Peroxidase Complex (ABC). High fat diet (a,b,d,e,f) and gemfibrozil (b and d) administration resulted in a positive reaction in the liver (a,b,c,d), mostly cytoplasmic and granular. In the kidneys (e and f) both cytoplasmic and nuclear positive reactions are seen. There is no difference between the groups in terms of positive number of stained cells and intensity of staining. (Bar c,d and f = 30 µm, a,b and e = 20 µm).

Positive reaction in heart muscle was in cardiac myocytes and granular style. Cytoplasmic and nuclear staining was also noted in adipocytes in the adipose tissue and stomach and intestinal epithelial cells. In the central nervous system, it was nuclear localization in almost all neurons and sometimes in glia cells (Figure 4 d).

Weak cytoplasmic localized immunoreactivity was observed in beta cells of the pancreas Langerhans islets. In the alpha cells located in the periphery of the islets, intense cytoplasmic and nuclear staining was

observed. In some cases, a mild positive reaction with nuclear localization was observed in exocrine pancreatic acinar cells.

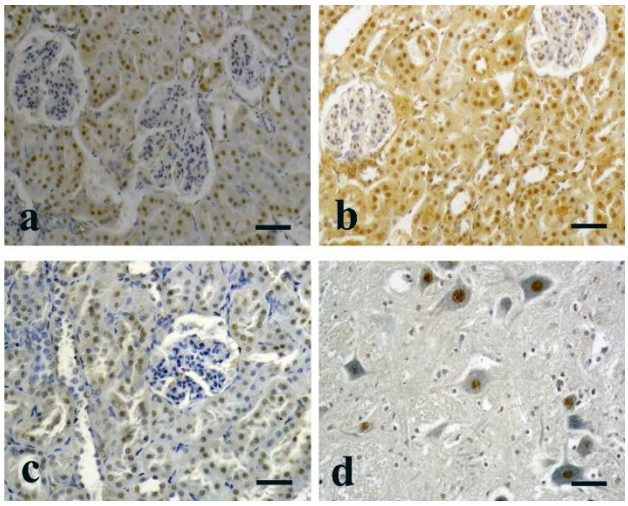

Figure 4. Stomach (a), heart (b), pancreas (c ve d), cerebellum (e) and brain stem (in neurons) (f) cytoplasmic and nuclear PPAR-β expression. (Bar a,b and c = 100 µm, d = 50 µm).

DISCUSSION

In this study, the effects of Gemfibrozil an antihyperlipoproteiemic fibrate, and L-carnitine were investigated on obesity, fattening and distribution of PPARs in tissues histopathologically (macroscopic, microscopic and histochemical staining methods) and biochemically on a model for obesity developed by feeding high fat diet in rabbits. In this study, exogenous administration of gemfibrozil and L-carnitine had various effect on plasma total lipid concentrations and fattening. The relationship between hepatic lipidosis, oxidative stress and Gemfibrozil and L carnitine was discussed elsewhere (Selek et al., 2011)

Gemfibrozil is reported to be used as a lipid regulator in human as it causes elevation in cholesterol, triglycerides, VLDL, LDL levels and cholesterol/HDL cholesterol, and decreases in cholesterol HDL levels (Tunca and Devrim, 2007; Schoonjans et al., 1996; Costet et al., 1998; Stalenhoef et al., 2000; Barter and Rye, 2006). The hypothesis that the effect of gemfibrozil on triglyceride level reduction is due to the lipid-regulating role of fibrates in increasing the activation of PPARs and consequently triglyceride catabolism (Mussoni et al., 2000; Schoonjans et al., 1996; Zambon et al., 2007) has been suggested by researchers.

Mondola et al. (1992) found that L-carnitine addition reduced serum triglycerides, cholesterol and free fatty levels in their studies in rats. It has also been reported that gemfibrozil in combination with other drugs leads to lower levels of serum triglycerides and other lipid profile parameters and is a good lipid regulator in this respect (Wägne et al., 2003). In our study, in Group VIII, in which L-carnitine and gemfibrozil were administered together, a much lower lipid and abdominal fat as compare to Group V-VII was thought to be due to the synergistic effect of these two drugs as this positive synergistic effect between the two drugs was first demonstrated in our study. This synergistic effect supports the findings of the study, in which Ringseis et al. (2008) reported that clofibrate, synthetic PPAR activator, increased intestinal carnitine absorption which in turn had additive effect.

Beta Oxidation

In the present study, beta oxidase was increased by 10% in Group II, 30% in Group III, approximately 40% in Group IV, 20% in Group VI, 40% in Group VII and approximately 50% in Group VIII. Gemfibrozil and L-carnitine addition (Group VIII) caused marked increase in beta-oxidation compared to all other groups.

Increases in β-oxidation rates due to gemfibrozil administration were compatible with the previous studies where gemfibrozil is shown to play an important role in fatty acid catabolism by increasing the gene expression responsible for PPAR-α mitochondrial fatty acid oxidation activated with fibrates, peroxisomal fatty acid oxidation and other fatty oxidation in the cell (Akbiyik et al., 2004; Mandard et al., 2004; Huang et al., 2009). PPAR-α agonists have been widely used for

decreasing plasma triglyceride and low density lipoprotein cholesterol and increasing high density lipoprotein levels (Guerre-Milloet al., 2000). Increasing β-oxidation in the liver and skeletal muscle is one of the reasons that leads to the effects mentioned previously (Minnich et al., 2001). Moreover PPAR-α also regulates liver metabolism by increasing glycolysis and reducing gluconeogenesis in the liver (Atherton et al., 2006) and it is shown that PPAR-α agonists have potent ability to lower glucose concentrations in blood plasma (Kim et al., 2003). In this context, free fatty acids are thought to increase the oxidation of fatty acids in the liver by increasing PPAR-α expression (Akbiyik et al., 2004; Mandard et al., 2004; Huang et al., 2009). These beneficial effects of PPAR-α ligands have been attributed by researchers to the fact that they increase rate-limiting peroxisomal (AOX) and mitochondrial (AD) β-oxidation enzyme activities in hepatocyte mitochondria and peroxisomes and lead to increased oxidation of fatty acids (Akbiyik et al., 2004; Lawrence et al., 2001).

The role of L-carnitine in transporting long chain fatty acids to mitochondria for β-oxidation has long been known (Kopec and Fritz, 1973; Lohninger et al., 1987; McCarty, 2001; Longo et al., 2016). Fritz (1955), for the first time in a mammalian animal study, has shown that L-carnitine increases the oxidation of fats in the liver. We are in the opinion that the findings of β-oxidation increase in our study, especially in Group VIII, are compatible with both other investigators and other findings of our study which led us to think that these two drugs have a positive synergistic effect.

PPAR Expression

High fat diet and gemfibrozil administration caused a significant increase in PPAR-α expression. On the other hand, L-carnitine administration was found to have no effect on PPAR-α expression in tissues, either positively or negatively. There was a difference in PPAR-α expression between gemfibrozil and high fat diet groups and non-high fat diet groups. High fat diet and gemfibrozil administration also caused more PPAR-α expression than gemfibrozil administration alone. This finding supports the data that clofibrate administration was more likely to cause PPAR-α activation than high fat diet administration in rats (Akbiyik et al., 2004) and mice (Tunca and Devrim 2007).

The increase in PPAR-α expression in the liver with the administration of high fat diet is probably been related to the increase in fatty acid concentrations in hepatocytes. Free fatty acids, especially palmitic acid, oleic acid, linolenic acid and arachidonic acid, are known to be natural ligands of PPAR-α (Tunca and Devrin, 2007; Yu et al., 1995). Free fatty acids not only increase the number of these receptors but also activate this receptor, leading to peroxisome proliferation (Akbiyik et al., 2004; Tunca and Devrim 2007). The target genes of PPAR-α are associated with the oxidation of mitochondrial and peroxisomal fatty acids and other lipid metabolites in the cell (Mandard et al., 2004). Thus, free fatty acids increase the PPAR-α expression and increase the oxidation of fatty acids in the liver (Akbiyik et al., 2004; Mandard et al., 2004).

The increase in PPAR-α and β expression in the liver was immunohistochemically increased in the animals fed with the high fat diet, whereas PPAR-γ expression was not affected. While there was no striking difference in the number of cells stained between the groups in terms of the number of cells stained with PPAR- β primer antibody in tissues, the density of staining especially in the liver was more prominent in rabbits fed the high fat diet. Although PPAR-β immunoreactivity was present in almost all tissues, no positive staining was observed in immunohistochemical tissue staining with PPAR-γ primer antibody. As in

PPAR-α, unsaturated fatty acids are natural ligands of PPAR-γ (Lehrke and Lazar, 2005).

Although it has been reported that the target genes of PPAR-γ are responsible for adipocyte differentiation, lipid storage and glucose metabolism, and are important for adipocyte differentiation both in vitro and in vivo (Xu et al., 2003), in this study no positive staining was observed in tissue staining with PPAR-γ primer antibody. The fact that PPAR-α was not affected by high fat diet administration resulted in the ineffectiveness of fatty acids in PPAR-β activation, although PPAR-α had a significant role in the oxidation of fatty acids as opposed to PPAR-γ in the study.

Animal Weight and Organ Weights

High fat diet administration (Group V) caused a significant increase in abdominal fat tissue. A more significant reduction in abdominal fat tissue mass occurred in groups of rabbits given gemfibrozil and L-carnitine separately (Group VI, VII) as well as togather (Group VIII).

It was determined that the liver weights obtained in group VI were higher than the other groups (Group V, VII and VIII). Macroscopically, the most striking finding was an increase in abdominal fat tissue in group only fed high fat diet (Group V). However, gemfibrozil administration resulted in a significant decrease in abdominal fat tissue in all groups. This decrease was much more pronounced in animals treated with gemfibrozil and L-carnitine (Group VIII) than all other groups. Total abdominal fat, abdominal fat/animal weight and abdominal fat/carcass ratios were significantly lower in Group VIII than in Groups V, VI and VII. The animals fed with high fat diet (Group V) had a higher omental fat, upper renal fat, total abdominal fat amount, abdominal fat/ animal weight ratio and abdominal fat/ carcass ratio than the group fed on the normal diet (Group I). This findings are in agreement with those studies where the effect of PPAR-α on obesity was investigated (Tunca and Devrim, 2007). Studies in null mice have reported the lower capacity of PPAR-α to metabolize long-chain fatty acids and consequently, dyslipidemia (Peters et al., 1997) and subsequent occurance of fat deposition (Costet et al., 1998). PPAR-α, activated by synthetic agonists such as unsaturated fatty acids and eicosanoids and synthetic agonists such as fibrates, plays an important role in the catabolism of fatty acids by increasing the expression of the gene responsible for PPAR-α mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation and other fatty oxidation in the cell (Mandard et al., 2004). Similarly, the lower amount of abdominal fat determined in Group VIII might be due to both the activation of gemfibrozil PPARs and the role of L-carnitine in fat transport to mitochondria (Kopec and Fritz, 1973; Lohninger et al., 1987; McCarty, 2001). Studies in food animals (cattle and pigs) reported increased the digestibility of feed and feed oils (LaCount et al., 1995), decreased the amount of fat in the gluteal region (Smith et al., 1994) and thus markedly increased the meat / fat ratio in the carcass (Owen et al., 1996) following administration of exogenous L-carnitine. Our findings are in line with the previous researchers (Keene, 1991). As PPAR-α ligands such as clofibrate, bezfibrate and gemfibrozil already reduced liver fat (Akbiyik et al., 2004; Toda et al., 2003; Tunca and Devrim, 2007).

CONCLUSION

As a result, gemfibrozil and L-carnitine application in this experimental model had positive effects on abdominal and hepatic fattening and lipid profile improvement. In this model, gemfibrozil administration caused a significant increase in PPAR-α expression in the liver, whereas PPAR-β and PPAR-γ expression were not affected. High fat diet

application increased PPAR-α expression but was ineffective on PPAR-γ and PPAR-β expression. However, L-carnitine administration had no significant enhancing effect on PPARs expression in both normal and high fat diet. It may be suggested that hepatic lipidosis due to high fat diet in rabbits might have been inhibited by gemfibrozil and L-carnitine administration. In addition, abdominal fat (obesity) due to high fat diets could be prevented by gemfibrozil, a synthetic PPAR-a ligand, and L-carnitine, and consequently reduces associated health problems.

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Ethical approval:

The study was approved from the Kafkas University Faculty of Veterinary Animal Experiments Local Ethics Committee
(Protocol number: 20.12.2005/27).

Conflict of interest: All authors have contributed to the design, conduct of the study/data collection, interpretation of data and writing of the manuscript.

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