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RESEARCH ARTICLE

The Effects of Gemfibrozil and Ovariectomy on the Peroxisome Proliferator Activated Receptors (PPARs) in Mice with Experimentally Induced Obesity [1]

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Summary

Effects of gemfibrozil and ovariectomy on Peroxisome Proliferator Activated Receptors (PPARs) activity were studied in obese mice. Ovariectomised and sham operated mice were fed with high fat diet and low fat diet for 15 weeks. Furthermore, gemfibrozil (100 mg/kg) was orally with high fat and low fat diets to the ovariectomised and sham operated mice. Body weights significantly increased in ovariectomised mice compared to sham operated mice ($P<0.001$) while gemfibrozil supplementation prevented such increase. Steatosis was as well more pronounced in the livers of ovariectomised mice fed with high fat diet compared to sham operated mice. However, liver steatosis was not seen in gemfibrozil supplemented high fat diet fed mice. On the other hand gemfibrozil supplementation slightly increased the liver weights. High fat diet significantly increased liver AST and ALT enzyme levels and serum total cholesterol, triglyceride and VLDL levels while gemfibrozil lowered these parameters. Immunohistochemically, while tissue PPAR-α and PPAR-γ expressions were affected by ovariectomy, diet and gemfibrozil, PPAR-β expression unchanged. While high fat diet incremented liver PPAR-α and PPAR-γ expressions their levels were reduced following ovariectomy operation. Only high fat diet and only gemfibrozil application increased PPAR-α expression level. PPAR-α expression levels were higher in gemfibrozil and high fat diet combined groups compared to all other groups. In conclusion, gemfibrozil reduced abdominal and hepatic fat deposition in mice with high fat diet and ovariectomy.

Keywords: *Gemfibrozil, Ovariectomy, PPAR-α, PPAR-β, PPAR-γ, High fat diet*

Deneysel Obezite Oluşturulan Farelerde Ovarioektomi Operasyonu ve Gemfibrozilin Peroksizom Proliferatörleri ile Aktive Olan Reseptörler (PPARs) ve Obezite Üzerine Etkilerinin Araştırılması

Özet

Ovarioektomi uygulanan farelerde deneysel olarak oluşturulan obezitenin peroksizom proliferatörleri ile aktive olan reseptörler (PPARs) üzerine etkisi araştırılmıştır. Bu amaçla ovarioektomi ve sham uygulanan fareler yağlı diyet ve düşük yağlı diyetle 15 hafta süre ile beslendi. Ayrıca, ovarioektomi ve sham uygulanan farelere farklı diyetlerle beraber 100 mg/kg dozunda oral gemfibrozil uygulandı. Çalışma sonunda ovarioektomi operasyonu uygulanan farelerde sham uygulanan farelere göre istatistiksel olarak anlamlı ($P<0.001$) canlı ağırlık artışı şekillenirken gemfibrozil uygulaması bu canlı ağırlık artışını engelledi. Yüksek yağlı diyet uygulanan farelerde, ovarioektomi uygulanan grupta, sham uygulanan farelerin karaciğerlerine göre daha belirgin bir steatozis tablosu görüldü. Diğer yandan yüksek yağlı diyet ve gemfibrozil uygulanan gruptaki fare karaciğerlerinde steatozis görülmedi. Buna karşın gemfibrozil uygulaması karaciğer ağırlığında hafif şiddette artışa neden oldu. Yüksek yağlı diyet uygulaması karaciğer AST ve ALT enzim seviyeleri ile plazma toplam kolesterol, trigliserid ve VLDL seviyelerinde artışa neden olurken gemfibrozil uygulaması bu parametrelerde iyileşme ile sonuçlandı. İmmunohistokimyasal olarak dokulardaki PPAR-α ve PPAR-γ ekspresyonu ovarioektomi operasyonu, diyet ve gemfibrozil uygulamasından etkilenirken PPAR-β ekspresyonu etkilenmedi. Yağlı diyet uygulamasında PPAR-α ve PPAR-γ ekspresyonu artarken ovarioektomi operasyonunda azaldı. Yalnızca yağlı diyet ve yalnızca gemfibrozil uygulanan gruplarda PPAR-α ekspresyonunda artış şekillendi. Yağlı diyet ile gemfibrozilin beraber uygulandığı gruplarda ise PPAR-α ekspresyonu diğer tüm gruplara göre daha fazla arttı. Çalışma sonunda gemfibrozil uygulamasının yüksek yağlı diyet ve ovarioektomi oluşturulan farelerde abdominal ve hepatik yağlanmanın azalmasında olumlu etkileri olduğu görüldü.

Anahtar sözcükler: *Gemfibrozil, Ovarioektomi, PPAR-α, PPAR-β, PPAR-γ, Yağlı diyet*

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the family of ligand-inducible nuclear receptors. Three isotypes called PPAR-α, -β, and - γ have been identified in lower vertebrates and mammals [1,2]. While PPAR-α is expressed in the liver, heart and kidneys PPAR-β is mainly present in the adipose tissue, skin and brain, and PPAR-γ is present in adipose tissue, large intestines, heart, kidneys, pancreas and spleen [3-5]. PPAR-γ is activated by prostaglandin J_2 whereas PPAR-α is activated by leucotrien B_4 [6,7]. PPARs are known to regulate glucose metabolism, energy balance and body weight. These effects are partially related to beta-oxidation of fatty acids leading to fatty acid degradation in the liver [8,9].

Activation of PPAR-α can occur as a result of treatment with hypolipidemic fibrate class of drugs (fenofibrate, gem-fibrozil etc) [3,9]. Fibrates organize a series of genes responsible for lipid and lipoprotein synthesis. Activated PPAR ligands induce heterodimerization with retinoid X receptor, and the subsequent interaction with steroid receptor co-activators, followed by binding to PPAR response elements [3,10]. Fibrates regulate energy homeostasis. A diet rich in energy results elevated plasma triglyceride and cholesterol levels. Consequently, high levels of triglycerides present in the circulation cause lipocyte hypertrophy and hyperplasia [11-13]. Therefore, it was suggested that obesity could be prevented by fibrates that have the ability to reduce plasma triglyceride and fatty acids levels [11,12].

Studies in humans and laboratory animals indicate that estrogen has a significant role in the adipose tissue regulation [14,15]. It has been reported that ovariectomy increases adiposity in rodents, and administration of estrogen derivatives reduce fat deposition in these animals [16]. Similarly, following menopause total body fat increases in women and of estrogen-replacement therapy attenuates the accumulation of fat in postmenopausal woman [15]. D'Eon et al.[17] reported in their ovariectomized mice model of menopause, estrogen replacement up-regulates PPAR-δ mRNA expression. Estrogen replacement also up-regulates PPAR-α gene expression [18] and increases lipid peroxidation in genetically PPAR-α deficient mice [19].

In the present study, the effects of gemfibrozil administration for the treatment of lipid metabolism disorders due to decreased estrogen levels was investigated in ovariectomized rodent model of menopause. Furthermore, the effects of various treatment and intervention methodologies including high fat diet, gemfibrozil treatment and ovariectomy in the tissue distribution and expression levels of PPARs were investigated by immunohistochemistry.

MATERIAL and METHODS

Animals and Husbandry

The experiment and study design were approved by the Experimental Ethics Committee, Kafkas University, Kars, Turkey. All animals were maintained in accordance to university policies. The material was consisted of 80 female (12-14 weeks old, 27±2.33 g bw) Swiss albino mice supplied from Ataturk University. The animals were housed in a well-ventilated, temperature-controlled room (23±2ºC), at 55% relative humidity under a 12 h light/dark cycle until the end of experiment, which lasted 15 weeks.

Eighty mice were initially divided equally into two main groups. Fourty mice in the first group were further subdivided into four groups (Group 1, 2, 3 and 4; *n*=10). Mice in these groups were ovariectomized by surgery performed from the median line. Mice from other main part were also subdivided into four groups (Group 5, 6, 7 and 8; *n*=10) and they were only sham operated from the median line.

Diet Preparation

Low fat diet (2.500 Kcal metabolic energy and 4.5% crude fat) were given *ad libitum* to the groups 3, 4, 7, and 8 througout the experiment. High fat diet (16.03 g margarine containing 80% vegetable fat melted at 50ºC and mixed with 83.97 g rodent chow containing 4.5% crude fat) were given *ad libitum* to the groups 1, 2, 5, and 6. The final composition of the high fat diet was calculated to posses 3109 Kcal metabolic energy and 15% fat.

Experimental Design and pathological examinations

Following 20 days of convalescence period, ovariectomized mice were divided into 4 groups (*n*=10; Group 1, 2, 3, and 4). Mice in Group 1 were given only high fat diet (15% fat) for 15 weeks. Group 2 received high fat diet plus gemfibrozil (Lopid®, Pfizer). Group 3 was fed with low fat diet (4.5% fat) plus gemfibrozil. Mice in the Group 4 were fed only with low fat diet (4.5% fat) for 15 weeks. Sham operated mice were also allocated in 4 groups (*n*=10; Group 5, 6, 7, and 8) and these groups were also received high fat diet, high fat diet plus gemfibrozil, low fat diet plus gemfibrozil and only low fat diet, respectively. Gemfibrozil was given orally (100 mg/kg). During the experimental period, animals were monitored three times a day, 30 minutes each and, weighed weekly.

At the end of the 15th, week mice were euthanized by cervical dislocation. Before sacrifice blood samples were collected from the abdominal aorta and necropsy was performed. Tissue samples were fixed in 10% neutral buffered formalin. After fixation, the sections were embedded in paraffin wax, sectioned at 4-6 µm, and stained with haematoxylin-eosin (H&E). Toluidine blue was stained in semi thin sections of liver to demonstrate fat globules.

Immunohistochemistry and Evaluation of Immunostaining

Tissue sections were labelled immunohistochemically by the streptavidin-biotin-peroxidase complex (ABC) technique for detection of the polyclonal rabbit anti-PPAR-α (1/100; sc-9000; Santa Cruz, California, USA), polyclonal goat anti-PPAR-β (1/100; sc-1987; Santa Cruz), and monoclonal mouse anti PPAR-γ (1/100; sc-7273; Santa Cruz) markers.

PPAR-α, -β and -γ immunoreactivities were evaluated on a semiquantitative grading scheme on which the number of positive cells in a certain area were counted. All sections were scanned at low magnification and analysis was commenced from the area where the staining intensity was the highest. Every sample was analysed under a light microscope with a 10x ocular (10x10 grid mounted) and an x 40 objective from a total of 10 different area (area of total analysis 0.025 mm^2). Furthermore, the staining intensity of relevant markers was also assessed as follows: (0) none; (1) weak; (2) moderate; (3) intense immunolabelling.

Serum Assays

Serum aspartat aminotransferase (AST) and alanine aminotransferase activities and total cholesterol, triglyceride and very low density lipoprotein (VLDL) concentrations were measured spectrophotometrically (Spectramax®; Plus 384) with use of commercial kits (Spinreact®, Spain).

Statistical Analysis

For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and the Tukey test using SPSS for Windows version 10.0.

RESULTS

Body Weight Changes and Gross Pathological Findings

Weekly average body weight changes were shown in detail in *Table 1*. Ovariectomy, the feed consumption and gemfibrozil administration were effective for body weight changes. Group 1 and 5, significant increases in body weight (mean; Group 1 and Group 5 was 30.74%: 21.92%) were observed. In Group 3 and 7, body weights were significantly decreased (median, Group 3: - 12.63%, and Group 7: -14.36%). In Group 2 and 6 body weight gain was 1.02%, and 0.28%, respectively. The body weight gain rate in sham operated mice in Group 4 was 2.74% while it was 6.5% in ovariectomized mice in Group 8.

Most remarkable gross finding was an evident increase of abdominal adipose tissue in the only high fat diet given groups (Group 1 and 5). On the other hand gemfibrozil administration significantly reduced abdominal fat content in groups 2, 3, 6, and 7. Differences between organ weights and their statistical significance were summarised in *Table 2*. Organ weight to body weight ratio showed that high fat diet and gemfibrozil administration significantly increased liver weights. Abdominal adipose tissue significantly increased in ovariectomy and high fat diet groups. Gemfibrozil administration significantly reduced abdominal fat contents of mice.

Table 1. *Weekly average body weights (g) changes and statistical significance between the groups*
Tablo 1. *Haftalık canlı ağırlık değişimleri ve gruplar arası istatistiksel farklılıklar*

Weeks	Groups								P
	1	2	3	4	5	6	7	8	
1	26.6±1.8	27.9±1.6	27.6±3.1	26.9±2.0	26.9±2.8	26.3±1.8	26.9±2.5	26.9±2.2	NS
2	27.5±1.8	28.7±1.7	28.4±3.3	27.6±2.3	28.1±2.8	28.4±2.1	28.1±2.8	27.8±1.9	NS
3	28.2±2.0	28.4±1.5	27.3±3.3	28.1±2.4	28.3±3.2	27.9±1.7	27.0±3.0	27.9±1.8	NS
4	28.8±2.0	27.6±1.1	27.0±3.5	27.6±2.9	28.9±3.3	27.0±2.1	27.9±4.4	27.4±1.9	NS
5	28.8±1.6	27.1±1.2	26.2±3.1	26.8±2,8	29.3±3.6	26.8±1.9	26.3±3.1	27.6±1.7	NS
6	$29.9±1.9^{A}$	$27.0±1.3^{ABC}$	$25.2±3.3^{BC}$	$27.6±2.7^{AB}$	$29.9±3.6^{A}$	$27.1±1.7^{AB}$	$24.6±2.7^{C}$	$27.3±2.1^{AB}$	P<0.001
7	$30.4±1.9^{A}$	$27.2±1.4^{BC}$	$26.2±3.3^{C}$	$27.8±2.8^{ABC}$	$29.8±4.1^{AB}$	$27.4±1.9^{ABC}$	$26.4±3.0^{C}$	$26.6±1.6^{BC}$	P<0.017
8	$31.1±1.6^{A}$	$27.4±1.1^{BC}$	$24.6±3.5^{C}$	$27.5±2.4^{BC}$	$30.0±3.9^{AB}$	$27.3±1.8^{BC}$	$25.3±2.9^{C}$	$26.5±2.0^{C}$	P<0.001
9	$32.1±1,7^{A}$	$27.3±1.3^{BCD}$	$25.8±3,1^{CD}$	$27.7±2.7^{BC}$	$30.5±3.2^{AB}$	$27.2±1.8^{BCD}$	$25.0±3.0^{D}$	$26.8±2.2^{CD}$	P<0.001
10	$32.3±2.2^{A}$	$27.7±1.3^{BC}$	$24.0±3.1^{D}$	$27.9±2.8^{BC}$	$30.8±3.4^{AB}$	$27.4±2.8^{BC}$	$25.4±3.0^{CD}$	$27.4±2.3^{BC}$	P<0.001
11	$33.3±2.1^{A}$	$27.5±1.3^{B}$	$24.3±2.8^{C}$	$27.7±2.7^{B}$	$31.3±3.5^{A}$	$27.4±2.5^{B}$	$24.4±3.0^{C}$	$27.9±2.1^{B}$	P<0.001
12	$34.0±2.1^{A}$	$27.0±1.4^{C}$	$24.3±2.8^{D}$	$27.2±2.6^{C}$	$31.6±3.1^{B}$	$26.9±1.9^{C}$	$23.2±3.1^{D}$	$27.3±2.4^{C}$	P<0.001
13	$34.7±1.4^{A}$	$27.7±1.6^{C}$	$23.3±2.4^{D}$	$27.8±2.5^{C}$	$32.0±2.8^{B}$	$26.8±2.2^{C}$	$23.5±2.8^{D}$	$27.6±2.4^{C}$	P<0.001
14	$34.7±2.1^{A}$	$27.9±1.8^{C}$	$24.2±2.6^{D}$	$28.3±3.1^{C}$	$32.4±3.2^{B}$	$26.4±1.4^{C}$	$23.2±2.7^{D}$	$28.0±2.5^{C}$	P<0.001
15	$34.8±2.3^{A}$	$27.9±1.9^{C}$	$24.1±2.2^{DE}$	$28.6±3.1^{C}$	$32.8±2.8^{B}$	$26.5±2.1^{CD}$	$23.0±2.8^{E}$	$27.6±2.4^{C}$	P<0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same column are statistically significant, ***NS:*** *Not Significant*

Table 2. *Between the groups organ weights (g) and the statistical significance of the differences*
Tablo 2. *Gruplar arasında, organ ağırlıklarındaki (g) farklılıklar ve istatistiksel önemi*

Groups	Organ Weights (g)						
	Liver	Kidneys	Spleen	Heart	Lungs	Brain	Abdominal Adipose Tissue
1	1.910±0.36^{A}	0.422±0.09	0.198±0.08^{A}	0.145±0.03	0.351±0.05^{AB}	0.452±0.03^{A}	0.882±0.158^{A}
2	1.793±0.24^{AB}	0.382±0.06	0.126±0.04^{B}	0.135±0.03	0.329±0.06^{ABC}	0.393±0.03^{D}	0.350±0.104^{D}
3	1.658±0.23^{BC}	0.400±0.12	0.165±0.05^{A}	0.153±0.05	0.383±0.16^{BC}	0.457±0.02^{A}	0.187±0.051^{E}
4	1.337±0.06^{DE}	0.388±0.07	0.128±0.03^{B}	0.157±0.29	0.274±0.04^{A}	0.437±0.02^{AB}	0.554±0.110^{C}
5	1.520±0.11^{CD}	0.360±0.03	0.122±0.03^{B}	0.130±0.02	0.278±0.05^{C}	0.396±0.04^{D}	0.731±0.200^{B}
6	1.434±0.24^{DE}	0.333±0.08	0.138±0.05^{B}	0.124±0.03	0.257±0.06^{C}	0.407±0.03^{CD}	0.342±0.107^{D}
7	1.360±0.18^{DE}	0.378±0.06	0.129±0.05^{B}	0.133±0.03	0.295±0.09^{BC}	0.435±0.02^{ABC}	0.178±0.052^{E}
8	1.230±0.11^{E}	0.364±0.08	0.126±0.03^{B}	0.128±0.03	0.264±0.03^{C}	0.420±0.02^{BCD}	0.310±0.055^{D}
P	0.001	NS	0.010	NS	0.003	0.001	0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same column are statistically significant, ***NS:*** *Not Significant*

Histopathological Findings

The hepatic accumulation of lipid was evident in the high fat diet group (Group 1 and 2). Macro and micro-vesicular type lipid vacuoles were clearly seen by light microscopy. Semi-thin sections also revealed lipid globules present in hepatocyte cytoplasms *(Fig. 1)*. Lipid vacuoles were more prominent in the periacinar area and it was mostly macrovesicular type. Hepatic accumulation of lipid was considerably higher in the ovariectomised and the high fat diet-fed mice than in the low fat diet controls. However, gemfibrozil supplemented high fat diet inhibited fat accumulation in the liver of mice. Gemfibrozil supplemented diet-fed mice exhibited signs of mild hydropic degeneration in the hepatocytes accompanied by dilated central veins and mild anisocytosis.

Mild hyperplasia was seen in the Langerhans islets of the pancreas in the high fat diet-fed mice. Size of Langerhans islets increased with irregular contours, in some cases islets were expanding into surrounding exocrine pancreatic tissue. Acinar cells were vacuolated in the high fat diet-fed mice (Group 1 and 5) while no acinar vacuolization was detected in the other groups. Cytoplasmic basophilia

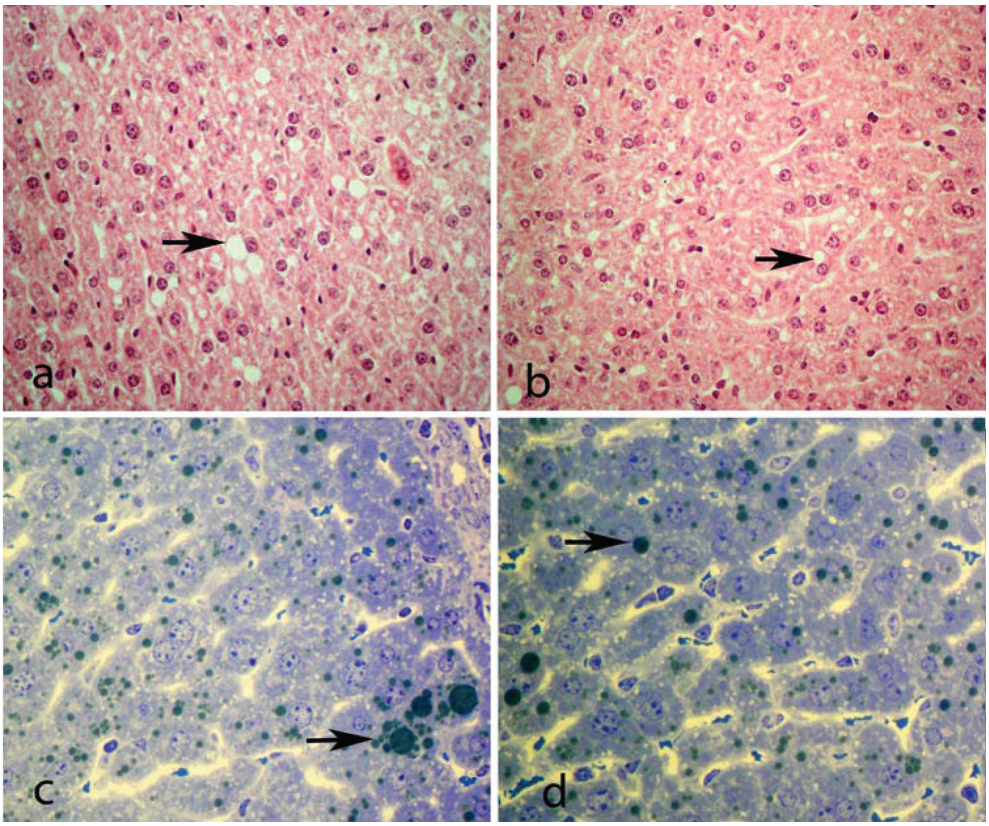

Fig 1. The histological appearance of fatty liver in the high fat diet groups. In Group 1 (a), and (b) 5 are showing sharply bounded fat vacuoles in hepatocytes in mice *(arrows)*. (H & E x 260). In (c) Group 1, and (d) 5 are semi-thin sections lipid globules *(arrows)* present in hepatocyte cytoplasms (toluidine blue x 750)

Şekil 1. Yüksek yağlı diyet uygulanan farelerde şekillenen karaciğer yağlanmasının histopatolojik görünümü. (a) Grup 1'deki ve (b) 5'teki farelerde hepatositlerdeki keskin sınırlı yağ vakuolleri *(oklar)*. (H&E x 260). (c) Grup 1'deki ve (d) 5'teki farelerde yarı ince kesitlerde hepatositlerdeki yağ globülleri *(oklar)* (Toluidin mavisi x 750)

increased in the pancreatic acinar cells of the gemfibrozil supplemented groups (Groups 2, 3, 6, and 7).

Lipocytes in ommental fat tissue, fat layer covering the outer surface of the kidneys and inguinal fat were microscopically hypertrophied in the high fat diet-fed mice. Adipose tissue was highly vascularised in the high fat diet-fed mice than in the other groups.

Immunohistochemistry

Ovariectomy, high fat diet and gemfibrozil supplementation altered tissue PPAR-α and -γ expression levels and reaction densities. However, PPAR-β signals were not affected by ovariectomy, high fat diet and gemfibrozil supplementation. PPAR-α, -β, and -γ positive cells (%) and their statistical significances were summarised in *Table 3*. PPAR-α signals were pronounced particularly in the liver, kidney and heart of the mice.

PPAR-α levels were significantly increased following high fat diet and gemfibrozil supplementation. However, ovariectomy reduced tissue PPAR-α expression levels. PPAR-α positive signals were detected in both hepatocyte cytoplasm and nuclei. PPAR-α staining intensity and distribution differed between groups.

PPAR-α positive cell numbers and staining intensity were higher in the livers of mice in Group 2 compared to that of Group 1 *(Fig. 2)*. In Groups 1, 2, 5, and 6 PPAR-α positive hepatocyte numbers and staining intensities were higher than remaining Groups of 3, 4, 7 and 8. In Group 3, PPAR-α positive signals were mostly present in the periacinar area. In contrast to this finding, high fat diet caused a partial shift of staining from periacinar area to periacinar and midzonal region and partly towards hepatocytes located in the periportal area. Hepatocytes in high fat diet plus gemfibrozil given groups revealed that almost all hepatocytesin liver parenchyma stained positively.

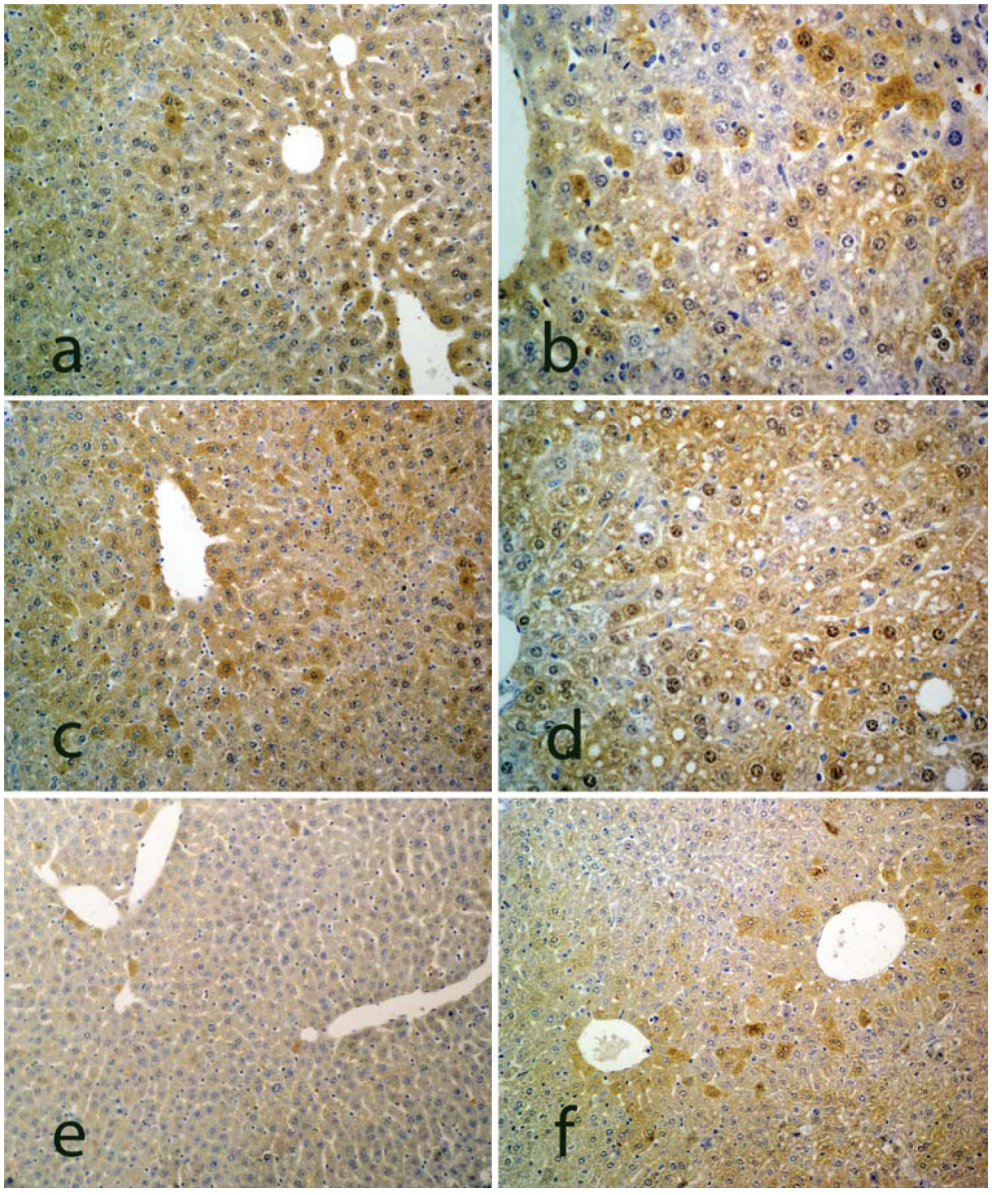

Fig 2. Figures are showing PPAR-α immunoreactivitiy in the liver of mice with the high fat diet (a, b, c and d), low fat diet (e and f), gemfibrozil (b and f), ovariectomised (a and c) and sham operated (b and d). Avidine biotine peroxidase complex (ABC). PPAR-α staining intensity and the number of positively stained cells were greater in gemfibrozil treated mice compared to the mice with no gemfibrozil treatment (b>a, d>c, e>f), and also in the high fat diet fed mice compared to the low fat diet fed mice (a, b, c, and d>e and f). Ovariectomy application in the high fat diet fed mice reduced the PPAR- α expression in liver (c>a, d>b). Magnification: a,c and f: x180; b and d: x260; e: x90)

Şekil 2. Şekillerde yüksek yağlı diyet (a, b, c ve d), düşük yağlı diyet (e ve f), gemfibrozil (b ve f), ovariektomi (a ve c) ve sham operasyonu uygulanan (b ve d d) farelerin karaciğerindeki PPAR-α immunoreaktivitesi görülüyor. Avidin biotin peroksidaz Kompleks (ABC). PPAR-α boyanma yoğunluğu ve pozitif boyanan hücre sayısı gemfibrozil uygulananlarda (b>a, d>c, e>f) gemfibrozil uygulanmayanlara göre, yüksek yağlı diyet ile beslenenlerde (a, b, c, d>e,f) düşük yağlı diyetle beslenenlere göre daha fazla. Yüksek yağlı diyetle beslenen farelere (a,b,c,d) ovariektomi uygulaması karaciğerde PPAR-α ekspresyonunu azalttı (c>a, d>b). (Büyütme: a,c ve f: 180; b ve d: x260; e: x 90)

In kidneys, PPAR-α positive signals were detected mainly in the proximal tubular epithelial cells. In all groups, almost all proximal tubular epithelial cells were stained for the PPAR-α antibody. Gemfibrozil supplemented high fat diet extended PPAR-α staining to distal tubular epithelial cells.

PPAR-α positive signals were also detected in the stomach and intestinal epithelial cells, adreno-cortical cells, heart muscle and smooth muscle cells, adipocytes and macrophages.

No significant difference was detected for PPAR-β expression amon the groups. PPAR-β immunoreactivity was seen in almost all tissue samples. Both cytoplasmic and nuclear, granular type PPAR-β signals were mostly

Table 3. *PPAR-α, -β, and -γ positive cells (%) in the liver and their statistical significances*
Tablo 3. *Karaciğerde PPAR-α, PPAR-β ve PPAR-γ primer antikorlarıyla pozitif boyanan hücre yüzdesi ve gruplar arası istatistiksel farklılıklar*

Immunoreactivity (Positivity, %)	Groups								P
	1	2	3	4	5	6	7	8	
PPAR-α	28.4±3.1^{F}	55.5±2.2^{B}	37.2±5.2^{E}	6.1±1.6^{H}	48.7±4.5^{C}	66.8±5.3^{A}	44.4±5.1^{D}	11.5±2.3^{G}	0.001
PPAR-β	29.7±8.4	28.0±13.1	27.9±6.2	28.6±9.1	30.5±8.1	29.0±12.4	27.8±10.7	29.7±9.7	NS
PPAR-γ	8.2±0.9^{BC}	8.8±0.7^{B}	5.8±0.7^{D}	5.2±1.0^{D}	10.9±1.5^{A}	11.0±1.8^{A}	8.1±1.3^{BC}	7.3±1.0^{C}	0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same line are statistically significant, ***NS:*** *Not Significant*

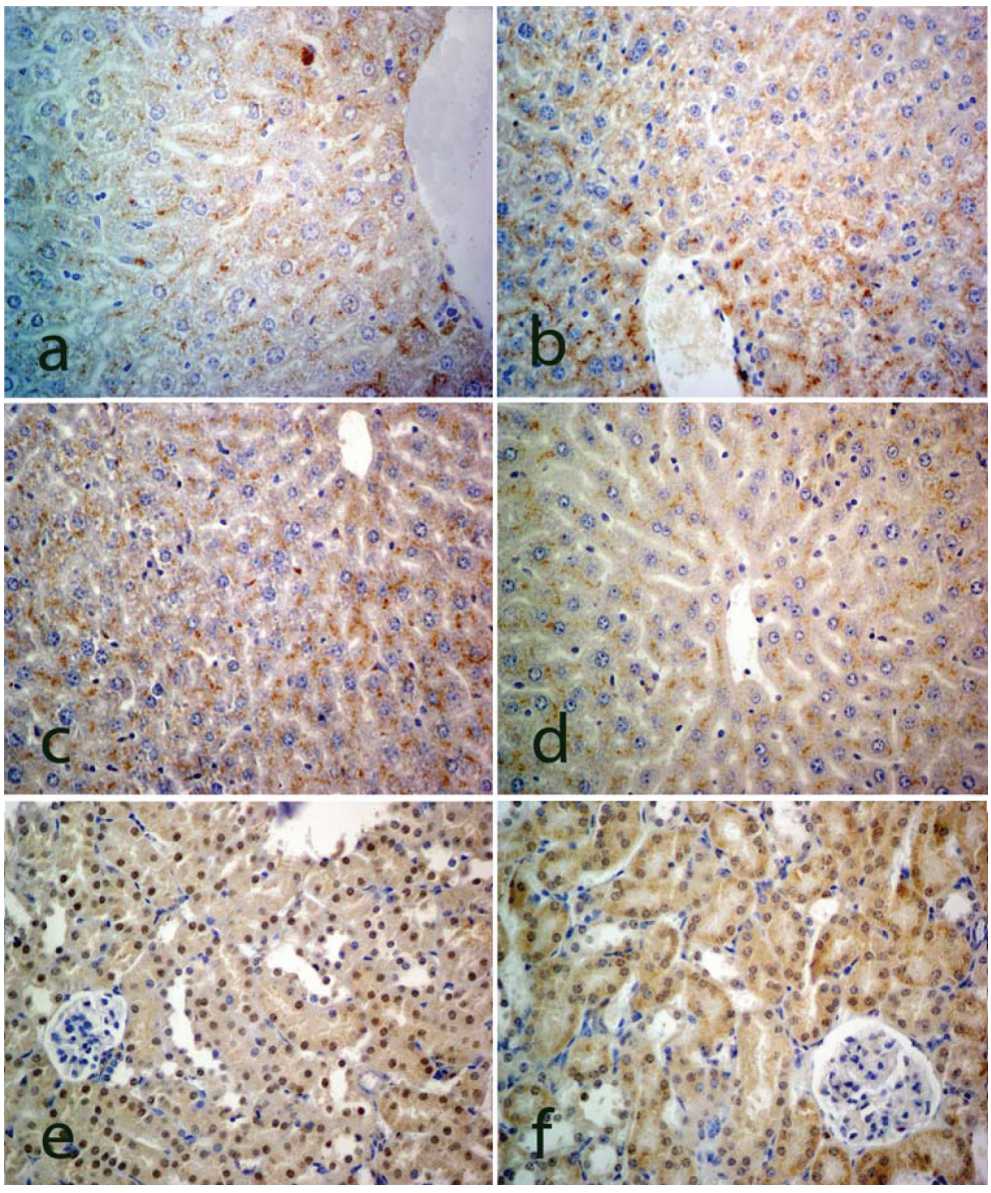

Fig 3. PPAR-β expression in the liver and kidneys. Avidin-biotin peroxidase complex (ABC).Following ovariectomy (a, c, d), sham (b, d, f), high fat diet (a, b, d, e, f) and gemfibrozil (b and d) administration (a, b, c, d) hepatocytes mostly revealed granular cytoplasmic and positive immunoreaction while in kidneys the staining was (e, and f both cytoplasmic and nuclear. There is no difference between groups in terms of staining intensity and the number of positively stained cells (Magnification: a, b, c, d and f: x 260)

Şekil 3. Karaciğer ve böbreklerdeki PPAR-β ekspresyonu. Avidin Biotin Peroksidaz Kompleks (ABC). Ovariektomi (a, c, d), sham (b, d, f), yüksek yağlı diyet (a, b, d, e, f) ve gemfibrozil (b ve d) uygulamaları sonunda karaciğerde (a, b, c, d) çoğunlukla sitoplazmik ve granüler tarzda pozitif reaksiyon görülürken böbreklerde (e ve f) hem sitoplazmik hem nükleer pozitif reaksiyon görülmekte. Pozitif boyanan hücre sayısı ve boyanma yoğunluğu açısından ise gruplar arası farklılık yok (Büyütme: a, b, c, d ve f: x 260)

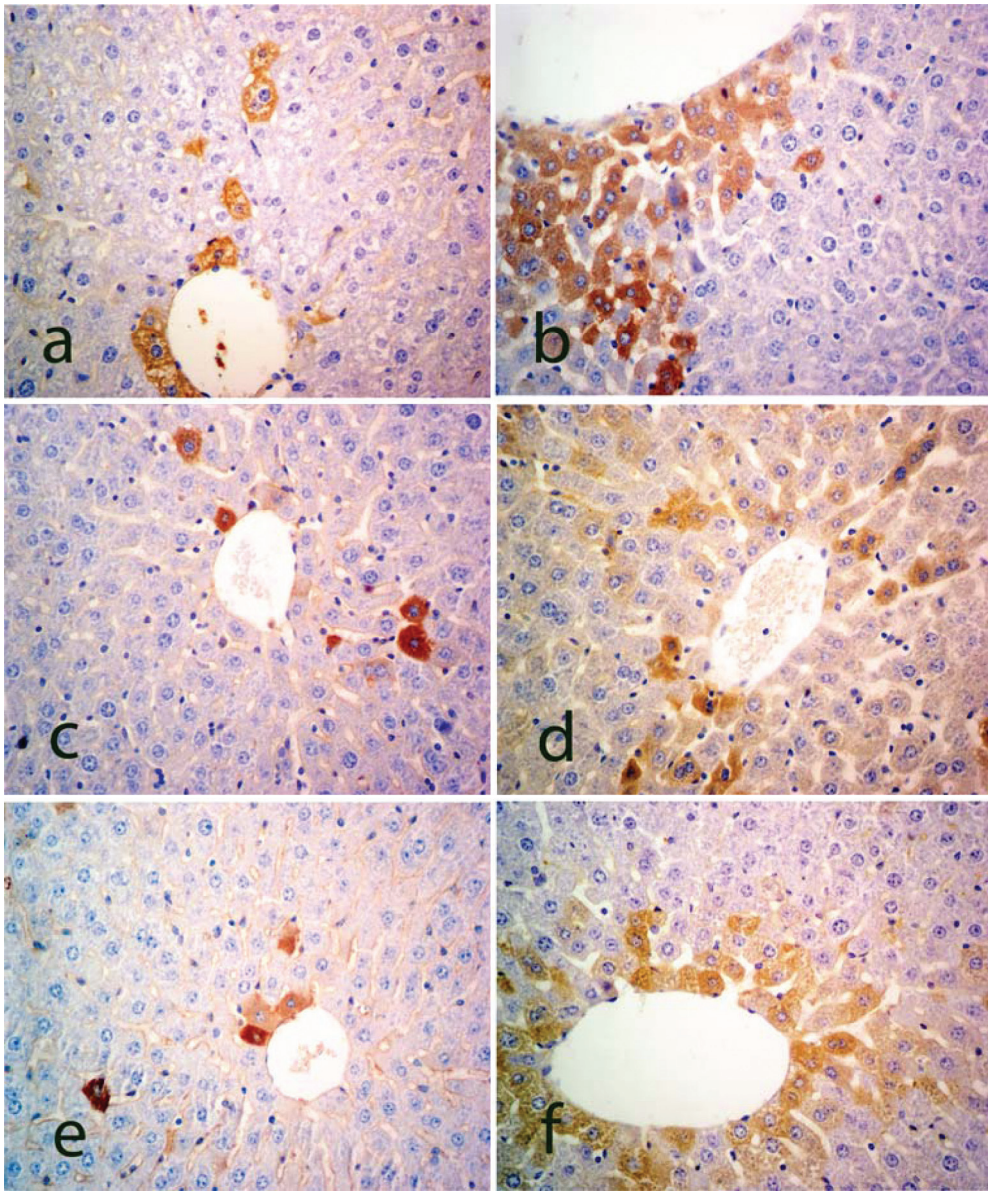

Fig 4. PPAR-γ expression in the liver. Avidin-biotin peroxidase complex (ABC). While there is no difference for the positively stained cells and staining intensity of PPAR-γ in the ovarioectomised (a, c, e), sham (b, d, f), high fat diet (a and b) and high fat diet plus gemfibrozil (c and d) applied mice groups staining intensity is elevated in the low fat diet and gemfibrozil (e and f) administered groups (Magnification: a, b, c, d, e and f: 260)

Şekil 4. Karaciğerde PPAR-γ ekspresyonu. Avidin Biotin Peroksidaz Kompleks (ABC). Ovarioektomi (a, c, e), sham (b,d,f), yüksek yağlı diyet (a ve b) ile yüksek yağlı diyet ve gemfibrozil (c ve d) uygulanan farelerde pozitif boyanan hücre sayısı ve boyanma yoğunluğu açısından fark görülmezken düşük yağlı diyet ve gemfibrozil (e ve f) uygulanan farelere göre PPAR-γ ekspresyonu daha fazla (Büyütme: a, b, c, d, e ve f: 260)

Table 4. *Biochemical alterations in the serum of treatment groups and their statistical significance*
Tablo 4. *Çalışma da kullanılan farelerin serum örneklerindeki biyokimyasal değişiklikler ve gruplar arası istatistiksel farklılıklar*

Gorups	AST (U/L)	ALT (U/L)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	VLDL (mg/dl)
1	280.62±10.05^{A}	86.21±6.09^{A}	131.81±8.71^{A}	178.17±20.67^{A}	30.84±5.81^{A}
2	120.80±20.07^{C}	55.70±11.38^{CD}	98.60±15.03^{BC}	138.40±20.33^{B}	25.90±4.86^{B}
3	86.79±15.80^{D}	50.01±10.57^{DE}	80.00±13.06^{EF}	69.38±14.06^{D}	16.45±1.88^{C}
4	147.00±21.28^{B}	61.43±15.05^{C}	86.00±8.93^{DE}	150.14±15.02^{B}	26.29±4.64^{B}
5	275.60±22.16^{A}	70.80±7.73^{B}	107.50±11.91^{B}	176.70±13.12^{A}	30.20±4.16^{A}
6	85.54±15.82^{D}	42.63±11.26^{EF}	71.58±8.82^{F}	69.30±18.76^{D}	15.79±3.65^{C}
7	87.46±16.89^{D}	48.31±12.96^{DEF}	94.08±12.74^{CD}	94.46±17.71^{C}	18.77±5.73^{C}
8	132.30±17.28^{BC}	38.80±6.91^{F}	74.00±12.62^{F}	143.40±15.74^{B}	25.00±2.54^{B}
P	0.001	0.001	0.001	0.001	0.001

Values presented as mean ± SE. Means denoted with different superscripts are within the same column are statistically significant, ***AST:*** *aspartate amino transferase,* ***ALT:*** *alanine aminotransferase,* ***VLDL:*** *very low density lipoprotein*

localised in the periacinar hepatocytes *(Fig. 3)*.

Diffuse and granular type PPAR-β staining was observed in the cardiac myocytes and adrenal cortical cells. Both cytoplasmic and nuclear PPAR-β immunopositivity were also recorded in the stomach and intestinal epithelial cells and adipocytes.

β cells of islets of Langerhans revealed weak cytoplasmic immunoreactivity againt PPAR-β antibody while α cells located at the periphery of the islets exhibited rather dense cytoplasmic and nuclear reaction. Nuclear PPAR-β staining was detected in the acinar cells from the exocrine component of the pancreas.

PPAR-β reaction was detected in the central nervous system particularly in the dentate gyrus, hippocampus, thalamic nuclei, cerebellum and telencephalic cortical neurons.

PPAR-γ staining was detected in the capillary endothelial cells, macrophages, adipocytes, Kupffer cells and hepatocytes. PPAR-γ staining was mostly observed in the periacinar hepatocytes and to less extend in the cytoplasm of hepatocytes present in the midzonal and periportal areas, Kupffer cells and vascular endothelial cells *(Fig. 4)*. While gemfibrozil supplementation did not alter PPAR-γ immunoreactivity ovariectomy and high fat diet ameliorated PPAR-γ expression levels. The number of PPAR-γ positive hepatocytes and staining intensities were higher in the high fat gemfibrozil supplemented diet group (Group 2) and only high fat diet group (Group 1) compared to only high fat diet applied group (Group 1) and low fat diet group (Group 3), respectively. PPAR-γ positive hepatocytes were generally localised in the periacinar region. However, individual hepatocytes present in the midzonal and periportal areas were reacted positively for the PPAR-γ antibody.

PPAR-γ immunoreactivity was detected in other organs including submucosally located macrophages present in the stomach and intestines, alveolar macrophages, glomerular parietal and visceral epithelial cells, adipocytes and vascular endothelial cells of all the organs subjected to this study.

Serum Assay Results

Serum AST, ALT, total cholesterol, triglyceride, and VLDL levels were higher in the high fat diet fed groups compared to low fat diet fed groups. However, gemfibrozil supplementation to high fat diet significantly reduced serum AST, ALT, total cholesterol, triglyceride, and VLDL concentrations, compared to only high fat diet fed mice. Ovariectomy and sham operated mice serum AST and ALT levels were not significantly altered. However, serum cholesterol, triglyceride and VLDL levels were higher than that of the sham operated mice. Serum assay results and their statistical significance were summarised in *Table 4*.

DISCUSSION

It has long been known that estrogen has an effect on adiposity in humans, rodents and other species. Decrease of estrogen levels upon senescence or ovariectomy in humans and rodents alter metabolic processes and cause an increase in adiposity [20]. In the present study, body weights of ovariectomised mice from Groups 1 and 4 increased 30.4% and 6.5%, respectively. Increased body weight following ovariectomy could be explained by direct effects of the estrogen on lipid metabolism and energy homeostasis [16,20,21]. Elevated food consumption of ovariectomised mice versus sham operated mice of the present study supports this hypothesis. PPAR-α like estrogen, also plays significant role in the β-oxidation of fatty acids [8,22]. Long chain fatty acid catabolism capacities of PPAR-α null mice are rather low and consequently dyslipidemia occur [23,24] and in the long term body fat content elevates [25]. In the present study, gemfibrozil supplementation prevented mice from weight gain despite high fat diet continued for 15 weeks. Gemfibrozil effectively prevented weight gain due to estrogen deficiency which occurred following ovariectomy. Moreover, gemfibrozil supplemented low fat diet reduced body weights in Group 3 (12.7%) and Group 7 (14.4%). It was suggested that PPAR-α agonists reduce food intake and thus contribute to weight loss [26]. However, in our study gemfibrozil supplementation, instead of reducing feed consumption, increased feed intake in the gemfibrozil supplemented high fat diet group (Group 6) compared to only high fat diet applied group (Group 5). However, there was no difference between gemfibrozil supplemented low fat diet group (Group 3) and only low fat diet group (Group 4) in terms of feed consumption. Gemfibrozil supplementation reduced abdominal fat content regardless ovariectomy operation. Present study showed that abdominal adiposity which could occur as a result of hypoestrogenism could be prevented by gemfibrozil, a well-known exogenous ligand for PPAR-α.

Estrogen inhibits lipogenesis and adiposity by reducing lipoprotein lipase enzyme activity which regulates lipid storage in the adipocytes. However, ovariectomy induces lipoprotein lipase enzyme activity and consequently increases lipid storage in the adipocytes and adiposity [27]. Hormone replacement therapy in the post-menopausal women reduces the incidence of cardiovascular disease [28,29]. Moreover, in women hormone replacement therapy reduces circulating LDL levels while increasing HDL and as a result of this cardiovascular disease possibility decreases significantly [28]. In our study, ovariectomy partly increased plasma triglycerides, total cholesterol and VLDL levels. This increase was most prevalent in the high fat diet fed group. However, gemfibrozil regulated plasma lipid profile.

Implementation of the high fat diet for the 15 weeks led to mild to moderate liver steatosis in these animals. Hepatic

steatosis was more prominent in the ovariectomised mice. This was associated with increased PPAR-α and γ expression levels while PPAR-β expression level did not alter. High fat diet associated with elevated PPAR-α expression levels is probably related to the increase in the density of fatty acids in hepatocytes [24]. PPAR-α target genes are related to the mitochondrial and peroxisomal oxidation of fatty acids and intracellular other lipid metabolisms [4,9,24]. In this context, it could be suggested that free fatty acids could induce PPAR-α expression and consequently increase oxidation of fatty acids in the liver [4,8]. Unsaturated fatty acids are natural ligands of the PPAR-γ as in PPAR-α [30]. PPAR-γ target genes are in charge of adipocyte differentiation, lipid storage and glucose metabolism [30,31]. PPAR-γ is very important for adipocyte differentiation both *in vitro* and *in vivo* [30-32]. In the present study, high fat diet increased the weight of abdominal fat tissue was possibly associated with increased expression of PPAR-γ. In the study, in contrast to PPAR-α and PPAR-γ PPAR-β expression level did not change following high fat diet although it has significant role in the oxidation of fatty acids. These findings possibly indicate that PPAR-β is not activated by fatty acids.

Interestingly, ovariectomy reduced PPAR-α and-γ expression levels. Various mechanisms could be put forward to explain this finding. Because estrogen alone, is not a ligand of PPAR-α and –γ [33] also they do not form in heterodimers of PPAR and estrogen *in vivo* [34]. Therefore, the relationship between estrogen and PPARs could be explained by indirect mechanisms. For example, PPAR-γ is activated by prostaglandins, and especially the J2 series of prostaglandins [35]. Estrogen regulates the synthesis of prostaglandins in the target tissues such as uterus [36]. Estradiol increases arachidonate which is one of precursor of the prostaglandins [37]. PPAR-γ expression decreased with ovariectomy could be related to reduced effects of estrogen on the prostaglandin synthesis in target tissues. On the other hand, changes in the PPAR-γ and -α expression levels could also be related to estrogen receptors.

In this study gemfibrozil which is an exogenous ligand for PPAR-α, induced PPAR-α expression more potently than that of high fat diet. Gemfibrozil supplemented high fat diet increased PPAR-α expression more significantly in comparison with gemfibrozil alone. This finding was supported by our previous study in which Wistar-Albino rats were fed high fat diet plus clofibrate and we reported that clofibrate alone increased PPAR-α expression more effectively in comparison with high fat diet alone [8]. However, in the previous study [8], there was no significant difference in between clofibrate plus high fat diet and control diet supplemented with clofibrate which is differed from our present findings. This difference could originate either from species specific difference of ligand-mediated activation of PPAR-α or gemfibrozil application and implementation of high fat diet could create a synergism to activate PPAR-α more potently.

In the present study, gemfibrozil administration ameliorated hepatic steatosis in ovariectomised and high fat diet fed mice as well as sham operated and high fat diet fed mice. Although, hepatic steatosis is not yet fully elucidated, the beneficial effects of PPAR-α ligands were attributed to enhanced oxidation of fatty acids by inducing enzymatic activities of rate-limiting peroxisomal (AOX) and mitochondrial (ACD) β-oxidation in the hepatocyte mitochondria and peroxisomes [8,45].

In conclusion, the high fat diet and ovariectomy induced fatty liver model of mice showed that gemfibrozil supplementation was effectively ameliorated hepatic steatosis and abdominal adiposity. Our ovariectomised mice model suggests that gemfibrozil potently activates PPAR-α whereas it does not effect the expression of PPAR-β and -γ. Furthermore, high fat diet induced the expression and activation of PPAR-α and -γ whereas it was ineffective for the PPAR-β. On contrary, ovariectomy reduced the expression of PPAR-α and -γ. And also gemfibrozil administration effectively prevented liver steatosis in mice.

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