FABAD J. Pharm. Sci., 43, 3, 233-240, 2018

RESEARCH ARTICLE

The Effect of Diabetes and High-Fat Diet on Aldose Reductase Enzyme Activity: Alterations in TNF-α and IL-6 Levels.

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SUMMARY

Obesity is the most common nutritional disorder and associated with an increased risk of developing insulin resistance and type 2 diabetes. There have been several reports related with inflammation in diabetes and obesity, but the relationship between inflammatory status, lipid profile and aldose reductase (AR) enzyme activity in diabetic and high-fat diet (HFD) is not evaluated before. The objective of the present study is to determine the AR activity, cytokine (tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6)), cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride and atherogenic index levels in control, diabetes and HFD groups and evaluate the correlation between them. Blood glucose levels and body weights of the Wistar albino rats were monitored at the 1st and at 6th weeks of the diabetes and HFD groups. AR is isolated from rat livers and AR activities of these groups were determined by a spectrophotometric method. Cytokine levels were measured by ELISA. Blood total cholesterol, HDL, LDL and triglyceride levels were measured by colorimetric diagnostic kits. The AR enzyme activity is 0.249 U/L in control group, 0.389 U/L in diabetes and 0.356 U/L in HFD group ($p \leq 0.05$). Increased TNF-α and IL-6 levels were detected in both diabetes and HFD groups. Serum cholesterol, LDL, triglyceride and atherogenic index levels were also found higher in diabetes and HFD groups. Enhancement of AR enzyme activities correlated with the increased cytokine levels and also with corrupted lipid profiles in both diabetes and HFD groups. HFD might have similar pathophysiological mechanisms that result in several similar metabolic complications like diabetes.

Key Words: *Diabetes, Obesity, High-Fat Diet, TNF- α, IL-6, Aldose Reductase*

Diyabet ve yüksek yağlı diyetin aldoz redüztaz enzim aktivesine etkisi: TNF-α ve IL-6 düzeylerindeki değişimler

ÖZET

İnsülin direnci ve tip 2 diyabet geliştirme riski ile yakından ilişkili olan obezite, en sık görülen beslenme bozukluklarından bir tanesidir. Diyabet ve obezite'de inflamasyon ile ilişkili birçok çalışma bulunmaktadır. Ancak diyabet ve yüksek yağlı diyette (HFD) inflamatuvar durum, lipid profili ve aldoz redüktaz (AR) enzim aktivitesi arasındaki ilişki daha önce değerlendirilmemiştir. Bu çalışmanın amacı, AR aktivitesini ve sitokin (tümör nekroz faktörü-α (TNF-α) ve İnterlökin-6 (IL-6)), kolesterol, yüksek dansiteli lipoprotein (HDL), düşük dansiteli lipoprotein (LDL), trigliserit ve aterojenik indeks düzeylerini kontrol, diyabetik ve HFD gruplarında belirlemek ve aralarındaki korelasyonu değerlendirmektir. Bu amaçla Wistar albino sıçanlarının kan şekeri düzeyleri ve vücut ağırlıkları diyabet ve HFD gruplarında 1. ve 6. hafta aralarında izlendi. AR, sıçan karaciğerlerinden izole edildi ve bu grupların AR aktiviteleri spektrofotometrik bir yöntemle ölçüldü. Sitokin seviyeleri ise ELISA yöntemi ile belirlendi. Kolorimetrik tanı kitleri ile ise kan total kolesterol, HDL, LDL ve trigliserit düzeyleri ölçüldü. AR enzim aktivitesi kontrol grubunda 0.249 U / L, diyabetik grupta 0.389 U / L ve HFD grubunda 0.356 U / L olarak bulundu ($p \leq 0.05$). Hem diyabet hem de HFD gruplarında artmış TNF-α ve IL-6 düzeyleri tespit edildi. Ayrıca diyabetik ve HFD gruplarında serum kolesterol, LDL, trigliserit ve aterojenik indeks düzeyleri de yüksek bulundu. AR enzim aktivitesinin artışı, hem diyabetik hem de HFD gruplarında artmış sitokin seviyeleri ve aynı zamanda bozulmuş lipit profilleri ile ilişkilidir. Sonuç olarak, HFD'in, diyabet gibi çeşitli benzer metabolik komplikasyonlarla sonuçlanan benzer patofizyolojik mekanizmalara sahip olabileceğini düşünmekteyiz.

Anahtar Kelimeler: *Diyabet, Obezite, Yüksek Yağlı Diyet, TNF-α, IL-6, Aldoz Redüktaz*

Received: 20.04.2018
Revised: 14.07.2018
Accepted: 31.07.2018

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INTRODUCTION

Obesity and diabetes are the major health problems in the world. The prevalence of obesity has been increasing in the last decades. Obesity is also associated with an increased risk of developing insulin resistance and type 2 diabetes (Kahn, Hull, & Utzschinder, 2006). Excess consumption of macronutrients such as carbohydrates and lipids coupled with increasing adiposity lead to the progression of type 2 diabetes mediated principally via their negative influence on insulin action and intermediary metabolism between obesity and type2 diabetes. This relation lies in a multitude of factors, which includes the changes in adipose tissue distribution, muscle and liver metabolism as well as alterations in levels of carbohydrates, fatty acids and adipocyte derived cytokine like factors including leptin, TNF-α and adiponectin (Keller, 2006). Both obesity and type 2 diabetes are associated with increased insulin resistance. Increased release of TNF-α, IL-6 and products of macrophages might have a role in the development of insulin resistance. These cytokines are the potential mediators of the inflammation that can lead to insulin resistance (Fain, Bahouth, & Madan, 2004; Wellen & Hotamisligil, 2005).

TNF-α and IL-6 are multifunctional cytokines, they have important roles in liver and adipocyte degeneration/regeneration (Lee & Pratley, 2005; Fujiyoshi & Ozaki, 2011). TNF-α has been demonstrated to regulate or interfere with adipocyte metabolism at numerous sites including transcriptional regulation, glucose and fatty acid metabolism and hormone receptor signaling. Thus, particularly in experimental models, it is clear that overproduction of TNF-α in adipose tissue is an important feature of obesity and contributes significantly to insulin resistance and metabolic disorders. In addition to immune cells, adipocytes have cytokine secretion capacity (Sethi & Hotamisligil, 1999). Lipid and glucose metabolisms are under control of inflammatory level of the body, inflammation is the link between diabetic complications and metabolic disorders (Zhang, Dellsperger, & Zhang, 2012).

Besides the change in cytokine and lipid levels in obesity and diabetes, enzyme activities also differ from the healthy subjects. AR is one of the enzymes which is affected from diabetes. AR is a member of AKRB1 subfamily and catalyzes the first step in the polyol pathway that has been a focus of interest due to its implication in the pathogenesis of diabetic complications (Hers, 1956; Srivastava, Ramana, & Bhatnagar, 2005; Kumar & Reddy, 2007). The elevated glucose concentration in blood activates the polyol pathway, through AR enzyme (Kador, 1988; Tomlinson, Stevens, & Diemel, 1994; Srivastava et al., 1998). The excess accumulation of intracellular sorbitol is linked to the pathogenesis of diabetic complications. There have been several reports related with inflammation in diabetes and obesity but the relationship between inflammatory status, lipid profile and AR activity in diabetic and HFD rats is not evaluated before.

The aim of this study is to determine the AR enzyme activity, cytokine, lipid and atherogenic index levels in control, diabetic and obese stage and make correlations between groups.

MATERIALS and METHODS

Animals

Wistar male albino rats weighing 200-250 g were used for experiments. The studies were approved by the animal experiment local ethics committee, Ankara University. All procedures were performed consistently by the same investigator. Six weeks old albino Wistar rats were used as models of diabetes, HFD and control group. Control and diabetic groups were received standard diet. Each group consists of 5 rats. Diabetes was induced in Wistar albino rats by intra-peritoneal injection of streptozotocin (STZ; 45 mg/kg body weight) (Wang, Zhang, Zhang, Cao, Zheng, & Yu, 2017).

On the other hand, rats were received HFD which contains vegetable oil 3,0 %, animal oil 37,0 %, corn 30,0 %, yellow casein 20,0 %, di-calcium phosphate 1,7 %, dl-methionine 0,2 %, lime stone 1,6 %, salt 0,5 %, complex of vitamin-mineral 1,0 %. Rats were sacrificed at 1st and 6th weeks of the experiment and livers were surgically extracted from all groups. All procedures involving animals conformed to the institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research. Fasting blood glucose (FBG), total cholesterol (total-C), HDL, LDL and triglyceride levels were analyzed by using commercially available colorimetric diagnostic kits (IL TestTM, Instrumentation Laboratory, Milano, Italy).

Isolation of Aldose Reductase Enzyme

Livers which were obtained from control, diabetic and HFD albino rats, were thawed on ice and homogenized with 3 volume of distilled water, homogenate were centrifuged at 10.000g for 20 minutes (Sigma

2K15; Sigma-Aldrich; St-Louis, USA). Saturated ammonium sulfate (AS) was added to the supernatant for 40 % saturation. The thick suspension was stirred for 15 minutes, was centrifuged at 10.000g for 20 minutes. The inert protein left in the supernatant was removed by increasing the AS concentration to 50 % saturation followed by centrifuging the mixture at 10.000g for 20 minutes. The AR enzyme was precipitated from the 50 % saturated solution by adding powdered AS to 75 % saturation and was recovered by centrifugation at 10.000g for 20 minutes (Cerelli et al., 1986). Protein concentrations were measured by the method of Bradford using bovine serum (BSA) as a standard (Bradford, 1976).

Determination of Aldose Reductase Activity

AR activity of the freshly prepared supernatant was assayed spectrophotometrically by determining the decrease in NADPH concentration at 340 nm by a UV-1700 Visible spectrophotometer (UV-1700 Visible Spectrophotometer, Shimadzu, Kyoto, Japan) (Cerelli et al., 1986). DL-glyceraldehyde was used as a substrate. The enzyme was dissolved in 10 mL 0,05 M NaCI solution. 0,2 mL enzyme was added to incubation medium which contains 0,1 mL phosphate buffer (0,067 M, pH: 6,2), 0,1mL NADPH (210^{-5} M final concentration) and 2,4 mL distilled water to obtain final volume 2,9 mL. The reaction was started by the adding of 0,1 mL DL-glyceraldehyde (510^{-5} M final concentration) to the incubation medium and the decrease in NADPH concentration was recorded at 340 nm for 5 minutes at 37 °C. Readings were taken at intervals in the periods when the changes in absorbance were linear.

1 unit (U) is the amount of AR enzyme that catalyzes the reaction of 1 umol of NADPH per minute.

The AR activity was calculated as:

$$\frac{U}{m}L = \frac{(\Delta a340nmTest - \Delta a340nmBlank).(VT).(df)}{(\varepsilon).(VE)}$$

where, 6.2 is micromolar extinction coefficient of NADPH at 340 nm.

Measurement of TNF-α and IL-6 Levels

Protein extracts were prepared from fresh tissue samples in Brij 150 lysis solution, which contains a cocktail of protease inhibitors aprotinin (2 mg/mL), leupeptine (2 mg/mL), pepstatine (2 mg/mL) and phenyl methane sulfonyl fluoride (PMSF) (2 mg/mL). Chemicals were obtained from Sigma unless otherwise stated. Lysis solution was added as two folds of tissue weight by volume and homogenized (Powergen 125, Fisher Scientific, PA, USA). Tissue supernatants were analyzed for TNF-α and IL-6 in triplicate using an ELISA kit (Cytolab/PeproTech, Israel). The lower detection limits of the assay were 15 and 30 pg/mL for TNF-α and IL-6.

Calculation of Atherogenic Index

Atherogenic index of plasma (AIP) is calculated as log (TG/HDL-C) and HDL-cholesterol in AIP reflects the balance between the atherogenic and protective lipoproteins. AIP level predicts cardiovascular risk. Clinically AIP related risk evaluation is as below (Dobiášová & Frohlich, 2001; Frohlich & Dobiášová, 2003).

AIP < 0,11 low risk

AIP: 0,11-0,21 intermediate risk

AIP > 0,21 increased risk

Statistics

All the data which are collected from the experiments were coded, recorded, and analyzed by using SPSS 10.0.1 for Windows (SPSS Inc. Chicago, IL, USA). The one-way analysis of variance (ANOVA) was used to compare differences among groups. When analysis of variance showed a significant difference, the post hoc multiple comparison test was applied to demonstrate the differences. In each test, the data were expressed as the mean value ± S.D. and p<0,05 was accepted as statistically significant.

RESULTS

Wistar albino rats which are fed by HFD were exhibited significant increase in body weight but diabetic rats were exhibited a drastic reduction in the body weight compared to control group in the sixth week (**Figure 1**, p<0,05).

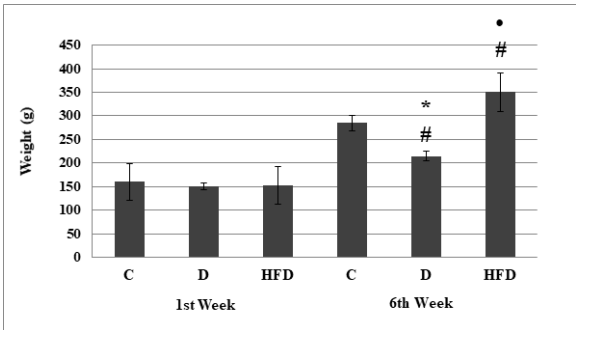

Figure 1: The weigt change of control (C), diabetic (D) and high fat diet (HFD) rats in 1st, 3rd and 6th week. Data is given as mean±SD. #Significantly different from control group, ●Significantly different from diabetic group, *Significantly different from HFD (p<0,05).

Plasma glucose levels were increased in the 6th week of diabetes thus producing the frank hyperglycemia compared to control and HFD-fed rats (**Figure 2**, p<0,001). Although fasting blood samples were taken from tail vain of all rats at 15 day intervals through the experiment, the results in **Figure 2** represents only the first and last sampling.

Total-C, HDL, LDL and triglyceride levels were analyzed in serum samples in control group and 6th week of diabetes and HFD group (**Table 1**). Lipid profiles of HFD and diabetic animals are found disturbed and both have increased Atherogenic Index whereas control group has very low risk (in fact negative risk) for atherogenity.

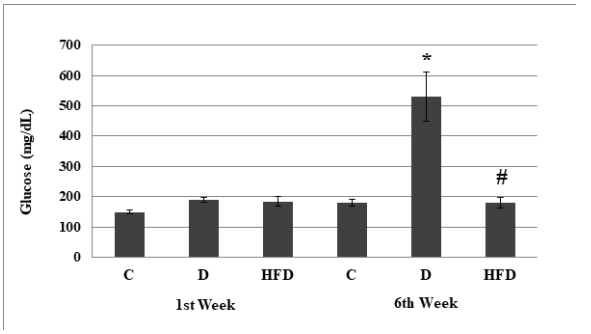

Figure 2: Blood glucose levels of control (C) , diabetic (D) and high fat diet (HFD) rats in 1st and 6th week. Data is given as mean±SD. *Significantly different from control group, #Significantly different from diabetic group (p<0,05).

Table 1. Lipid profiles of different groups at the end of the experimental period.

Groups	Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Triglyceride (mg/dL)	AIP
Control	75±8	38±4	23±4	65±3	-0,127±0,04
High Fat	93±6*	28±5*	31±5*	97±4*	0,195±0,04*
Diabetic	136±12*	35±4	35±4*	136±5*	0,242±0,05*

Data are mean ±SEM - *p<0,05 (between control and other groups)

(HDL: High-density lipoprotein, LDL: Low-density lipoprotein, AIP: Atherogenic index of plasma)

Pro-inflammatory cytokine levels were determined by the measurement of TNF-α and IL-6 levels. TNF-α and IL-6 levels were found increased in both diabetic and HFD groups (p<0,001, **Figure 3**), and an increased AR enzyme activity was found in diabetes and HFD groups as well (p<0,001, **Figure 4**). AIP of diabetic and HFD groups were found correlated with liver TNF-α levels (p<0,01, r = 0,921) and AR enzyme levels (p<0,001, r = 0,988). There was a significant correlation between liver TNF-α and AR enzyme levels (p<0,01, r = 0,91).

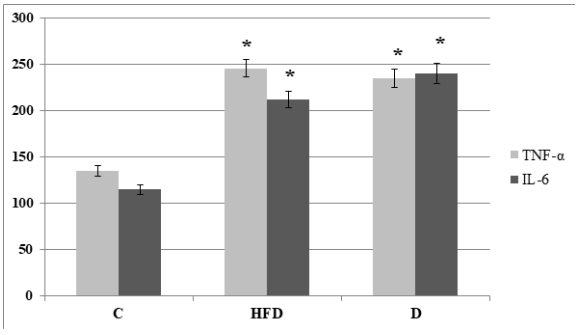

Figure 3: Liver tissue TNF-α and IL-6 levels. Data is given as mean±SD. *Significantly different from control group (p<0,01).

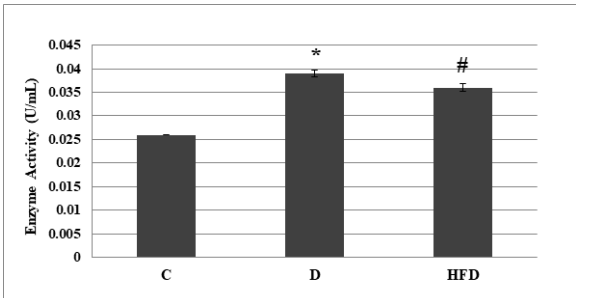

Figure 4: Enzyme activity levels of control (C) , diabetic (D) and high fat diet (HFD) rats. Data is given as mean±SD. *Significantly different from control group, #Significantly different from control group (p<0,05).

DISCUSSION

Liver tissue plays a major role in energy metabolism, particularly glucose and lipid homeostasis. Disorders of glucose metabolism is often associated with lipid accumulation in liver tissue might lead to hepatic steatosis and nonalcoholic fatty liver diseases (NAFLD). A number of factors like oxidative damage, increased inflammation are linked with NAFLD. Therefore, the liver appears to be a preferred source and target of cytokine signalling and metabolic disorders. It is shown that in streptozotocin-induced diabetic mice, deficiency or inhibition of AR resulted in

reductions in serum lipid levels by dephosphorylation and activation of hepatic transcriptional factor peroxisome proliferator-activated receptor alpha (PPARα) (Qiu et al., 2012), but the relationship between inflammatory cytokine levels, AIP and AR enzyme activity is not demonstrated yet.

The relationship between AIP, inflammatory status and AR activity were evaluated in this study. We found increased AIP in both HFD and diabetic groups (Table 1). AR activity is found increased in the HFD and diabetic groups independently from blood glucose levels and found correlated with TNF-α levels of liver tissue. Erbel et al (2016) found that AR gene expression AKR1B1 mRNA expression correlated positively with the M1 markerTNF-α and and the AR expression in M1 macrophages was hyperglycemia dependent. Yuan et al (2018) showed that AR is important in the progression of atherosclerosis in diabetic mice, by effecting plaque macrophage inflammation.

As it is known that obesity, lipids, constant stress and inflammatory conditions are the main risk factors for the development of type 2 diabet and dyslipidemia related atherogenic vascular problems (Bastard, Maachi, Lagathu, Kim, & Caron, 2006). Dyslipidemia, as associated with diabetic metabolism and the metabolic syndrome, is characterized by a so-called proatherogenic blood lipid profile, comprising low levels of HDLs, increased LDLs, serum triglycerides associated with very low-density lipoprotein (VLDL). Indeed, hepatic VLDL release is increased in diabetes and is thought to drive other aspects of the dyslipidemia associated with this disorder. In fact, hypertriglyceridemia is considered to represent an important risk factor for atherosclerosis and subsequent cardiovascular complications in type 2 diabetic patients (Ginsberg, 1996; Onat, Can, Kaya, & Hergenç, 2010). The atherogenic index has recently been proposed as a marker of plasma atherogenicity and it is increased in people with higher risk of coronary heart disease and is inversely correlated with lipoprotein particle size (Alexander, Landsman, Teutsch, & Haffner, 2003; Vinik, 2005; Onat, Can, Kaya, & Hergenç, 2010). It is known that insulin resistant state promotes vascular oxidative stress, inflammation, apoptosis, atherogenesis and impaired endothelial function (Wellen & Hotamisligil, 2005; Zhang, Dellsperger, & Zhang, 2012).

AR is the key enzyme in polyol pathway which catalyses the oxidation of glucose to sorbitol. Sorbitol is another toxic compound produced by abnormal metabolic pathways in diabetes, results from increased activity of the polyol pathway. Increased reactive oxidative and nitrosative products serves as a final common pathway of hyperglycemia-induced complications. In addition to a potential role for AR in diabetes also in atherosclerosis was demonstrated in LDL receptor knock-out mice overexpressing human AR (Vikramadithyan et al., 2005).

The effects of both the human AR (hAR) transgene and STZ treatment were found evident in studies of cholesterol/cholic acid-containing (CCA) diet-fed Ldlr+/– mice. Total lesion area was found similar in nondiabetic and STZ-treated animals, and hAR increased lesion size in the diabetic mice. Aortic root lesions were found greater in diabetic mice, even without hAR expression, though hAR increased the lesion size even more. This cholic acid-containing diet is known to increase inflammation (Vikramadithyan et al., 2005).

AR activity is important in oxidative, hyperglisemic and dyslipidemic conditions. Increased oxidative status induces inflammation in many conditions and inhibition of AR activity reduces inflammation in diabetes (Lee & Pratley, 2005; Srivastava, Ramana, & Bhatnagar, 2005). Our results showed that AR activity is correlated with TNF- α levels independent of glucose levels. Lipid profile might be more important for this correlation than glucose levels. We found increased AR activity in diabetic stage and HFD group (**Figure 4**). Cytokine levels were also increased and correlated in diabetes and obese group (**Figure 3**). These results were also paralel with the elevated cholesterol, LDL, TG and AIP and low HDL levels. Increased AR activity might be direct cause or result of concomitant events. Atay et al (2017) showed that IR is important and one of key factor in progression of NAFLD. It is known that excessive intake of dietary fat, fructose and cholesterol has been linked to the development. Maccari et al (2018) reported high fat diet rats have higher levels of plasma triglycerides, hepatic dysfunction and inflammation compared to high carbohydrate groups. We also found highest level of inflammation in HFD group.

Gleissner et al found that hyperglycemia-related upregulation of AR gene expression and activity as a new proinflammatory mechanism of oxidized-LDL (oxLDL) in human macrophages with potential relevance for atherogenesis, by this study they identified a new potential link between diabetes and hyperlip-

idemia (Gleissner, Sanders, Nadler, & Ley, 2008). In addition to these findings, they reported that ox-LDL-induced AR upregulation increases oxidative stress and the protective effects of AR inhibition on reactive oxygen species (ROS) formation seem to be more pronounced under hyperglycemic conditions. The researchers suggested that harmful effects of AR require oxLDL as well as elevated glucose levels. Ox-LDL is important for development of atherogenesis. Our results are overlapped to these findings and AR levels were found correlated with AIP of diabetic and HFD rats. Nowadays it is widely accepted that AR could be a target for the treatment diabetes, its complications and other inflammatory processes (Maccari & Ottanà, 2015).

Besides reducing glucose to sorbitol, AR reduces a wide range of aldehydes and their conjugates as well (Srivastava, Chandra, Bhatnagar, & Srivastava, 1995). AR has pivotal function in diabetes and it has also a beneficial role on the detoxification of toxic lipids and aldehydes. AR catalyzes lipid-derived aldehyde metabolites which are the mediators of cytokine-, chemokine-, growth factor- and LPS-induced cellular cytotoxicity (Ramana, Friedrich, Bhatnagar, & Srivastava, 2003; Ramana, Bhatnagar, & Srivastava, 2004; Ramana et al., 2006).

The increase in vascular disease in diabetic patients is thought to be due to the deleterious effects of similar metabolic abnormalities, increased inflammation, dyslipidemia and advanced glycation end products. Vascular damage, which results from lipid deposition and oxidative stress to the vessel wall, triggers an inflammatory reaction, and the release of chemoattractants and cytokines worsens the insulin resistance and endothelial dysfunction and causes to the development of obesity related disorders, multiple sclerosis, atherosclerosis and cardiovascular diseases.

As a result, the AR enzyme activity is found elevated both in hyperglycemic and lipid dysfunction conditions while blood glucose levels are not found increased in HFD group. Our findings suggested that the elevation of the AR enzyme activity and inflammation is not dependent on the blood glucose levels. Lipid level changes are important as glucose metabolism in metabolic disorders. On the other hand, there is strong relationship between AR enzyme expression and inflammatory status of organ systems. Cytokines, especially TNF and IL-6 both disrupts insulin signaling in hepatocytes and fat cells, systemically they have been assigned a role in the pathogenesis of diabetes mellitus. For this reason, treating obesity must be more than a reduction of excess fat and weight loss; it must be also the treatment of obesity's comorbidities/complications, counteract derangements induced by adipokine and cytokine excesses. Elevated lipid levels, high AIP, increased cytokine levels and AR enzyme activity in HFD and diabetes might be the causes of the complications in both conditions. Further studies are needed to explain underlying mechanism of dyslipidemia and inflammation in metabolic syndrome and diabetes.

Ethics Committee Approval: Ethics committee approval was received fro this study from Ankara University Ethics Committee for Animal Experimentation (Permit no: 2010-275)

Acknowledgements: This study was funded by Ankara University Scientific Research Project Coordinatorship (Project Code: 11B3330002), Ankara, Turkey.

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