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Effect of Resveratrol on Fatty Acid Compositions and Lipophilic Vitamins of Fructose Induced Nonalcoholic Fatty Liver

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ABSTRACT

İn this study, the effect of resveratrol on the liver of rats induced by fructose was investigated. Adult male Winstar rats were randomly divided into four groups; control, resveratrol, fructose, and resveratrol plus fructose. Resveratrol was administered 30 mg/kg intraperitoneally. Fructose (10% w/v) was administered with drinking water for 56 days every alternate day. Fructose administration increased the hepatic omega-6/omega-3 and 16:1 n7 / 16:0 ratios ($p<0.001$, $p<0.001$), but decreased the retinol and alfa tocoferol levels ($p<0.01$) compared with control group. Resveratrol administration to fructose treated rats decreased the retinol and cholesterol levels ($p<0.01$, $p<0.05$), and increased alfa tocoferol and vitamin d_3 levels ($p<0.01$) in the liver. Our results suggest that resveratrol act as a bioregulator for some biochemical parameters in fructose induced non-alcoholic fatty liver.

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MG contributed in project, experimental aplication and laboratory work. ANG helped in designing the project and experiments. ÖY contributed in laboratory work. MT contribute in experimental application.

Key words
Resveratroll, Fructose, Fatty acids, Alfa-tocoferoll, Vitamin D3, Cholesterol, Retinol.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease that affects 20–30% of adults and 10% of children especially in industrialized countries, the prevalence of which is increasing all over the world. Prevalence of NAFLD is 15–40% in Western countries and 9–40% in the Asian population. NAFLD encompasses a spectrum of liver disease that ranges from steatosis to its progressive subtype nonalcoholic steatohepatitis (NASH), which can progress to hepatocellular carcinoma and liver cirrhosis. NAFLD is linked to insulin resistance, hypertriglyceridemia (Tessari *et al*., 2009) and it is considered to be the hepatic manifestation of the metabolic syndrome which includes obesity, hypertension and dyslipidemia (McCarthy and Rinella, 2012; Bernal *et al*., 2013).

Lipid peroxidation, inflammation and chronic oxidative stress is associated with pathogenesis of NASH. Higher free radical activity with overproduction of free radicals overcomes the antioxidant defenses and depleted antioxidants levels via lipid peroxidation which prediposes the liver to be more susceptible to oxidative damage (Rhee *et al*., 2013; Browning and Horton, 2004; Oliveira *et al*., 2002; Yu *et al*., 2010; Serviddio *et al*., 2013).

Herbal medicines and their derivatives are widely used for the treatment of NAFLD (Mao *et al*., 2012; Yang *et al*., 2011; Dong *et al*., 2012). Natural products such as *Lycii fructus (*wolfberry derivated), *Silybum marianum* derivatives (silymarin and silibinin), curcumin, garlic**,** green tea and grape derivated resveratrol are used in experimental liver injuries (Xiao *et al*., 2012, 2013; Kang *et al*., 2011; Masterjohn and Bruno, 2012). Several mechanism as such as antioxidant properties, insülin sensitivity, antidyslipidemic properties of herbal have beneficial effects on NAFLD (Mao *et al*., 2012; Yang *et al*., 2011; Dong *et al*., 2012; Kang *et al*., 2011; Masterjohn and Bruno, 2012). Resveratrol is a naturally ocurring polyphenol compound belonging to the stilbene class of aromatic phytochemicals, found in the skin of grapes, berries and peanuts and *Polygonum cuspidatum* roots (Kim *et al*., 2011). It is found in nature as cis and trans isomers. Trans forms are biologically active and most abundant. They are produced by plants as a defensive response to microbial injury, fungal infection, or abiotic (*i.e.* environmental) stress. They have beneficial effects on hepatic, neurological, diabetes, cancer, cardiovascular diseases. They also improve insulin sensitivity and lower body and liver weight and also alleviates steatosis (Xiao *et al*., 2013; Shang *et al*., 2008; Gomez-Zorita *et al*., 2012; Xin *et al*., 2013).

Omega-3 PUFAs are unable to be synthesised de novo and essential in human diet (Zivkovic *et al*., 2007). According to Toshimitsu *et al*. (2007) and Cortez-Pinto *et*

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al., (2006) individuals with NAFLD have lower dietary intake of omega-3 fatty acids and their hepatic long chain fatty acid composition altered. Levels of n−3 PUFA (polyunsaturated fatty acids) decreased and n_6/n_3 ratio is increased. This alterations in the fatty acid compositon of individuals with NAFLD is associated with a pro-inflammatory state and increased lipogenesis leading to steatosis (Parker *et al*., 2012; Serviddio *et al*., 2013).

n-3 LC-PUFAs decrease liver steatosis by decreasing the expression of delta 9 desaturase enzymes, lipogenesis and stimulating fatty acid oxidation in the liver (Parker *et al*., 2012).

We aimed to study the effect of resveratrol on some lipophilic vitamins and fatty acids levels of fructose induced non-alcholic fatty liver.

MATERIALS AND METHODS

Animals

A total of 28 male Wistar rats (n=28, 8 weeks), supplied by Elazıg Animal Diseases Research Center of the Agricultural Ministry of Turkey, were used in this study. Rats were housed in cages where they had ad libitum rat chow and water in an air-condition room with 12-h light/12-h dark cycle. Animals were randomly divided into four groups**:** The first group was (i) Negative control (n = 6) nutriet with standard diet without resveratrol and fructose, (ii) second group(ii) positive control (n=6) nutrient with standard diet plus resveratrol without fructose (30 mg/kg live weight daily i.p.); (iii) Third group: nutrient with standard diet plus fructose (10% w/v drink water); (iv) Fructose plus resveratrol group; nutriet with fructose and resveratrol. Fructose was given to animal with drinking water by a ratio of 10% w/v. Resveratrol is injected to animal 30 mg/kg intraperitoneally. These treatments were continued for seven weeks after which, each experimental rat was anesthetized with ether. Liver tissue samples were collected and stored in -85°C prior to biochemical analyses. Liver fatty acid levels were determined using Gas chromatography, the vitamin levels in liver were determined by HPLC.

Lipid extraction

The lipid of liver tissue samples were extracted by the method of Hara and Radin (1978). Tissue samples were homogenized with the mixture of hexane: isopropanol (3:2 v/v) in the homogenizator. Aliquots of the lipid extract were esterified with 2% H_2SO_4-methanol (Christie, 1992) and the fatty acid composition was determined by gas chromatography (GC).

Fatty acid composition analayses

Fatty acid methyl ester forms (FAME) were extracted with n-hexane. Gas chromatography analysis was employed GC-17A instrument with FID and AOC-20i Autoinjector and Autosampler from Shimadzu (Kyota, Japan). FAMEs were separated by fused silica capiller column, 25 m length and 0.25 mm diameter, Permabond (Machhery–Nagel, Germay). Column temperature was programmed between 120–220C, 5°C/min and the final temperature was held for 15 min. Injector and FID temperatures were 240 and 280°C, respectively. Nitrogen was used as carrier gas under head pressure of 50 kPa (corresponding to 1.2 ml/min, 43 cm/s column flow rate). Identification of the individual methyl esters was performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Class GC 10 software version 2.01 assisted in working of the data.

Determination of lipid soluble vitamins

Liver tissue samples (500mg) were homogenized in 5 ml acetonitrile/methanol/isopropyl alcohol (2:1:1 v/v/v)-containing tubes and the samples were vortexed for 30 s and centrifuged at 6000×g for 10 min at 4°C. Supernatants were transferred to autosampler vials of the HPLC instrument (Shimadzu, Kyoto Japan). For lipophylic vitamins, the mixture of acetonitrile/methanol (75/25 %) was used as the mobile phase and the elution was performed at a flow-rate of 1 ml/min. The temperature of analytical column was kept at 30°C. (Bragagnolo and Rodriguez-Amaya, 2003). Supelcosil™ LC 18 DB column (250 × 4.6 mm, 5 µm; Sigma, USA) was used as the HPLC column and detection were performed at 320 nm for retinol (vitamin A) and retinol acetate, and 215 nm for δ-tocopherol, vitamins D2, D3 and K1, α-tocopherol, α-tocopherol acetate (Katsanidis and Addis, 1999). Identification of the individual vitamins was performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Quantification was carried out by external standardization using Class VP software. The results of the analysis were expressed as µg/g wet cell pellet (Katsanidis and Addis, 1999).

Cholesterol analysis

Liver tissue samples (500 mg) were extracted in 5 ml acetonitrile/isopropyl alcohol (70:30 v/v) containing tubes and the mixture were vortexed for 30 s and centrifuged at 6000×g for 10 min at 4°C. Supernatants were transferred to autosampler vials of the HPLC instrument (Shimadzu, Kyoto Japan). Acetonitrile-isopropyl alcohol (70:30 v/v) was used as a mobile phase at a flow rate of 1 ml/min (Bragagnolo and Rodriguez-Amaya, 2003). Supelcosil LC 18™ DB column (250 × 4.6 mm, 5 µm; Sigma, USA) was used as the HPLC column. Detection was performed by UV at 202 nm and 30°C column oven (Katsanidis and

Addis, 1999). Quantification was carried out by external standardization using Class VP software. The results were expressed as µg/g.

Statistical analysis

The experimental results were reported as means±SD. Statistical analysis was performed using SPSS Software. Analysis of variance (ANOVA) and an LSD test were used to compare the experimental groups with the controls.

RESULTS

In the study, the effects of resveratrol on the levels of the fatty acids and some lipophilic vitamins in the liver of male Winstar rats by induced-fructose were examined. Palmitic acid (16:0), palmitoleic acid (16:1n7), heptadeconoic acid (17:0), stearic acid (18:0), oleic acid 18:1n7, n9) linoleic acid (18:2, n-6), linolenic acid (18:3, n-3) eicosanoic acid (FA20:2 n-6), eicosatrienoic acid (20:3, n-6), arachidonic acid (20:4 n6), eicosapentaenoic acid (20:5, n-3), docosatetraenoic acid, docosapentaenoic acid (22:5 n6) and docosahexaenoic acid (22:6, n-3) were observed in fatty acid components of the liver. Of these, palmitic, stearic, oleic, linoleic and arachidonic acids were present in higher quantities in animals of the control group (Table I).

The fatty acid composition of liver is shown in Table I. The liver palmitic acid level (16:0), linoleic acid(18:2, n-6) and eicosatrienoic acid (20:3, n-6) levels were significantly higher in the F groups than the C group ($p < 0.05$). Palmitic acid level (16:0), palmitoleic acid (16:1 n7), eicosatrienoic acid (20:3, n-6) and stearic acid (18:0) levels were significantly higher in the R groups than the C group ($p < 0.001$, $p < 0.05$).

Oleic acid 18:1n7, n9), linolenic acid (18:3, n-3) docosahexaenoic acid (22:6, n-3) and levels were significantly lower in the F and R groups than the C group ($p<0.05$, $p<0.01$, $p<0.001$). Monosaturated fatty acids (MUFA) levels and n6/n3 ratios were significantly higher in the R groups than the C group ($p<0.01$, $p<0.001$). MUFA/ PUFA ratio were significantly lower in F groups than the C group ($p<0.01$) and ome-3 (n3) levels were significantly lower in the F and R groups than the C group ($p<0.001$). omega 6 (n6), arachidonic acid (20:4 n6) levels has decreased ($p<0.05$) and pufa (polyunsaturated fatty acids) levels has meaningless changes statistically ($p>0.05$).

In the liver tissue, retinol, α-tocopherol levels were decreased in the F groups when compared to C group ($p<0.01$, $p<0.01$,). Retinol, levels were decreased, α-tocopherol levels were increased in R groups when compared to C group ($p<0.01$, $p<0.01$). Vitamin D_3 and cholesterol levels were increased in the F groups when compared to C group ($p<0.01$, $p<0.01$). Vitamin D_3 levels were increased and cholesterol levels were decreased in the (R) groups when compared to C group ($p<0.01$, $p<0.01$).

Table I.- Fatty acıds composıtıon of liver (%).

Yağ asitleri	Control	Fructose	Fructose + Resveratrol
FA16:0	17.49±0.50[a]	18.97±0.41[b]	20.37±0.16[d]
FA16:1n7	1.11±0.14[a]	1.66±0.12[c]	2.09±0.11[d]
FA17:0	0.67±0.03[a]	0.59±0.03[a]	0.57±0.02[a]
FA18:0	19.32±0.33[a]	19.63±0.44[a]	17.60±0.46[c]
FA18:1 n7	3.20±0.07[a]	3.20±0.07[a]	4.34±0.13[d]
FA18:1 n9	5.45±0.78[a]	5.80±0.36[a]	6.34±0.42[a]
FA18:2 n6	15.13±0.67[a]	16.78±0.4[b]	17.42±0.43[c]
FA18:3 n3	0.24±0.04	0.11±0.03[b]	0.04±0.04[c]
FA18:3 n6	0.81±0.05	0.61±0.16	0.34±0.13[b]
FA20:2 n6	0.29±0.01	0.26±0.01	0.34±0.02
FA20:3 n6	0.32±0.06	0.64±0.05[b]	0.84±0.11[d]
FA20:4 n6	27.14±0.18	25.09±0.77[b]	25.08±0.41[b]
Fa22:5 n6	0.28±0.02	0.25±0.01	0.26±0.04
FA22:6 n3	4.04±0.13	1.94±0.07[d]	2.5±0.07[d]
n3	4.58±0.06	2.58±0.11[d]	2.73±0.11[d]
n6	43.99±0.017	43.61±0.96	44.63±0.42
mufa	9.77±0.084	10.71±0.29	12.44±0.17[c]
pufa	48.43±0.83	46.22±0.92	46.64±0.65
n6/n3	9.62±0.2	17.14±1.02[d]	16.48±0.59[d]
Mufa/ pufa	5.20±0.04	4.31±0.19 b	3.74±0.06[c]
FA16:1/ FA16:0	0.06±0.01	0.08±0.03[b]	0.10±0.04[c]
FA18:1 / FA18:0	0.28±0.02	0.29±0.02	0.36±0.04[c]
FA18:3/FA18:2	0.05±0.01	0.03±0.01[b]	0.01±0.0[d]
FA20:4 / FA20:3	84.81±0.83	39.20±0.86[d]	29.85±0.48[d]

a, $p > 0.05$; b, $p < 0.05$; c, $p < 0.01$; d, $p < 0.001$.

Table II.- Some lipophilic vitamin parameters of liver.

	Control	Fructose	Fructose + Resveratrol
VitaminD_3 (µg/g)	3.37±0.19	3.95±0.07[b]	14.37±0.48[d]
Alfa-tocoferoll (µg/g)	16.89±0.26	13.30±0.12[b]	17.23±0.29
Cholesterol (µg/g)	2661.36± 12.08	3430.20± 19.03[d]	3390.53± 13.09[d]
Retinol (µg/g)	443.33± 9.68	409.66± 7.69[b]	354.90± 15.73[d]

a, $p > 0.05$; b, $p < 0.05$; c, $p < 0.01$; d, $p < 0.001$.

DISCUSSION

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring polyphenol present in grapes, berries, peanuts and other food vegetables. Resveratrol has many remarkable effects in mammals. Supplementation of resveratrol into mice on high fat diets increases mitochondrial content/activity in liver and improves hepatosteatosis (Baur *et al*., 2006; Mercader *et al*., 2011; Lagouge *et al*., 2006; Ahn *et al*., 2008). Decreased leptin levels and increased IL 6 and TNF alfa levels were shown in NAFLD, and resveratrol reduced the elevated levels of TNF-α and IL 6 (Kang *et al*., 2010).

Although chronic inflammation has been implicated in its pathogenesis and severity of nonalcoholic fatty liver, oxidative stress plays an important role in nonalcoholic fatty liver development and progression (Angulo, 2007; Botella-Carretero *et al*., 2010). Chronic liver diseases are often associated with decreased antioxidant defenses. Different antioxidants exhibit positive effects on liver damage (Harrison *et al*., 2013; Kugelmas *et al*., 2003; Thong Ngam *et al*., 2007).

Resveratrol is the one of the important antioxidant, it has antioxidant properties as an reactive oxygen species scavenger, metal chelators and have rewarding effects on diabetics and obeses. Resveratrol exhibit beneficial biological activities in human health as antiplatelet, anticancer, neuroprotector, cardioprotector, vasoprotective, anti-inflammator and anti-aging (Rhee *et al*., 2013; Barchetta *et al*., 2013; Kitson and Roberts, 2012; White and Cooke, 2000; Wu *et al*., 2013; Wilson *et al*., 1996; Karlsson *et al*., 2000; Zern *et al*., 2003; Jeandet *et al*., 2012).

Cell proliferation and differentiation is regulated by Vitamin D. Vitamin D possess anti-inflammatory, anti-fibrotic and immunomodulatory properties (Kitson and Roberts, 2012). Vitamin D deficiency may result in insulin resistance, metabolic syndrome and cardiovascular diseases and cancer (Rhee *et al*., 2013). Vitamin D deficiency and NAFLD is affiliated with inflammation and oxidative stress. Inflammation is the common pathogenic mechanism for the two diseases. And both diseases are related with cardiovascular disease, type 2 diabetes and insulin resistance. Studies has shown that low Vitamin D levels were affiliated with the improvement of NAFLD (Yuan *et al*., 2006; Yanagitani *et al*., 2004; Schwedhelm *et al*., 2003; Ross and Zolfaghari, 2004; Mecradır *et al*., 2011; Lagouge *et al*., 2006; Rhee *et al*., 2013). NAFLD individuals have decreased serum 25(OH)D concentrations that perform a function in the development and improvement of NAFLD (Targher *et al*., 2007; Eliades *et al*., 2013; Jablonski *et al*., 2013).

Barchetta *et al*. (2011) all have shown decreased vitamin D levels are related with the existance of NAFLD separately from metabolic syndrome, diabetes and insulin-resistance profile. High vitamin D level reduce the risk for nonalcoholic fatty liver (Rhee *et al*., 2013). Decreases in DBP titers are seen in patients with advanced liver or kidney disease (McCarthy and Rinella, 2012) and this may help us to understand the little increase in our fructose fatty liver group. But increased vitamin D3 level in fruc+Res group is a conflicting result.

α-tocopherol fulfils as a pyroxyl radical scavenger and chain-breaking antioxidant. It interacts directly with the oxidizing radicals (Burton and Ingold, 1986; Jones *et al*., 1995) and protect the cells from reactive oxygen species (Cortez-Pinto *et al*., 2006; Burton and Ingold, 1986). Also polyunsaturated fats and low-density lipoproteins (LDL) in cell membranes protects the cell from oxidation by the α-tocopherol (Botella-Carretero *et al*., 2010). Decreased antioxidant defenses are often observed with chronic liver diseases. Vitamin E levels have been shown to be decreased in chronic liver diseases which is confirmed with our study and resveratrol increased α-tocopherol levels in our study, but the role of Vitamin E supplementation in clinical conditions is still debated (Patra *et al*., 2001; Di Sario *et al*., 2007). The use of Vitamin E, slows the progression of NAFLD to NASH (Yu *et al*., 2010).

Retinol plays important roles in metabolism. It controls cellular growth and differentiation (Dong *et al*., 2012; Xiao *et al*., 2012), it inhibits hepatic cell transformation and helps in the production of proinflammatory cytokines in macrophages (Yang *et al*., 2011; Botella-Carretero *et al*., 2010). Retinol is a protective antioxidant. It also suppress proliferation of hepatoma cells, reduce inflammatory reactions and neutralizes reactive oxygen species (Di Sario *et al*., 2007). Utilization/metabolism of retinol is greatest when liver retinol stores and circulating holo-RBP4 concentrations are high (Cifelli *et al*., 2008; Alapatt, 2013).

In our result, resveratrol has decreased levels of hepatic retinol. Mercader *et al*. (2011) have shown that resveratrol reduces Retinol-binding protein 4 expression in white adipose tissue. Botella-Carretero *et al*. (2010) and Villaca Chaves *et al*. (2008) have shown relation of nonalcoholic fatty liver with Vitamin A. Also, a study made by Musso *et al*. (2007) has shown pathogenic role of decreased intakes of Vitamin A in nonalcoholic fatty liver. İncreased concentrations of serum retinol lower the risk of hepatocellular carcinoma and transgenic mice with hepatic loss of retinoic acid receptor function which causes liver tumors and steatohepatitis (Botella-Carretero *et al*., 2010; Yuan *et al*., 2006; Yanagitani *et al*., 2004). Pathogenesis of fatty liver is may be associated with Decreased hepatic

retinol levels (Botella-Carretero *et al*., 2010; Schwedhelm *et al*., 2003; Ross and Zolfaghari, 2004).

A study made by Chen *et al*. (2012) has shown that high-fat fed C57BL/6J mice liver cholesterol level is decreased by dietary resveratrol of which confirm our result. Laden and Porter (2001) have shown that resveratrol reduced cholesterol synthesis by inhibiting squalene monooxygenase *in vitro*, a rate-limiting enzyme in cholesterol biosynthesis. Zhu *et al*. (2008) and Do *et al*. (2008) has reported that two doses of dietary resveratrol, 0.02% and 0.06% w/w, inhibit hepatic HMG-CoA reductase, which may reduce the hepatic cholesterol pool and prevent liver from cholesterol accumulation. İncreased synthesis of cholesterol and expression of fatty acid synthase (FAS) is associated with copper deficiency and in intestine Copper uptake protein Ctr1 is depleted by high dietary fructose (Burkhead *et al*., 2013).

Many of the chronic diseases such as, obesity, autoimmune diseases, cardiovascular disease, diabetes, cancer, rheumatoid arthritis, asthma, depression and NAFLD are associated with over production of IL-6, TNF. These factors increase by increases in omega-6 fatty acid intake and decrease by increases in omega-3 fatty acid intake, either ALA or EPA and DHA (Simopoulos, 2004).

In the liver of the obese, NAFLD patients is n-3 long-chain polyunsaturated FA (n-3 LCPUFA), depletion is observed with substantial diminution in eicosapentaenoic acid (20:5, n-3; EPA) and docosahexaenoic acid (22:6, n-3; DHA) levels (Rhee *et al*., 2013; Kitson and Roberts, 2012) which has been related to higher utilization of n-3 LCPUFA due to oxidative stress and defective (White and Cooke, 2000; Pettinelli *et al*., 2013). In our study; n-3 long-chain polyunsaturated FA (n-3 LCPUFA) depletion is observed but not changed by resveratrol inducing.

The desaturating enzymes, Δ9-desaturase [stearoyl-CoA-desaturase (SCD)], Δ6-desaturase (Δ6D) and Δ5-desaturase (Δ5D), introduce *cis*-double bonds in the carbon chain of long chain fatty acids (Nakamura and Nara, 2004). Stearoyl-CoA desaturase is a key enzyme expressed in the liver and is involved in the synthesis of monounsaturated long-chain FAs from saturated fatty acyl-CoAs. It catalyzes palmitoyl and stearoyl-CoA to palmitoleoyl and oleoyl-CoA, respectively (Toshimitsu *et al*., 2007; Ntambi *et al*., 2002).

The ratio of C16:1 to C16:0 or C18:1 to C18:0 is used as D9 desaturation index (Lee *et al*., 1998). Incresed ratio of C16:1 to C16:0 or C18:1 to C18:0 fructose and fructose plus resveratrol group shows increased SCD activity, sign of fat accumulation in present study.

The velocity restriction step in the production of 20:4 and 22:6 is the desaturation of 18:2 (n6) and 18:3 (n3) by D6 and D5 deasaturases (Youdim *et al*., 2002). Both linolenic acid (18: 3 n-3) and linoleic acid (18:2 n-6) are metabolized by longer chain fatty acids, substantially in the liver. 18:3 is converted to eicosapentaenoic acid and hence to docosahexaenoic acid and 18:2 is the metabolic pioneer of arachidonic acid (AA). In the present study (fruc and fruc+res group), arachidonic and docosahexaenoic acids, delta 6 and delta 5 desaturase enzymes products levels are decreased as a result of fat acumilation as sign of decreases in delta 6 and delta 5 desaturase enzymes.

CONCLUSIONS

Acording to our results resveratrol of which is a naturally occuring antioxidant, has diverse effects on fatty acid composition and lipophilic vitamin of fructose induced nonalcoholic fatty liver. Further researches are needed on effect of resveratrol on nonalcoholic fatty liver disease.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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