Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2021;46(1):125-131
DOI: 10.17826/cumj.788377

ARAŞTIRMA / RESEARCH

Silibinin'in yüksek kolesterol diyeti ile beslenen sıçanlarda hiperlipidemi üzerine etkisi

Effect of silibinin on the hyperlipidemia in rats fed with high cholesterol diet

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Cukurova Medical Journal 2021;46(1):125-131

Abstract

Purpose: Despite current hypolipidemic drugs, the search for a more effective hypolipidemic agent is ongoing. In this study, it was aimed to investigate the effect of Silibinin on hyperlipidemia in rats fed high cholesterol diet (HCD).
Materials and Methods: Rats were made obese. Rats were given egg yolk for 60 days and then 50 mg/kg, 100 mg/kg Silibinin were applied i.p. for 7 days.
Results: The first and last weights of the rats were significantly different. While total cholesterol (TC), LDL, TG and VLDL levels increased significantly in the groups fed with HCD, HDL level reduced compared to control group (CG). OxLDL and TAS were significantly different between groups.
Conclusion: The effects of Silibinin on serum LDL, TC, VLDL, HDL, TG, OxLDL levels and to observe the antioxidant effect, TAS and TOS were investigated in experimental obese rat models. It was concluded that Silibinin plays an effective role in lowering TG and LDL levels, increasing HDL levels and decreasing hepatic lipid accumulation in HCD rats at 100 mg/kg dose. The use of Silibinin does not cause antihyperlipidemic effect but has antioxidant effect.

Keywords:. Silibinin, hyperlipidemia, antioxidant, atherosclerosis

Öz

Amaç: Hipolipidemik ilaçlar mevcut olmasına rağmen, daha etkin bir hipolipidemik ajan arayışı devam etmektedir. Bu nedenle, bu çalışmada yüksek kolesterol diyeti ile beslenen sıçanlarda Silibinin hiperlipidemiye olan etkisinin araştırılması amaçlanmıştır.
Gereç ve Yöntem: Öncelikle deney grubundaki sıçanlar obez hale getirilmiştir. Sıçanlara 60 gün boyunca yumurta sarısı verilmiş ve daha sonra 50 mg/kg ve 100 mg/kg Silibin i.p. olarak 7 gün boyunca uygulanmıştır.
Bulgular: Sıçanların ilk ve son kiloları arasında anlamlı bir fark bulunmuştur. Çalışmamızda yüksek kolesterollü diyet ile beslenen grupların total kolesterol (TK), LDL ve trigliserit (TG), VLDL seviyeleri, kontrol grubuna kıyasla anlamlı bir şekilde yükselirken, HDL seviyesinde kontrol grubuna göre anlamlı bir azalma görülmüştür. Gruplar arasında OxLDL ve TAS değerleri bakımından anlamlı bir fark olduğu tespit edilmiştir.
Sonuç: Bu çalışmada deneysel obez sıçan modellerinde Silibinin, serum LDL, TK, VLDL, HDL, TG, OxLDL düzeylerine etkisi incelenmiştir. Silibinin, yüksek kolesterol diyetle beslenen (YKD) sıçanlarda 100 mg/kg dozda kullanıldığında, TG ve LDL seviyelerini düşürmede, HDL seviyesini arttırmada ve hepatik lipid birikimini azaltmada etkili bir rol oynadığı sonucuna ulaşılmıştır. Silibin kullanımının, antihiperlipidemik etkiye yol açmadığı, ancak antioksidan etkiye neden olduğu ortaya konulmuştur.

Anahtar kelimeler: Silibinin, hiperlipidemi, antioksidan, ateroskleroz

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Geliş tarihi/Received: 03.09.2020 Kabul tarihi/Accepted: 11.11.2020 Çevrimiçi yayın/Published online: 10.01.2021

INTRODUCTION

The diseases causing the most deaths in the world such as the coronary heart disease (CHD), ischemic cerebrovascular, and peripheral vascular disease[1] are associated with the increased atherogenic risk. The main causes of the atherosclerosis are the hypercholesterolemia and the low levels of HDL (High Density Lipoprotein)[2]. In contrast to the atherogenic potential of the IDL (Intermediate Density Lipoprotein) and LDL (Low Density Lipoprotein), the HDL particles are protective against the atherosclerotic plaque formation. The high levels of HDL are associated with the low CHD incidence [3]. HDL serves as a means for the reverse transport of the cholesterol from the peripheral tissues to the liver and plays a role as the local anti-inflammatory and anti-oxidative agent[3,4]. LDL is protected against the oxidation by various antioxidants (e.g. lycopene, β carotene, and probucol). It was reported that the LDL isolated from the atherosclerotic plaques differed from the native LDL in terms of structure and biological properties, but it was similar to the modified Ox-LDL, and the Ox-LDL accumulated in the atherosclerotic plaques[5]. It was asserted that the plasma LDL oxidation levels increased in the patients with cardiovascular disease. The plasma level of OxLDL is accepted as the indicator for CHD risk. In the rats fed with saturated diet, the plasma level of the OxLDL showed a temporary increase before the onset of atherosclerosis[6,7]. In addition to the lifestyle modification, the patients in the secondary prevention and with risk factors (Diabetes, Hypertension, Low HDL, high LDL, and family history) need a lipid-lowering medication. Hyperlipidemia medication is a safe and effective method in reducing CHD risk and total mortality. The relative risk of CHD can be reduced by 25-30% through treating the hyperlipidemias with the lipid-lowering drugs[8]. The hypolipidemic drugs such as statins, bile acid sequestrants, nicotinic acid, and fibrates have side effects like myopathy. Due to the side effects of the hyperlipidemic drugs, we are still searching for safer drugs[8,9].

Silymarin is a group of flavonoid mixture extracted from the milk thistle (Silybum marianum), an edible plant, and has been used as an herbal medicine in the treatment of liver disorders for more than 2000 years[10]. Silymarin consists of four flavonoid isomers; silibinin, isosilibinin, silicristin, and silidianin. All of these compounds are polyphenolic substances. Of these, Silibinin is the most active substance and largely responsible for the claimed benefit of silymarin[11,12]. It binds to the receptor site in hepatocytes and causes the followings: hepatotoxin inhibition, decrease in the glutathione oxidation, antioxidant activity, ribosomal RNA polymerase stimulation, and then protein synthesis and hepatocyte increase. Silymarin has some metabolic and cell-regulatory effects such as carrier-mediated regulation of the permeability of the cell membrane, inhibition of the 5-lipoxygenase pathway, clearance of reactive oxygen species (ROS) of the R-OH species, and the NF-κB (nuclear factor kappa B) regulation on gene expression[12,13,14].

Although hypolipidemic drugs are available in the market, there is an ongoing search for a more effective hypolipidemic agent. Therefore, the purpose of this study was to examine the effect of silibinin on the hyperlipidemia in rats fed with high cholesterol diet (HCD).

MATERIALS AND METHODS

The study protocol and experimental method were approved by the Local Ethics Committee on Animal Experimentations of HMKU. (Ethical Approval No: 2017/7-1). The study was conducted in accordance with the Declaration of Helsinki.

Procedure

For the Total Antioxidant/Oxidant Level, the total antioxidant status (TAS) and total oxidant status (TOS) levels of the samples were measured using the Rel Assay brand commercial kits (Rel Assay Kit Diagnostics, Turkey) developed by Erel[15, 16]. Trolox, a water-soluble analogue of vitamin E, was used as a calibrator.

In order to measure the Oxidized LDL level, the ELISA method was applied using the Rat OxLDL ELISA Kit (Sandwich ELISA). Silibinin (siliphos) was obtained from Swanson Health Products (Indena SpA., Italy) USA. Each capsule of 300 mg contains 89–109 mg Silibinin [17].

The rats were obtained from Research and Application Center for Experimental Researches, Hatay Mustafa Kemal University (HMKU). 32 male Wistar albino rats (4 groups, 8 rats in each group) with the weight ranging between 300g and 350g, at the age of 12-16 months were used in this study. The

rats were kept in wire cages (4 animals in each cage) in an alternating 12-hour light-dark cycle at an ambient temperature of 20-24 ˚C and a humidity of 40-50%.

The diets given to the groups and the drug doses administered were as follows [18]. Group 1 (control group): The rats in this group were fed ad libitum with standard rat feed and distilled water [18], and no procedure was administered to them during the study. At the end of the study, they were sacrificed under anesthesia by taking blood from the heart with syringe [18]. Group 2 (high cholesterol diet (HCD) rats): The rats in this group were fed with egg yolk for 60 days before the experiment and during the experimental period (7 days) [18]. Group 3 (high cholesterol diet + silibinin 50 mg/kg): The rats in this group were fed with egg yolk for 60 days before the experiment and during the experimental period (7 days), and the saline solution (0.9% NaCl) and silibinin (50 mg/kg, i.p.) were injected to the rats during the experimental period for 7 days [18]. Group 4 (high cholesterol diet + silibinin 100 mg/kg): The rats in this group were fed with egg yolk for 60 days before the experiment and during the experimental period (7 days), and the saline solution (0.9% NaCl) and silibinin (100 mg/kg, i.p.) were injected to the rats during the experimental period for 7 days. Then, the rats were sacrificed under anesthesia by taking blood from the heart with syringe [18].

Blood samples

At the end of the experiment, the samples were taken into the gel biochemistry tubes and then centrifuged at 1500 × g for 10 minutes to be used in the analyses of total cholesterol, HDL-LDL cholesterol, and triglyceride. The serum samples were portioned in Eppendorfs and stored at -80 ˚C for the biochemical analysis.

Lipid measurements from the serum samples

In order to measure the Oxidized LDL level from the blood samples, the ELISA method was applied using the Rat OxLDL ELISA Kit (Sandwich ELISA) (2017). The optical density (OD) in the plates was assessed by reading at 450 nm. The concentrations were calculated using the logarithmic calibration curve. The result was expressed in ng/mL. Triglyceride, Total Cholesterol, LDL, and HDL Cholesterol Analyses were carried out using an Abbott Architect c8000 autoanalyzer in the Central Biochemistry Laboratory of HMKU Health Research and Practices Hospital. The measurements were carried out on a spectrophotometer at 500 nm by enzymatic colorimetric method using commercially available kits (BioSystems®). The following formula was used for VLDL:$(\frac{TG}{5})$. The measurement of the TAS/TOS parameters were also carried out using an Abbott Architect c8000 autoanalyzer in the Central Biochemistry Laboratory of HMKU Hospital. The total antioxidant levels (TAS) of the samples were measured using the Rel Assay brand commercial kits developed by Erel (Rel Assay Kit Diagnostics, Turkey). Trolox, a water-soluble analogue of vitamin E, was used as a TAS calibrator and the results were expressed in μmol Trolox equivlt. Hydrogen peroxide was used as the calibrator for TOS. The results were expressed in H_2O_2[16].

Statistical analysis

In our study, the data were analyzed using SPSS 21 (SPSS Inc. Chicago, Illinois, USA) package program at the confidence interval of 95%. Mean ± standard deviation from the measures of central dispersion was used for continuous variables. Shapiro-Wilk test was used for the normality analysis of the variables. Based on the result of this test, it was decided to carry out the parametric and non-parametric tests. The tests used in our study were as follows; Kruskal Wallis; Mann Whitney U test (Bonferroni corrected) for the paired comparison (since 6 paired comparisons were made p=0.05/6=0.008; and Wilcoxon Signed Rank test. Wilcoxon Signed Rank test was used for Initial and Final weight (g) comparison. Kruskal Wallis test was used to compare Control, HCD, 50 mg Silibinin+HCD, 100 mg Silibinin+HCD groups in terms of lipids and other parameters. After Kruskal Wallis test, Mann Whitney U test with Bonferroni correction was used for the double comparison of the mentioned groups (Control, HCD, 50 mg Silibinin+HCD, 100 mg Silibinin+HCD). The statistical significance was set at p=0.05.

RESULTS

In our study, the initial and final weights, the biochemical parameters (HDL, LDL, TG, TC, VLDL), and the oxidant/antioxidant parameters, that is, oxLDL, TAS, and TOS were measured. Whereas there was no significant difference between the initial and final weights in the control group (p=0.111), there was a significant difference (p=0.012) between the initial and final weights in other groups (HCD, 50

mg silibinin + HCD, 100 mg silibinin + HCD). The groups were compared in terms of LDL, TC, HDL, TG, VLDL, OxLDL, TAS, and TOS. The result of the comparisons was given in the Table 2. As can be seen in the Table 2, at least one group was found to be different from the others in the comparison between the groups (control, HCD, 50 mg+HCD, and 100 mg+HCD) in terms of LDL, TC, TG, VLDL, and TAS (p=0.001).

As can be seen in the Table 3, there was a significant difference between the control group and the other groups (HCD, 50 mg Silibinin+HCD, and 100 mg Silibinin+HCD) in terms of LDL, TC, TG, and VLDL (p = 0.001). However, when we compared the HCD group with the 50 mg Silibinin+HCD group and 100 mg Silibinin+HCD group, no significant difference was found.

Table 1. Initial and final weights of the groups

Mean+SD	Initial Weight (g)	Final Weight (g)	p*
Control	308.37±53.96	307.12 ± 53.78	0.111
HCD	320.12±60.91	455.75±57.66	0.012
50 mg Silibinin + HCD	315.62±25.17	423.25±52.46	0.012
100 mg Silibinin + HCD	322.00±36.81	401.00 ± 51.30	0.012

Wilcoxon Signed Rank test results used in the comparison of the initial and final weights.

Table 2. Results obtained for four independent groups according to Kruskal Wallis analysis. (mg/dL)

Mean ± SD	Control	HCD	50 mg Silibinin+ HCD	100 mg Silibinin + HCD	P
LDL	7.50 ± 1.97	52.42 ± 17.17	38.07 ± 23.22	40.80 ± 8.82	0.001
TC	33.67 ± 9.20	104.68 ± 22.42	85.07 ± 29.80	81.43 ± 9.82	0.001
HDL	21.05 ± 8.27	27.11 ± 4.77	32.11 ± 6.77	25.82 ± 4.21	0.058
TG	31,34 ± 9,06	241.86 ± 189.3	133.33 ± 53.24	102.20 ± 22.25	0.001
VLDL	6.26 ± 1.81	48.37 ± 37.87	26.66 ± 10.64	20.44 ± 4.45	0.001
OxLDL	20.09 ± 14.53	47.22 ± 24.45	30.19 ± 20.64	6.12± 3.45	0.004
TAS	1.48 ± 0.27	0.77 ± 0.34	1.06 ± 0.06	1.16 ± 0,011	0.001
TOS	51.21 ± 18.36	104.20 ± 79.91	81.14 ± 42.36	71.03 ± 20.09	0.164

*: Kruskal Wallis test was used. (Lipids were expressed in mg/dL, TAS in μmol/L, TOS in μmol/L.); HCD; High cholesterol diet, LDL; Low density lipoprotein, TC; Total cholesterol, HDL; High density lipoprotein, TG; Triglyceride, VLDL; Very-low density lipoprotein, OxLDL; Oxidized low density lipoprotein, TAS; Total antioxidant status, TOS; Total oxidant status, SD; Standard deviation.

Table 3. In the LDL, TC, TG, and VLDL parameters, Mann Whitney U test (Bonferroni corrected) was used for the paired comparison (since 6 paired comparisons were made p=0.05/6=0.008). (Expressed in mg/dL)

Groups	Groups	LDL(p)	TC(p)	TG (p)	VLDL (p)
Control	HCD	0.001	0.001	0.001	0.001
Control	50 mg Silibinin + HCD	0.002	0.001	0.001	0.001
Control	100 mg Silibinin+HCD	0.001	0.001	0.001	0.001
HCD	50 mg Silibinin+HCD	0.208	0.208	0.208	0.208
HCD	100 mg Silibinin + HCD	0.248	0.036	0.046	0. 046
HCD + 50mg Silibinin	100 mg Silibinin +HCD	0.916	0.834	0.093	0.093

HCD; High cholesterol diet, LDL; Low density lipoprotein, TC; Total cholesterol, TG; Triglyceride, VLDL; Very-low density lipoprotein.

Table 4. Results of Mann Whitney U test (Bonferroni corrected; since 6 paired comparisons were made, p=0.05/6=0,008) in the OxLDL and TAS parameters (expressed in mg/dL)

Groups	Groups	OxLDL(p)	
Control	HCD	0.035	0.002
Control	50 mg Silibinin+HCD	0.298	0.002
Control	100 mg Silibinin +HCD	0.063	0.001
HCD	50 mg Silibinin+HCD	0.165	0.059
HCD	100 mg Silibinin+HCD	0.003	0.008
50 mg Silibinin+HCD	100 mg Silibinin+ HCD	0.001	0.105

HCD; High cholesterol diet, **TAS;** Total antioxidant status, **OxLDL;** Oxidized low density lipoprotein.

As seen in the Table 4, a significant difference was found between the rats in the HCD group and the 100 mg Silibinin+HCD group in terms of OxLDL levels (p=0.003). Furthermore, it was found that there was a significant difference between the control group and the HCD group, the control group and the 50 mg Silibinin+HCD group, the control group and the 100 mg Silibinin+HCD group, and the HCD group and the 100 mg Silibinin+HCD group in terms of the TAS parameter (p<0.008). It can be asserted that Silibinin increases the antioxidant capacity.

DISCUSSION

Due to the fact that hypercholesterolemia is one of the most important risk factors in the development of CHD, many animal experiments have been carried out to better understand the relationship between cholesterol metabolism disorders and atherogenesis [19]. In this study, the effect of Silibinin, on which studies are going on to reveal whether it has a lipid-lowering effect, on hyperlipidemia, antioxidant properties, and OxLDL were examined in the experimentally generated obese rat model. Since the formation of OxLDL is required in the development of atherogenesis, it is assumed that silibinin in the milk thistle can inhibit the generation of OxLDL and the interactions with monocyte-expressed scavenger receptor (SR) and Fc gamma receptors (FcγR) [20, 21]. It has been stated in the literature that silymarin exhibits an antioxidant effect by means of inhibiting the radical formation, binding certain radicals (radical scavenger), preventing the lipid peroxidation of membranes (thereby preserving membrane permeability), and increasing the number of intracellular radical scavengers [22]. Another reason for the antioxidant effect of silibinin is that it acts as an iron chelate. Thus, it reduces the production of hydroxyl radicals by inhibiting the Fenton reaction[23].

Metwally et al., the obese model of whom we took as a reference, compared the rats fed with a high-control diet with the rats in the control group and found a significant increase in the serum triglycerides, cholesterol, LDL, HDL, and VLDL. Metwally et al. managed to create an obese rat model by feeding the rats with egg yolk for 60 days and found a significant increase in their initial and final weights. Also in the obesity model we created by feeding the rats with egg yolk for 60 days, which was similar to the study of Metwally et al., a statistically significant difference was found between the initial and final weights of the rats; so, we were successful at making the rats obese (p=0.012). Furthermore, Metwally et al. found a significant decrease in the serum lipid levels (LDL, HDL, VLDL, cholesterol, TG) in the group treated with Silymarin (p<0.001). In our study, after the rats were exposed to Silibinin for 7 days, a significant difference was found between the high-cholesterol group and the control group (Table 2) in terms of serum TC, TG, LDL, and VLDL (p=0.001). In the comparison of the lipid parameters between the obese groups, the TG, TC, and HDL values were found to be lower in the groups exposed to 50 mg and 100 mg silibinin for 7 days compared to the hypercholesterimic group. However, this decrease was not found to be statistically significant (p=0.208, p=0.036). Our results were not in line with the literature. We are of the opinion that if the silibinin had been given a little longer, the decrease might have been significant.

Senthil et al. used a commercial cholesterol feed as a high-fat diet in their study. Like our study, they reported that a significant difference was found between the initial and final weights of the rats and there was significant increase in the serum TC, TG, LDL, and VLDL and a decrease in HDL in the rats fed with a high cholesterol diet (p<0.005). As seen in the Table 2, similar to the findings of Senthil et al., a significant difference was found between the groups in terms of serum total cholesterol, TG, LDL, and VLDL (p=0.001). On the other hand, unlike the study by Senthil et al. (2016), the HDL levels did not decrease and even insignificantly increased (p=0.058). In their study, Wallace S. et al. reported that the silymarin was effective in inhibiting the OxLDL generation and the subsequent scavenger receptor (SR) mediated monocyte adherence to OxLDL. Our study was designed differently from the study carried out by Wallace et al. in terms of model and practice. As can be seen in the Table 4, although no significant difference was observed between the groups exposed to silibinin at different doses and the control group in terms of OxLDL (p=0.165); a significant difference was observed in the hypercholesterolemic group (p<0.05). It can be asserted that, similar to the study by Wallece, our silibinin dose suppressed the effect of hypercholesterolemia on the oxidation.

In their study, Nina et al. found that, compared to the control group, there was an increase in terms of Thiobarbituric Acid Reactive Substances (TBARS) and the conjugated dienes, which are used as the serum/plasma oxidation indicators, and a decrease in

terms of the levels of glutathione peroxidase, superoxide dismutase, and reduced glutathione (antioxidants) in the group fed with a high-sucrose diet. They found that, after the administration of silymarin, there was a decrease in both TBARS and conjugated dienes, but there was a significant increase in the levels of superoxide dismutase and reduced glutathione [25]. In our study, TAS and TOS were used as the serum oxidation indicators. While in the control group TOS was measured as 51.21±18.36 μmol/L, in the hypercholesterolemic group it was 104.20±79.91μmol/L; in other words, the increase was not statistically significant. However, while TAS was found to be 1.48 ±0.27 μmol/L in the control group, it was measured to be 0.77 ± 0.34 μmol/L in the hypercholesterolemic group, which meant that there was a statistically significant decrease (p=0.002). Furthermore, TAS was 1.06 ± 0.06 μmol/L in the 50 mg Silibinin-injected group, which meant that there was no statistically significant increase compared to the HCD group (p=0.105). On the other hand, TAS was 1.16±0.11 μmol/L in the 100 mg Silibinin-injected group, and there was a statistically significant increase in this regard.

In natural environment, active substances are found in small amounts in medicinal plants; therefore, using these active substances in pure form and in large quantities may cause some undesirable effects [26]. When our findings on the hyperlipidemic properties of silibinin were statistically analyzed, it was found that whereas they were statistically significant according to Kruskal Wallis test (0.05), they were not statistically significant according to Mann Whitney U test (Bonferroni corrected; since 6 paired comparisons were made, p=0.05/6=0.008) It was revealed that silibinin did not cause an antihyperlipidemic effect; however, it had an antioxidant effect.

In conclusion, in our study it was found that silibinin played a significant role in decreasing the TG and LDL levels, increasing the HDL levels, and decreasing the hepatic lipid accumulation when used at the dose of 100 mg/kg in the hypercholesterolemic rats. When we look at the antioxidant parameters between the groups fed with high cholesterol and the control group; There was also a significant difference in OxLDL and TAS (p = 0.004 p = 0.001) levels (p <0.008). In our study, silibinin was observed to have both antioxidant and lipid-lowering effects. The mechanism underlying the cholesterol-lowering effect was most probably that the clearance of ROS led to the inactivation of HMG-CoA reductase and the inhibition of the endogenous cholesterol synthesis [27]. These observations show that silibinin may also be examined as a useful therapeutic drug in treating hypercholesterolemia. There were some limitations in our study. First limitation was about the duration of our study. In order to reveal the changes in lipid metabolism, there is need for the studies in which drug is administered more than one week. Second, it would have been more appropriate to feed the rats with a diet having a more standard cholesterol content rather than egg yolk.

Yazar Katkıları: Çalışma konsepti/Tasarımı: AA, DD; Veri toplama: DD, ED; Veri analizi ve yorumlama: AA, DD, ED, HSB; Yazı taslağı: AA, DD; İçeriğin eleştirel incelenmesi: ED, HSB; Son onay ve sorumluluk: AA, DD, ED, SB, HSB; Teknik ve malzeme desteği: DD, HSB; Süpervizyon: AA; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu çalışma protokolü ve deneysel yöntem HMKU Hayvan Deneyleri Yerel Etik Komitesi tarafından onaylandı. (Etik Onay No: 2017/7-1). Çalışma Helsinki Bildirgesi'ne uygun olarak yapıldı.
Hakem Değerlendirmesi: Dış bağımsız.
Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.
Finansal Destek: Yazarlar finansal destek beyan etmemişlerdir.
Yazarın Notu: Bu çalışma, Hatay Mustafa Kemal Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü tarafından desteklenmiştir. Proje no: 18.YL.023

Author Contributions: Concept/Design : AA, DD; Data acquisition: DD, ED; Data analysis and interpretation: AA, DD, ED, HSB; Drafting manuscript: AA, DD; Critical revision of manuscript: ED, HSB; Final approval and accountability: AA, DD, ED, SB, HSB; Technical or material support: DD, HSB; Supervision: AA; Securing funding (if available): n/a.
Ethical Approval: The study protocol and experimental method were approved by the Local Ethics Committee on Animal Experimentations of HMKU. (Ethical Approval No: 2017/7-1). The study was conducted in accordance with the Declaration of Helsinki.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: Authors declared no financial support
Acknowledgement: This study was supported by Hatay Mustafa Kemal University, Coordinatorship of Scientific Research Projects. Project no: 18.YL.023

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