

Effect of Rosuvastatin on Arginase Enzyme Activity and Polyamine Production in Experimental Breast Cancer

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Background: Breast cancer is the most common malignant tumour of women around the world. As a key enzyme of the urea cycle, arginase leads to the formation of urea and ornithine from L-arginine. In the patients with several different cancers, arginase has been found to be higher and reported to be a useful biological marker.

Aims: The aim of this study was to investigate the effect of rosuvastatin on serum and cancer tissue arginase enzyme activity, and ornithine and polyamine (putrescine, spermidine, spermine) levels.

Study Design: Animal experiment.

Methods: In this study, 50 male Balb/c mice were used. Erchlich acid tumour cells were injected into the subcutaneous part of their left foot. The mice were divided into five groups: healthy control group, healthy treatment, tumour control, treatment 1 and treatment 2. Then, 1 mg/kg and 20 mg/kg doses of rosuvastatin were given intraperitoneally. Serum and tissue arginase enzyme activities and tissue ornithine levels were de-

termined spectrophotometrically. HPLC measurement of polyamines were applied.

Results: Increased serum arginase activity and polyamine levels were significantly decreased with rosuvastatin treatment. In the tumour tissue, arginase activity and ornithine levels were significantly decreased in treatment groups compared to the tumour group. Tissue polyamine levels also decreased with rosuvastatin treatment.

Conclusion: We suggest that rosuvastatin may have some protective effects on breast cancer development as it inhibits arginase enzyme activity and ornithine levels, precursors of polyamines, and also polyamine levels. This protective effect may be through the induction of nitric oxide (NO) production via nitric oxide synthase (NOS). As a promising anticancer agent, the net effects of rosuvastatin in this mechanism should be supported with more advanced studies and new parameters.

Keywords: Arginase, breast cancer, ornithine, polyamines, rosuvastatin

Breast cancer is still the commonest type of cancer in women. It accounts for about 20% of all cancer-related mortality. Therefore, great medical and scientific efforts are continually invested into understanding the disease's pathology and finding new methods for its early diagnosis, prevention and treatment.

In cells, L-arginine is embraced in protein synthesis. Arginine is also used by arginase, arginine decarboxylase, nitric oxide synthases (NOSs) and glycine transaminase. Arginase is

a crucial enzyme that is chargeable for nitrogen metabolism. Arginine is its main substrate; from this, urea and L-ornithine are formed (1). There are two types of arginase: Arginase I is cytosolic and mostly found in the liver; it is considered to be primarily liable for the detoxification of ammonia. The other isoenzyme, arginase II, is engaged in ornithine creation. Ornithine is the precursor of some products which take place in cell growth: glutamate, proline and polyamines (spermine,

This study was presented as a poster at the congress of IFCC WorldLab, 22-26 June 2014, İstanbul, Turkey.

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Received: 11.06.2014 Accepted: 08.10.2014 • DOI: 10.5152/balkanmedj.2015.15611

Available at www.balkanmedicaljournal.org

Cite this article as:

Erbaş H, Bal O, Çakır E. Effect of rosuvastatin on arginase enzyme activity and polyamine production in experimental breast cancer. *Balkan Med J* 2015;32:89-95.



spermidine and putrescine) (2). As polyamines are crucial for cell proliferation, it is probable that the elevated level of ornithine, due to increased arginase activity, could lead to the development of cancer (3).

Nitric oxide (NO) production is dependent on NOSs which are the enzymes that synthesis the oxidation of arginine to NO and citrulline. Three different types of NOS are available: neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3). eNOS and nNOS are believed to be constitutive (cNOS) (4). NO has several aspects in cells and its action may vary depending on its level (5). Studies have proposed that in cancer growth, NO may have an important action as a preventative and therapeutic agent. The use of NO donors as cancer therapeutics had been shown as a new venue that has not been appreciated in the past, because NO was primarily used for the treatment of blood vessel-related disorders and in other non-cancer conditions. In cancer cells, the indication of NO-mediated cytotoxicity through its anti-proliferative and chemo-sensitising behaviour supported the idea of their usage in cancer therapy (5).

Many different studies conducted on various types of cancer arginase levels in the serum and tissues examined have associated arginase enzyme activity with cancer. Some researchers even emphasised of arginase enzyme activity as an important determinant even in patients with breast cancer (6).

Studies have shown that polyamines are the polycationic compounds necessary for growth and division in all cells (7). They are aliphatic molecules that are responsible in various fields such as cell division, protein-DNA interactions, cell differentiation, embryonic development, immunological interactions, signal transduction, apoptosis, DNA and RNA conformational change tasks and cancer (8). Although the increase in level of the polyamine concentration known to be associated with cell proliferation, in recent studies, these molecules have been shown to play an important role in both normal and cancer growth (9).

As a synthetic molecule, rosuvastatin was chosen; this is a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase inhibitor (10). Rosuvastatin selectively, reversibly and competitively inhibits the enzyme which catalyses the formation of mevalonic acid from HMG-CoA as a rate-limiting step in cholesterol synthesis (10).

As with other statins, rosuvastatin has some other effects besides the inhibition of HMG CoA reductase. These are the improvements in endothelial function, anti-inflammatory, antithrombotic and antioxidant effects (10). It has been emphasised that rosuvastatin decreased the oxidative stress by increasing antioxidant capacity (11). It has also been suggested that it reduced the free radical damage to the DNA through some antioxidant mechanisms such as the down-regulation of NADPH oxidase and increased expression of the enzymes which contribute to glutathione metabolism (12). Statins are

emphasised to regulate the endothelial and cardiovascular functions by increasing the formation of NO (13).

In this study, we aimed to investigate the possible positive effects of rosuvastatin, a statin for which the antioxidant and anti-carcinogenic effects have been shown in previous studies, on breast cancer development. For this purpose, we determined the breast cancer serum and tissues arginase enzyme activities, as well as ornithine and polyamine levels in mice.

MATERIALS AND METHODS

Animals and tumour

This study was performed in the Experimental Animal Breeding and Research Unit of the University upon approval by the local animal ethics committee (2011.09.05/09). Fifty adult male inbred BALB/c mice (8 weeks old) were used in the experiment. The weight of the animals was 30±3 g. Ehrlich ascites tumour cells which were derived from a spontaneous murine mammary adenocarcinoma were used obtaining the breast carcinoma.

Experimental design

In the eighth week, five groups were formed. The first group was healthy controls. The other groups were breast cancer. To induce the formation of tumours, subcutaneous inoculation of Ehrlich ascites cells into the mice's left footpad was performed. Tumour development was assessed after 7 days by measuring their footpad thickness. On the 7th day, treatments were started. Group 2 was formed with healthy animals (healthy control+treatment, 1 mg/kg/day). Group 3 was a tumour control group and received saline solution (tumour control). Group 4 received 1 mg/kg/day rosuvastatin (tumour+treatment 1). Rosuvastatin was a gift from Sanovel (İstanbul, Turkey). Group 5 received 20 mg/kg/day rosuvastatin (tumour+treatment 2) for 15 days intraperitoneally (ip). At the end of the treatment period, the animals were sacrificed under anaesthetic. Serum and tissue samples were taken and stored at -80°C.

Determination of Arginase enzyme activity in serum and tissue

Serum arginase enzyme activity was determined spectrophotometrically according to Munder (14).

Breast cancer tissue arginase activity was measured spectrophotometrically according to the thiosemicarbazide diacetylmonoxime urea (TDMU) method (15).

Breast tissue ornithine levels were determined spectrophotometrically with the Chinard Method (15).

HPLC measurement of polyamines

Before starting to work, serum samples were removed from -80°C and dissolved at room temperature. For the tissue homogeni-

TABLE 1. Serum and tissue arginase enzyme activities, putrescine, spermidine and spermine levels in groups (mean±SEM). [#]Serum comparisons between group 1 and group 3, [§]Serum comparisons between group 3 and group 4, [¶]Serum comparisons between group 3 and group 5, [^]Tissue comparisons between group 3 and group 4, [†]Tissue comparisons between group 3 and group 5, [‡]Tissue comparisons between group 4 and group 5, *p<0.05, **p<0.01, ***p<0.001

	Cont.	Cont+treat	Tumour	Tumour+treat 1	Tumour+treat 2	
Serum	Arginase (U/L)	2.89±0.15	2.84±0.20	3.71±0.13 ^{#**}	2.94±0.13 ^{§***}	2.59±0.13 ^{¶***}
	Putrescine (nmol/mL)	225.4±83.6	165.7±72.9	1070±251 ^{#*}	16.45±9.34 ^{§***}	3.62±0.99 ^{¶***}
	Spermidine (nmol/mL)	32.66±8.63	37.22±12.46	409±217 ^{#*}	3.21±1.38 ^{§***}	2.92±0.93 ^{¶***}
	Spermine (nmol/mL)	30.71±6.06	31.55±7.56	355.6±187 ^{#***}	10.44±4.93 ^{§***}	4.07±1.34 ^{¶***}
	Tumour	Tumour+treat 1	Tumour+treat 2			
Tissue	Arginase (U/mg pr.)	39.4±3.98	21.9±2.74 ^{^**}	20.7±2.75 ^{†**}		
	Ornithine (nmol/mg pr.)	72±10	45±3 ^{^*}	39±2 ^{†***}		
	Putrescine (nmol/mg pr.)	7.51±1.45	4.51±1.27	3.26±0.89 ^{†**}		
	Spermidine (nmol/mg pr.)	40.6±9.42	30.0±10.4	2.15±0.15 ^{†***, ‡***}		
	Spermine (nmol/mg pr.)	22.5±3.51	10.41±3.05	1.31±0.16		

sation, phosphate buffer was used. Samples were homogenised with 0.05 M phosphate buffer (5 times the weight, pH: 7.2).

Here, 167 µL of the sample was taken into the test tubes and 100 µL of 10% trichloroacetic acid was added. Then, the samples were centrifuged for 10 minutes at 3000 g and the ultrafiltrate was obtained. For 200 µL of sample ultrafiltrate, 25 µL hexamethylenediamine, 25 µL saturated sodium carbonate and 50 µL dansyl chloride were added and vortexed. The mixture was kept at 50°C for 30 minutes in a water bath and subsequently centrifuged for 5 minutes at 4000 g and applied to the HPLC system (16).

In this method, Waters Alliance 2690 separation module, Model 474 fluorescence detector and Waters Millennium 32 software were used. Chromatography was performed on a µBondapak C18 cartridge (4.6 x 250 mm, 5 µM particle size) with a guard column (3.9 x 20 mm) packed with the same stationary phase (Waters, Milford, MA, USA). The column temperature was 50°C and flow rate was 1 ml/min. Fluorescence was measured at excitation and emission wavelengths of 370 and 506 nm, respectively.

Statistical analysis

The statistical analyses were performed with the SPSS software version 19.0 (IBM, New York, USA). The results were reported as mean ± standard error of mean. The data were first analysed with the Shapiro-Wilk's test and were found to be nonparametric. Differences between the control and treatment group arginase enzyme activities and ornithine and polyamine levels were evaluated by Kruskal-Wallis test and compared with the Mann-Whitney U test. P values below 0.05 were recognised to be statistically significant.

RESULTS

In this study, serum arginase enzyme activities and ornithine and polyamine levels and tissue arginase enzyme activity and

ornithine and polyamine levels were determined. All of those parameters are shown in Table 1.

In serum samples, a statistically significant decrease in arginase activity, putrescine, spermidine and spermine levels was observed for the rosuvastatin treatment compared to the tumour group, which was also significantly higher than the healthy controls (Figure 1). This decreasing effect was parallel with the dose of the rosuvastatin.

In tumour tissue, there was still a significant decrease in arginase activity, ornithine, putrescine, spermidine and spermine levels in the rosuvastatin treatment compared to the tumour group (Figure 2). This reduction was more relevant with the dose of the treatment.

DISCUSSION

In this study, for the first time in the current literature, we investigated the effects of rosuvastatin on the arginase enzyme system and polyamines in the breast cancer model. We clearly demonstrated that rosuvastatin had some beneficial effects on cancer progress as it significantly reduced the arginase enzyme activity and polyamine formation in breast cancer.

Nitric oxide synthases (NOSs) and arginase can compete for their common substrate, L-arginine; this interaction between the two enzymes represents a potentially important factor in the regulation of NO production. Elevated arginase activity could limit NO synthesis by reducing L-arginine availability for NOS (17). It has been documented that arginase activity is 5-fold greater than NOS activity and arginase was found to be present in the major pathway of L-arginine metabolism in nephritic glomeruli (18); likewise, it was suggested that while arginase activity increases, NOS activity decreases in the erythrocytes of the patients with chronic renal failure (19). It was also reported that for the mammalian arginase, the Km for

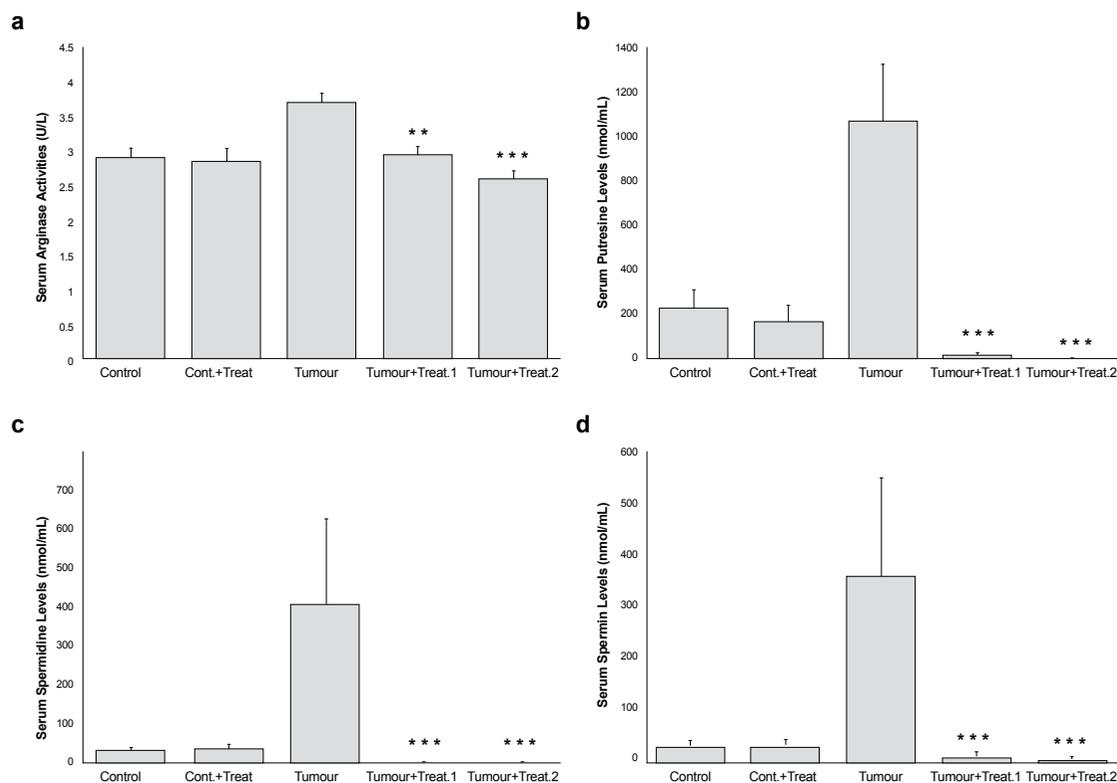


FIG. 1. a-d. Serum Arginase enzyme activities (a), Putrescine levels (b), Spermidine levels (c), Spermine levels in groups (d) (mean±SEM, *p<0.05, **p<0.01, ***p<0.001, comparisons between tumour control vs treatment groups)

L-arginine was 2-20 mM and 1-20 mM for the various NOS isoenzymes (20). However, at physiological pH, the V_{max} of each of the NOS enzymes was less than 1000-times that of arginase (21); therefore, it was concluded that both NOS and arginase could use arginine (22). Previously, we also showed that there was a negative relation between arginase enzyme activities and NO levels in breast cancer (15).

Nitric oxide is a pervasive molecule that employs many biological outcomes (5). The decisive effect of NO on tumour growth is complicated and remains largely unclear. While low levels of NO have been suggested to stimulate tumour cell proliferation and growth (23), and to increase the metastatic ability of tumour (24), high NO concentration may inhibit tumour growth (25, 26) and induce apoptosis in tumour cells (27). Studies have reported that the duration, expression level and timing of NO delivery, the microenvironment, the cell type and the genetic background might regulate NO sensitivity and the total effect of NO (28). NO can be used as a chemo-sensitising and immuno-sensitising agent (5), and thus, we believe its clinical application may help in the treatment of cancer.

Arginase has been found to be present in many non-hepatic tissues such as the mammary gland, intestine, kidney, brain

and lungs (29). Extrahepatic arginase could be connected in the regulation of tissue repair and cell growth (1). Elevated arginase activity was found in a variety of established human breast cancer cells (6). Previous studies have demonstrated high levels of serum arginase activity in several different carcinomas including gastric, colorectal, large bowel, prostate, lungs and breast cancers, suggesting that this enzyme might serve as a useful biomarker in cancer and cancer progression (6). L-ornithine could be converted to proline and glutamate by ornithine aminotransferase. In mammalian cells, ornithine is substrate for the formation of glutamate, proline and polyamines (30). Polyamines have also been indicated to play an important part in cellular proliferation and growth (30, 31). They have also been associated with carcinogenesis (9). The exact mechanism(s) for arginase activity in cancer tissues are unknown. On the other hand, one possible mechanism would be dependent upon the elevated concentration of ornithine, a precursor of polyamines, and might result from an increase in extrahepatic arginase. Increased tissue arginase enzyme activity and ornithine levels together appear to enlarge polyamine formation, which could lead to cancer development.

Supporting this theory, in our study, we have demonstrated that serum arginase activity and polyamine levels increased in

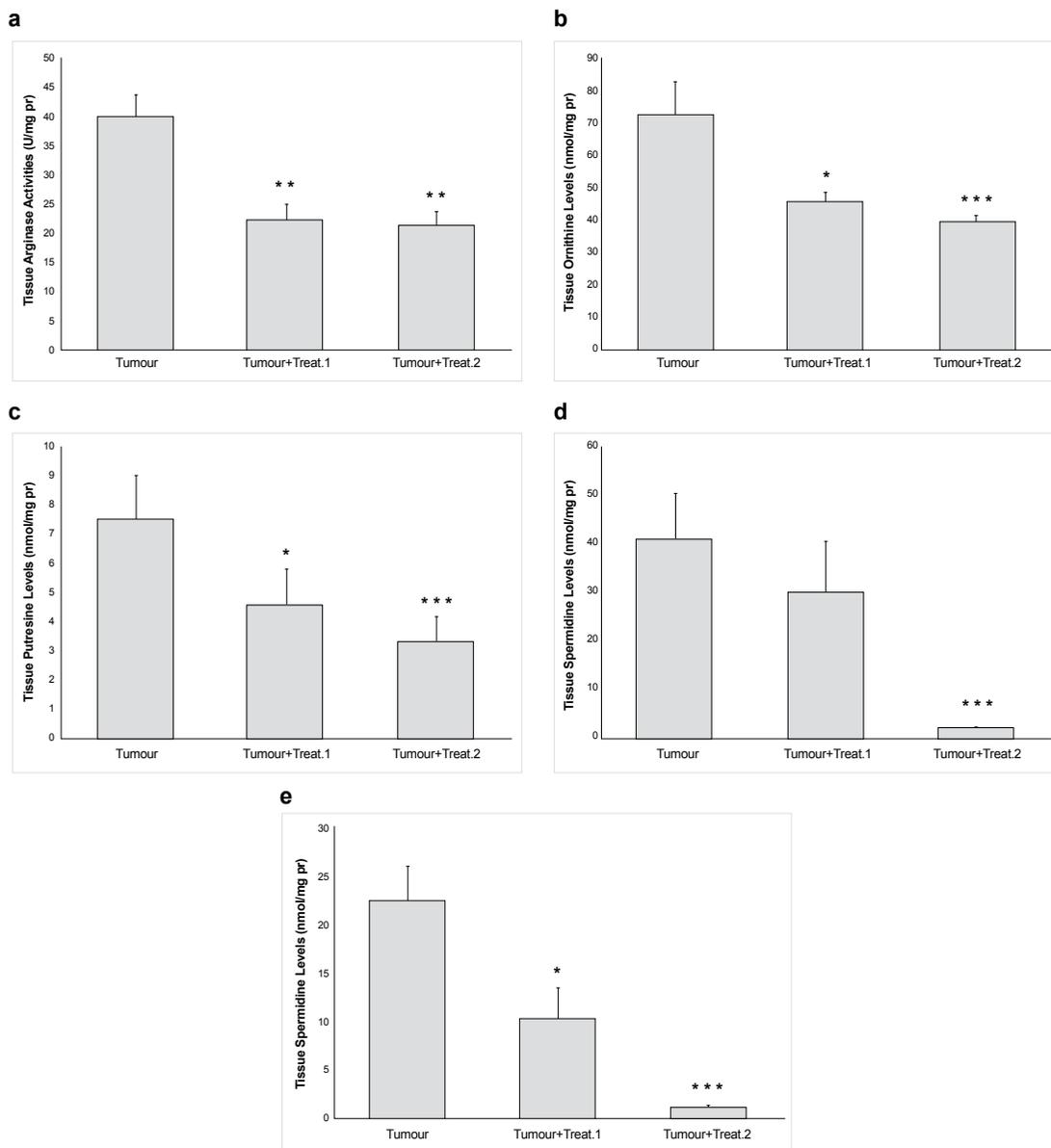


FIG. 2. a-e. Tissue Arginase enzyme activities (a), Ornithine levels (b), Putrescine levels (c), Spermidine levels (d), Spermine levels in groups (e) (mean±SEM, *p<0.05, **p<0.01, ***p<0.001, comparisons between tumour control vs treatment groups)

breast cancer and that rosuvastatin treatment significantly reduced these markers. Moreover, in tumour tissue, we had a similar action of reduction in the concentrations of cancer molecules, including ornithine. This reduction has been more relevant with the increased concentration of rosuvastatin.

It has been shown that statins inhibit cancer cell proliferation by stopping the cell cycle in G1-S phase and inducing apoptosis (13). In a study of experimental pancreatic cancer, the administration of statins has been shown to prevent cancer cell growth (32). A colorectal cancer study showed that statins had a 47%

decrease in the probability of cancer progression (33). In a recent study on hepatocellular cancer, it was also shown that the use of statins reduced the risk of hepatocellular cancer (34).

In cancer development, one of the possible protective mechanisms could be NO production. While the inhibition of arginase will lead to the lower production of ornithine and polyamines, at the same time it would stimulate the NOS action and therefore the formation of NO. In one study, researchers also strongly emphasised that the action of the statins in MCF-7 breast cancer cells should be through the higher production of NO via increasing NOS expression (13).

As we know, statins like rosuvastatin inhibit HMG CoA reductase; this inhibition also leads to a reduction in the usage of its coenzyme, NADPH, which will lead to the formation of a high concentration of NADPH. NADPH is also a coenzyme for NOS and stimulates NOS activation. At the end, this mechanism would result in an increased NO formation. In other words, the high level of NADPH could depress arginase enzyme activity and may activate the NOS production, therefore decreasing polyamine production in breast cancer.

In polyamine metabolism, the main enzyme group is polyamine oxidases. The action of these enzymes involves hydrogen peroxide production. Hydrogen peroxide is also an effective negative feedback regulator of the enzyme at high concentrations and inhibits the catabolism of polyamines via reducing enzyme activities (35). One of the enzymes in hydrogen peroxidase detoxification is glutathione reductase, which needs NADPH for regeneration. In the NADPH high environment, hydrogen peroxide production would be inhibited, which would lead to a reduction in polyamine levels through the increase in polyamine catabolism. Another possible mechanism behind the finding of decreased polyamine levels in this study may be hidden at this point.

As a conclusion, we may suggest that rosuvastatin may have some protective effects on breast cancer development as it inhibits arginase enzyme activity, ornithine levels, precursors of polyamines, and also polyamines, therefore inducing NO production via NOS. As a promising anticancer agent, the net effects of rosuvastatin in this mechanism should be supported with more advanced studies and new parameters.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Trakya University Animal Ethics Committee (2011.09.05/09).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.E., O.B., E.Ç.; Design - H.E., O.B., E.Ç.; Supervision - H.E., E.Ç.; Resource - H.E., O.B.; Materials - H.E., O.B.; Data Collection&/or Processing - O.B.; Analysis&/or Interpretation - H.E., O.B.; Literature Search - H.E., O.B.; Writing - H.E., O.B., E.Ç.; Critical Reviews - H.E., E.Ç.

Acknowledgements: We thank to Sanovel for supplying rosuvastatin as a gift.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received financial support from Trakya University Research Project (TUBAP 2012/42).

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