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Determining the Response to Statins in Rat Aorta Using High Fat Diet

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Abstract

Atorvastatin and rosuvastatin, statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase are widely prescribed as lipid-lowering drugs. They also inhibit the RhoA-Rho-associated kinase pathway in vascular smooth muscle cells and inhibit smooth muscle function. It remains presently unknown whether vascular reactivity is influenced with a high fat diet. This study aimed to investigate the vascular responses to statins when rats were fed high fat diet. Adult rats were fed a normal diet or a high-fat diet (HFD) for four weeks. The relaxant effects of atorvastatin and rosuvastatin (10^{-9}-10^{-3} M) were tested against serotonin (5-HT; 10^{-6} M)-induced tone. To analyze the role of nitric oxide on the responses to statins, the concentration-response curves were also obtained in the presence of nitric oxide synthase inhibitor L-NAME (10^{-4} M). Statins, concentration-dependently relaxed aorta rings compared to control and fed a high-fat diet. The pIC_{50} values of aortas from rats fed a high fat diet to both atorvastatin and rosuvastatin were significantly lower than control groups. Preincubation with L-NAME significantly decreased the sensitivity to both statins. This finding suggests that acute vascular relaxant effects of these statins are importantly related with endothelial nitric oxide. Compared to the control group, high fat diet significantly reduced the luminal area of the aorta. Furthermore, the aorta wall thickness was significantly increased in high fat diet group. The results indicate that high fat diet affects vascular sensitivity to statins and morphology in rat aorta.

Keywords: Atorvastatin, High fat diet, Nitric oxide, Rat aorta, Rosuvastatin.

1. Introduction

High fat diet-induced hyperlipidemia is one of the most common risk factors worldwide associated with the development of atherosclerosis and coronary heart disease. Statins are the most widely prescribed medications for treatment of hyperlipidemia for over 20 years (Izadpanah and Schächtele, 2015).

Statins also have effects on vascular wall independent of their cholesterol lowering properties. Among the many effects attributed to statins, increased nitric oxide synthesis, vascular dilatation, anti-inflammatory and antithrombotic effects were described. Early findings obtained from clinical trials and experimental studies revealed that their beneficial effects are due to restoration of endothelial function (Beckman and Creager, 2006; Adam and Laufs, 2008). In vitro findings strongly suggested that increased bioavailability of, by upregulating and activating endothelial nitric oxide synthase or by decreasing its oxidative inactivation, mediate direct vasculoprotective effects of statins (Wagner *et al.*, 2000; Bonetti *et al.*, 2003). A recent study showed that lovastatin and pravastatin stimulated endothelial nitric oxide production in bovine aortic endothelial cells (Datar *et al.*, 2010).

Lopez *et al* (2008) reported that, rosuvastatin, like other statins (Uydes-Dogan *et al.*, 2005), has vasodilator properties in rat aortic rings. Although, it is known that arteriosclerosis is closely associated with endothelial dysfunction and the consequent reduced production of its relaxant factors, especially nitric oxide, there is no report related to the role of high fat diet on rat aortic responses to statins. The investigators (Lopez-Canales *et al.*, 2015) also reported that vasorelaxant response to rosuvastatin was significantly greater in endothelium-intact than denuded aortic rings, which suggests that endothelium plays an important role in the vasorelaxant effect produced by rosuvastatin. Moreover, the fact that the vasorelaxant response to rosuvastatin was significantly greater in aortic rings from rats with a standard diet than in aortic rings from rats with a cafeteria-style (CAF) diet suggests that endothelial dysfunction could be involved in the inhibitory effect produced by a CAF diet. Moreover, Qin *et al* (2018) reported that high fat diet induced significant attenuation in the nitric oxide-dependent relaxation to acetylcholine of rat aortic rings. The investigators also suggested that nitric oxide produced by endothelium was inhibited by high-fat diet. However, no study has yet analysed the effect of high fat diet on the statins-induced vascular responses.

Although statins have some pleiotropic effects on vascular wall, and these nonlipid-properties might be important to prevent some cardiovascular events, there is no study on how high-fat diets affect the direct vascular responses to these drugs. Therefore, in this study we aimed

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to investigate whether high fat diet has effects on atorvastatin and rosuvastatin-induced vasodilation in thoracic aorta rings of high fat diet-treated rats.

2. Materials and Methods

2.1. Drugs

Serotonin chloride, N^G nitro-L-arginine methyl ester, acetylcholine chloride (dissolved in distilled water) and atorvastatin, rosuvastatin (dissolved in dimethyl sulphoxide) were used. The concentration of dimethyl sulphoxide in the tissue bath was always kept below 0.4%. Serotonin chloride, N^G nitro-L-arginine methyl ester and acetylcholine chloride were obtained from Sigma (St. Louis, MO, USA). Atorvastatin and rosuvastatin were kindly provided by Abdi İbrahim Drug Industry (Istanbul, Turkey).

2.2. Animals and preparation of aortic rings

Fourteen male Wistar rats, weighing 259,36 ± 28,3 g and aged 8-12 weeks, were obtained from Necmettin Erbakan University Experimental Medicine Research and Application Center (Konya, Turkey). The rats were housed in a climate controlled room (22 ± 2 °C temperature and 50 ± 5% humidity) on a 12/12 h light/dark cycle (lights on between 07:00 and 19:00), with ad libitum food and fresh water. The experimental animals were randomly divided into two groups (n=7 for each group) and fed with either standard rat chow, or high-fat diet (Bravo *et al.*, 2014), for a period of 4 weeks. The caloric value of food intake was determined on the basis of 35% of total calorie intake for the high fat diet group rats (Table 1). Body weights before and after four weeks were recorded (Table 2). All animals were sacrificed after an overnight fasting by cervical dislocations.

Prior permission for animal experimentation was obtained from the Experimental Medicine Research and Application Center Ethics Committee of Necmettin Erbakan University (15-020).

2.3. Experimental design

Rats were sacrificed by cervical dislocations. The descending thoracic aorta was quickly isolated, cleaned and sectioned into 3- to 4-mm-long rings. The rings were then placed in organ baths containing 15 ml Krebs-Henseleit solution (KHS, mM: NaCl 119, KCl 4.70, $MgSO_4$ 1.50, KH_2PO_4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25, Glucose 11), which were thermoregulated at 37 ºC and aerated (95% O_2 and 5% CO_2).

Changes in isometric tension of aortic rings were recorded using a four-channel force-displacement transducer (BIOPAC MP36, Santa Barbara, California, USA) connected through amplifiers to a ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey). Endothelium intact rings were used. After the stabilization period, isometric contraction was induced by serotonin (5-hydroxytryptamine, 5-HT) (10^{-6} M) and acetylcholine (ACh, 10^{-6} M) was added to verify the integrity of the endothelium. The vascular endothelium was considered complete when the aortic rings showed relaxation ≥ 50%. Then, the rings were washed and rested; to evaluate the effect of atorvastatin or rosuvastatin on vascular tension, rat aorta rings from seven different preparations were exposed to 5-HT (10^{-6} M) to induce constriction. Atorvastatin or rosuvastatin (10^{-9}-10^{-3} M) was added after the maximal vasoconstrictive response to 5-HT had been achieved. To determine the role of nitric oxide, the relaxant effect of atorvastatin or rosuvastatin was investigated in the presence of nitric oxide synthase inhibitor N^G nitro-L-arginine methyl ester (L-NAME, 10^{-4} M). L-NAME had been added to the organ bath 20 min before statin concentration-response curves were obtained.

In another part of the study, the same procedure was repeated in the aortas from HFD-treated rats in the absence and presence of L-NAME (10^{-4} M).

2.4. Analysis of blood parameters

Blood was immediately collected at the end of experiment and centrifuged at 3000 rpm for 15 min at 4 °C. Serum was stored at -70 °C until used in assays. Serum triglyceride (TG), cholesterol (Chol) and HDL levels were determined using commercial kits (Table 2).

2.5. Histological morphometric analysis of thoracic aorta

Thoracic aorta samples were obtained after rats were sacrificed at the end of experiment. After formaldehyde fixation, the entire thoracic aorta was sectioned at 5 segments of 4 mm each. Tissues were embedded in paraffin, cut into 4-6 µm thick sections and stained by Hematoxylin and Eosin. Measurements of the thoracic aorta cross-sectional area, were made in a single-blind fashion by pathologist.

Morphometric measurements on all five segments of aorta were performed by using the Image Analysis System (BAB Bs200ProP Image Processing and Analysis System, Ankara, Turkey). The luminal area (μm^2) and wall thickness (µ) were calculated from the perimeter of the luminal border. The luminal area and wall thickness for each thoracic aorta were obtained by averaging these measurements. The mean ± SE value for a particular vessel, after averaging five consecutive thoracic aorta segment values for cross-sectional area, was obtained.

2.6. Statistical analysis

Relaxation responses to atorvastatin or rosuvastatin were expressed as percentages of the 5-HT (10^{-6} M) induced contraction. Concentrations of atorvastatin or rosuvastatin causing 50% of the maximal response (IC_{50}) were calculated from each individual concentration-response curves; pIC_{50} values were determined as -log IC_{50}.

Statistical analysis was performed by using Mann-Whitney U and unpaired Student's t-test where appropriate. Statistical significance was set at $P < 0.05$. All the data were expressed as mean ± SEM.

3. Results

3.1. Body weights and plasma lipid profile of rats fed a standard or high-fat diet

The effect of a high fat diet on body weight and the serum lipid profile levels in male rats is shown in Table 2. A modest nonsignificant increase in body weight was observed in rats fed a high-fat diet for a period of four weeks compared with rats fed a standard diet. A significantly higher level of plasma cholesterol and triglyceride levels were observed in rats fed with a high fat

diet ($P < 0.05$). No difference was observed in high-density lipoprotein (HDL) levels between groups ($P > 0.05$).

3.2. Effect of atorvastatin on 5-HT-precontracted rat aortic rings

Figure 1 shows the effects of cumulative addition of atorvastatin on 5-HT-pre-contracted aortic rings from rats with either a standard diet or a high fat diet. In all animals, (10^{-9}-10^{-3} M) atorvastatin elicited concentration-dependent vasorelaxation in aortic rings, regardless of whether the animals were fed a standard diet or a high fat diet. The pIC_{50} value in 5-HT-precontracted aortic rings from rats given a standard diet was 5.27 ± 0.20. Pre-treatment with nitric oxide synthase inhibitor L-NAME (10^{-4} M) produced a rightward shift of the atorvastatin curve in control group (pIC_{50} value was 4.77 ± 0.15). In aortas from rats fed high fat diet the pIC_{50} value (4.60 ± 0.10) was significantly lower than control group. In high fat diet group, pre-incubation with L-NAME significantly decreased the pIC50 value to atorvastatin (4.24 ± 0.20, Table 3).

3.3. Effect of rosuvastatin on 5-HT-precontracted rat aortic rings

As shown in Figure 2, the effect of cumulative addition of rosuvastatin on 5-HT-precontracted aortic rings from rats with either a standard diet or a high fat diet. In all animals, rosuvastatin (10^{-9}-10^{-3} M) elicited concentration-dependent vasorelaxation of aortic rings, regardless of whether the animals were fed a standard diet or a high fat diet. In control rats the pIC_{50} value was 5.82 ± 0.10. L-NAME- pretreatment significantly decreased the sensitivity to rosuvastatin in control group (Table 3). In rats fed high fat diet, the pIC_{50} value was significantly lower than control (4.95 ± 0.23, $p < 0.05$). The pIC_{50} value (4.29 ± 0.16) of aortic rings after L-NAME treatment significantly reduced in high fat diet rats. These results demonstrate that inhibition of nitric oxide synthesis significantly decreased the sensitivity of aorta to rosuvastatin in both control and high fat diet rats.

3.4. Analysis of aorta wall thickness and lumen diameter

The mean values for the measurements of aortic wall thickness and lumen diameter were analysed statistically; confidence interval was assessed at 95%. The aorta wall thickness was significantly increased in high fat diet group (99.3 ± 2.8 µm) compared to control (88.0 ± 2.6 µm) ($P < 0.05$). The average luminal areas of rat aorta were found to be 1612290.0 ± 18090.4 μm^2 in control and 1501073.0 ± 26016.2 μm^2 in high fat diet group (Figure 3). The luminal area of the high fat diet group decreased significantly compared to the control ($P < 0.05$).

Table 1. Compositions and energy loads of the diets.

	Standard		High Fat	
	% of weight	% of total energy	% of weight	% of total energy
Casein	20	25.6	20	22.5
Starch	63	59.4	51.3	42.5
Vegetable oil/tallow*	7	15	18.7	35
Fibers	5	-	5	-
Vitamin and mineral mix	4.5	-	4.5	-
Total energy (gross kcal/g)	4.4	-	5	-

*Vegetable oil was used in the standard diet, whereas in the high-fat diet tallow was used.

Table 2. Characteristics of rats after four weeks of normal (control) and high-fat diet (HFD).

	Control	HFD
Triglyceride (mg/dl)	60.71 ± 5.8	93.57 ± 9.6*
Cholesterol (mg/dl)	53.85 ± 2.6	60.71 ± 1.5*
HDL (mg/dl)	32.67 ± 2.1	33.72 ± 1.2
Initial body weight (g)	267.43 ± 11.2	265.00 ± 9.9
Final body weight (g)	339.14 ± 16.9	343.86 ± 11.7
Weight gain (g)	71.71 ± 21.6	78.85 ± 17.6

Values are means ± SD. Each value is derived from seven rats. *$P < 0.05$, significant difference.

Table 3. pIC_{50} (- log IC_{50}) values for atorvastatin and rosuvastatin (10^{-9}- 10^{-3}M) in 10^{-6} M 5-HT-precontracted aortas from control, L-NAME-incubated, HFD-treated and L-NAME-incubated HFD-treated rats.

	pIC_{50}	
	Atorvastatin	Rosuvastatin
Control	5.27 ± 0.20	5.82 ± 0.10
L-NAME	4.77 ± 0.15*	4.95 ± 0.23*
HFD	4.60 ± 0.10*	4.54 ± 0.25*
HFD-L-NAME	4.24 ± 0.20**	4.29 ± 0.16**

Values are mean ± SD. Each value is derived from seven experiments.

*Statistically significant ($P < 0.05$) compared with control.

** Statistically significant ($P < 0.05$) compared with HFD.

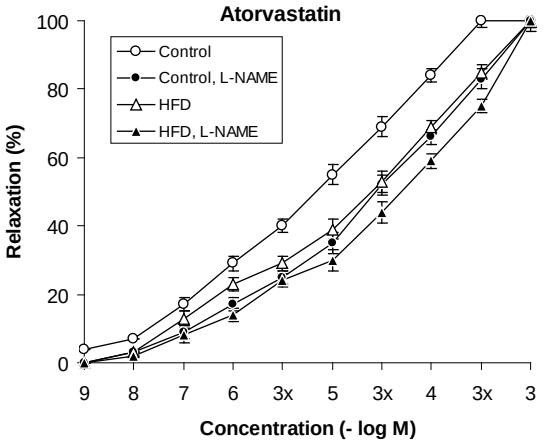

Figure 1. Concentration-response curves showing relaxations induced by atorvastatin (10^{-9}- 10^{-3} M) in 5-HT-precontracted rat thoracic aortas from control, L-NAME-incubated, HFD-treated and L-NAME-incubated HFD-treated rats. Each point represents the mean ± SD expressed as percentage of the tension developed by 10^{-6} M 5-HT. Each value is derived from seven experiments.

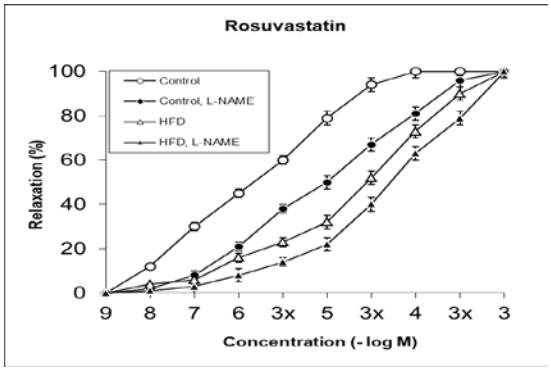

Figure 2. Concentration-response curves showing relaxations induced by rosuvastatin (10^{-9}- 10^{-3} M) in 5-HT-precontracted rat thoracic aortas from control, L-NAME-incubated, HFD-treated and L-NAME-incubated HFD-treated rats. Each point represents the mean ± SD expressed as percentage of the tension developed by 10^{-6} M 5-HT. Each value is derived from seven experiments.

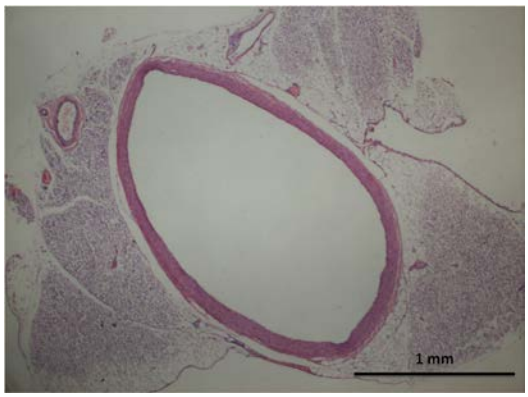

Figure 3 A. Luminal area in control rat aorta.

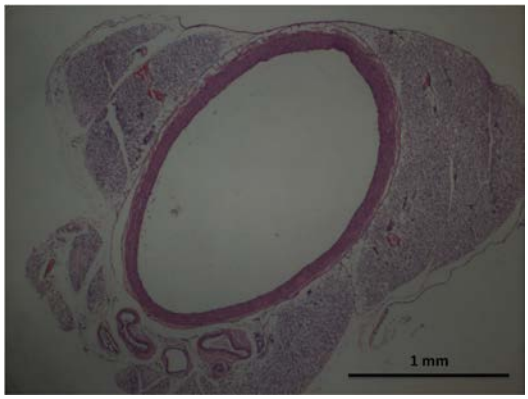

Figure 3 B. Luminal area in high fat diet treated rat aorta.

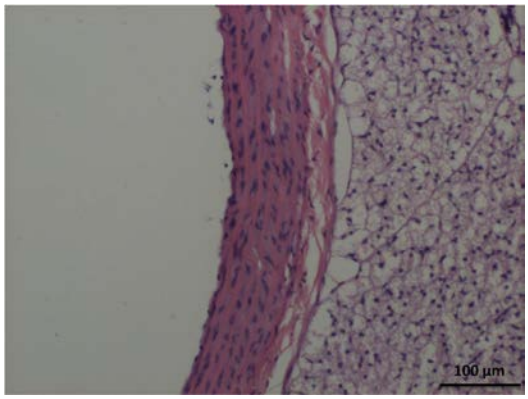

Figure 4 A. Wall thickness in control aorta.

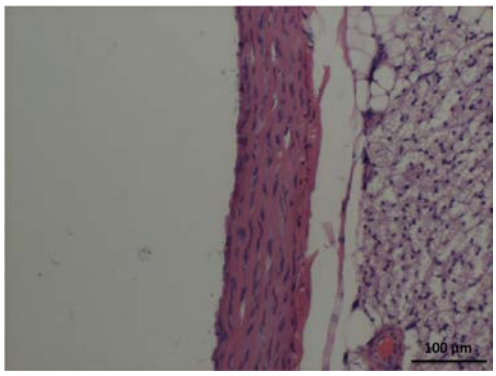

Figure 4 B. Wall thickness in high fat diet treated aorta.

4. Discussion

Atorvastatin and rosuvastatin have acute relaxant effect on rat thoracic aorta smooth muscle, which is in agreement with previous reports evaluating the vascular effects of different statins on isolated rat aortic rings (Lopez *et al.*, 2008). Furthermore, the principal finding of this study was high-fat diet could influence the responses to statins in vascular smooth muscle. We observed that the sensitivities to both atorvastatin and rosuvastatin were decreased in rats fed with a high fat diet. Overall, this study indicates that high fat diet for 4 weeks promotes vascular and histologic alterations.

Statins induce acute vasorelaxation that is independent of their lipid-lowering action which may contribute to the overall benefits of these drugs in the treatment of cardiovascular disease (Sun *et al.*, 2009; Qin *et al.*, 2018). It has even been reported that the effects of statins appear to be diverse and depend upon the vasculature studied (Corsini *et al.*, 1998; John *et al.*, 2001). Our results indicate that both atorvastatin and rosuvastatin-induced concentration-dependent relaxation of 5-HT-contracted rat aortas. Furthermore, the potencies of both statins were similar in each groups. 5-HT induces smooth muscle contraction and 5-HT2A, a G protein-coupled receptor, mediates 5-HT contractions in rat thoracic aorta (Banes *et al.*, 1999). It activates phospholipase C via Gq resulting in IP_3 and DAG formation, which induces protein kinase C and Ca^{2+} entry through voltage-operated Ca^{2+} channels. In the present study, atorvastatin and rosuvastatin both inhibited the contractions induced by 5-HT in a concentration-dependent manner. This result suggests that atorvastatin and rosuvastatin can inhibit the vasocontraction induced by extracellular Ca^{2+} entry via the voltage-operated Ca^{2+} channel pathway. The result of the present study is in agreement with previous reports evaluating the vascular effects of different statins on isolated rat aortic rings (Uydes-Dogan *et al.*, 2005; López *et al.*, 2008; Lopez-Canales *et al.*, 2015; Nurullahoglu-Atalik *et al.*, 2017). Furthermore, in this study both atorvastatin and rosuvastatin produced similar responses.

It is known that endothelial nitric oxide is important for vasodilation, and it has been well established that statins improve the endothelial function, especially by enhancing nitric oxide production in a manner not only dependent on but also independent of its cholesterol-lowering effect in humans and animal models (Bellosta *et al.*, 2000; Lefer *et al.*, 2001). In this study we have shown that incubation with the nitric oxide synthase inhibitor, L-

NAME, abolished the vascular smooth muscle relaxation elicited by atorvastatin and rosuvastatin in control rings. Similarly, Uydes-Dogan *et al* (2005) reported that incubation of the aortic rings with nitric oxide synthase inhibitor L-NOARG significantly attenuated the acute vasorelaxation induced by pravastatin, atorvastatin and cerivastatin on isolated rat aorta. Furthermore, lovastatin (Bravo and Herrera, 1998), serivastatin (Mukai *et al.*, 2003) and fluvastatin (Yaktubay-Dondas *et al.*, 2011) also produced vascular relaxations on isolated vessel rings; however, a significant differential was observed in their response profile in terms of endothelium dependency. Bravo and Herrera (1998) reported that lovastatin is capable of relaxing the rat aorta independently of the presence of endothelium. On the other hand, we previously observed that rosuvastatin induced endothelium-dependent relaxations in calf cardiac vein (Nurullahoglu-Atalik *et al.*, 2015). Likewise, in this study, our findings suggested that both atorvastatin and rosuvastatin-induced responses were, partly nitric oxide-dependent.

In the present study, the high fat diet produced a rightward shift in atorvastatin and rosuvastatin concentration-response curves with no suppression of maximum effect. Although, it is known that endothelial dysfunction often accompanies the hyperlipidemic state, there is no in vitro report related to the role of high fat diet on rat aortic responses to statins. In literature, the effects of high fat diet on vascular responses to different agents were investigated in statin-treated rats for a while (Choi *et al.*, 2016; Xu *et al.*, 2014), but in this study direct effects of statins in aortas from rats fed with high fat diet those pre-contracted with 5-HT were analyzed. Therefore, to the best of our knowledge, this is the first study that suggested the direct vascular effects of atorvastatin and rosuvastatin in rats fed with high fat diet for four weeks.

Changes in the aorta wall thickness are significantly associated with serum lipid profiles and hypertension, which begins in childhood and may develop into cardiovascular disease (Davis *et al.*, 2001; McGill *et al.*, 2001). Atherosclerotic plaque formation in the vessel causes increased wall thickness. In this study, the aorta wall thickness was only slightly increased in high fat diet group than control. This suggests that high fat diet treatment may have led to the development of atherosclerotic plaques. Our results also demonstrate that rats fed with high fat diet decreased the luminal areas of rat aortas compared to the control groups. To the best of our knowledge, the results are the first demonstration that high fat diet-treatment significantly affected the aortic morphology and relaxations induced by atorvastatin and rosuvastatin in rats but we have no explanation for this phenomenon. The results of this study also showed an increase in vessels wall thickness, as Pashaie *et al.*(2017) reported in aortas from rats fed with a high cholesterol diet for six months.

5. Conclusion

In the present study, we demonstrated that atorvastatin and rosuvastatin can acutely induce vasorelaxation on precontracted aortic rings via nitric oxide-dependent mechanisms. The results indicate that high fat diet treatment affects vascular sensitivity and morphology in rat aorta.

Conflict of interest

The Authors declare that there is no conflict of interest with this work and the preparation of the paper.

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