



# DUAL EFFECT OF ATORVASTATIN ON ENDOTHELIUM; REDUCTION IN LDL AND TXA<sub>2</sub> LEVELS BUT UNALTERED HCY LEVELS IN OBESE HYPERLIPIDEMIC RATS.

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**ABSTRACT... Objective:** The objective of the study was to observe the effect of lipid lowering therapy on homocysteine and TXA<sub>2</sub> concentration in obese hyperlipidemic Sprague Dawley rats. **Design:** Randomized Control Trial (RCT). **Place and Duration of study:** The study was conducted in Department of Physiology and Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi; and National Institute of Health (NIH) Islamabad over a period of 12 months. **Methodology:** Ninety healthy Sprague Dawley rats divided into three equal groups. Group I (n=30) were healthy controls, group II (n=30) were made obese and group III (n=30) were obese treated (atorvastatin 10 mg/kg/day orally by gavage method for three weeks). Body weight was recorded thrice weekly, lipid profile was measured by colorimetric method on microlab and homocysteine and TXA<sub>2</sub> were measured by Enzyme Linked Immunosorbant Assay. **Results:** Serum low density lipoproteins and TXA<sub>2</sub> decreased after three weeks of atorvastatin administration, elevated HCY concentration in obese hyperlipidemic rats however was not significantly affected. **Conclusion:** Atorvastatin apart from lowering lipid levels in the body also reduces TXA<sub>2</sub> concentration which is a vasoprotective. Elevated HCY concentration which is deleterious to the endothelium however is not affected.

**Key words:** Atorvastatin, Thromboxane-A<sub>2</sub>(TXA<sub>2</sub>), Endothelium, Obesity, Hyperlipidemia.

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## INTRODUCTION

The endothelium consists of a single layer of cells that lines all the blood vessels and performs various important physiological functions. It controls the trafficking of blood cells, tone and permeability of vessels, and hemostatic balance. It causes release of vasodilator (e.g. nitric oxide) and vasoconstrictor substances (e.g. endothelin-1) in response to physical and chemical stimuli.<sup>1</sup> Endothelial dysfunction causes the impairment of regulation of vascular tone, inflammation, and hemostasis.<sup>2</sup> One of the mechanisms by which obesity and hyperlipidemia predispose to thrombotic conditions is disturbed endothelial function and ongoing atherosclerotic process. Cytokines released from adipose tissue in obesity disrupt the normal functioning of endothelial cells and act as protagonists in development of atherosclerosis.<sup>3</sup> Hypercholesterolemia with increased LDL levels causes the impairment of endothelial function both in coronary as well as in

peripheral vessels by the decreased production of endothelial nitric oxide.<sup>4</sup>

Consumption of high fat diet leads to an increase in homocysteine concentration. Individuals taking diet having more fat content were found to have high homocysteine concentration. Hordaland homocysteine study revealed the influence of different diets on homocysteine concentration.<sup>5</sup> Elevated homocysteine concentration has been implicated in the pathogenesis of cardiovascular disease by disrupting normal endothelial function. Homocysteine has been documented to act either as a causative agent or risk factor in the development of coronary heart disease in a systematic review and meta-analysis, where relationship between homocysteine and coronary heart disease was studied.<sup>6</sup> The exact pathway by which homocysteine causes endothelial dysfunction is controversial, and several mechanisms have been proposed.

Homocysteine is detoxified by nitric oxide released by normal endothelial cells which in the presence of oxygen combines with homocysteine to form S-nitroso homocysteine. Generation of sulfhydryl dependent hydrogen peroxide is inhibited by nitrosation of homocysteine sulfhydryl group. Long term endothelial homocysteine exposure limits its nitric oxide production and compromises the protective effect of nitric oxide. The endothelium therefore becomes vulnerable to oxidative injury produced by homocysteine.<sup>7</sup>

Thromboxane was first identified in 1975 by Hamberg, Svensson, and Samuelsson as an agent that caused aggregation of platelets and constriction of vessel wall. It is a metabolite of arachidonic acid which is synthesized by oxidation of arachidonic acid to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) by either cyclooxygenase 1 (COX-1) or cyclooxygenase 2 (COX-2) as the first step, and then metabolism of PGH<sub>2</sub> to TXA<sub>2</sub> by thromboxane synthase as the final step. Due to short half-life and rapid metabolism, it is thought to have only localized effects close to its site of synthesis.<sup>8</sup> Increased TXA<sub>2</sub> biosynthesis is implicated in the pathogenesis of cerebrovascular and cardiovascular diseases. In a clinical trial, where TXA<sub>2</sub> levels in patients having ischemic stroke were determined, majority had increased levels.<sup>9</sup>

Dyslipidemia is one of the serious consequences of obesity. Elevated triglycerides and reduced HDL predispose to the development of atherosclerosis,<sup>10</sup> Elevated levels of lipids in blood, predispose to the development of coronary artery disease that needs effective treatment. Different lipid lowering drugs are available to normalize lipid levels, however based on efficacy, popularity, importance and medical use, statins and fibrates are preferred over others.<sup>11</sup>

Improvement of endothelial dependent dilation of peripheral and coronary arteries by statins in hypercholesterolemic subjects is known since 1994. A 6 month pravastatin treatment trial (10 -20 mg/day) in hypercholesterolemic patients resulted in improvement of endothelium dependent coronary vasomotion.<sup>12</sup>

Although improvement in endothelial function has been observed in various clinical trials secondarily to reduction in cholesterol levels. Statins effect on endothelial function outweigh its effect on lipids which suggest involvement of a mechanism independent of lipid lowering more commonly called pleiotropic effects of statins. A proposed mechanism for this improved endothelial function is increased nitric oxide production resulting from increased bioavailability of the enzyme endothelial nitric oxide synthase.<sup>13</sup>

## METHODOLOGY

The study was a randomized control trial conducted in Department of Physiology and Centre for Research in Experimental and Applied Medicine (CREAM), Army medical College, Rawalpindi in collaboration with National Institute of Health (NIH) Islamabad. The rats were randomly divided in three groups; each comprising of 30 rats. Each group was further divided into male and female subgroups with 15 rats in each subgroup.

Group I: (Control; n=30) Rats in this group were fed normal pellet diet and water ad libitum for a period of three weeks.<sup>14</sup>

Group II: (Obese; n=30) Rats in this group were fed high fat diet and water ad libitum for a period of three weeks<sup>14</sup> in order to induce obesity.

Group III: (Treated; n=30) Rats in this group were treated similar to the group II to induce obesity. Atorvastatin was administered for a period of three weeks in the dose of 10 mg/kg/day<sup>15</sup> orally by gavage method. 0.5 percent weight by volume carboxy methyl cellulose (CMC) was used as a suspension vehicle to administer the drug<sup>16</sup> after the induction of obesity along with the continuation of high fat diet. Terminal intra-cardiac sampling was done at the end of 3 weeks in group I and II rats and at the end of 6 weeks in group III rats. 4.5 – 5 ml blood was obtained from each rat after intra cardiac sampling. Blood was transferred to the serum gel and clot activator tubes. Tubes were placed in the eppendorf centrifuge machine (5810R), balanced by equalizing the number of empty spaces and spaces containing tubes in the

centrifuge machine. The blood was centrifuged for 10 minutes at the speed of 3000 rpm.

After centrifugation, serum was pipetted out of the serum gel and clot activator tubes by means of micropipette and transferred into the eppendorf storage tubes. These tubes were

stored at a temperature of -80°C in CREAM lab, Army Medical College for estimation of TXA<sub>2</sub>, homocysteine, and lipid profile at the end of the study. Lipid profile was analyzed on microlab by the principle of colorimetric method and LDL was calculated indirectly from other lipid parameters using Friedwald's formula.<sup>17</sup>

Parameter	Male	Female	Combined
	Mean ± SD (n = 430)	Mean ± SD (n = 242)	Mean ± SD (n = 672)
Cranial length	188.03 ± 9.71*	173 ± 7.87	180.30 ± 8.84
Cranial breadth	151.72 ± 6.33*	144.14 ± 5.42	148 ± 5.76
Auricular head height	144 ± 9.87*	139 ± 7.98	141 ± 8.43
Horizontal cephalic index (HCI)	79.13 ± 5.56*	78.32 ± 6.40	78.54 ± 5.55
Vertical cephalic index (VCI)	78.31 ± 5.19	78.32 ± 4.67	78.27 ± 6.43
Transverse cephalic index (TCI)	103 ± 7.78*	100 ± 8.67	101.81 ± 8.02

Table-I. Cranial dimensions and cephalic indices

\*P < 0.05

$$LDL = TC - [HDL + (TG / 5)]$$

TXA<sub>2</sub> and homocysteine were measured by Enzyme linked immunosorbant assay (ELISA).

## RESULTS

### LDL

LDL levels in the control group were 40.09 ± 7.28 mg/dl. High fat diet induced obesity resulted in significant (p=0.000) increase in LDL levels i.e. 89.76 ± 10.94 mg/dl with a mean difference of 49.673 mg/dl. Atorvastatin administration significantly (p=0.000) decreased LDL levels to a mean value of 42.59 ± 6.04 mg/dl so that there was insignificant difference p=0.481 between the control and atorvastatin treated group.

### TXA<sub>2</sub>

The pattern of increase and decrease in thromboxane A2 levels was similar to that of LDL. Control group rats had a mean TXA2 level of 37.63 ± 15.37 pg/ml. There was a significant increase (p=0.000) in obese rats (mean value 101.08 ± 14.95 pg/ml) that was significantly (p=0.000) reduced by atorvastatin administration (mean value 57.59 ± 13.27).

### HCY

HCY levels (control group 17.47 ± 3.01 pmol/ml) significantly increased (p=0.000) in the

obese group (mean value 53.82 ± 5.33 pmol/ml). Atorvastatin administration however did not significantly affect HCY levels (p=0.569) and the mean difference of homocysteine level in the obese and atorvastatin treated group came out to be 1.232 pmol/ml.

## DISCUSSION

### LDL

In our study there was a significant increase in serum LDL levels in obese group, as observed in most of the studies. Hyperlipidemia was also observed by Balamurugan and Shantha in obese rat model. The intake of high fat diet for 90 days resulted in significant increase in body weight accompanied by significant elevation in serum total cholesterol, triglycerides, LDL and VLDL levels along with decreased HDL levels; hence hyperlipidemia was induced in parallel with obesity.<sup>18</sup>

The association of obesity with hyperlipidemia has also been studied in humans who revealed a positive correlation. Obesity and associated insulin resistance result in increased influx of free fatty acids and glucose into the liver cells resulting in production of VLDL and TG's by hepatocytes. Increased VLDL synthesis along with its decreased clearance due to impaired LDL

receptors result in increased VLDL levels in blood. Increased VLDL levels in turn correlate directly with increased LDL and decreased HDL levels.<sup>19</sup>In the present study to see whether normalization of lipid profile could reverse the enhanced platelet reactivity and endothelial dysfunction or not, the obese rats were administered atorvastatin as a lipid lowering drug. Atorvastatin is a newer, third generation statin with greater half-life that has been documented as an effective drug in normalizing lipid profile because of its higher affinity and inhibition of enzyme 3-hydroxy-3-methylglutaryl coenzyme A for longer duration.<sup>20</sup>Moreover, it could be administered orally. The published data on animal models have shown more than 30 to 50 % reduction in plasma cholesterol and significant reduction in LDL. Statins not only decrease cholesterol synthesis but also increase its clearance from the blood stream.<sup>21</sup>

### TXA<sub>2</sub>

In our study, there was a significant increase in TXA<sub>2</sub> levels in obese hyperlipidemic male and female Sprague Dawley rats. The increase was greater in female rats corresponding with increased LDL levels. This result was similar to the results of other studies conducted both in animal models as well as humans.

Truape et al conducted a study to investigate the effect of obesity on vascular function and genes involved in prostanoid action in lean and diet induced obese mice. Thromboxane receptor gene analysis by means of real time quantitative polymerase reaction revealed a significant up regulation in obese mice. The inference drawn from their data was that obesity enhanced prostanoid mediated constriction of vessels along with increased gene expression for thromboxane receptor.<sup>22</sup>

Boger et al studied the effect of chronic dietary supplementation of L-arginine on platelet aggregation and TXA<sub>2</sub> synthesis in hypercholesterolemic rabbits. The study was conducted in rabbits fed either a normal rabbit chow, or chow enriched with cholesterol or chow enriched with cholesterol and arginine. In their

study when thromboxane levels were compared between normal and high cholesterol diet group, the later had significantly raised thromboxane levels due to increased synthesis owing to hypercholesterolemia. Arginine decreased platelet reactivity in rabbits which were fed chow enriched with cholesterol and arginine due to restoration of nitric oxide release by endothelium which is an inhibitor of platelet aggregation and showed decreased thromboxane production.<sup>23</sup>

Graziani et al measured the levels of a stable metabolite (TXB<sub>2</sub>) of TXA<sub>2</sub> in 17 lean subjects having a BMI of  $22.9 \pm 1.6$  kg/m<sup>2</sup>, 25 obese subjects having a BMI of  $32.6 \pm 2.4$  kg/m<sup>2</sup> and 23 morbidly obese subjects having a BMI of  $48.6 \pm 7.1$  kg/m<sup>2</sup> who did not have insulin resistance, diabetes or cardiovascular disease. Obese subjects were found to have significantly higher TXB<sub>2</sub> levels than the lean subjects. Morbidly obese subjects manifested unexpected results, cause of which remained unexplained. TXB<sub>2</sub> levels were significantly lower than obese subjects and they were insulin sensitive. The decrease in TXB<sub>2</sub> levels accompanied by insulin sensitivity was suggested to play a protective role in these subjects from atherosclerosis.<sup>24</sup>

In our study, there was a significant decrease in TXA<sub>2</sub> levels in the treated group. Effect of statins on TXA<sub>2</sub> levels is not studied in animal models, results of studies however conducted in humans showed a decrease in TXA<sub>2</sub> levels after statin administration.

Sik et al conducted a study to see the effect of statins on urinary metabolite of thromboxane i.e. 11-dehydrothromboxane. They took 58 patients and administered standard dose of three different lipid lowering drugs for the period of three months. Urinary levels of 11-dehydrothromboxane levels were measured before and after therapy and these were found to be significantly reduced after the therapy.<sup>25</sup>In another similar study, Puccetti et al studied the effect of two lipid lowering drugs on thromboxane dependent platelet activation in hypercholesterolemia. Sixty hypercholesterolemic subjects were included in the study and were

administered either atorvastatin 20mg/day or rosuvastatin 10mg/day for the period of eight weeks and 11-dehydrothromboxane B2 levels were measured. Administration of both the lipid lowering drugs resulted in significant reduction in 11-dehydrothromboxane B2 levels.<sup>26</sup>

**Hcy**

Serum homocysteine (Hcy) concentration was found increased in obese rats of our study, which could be associated with the endothelial dysfunction. The published data regarding hcy concentration in obese animal models is not available. Hcy concentration in human obesity however, had been studied which revealed the positive correlation between the two.

Vaya et al found increased hcy levels in obese patients who suffered stroke. Their study was directed to find out the role of hyperhomocysteinemia in patients who suffered stroke and to assess the contribution of obesity in this condition. The comparison of hcy levels between patients and controls revealed that such patients had significantly higher BMI and hcy levels as compared to healthy controls.<sup>27</sup>

Karatela and Sainani compared hcy levels in three subgroups of normotensives and three subgroups of hypertensives(normal weight, overweight and obese). The data regarding normotensive subgroups revealed that obese and overweight subjects had significantly higher hcy levels as compared to normal weight subjects. The results suggested that increase in BMI was associated with elevated hcy concentration and decreased vitamin B12 and folic acid levels in obese normotensives.<sup>28</sup>

Increased serum hcy concentration in obese rats

was not affected by atorvastatin. This finding was similar to the results of most other studies in which statin administration did not affect homocysteine concentration in hyperlipidemic subjects.

Gival et al conducted a study to evaluate the effect of two different lipid lowering agents (statins and fibrates) on hcy concentration in patients of mixed hyperlipidemia and found that atorvastatin did not significantly affect hcy concentration. Fibrates administration, however resulted in increased hcy concentration. 29Dierkes et al reviewed the effects of lipid lowering and antihypertensive drugs on hcy levels. The data regarding lipid lowering drugs revealed that fibric acid derivatives increased hcy concentration whereas statins did not influence hcy concentration significantly.<sup>30</sup>

Bhandari et al studied the effect of atorvastatin in methionine induced hyperhomocysteinemic rats. Albino rats were administered methionine 1g/kg by oral route for 30 days to induce hyperhomocysteinemia. Administration of 0.2 mg/kg/day of atorvastatin significantly decreased hcy levels.<sup>31</sup>

Mostofthestudiesregardingeffectofadministration of lipid lowering drug on hcy concentration resulted in reduction or no significant effect on hcy concentration. However, study conducted by Loo et al revealed different results. In their study, administration of atorvastatin in the dose of 80 mg/day for a period of 6 months to patients of peripheral arterial disease resulted in significantly increased plasma hcy levels. The folic acid levels in these patients were also elevated. The mechanism of hyperhomocysteinemia in the presence of increased folic acid levels however could not be elucidated.<sup>32</sup>

Groups	LDL (mg/dl)	TXA <sub>2</sub> (pg/ml)	Hcy (pmol/ml)
Group I	40.09 ± 7.28	37.63 ± 15.37	17.47 ± 3.01
Group II	89.76 ± 10.94	101.08 ± 14.95	53.82 ± 5.33
Group III	42.59 ± 6.04	57.59 ± 13.27	55.05 ± 5.35

**Table-I. Comparison of LDL, TXA<sub>2</sub> and homocysteine between the three groups at the end of study.**  
*All values expressed as mean ± standard deviation.*

Group Comparisons	LDL (mg/dl)		TXA <sub>2</sub> (pg/ml)		HCY (pmol/ml)	
	Mean Difference	p-Value	Mean Difference	p-Value	Mean Difference	p-Value
Group I vs. II	49.673	0.000*	63.455	0.000*	36.348	0.000*
Group II vs. III	47.175	0.000*	43.494	0.000*	1.232	0.569
Group I vs. III	2.498	0.481	19.960	0.000*	37.580	0.000*

**Table-II. Comparison between groups of LDL, TXA<sub>2</sub> and homocysteine.**

\* Significant at  $p < 0.05$ , 95% confidence interval.

## CONCLUSION

Atorvastatin apart from lowering lipid levels in the body also reduces TXA<sub>2</sub> concentration which is a vasoprotective. Elevated homocysteine concentration which is deleterious to the endothelium however is not affected.

## CONFLICT OF INTEREST

Research for the study was funded by Higher Education Commission (HEC), Pakistan. Present study is a part of FCPS dissertation submitted to CPSP, Karachi. Atorvastatin pure active salt was provided free of cost for research purposes only by Nimrall Laboratories, RCCI Rawat, Islamabad. We hereby declare no conflict of interest.



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