

**CARDIOPROTECTIVE ROLE OF ATORVASTATIN IN HYPERHOMOCYSTEINEMIC RAT HEARTS**ANKUR ROHILLA\*<sup>1</sup>, M.U. KHAN<sup>2</sup>, RAZIA KHANAM<sup>3</sup>

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**ABSTRACT**

Hyperhomocysteinemia (Hhcy) has been considered as an independent risk factor for various cardiovascular diseases. The present study has been designed to investigate the cardioprotective effect of Atorvastatin, a 3-hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitor, in hyperhomocysteinemic rat hearts. Rats were administered L-methionine (1.7 g/kg/day orally) for 4 weeks to produce Hhcy. Isolated Langendorff-perfused normal and hyperhomocysteinemic rat hearts were subjected to global ischemia (I) for 30 min followed by reperfusion (R) for 120 min. Myocardial infarct size was assessed using triphenyltetrazolium chloride staining. Coronary effluent was analyzed for the release of creatine kinase MB (CK-MB) and lactate dehydrogenase (LDH) to assess the degree of myocardial injury. Moreover, oxidative stress in the heart was assessed by measuring lipid peroxidation, superoxide anion generation and reduced glutathione. Ischemia-reperfusion (I/R) was noted to produce myocardial injury, assessed in terms of increase in myocardial infarct size, LDH and CK in coronary effluent and oxidative stress. Additionally, hyperhomocysteinemic rat hearts showed enhanced myocardial injury in comparison with normal rat hearts with high degree of oxidative stress. Treatment with Atorvastatin (50 µM) afforded cardioprotection against I/R-induced myocardial injury in normal and hyperhomocysteinemic rat hearts as assessed in terms of reductions in myocardial infarct size, LDH and CK levels in coronary effluent and oxidative stress. It may be concluded that reductions in the high degree of oxidative stress may be responsible for the observed cardioprotective potential of Atorvastatin against I/R-induced myocardial injury in normal and hyperhomocysteinemic rat hearts.

**Keywords:** Hyperhomocysteinemia, Atorvastatin, Oxidative stress.

**INTRODUCTION**

Despite the unambiguous advantage of reperfusion of blood to an ischemic tissue, reperfusion brings out a series of adverse reactions that paradoxically damage the cardiac tissue, known as I/R injury<sup>1</sup>. Various mechanisms have been already reported to be involved in I/R-induced myocardial injury that include oxidative stress, intracellular calcium overload, apoptotic and necrotic myocytes death<sup>2-4</sup>. Hhcy refers to elevated homocysteine concentrations in the blood and is considered to be an independent risk factor for cardiovascular diseases<sup>5,6</sup>. Hhcy is associated with an increased risk of cardiovascular complications such as atherosclerosis, endothelial dysfunction, hypertension, myocardial infarction, chronic heart failure and obesity<sup>7,8</sup>. In addition, Hhcy increases the generation of reactive oxygen species (ROS) by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase<sup>9</sup>. Moreover, Hhcy has been noted to downregulate endothelial nitric oxide synthase (eNOS) and consequently reduces the generation of NO to produce cardiac dysfunction<sup>10</sup>. Additionally, Hhcy has been noted to increase the production of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by activating nuclear factor NF-kappa-B (NF-kB), which may affect the function of coronary endothelium. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins, are extensively prescribed for treating hypercholesterolemia and for reducing cardiovascular mortality and morbidity. Atorvastatin, a potent inhibitor of HMG-CoA reductase, has been well reported to possess various beneficial effects in the treatment of various cardiovascular diseases such as myocardial infarction, stroke, unstable angina and revascularization<sup>11-13</sup>. Various studies have reported Atorvastatin to reduce the infarct size in isolated Langendorff-perfused heart model by activating pro-survival kinases and nitric oxide (NO) levels<sup>14,15</sup>. Atorvastatin treatment has been noted to reduce thiobarbituric acid reactive oxygen substances (TBARS) levels and lipid peroxidation levels, the oxidative stress markers, which further evidenced its antioxidant action in affording cardioprotection<sup>16</sup>. In addition, treatment with atorvastatin has been reported to reduce vascular and cardiac free radical formation, normalize the expression of the NADPH oxidase and thus show anti-oxidative properties<sup>11,17</sup>. Therefore, the present study has been undertaken to investigate the cardioprotective effect of Atorvastatin against I/R-induced myocardial injury in normal and hyperhomocysteinemic rat hearts.

**MATERIALS AND METHODS****Experimental Animals**

The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. Wistar albino rats of either sex weighing 175-225 gm were used. They were housed in Institutional animal housing and were maintained on rat feed (Kisan Feeds Ltd., Chandigarh, India) and tap water ad libitum.

**Isolated Rat Heart Preparation**

Rats were heparinized (500 IU i.p.) and sacrificed by stunning. The heart was rapidly excised and immediately mounted on a Langendorff apparatus<sup>18</sup>. The heart was enclosed in a double walled jacket, the temperature of which was maintained at 37°C by circulating hot water. The preparation was perfused with Krebs-Henseleit (K-H) solution (NaCl 118 mM; KCl 4.7 mM; CaCl<sub>2</sub> 2.5 mM; MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2 mM; NaHCO<sub>3</sub> 25 mM; KH<sub>2</sub>PO<sub>4</sub> 1.2 mM; C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> 1 mM) pH 7.4, maintained at 37 °C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The coronary flow rate was maintained at around 7 mL/min, and the perfusion pressure was kept at 80 mmHg. Global ischemia was produced for 30 min by blocking the inflow of physiological solution and it was followed by perfusion for 120 min.

**Laboratory Assays**

Myocardial infarct size was measured macroscopically using triphenyl tetrazolium chloride (TTC) staining employing volume method<sup>19</sup>. The myocardial injury was assessed by measuring the release of LDH and CK-MB in the coronary effluent using the commercially available enzymatic kits (Vital Diagnostics, Thane, Maharashtra, India). The level of TBARS, an index of lipid peroxidation in the heart was estimated according to the method of Ohkawa et al.<sup>20</sup>. The superoxide anion generation was assessed by estimating the reduced nitro blue tetrazolium (NBT) using the method of Wang et al.<sup>21</sup>. Moreover, the reduced glutathione (GSH) content in each heart was estimated using the method of Beutler et al.<sup>22</sup>.

**Experimental Protocol**

Five groups of 8-10 animals each were employed in the present study. In all groups, each isolated perfused heart was allowed to stabilize for 10 min by perfusing with K-H solution.

**Group I (Normal Control):** Isolated normal rat heart was perfused for 150 min using K-H solution after 10 min of stabilization.

**Group II (I/R-Control):** Isolated normal rat heart after 10 min of stabilization was subjected to 30 min of global ischemia followed by 120 min of reperfusion

**Group III (Ator per se Normal Control):** After 10-min stabilization, the isolated normal rat heart was infused with Atorvastatin (50  $\mu\text{M}$ ) for 10 min. Then the heart was perfused for 150 min using K-H solution

**Group IV (Ator Treated I/R-Control):** After 10 min of stabilization, isolated normal rat heart was infused with Atorvastatin (50  $\mu\text{M}$ ) for 10 min. The heart was then subjected to 30 min of global ischemia followed by 120 min of reperfusion.

**Group V (Hhcy control):** The isolated Hhcy rat heart was perfused for 150 min using K-H solution after 10-min stabilization.

**Group VI (Hhcy-I/R control):** Isolated Hhcy rat heart after 10 min of stabilization was subjected to 30 min of global ischemia followed by 120 min of reperfusion

**Group VII (Ator per se Hhcy-control):** After 10-min stabilization, the isolated Hhcy rat heart was infused with Atorvastatin (50  $\mu\text{M}$ ) for 10 min. Then the heart was perfused for 150 min using K-H solution

**Group VIII (Ator Treated Hhcy-I/R Control):** After 10 min of stabilization, isolated Hhcy rat heart was infused with Atorvastatin (50  $\mu\text{M}$ ) for 10 min. The heart was then subjected to 30 min of global ischemia followed by 120 min of reperfusion

#### Statistical Analysis

The results were expressed as mean  $\pm$  SD. The data obtained from various groups were statistically analyzed using two-way ANOVA followed by Tukey's multiple-comparison test. A P value < 0.05 was considered to be statistically significant.

#### Drugs and Chemicals

The LDH and CK enzymatic estimation kits were purchased from Vital Diagnostics, Thane, Maharashtra, India. DTNB and NBT were

obtained from Loba Chem, Mumbai, India. Atorvastatin, 1,1,3,3-tetramethoxy propane and reduced glutathione were procured from Sigma-Aldrich, USA. All other reagents used in this study were of analytical grade.

#### RESULTS

Rats fed with L-methionine (1.7 g/kg/day, p.o.) for 4 weeks via oral gavage produced hyperhomocysteinemia (21.25 $\pm$ 1.93  $\mu\text{M}/\text{L}$ ) when compared with normal rats (4.51 $\pm$ 0.61  $\mu\text{M}/\text{L}$ ). In addition, L-methionine administration did not produce mortality in rats.

#### Effect of Atorvastatin in I/R-induced myocardial injury in normal and hyperhomocysteinemic rat hearts

Global ischemia followed by reperfusion significantly increased LDH and CK release in the coronary effluent in normal and hyperhomocysteinemic rat hearts. Maximum release of LDH was noted immediately after reperfusion, while maximum release of CK was noted at 5 min of reperfusion (Table 1). In addition, I/R was noted to increase the infarct size in normal and hyperhomocysteinemic rat hearts (Table 1). Hyperhomocysteinemic rat hearts showed enhanced myocardial injury when compared with normal rat hearts subjected to I/R. Treatment with Atorvastatin (50  $\mu\text{M}$ ) significantly attenuated I/R-induced myocardial injury in normal and hyperhomocysteinemic rat hearts, as assessed in terms of reduction in myocardial infarct size and decreased release of LDH and CK in coronary effluent (Table 1).

#### Effect of Atorvastatin in I/R-induced oxidative stress in normal and hyperhomocysteinemic rat hearts

Lipid peroxidation, measured in terms of TBARS, and superoxide anion generation, assessed in terms of reduced NBT, were significantly increased and levels of reduced GSH were found to be decreased in normal and hyperhomocysteinemic rat hearts subjected to I/R (Table 2). In addition, hyperhomocysteinemic rat hearts showed high oxidative stress when compared with normal rat hearts subjected to I/R. Atorvastatin treatment (50  $\mu\text{M}$ ) attenuated the I/R-induced oxidative stress in normal and hyperhomocysteinemic rat hearts, as assessed in terms of reduction in TBARS and superoxide anion generation, and the consequent increase in GSH (Table 1).

**Table 1: Effect of Atorvastatin in I/R-induced increase in Infarct size (I.S.), LDH and CK levels.**

Groups	I.S.(%)	Myocardial Injury parameter			
		LDH (U/L)		CK (U/L)	
		Basal	Imm Rep	Basal	5 min
Normal control	7.5 $\pm$ 1.1	37.5 $\pm$ 4.1	39.8 $\pm$ 4.2	22.9 $\pm$ 3.2	28.7 $\pm$ 4.1
I/R Control	51.1 $\pm$ 2.9 <sup>a</sup>	44.1 $\pm$ 4.6	283.5 $\pm$ 17.8 <sup>a</sup>	26.2 $\pm$ 4	165.2 $\pm$ 13.2 <sup>a</sup>
Ator Per se Normal control	7.1 $\pm$ 2.1	39.8 $\pm$ 2.5	44.6 $\pm$ 5.1	24.5 $\pm$ 4.2	25.9 $\pm$ 3.8
Ator Treated I/R Control	23.6 $\pm$ 3.6 <sup>b</sup>	42.3 $\pm$ 3.1	177.2 $\pm$ 11.2 <sup>b</sup>	26.3 $\pm$ 2.6	82.6 $\pm$ 6.1+6.1 <sup>b</sup>
Hhcy - Control	7.9 $\pm$ 2	46.6 $\pm$ 4.2	41.6 $\pm$ 5.5	24.7 $\pm$ 2.6	29.2 $\pm$ 5.7
Hhcy-I/R Control	59.1 $\pm$ 5.6 <sup>c,d</sup>	40.2 $\pm$ 3.8	333.3 $\pm$ 25.5 <sup>c,d</sup>	28.2 $\pm$ 3.5	196.1 $\pm$ 14.3 <sup>c,d</sup>
Ator Per se Hhcy-Control	7.8 $\pm$ 2.2	47.5 $\pm$ 2.8	35.9 $\pm$ 3.7	26.5 $\pm$ 4.1	28.2 $\pm$ 3.9
Ator Treated Hhcy-I/R Control	34.4 $\pm$ 3.2 <sup>c</sup>	44.6 $\pm$ 4.3	212.3 $\pm$ 13.2 <sup>c</sup>	25.4 $\pm$ 3.2	111.5 $\pm$ 8.2

Values are expressed as mean  $\pm$  S.D. a = p < 0.05 vs Normal Control; b = p < 0.05 vs I/R Control; c = p < 0.05 vs Hhcy-Control; d = p < 0.05 vs I/R control; e = p < 0.05 vs Hhcy-IR Control.

**Table 2: Effect of Atorvastatin in I/R-induced increase in TBARS and Reduced NBT with consequent reduction in GSH levels**

Groups	Oxidative Stress Parameters		
	TBARS (n M/gm)	Reduced NBT (pM/m in mg)	Reduced GSH ( $\mu\text{M}/\text{mg}$ )
Normal control	40.2 $\pm$ 4.2	17.8 $\pm$ 1.9	0.74 $\pm$ 0.03
I/R control	82.2 $\pm$ 6.1 <sup>a</sup>	67.3 $\pm$ 4.1 <sup>a</sup>	0.577 $\pm$ 0.04 <sup>a</sup>
Ator per se Normalcontrol	44.6 $\pm$ 4.4	20.1 $\pm$ 2.2	0.756 $\pm$ 0.048
Ator Treated I/R control	56.3 $\pm$ 3.8 <sup>b</sup>	37.2 $\pm$ 2.4 <sup>b</sup>	0.933 $\pm$ 0.031 <sup>b</sup>
H hcy Control	46.1 $\pm$ 5.1	23.9 $\pm$ 2.4	0.689 $\pm$ 0.042
Hhcy -I/R control	103.4 $\pm$ 6.6 <sup>c,d</sup>	92.5 $\pm$ 5.9 <sup>c,d</sup>	0.413 $\pm$ 0.034
Ator Per Hhcy-Control	46.4 $\pm$ 3.7	21.7 $\pm$ 3.9	0.733 $\pm$ 0.037
Ator Treated Hhcy-I/R Control	68.4 $\pm$ 4.4 <sup>e</sup>	52.1 $\pm$ 3.5 <sup>e</sup>	0.672 $\pm$ 0.039 <sup>e</sup>

Values are expressed as mean  $\pm$  S.D. a = p < 0.05 vs Normal Control; b = p < 0.05 vs I/R Control; c = p < 0.05 vs Hhcy-Control; d = p < 0.05 vs I/R control; e = p < 0.05 vs Hhcy-IR Control.

## DISCUSSION

Myocardial ischemia denotes a condition of restriction of blood supply to the myocardial tissue with resultant damage or dysfunction in the myocardium<sup>4,23</sup>. The tissue damage caused when blood supply returns to the cardiac tissue after a prolonged period of ischemia resulting in inflammation and oxidative damage is known as I/R injury. It has been previously demonstrated that the increase in infarct size and the release of LDH and CK are documented to be an index of I/R-induced myocardial injury<sup>24,25</sup>. In the present study, 30 min of ischemia followed by 120 min of reperfusion produced myocardial injury, as assessed in terms of increased infarct size in the heart and elevated release of LDH and CK in the coronary effluent. The maximal release of LDH was noted immediately after reperfusion, whereas peak release of CK was observed after 5 min of reperfusion which are in accordance with our earlier studies<sup>19,25</sup>. Moreover, increase in lipid peroxidation and superoxide anion generation have been reported to be the indicators of oxidative stress<sup>26,27</sup>. In addition, the concentration of GSH has been found to be decreased during enhanced oxidative stress<sup>28</sup>. As a result of I/R, the GSH level was decreased and lipid peroxidation, measured in terms of TBARS, and superoxide anion generation, assessed in terms of reduced NBT, were noted to be increased. These indicators suggest the development of I/R-induced oxidative stress, which may be responsible for the noted I/R-induced myocardial injury.

Administration of L-methionine (1.7 g/kg/day orally) in rats for 4 weeks produced Hhcy<sup>29</sup>. In the present study, a marked increase in infarct size and release of LDH and CK were noted in the hyperhomocysteinemic rat heart when compared with the normal rat heart. Hhcy has been noted to downregulate NO bioavailability by accumulating asymmetric dimethylarginine, which is an endogenous inhibitor of eNOS<sup>30</sup>. In addition, Hhcy has been reported to produce high oxidative stress in the heart by activating NADPH oxidase-mediated ROS generation<sup>10</sup>. Hhcy-induced oxidative stress may occur as a result of decreased expression and activity of key antioxidant enzymes, as well as increased enzymatic generation of superoxide anion<sup>31</sup>. Thus, development of high degree of oxidative stress may be responsible for the observed marked increase in myocardial injury in the hyperhomocysteinemic rat heart. This contention is supported by the fact that a marked increase in lipid peroxidation and superoxide anion generation and a subsequent decrease in GSH levels were noted in the hyperhomocysteinemic rat heart when compared with the normal rat heart.

The HMG-CoA reductase inhibitors, known as statins, are potent inhibitors of cholesterol biosynthesis and possess valuable effects in the prevention of coronary artery disease<sup>32</sup>. Additionally, statins have been noted to exert extrahepatic, cholesterol independent effects, referred to as pleiotropic effects, that include direct effects on vascular tissue, heart, kidney, bone and glucose metabolism<sup>32,33</sup>. Atorvastatin has been reported to reduce the infarct size in isolated Langendorff-perfused heart model by activating pro-survival kinases such as phosphatidylinositol 3-kinase/protein kinase B (PI3-Akt) and increasing NO levels<sup>14,15</sup>. The present study investigated the cardioprotective potential of atorvastatin against I/R injury in rat hearts administered at the onset of reperfusion. The data demonstrates that atorvastatin (50 µM) administered as an adjunct to reperfusion afforded cardioprotection by significantly attenuating I/R-induced myocardial injury in rat hearts as assessed in terms of reductions in myocardial infarct size and decreased release of LDH and CK in coronary effluent, which is in accordance of our recent study<sup>34</sup>. Moreover, it has been recently reported that treatment with Atorvastatin lowered the increased levels of serum homocysteine in rats providing an evidence of inhibitory role of Atorvastatin in Hhcy<sup>35</sup>. In addition, a number of studies have reported that Atorvastatin exerts protective effects against oxidative stress in order to afford mimic cardioprotection. Treatment with Atorvastatin has been noted to reduce the oxidative stress markers such as TBARS and lipid peroxidation levels<sup>36</sup>. Moreover, Atorvastatin has been reported to reduce vascular and cardiac free radical formation, normalize the expression of the NADPH oxidase and thus show anti-oxidative properties<sup>37</sup>. In addition, Atorvastatin has been reported to induce a significant decrease in malondialdehyde (MDA) levels alongwith a significant increase of superoxide dismutase (SOD)

activity that accounts for its cardioprotective and antioxidant action<sup>38</sup>. In the present study, Atorvastatin treatment (50 µM) was noted to reduce I/R-induced myocardial injury in the normal and hyperhomocysteinemic rat heart, as assessed in terms of reduction in infarct size, release of LDH and CK, and oxidative stress. This strongly suggests that the high degree of oxidative stress in hyperhomocysteinemic rat hearts in response to I/R injury was attenuated by Atorvastatin in order to show cardioprotection in normal and hyperhomocysteinemic rat hearts

On the basis of the above discussion, it may be concluded that Hhcy modulate the myocardium vulnerable to I/R-induced oxidative stress, and that this high degree of stress produced by the hyperhomocysteinemic rat heart in response to I/R may be responsible for the prevention of cardioprotection. Treatment with Atorvastatin afforded cardioprotection in normal and hyperhomocysteinemic rat hearts by abolishing the high degree of oxidative stress in Hhcy. Further studies are undergoing in our laboratory to explicate the cardioprotective mechanisms of statins in

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