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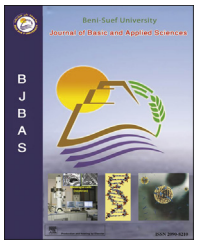

Full Length Article

Rosuvastatin and ellagic acid protect against isoproterenol-induced myocardial infarction in hyperlipidemic rats

Mai A. Elhemely [a], *Hany A. Omar* [a,b,*], *Afaf A. Ain-Shoka* [c], *Hekma A. Abd El-Latif* [c,d], *Amira M. Abo-youssef* [a], *Gamal A. El Sherbiny* [e]

[a] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt*
[b] *Sharjah Institute for Medical Research, Department of Pharmacology, College of Pharmacy, University of Sharjah, Sharjah 27272, United Arab Emirates*
[c] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Egypt*
[d] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Umm Al-Qurah University, Saudi Arabia*
[e] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Kafr El-Sheikh University, Egypt*

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ABSTRACT

Hyperlipidemia (HL) with subsequent coronary atherosclerosis is the major trigger of ischemia and hence, myocardial infarction (MI) occurs. The present study aimed to elucidate the effects of pretreatment with rosuvastatin and ellagic acid, as well as their combination on isoproterenol-induced MI in hyperlipidemic rats. Adult rats were fed with a cholesterol-rich diet for seven weeks and received rosuvastatin (10 mg/kg) and/or ellagic acid (30 mg/kg) by oral gavage daily starting from the fifth week then subcutaneously injected with two doses 24-h apart of 100 mg/kg isoproterenol in the last two days. ECG pattern was monitored and both cardiac biomarkers (cTnI, CK-MB, LDH and AST) and lipid profile (TC, TG, HDL-c and LDL-c) were measured in serum. MDA and GSH levels were quantified in cardiac homogenates and heart tissue damage was examined by histopathology. Furthermore, the expression levels of iNOS, eNOS, Bax and Bcl-2 in heart samples were assessed by western blotting. Three-week pretreatment with rosuvastatin and/or ellagic acid markedly ameliorated HL- and isoproterenol-induced alterations in ECG, cardiac markers, oxidation markers, lipid profile and heart architecture. Both drugs downregulated iNOS and upregulated eNOS, while only rosuvastatin and the combination downregulated Bax. This study provides evidence that rosuvastatin and ellagic acid possess cardioprotective effect on the hyperlipidemic-myocardial infarction rat model and the combination does not offer extra protection than monotherapy.

* *Corresponding author*. Department of Pharmacology and Toxicology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt. Tel.: +20 01000882828; fax: +20 082 2317958.
E-mail address: omar.22@buckeyemail.osu.edu (H.A. Omar).
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1. Introduction

Myocardial infarction (MI) is an acute necrotic disorder of the heart which is expected to be the main reason for mortality in the world by the year 2020 (Patel et al., 2010). It results from sudden blockage of one or more of the coronary arteries that deprives a myocardial segment from blood supply. The most common recognized scenario for MI is coronary atherosclerosis that is strongly correlated to hyperlipidemia (HL), an important risk factor for acute coronary syndromes. HL increases myocardial infarct size in experimental ischemia/reperfusion injury as a consequence of the exaggerated stimulation of myocardial apoptosis, thrombosis and inflammatory events (Ferdinandy, 2003; Balakumar and Babbar, 2012; Ma et al., 2013).

Oxidative stress is an important pathogenic event in a variety of cardiovascular diseases such as atherosclerosis, hypertension, ischemic heart disease and heart failure (Dhalla et al., 2000; Mehany et al., 2013). It occurs when excessive generation of reactive oxygen species (ROS) cannot be adequately neutralized by endogenous antioxidants leading to cell membrane injury and cell death (Takimoto and Kass, 2007; Galaly et al., 2014). In addition, increased ROS production lessens the bioavailability of the vasodilator nitric oxide (NO) which significantly affects the heart at both physiological and pathological levels (Förstermann, 2010). Low level of NO produced by endothelial nitric oxide synthase (eNOS) regulates vascular tone and provides cardioprotection from ischemic damage (Jones and Bolli, 2006). On the other hand, large amounts of NO, mediated by inducible nitric oxide synthase (iNOS), are cardiotoxic due to suppression of myocardial contractility and further increase of ROS generation and cardiomyocyte apoptosis (Haywood et al., 1996; Wollert and Drexler, 2002).

The apoptosis process is programmed and regulated by numerous proteins such as Bcl-2 and Bax. Bcl-2, an anti-apoptotic protein, is expressed shortly after MI onset, whereas Bax, a pro-apoptotic protein, is overexpressed at the old stage of MI (Krijnen et al., 2002). The role of apoptosis in triggering MI is ambiguous, however, the major effect of apoptosis appears to be on late cardiomyocyte loss and ventricular remodeling after MI (Abbate et al., 2008).

So, the optimal therapeutic strategies to protect the heart against MI should involve antihyperlipidemic, antioxidant, anti-apoptotic and/or eNOS-mediated NO producing drugs. In the current study, we selected rosuvastatin and ellagic acid as cardioprotective candidates.

Rosuvastatin, a lipid-lowering agent in clinical practice, has an appreciable anti-atherogenic property which is due to the improvement of endothelial dysfunction as well as its anti-thrombotic, anti-inflammatory and antioxidant effects (Qu et al., 2009; Rondi et al., 2014). Rosuvastatin has been reported to prevent MI induced experimentally by occlusion of left coronary artery in rats (Dourado et al., 2011). Ellagic acid is a polyphenolic compound that is found in strawberries, pomegranates and grapes (Mari Kannan and Darlin Quine, 2012). It possesses a number of biological activities such as antioxidant, antihyperlipidemic, anti-inflammatory and hepatoprotective effects (Rogerio et al., 2008). Ellagic acid has been recognized to have a cardiprotective effect against experimentally induced MI (Kannan and Quine, 2013).

Catecholamines have a key role in normal cardiac function. However, isoproterenol, a synthetic catecholamine, liberates ROS and causes an infarct-like necrosis of the myocardium resembling MI in humans at toxic doses (Nagoor Meeran et al., 2012).

The current study was executed in an effort to explore whether rosuvastatin or ellagic acid alone or in combination, could achieve cardioprotection against isoproterenol-induced MI in cholesterol-rich diet fed rats.

2. Materials and methods

2.1. Animals

Male Wistar rats of 140–160 g weight were provided from the animal house of Faculty of Pharmacy, Beni-Suef University. They were housed in plastic cages and kept under suitable laboratory conditions that meet the guidelines of the Ethics Committee, Faculty of Pharmacy, Cairo University.

2.2. Drugs and chemicals

Cholesterol and isoproterenol hydrochloride were bought from Lobachemie (India) and Sigma–Aldrich (USA), respectively. Rosuvastatin was obtained kindly from Apexpharma (Egypt), whereas ellagic acid was purchased from Acros (USA). Test agents were prepared in 1% Tween 80 so that each 100 g animal body weight received 0.5 ml orally of the respective preparation.

2.3. Induction of HL

Rats were fed with a cholesterol-rich diet (1% cholesterol, 0.2% cholic acid, 0.2% methyl thiouracil, 7% egg yolk, 4% beef tallow, 1% sodium chloride, 45% wheat flour, 6.6% wheat bran, 35% corn starch) for seven weeks (Pengzhan et al., 2003).

2.4. Induction of MI

Two doses 24-h apart of 100 mg/kg isoproterenol, prepared in saline, were injected subcutaneously in rats (Zaafan et al., 2013). The injected area was massaged for 30 s after injection.

2.5. Experimental design

At random, rats were divided into five groups ($n = 8$). Groups I & II, normal and hyperlipidemic-myocardial infarction (HM) control groups, respectively, received 1% Tween 80 (p.o.). Group III received rosuvastatin (10 mg/kg; p.o.) (Zaitone and Abo-Gresha, 2012), while group 1V received ellagic acid (30 mg/kg; p.o.). The combination of rosuvastatin with ellagic acid was received by group V.

Except group I that was fed with a standard pelleted rat chow, all groups were fed with the cholesterol-rich diet for 7 weeks (the whole experimental period) and were injected with isoproterenol (100 mg/kg; s.c.) once daily in the last 2 days of

the experiment. Tween 80 and the test agents were administered starting from the 5th week.

At the end of the experiment, the animals were fasted overnight for 12 h but had free access to water *ad libtium*. In the morning, the rats were anaesthetized with ketamine HCl and xylazine (50 mg/5 mg/kg; i.p.) simultaneously for ECG recording (Struck et al., 2011). Thereafter, blood samples were collected, the animals were sacrificed by decapitation and the hearts were removed rapidly. The collected blood samples, heart homogenates (25% w/v in ice-cold saline) and heart samples were used for the assessment of the chosen parameters.

2.6. ECG monitoring

Four needle electrodes were inserted under the anesthetized rat's limb skin for ECG recording at lead II. Alterations in ST segment were determined.

2.7. Biochemical analyses

The enzyme linked immunoassay (ELISA) technique was performed to measure the serum level of troponin-I (cTnI) using a standard kit (Glory Science Co., Ltd, USA). The serum activity of creatine kinase MB (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) was determined using Biosystems kits (Spain). The serum levels of total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL-c) were quantified using Spinreact kits (Spain). For measuring low density lipoprotein cholesterol (LDL-c) level in serum, Friedewald equation [LDL-c = TC − (HDL-c + TG/5)] was employed (Friedewald et al., 1972).

In the cardiac homogenate, malondialdehyde (MDA), an indicator of lipid peroxidation, and reduced glutathione (GSH) were estimated as mentioned before (Sedlak and Lindsay, 1968; Ohkawa et al., 1979).

2.8. Western blotting for iNOS, eNOS, Bax and Bcl-2

To extract proteins from cardiomyocytes, about 300 mg of heart tissue was added to 150 μl of RIPA lysis buffer (Sigma–Aldrich) and thoroughly ultra-homogenized (Omar et al., 2009). After brief centrifugation, equal amounts of total proteins were separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with TBST (10 mM Tris–HCl, 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated with a specific primary antibody (1:1000 dilution in 5% nonfat milk in TBST) overnight at 4 °C for each of iNOS, eNOS, Bax and Bcl-2 (Proteintech, USA). After three washes with TBST, the membrane was probed with rabbit IgG-horseradish peroxidase conjugated secondary antibody (R&D Systems, USA) for 2 h at room temperature. The immunoblots were visualized by enhanced chemiluminescence system. β-actin was assessed as the internal control.

2.9. Histopathological examination

Tissue samples were taken from rat hearts from different groups. These samples were fixed in 10% formalin prepared in saline for about 48 h. Tissues were embedded in paraffin, sectioned at 5 μm by slidge microtome and stained with hematoxylin and eosin (H&E). Photomicrographs were obtained after examining the sections under light microscope.

2.10. Statistical analysis

Statistical analysis was carried out using GraphPad Prism statistical software. Measurements were displayed as mean ± SEM. Groups were compared by one-way ANOVA and subsequent Tukey–Kramer test. At p value <0.05, differences were considered significant.

3. Results

3.1. Effect of three-week administration of rosuvastatin and/or ellagic acid on ECG pattern

As compared to normal group, a noticeable elevation in ST-segments was observed in HM group. Such alterations were reinstated to proximate normal in rats pretreated with rosuvastatin, ellagic acid or their combination when compared to HM group (Fig. 1).

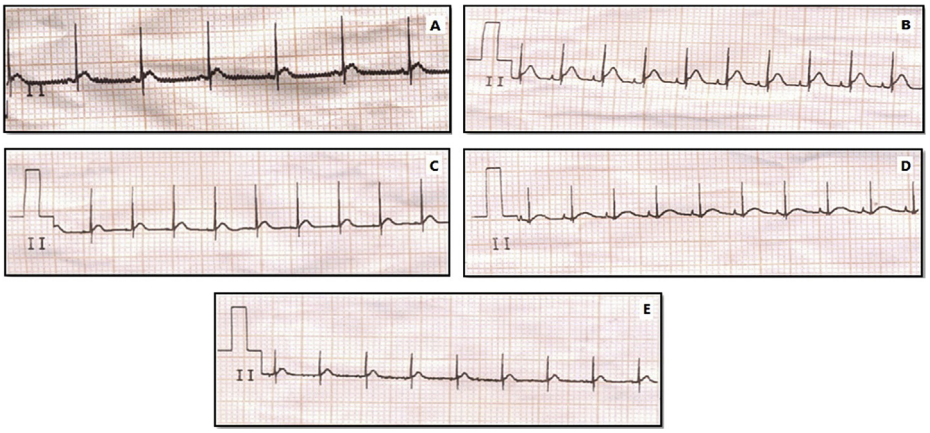

Fig. 1 – Lead II ECG configuration of normal rat (A), HM rat (B), rosuvastatin-pretreated HM rat (C), ellagic acid-pretreated HM rat (D), and rosuvastatin plus ellagic acid-pretreated HM rat (E).

Table 1 – Effect of three-week administration of rosuvastatin and/or ellagic acid on serum cardiac markers.

Groups	cTnI (ng/L)	CK-MB (U/L)	LDH (U/L)	AST (U/L)
Normal control	53.6 ± 1.41	216.9 ± 8.39	1623 ± 104.1	26.5 ± 1.06
HM control	74.9 ± 2.29*	512.9 ± 19.73*	4707 ± 313.7*	55.0 ± 2.35*
Rosuvastatin	53.6 ± 2.06@	341.3 ± 7.06@	1897 ± 164.4@	36.1 ± 2.30@
Ellagic acid	59.0 ± 1.15@	225.6 ± 19.29@	1849 ± 148.8@	36.7 ± 2.69@
Rosuvastatin + Ellagic acid	62.3 ± 1.04@	220.4 ± 16.45@	2195 ± 111.2@	37.1 ± 3.04@

*$p < 0.05$ compared to normal group and @$p < 0.05$ compared to HM group.

3.2. *Effect of three-week administration of rosuvastatin and/or ellagic acid on cardiac markers*

The serum level of cTnI, CK-MB, LDH and AST elicited a significant rise in HM group as compared to normal group. Rosuvastatin pretreatment decreased cTnI, CK-MB and LDH levels by 28.5%, 33.5% and 59.7%, respectively relative to HM group. Prior administration of ellagic acid produced a more potent suppressant effect on the serum activities of CK-MB, LDH and AST by 56.0%, 60.7% and 34.2%, respectively as compared to HM group. Combination of rosuvastatin with ellagic acid didn't show any appreciable difference than the individual treatments (Table 1).

3.3. *Effect of three-week administration of rosuvastatin and/or ellagic acid on cardiac oxidative stress markers*

Compared with normal group, HM group had a significant elevation in cardiac MDA content, whereas no difference was detected with respect to the cardiac content of GSH. Pretreatment with rosuvastatin reduced MDA by 34.5% with a parallel increase in GSH by 96.5% as compared to HM group, while ellagic acid pretreatment resulted in a 33.3% decrease in MDA coupled with 59.8% increase in GSH. Upon administration of rosuvastatin combined with ellagic acid, MDA was decreased and GSH was increased by 32.5% and 99.8%, correspondingly relative to HM group (Table 2).

3.4. *Effect of three-week administration of rosuvastatin and/or ellagic acid on lipid profile*

In HM group, compared with normal group, the levels of TC, TG and LDL-c were markedly increased and HDL-c level was markedly decreased. The lipid lowering capacity of ellagic acid was the same as rosuvastatin. Regarding HDL-c level, rosuvastatin and ellagic acid significantly elevated it by 102.5% and 284.2%, respectively as compared to HM group (Table 3). But as a whole, there was no remarkable difference in the lipid lowering activity upon administration of rosuvastatin, ellagic acid or combination of rosuvastatin with ellagic acid.

Table 2 – Effect of three-week administration of rosuvastatin and/or ellagic acid on cardiac oxidative stress markers.

Groups	MDA (nmol/g wet tissue)	GSH (μg/g wet tissue)
Normal control	26.46 ± 1.062	121.7 ± 6.614
HM control	55.02 ± 2.353*	141.9 ± 4.886
Rosuvastatin	36.05 ± 2.304@	278.9 ± 17.65*@
Ellagic acid	36.71 ± 2.692@	226.7 ± 20.69*@
Rosuvastatin + Ellagic acid	37.11 ± 3.041@	283.7 ± 25.00*@

*$p < 0.05$ compared to normal group and @$p < 0.05$ compared to HM group.

3.5. *Effect of three-week administration of rosuvastatin and/or ellagic acid on iNOS, eNOS, Bax and Bcl-2 protein expression in cardiomyocytes*

In comparison with normal group, HM group revealed a significant increase in the expression level of iNOS with a parallel decrease in eNOS level and provoked apoptosis as indicated from higher level of Bax expression. There was no significant difference among all groups regarding Bcl-2 protein level. Pretreatment with rosuvastatin, ellagic acid or their combination markedly decreased the expression level of iNOS and raised eNOS expression level as compared to HM group. Regarding Bax, rosuvastatin and the combination effectively downregulated its expression. Conversely, ellagic acid pretreatment resulted in no notable difference in Bax expression, in comparison to HM group (Fig. 2).

3.6. *Effect of three-week administration of rosuvastatin and/or ellagic acid on myocardial architecture*

On histopathological examination, HM group elicited necrotic changes; nuclear pyknosis, karyorrhexis, karyolysis and cytoplasmic eosinophilia. A massive granulation tissue was formed in HM group to replace the infarcted one. Pretreatment with either rosuvastatin or ellagic acid resulted in mild necrosis, very mild granulation tissue and a marked decrease in inflammatory cells infiltration. Heart of HM rat pretreated with rosuvastatin plus ellagic acid exhibited approximately normal architecture (Fig. 3).

4. Discussion

Despite the fact that coronary heart disease is a complicated disorder associated with hypertension, hyperlipidemia, diabetes and heart failure, most experimental studies of cardioprotection depend on induction of heart injury in absence of other related diseases. Thus, we employed a combined hyperlipidemic-myocardial infarction (HM) rat model that is relevant to the clinical case of MI in humans. The current

Table 3 – Effect of three-week administration of rosuvastatin and/or ellagic acid on serum lipid profile.

Groups	TC (mg/dl)	TG (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)
Normal control	65.7 ± 2.24	26.3 ± 2.04	29.26 ± 0.98	28.25 ± 2.51
HM control	371.8 ± 23.13*	58.3 ± 2.03*	15.38 ± 0.56*	219.9 ± 12.17*
Rosuvastatin	135.5 ± 8.627@	12.8 ± 0.52*@	31.18 ± 1.93@	125.6 ± 9.07*@
Ellagic acid	225.5 ± 18.37*@	14.0 ± 0.93*@	59.09 ± 3.89*@#	96.46 ± 4.53*@
Rosuvastatin + Ellagic acid	150.2 ± 10.39*@	9.5 ± 0.79*@	33.87 ± 1.104@	87.71 ± 5.61*@#

*$p < 0.05$ compared to normal control group, @$p < 0.05$ compared to HM control group and #$p < 0.05$ compared to rosuvastatin-pretreated HM group.

study is unique from previous literatures in the sense that it is the first to examine the cardioprotective potential of rosuvastatin, ellagic acid and, especially, their combination on isoproterenol-intoxicated rats in the presence of HL.

Alterations of ECG pattern, especially ST-segment elevation, represent the standard measure used to precisely diagnose MI in humans and animals (Panda et al., 2014). In our study, HM group demonstrated a significant ST-segment elevation which could be due to the deleterious effects of isoproterenol on cardiac cell membrane integrity with subsequent reduced mechanical capacity of the ventricles (Thippeswamy et al., 2009). Similar ECG changes were reported in isoproterenol-infarcted (Zaafan et al., 2013) and hypercholesterolemic isoproterenol-infarcted rats (Abo-Gresha et al., 2014).

Auto-oxidation of high doses of isoproterenol causes extreme generation of cytotoxic ROS and consequently impairment of integrity and function of myocardial membranes occurs (Rona, 1985; Garjani et al., 2011). We observed an increase in myocardial content of MDA in HM group which is in agreement with other studies (Evran et al., 2014). However, no change in cardiac GSH level was observed in HM group, as compared to normal group.

Isoproterenol-induced myocardial necrosis was evidenced by the significant leakage of cTnI, CK-MB, LDH and AST from the ruptured myocardial membranes into serum. Such cardiotoxic effect of isoproterenol was confirmed by the histopathological changes observed in HM group. These findings are consistent with previous reports (Abo-Gresha et al., 2014).

In accordance with previous studies (Sherief et al., 2008; Kannan and Quine, 2011), our findings have demonstrated

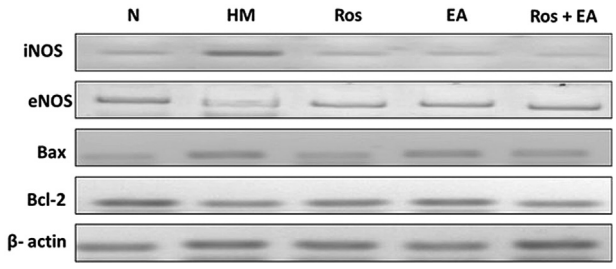

Fig. 2 – Western blots showing the effect of rosuvastatin and/or ellagic acid on the expression of iNOS, eNOS, Bax and Bcl-2 in heart tissues subjected to hyperlipidemia and isoproterenol-induced myocardial infarction (N, normal control; HM, hyperlipidemic-myocardial infarction control; Ros, rosuvastatin; EA, ellagic acid; Ros + EA, combination of rosuvastatin and ellagic acid).

that pretreatment of HM rats with rosuvastatin, ellagic acid and their combination successfully prevented the pathological ST-segment elevation and significantly lowered the leakage of the cardiac biomarkers. Such observations could be attributed to the potential role of rosuvastatin and ellagic acid in protecting the myocardial membranes and in alleviating the extent of myocardial damage. Owing to their known antioxidant nature, rosuvastatin and ellagic acid as well as their combination markedly reduced lipid peroxidation as observed from decreasing MDA and replenishing GSH levels in cardiomyocytes. In addition, the histopathological changes of pretreated groups showed retardation of inflammation and myonecrosis which could be attributed also to the antioxidant and anti-inflammatory properties of rosuvastatin and ellagic acid (Kannan and Quine, 2011; Dubé, 2014).

Both feeding with cholesterol-rich diet and injection of isoproterenol led to extensive alteration of lipid profile in HM group. This is in agreement with previous investigations (Nagoor Meeran et al., 2012; Abo-Gresha et al., 2014; Mahmoud et al., 2014). The observed increase in TC, TG and LDL-c levels in blood could initiate atherosclerosis with subsequent ischemic heart disease (Ferdinandy et al., 2007). Pretreatment with rosuvastatin, ellagic acid and their combination led to a significant improvement in the lipid profile. Similarly to rosuvastatin, ellagic acid's hypocholesterolemic effect is mainly mediated via HMG-CoA reductase inhibition (Cheng-Lai, 2002; Kannan and Quine, 2013).

Altered cardiac function and endothelial dysfunction were reflected in HM group by the high iNOS and the low eNOS expression levels. These results could be attributed to both the detrimental toxic effects of isoproterenol on cardiomyocytes and HL-induced myocardial oxidative stress (Cai and Harrison, 2000; Li et al., 2006; Ribeiro et al., 2009). The observations that rosuvastatin downregulated iNOS and upregulated eNOS were established previously in ischemic/reperfused rats (Di Napoli et al., 2005). To the best of our knowledge, the current study is the first one to demonstrate the effect of ellagic acid on iNOS and eNOS *in vivo*. Interestingly, pretreatment with ellagic acid downregulated iNOS and upregulated eNOS and subsequently, an improvement of the endothelial function and the coronary blood flow could be expected.

Apoptosis plays a role in the tissue damage process following MI and it was obvious in HM group from Bax overexpression in cardiomyocytes. The induced apoptosis could be due to isoproterenol-induced β-adrenergic stimulation and to some extent due to HL. It was reported that ROS, generated from

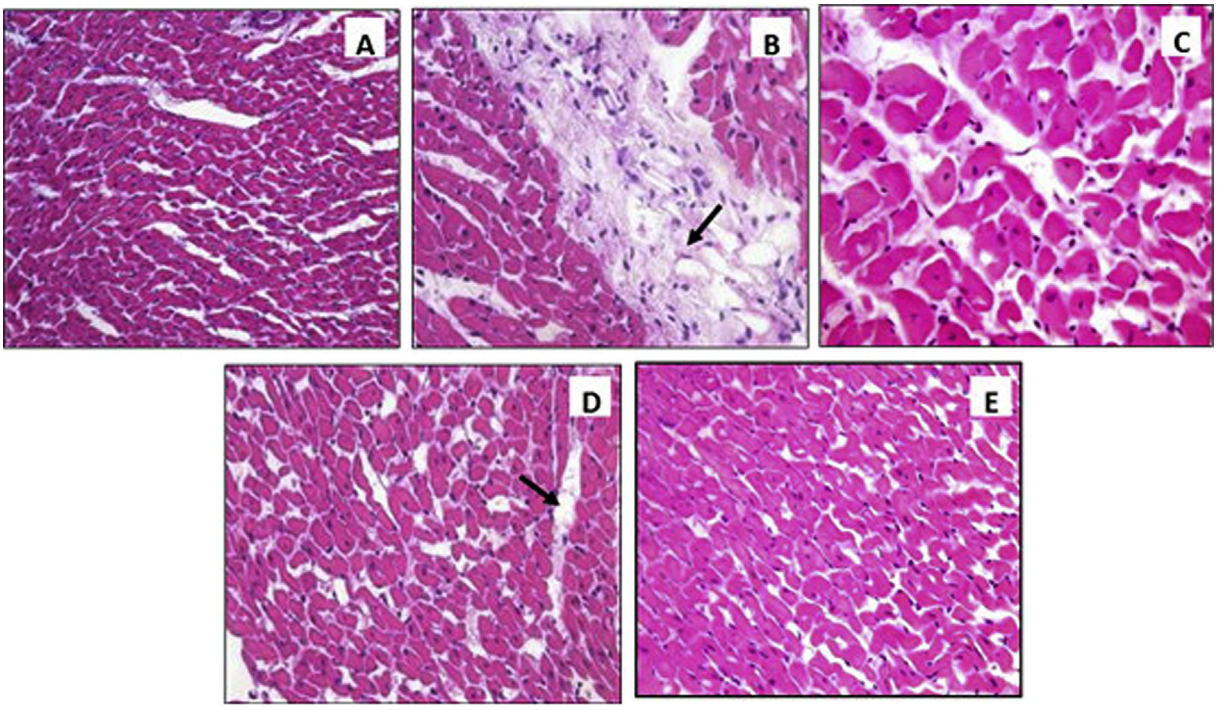

Fig. 3 – Photomicrographs of cardiac sections from: (A) Normal control rat showing normal histologic pattern (H&E-x100), (B) HM control rat showing necrotic changes and proliferated granulation tissue (arrow) (H&E-x200), (C) Rosuvastatin-pretreated HM rat showing mild necrosis and very mild infiltration of leucocytes (H&E-x400), (D) Ellagic acid-pretreated HM rat showing mild necrosis, very mild granulation tissue (arrow) (H&E-x200), and (E) Rosuvastatin plus ellagic acid-pretreated HM rat showing no infarction and restoring the normal heart architecture (H&E-x200).

excessive β-adrenergic stimulation by isoproterenol, play a central role in the regulation of myocyte apoptosis via activation of the mitochondrial death pathway (Remondino et al., 2003). HL-aggravated apoptosis could be due to lipid peroxidation and inflammation (Wang et al., 2002). Here, rosuvastatin reduced cardiomyocyte apoptosis by Bax downregulation and mild Bcl-2 upregulation. On the other hand, pretreatment with ellagic acid was unable to significantly protect the cardiomyocytes from apoptosis which contrasts with a report of anti-apoptotic effect of ellagic acid on rats with MI (Mari Kannan and Darlin Quine, 2012). In such report, ellagic acid was administered in smaller doses (7.5 & 15 mg/kg/day) for a shorter period (10 days), which could account for the disagreement with our study. In addition, the Bax discrepancy could be explained based on the reported apoptosis-inducing activity of ellagic acid on cancer (Han et al., 2006; Umesalma et al., 2014).

In conclusion, the collective antioxidant, lipid-lowering, anti-inflammatory, iNOS downregulating and eNOS upregulating properties of rosuvastatin and ellagic acid, and the anti-apoptotic effect of rosuvastatin enabled them to protect rat hearts from myocardial infarction in presence of hyperlipidemia. Unexpectedly, the combination did not show any additional effect over the individual drugs.

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