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Influence of Simvastatin Chronotherapy on Erythrocytes Nitric Oxide Synthase Activity

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ABSTRACT
This study was undertaken to investigate the impact simvastatin chronotherapy on erythrocytes Nitric Oxide Synthase (NOS) activity. In the present study we select 24 adult human subjects have plasma total cholesterol high borderline (200-230 mg dL^{-1}). Subjects were administrated simvastatin (20 mg day time) for 2 months. Afterward, they were recommended for washout period (2 weeks), then subjects were administered the same dose at night time for additional 2 months. The present results showed that both day time and night time simvastatin treatment regimen significantly decrease plasma atherogenic index, malondialdehyde and protein carbonyl levels. However, paraoxonase-1 activity and total thiol level were significantly increased. Moreover, simvastatin therapy improved nitrite (NO marker) levels in both plasma and erythrocytes compared to baselines. As well, simvastatin day time and night time dosing significantly increased erythrocytes NOS activity (46 and 64%, respectively) compared to baselines. Night time dosing induced marked increase of NOS activity (19%) compared to day time. This study confirms that night time dosing boost hypocholesterolemic, antioxidant and NO modulating effects of simvastatin compared to day time.

Key words: Hypercholesterolemia, chronotherapy, simvastatin, nitric oxide, paraoxonase-1

INTRODUCTION

Chronotherapy is either imitative or preventive therapy denotes to the utilization of circadian rhythmic cycles in the administration of drug in order to maximize efficiency and minimize side effects of the drug (Biswas *et al*., 2014). The suprachiasmatic nucleus drives the daily regulation of biological signals. Consequently, a marked oscillations of organ functions, biochemical pathways and other biological processes were observed during 24 h (Singh *et al*., 2004; Subash and Subramanian, 2007). So that understanding functions of biological clock and identification of modalities to manipulate this system enable us to establish chronotherapy (Biswas *et al*., 2014). Despite chronotherapy was documented in the treatment of many diseases such as bronchial asthma, angina pectoris, peptic ulcer, diabetes, hypertension and Hypercholesterolemia (HC) (Biswas *et al*., 2014). This topic is still a matter of research.

It has been reported that cholesterol biosynthesis, glucose homeostasis, oxidant/antioxidant balance and many other biochemical process have a marked diurnal variations (Singh *et al.*, 2004; Subash and Subramanian, 2007; Favero *et al*., 2014).

HC is one of the silent risks faced by many communities, it resulted from over activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that increase cholesterol

biosynthesis (Jones and Schoeller, 1990; Subash and Subramanian, 2007). It has been reported that the activity of HMG-CoA reductase follows diurnal variation in which maximal activity at early morning (Jones and Schoeller, 1990; Subash and Subramanian, 2007). Therefore, the time dependent targeting of HMG-CoA reductase peak is a good strategy for HC treatment.

Nitric oxide (NO) is mainly produced by endothelial Nitric Oxide Synthase (eNOS) and its bioavailability follow circadian dependent pattern (Reghunandanan and Reghunandanan, 2006; Antosova *et al*., 2009). NO is a major player in the regulation of the cardiovascular system and reduced NO bioavailability has been linked to cardiovascular disorders (Andrade *et al*., 2013). The decrease of NO bioavailability associated with erythrocyte dysfunction and cardiovascular complications (Mason *et al*., 2004). In the last years, erythrocyte was ordered as an additional source of NO biosynthesis by its own NOS (Kleinbongard *et al*., 2006). HC is associated with oxidative stress and endothelial dysfunction, this is attributable to endothelial and erythrocyte cells membrane become cholesterol-enriched (Mason *et al*., 2004; Eligini *et al*., 2013). This is resulted in loss of membrane integrity, calcium dysregulation, decreased NO production and increased risks of vascular diseases (Mason *et al*., 2004; Eligini *et al*., 2013).

Statins are HMG-CoA reductase inhibitors. They decreased cholesterol levels, modulate NOS activity and have pleiotropic effects (Mason *et al*., 2004; Lahera *et al*., 2007). These effects include regulation of erythrocytes membrane cholesterol homeostasis, rheology and antioxidant capacity (Forsyth *et al*., 2012; Uydu *et al*., 2012; Adekunle *et al*., 2013). Kamal (2011) suggested that evening statin dosing elicit more effective treatment than morning dosing.

Despite several studies investigated the effect of statins on erythrocytes, until now none of them addressed the effect of simvastatin chronotherapy (morning versus evening) on erythrocytes NOS activity and plasma oxidative status. Therefore, the aim of the present study was to assess the effect of simvastatin chronotherapy on plasma Malondialdehyde (MDA), Protein Carbonyl (PCO) level, Total Thiol (T-SH) and Paraoxonase-1 (PON1). Moreover, the effect of simvastatin administration on erythrocytes NOS activity was investigated.

MATERIALS AND METHODS

Chemicals: Paraoxon, 1,1,3,3-tetramethoxypropane and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were provided from (Sigma Aldrich, St Louis, MO, USA). The commercial diagnostic kits of Total Cholesterol (TC) and Triacylglycerol (TAG) were provided from (Randox Laboratories, Crumlin, UK). All other chemicals used of good quality and analytical grade.

Subjects: Experimental protocol was approved by our institutional guidelines followed to the guidelines set forth by the Declaration of Helsinki. Verbal consent was voluntarily obtained from all subjects prior to participation. Twenty four adult human male subjects participated in randomized two-period crossover experiment. Table 1 shows characteristics of subjects. Exclusion criteria included physical inactivity, bad dietary habit, smoking, obesity, diabetes and other diseases. Inclusion criteria based on plasma Total Cholesterol (TC) high borderline (200-230 mg dL^{-1}).

Table 1: Characteristics of subjects

Parameters	Values
Age (years)	57±4.1
Wight (kg)	75±6.8
Height (cm)	176±4.9
Body mass index (km^2)	25±2.1

Study protocol: Subjects were synchronized for 2 weeks with diurnal activity from about 07:00 to about 22:00 and nocturnal rest about 8 h. No drugs or food supplements that affect lipid levels or antioxidant status were taken for 2 weeks as wash out period. Breakfast was around 08:00, lunch was around 14:00 and dinner was around 20:30. Subjects were instructed to administer simvastatin tablets (20 mg day^{-1}) 2 h after breakfast for 2 months (Day time). Afterward, the volunteers were kept on 2 weeks as wash out period from simvastatin medication. The subjects were informed to administer simvastatin tablets at dose level of (20 mg day^{-1}) 2 h after dinner meal for another 2 month (Night time). The balanced diets and regular exercise at least (30 min per day) were recommended for all volunteers throughout the therapeutic protocol. Figure 1, shows the study protocol. Blood samples were collected by venipuncture at base lines and after 2 months of each treatment, before dinner meal day time (20:00) and before breakfast night time (8:00). The blood samples withdrawn into heparinized tubes centrifuged at 3000 rpm min for 5 min, plasma and buffy coat were carefully removed. The erythrocytes were isolated and subsequently washed three times with cold Phosphate-Buffered Saline pH 7.4 (PBS) containing 0.154 mM NaCl and 10 mM Na_2HPO_4. The supernatant and buffy coat were carefully removed after each wash. The plasma samples were analyzed for lipid profile in term of plasma TC, TAG, High Density Lipoprotein Cholesterol (HDL-C), LDL-C, paraoxonase-1(PON1 activity), lipid peroxides in terms of MDA, protein oxidation in terms of PCO, T-SH and NO in terms of nitrite. The washed erythrocyte samples were analyzed NOS activity and nitrite as index for erythrocytes NO production.

Lipid profile measurements: Plasma levels of TC, HDL-C and TAG were assayed enzymatically using commercial test kits (Randox Laboratories, Crumlin, UK). LDL-C was calculated using Friedewald equation. The AIP was calculated as log (TAG/HDL-C) using the Czech online calculator of atherogenic risk.

Determination of plasma PON1 activity: The PON1 activity in plasma samples was measured using paraoxon as substrate, the produced 4-nitrophenol was measured

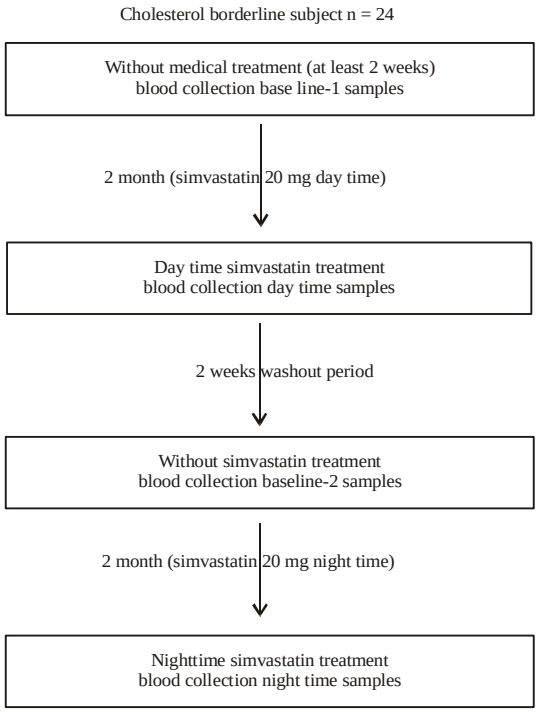

Fig. 1: Study protocol. The 24 Cholesterol borderline subjects were kept without medication for 2 weeks (baseline-1). Afterward, they were treated with simvastatin (20 mg day^{-1} time) for 2 months, afterward 2 weeks washout period and then treated with simvastatin (20 mg $night^{-1}$ time)

spectrophotometrically. Briefly, 50 µL of plasma were added to 1 mL of 100 mM Tris–HCl pH 8 buffer containing 20 mM $CaCl_2$ and 5.5 mM paraoxon. The blank sample containing the incubation mixture without plasma was run simultaneously to correct spontaneous substrate hydrolysis. The absorbance was monitored at 412 nm for 3 min. Enzyme activity was calculated using molar extinction coefficient 17100 M^{-1} cm^{-1}. One unit of PON1 activity is defined as 1 nM 4-nitrophenol formed per minute (Paragh *et al*., 1998).

Determination of plasma total thiol: Plasma total thiol (T-SH) concentration was determined using 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) as described by Hu (1994). Briefly, 50 µL of the sample were mixed with 1 mL of 0.1 M Tris EDTA pH 8.2. Forty microliter 10 mM DTNB in methanol were added and absorption was measured at 412 nm. The concentrations of thiol groups were calculated using a molar extinction coefficient of 13600 M^{-1} cm^{-1} and expressed as µM.

Measurement of plasma lipid peroxidation: Lipid peroxidation in terms of MDA interacted with thiobarbituric acid in hot acidic media to produce a pink color that can be measured spectrophotometrically. The determination procedure consists of the addition of 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid, 200 µL of 8.1% sodium dodecyl sulphate and 700 µL of distilled water to a 100 µL sample. The reaction mixture was heated in boiling water for 60 min. After cooling, 1 mL of distilled water and 5 mL of buthanol/pyridine mixture (14:1 v/v) were added to the samples. The samples were then centrifuged at 10000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. MDA concentration was calculated using tetramethoxypropane as standard. Results are given as nM of MDA (Ohkawa *et al*., 1979).

Determination of plasma protein carbonyl: Plasma PCO levels were measured spectrophotometrically using method of Levine *et al.* (1990). The proteins were precipitated by the addition of 10% trichloroacetic acid (TCA). The precipitated protein samples were resuspended in 1.0 mL of 2 M HCl containing 2% 2,4- dinitrophenylhydrazine. After incubation for 1 h at 37°C, the protein samples were washed with alcohol and ethyl acetate and reprecipitated by the addition of 10% TCA. The precipitated protein samples were dissolved in 6 M guanidine hydrochloride, and the absorbance was measured at 370 nm. The molar extinction coefficient 22×103 M^{-1} cm^{-1} was used to calculate the PCO level, which was then expressed as nM of carbonyl groups formed/mg protein. Total protein contents was measured spectrophotometrically using Folin kit (Sigma Diagnostics, St. Louis, MO, USA).

Measurement of plasma nitrite: This assay was based on the reduction of NO stable end product (nitrate) to nitrite by nitrate reductase as described by Green *et al*. (1982). Three hundred microliter of plasma were deproteinized by adding 600 µL of 75 mM $ZnSO_4$ solution. The mixture was stirred and centrifuged at 10000 rpm for 5 min at ambient temperature. Then 100 µL of supernatants were added to the reaction mixture containing 40 µL of nitrate reductase (20 mU), 50 µL of FAD (5 mM), 10 µL of NADPH (0.6 mM) and 250 µL of phosphate buffer (50 mM). The mixtures were incubated for 1 h at 37°C and then 150 µL o f the mixture was added to 450 µL of Griess reagent and incubated in the dark place for 30 min at room temperature. Absorbance was measured at 545 nm. The nitrites levels were calculated from sodium nitrite standard curve and expressed in µM.

Determination of erythrocytes NOS activity: The NOS activity assay was performed by monitoring the rate of conversion of L-ARG into citrulline as described by Mckee *et al*. (1994). The erythrocyte hemolysate was incubated with 0.2 mM L-ARG, 10 mM HEPES, 0.425 mM Ethylene Diamine Tetraacetic Acid (EDTA), 0.45 mM $CaCl_2$, 80 units of calmodulin, 1 µM tetrahydrobiopterin, 4 µM Flavin Adenine Dinucleotide (FAD), 4 µM Flavin Mononucleotide (FMN), 0.5 mM Dithiothreitol (DTT), 0.16 M sucrose and 1 mM NADPH. One unit of NOS was defined as the amount of enzyme required to catalyze the conversion of 1 µM of L-ARG into citrulline/min/mg protein under the assay conditions used.

Determination of erythrocytes nitrite level: The erythrocytes were hemolysed by addition of ice-cold distilled water and

hemoglobin was precipitated by addition of cold ethanol and chloroform. After vortex, the mixture was centrifuged at 10000 rpm for 10 min Carvalho *et al*. (2004). Clear supernatants were processed as plasma samples.

Statistical analysis: The data were analyzed using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. The data were expressed as the Mean±SD of each group. A probability value of $p \leq 0.05$ was used as the criterion for significance.

RESULTS

Lipid profile: The present results show that evening or morning administration of simvastatin resulted in significant decrease of plasma TC, TAG, LDL-C and AI ($p \leq 0.05$). However, HDL-C levels were increase by 18 and 16.3%, respectively compared to baseline values (Table 2). However, night administration of simvastatin elicits a marked decrease of TC, TAG, LDL-C and AI compared to day time.

Paraoxonase-1 activity: In the present study, night or day simvastatin supplementation significantly increased PON1 activity compared to those at baselines (43 and 26%, respectively). However, evening simvastatin administration caused marked increase of PON1 activity (17%) compared to morning intake (Fig. 2).

T-SH, PCO and MDA: As shown in Table 3 night or day simvastatin treatment significantly increased T-SH level (44.7 and 24.4%, respectively) compared to those at baselines. On the other hand, markers of protein oxidation (PCO) and lipid peroxidation (MDA) plasma levels decreased with both simvastatin treatments compared to baselines ($p \leq 0.05$).

However, evening simvastatin administration caused a significant increase in PCO and MDA compared to morning regimen.

Table 2: Effects of evening or morning simvastatin treatment on plasma levels of TAG, TC, HDL-C, LDL-C and AIP

Parameters ($mg\ dL^{-1}$)	Baseline-1	Day time	Base line-2	Night time
TAG	109.000±8.73	84.940±13.6[a]	107.500±9.73	79.810±15.6[a]
TC	218.900±7.63	180.500±13.4[a]	207.100±9.89	171.400±14.1[a]
HDL-C	65.400±5.58	76.050±5.92[b]	67.530±5.58	79.700±6.20[b]
LDL-C	117.700±13.2	78.680±8.66[a]	118.700±11.23	73.740±20.5[a]
AI	-0.136±0.14	-0.311±0.04[a]	-0.159±0.11	-0.360±0.02[a]

Data expressed in Mean±SD, n = 6, a: Significant decrease from baseline was observed, b: Significant increase from baseline was observed, p value≤0.05

Table 3: Effects of evening or morning simvastatin treatment on plasma levels of T-SH, MDA and PCO

Parameters	Base line-1	Day time	Base line-2	Night time
T-SH (μM)	451.300±70.7	561.500±60.1[a]	457.200±74.1	661.700±57.8[a]
MDA (nM)	5.483±0.95	3.315±0.51[b]	5.112±0.84	2.158±0.27[b]
PCO (nM/mg Pro)	0.598±0.14	0.407±0.06[b]	0.523±0.14	0.249±0.05[b]

Data expressed in Mean±SD, n = 6, a: Significant increase from baseline and/or Day time was observed, b: Significant decrease from baseline and/or Day time was observed and p value≤0.05

Nitric oxide: In the current results, morning or evening simvastatin medication significantly increased plasma nitrite level 46 and 88%, respectively compared to baselines. Furthermore, night time dosing of simvastatin induced significant increase of nitrite level compared to day dosing by 22%. Figure 3 displays the effect of simvastatin on plasma nitrite.

Regarding to erythrocyte NO markers the present results show that simvastatin day time and night time dosing significantly increased erythrocyte NOS activity (46 and 64%, respectively) compared to NOS activity in baselines samples. However, night time dosing induced significant increases in NOS activity (19%) compared to day time (Fig. 4). In respect to erythrocytes nitrite, day time and night time dosing of simvastatin administration increased nitrite level by 34 and 55% compared to baselines. On the other hand, night time dosing increased nitrite level by 17% compared to morning administration. Figure 5 represents erythrocyte nitrite level.

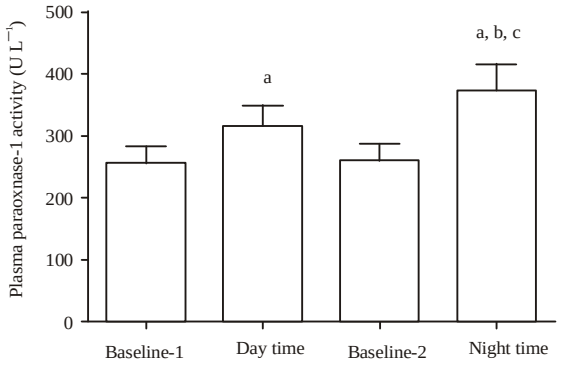

Fig. 2: Effects of day time and night time simvastatin treatment on plasma PON1 activity, Data is expressed as Mean±SD, n = 6, a: Significance increase from baseline-1 was observed, b: Significance increase from day time was observed and c: Significance increase from baseline-2 was observed

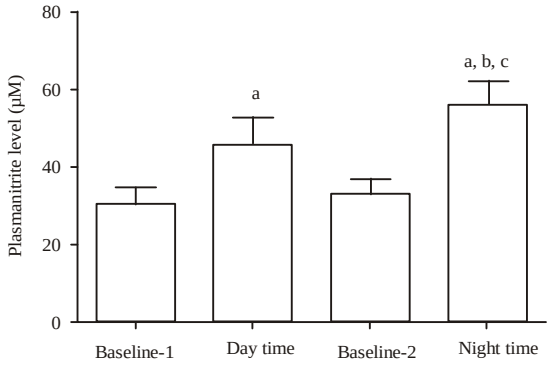

Fig. 3: Effects of day time and night time simvastatin treatment on plasma nitrite levels, Data is expressed as Mean±SD, n = 6, a: Significance increase from baseline-1 was observed, b: Significance increase from day time was observed and c: Significance increase from baseline-2 was observed

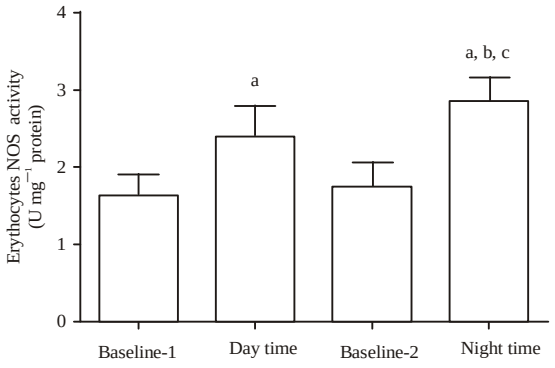

Fig. 4: Effects of day time and night time simvastatin regimen on erythrocytes NOS activity, Data is expressed as Mean±SD, n = 6, a: Significance increase from baseline-1 was observed, b: Significance increase from day time was observed and c: Significance increase from baseline-2 was observed

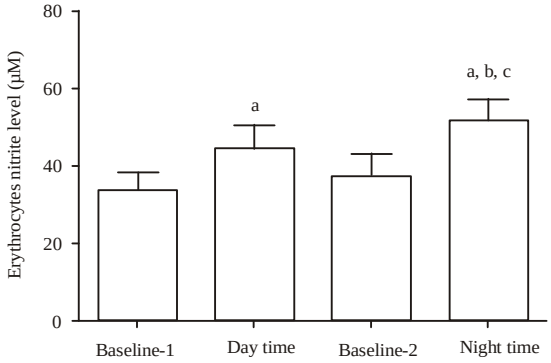

Fig. 5: Effects of day time and night time simvastatin treatment on erythrocytes nitrite levels, Data is expressed as Mean±SD, n = 6, a: Significance increase from baseline-1 was observed, b: Significance increase from day time was observed and c: Significance increase from baseline-2 was observed

DISCUSSION

The chronotherapy consistent with the body's circadian rhythm that matching the release of specific biological signal produce a good therapeutic effects and minimize the side effect of medications (Biswas *et al*., 2014). Melatonin is involved in biological clock function and its secretion reaches peak value after midnight. It has direct antioxidant activity: up regulates antioxidant enzymes, enhances non-enzymatic antioxidants and modulates NO bioavailability (Chakravarty and Rizvi, 2011; Xu *et al*., 2012).

Cholesterol biosynthesis has diurnal periodicity due to nocturnal increases of cholesterol biosynthesis precursors and increase activity of HMG-CoA reductase. Therefore, in this work night treatment with simvastatin gives pronounced effect than morning. Also, at night the high peak of melatonin decreases cholesterol biosynthesis and enhances its metabolism into bile acids (Favero *et al*., 2014). AI reflects atherogenic /anti-atherogenic lipoproteins balance and it is a measure of response to treatment. In this study, administration of simvastatin resulted in significant decrease of TC, TAG, LDL-C and AI; However, HDL-C levels were increased as compared to basal levels. These results are in agreement with several studies reported that statin therapy improves lipid profile and decreases atherogenic index (Pereira *et al*., 2004; Adekunle *et al*., 2013). Favero *et al*. (2014) reported that melatonin is biological atheroprotective agent, it ameliorates profile and antioxidant status. Therefore, the positive effects of simvastatin were significantly increased by evening dosing rather than in morning. The obtained results consistent with many workers reported that night time statin dosing gives a good therapeutic response (Adekunle *et al*., 2013; De Giorgi *et al*., 2013). In contrast, some studies reported that there is no significant difference between administration of simvastatin in morning or in evening manner (Yoon *et al*., 2011).

Additionally, diurnal fluctuations of antioxidant enzymes activity was documented in mammals including humans (Singh *et al*., 2005; Xu *et al*., 2012). The PON1 is one of antioxidant enzymes protecting against oxidative damage (Nezami *et al*., 2011). In the present study, administration of simvastatin evening or morning increased PON1 activity compared to baselines. Likewise, Costa *et al*. (2011) reported that simvastatin treatment was increased PON1 activity. However, evening simvastatin administration caused significant increase in PON1 activity compared to morning dosing. Similarly, Kamal (2011) demonstrated that evening treatment of statin induced better antioxidant profile than morning dosing. Moreover, melatonin plays a role in upregulation of antioxidant enzymes such as reductases and peroxidases (Berra and Rizzo, 2009; Chakravarty and Rizvi, 2011; Xu *et al*., 2012). Similarly, PON1 has peroxidase activity, therefore it may be induced by melatonin.

Several studies reported that there is a day and night fluctuation of oxidative stress parameters in animals and in humans (Singh *et al*., 2004; Subash and Subramanian, 2007; Chakravarty and Rizvi, 2011; Xu *et al*., 2012). Herein, simvastatin treatment preserves T-SH level compared to basal levels. These findings are consistent with the study of Zinellu *et al*. (2012) demonstrated that simvastatin therapy induced a significant increase of T-SH levels. Moreover, Chakravarty and Rizvi (2011) demonstrated that high level of thiols is in nocturnal samples. The protective roles of thiols against biomolecules oxidative damage are already established (Nagy *et al*., 2007). Therefore, plasma levels of PCO and MDA were decreased by simvastatin treatment. The effect of night dosing was more noticeable on PCO and MDA than day regimen. Likewise, Chakravarty and Rizvi (2011) founded that decreased lipid peroxidation in nocturnal samples. At night, level of melatonin is increased leading to direct antioxidant activity, up regulation of antioxidant systems and suppression of free radicals producing systems (Berra and Rizzo, 2009; Chakravarty and Rizvi, 2012). This is

the possible cause for decreasing lipid peroxidation and protein oxidation in the nocturnal samples in the present work. Moreover, several studies reported that treatment with statin decreased oxidative stress, correct antioxidant status and decreases oxidation of biomolecules (Shin *et al*., 2007; Aydin *et al*., 2009). The reduction of lipids and protein oxidation by simvastatin seems to be related to its antioxidant effect (Pereira *et al*., 2004).

Lipids abnormality increases cholesterol infiltration into the endothelial cells and erythrocytes (Mason *et al*., 2004; Eligini *et al*., 2013). In addition to endothelial cells, erythrocytes are involved in transport, metabolism and biosynthesis of NO (Ramirez-Zamora *et al*., 2013). Cholesterol enrichment associated with disruption of L-arginine active transport into erythrocytes and endothelial cells, so, the NO production was decreased (Mason *et al*., 2004; Eligini *et al*., 2013). Under HC condition, statins persevered structural and functional features of erythrocyte membranes and oxidant/antioxidant balance. This may attributed to statins therapy which (Tziakas *et al*., 2009) decreases cellular cholesterol inclusion (Mason *et al*., 2004; Forsyth *et al*., 2012; Uydu *et al*., 2012). Also, statins scavenge free radicals, preserve NOS cofactors, prevent NOS uncoupling and enhance NOS activity (Ozuyaman *et al*., 2008; Harisa *et al*., 2012; Li and Forstermann, 2013). Moreover, melatonin is endogenous agent ameliorate the fatty infiltration into endothelial cells and restores NO levels (Favero *et al*., 2014).

The increased NO in the present study with simvastatin treatment in congruence with several studies reported that statins improve NO bioavailability (Ozuyaman *et al*., 2008; Harisa *et al*., 2012; Li and Forstermann, 2013; Glutkina, 2013; Andrade *et al*., 2013). Moreover, it has been established that atheroprotective effect of statins is mediated by restoring the normal NO/peroxynitrite balance (Heeba *et al*., 2009). The night dosing of simvastatin resulted in an increase of NO level compared to day treating. Similarly, Antosova *et al.* (2009) confirmed that NO has circadian variations with a higher peak during morning hours. Also, melatonin is able to quench the superoxide, and decrease conversion of NO to peroxynitrite (Favero *et al*., 2014).

CONCLUSION

This study proves that night simvastatin dosing elicit more beneficial effects than day time dosing as indicated by decrease of AI and TC, MDA and PCO; however, it enhances T-SH and PON1 activity. These effects improve microenvironment for both eNOS and erythrocyte-NOS to produce NO that has beneficial effects on erythrocytes and endothelial cells. A relatively small sample size was the limitation of this study, large-scale prospective studies are needed to confirm this issue.

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