International Journal of Pharmacology

ISSN 1811-7775
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
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., 2004, Lahera et al., 2004). These effects of statins are 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). 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).

**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,HPO₄. 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 paraoxan as substrate, the produced 4-nitrophenol was measured
The concentrations of thiol groups were calculated using a molar extinction coefficient of 13600 M$^{-1}$ cm$^{-1}$. The reaction mixture was heated in boiling water for 60 min. After cooling, 1 mL of distilled water and 5 mL of butanol/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 precipitated protein samples were resuspended in 1.0 M 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 of 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.

**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 butanol/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).
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<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<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<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 AI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline-1</th>
<th>Day time</th>
<th>Base line-2</th>
<th>Night time</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG (mg dL⁻¹)</td>
<td>109.00±8.73</td>
<td>84.94±13.6³</td>
<td>107.50±9.73</td>
<td>79.81±15.6³</td>
</tr>
<tr>
<td>TC (mg dL⁻¹)</td>
<td>218.90±7.63</td>
<td>180.50±13.4³</td>
<td>207.10±9.89</td>
<td>171.40±14.1³</td>
</tr>
<tr>
<td>HDL-C (mg dL⁻¹)</td>
<td>65.40±5.58</td>
<td>76.05±5.92³</td>
<td>67.53±5.58</td>
<td>79.70±6.20³</td>
</tr>
<tr>
<td>LDL-C (mg dL⁻¹)</td>
<td>117.70±13.2</td>
<td>78.68±8.66³</td>
<td>118.70±11.23</td>
<td>73.74±20.5³</td>
</tr>
<tr>
<td>AI (mg dL⁻¹)</td>
<td>-0.13±0.14</td>
<td>-0.31±0.04³</td>
<td>-0.15±0.11</td>
<td>-0.36±0.02³</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline-1</th>
<th>Day time</th>
<th>Base line-2</th>
<th>Night time</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SH (µM)</td>
<td>451.30±70.7</td>
<td>561.50±60.1³</td>
<td>457.20±74.1</td>
<td>661.70±57.8³</td>
</tr>
<tr>
<td>MDA (nM)</td>
<td>5.48±0.95</td>
<td>3.31±0.51³</td>
<td>5.11±0.84</td>
<td>2.15±0.27³</td>
</tr>
<tr>
<td>PCO (nM/mg Pro)</td>
<td>0.59±0.14</td>
<td>0.40±0.06³</td>
<td>0.52±0.14</td>
<td>0.24±0.05³</td>
</tr>
</tbody>
</table>

Data expressed in Mean±SD, n = 6, a: Significant increase from baseline was observed, b: Significant increase from 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.

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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
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 regulation of antioxidant enzymes such as reductases and peroxidases (Berra and Rizzo, 2009; Chakravarty and Rizvi, 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 results are in agreement with the study of Zinellu et al. (2012) demonstrated that simvastatin therapy induced a significant increase of T-SH levels. 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 its metabolism into bile acids (Favero et al., 2014). AI reflects atherogenic and 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.
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., 2004; Aydin et al., 2009). The reduction of lipids and protein oxidation by simvastatin seems to be related to its antioxidant effect (Pereira et al., 2014).

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.

ACKNOWLEDGMENTS

The author extends his appreciation to the Deanship of Scientific Research at King Saud University, Saudi Arabia for funding the work through the research group project No. RGP-238. The authors declare that there are no conflicts of interest.

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