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Molecular and Biochemical Effects of Pravastatin on Male Albino Rats

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Abstract: Dyslipidemia is a major risk factor for coronary heart disease (CHD), its management is important in preventing the incidence of cardiovascular events. Statins (HMG-CoA reductase inhibitors) are widely used for the treatment of hypercholesterolemia. In the present work, we induced hypercholesteremia in experimental rats and studied the effect of different daily doses (5, 50 and 100 mg/kg/day) of pravastatin sodium salt on lipid profile and mRNA gene expression of glutathione peroxidase and copper/zinc-containing superoxide dismutase (Cu/Zn-SOD), two major enzymes of antioxidant system in hypercholesterolemic male albino rats. Our model resulted in no significant effect on lipid profile at 5mg/kg daily dose. However, increasing the dose can produce anticholesteremic action and affects the expression level of glutathione peroxidase and Cu/Zn-SOD. The study strengthened the idea about the pleiotropic effect of pravastatin.

Key words: Rat · Pravastatin · Dyslipidemia · Lipoprotein profile · Gene expression · SOD · GPX

INTRODUCTION

An association between dyslipidemia and risk of morbidity and mortality from cardiovascular disease has been demonstrated in epidemiological and observational studies [1,2]. Studies included different examinations as young men [3], middle aged men[4], women [5] and elderly patients [6] recommended the need of aggressive lipid-lowering therapy. Several, other trials have documented the use of statins in lowering of cholesterol. Statins inhibits HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase This could be used in the treatment of patients with hypercholesterolemia [7,8] and to reduce risk of death or cardiovascular events across wide range of cholesterol levels [9]. Borghi and his coworkers [10] reported that the inhibition of HMG-CoA reductase at the rate-limiting step of cholesterol biosynthesis leads to up-regulation of LDL receptors in the liver, enhancing LDL clearance from the plasma. In addition, statins decrease the hepatic production of VLDL and increase the catabolism of VLDL remnants in the plasma.

Among the HMG-CoA reductase inhibitors, pravastatin is a natural member and relatively liver specific as it has been previously demonstrated that after *i.v.* administration of the drug, liver accounted for most of the uptake compared with other tissues [11]. It decreases the intracellular cholesterol concentration which leads to compensatory up-regulation of receptors for low-density lipoprotein (LDL) cholesterol [12]. However, the response to statins is highly variable from patient to patient due to pharmacogenomics of statins [13].

Reactive oxygen species play a central role in vascular physiology and pathophysiology. Nitic oxide (NO), superoxide anion (O_2.-), the hydroxyl radical (OH.-), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO.-) are produced in the vasculature both under normal and under stress conditions such as hypercholesterolemia [14].

Superoxide anions can be generated by different enzymes (e.g. NADP(H) oxidase, xanthine oxidase, cyclo-oxygenases, NO synthases, CYP450 mono-oxygenases and enzymes of the mitochondrial respiratory chain) in virtually all cell types including vascular smooth muscle and endothelial cells. Then the produced superoxide anion either spontaneously or enzymatically [through dismutation by superoxide dismutase (SOD)] is reduced to the uncharged H_2O_2 which in the presence of the enzyme catalase or glutathione peroxidase is then dismutated into water and oxygen [15].

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Recent studies have suggested that increased vascular superoxide (O_2-) production by vascular NAD(P)H oxidase may play a critical role in the progression of atherosclerosis in patients with hypercholesterolemia [16]. Moreover, it was clarified that the predominant activity of SOD in the vasculature is attributed to Cu/ZnSOD, which may play an important role in the pathogenesis of atherosclerosis [17].

Many candidate genes involved in statin response were studied, either those involved in pharmacokinetics with their reflection on lipid response [18] and adverse events [19], or those involved in pharmacodynamics with their reflection on lipid response [20], cardiovascular events [21], adverse effects and effects on other systems [22,23].

The ability of statins to scavenge oxygen-derived free radicals was demonstrated in simvastatin by Day *et al*. [24], fluvastatin by Yamamoto *et al*. [25], atorvastatin by Giroux *et al*. [26], pravastatin and cerivastatin by Wagner *et al*. [27] in a variety of cell types, including macrophages, neutrophils, vascular smooth muscle cells and endothelial cells.

This study was designed to evaluate the effect of different doses of pravastatin on Lipid profile and Transcriptomic level (mRNA Gene expression) of two major enzymes of antioxidant system, the glutathione peroxidase and copper/zinc-containing superoxide dismutase (Cu/Zn-SOD) in hypercholesterolemic male albino rats with correlation between the results to give more understanding of statins effects, especially pleiotropic effects.

MATERIALS AND METHODS

Chemicals:

Cholesterol: (5-cholesterol-3B-ol) equivalent to USP\NF approx 95% (GC-Sigma).

Pravastatin Sodium: Pravastatin sodium (Bristol-Myers Squibb Pharmaceutical) structural formula (Figure 1)

Animals and Doses: A total of sixty male albino Rats (*Rattus norvegicus*)-weighing 130±20 g-were kept under standard laboratory conditions with free access to standard water and diet [28] before and during the experiment. Rats were divided randomly into two main groups, one is the negative control group (nc or Nd = 15 rats, kept at standard conditions), other is the treated group (hypercholesterolemia group = 45 rats kept at standard conditions for 4 weeks, yet they were changed into cholesterol-fortified diet 2% W/W diet for another 4 weeks). After 4 weeks the treated group was randomized into four groups 10 rats each, a positive control group (PC or Hc) fed hypercholesteremic food for further 4 weeks and other three groups (PI, PII and PIII) fed on cholesterol-fortified diet 2% (w/w) and pravastatin sodium at daily doses of 5, 50 and 100mg/Kg of body weight, respectively for 4 weeks. Dosages and method of pravastatin administration were chosen according to Li *et al*. [29] with some modifications.

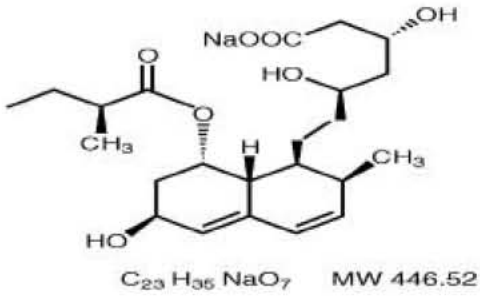

Fig. 1: *Structural formula* of pravastatin sodium.

Sampling: Two sampling times were applied to the experiment. After 4 weeks, 5 rats were taken from the negative control and 5 from the positive control group (before treatment with pravastatin). At the end of the experiment (after 8 weeks), five rats from each group (Nd, Hc, PI, PII and PIII). Samples were dissected, blood was taken from heart into sterile 5-ml tubes and 0.1g of liver tissue was taken into liquid nitrogen (the remaining rats were used for other parameters assays, data not shown).

Lipid Profile Assays: Serum from blood tubes were collected into 1.5 ml tubes and used for lipid profile assays. Fifty µl of samples was used in each test using UV/Visible spectrophotometer and Roche/Hitachi-912 automated analyzer. Total cholesterol was determined by Roche/Hitachi cholesterol kit according to Allain *et al*. [30] and Roeschlau *et al*. [31]. Triglycerides by Roche/Hitachi Triglycerides kit according to Siedel *et al*. [32] and Wahlefeld *et al*. [33]. HDL-cholesterol (high density lipoprotein) was determined with UV/VIS spectrophotometer V.530 at Wavelength λ 510nm. Low density lipoproteins (LDL and VLDL) and chylomicron fractions precipitate quantitatively by adding buffered PEG6000 HDL Cholesterol PEG6000 LR kit (SGM Italia co. Italy). After centrifugation, the concentration of HDL cholesterol fraction was determined and LDL-cholesterol was calculated according to Friedewald formula [34].

Table 1: The configuration of the used primers for the studied genes and their accession numbers; Supper Oxide Dismutase (SOD), Glutathione Peroxidase (GPX), Beta-Actin (B-Actin)

NO.	Name	Accession No		Primer Sequence (5'?3')	Product Size (bp)
1	SOD	M21060.1	Fwd.	GCAGAAGGCAAGGGGTGAAC	387
			Rev.	TAGCAGGACAGCAGATGAGT	
2	GPX	FQ210484.1	Fwd.	CTCTCCGCGGTGGCACAGT	290
			Rev.	CCACCACCGGGTCGGACATAC	
3	B-Actin	NM_031144.2	Fwd.	CCTGCTTGCTGATCCACA	726
			Rev.	CTGACCGAGCGTGGCTAC	

LDL concentration=Total cholesterol-(HDL concentration + triglyceride /5).

Semi-quantification of Gene Expression: Total RNA was extracted from approximately 100 mg liver using Trizol Reagent protocol (Gibco BRL) and according to manufacturer instructions. cDNA synthesis was done with random hexamers-primer and M-MLV-reverse transcriptase (from Quiagen) according to Ausubel *et al.* [35] and Nathan *et al.* [36]. Go Taq® Green Master Mix (Promega) was used to amplify the genes (Table 1) in a program (DNA Engine Dyad® Cycler, BioRad, USA) of 25 PCR cycles using 58°C as annealing temperature. PCR products were electrophoresed at 2% agrose gel at 100V current, then photographed by α-Gelfox™ 2D v.3.0 gel documentation unit (UK) and were scanned and quantitated using Alpha Ease software for windows v.4.0.0, band intensities were analyzed and corrected to those of β-actin.

$$\text{IDV of a sample gene} = \text{IDV}_{\text{of sample product}} / \text{IDV}_{\text{for Beta actin product of same sample}}$$

IDV denotes to Integrated Density Value as estimated by the Alpha Ease software from the photography of PCR products after agarose gel electrophoresis.

Statistical Analysis: The Mann-Whitney U-rank test was used to test for the differences in MT/β-actin ratios between the tested groups compared to the control group. In all the statistical tests, difference was considered significant when $P < 0.05$.The statistical analysis of the obtained data was done according to Kurtz [37] and the analysis was revised by SPSS for windows program v12.

RESULTS

Hypercholesteremic Induction: after 4 weeks of cholesterol enriched diet (Hc group), serum lipoproteins were significantly ($P < 0.05$) increased (Figure 2). Serum cholesterol was increased from 50.9 ± 0.3 to 161 ± 1.1, triglycerides from 35 ± 0.1 to 152.4 ± 0.8, HDL from 51.5 ± 0.3 to 55.5 ± 1.3 and LDL from 34± 0.5 to 119.4 ± 1.1.

Effects of Pravastatin Sodium on Lipid Profile (Figure 3): in the low dose pravastatin group (PI) Lipid profile showed an insignificant changes as compared to the normal diet control group (Nd). LDL increased from 120.13±1.31 to 147.13±1.26, HDL from 55.46±1.19 to 53.46±1.19, cholesterol from 161.86±0.95 to 160.93±.93 and triglycerides from 152.33±1.9 to 150.33±1.9. However, There was a very highly significant decrease ($P < 0.001$) in the dose of 50 mg/Kg/day (PII) as compared to Nd group, LDL from 120.13±1.31 to 76.72±.89, HDL from 55.46±1.19 to 34.6±1.0, cholesterol from 161.86±0.95 to 96.66±1.65 and triglycerides from 152.33±1.9 to 65.2±1.24.

There was a very highly significant decrease ($P < 0.001$) in of the high pravastatin dose (PIII) as compared to Nd group, LDL from 120.13±1.31 to 47.33±1.1, HDL from 55.46±1.19 to 33.66±1.23, cholesterol from 161.86±0.95 to 63.4±1.47 and triglycerides from 152.33±1.9 to 46.66±1.11. There was also a very highly significant difference ($P < 0.001$) in LDL from 76.72±.89 to 47.33±1.1, cholesterol from 96.66±1.65 to 63.4±1.47 and triglycerides from 65.2±1.24 to 46.66±1.11 between groups treated by 50, 100/kg/day, however we did not found any significant difference in HDL from 34.6±1.0 to 33.66±1.23 between mentioned doses.

Effects of Pravastatin on (mRNA) Gene Expression of Glutathione Peroxidase (Figures 4, 5): There was a non-significant decrease of GPX gene expression in the hypercholesteremic group (Hc) as estimated from means of IDV ratios (1.213 ± 3.1), as compared to Nd group (1.3 ± 1.1).However, GPX gene expression was non-significantly increased at dose of 5 mg pravastatin/Kg body weight/ day (PI) as estimated from means of IDV ratios (1.274 ± 2.3) as compared to that of Nd group.

Changes in mean IDV ratios (1.239 ± 2.1) due to the dose of 50 mg pravastatin /Kg/day were non-significant. However, There were highly significant (P value > 0.01) changes in mean IDV ratios (1.344 ± 0.9) at high dose of pravastatin (100mg/kg/day) as compared to positive control group.

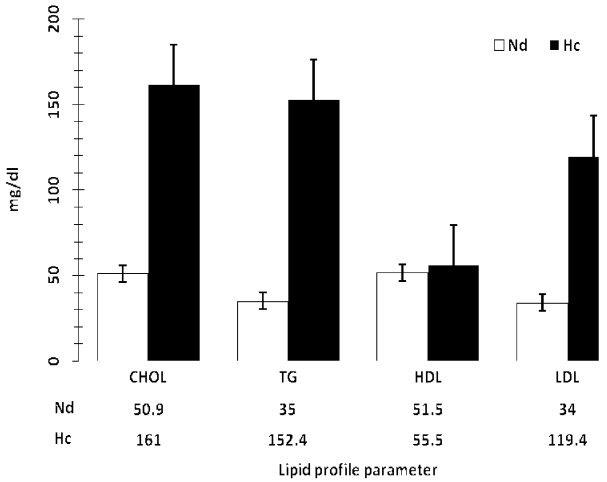

Fig. 2: Histogram represents the lipid profile due to hypercholesteremic induction (after 4 weeks of the experiment); Normal diet group (Nd),Hypercholesteremic group (Hc), cholesterol concentration (CHOL), Triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL). Hypercholesteremic parameters were significant for pc group ($p \leq 0.05$).

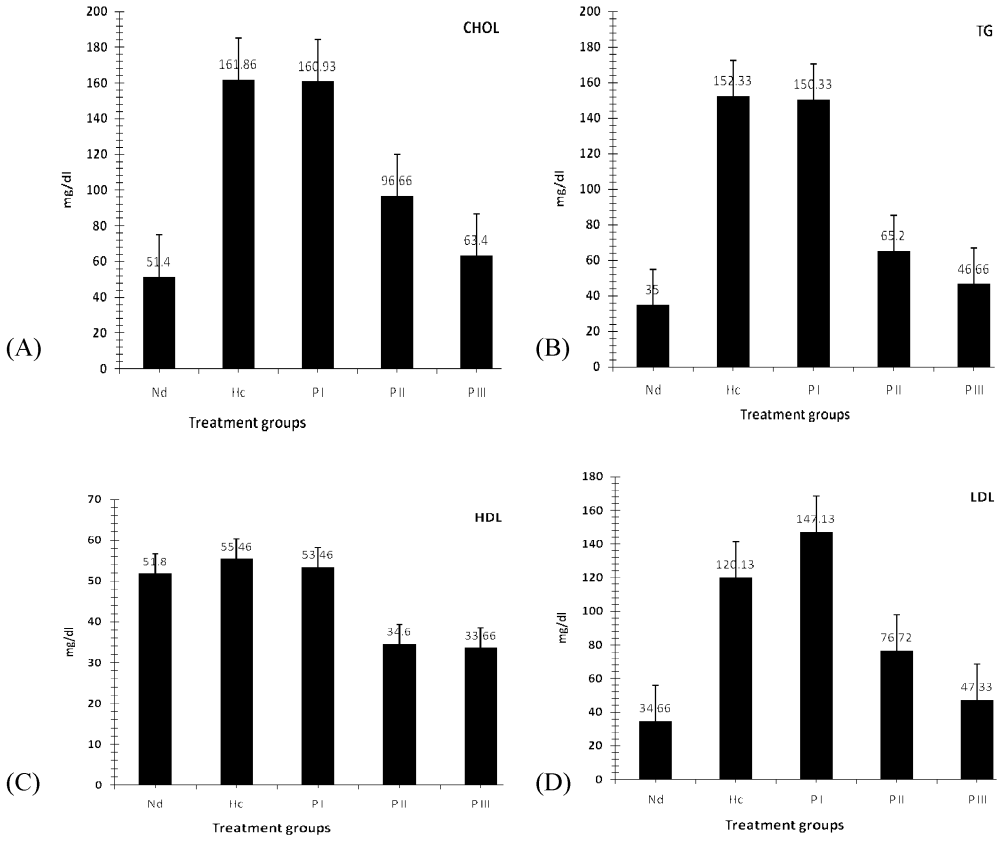

Fig. 3: Panel represents the lipid profile due to Pravastatin (after 4 weeks of pravastatin treatment); normal diet group (Nd), hypercholesteremic group (Hc) and the Pravastatin treated groups (PI, PII and PIII),A= cholesterol concentration (CHOL), B=Triglycerides (TG),C= high density lipoprotein (HDL), D=low density lipoprotein (LDL). All lipid profile readings for group PII and PIII were highly significant (P value < 0.001), except for, HDL (decreases were significant $p \leq 0.05$ at PII and insignificant at PIII)

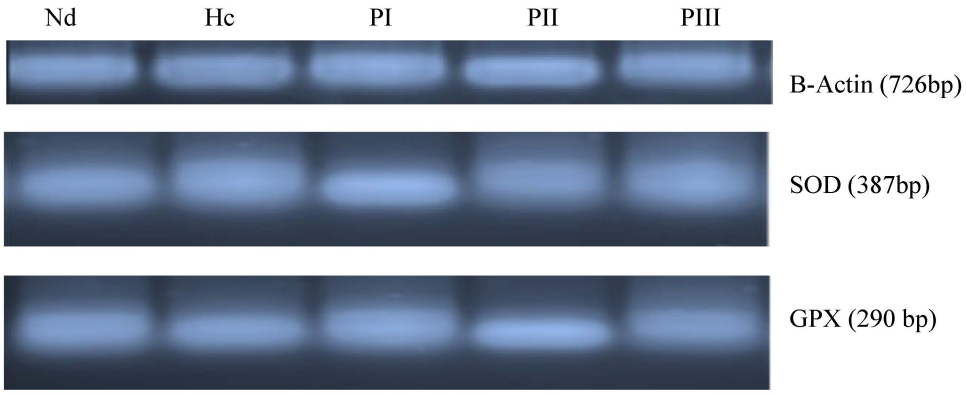

Fig. 4: Panel represents PCR products of the studied genes, Superoxide dismutase (SOD), Glutathione peroxidase (GPX) and the internal control gene Beta actin (B-Actin) for the groups, normal diet group (Nd), hypercholesteremic group (Hc) and the Pravastatin treated groups (PI, PII and PIII).

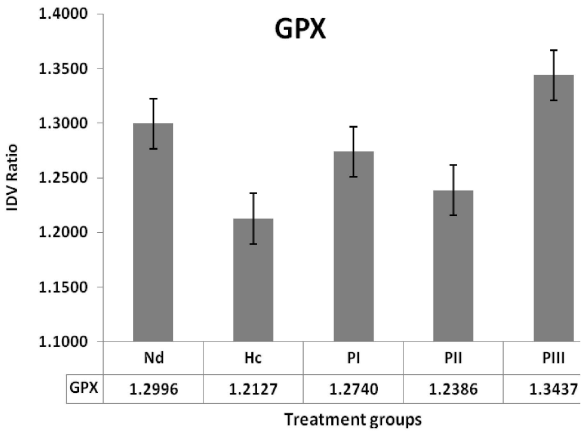

Fig. 5: Histogram for the Integrated denesity values (by Alpha Ease software v4.0) of GPX-PCR product correlated to those of B-Actin (internal control) in the normal diet group (Nd), hypercholesteremic group (Hc) and the Pravastatin treated groups (PI, PII and PIII). Increase at PIII was highly significant ($p \leq 0.01$).

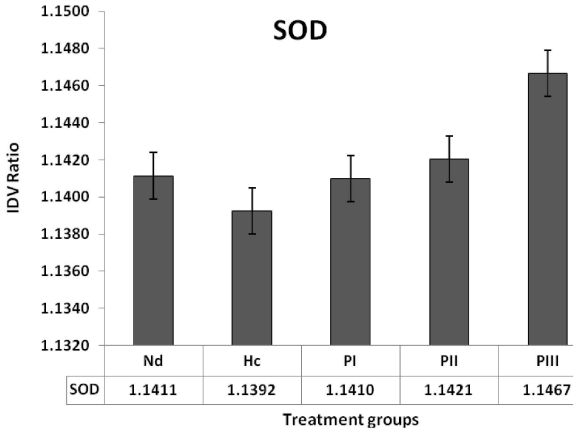

Fig. 6: Histogram for the Integrated denesity values (by Alpha Ease software v4.0) of SOD-PCR product correlated to those of B-Actin (internal control) in the normal diet group (Nd), hypercholesteremic group (Hc) and the Pravastatin treated groups (PI, PII and PIII). Increases at PII was significant ($p \leq 0.05$).

Effects of Pravastatin on Cu-Zn Superoxide Dismutase Gene (Figures 4, 6): There was a non-significant decrease in mean IDV ratios (1.139 ± 0.7) of hypercholesteremic group (positive control) as compared to normal diet (negative control) group (1.141 ± 1.4).

Pravastatin doses of 5 and 50mg/kg/day showed non-significant changes in means of IDV ratios (1.141± 3.1 and 1.142 ± 1.7, respectively), however a significant increase in mean IDV ratios (1.147 ± 1.6) at 100mg/kg/day dose of pravastatin as compared to positive control (hypercholesteremic) group.

DISCUSSION

In the present study, it was noticed that pravastatin in a dose of 5mg/kg/day had no significant effects on lipid profile (LDL, HDL, cholesterol and triglycerides) as compared to positive control group.These results are in agreement with previous studies [29,38,39] who reported that pravastatin in a small dose has no effect on lipid profile in rats. Other studies done by Bombig *et al*. [40], Kanda *et al.* [41] and Beltowski *et al.* [42], used Pravastatin in doses of 4,8,10mg/kg/day and found no effect of pravastatin on the lipid profile.

Increasing pravastatin dose to 50 mg/kg/day produced a very highly significant decrease in the level of serum Cholesterol, triglycerides and LDL. These results are in agreement with the study done by Li *et al.* [23] in rats who reported that pravastatin in dose more than 20mg/kg/day affects lipid profile in rats. Also, other studies done by Beltowski *et al.* [42] tested pravastatin in doses of 25,40 mg/kg/day and found that pravastatin at these doses affects lipid profile. However, studies by Daimon *et al.* [43], Li *et al.* [23] and Kivisto *et al.* [44] found no effect of 20mg/kg/day pravastatin on lipid profile in rats.

The more Increase in pravastatin dosage 100mg/kg/day produced a very high significant decrease in the level of serum cholesterol, triglycerides, low density lipoprotein as compared positive control group.

These results are in agreement with the study conducted by Pierno *et al.* [45] on pravastatin at 100mg/kg/day and showed that pravastatin produced high significant decrease in lipid profile.

Unexpectedly, in the present work it was noticed that pravastatin in a dose of (50mg/kg/day) significantly decreased HDL. as compared hypercholesteremic group. However, increasing pravastatin dose to 100mg/kg/day produced no further decrease in HDL level. These results are not in concordance with the study done by Shepherd *et al.* [46] who found that pravastatin increase HDL level in doses that affect lipid profile. The noticed effect of pravastatin on HDL level may be due to the morning administration of the drug and is in concordance with the recent clinical reports by Pappu and Illingworth [47], Wallace *et al.* [48] who found that the evening administration of statins significantly increase HDL level compared with the morning administration. This explained by Kamal *et al.* [49] who reported a difference due to the circadian rhythm of cholesterol biosynthesis

The current study reported significant increases ($P < 0.05$) in expression of Cu/Zn-SOD gene (1.421 ± 0.44 and 1.147 ± 0.98) with the doses of 50mg/kg/day and 100 mg/kg/day pravastatin, respectively as compared to positive control group (hypercholesteremic group). These results were in agreement with studies done by Rikitake *et al.* [50] who reported that hypercholesterolemia was associated with excess LDL oxidation and increased Reactive oxygen species.

The non-significant increase in expression of SOD due to low dose of pravastatin (5mg/Kg/day) was close to the normal value (1.1411 ± 1.08) of negative control (normal diet, Nd) group. Previous works [51,52] supported our data, they stated that different doses of pravastatin did not affect copper/zinc-containing superoxide dismutase (Cu/Zn-SOD).

It was also noticed that pravastatin at a dose 100 mg/kg/day produced a high significant increase in mRNA expression of glutathione peroxidase gene (1.344 ± 0.52).

These result were in agreement with studies done by Félétou and Vanhoutte [15] and Yilmaz *et al.* [53] who postulated that Lipid-lowering independent action of pravastatin play an important role in increasing endothelial NO biosynthesis and reducing generation of reactive oxygen species through inhibition of NAD(P)H oxidase activity and restoration of glutathione peroxidase activity. This results in reduced LDL oxidation and intracellular oxidative stress.

The underling mechanism can be explained according to Wagner *et al.* [27] who postulated that Rho family member Rac1 is a regulatory component of NAD(P)H oxidase and inhibition of Rac1 isoprenylation by statins inhibits release of reactive oxygen species in ECs. Furthermore, Vecchione and Brandes [54] demonstrated that withdrawal of statin therapy induces oxidative stress and endothelial dysfunction in mice. The mechanism underlying this involves activation of gp91phox-containing NAD(P)H oxidase by Rac-1 resulting in generation of superoxide anions, which scavenge eNOS.

Our study concluded that low daily dose of pravastatin has no effects on lipid profile. However, increasing the dose can produce anticholesteremic action and affects the expression level of some enzymes from the antioxidant system as glutathione peroxidase and Cu/Zn-SOD. The study also strengthened the idea about the pleiotropic effect of pravastatin.

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