Antimicrobial Effect and Immunomodulation of Atorvastatin

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Abstract: Objectives: Epidemiological studies of statins have suggested a link between statin therapy and a decreased risk of bacterial infection. It has been proposed that the mechanism underlying this protective effect of statins relates to their known immunomodulatory and anti-inflammatory effects. The aim of this study is to explore the antibacterial effect of atorvastatin, and its immunomodulation effect on tumor necrosis factor alpha, TNF α, and C reactive protein, CRP. **Method*:*** 20 serum samples were collected from patients who were under therapy with Atorvastatin for more than three months and 10 serum samples from control group who do not administer any statins. The serum samples were analyzed for TNF α quantitatively using ELISA kit and CRP semi quantitatively by agglutination kit. The antibacterial effect was tested against five clinical isolates for each of *E. coli, S. aureus, Proteus sp., and Bacillus sp.* **Results:** the concentration of TNF α and CRP were significantly decreased than the control group. Atorvastatin showed significant antibacterial effect against the tested bacterial isolates compared to that of the control except for *Proteus sp.* in addition to its effect on lipids profile. **Conclusion:** Atorvastatin shows antibacterial effect & reduces serum concentrations of TNF α & CRP, but still future studies are recommended to elucidate mechanism (s) by which atorvastatin is inducing its antibacterial effects.
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1. Introduction

Statins are a class of lipid lowering drugs that inhibit 3- hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase leading to decreased cholesterol and isoprenoid synthesis. Several recent studies have shown a link between statin use and a decreased risk of sepsis and inflammation. Statins possess a number of pleiotropic effects that are thought to have a beneficial effect on the cascade of detrimental events that characterize the sepsis syndrome (Kouroumichakis *et al.*, 2011). Statins have also been shown to decrease production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin- 1 (IL-1), and IL-6 present during sepsis (Zhang *et al.*, 2005). TNF α is a central mediator that stimulates leukocytes and vascular endothelial cells to release other cytokines to express cell- surface molecules that enhance neutrophil- endothelial adhesion at sites of infection, and to increase prostaglandin and leukotriene production (Anas *et al.,* 2010), so it was chosen to be determined in this study. There are some data that suggest that statins interfere with the recognition of microbial products by immune cells thus depressing the inflammatory cascade (Weitz-Schmidt *et al*., 2001). Another mechanism was suggested by Jerwood & Cohen (2008) that it would be due to direct antimicrobial effect.

Nine cohort studies addressed the effect of statins on various types of infection: bacteremia (n = 3), pneumonia (n = 3), sepsis (n = 2), and bacterial infection (n = 1).(Tleyjeh *et al.,* 2009). The aim of this study was to assess the direct antibacterial effect of Atorvastatin and immunomodulation on TNF-α and CRP in addition to its effect on lipids profile.

2. Materials & Methods:

Antibacterial effect:

It was done according to the method described by Jerwood and Cohen (2008). Briefly twenty bacterial isolates, (*Bacillus sp*., methicillin resistant *Staphylococcus aureus, E. coli* and *Proteus mirabilis*) were recovered from frozen or ambient saves by spreading on blood agar plates and inspected for contamination after overnight incubation. The test organisms from these plates were made up to a turbidity equivalent to that of a 0.5 McFarland standard then diluted 1:100 in Mueller–Hinton broth. These inocula were prepared immediately prior to each experiment. Testing was by a method based on the classical microtitre broth dilution recommended by the CLSI (formerly the NCCLS). Atorvastatin was obtained from the manufacturer as pure drug and dissolved in methanol to give an initial concentration of 1 g/L. The first test well therefore contains a concentration of 500 mg/L after the addition of isolate broth. There were eight further wells tested, each with

half the concentration of the previous and the concentration in the final well tested was 7.8 mg/L. The trays were read after 18–24 hrs of incubation at 37˚C in air. Positive growth and negative sterility controls, and a methanol control were included on each microtitre tray. The MIC of the statin was taken to be that at which there was no visible growth.

Tests applied on serum:

Twenty patients who were under atorvastatin treatment for more than three months were chosen as the test group and ten normal people were chosen as the control group. Briefly ten milliliters of venous blood was collected from both groups and the following tests were applied:

Effect on TNF- α:

TNF-α was measured quantitatively by Orgenium Laboratories human TNF-α ELISA kit according to the manufacturer's instructions. Briefly, prepare all reagents, samples and standards. Add 50 ul standards (starting from 500 pg/ml), test samples and sample diluents as a blank into the appropriate wells of the strips. Incubate one hour at room temperature then wash 5 times. Add 50 ul ready for use biotin antibody promptly to each well, incubate 30 minutes at room temperature then wash 5 times. Add 50 ul ready to use HRP- streptavidin solution, incubate for 30 minutes at room temperature then wash 5 times. Add 50 ul TMB substrate solutions to each well, incubate 20 minutes at room temperature. Finally add 25 ul stop solution to each well, read at 450 nm against 630 nm immediately. Calculation of the result is done by construction of a standard curve which is generated by plotting optical density for each of the standard concentrations versus the corresponding TNF α concentration. The concentration read from the standard curve must be multiplied by the dilution factor.

Effect on CRP:

CRP was measured semi quantitatively by CRP- latex slide agglutination, (SPINREACT, S.A.U Ctra. Santa Coloma, Spain), according to the manufacturer's instructions. Briefly make serial dilutions of the sample in 9 g/l saline solution of the sample and one drop of each positive and negative control into separate circles on the slide test. Add one drop (50 ul) next to the sample to be tested. Mix the drops with different stirrers for each sample to be tested. Place the slide on mechanical rotator at 80-100 r.p.m. for two minutes. Examine microscopically the presence of or absence of visible agglutination immediately after removing the slide from the rotator. The presence of agglutination indicates a CRP concentration equal or greater than 6mg/l. the titre is defined as the highest dilution showing a positive result. The approximate CRP concentration in the patient serum is calculated as 6 X CRP titre = mg/l

Determination of lipids profile:

a) Measurement of Triacylglycerols (TAG):

The TAG levels were determined according to the method of *Siedel et al. (1983)* using diagnostic kit provided by Spectrum Diagnostics (Egypt).

b) Measurement of Total Cholesterol (TC):

The TC levels were determined according to the method of *Richmond, (1973)* using diagnostic kit provided by Spectrum Diagnostics (Egypt).

c) Measurement of High Density Lipoprotein-Cholesterol (HDL-C)

The HDL-C levels were determined by Spectrum Diagnostics kits according to the method of *Burstein et al. (1970).*

d) Measurement of Low Density Lipoprotein-Cholesterol (LDL-C)

Serum LDL-C levels were determined according to the method of *Richmond (1973)* using diagnostic kit provided by Biosystem (Egypt).

Statistical analysis was done by SPSS 17.0 programme. to determine if there was significant difference between the test group and the control group

3. Results:

Antibacterial effect

There is significant difference between MIC of atorvastatin and that of methanol (Control) as shown in tables (1& 2). On the contrary, Atorvastatin has no effect on *Proteus sp.* as the mean MIC of atorvastatin was the same as methanol.

Effect on TNF α:

Atorvastatin significantly reduced the concentration of TNF- α in serum of patients treated with it compared to the control group. Results are shown in table (3).

Effect on CRP:

CRP concentration in serum of patients treated with Atorvastatin was significantly reduced than the control group. Results are shown in table *(3).*

Effect on Lipids Profile:

Atorvastatin significantly affected the concentration of cholesterol, HDL & LDL, while it has no significant effect on triglyceride (Tables 4 & 5)

MIC: minimum inhibitory concentration; N: number of tested isolates.

Table (1): Report on MIC of Atorvastatin & Methanol

Bacteria		Atorvastatin MIC mg/L	Methanol MIC mg/L
Bacillus sp.	Mean	43.7500	125.0000
	N	5	5
	Std. Deviation	17.11633	.00000
E. coli	Mean	75.0000	125.0000
	N	5	5
	Std. Deviation	27.95085	.00000
Proteus sp.	Mean	125.0000	125.0000
	N	5	5
	Std. Deviation	.00000	.00000
Staph. Aureus	Mean	37.5000	62.5000
	N	5	5
	Std. Deviation	13.97542	.00000

Table (2): Correlation between MIC of Atorvastatin & Methanol

		MIC Atorvastatin	Methanol MIC
MIC Atorvastatin	Pearson Correlation	1	.497*
	Sig. (2-tailed)		.026
	N	20	20
Methanol MIC	Pearson Correlation	.497*	1
	Sig. (2-tailed)	.026	
	N	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

Table (3): One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
CRP	13.714	29	.000	11.40000	9.6998	13.1002
TNF	12.859	29	.000	9.08333	7.6387	10.5280

Table (4) mean concentration, number of samples & standard deviation for CRP, TNF, Cholesterol, LDL & Triglyceride.

Patients		CRP	TNF	Cholesterol	HDL	LDL	Triglyceride
Control group	Mean	9.0000	10.9900	217.0000	30.9000	138.4000	140.8000
	N	10	10	10	10	10	10
	Std. Deviation	3.16228	2.65433	48.12715	9.84829	35.37168	51.20937
Test group	Mean	12.6000	8.1300	150.4500	46.7500	85.4500	112.7000
	N	20	20	20	20	20	20
	Std. Deviation	4.72841	4.07910	45.75734	8.49071	38.62298	52.53480
Total	Mean	11.4000	9.0833	172.6333	41.4667	103.1000	122.0667
	N	30	30	30	30	30	30
	Std. Deviation	4.55313	3.86889	55.75592	11.62261	44.83483	52.94886

Table (5): ANOVA Table

			Mean Square	F	Sig.
Cholesterol * Patients	Between Groups	(Combined)	29526.017	13.636	.001
		Within Groups	2165.248		
HDL * Patients	Between Groups	(Combined)	1674.817	20.910	.000
		Within Groups	80.095		
LDL * Patients	Between Groups	(Combined)	18691.350	13.215	.001
		Within Groups	1414.405		
Triglyceride * Patients	Between Groups	(Combined)	5264.067	1.938	.175
		Within Groups	2715.707		

4. Discussion:

Statins have a proven role in cardiovascular patients primarily via their cholesterol-lowering ability but also possess antiinflammatory and immunomodulatory pleiotropic effects that have been postulated to be beneficial in patients with sepsis and/or infection.

This study was done to assess that the antibacterial effect of Atorvastatin is due to immunomodulatory effect, especially on TNF- α, or due to its direct antibacterial effect. HMG-CoA reductase, the target of statins, is essential in prokaryotes but it is required for the biosynthesis of isoprenes, not sterols as in eukaryotes. Furthermore, the bacterial HMG-CoA reductase is of a different structural class with an affinity for statins that is _10 000 times weaker than the enzyme found in eukaryotes (Friesen & Rodwell, 2004) Thus, it is highly unlikely that the antimicrobial effect we have seen can be attributed to a known mechanism of action of statins.

In another work , statins decreased lipopoly saccharide toxicity by reduction of NF- Kappa B activation and subsequent release of TNF by modulating 3- hydroxyl – 3- methylglutaryl coenzyme A reductase activity (Fraunberger *et al*., 2009). This work is in agreement with this study as TNF is significantly decreased in the test group than in the control group (Table 3).

A meta-analysis regarding the use of statins for the prevention and treatment of infections has indicated that the use of statins might be beneficial ((Tleyjeh *et al.,* 2009). Several mechanisms could explain this protective effect. Statins are known to have immunomodulatory properties (Kwak *et al*., 2000). In contrast some studies stated that the antimicrobial effect of statins is due to direct effect; Lovastatin reduces the intracellular growth of *Salmonella typhimurium* (Catron *et al*., 2004); and reduces HIV-1 viral load (del Real *et al.*, 2004). Fluvastatin significantly reduces cytomegalovirus DNA concentration and production of viral particles (Potena *et al.*, 2004). Similarly, a number of studies have found that statins exhibit direct antifungal activity (Gyetvai *et al.*, 2006).

Atorvastatin has an unexpected antimicrobial effect *in vitro* but requires concentrations that are far higher than are probably achieved *in vivo* with traditional indications for statins. Therefore, statins probably do not exert a significant antimicrobial effect in patients. However, since multiple dose statins are known for their favorable effect on the course of bacterial infections (Thomsen *et al.,* 2006), it is possible that statins undergoes accumulation at target human tissues upon multiple dosing, or there could be a formation of relevant breakdown products *in vivo*. Other possible mechanisms could be related to the pleiotropic properties of statins. For example, multiple statins including atorvastatin and simvastatin, were shown to be cytotoxic, to suppress cells growth, and to promote apoptosis (Muck *et al.*, 2004; Yamazaki *et al.,* 2006; Tapia-Perez *et al.,* 2011). It is possible that the currently reported antibacterial activity of statins is related to such effects.

Results of the current work showed that Atorvastatin has antibacterial effect against *Bacillus sp. , Staph. aureus and E. coli*, but it showed no activity against *Proteus sp.*

Also Atorvastatin decreased serum concentration of TNF α and CRP in patients treated with it in addition to its effect on lipids. In conclusion future studies are recommended to elucidate mechanism (s) by which atorvastatin is inducing its antibacterial effects.

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