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REVIEW ARTICLE

Inhibitory effect of Atorvastatin on the secretion of extracellular virulence products by Methicillin resistant *Staphylococcus aureus*

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A B S T R A C T:

Statins are lipid-lowering therapeutic agents that have displayed useful anti-inflammatory and antibacterial properties. The focus of this research study was to understand the role of statins in controlling bacterial pathogenicity. Haemolysins and some other substances secreted by *S. aureus* were inhibited by Atorvastatin. It was found that Atorvastatin had a negligible effect on the growth of the bacteria at subminimal inhibitory concentrations, meanwhile it significantly reduced the secretion of haemolysins, coagulase and catalase. Thus, Atorvastatin could be used in conjunction with some antibiotics in controlling Methicillin resistant *S. aureus* MRSA infections and immunomodulation of the defense system.

KEY WORDS: MRSA, Atorvastatin, Haemolysin, α-Toxin.
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1. INTRODUCTION:

Methicillin resistant *S. aureus* can cause serious health issues, and is responsible for both nosocomial and cosmopolitan infections (Naimi, 2003; Lakhundi and Zhang, 2018; Siddiqui and Koirala, 2020). In the past four decades considerable attention has been focused on the mechanisms of protein secretion in bacteria, numerous observations have suggested that there is a link between lipid synthesis, membrane topography, integrity, regulatory determinants and the formation and secretion of haemolysins, toxins and enzymes in *S. aureus* and other microorganisms (Altenbern, 1977; Fishman *et al.*, 1978; Caulfield *et al.*, 1979; PATON *et al.*, 1980; Engels and Kamps, 1981; MÄntsälä, 1982; Saleh and Freer, 1984; Berkeley *et al.*, 1987; Hiltunen and Söderhäll, 1992; Price *et al.*, 2001; Adhikari and Novick, 2005; Welsh *et al.*, 2009; Manalo *et al.*, 2017 A. Khan *et al.*, 2018; T. J. Khan *et al.*, 2018; Rana *et al.*, 2019).

Atorvastatin (Pfizers, lipitor) a 3- hydroxy – methylglutaryl coenzyme A reductase inhibitor which lowers serum cholesterol and lipoprotein (Ciaravino *et al.*, 1995)
(Guerin Maryse *et al.*, 2000) has also been shown to inhibit inflammatory properties of *S. aureus* and other bacteria (Pruefer Diethard *et al.*, 1999; Liu *et al.*,2005, 2008; Graziano *et al.*, 2015; Ko *et al.*, 2017). It has been shown that patients on a statin regiment manifested decreased risk of death due to bacterial infections and sepsis (Horn *et al.*, 2008, Hennessy *et al.*, 2016).

During the COVID-19 pandemic, patients on statins have been found to be more resistant to viral infections(Castiglione *et al*., 2020), they also showed a decreased risk of death due to bacterial sepsis (Horn *et al.*, 2008; Zhang *et al.*, 2020). This study attempted to evaluate the effect of atorvastatin on the secretion of virulence factors by MRSA.

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2. Materials and Methods

2.1 Organism and growth conditions

A Methicillin resistant strain of *Staphylococcus aureus* (MRSA252, genotype EMRSA, from NARSA71, www.narsa.net) was maintained by a weekly transfer on nutrient agar plates. For cultivation, it was transferred to human blood agar plates (5%) then incubated at 37Cº overnight. Exponential phase cultures were prepared by inoculating a haemolytic colony that showed haemolysis on blood agar into 50 ml of brain heart infusion broth in a 250 ml Erlenmeyer conical flask and incubated at 37Cº on an orbital shaker (Gallenkamp UK) at 150 revolution per minute (RPM) to an optical density at 600 nm (OD 600) for 15-16 hours.

2.2 Identification

S. aureus was identified by mannitol fermentation, gram-stain, coagulase, catalase, golden colour, haemolysins production and by its resistance pattern to methicillin.

2.3 Preparation of Atorvastatin stock solution

Atorvastatin 40 mg tablets were obtained from Pfizer (Lipitor). A stock solution of Atorvastatin (40 mg tablet/ 10 ml (4000 µg/ml) in sterilized distilled water) was prepared.

From the stock solution 10, 20, 40 and 80 µl were aseptically added to 20 ml of rabbit blood agar (5%) medium in Petri dishes to reach a final concentrations of 2, 4, 8 and 16 µg/ml.

In broth culture experiments, 100 and 200 µl of Atorvastatin stock solution were added to 50 ml of brain heart infusion broth BHI in a 250 ml Erlenmeyer conical flasks to achieve final concentrations of 8 and 16 µg/ml respectively.

2.4 Inoculation of *S. aureus* onto Atorvastatin treated culture plates

Fresh cultures of *S. aureus* were subsequently subcultured onto both control and Atorvastatin treated rabbit blood agar plates. The plates were then incubated overnight at 37Cº. Zone of hemolysis around the colonies on Atorvastatin treated plates were then compared with zones around control colonies.

Coagulase production by both control and Atorvastatin treated cells were screened by emulsifying a colony of *S. aureus* in a drop of citrated rabbit plasma and observed for agglutination. Catalase production was monitored by emulsifying a colony of *S. aureus* from control and Atorvastatin treated cultures into a drop of H_2O_2 on a slide and observed for bubble formation.

2.5 Measurement of bacterial growth in control and Atorvastatin treated broth cultures

Exponential- phase cultures were prepared by inoculating a haemolytic colony into 50 ml of brain- heart infusion broth (BHI) in a 250 ml Erlenmeyer conical flask.

The flasks were incubated at 37Cº on a orbital shaker set at a speed of 150 RPM (Glallenkamp, UK). The flasks were monitored until they reached an optical density (OD) of 600 nm for $\pm$ 16 hours. An aliquot of 100 µl of this growing culture were inoculated into three 250 ml Erlenmeyer conical flasks (50 ml BHI medium), one flask was kept as control whereas the other two were treated with 8 and 16 µg/ml Atorvastatin. The experiments were performed in duplicate flasks of BHI grown at 37Cº with shaking at 150 RPM for a period of 16 hours, and repeated twice. The OD of the shaker growth was measured after an incubation period of 16 hours (Saleh and Freer, 1984).

2.6 Effect of Atorvastatin on haemolysin (alpha toxin), catalase and coagulase production during growth

The effect of Atorvastatin on haemolysins, catalase and coagulase production was monitored during growth as cited previously. Optical Density readings at 600nm were taken at 2 hour intervals. Duplicate 1 ml samples of growth cultures were taken and centrifuged at 15,000 RPM in an MSE microcentrifuge (Eisons,UK). Supernatants were assayed for haemolysins production (especially alpha haemolysin (α-toxin)) by doubling dilution titration assay against 1% v/v rabbit blood (which is highly sensitive to α- toxin) performed in phosphate buffered saline PBS in titration plates. The pellets were used for coagulase and catalase tests. To reach an optical density of 600 nm, dilutions were made in BHI broth when necessary. All experiments were repeated twice and all samples were analyzed in duplicate. To perform coagulase and catalase tests, the pellets were washed twice in PBS, centrifuged 15000 RPM, and then emulsified in 100 µl PBS.

An aliquot of 50 µl of this emulsified materal was added to a drop of citrated rabbit plasma or H_2O_2 on microscopic slides, and observed for agglutinations or bubble formations respectively.

2.7 Erythrocytes preparation and Haemolytic assay

An assay involving the lysis of rabbit erythrocytes were used to determine the presence of biologically active haemolysins (especially α-toxin of *S. aureus).* (Saleh and Freer, 1984).

Prior to use, the erythrocytes were washed three times in phosphate buffered saline (0.01 M sodium Phosphate in 0.015 M NaCl, pH 7.0, PBS). The Erythrocytes were then packed via centrifugation at 2000 RPM for 10 minutes in a Hereaus Sepatch Centrifuge (Germany)), prior to the preparation of a 1.0% v/v suspension.

Aliquots of 0.5 ml of culture supernatant were serially diluted in 0.5 ml of PBS in titration plates (Flow Laboratories, UK).

Rabbit erythrocytes (0.5 ml of 1% v/v suspension) were added to each well and the plates were incubated at 37Cº for 30 minutes. An end point at 50% haemolysis was determined visually. The point at which 50% haemolysis occurred was used to determine the haemolysins titration. This was subsequently called a ***a titre haemolytic unit per ml or HU/ml*** (Saleh and Freer, 1984).

2.9 Statistical analysis

Analysis of data was performed by using a SPSS (Version 10) Software Program. Results are expressed as mean ± SE.

3. Results

3.1 Effect of Atorvastatin on haemolysin (alpha toxin) , catalase and coagulase production by *S.aureus* grown on rabbit blood agar culture plates

Atorvastatin at concentrations of 2 and 4 µg/ml had insignificant effect on haemolysins , catalase and coagulase production, whereas, concentrations of 8 and 16 µg/ml had a significant effect on the production of haemolysins, coagulase and catalase, in fact 8 µg/ml **Atorvastatin** reduced the haemolytic zone diameters around the colonies by 50% whereas 16 µg/ml led to complete inhibition or the disappearance of haemolysins. Figure 1 represents the results, all experiments were repeated three times . **Atorvastatin** effect on catalase and coagulase production was profound since significant inhibition of coagulase and catalase were observed at 8 µg/ml , whereas complete inhibition of both enzymes were observed at 16 µg/ml of **Atorvastatin**.

The observations made via light microscopy following gram staining revealed some variations in the shape and integrity of **Atorvastatin** treated cells at a concentration of 8 and 16 µg/ml when they were compared with non-treated cell cultures. **Atorvastatin** treated cells appeared to be somehow smaller than control or non –treated cells. It was additionally observed that their grape like arrangements seemed to be distorted. These cells appeared to be more dispersed especially when involving samples taken from colonies grown on blood agar plates (some irregular clumps have been observed). It's noteworthy to mention that the grape-like arrangements were significantly affected by Atorvastatin treatment**.**

3.2 Effect of Atorvastatin **on the production of haemolysins, catalase and coagulase by *S.aureus* during growth cycle in Brain Heart Infusion broth BHI medium**

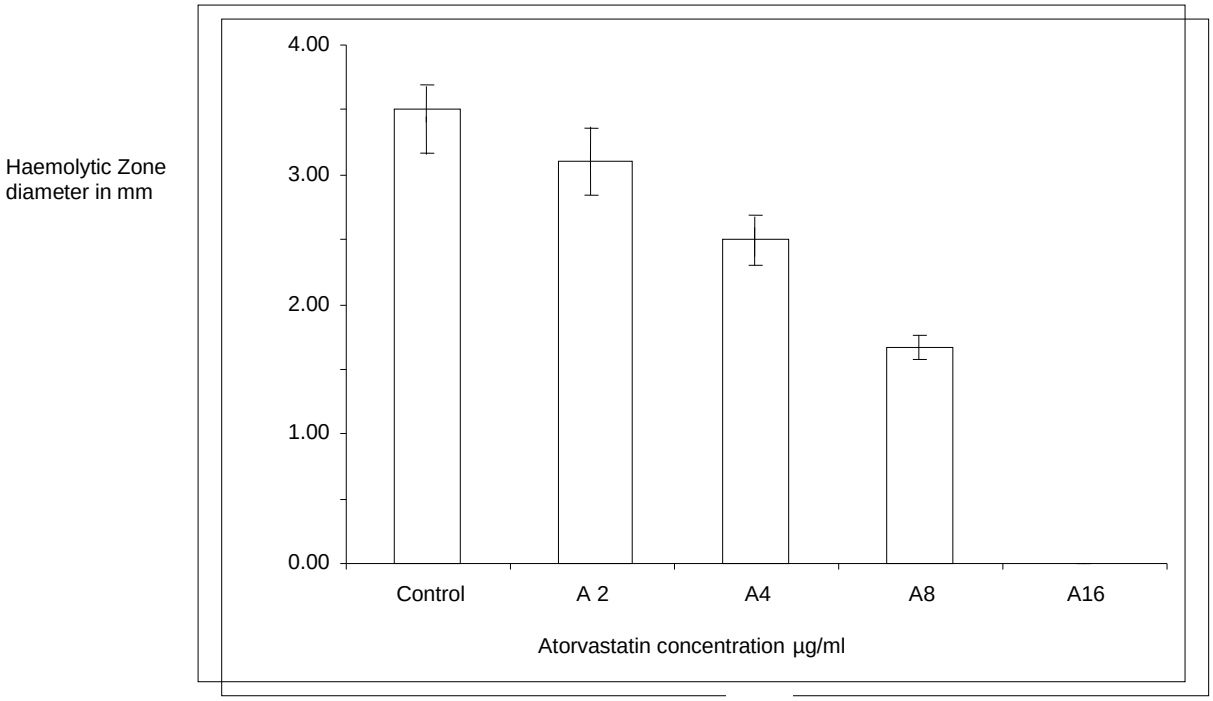

Figure 1 Means and standard errors of the effect of A2, A4, A8 and A16 µg/ml Atorvastatin on the haemolytic zones produced by MRSA grown on rabbit blood agar plates

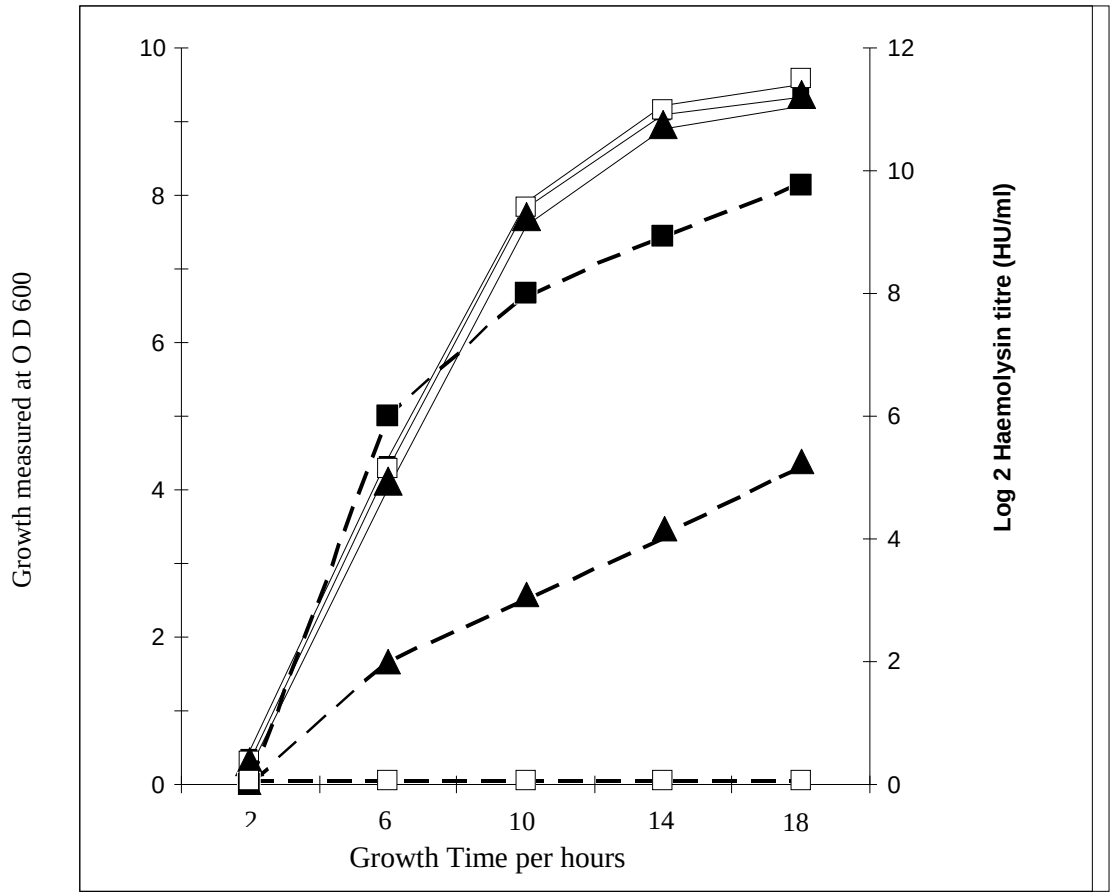

Figure 2 Effect of Atorvastatin on growth and production of haemolysins (α- toxin) by MRSA:

_____ growth measured at O D 600 ; ---------- haemolysin titre in culture supernatant ;

■ Control culture; ▲ culture containing Atorvastatin 8 µg/ml ; □ culture containing Atorvastatin 16 µg/ml

4. Discussion

The virulence characteristics and multi-drug resistance mechanisms in bacteria have been focused on for many years. Researchers have been investigating alternatives to antibiotics in order to combat this phenomenon.

The options investigated included a variety of agents among which were natural plant extracts, membrane perturbing agents, bacteriophages and lipid lowering agents. It was important to investigate the effect of lipid synthesis inhibiting agents, because they inhibit the synthesis of phospholipids in eukaryotic cells and their effects on the secretion of virulence factors by bacteria. Secretions of several exoproteins produced by several gram-position bacteria including staphylococcal enterotoxins, staphylocoagulase, penicillinase, levansucrase, α-amylase, protease, α-toxin and golden colour pigmentation in *S. aureus* have been shown to be sensitive to lipid synthesis inhibitors and membrane- perturbing agents (Altenbern, 1977; Fishman *et al.*, 1978; Caulfield *et al.*, 1979; PATON *et al.*, 1980; Engels and Kamps, 1981; MÄntsälä, 1982; Saleh and Freer, 1984; Berkeley *et al.*, 1987; Liu *et al.*, 2005, 2008; Cauz *et al.*, 2019; Chung, 2020). This investigation had focused upon evaluating the effect of Atorvastatin on the production of toxins and virulence factors by *S. aureus*.

In our research, it was found that the concentrations of Atorvastatin as high as 16 µg/ml of Atorvastatin had no significant effect on growth rate or final OD readings at 600nm, whereas it progressively inhibited the production of haemolysins especially α-toxin with the titer falling from 1024 HU/ml when compared with control cell culture. There was a complete inhibition of toxin production in the Atorvastin treated cultures at concentrations of 16µg/ml.

There also occurred a total inhibition of coagulase and catalase secretions at a concentration of 16µg/ml. The results obtained in our investigation were consistent with previous reference literature citing in this field. These earlier reports had suggested mechanisms that combined between synthesis of lipids or changes which had been induced by statins in physicochemical environments. The changes induced by statins include changes in: membrane integrity, topography or at the level of regularity determinants required for protein secretions (Petit Glatron and Chambert, 1981; Jacques, 1983; Silhavy *et al.*, 1983; Saleh and Freer, 1984; Adhikari and Novick, 2005).

The Atorvastatin effect on the shape and integrity of *S.aureus* cells may be attributed to its effect on the phospholipid and cell wall synthesis. The decrease in bacterial cell size observed in Atorvastatin treated cells may be due to a decrease in the amounts and proportion of different types of phospholipids integrated into these membranes. The dispersions of the cells could reflect the effect of Atorvastatin on the synthesis of teichoic acids which might have a role in binding or holding bacterial cells together through their role as ion chilators.

The recent literature citations associated with the emergence of MRSA have reported numerous investigations which had focused on the role of Atorvastatin in combating these hardy virulent MRSA strains (Pruefer Diethard *et al.*, 1999; Adhikari and Novick, 2005; Liu *et al.*, 2005, 2008;; DeMars *et al.*, 2020; Wang *et al.*, 2020). Recently it has also been found that statins may have a positive role in treating the symptoms of Coronavirus COVID-19 which is caused by SARS Cov-2 virus. COVID-19 supposedly enters the pulmonary cells through the action of the angiotensin converting enzyme 2 ACE2 and a common determinant CD147. The end result being an acute life threatening distress syndrome ARDS (Hirano and Murakami, 2020; Ragia and Manolopoulos, 2020).

Statins have been found to have pleiotropic effects on inflammation and antioxidative stress, which modulate immune response at different levels including immune cell adhesion, migration, antigen presenting, cytokine production and increase antioxidation (Castiglione *et al.*, 2020; Sarkeshikian *et al.*, 2020; Zhang *et al.*, 2020). In this case, Atorvastatin could have changed the lipid/protein proportion of the biological membrane which may have led to a change in the integrity and stoichemistry of ACE2 or CD receptors of SARS Cov-2 virus, therefore the virus may not be able to recognize its protein receptor. A similar scenario in our case would be explained in either of two hypotheses. Firstly, the changes that have been triggered by Atorvastatin in the biosynthesis of bacterial membrane lipids may have caused a change in the topography and stoichemistry of the membrane which may have interfered with the nascent signal protein synthesis on free ribosomes not being able to recognize their receptors. Secondly, a direct effect of Atorvastatin on the transcription of m RNA of extracellular proteins is predictable (Adhikari, R.P., Novick, R.P., 2005). Bearing these in mind, Atorvastatin may be a good tool for controlling bacterial pathogenicity.

Our investigation showed a profound effect of Atorvastatin at sub minimal inhibitory concentrations on the growth and secretion of alpha toxin, coagulase, and catalase by *S.aureus*.

Thus, we suggest that Atorvastatin could be thought of as an agent in the treatment of bacterial sepsis and infections.

This approach may provide the basis for protection at the level of the host in invasive infections by *S. aureus.* In recent years there has been increased use of statins to protect patients from arteriosclerosis.

5. Conclusion

The results obtained in our study lead us to conclude that statins, with their pleotropic and immunomodulatory characteristics and their significant effect on the secretion of virulence factors by MRSA, could be used in conjunction with other antibiotics in combating the severity of MRSA infections.

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Conflict of interest

This is an original work of the author and there is no conflict of interest to be mentioned.

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