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The Pharma Innovation

ISSN: 2277- 7695
TPI 2017; 6(2): 105-109

www.thepharmajournal.com
Received: 17-12-2016
Accepted: 18-01-2017

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Original Research Paper

Evaluation of immunomodulatory action of atorvastatin calcium in mice

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Abstract
Objectives
1. To evaluate immunomodulatory activity of atorvastatin calcium by using host resistance model against *E. coli* induced sepsis in mice
2. To elucidate probable mechanism of action of immunomodulatory activity of atorvastatin calcium & septilin syrup.

Methods: Immunomodulatory activity of atorvastatin calcium was evaluated by using host resistance model against *E.coli* induced sepsis. The mechanism of action was studied in survived mice from sepsis, by studying histopathology of spleen, thymus along with evaluation of bone marrow.
Results: The atorvastatin calcium have increased the survival in sepsis induced mice & also decreased apoptosis of T & B cells by stimulating cellularity in PALS, marginal zone & follicles of spleen as well as cortex, medulla of thymus. The test drugs also increased myeloid+lymphoid cells in bone marrow. All the results were statistically significant as compared with control & were comparable with septilin syrup group which was used as positive control.
Conclusion: Pretreatment of atorvastatin calcium as well as Septilin syrup before induction of sepsis have shown prominent increase in the survival rate due to their immunostimulant activity.

Keywords: Atorvastatin, septilin, sepsis, spleen, thymus, bone marrow

Introduction
Sepsis and septic shock are formidable medical problems which challenge physicians today [1]. Sepsis is a complex, pleiotropic inflammatory response [2]. It is also reported that sepsis causes multiple organ failure & extensive apoptosis of T cells in thymus, spleen and bone-marrow which result into immune-deficient state in patients [3]. Current sepsis treatment includes use of broad spectrum antibiotics along with short term corticosteroid therapy, which produces severe immunosuppression. To overcome immunosuppression, immunoglobulines, interleukin-7, granulocyte–macrophage colony stimulating factor, or interferon-γ [4] are used. Despite of this type of high treatment expenditures, septicemia and sepsis are often fatal & are leading cause of death. Patients who survive severe sepsis are more likely to have permanent organ damage, cognitive impairment, and physical disability [5].
Bhaisare *et al* studied several samples of septic neonates, they found Gram negative organisms are more common and are mainly Klebsiella, Escherichia coli, Pseudomonas, and Salmonella [6]. Therefore in this study Escherichia coli were used to induce sepsis in mice.
3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (HMGCoARI), are known to exert anti-inflammatory and immunomodulating properties in addition to their lowering effect on cholesterol levels. There has been speculation that the administration of statins may alter the inflammatory response to infection, suggesting that they may represent a potentially important adjunct to therapy. Data suggests that critically ill patients may benefit from statins, and observational and retrospective studies have suggested that patients taking statins prior to the development of sepsis may have improved sepsis outcomes [1].
Septilin is a marketed immunostimulant preparation. Sharma S.B *et al* have recommended use of sept ilin for immunosuppressed high risk patients [7]. Many scientists, have studied immunomodulatory activity of septilin in animals [7-9]. Therefore in this study septilin syrup has been used as standard drug.
This study evaluated immunomodulatory activity of atorvaststin calcium in sepsis model & its mechanism of action. The parameters studied were % survival, evaluation of lymphoid+myeloid cells in bone marrow, histopathology of spleen, thymus to check immunostimulant activity of the study treatments.

Materials and Methods

Experimental protocol was approved by Institutional Animal Ethical Committee (IAEC).

Swiss Albino mice weighing 20-25 g housed in polypropylene cages were used. They were fed pellet diet and water *ad-libitum*. They were maintained under standard conditions of temperature (25 ºC ±5ºC) and relative humidity (55±10%) along with 12 hours night & day cycle. Animals of either sex were used.

Study Treatments

1. Atorvastatin calcium

Atorvastatin calcium was received from Emcure Pharmaceuticals Pvt. Ltd., Bhosari, Pune as a gift sample.

2. Septilin syrup

Manufactured by The Himalaya Drug Company was purchased from market.

Experimental Design

Animals were divided into three groups, with thirty mice in each group.

Group I: Vehicle for Control (0.5 % Sodium carboxy methyl cellulose in distilled water) [10]

Group II: Septilin Syrup (Dose 2 ml/ kg) (Positive control).

Group III: Atorvastatin calcium (Dose 10 mg/ kg).

All the three groups received the respective study treatments daily for 28 days by oral route. On 29th day, abdominal sepsis was induced in the test mice by challenging them, intraperitoneally, with 3 x 10^8 CFU /ml of *E. coli* suspension. The test mice were observed for 7 days. The alive & dead mice were noted down, to calculate the % survival from sepsis. On the 8th day of survival, the protected mice were sacrificed. Spleen & thymus were removed and fixed in 10 % formalin. After processing, five micrometer sections were stained with H&E and analyzed by a pathologist who was blinded for groups. Cellular density was studied in compartments of spleen like : - a)periarterial lymphoidal sheath (PALS) b)marginal zone (MZ) and c)follicles. Cellular density was studied in compartments of thymus like : - a) cortex and b) medulla. Each parameter was graded on a scale from 0 to 4, as follows: 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. The total immunostimulant score was expressed as the sum of the scores for all parameters; the maximum values were 16 [11].

To evaluate the myeloid+lymphoid cells, slides were prepared from bone marrow of femur of the same mice. The slides were dried, fixed with methanol, stained with May Grunwald- Giemsa stain & the myeloid+lymphoid (granulocytic) cells were calculated [12].

Statistical analysis

Data were analysed using the statistical software program Prism (GraphPad). The host resistance against E. coli induced abdominal sepsis results were analysed using the Chi-square test. The effect of test drugs on spleen & thymus results were analysed using kruskal wallis test followed by Dunns test for multiple comparison. The effect of test drugs on myeloid cells were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test. For all tests $p<0.05$ was considered as statistically significant.

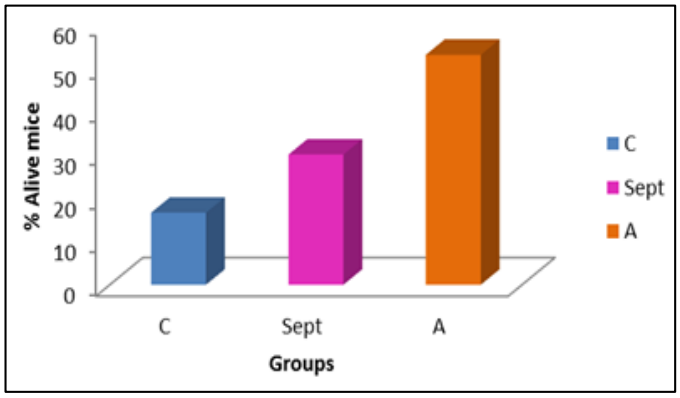
Results

Table I: Effect of test drugs on *E. coli* induced sepsis in mice (n = 30 per group) Results expressed as % survival.

Treatment	Dead	Alive	% survival
Vehicle treated	25	05	16.60
Septilin syrup	20	10	33.00***
Atorvastatin calcium	14	16	53.00***

***= comparison with vehicle treated group ($P<0.001$).

Statistically significant increase in survival was seen with test drug ie atorvastatin calcium treated group when compared to control group & the increase was comparable with the septilin syrup treated group used as a positive control. Table I, Fig. 1.



***= comparison with vehicle treated group ($P<0.001$)

Fig 1: % survival from sepsis

Periarterial lymphoidal sheath (PALS) was screened for number of lymphocytes.

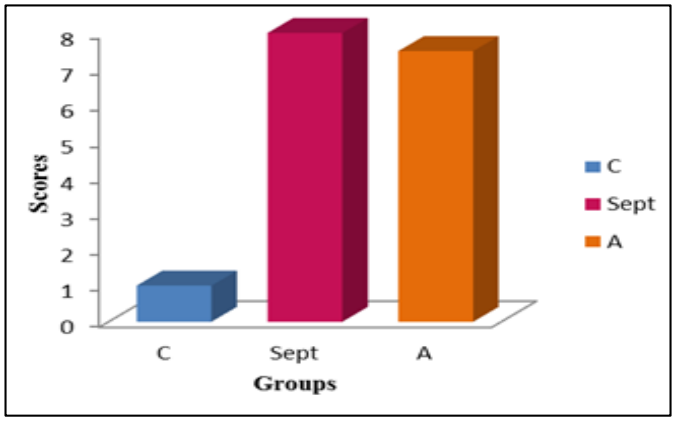
(n= 16 per group) Results expressed in median.

Table II: Effect of test drugs on Periarterial lymphoidal sheath of spleen

Groups	n	Number of lymphocytes
Vehicle treated	5	1.00
Septilin syrup	10	8.00 **
Atorvastatin calcium	16	7.50**

** = Comparison with vehicle treated group ($P< 0.01$)

There was significant increase in number of lymphocytes of periarterial lymphoidal sheath of spleen with atorvastatin calcium as compared to control & the increase was comparable with the septilin syrup treated group used as a positive control. Table II, Fig. 2.



**= comparison with vehicle treated group ($P< 0.01$).

Fig 2: Effect of drugs on periarterial lymphoidal sheath of spleen

Marginal zone was screened for number of lymphocytes.

Results are expressed in median.

Table III: Effect of study treatments on marginal zone of spleen

Groups	n	Number of lymphocytes
Vehicle treated	5	1.00
Septilin syrup	10	4.50 ***
Atorvastatin calcium	16	4.50***

*** = Comparison with vehicle treated group (P< 0.001)

There was highly significant increase in the number of lymphocytes of marginal zone of spleen with atorvastatin calcium treated group as compared to control. However there was no significant difference between positive control i. e. septilin syrup treated group, they were comparable. Table III, Fig. 3.

A follicle was screened for number of lymphocytes and germinal centres.

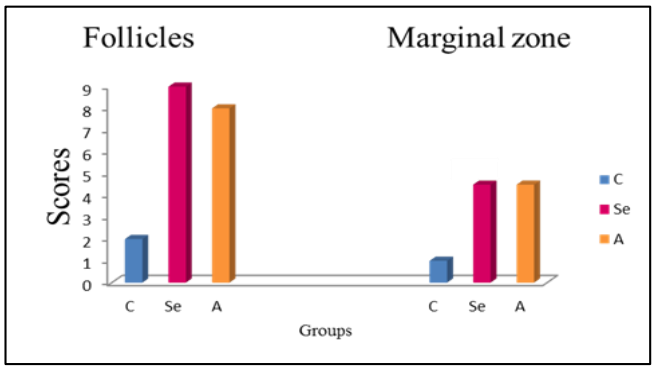
Results are expressed in median.

Table IV: Effect of study treatments on follicles of spleen

Groups	N	Number of lymphocytes & germinal centres
Vehicle treated	5	2.00
Septilin syrup	10	9.00 ***
Atorvastatin calcium	16	8.00***

*** = Comparison with vehicle treated group (P< 0.001)

Score of follicles of spleen in atorvastatin calcium treated mice was significantly higher as compared to vehicle treated group. However there was no significant difference between positive control i. e. septilin syrup treated group, they were comparable. Table IV, Fig. 3.



*** = Comparison with vehicle treated group (P< 0.001)

Fig 3: Effect of study treatments on Follicles & marginal zone of spleen

Thymus cortex was screened for number of lymphocytes.

Results are expressed in median.

Table V: Effect of study treatments on cortex of thymus

Groups	n	Number of lymphocytes
Vehicle treated	5	1.0
Septilin syrup	10	4.0**
Atorvastatin calcium	16	4.0 ***

** = Comparison with vehicle treated group (P< 0.01)
*** = Comparison with vehicle treated group (P< 0.001)

Atorvastatin calcium treated group has shown significantly higher score in cortex of thymus in protected mice when compared to vehicle treated group. The results were comparable with septilin syrup treated group which was used as positive control. Table V, Fig.4.

Thymus medulla was screened for number of lymphocytes.

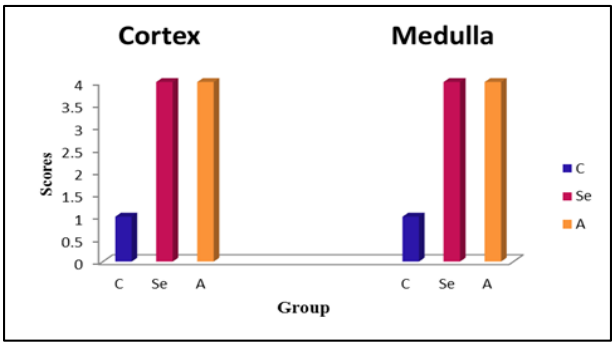
Results are expressed in median.

Table VI: Effect of study treatments on medulla of thymus

Groups	n	Number of lymphocytes
Vehicle treated	5	1.00
Septilin syrup	10	4.0***
Atorvastatin calcium	16	4.0 ***

*** = Comparison with vehicle treated group (P< 0.001)

There was highly significant increase in number of lymphocytes in medulla of thymus with atorvastatin calcium treated group as compared to control. The results were comparable with septilin syrup treated group which was used as positive control. Table VI, Fig. 4.



** = Comparison with vehicle treated group (P< 0.01)
*** = Comparison with vehicle treated group (P< 0.001)

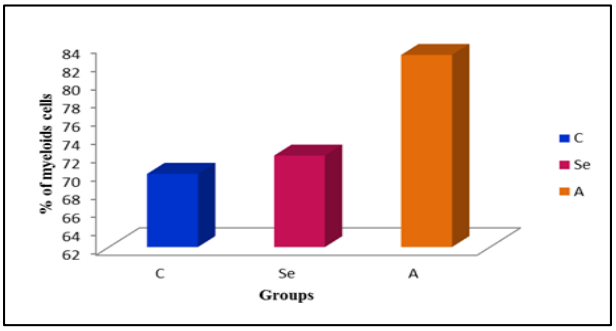
Fig 4: Effect of study treatments on cortex & medulla of thymus

Table VII: Effect of study treatments on myeloid & lymphoid cells
Results expressed in mean ± SD.

Groups	n	% leucocytes
Vehicle treated	5	70±4.6
Septilin syrup	10	72±5.8
Atorvastatin calcium	16	83±5.9 ** $$$

** = Comparison with vehicle treated group (P< 0.01)
$$$ = Comparison with septilin syrup treated group (P<0.001)

There was statistically significant increase in myeloid & lymphoid cells in atorvastatin calcium treated group as compared to control group as well as septilin syrup treated group which was used as positive control. Table VII Fig. 5.



** = Comparison with vehicle treated group (P< 0.01)
$$$ = Comparison with septilin syrup treated group (P<0.001)

Fig 5: Effect of study treatments on myeloid & lymphoid cells

Discussion

Sepsis is the leading cause of death in critically ill patients in the developed world. The overall mortality rate in patients of sepsis is 25–30% and mortality in patients with abdominal sepsis can be as high as 60% [13].

The most important finding of our study is that atorvastatin calcium pretreatment for 28 days daily prior to sepsis has improved survival in mice. The results were comparable with septilin syrup, ie. positive control.

In addition to this, histopathology of spleen, thymus & evaluation of bone marrow cells were carried out in protected mice from sepsis to elucidate probable mechanism of action of atorvastatin calcium along with septilin syrup. It may be noted that 'n' differ in different groups due to different % of survival in these groups.

Maronpot R *et. al.* [14] have stated that bone marrow is critical primary lymphoid tissue & main site of granulopoiesis & lymphopoiesis. Myeloid+ lymphoid (M+L) cells can be evaluated for assessing the cellularity of bone marrow.

Significant increase in myeloid & lymphoid cells of bone marrow in atorvastatin calcium group & septilin syrup treated group, indicates stimulation of production of leucocytes in bone marrow. Therefore we suggest that this indicates enhanced specific as well as nonspecific immunity with atorvastatin calcium & septilin syrup.

Cesta M.F.*et al* [15] reported that the spleen is the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens and thus can respond to systemic infections[16-17]. In rodents, it is a site of hematopoiesis.

Elmore S. *et. al.* [18] reported that PALS of spleen represents mainly T lymphocyte area. B lymphocytes & few macrophages are present in marginal zone as well as in follicles of spleen. Thus our results shows statistically significant stimulation in T & B cell areas of spleen. This indicates an enhancement of cell as well as humoral mediated immunity in atorvastatin calcium as well as septilin treated group.

Peters R *et. al.* [19] reported that thymus is an important organ of immune system & plays important role in maturation of lymphocytes. T cell progenitors are produced in the bone marrow and, enter the thymus, differentiate, undergo selection, and eventually mature into functional T cells [20-21]. They provide cellular immunity against intracellular microorganisms e.g. bacteria, viruses.

Gail Pearse *et. al.* [22] reported that the cortex of thymus represents mainly premature T lymphocytes & medulla of thymus mainly contains mature T lymphocytes & few B lymphocytes.

Thus our results on thymus histopathology, indicates that atorvastatin calcium & septilin syrup treated groups shows prominent immunostimulant activity which is the result of stimulation of cell mediated immunity.

We suggest that, stimulation of immune system in mice has helped them to overcome the immunosuppression caused due to sepsis which result in the increased survival rate in sepsis induced mice [8].

Conclusion

Pretreatment of atorvastatin calcium as well as Septilin syrup before induction of sepsis have shown prominent increase in the survival rate due to their immunostimulant activity. Therefore therapy with these test drugs may represent a novel approach in the treatment of this highly lethal disorder, as it improves survival by prevention from apoptosis of lymphocytes in sepsis. These drugs can also be evaluated for other immunocompromised conditions like AIDS, cancer chemotherapy. They can also be used prophylactically to prevent the occurrence of the infections as all the test drugs have stimulated immune system under normal conditions.

Acknowledgement

The authors thank Emcure Pharmaceutical Pvt. Ltd., Pimpri, Pune for providing the gift sample of atorvastatin calcium. Microbiology & Pathology Departments, Padmashree Dr.D.Y.Patil Medical College, Dr. D.Y. Patil University, Pimpri, Pune-18, for providing the facility. We also thank Dr. Wangikar Prado – Preclinical Research and Development Organisation, Punawale road, Tathawade, Pune-33, for his valuable inputs.

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