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Evaluation of Immunoprotective Activity of Septilin against Cyclosporine A induced Immune Suppression

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ABSTRACT

Immunoprotective activity of Septilin evaluated against Cyclosporine A induced immunosuppression in wistar albino rats. An equivalent of 100mg of Septilin tablet extract administered as an oral dose and its Immunoprotective activity assessed by determining of host resistance against *E.coli*, determining hematological parameters (Total Leukocyte Count), determining *in vivo* phagocytic test by carbon clearance, determining humoral response (by measuring antibody titre) and cell mediated immunity (by measuring foot pad thickness). The present study suggests that Septilin significantly protected the animals by increasing the immunoprotective activity in immune compromised conditions. In Septilin treated, immunosuppressed rats, there was 33.3% mortality against 100% mortality in immunosuppressed rats after 48hrs of infection. 16.6% mortality was observed in Septilin treated rats against 66.6% in immunosuppressed rats after 24hrs of infection. Furthermore, Septilin treatment raised the TLC significantly ($P<0.01$) when compared with control immunosuppressed rats. It was also found that Septilin treatment potentiated the phagocytic activity in immunosuppressed animals. The antibody titre determination study suggested that Septilin enhances both primary and secondary responses in rats immunized with BSA. In normal rats, Septilin increased the primary antibody titre values by 2.4 times and the secondary antibody titre values by 1.56 times. In immune suppressed rats, Septilin increased the primary antibody titre values by 2.5 times and secondary antibody titre values by 1.81 times. Septilin also showed the protective activity against impaired DTH conditions.

Keywords: Herbal drugs, Percent mortality, Total Leukocyte Count, Carbon clearance test, Antibody titre value, Delayed type hypersensitvity.

1. INTRODUCTION

Clinically, immunosuppressants are used in various pathophysiological conditionslike organ transplantation, to treat allograft rejection and autoimmune diseases in whichthey non-specifically suppress the immune system. Even though various regimens are used, this therapy has major drawbacks of increased risk of bacterial, fungal, viral (speciallyCMV) as well as opportunistic infections, development of lymphomas and relatedmalignancies and myeloma suppression etc. [1,2] Recent therapeutic approaches are aimed to boost the host's defense mechanism to assist the eradication of opportunistic infection during the immunosuppression therapy and to overcome the other drawbacks. The basic areas of immuno-modulators are currently receiving an adequate attention. A number of plant products are being investigated for immune response modifying activity.[3] Immunostimulation in a drug induced immunosuppression model and immunosuppression in experimental hyper reactivity model by same preparations is said to be true immunomodulation.[5] Apart from being specifically stimulatory (or) suppressive, certain agents have been shown to possess activity to normalize (or) modulate patho-physiological process and hence called as immunomodulatory agents. [6.7]

The present study is one of such approach, which involves the evaluation of various immune responses of Septilin against cyclosporineA induced immune suppression. CyclosporineA is widely used in various clinical and immuno pathological conditions and is approved for use in organ transplantation to prevent graft rejection in kidney to prevent rejection following bone marrow transplantation and in the prophylaxis of host-versus-graft disease. It is also used in the treatment of psoriasis, atopic dermatitis, rheumatoid arthritis and nephrotic syndrome.[8]

Septilin is a poly herbal formulation marketed as both in syrup and tablet form. Each tablet contains powder of guggulu 324 mg and shankhbhasma 64 mg and extracts of *Maharasanadiqoath*, 130 mg *Guduchi* 98 mg, *Manjishtha* 64 mg, *Amalaki* 32 mg, Shigru 32 mg and Yashti-Madhu 12 mg. Septilin is widely used as a daily health supplement in India.

In this study various parameters will be studied to determine the efficacy of Septilin against CyclosporineA induced immune suppression.

i. Determination of host resistance against *E. coli*

ii. Determination of hematological parameters (TLC & DLC)

iii. Determination of *in vivo* phagocytic test by carbon clearance

iv. Humoral response (by measuring antibody titre)

v. Cell mediated immunity (by measuring foot pad thickness)

2. MATERIALS AND METHODS

2.1 Drug and chemicals used

Septilin tablets (Batch No.30812 RL, Himalaya Drugs Ltd., Bangalore, INDIA) were purchased from market. CyclosporineA was supplied by Panacea Biotec Ltd., INDIA as gift sample. Eagles Minimum Essential Medium (CDH India.), Hank's Balanced Salt Solution (CDH India.),Rabbit anti rat IgG-HRP (GENEI India), TMB/H2O2 for ELISA (20 x Conc) (GENEI India),Bovine Serum Albumin(LOBA India.) of analytical grade were purchased from local commercial supplier (UV scientifics, Hyderabad, Telangana state, INDIA.). *E. coli* for Infection (MTCC No. 47) was obtained from Institute of Microbial Technology, Chandigarh, India.

2.2 Animals

The study was carried out on wistar albino rats (120-150 gms.) obtained from NIN, Hyderabad. They were housed in departmental animal house, in polypropylene cages with light and dark cycles and temperature (22±2ºC, 60±2%RH) controlled conditions, the feed (Pellet diet, Gold Mohar brand, Lipton India) and water available *ad libitum.* The protocol of the study was approved by the Institutional Animal ethics committee and the care of animals was as per the 'Guidelines for the care and use ofanimals in scientific research' prepared by Indian National Science Academy, New Delhi.

Animals were acclimatized for one week. After one week, animals were divided into four groups. A. Control group, B. Control immunosuppressed group, C. Treated group and D. Treated immunosuppressed group. Animals were immunosuppressed by three days oral administration of CyclosporineA (20mg/kg).

2.3 Drug Extract

6 gms of Septilin tablet powder was boiled in 600 ml of purified distilled water for 30 min. It was centrifuged and supernatant was evaporated to dryness on a water bath and the residue was resuspended in 45 ml of purified distilled water. 0.75 ml of this extract was used for oral administration and it contained the extract from 100 mg of Septilin. [9]

2.4 E. coli induced abdominal sepsis

The pathogenic strain of *E. coli* was obtained from IMTECH-Chandigarh INDIA and it was sub-cultured in nutrient broth for 48 hrs and the transmittancy of the culture was determined at 540 nm.Based on the transmittancy, the dose of viable cells to cause the death in animals was found to be 1ml, which is equivalent to 5 x 10^8 cells.[10] Control and treated groups were fed with tap water and 0.75ml of Septilin extract respectively for 14 days. B and D groups were administered with CyclosporineA from 12thday to 14thday. On day 14, 3Hrs after the last dose of Septilin extract, blood samples for TLC were collected and 1ml of *E. coli* (5 x 10^8 cells) was injected intraperitonially for all the groups and percent mortality in all the groups was observed up to 48 hrs. During the experiment, the blood samples were collected on 0, 7 and 14day for TLC count. The survivors, if any, were observed for further 3days. Throughout the study, the intensity of abdominal sepsis caused by viable *E. coli* in dead animals was determined by culturing on Mac Conkey Agar medium and identified by gram staining, motility and biochemical tests. [11,12]

2.5 Carbon Clearance Test for Phagocytic Response

The non-specific immunity by the change in macrophage phagocytic activity through Reticulo-Endothelial System (RES) was determined by carbon clearance test.[13] Control and treated groups were fed with tap water and 0.75 ml of Septilin extract respectively for 7days. B and D groups were administered with CyclosporineA from 5th day to 7th day. On day 7, 3 Hrs after the last dose, all groups were administered with 0.2ml of Indian ink

intravenously through tail vein individually. Blood samples were collected from retro orbital plexus immediately before and up to 30 min with 5 min interval of injection of Indian ink. An aliquot of each and 25±l of blood sample was lysed with 2 ml 0.1% v/v acetic acid and absorbance was observed at 675 nm. The graph of absorbance against time was plotted. [14,15]

2.6 Humoral response by measuring antibody titer

In experimental animals, the effect of immunomodulators on antigen specific humoral response can be measured by measuring the specific antibody titre value by ELISA method in which the absorbance is directly proportional to the specific immunoglobulin (IgG) concentration. [16]

2.7 Immunization protocol

On day 0, all the animals were immunized by intra peritoneal administration of 0.2 ml of 1% w/v BSA in PBS. Before immunization, blood samples from all animals were withdrawn and the IgG levels were determined and considered as negative control. After immunization, Control and treated groups were fed with tap water and 0.75 ml of Septilin extract respectively for 7days. B and D groups were administered with CyclosporineA. from 5^{th} day to 7^{th} day. On day 7, 3 hrs after the last dose, blood samples were collected and subjected for ELISA to determine IgG and recorded as primary antibody levels. C and D group animals were administered with 0.75ml of Septilin extract for 14days. B and D groups were administered with CyclosporineA from 12^{th}day to 14^{th}day. On 14^{th} day, 3 hrs after the last dose, animals were challenged with 0.2 ml of 1 % w/v BSA and on 21st day, blood samples were collected and subjected for ELISA to determine IgG levels and recorded as secondary antibody levels.

2.8 Measurement of IgG by ELISA

Specific anti IgG antibody levels in the serum were determined by ELISA using slightly modified method of Michalek.[17] The wells of ELISA Plates (TARSONS,INDIA) were coated with 100µl of 1%w/v BSA in PBS and incubated at 37ºC for 1hr.The unbounded BSA was removed by washing three times with PBS–T solution. 25µl of 1000folds diluted serum samples were added accordingly in corresponding wells and incubated for 1hr. The unbounded serum antibodies were removed by washing three times with PBS–T solution. 50µl of Rabbit Anti rat IgG-HRP was added to all wells and incubated for 1hr. All wells were washed thrice to remove the unbounded materials and 50 µl of substrate (TMB $H_2O_2$for ELISA) was added and incubated for 5min. The Enzyme –Substrate reaction was stopped by adding 50 µl of 5N H_2SO_4 and absorbance was read at 450 nm. The absorbance is directly proportional to IgG levels present in serum.

2.9 Cell mediated immunity-by measuring foot pad thickness

In experimental animals, the effect of immunomodulators on antigen specific cellular immunity can be measured by calculating the degree of delayed type hypersensitivity response by using foot pad swelling. The percentage increase in foot pad thickness is taken as measurement for the DTH responses. Animals were divided into four groups viz. A. Control group, B. Control with CyclosporineA, C. Treated and D. Treated with CyclosporineA group. SRBC were collected in Alsever's solution and washed thrice with large volumes of normal saline and the cells were adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenging. On day 0, all groups were immunized by subcutaneous administration of 1 ml of SRBC. [18] Cell suspension which is equivalent to 0.5×10^9 cells into right hind paw and all groups were fed with tap water and 0.75 ml of Septilin extract respectively for 14 days. B and D groups were administered with CyclosporineA from 12^{th}day to 14^{th}day. On day 15, all groups were challenged by injecting 0.5 ml of cell suspension into left hind paw via subcutaneous route and the footpad thickness was measured immediately, after 24 hrs and after 48 hrs by Plethysmometer. Paw volume before challenging was taken as blank and percentage increase in paw volume was determined.

2.10 Statistical Analysis

All results were expressed as mean ±SEM. The data generated were analysed statistically using Instat 2 (Graph Pad Software Inc., USA). Statistical significance was calculated by applying one way ANOVA followed by post hoc (Tukey's Method) test. P values less than 0.05 were considered as significant.

3. RESULTS AND DISCUSSION

3.1 Survival Study

Measuring the mortality rate in *E. coli* induced abdominal sepsis can assess the positive immune-prophylactic efficacy of an immunomodulatory formulation. Intra-abdominal sepsis continues to be a major cause of morbidity and mortality following trauma and abdominal surgery for bowel perforation. Treatment of this condition has always focused on appropriate surgery, antibiotics and nutritional support. But in spite of this, fatal complications have been reported. A factor, which influences the recovery from such an infective process, is the host defense mechanism. In normal rats, Septilin protected rats against *E. coli* induced abdominal sepsis. In Septilin treated rats there was 16.6% mortality with a significant reduction against 66.6% in control rats after 48hrs of infection. No mortality was observed in Septilin treated rats, where as 50% was observed in control group after 24 hrs of infection.

In immunosuppressed rats, Septilin significantly protected the animals. In Septilin treated,immunosuppressed rats, there was 33.3% mortality against 100% mortality inimmunosuppressedrats after 48hrs of infection. 16.6% mortality was observed in Septilin treated rats against 66.6%in immunosuppressed rats after 24hrs of infection (Fig. 1).

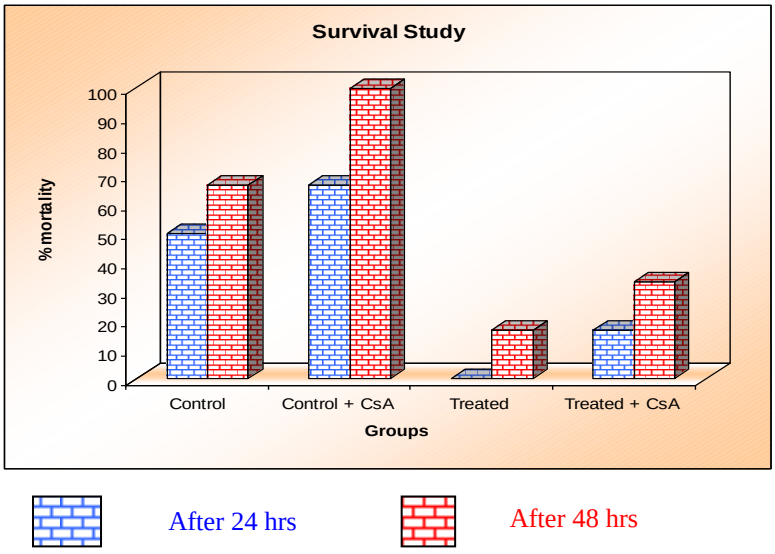

Fig.1. Immunoprotective activity of Septilin against CyclosporineA induced immunosuppression on percentage mortality in *E.coli* induced abdominal sepsis (n=6)

3.2 Haematological Parameters

Immunomodulatory action is mainly concerned with the cellular involvement of haemopoetic and lymphoitic tissue. The two major groups of WBCS are granular leukocytesand agranular leukocytes. Neutrophils and macrophages are active in phagocytosis, they caningest bacteria and destruct bacteria with lysozyme defensins and strong oxidants such assuperoxide anion, Hydrogen peroxide and hypochlorite anion and dispose the matter.The two kinds of agranulocytes are lymphocytes and monocytes. Lymphocytesdevelop from lymphoblasts and monocytes develop from monoblasts. Lymphocytes mainlymediate the immune responses including Ag-Ab reactions. B lymphocytes develop intoplasma cells which secrete antibodies. T-lymphocytes attack invading viruses, cancer cellsand transplanted tissues. Natural killer cells attack a wide variety of infection and certainspontaneously arising tumor cells. Monocytes mainly involve in phagocytosis aftertransforming into fixed/wandering macrophages.[19]

In normal rats, Septilin (100mg/day/rat, 14 days) treatment increased TLC significantly (P<0.001) as compared with control rats. Septilin treatment also raised the lymphocyte count and neutrophil count significantly (P<0.001) which is further confirmed by increased phagocytosis and survivalence against inert particles and bacterial infections respectively.

Similarly, in immunosuppressed rats with CyclosporineA (20mg/kg/day, oral for 3days) Septilin raised the TLC significantly (P<0.01) when compared with control immunosuppressed rats in which CyclosporineA may inhibit the T-lymphocyte count which results in decrease in TLC. Septilin also raised significantly (P<0.05) the lymphocyte and neutrophil counts (P<0.05) in immunosuppressed rats as compared with control immunosuppressed rats, which is further confirmed by raised antibody titre values and survivalence (Fig. 2).

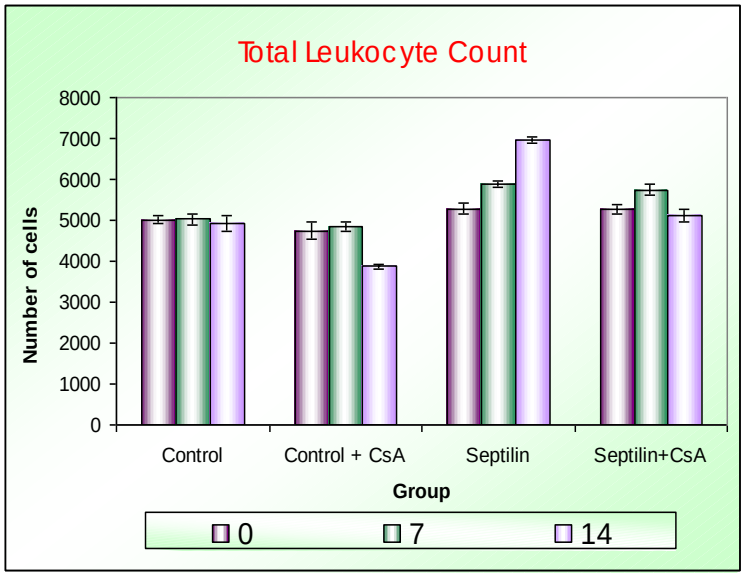

Fig. 2. Effect of Septilin on total leukocyte count in normal and CyclosporineA induced immunosuppressed rats (n=6)

3.3 Carbon Clearance Test

In experimental animals Carbon Clearance Test can determine the influence of immunomodulators on change in macrophage phagocytic activity through Reticulo-Endothelial System. The absorbance of lysed blood samples at 675 nm was proportional to the amount of residual carbon particles (Indian ink) in the blood. In control rats and CyclosporineA treated rats the absorbance of lysed blood samples after 30minutes of intravenous injection of Indian ink at 675 nm was found to be 0.101±0.002and 0.118±0.009 respectively which indicates the amount of residual foreign particles in CyclosporineA treated rats is insignificant (P>0.05) in comparison with that of control rats. In Septilin treated animals, the absorbance of lysed blood samples after 30minutes of i.v. injection of Indian ink at 675 nm was found to be 0.048±0.004

which is very significantly (P<0.001) less in comparison with that of control rats. It clearly indicates that 7 days treatment of Septilin potentiates the elimination of foreign particles from its surrounding by enhancing the phagocytic activity. In animals treated with Septilin for 7 days and CyclosporineA on 5th, 6th and 7th day, the absorbance of lysed blood samples after 30minutes of i.v. injection of Indian ink at 675 nm was found to be 0.053±0.001 which is significantly (P<0.001) less from that of animals treated with CyclospoirneA only (Fig. 3). It clearly indicates that the rate of elimination of carbon particles is more in Septilin treated group when compared with immune suppressed animals. This study demonstrates that Septilin potentiates the phagocytic activity in immune suppressed animals also.

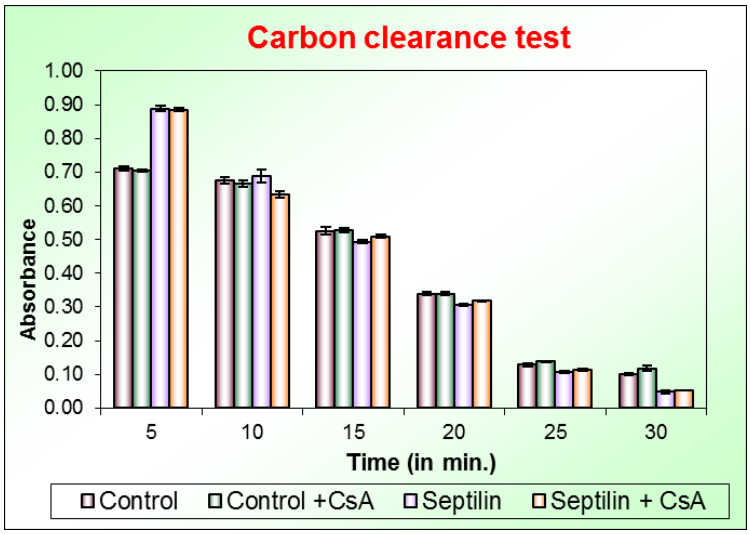

Fig. 3. Effect of Septilin on Carbon clearance rate in normal andCyclosporineA induced immunosuppressed rats (n=6)

3.4 Antibody Titre Values

The influence of immunomodulators on Humoral immune response in experimental animals can be determined by measuring specific antigen (BSA) anti IgG levels by ELISA methods. The absorbance will give the measurement of anti IgG serum levels against BSA. In control rats, the absorbance due to 1000 folds dilution samples was found to be0.341±0.010. The negative controls were found to be 0.046±0.004. In rats treated with CyclosporineA before primary immunization, the absorbance was found to be reduced significantly (p<0.001) to 0.181±0.003, which indicates the CyclosporineA suppressed the humoral immune response against primary exposure of antigen. Absorbance due to primary IgG in Septilin treated animals (0.75/ml/day/rat, oral) for one week was found to be 0.819±0.010 which is very significant when compared with control 0.341±0.010. Absorbance due to primary IgG in immunosuppressed Septilin treated animals (0.75/ml/day/rat, oral) for one week was found to be 0.453±0.003 which is very significant when compared with control immunosuppressed animals 0.181±0.003.

Absorbance due to secondary IgG in Septilin treated animals (0.75/ml/day/rat, oral) for 14days was found to be 2.351±0.06, which is very significant when compared with control 1.502±0.039. Absorbance due to secondary IgG in immunosuppressed Septilin treated animals (0.75/ml/day/rat, oral) for 14 days was found to be 1.493±0.040 which is significant when compared with control immunosuppressed animals 0.827±0.020 (Fig. 4).These studies indicated that CyclosporineA exhibited a significant suppression activity on Humoral immune response and Septilin potentiates the humoral immune response in immunosuppressed group also.

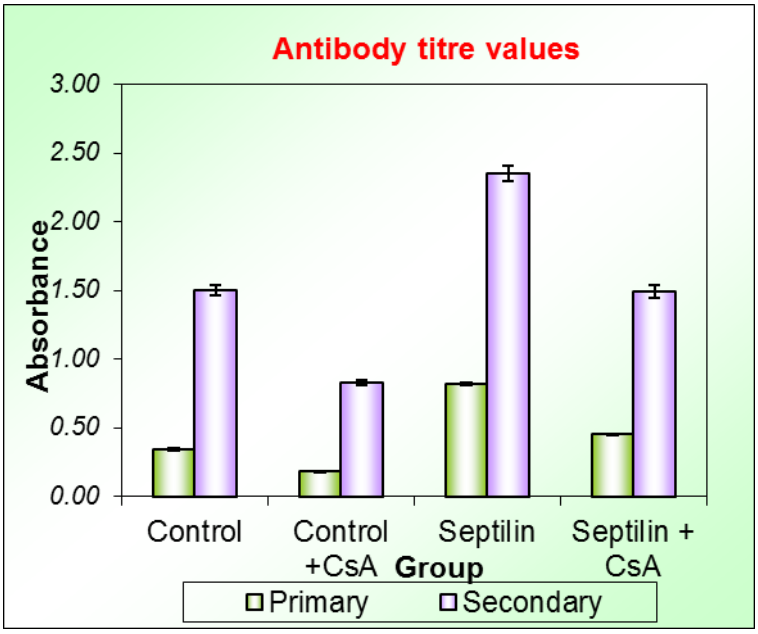

Fig 4: Effect of Septilin on primary and secondary antibody titrevalues in normal and CyclosporineA induced immunosuppressedrats (n n=6)

3.5 Delayed Type Hypersensitivity (DTH)

Measuring the delayed type hypersensitivity reaction can assess the influence of immunomodulators on T cell function. SRBCs were used for inducing the hypersensitivity reaction. The delayed type hypersensitivity was the first experimental evidence of transferable immunity carried only by immune cells. In control rats, the edema due to SRBC induced DTH was found to reach a peak value of 23.6±0.4 % at 24hr after challenge and it was reduced to 15.3±0.3 % after 48 hrs. The immediate hypersensitivity due to influx of neutrophils and macrophages was found to be

20.1±0.7 % in control rats. In immunosuppressed rats, the immediate hypersensitivity due to SRBC challenge was found to be 17.9±0.4%. The reduction as compared with control rats may be due to CyclosporineA induced damage of the short lived suppressor T-cells in immune regulatory system. The inflammation was found to reach its peak value of 26.2±0.4 % after 24 hrs challenge due to influx of neutrophils and macrophages, and it continued even after 48hrs22.7±0.5 % (P<0.001) which may be due to the immune suppressed activity of CyclosporineA. In Septilin treated groups, the immediate hyper sensitivity after challenge of SRBC was found to be 39.7±0.9 % (P<0.001) which may be due to the potentiating activity of Septilin on short lived T-cells and after 24hrs, the inflammation was reduced significantly to 25.0±0.6% (P<0.001) which may be due to the release of lymphokines and migration of macrophages from the site which clearly indicates that Septilin potentiated the DTH reaction which is further confirmed by a significant (P<0.001) reduction in paw volume 2.9±0.4% after 48 hrs challenge. In immunosuppressive group, Septilin showed the protective activity against impaired DTH conditions. The immediate hypersensitivity in this group was found to be 30.9±0.8% (P<0.001), which indicates that Septilin prevented the damage of short lived suppressor T-cell against CyclosporineA treatment. The inflammation was significantly reduced to 18.7±0.7 % after 24 hrs of challenge which is further reduced to 10.1 % (P<0.001) which clearly indicates the immunoprotective activity of Septilin against CyclosporineA induced cellmediated Immunosuppression (Fig. 5).

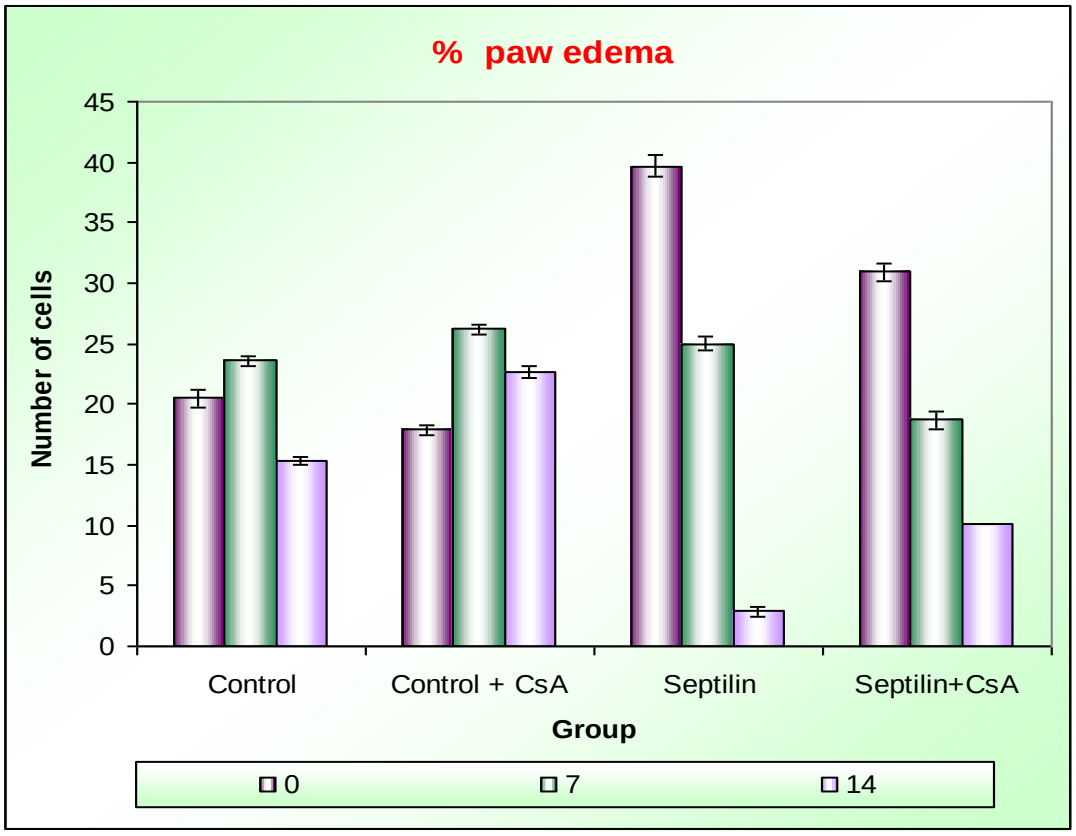

Fig. 5: Effect of Septilin on Percent paw edema in normal and CyclosporineAinduced immunosuppressed rats (n=6)

4. CONCLUSION

The survival study suggested that, Septilin reduces the risk of opportunistic infections during the immunosuppressive therapy, which is clearly indicated by significant reduction in the mortality rate. In CyclosporineA treated rats, Septilin significantly protected the animals against *E. coli* induced abdominal sepsis as compared to that of controls. The leucocytosis which probably occurs due to secretion of interleukin-1 and colony stimulating factors from activated macrophages along with increased number and functional capabilities of peritoneal macrophages [20] appears to be the underlying mechanism of protection offered by Septilin. [21,22] Septilin might have enhanced the capacity of monocyte-macrophage system, which is further proved by a significant rise in TLC ,Neutorphil count both in normal as well as immune suppressed rats.

Virtually every cell except the mature erythrocyte ingests particulate materials from its surrounding by receptor-mediated pinocytosis. Phagocytic ability is an important element of cellular immunity, and it differs from pinocytosis by bigger size of ingested particles and stronger dependence on the inhibitory effects of cytochalasins and low temperature. Phagocytosis provides the first line of defense of the host against infectious microorganisms. In the body of higher species, there are two mostly recognized 'professional' phagocytes: polymorphonuclear (PMN) leukocytes (neutrophils and eosinophils) and mononuclear phagocytes (monocytes and macrophages). Macrophages have a major role inimmunomodulation. The primary target of most of immunomodulators is believed to be macrophages which play a major role by engulfing pathogens (or) foreign particles and initiating innate immune response which in turn orchestrate the adaptive response[25,26]. The PMN cells emerge from the marrow as mature cells, which circulate in the blood for about 10hrs before migrating into the tissues where they perform their effector functions for 1 or 2 days. In contrast, mononuclear phagocytes emerge from them arrow as immature cells monocytes, circulate in the blood and then enter tissues.

The carbon clearance test, suggests that Septilin accelerates the elimination colloidal carbon particles from blood stream, which clearly confirms the enhanced phagocytic activity due to the oral administration of Septilin, which indicates the stimulation of Reticulo-Endolthilial System (RES).

Immunoglobulins are the antigen binding proteins that are present on B-cell membrane and they are also secreted by plasma cells and functions as antibodies. Secreted antibodies circulate inblood, they serve as the effectors of humoral immunity by searching out and neutralizing antigens (or) by making them for elimination. [24] The antibody titre determination study suggest that Septilin enhances both primary and secondary responses in rats immunized with BSA. In normal rats, Septilin increased the primary antibody titre values by 2.4 times and the secondary antibody titre values by 1.56 times. In immune suppressed rats, Septilin increased the primary antibody titre values by 2.5 times and secondary antibody titre values by 1.81times. This study concludes that Septilin enhances the IgG production and counteracts the IgG suppression when administered orally along with immunosuppressive drug (CyclosporineA) i.e. it counteracts the CyclosporineA induced humoral immune-suppression. The influence of immunomodulators on T cell function can be assessed by measuring the delayed type hypersensitivity reaction. SRBCs were used for inducing the hypersensitivity reaction. The delayed type hypersensitivity was the first experimental evidence of transferable immunity carried only by immune cells. The reaction was first discovered in 1882 by Robert Koch, but it was not until 1940's that Land Steiner and Chase proved that the reaction was mediated by the cellular and not the humoral arm of immune system.[23]. In the present study, animals treated with CyclospoirneA showed maximum potentiation of DTH as observed from percentage increase in foot pad thickness. This may be due to influx of neutrophils and macrophages at the site of antigen exposure. The elimination of lymphokines, neutrophils and macrophages from the site is reduced in animals treated with CyclosporineA, which is confirmed by the persistent increase in percentage paw volume after 48hrs. Septilin showed protective activity against DTH, which is confirmed by a significant reduction in inflammation after 48hrs.The present studies thus distinctly corroborated and clearly demonstrated that Septilin treatment:

(a) Prevented the mortality from *E. coli* induced abdominal sepsis.

(b) Augmented significantly TLC and number of neutrophils and lymphocytes in bothnormal and immune suppressed animals.

(c) Accelerated the elimination of colloidal carbon particles

(d) Potentiated the humoral immunity in rats and counteracts the CyclospoirneA inducedhumoral immune suppression in rats.

(e) Enhanced the cell mediated immunity

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