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Effect of *Ocimum sanctum* on the development of protective immunity against *Salmonella typhimurium* infection through cytokines

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ABSTRACT

Objective: To investigate the protective role of *Ocimum sanctum (O. sanctum)* leaves against *Salmonella typhimurium(S. typhimurium)* infection in rats by inducing TNF- α , IFN- γ & IL-2 cytokines. **Methods:** Wistar albino rats were fed with aqueous extract of *O. sanctum* leaves using 250 mg/kg body weight dose once a day for 20 consecutive days. Control rats were fed with placebo. Rats were infected with LD_{50} dose of *S. typhimurium* infection and monitored for their survival. Bacterial blood burden in both the groups was compared and numbers of activated peritoneal macrophages were counted. Concentration of TNF- α , IFN- γ and IL-2 cytokines in serum during different time intervals was assayed by sandwich ELISA. **Results:** Rats of control group showed a high mortality rate and had higher bacterial blood burden when compared with *O. sanctum* extract fed rats. There was a significant increase in the number of *S. typhimurium* engulfed peritoneal macrophages in the peritoneal fluid of *O. sanctum* fed animals. The protective control against bacterial infection in *O. sanctum* fed rats was associated with elevated level of TNF- α , IFN- γ and IL-2 cytokines in serum. **Conclusions:** These findings suggest that orally administered *O. sanctum* leaves extract effectively enhanced activation in macrophage and lymphocytes, depicted by the elevated serum concentration of TNF- α , IFN- γ and IL-2 cytokines, leading to induce a protective resistance against *Salmonella typhimurium* infection.

1. Introduction

Salmonella enteric serovar Typhimurium (*S. typhimurium*), a gram negative intracellular baterium, has been used extensively as a model for knowing the mechanism of Salmonella pathogenesis[1]. Due to emergence of drug resistant Salmonella bacteria[2,3] as well as inadequate understanding of host resistance to Salmonella which hampers the vaccine design[4], there is a need to evaluate the efficacy of the natural plant products which can be used against the disease.

Ocimum sanctum (O. sanctum) has been widely used for its diverse utility and multidirectional therapeutic uses since the Vedic time. Aqueous and ethanolic extracts of *O. sanctum* showed *in vitro* antimicrobial activity against different bacterial strains like *Staphylococcus aureus, Bacillus pumilus, Escherichia coli, Salmonella typhi murium, Pseudomonas aeruginosa* etc[5,6]. *O. sanctum* leaves extract also possesses potent immune modulating activity[7,8]. Recently, immune modulating effect of plant extracts has now been evaluated on the basis of induction of different cytokines[9]. TNF- α and IFN- γ play a crucial role against *Salmonella* infection[10]. TNF- α , IFN- γ and IL-2 are found essential for the suppression of bacterial growth during sub lethal *Salmonella* infection up to 10^8 CFU[11] and are specially required for macrophage activation[12]. Macrophages and natural killer (NK) cells, capable of producing a variety of cytokines, are important for host survival during the primary response to *Salmonella* infection[12-15]. Data from our previous ex vivo experiments[16-18] showed that aqueous extract of *O. sanctum* leaves up regulated the level TNF- α , IFN- γ and IL-2 cytokines and this led us to examine the efficient immune response in clearance of *Salmonella* infection. Thus, in this study the protective role of *O. sanctum* against *S. typhimurium* infection, by altering the level of TNF- α , IFN- γ and IL-2, has been investigated.

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2. Materials and methods

2.1. Chemicals and reagents

Cytokine kits of IFN-γ & IL-2 from R&D system, USA; ELISA kit for TNF-α (BD. Biosciences, USA), RPMI-1640 medium & fetal bovine serum (SIGMA chemicals, USA), nitroblue tetrazolium dye (NBT dye) (Loba chemicals, INDIA) & Luria-Bertani agar medium (Hi Media Labs, Bangalore)

2.2. Animals

Wistar albino rats of 60-80 g, purchased from IVRI, Izaitnagar, U.P. India, were used for all the experiments. Rats were maintained under controlled condition of light (14 h) and temperature [(24+ 2) ℃]. They were provided a standard diet as prescribed by Indian Council Medical Research (ICMR), water *ad libetum* and acclimatized to their environment at least one week before the start of experiment. The experiments were approved by Institute's Animal Ethics Committee constituted as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the purpose of control and supervision of experiments on animals.

2.3. Plant material

Leaves of *O. sanctum* were collected from garden of College of Veterinary Sciences, DUVASU, Mathura and authenticated from National Botanical Research Institute; Lucknow. Voucher specimen was preserved.

2.4. Preparation of herbal drug extract

Aqueous leaves extract was prepared by dipping the 100 g dry powder with 500 mL of triple distilled water at room temperature for 3 days with few drop of chloroform to avoid the fungal growth. The suspension was filtered through muslin cloth and then Whatman No. 1 filter paper and finally dried in lyophilizer under vacuum. The yield of the extract was approximately 12%-15% in terms of dry powder.

2.5. Preparation of S. typhimurium suspension

Smooth colonies of *S. typhimurium*, maintained in the Department of Microbiology & Immunology, grown on Luria-Bertani agar medium were selected and inoculated in Luria broth. Overnight incubated broth was centrifuged at 3 000 rpm and cell pellet was resuspended in PBS. Bacterial cell concentration was determined by matching with Mc Ferland's nephelometer and viable count method using pour plate of LB agar.

2.6. Determination of 50% lethal dose of S. typhimurium infection in rats

The LD_{50} of *S. typhimurium* was determined by the method of Reid and Munch[19]. Four groups having 10 rats in each group were made and 1 mL of suspension of 4 different concentrations i.e.2.10×10^9, 1.05×10^9, 2.10×10^8 and 1.05×10^8 cells were administered intraperitoneally in rats of respective group. Following infection, mortality was scored over a 20 days period. The dose causing 50% mortality was recorded and considered as LD_{50}.

All the rats in group I challenged with 2.10×10^9 CFU of *S. typhimurium* were died within 4 days where as in group II i.e.1.05×10^9 of infection caused 50% animals mortality on day 10^{th}. In group III, having 2.10×10^8 CFU, only 20% death was recorded while at the 1.05×10^8 CFU of *S. typhimurium* all the rats survived. Thus 1.05×10^9 CFU of *S. typhimurium* was calculated as LD_{50} dose.

2.7. Effect of O. sanctum on S. typhimurium infection

Ten animals were divided equally into two groups. Group A (control) and group B (experimental group) which was prefed with *O.* sanctum extract at 250 mg/kg body weight for 20 days. On 21st day both groups were challenged with LD_{50} dose i.e. 1.05×10^9 CFU of *S. typhimurium* by intraperitoneal route.

2.7.1. Survival

Animals were observed for the development of clinical sign and mortality, post challenge up to 20 days.

2.7.2. Bacterial load in blood

Blood was collected at different intervals i.e. 24 h, 48 h and 72 h following *S. typhimurium* infection as designed above. A total of 0.5 mL from 10 fold serially diluted blood was plated on Luria Bertoni agar plate and incubated at 37 ℃ for 24 h to measure viable count.

2.7.3. Phagocytic activity of peritoneal macrophages (nitroblue tetrazolium test)

After 24 h of infection, nitroblue tetrazolium test (NBT) was performed as per method by Tanaka[20]. Peritoneal fluids were collected and glass adherent macrophages were separated. They were suspended in RPMI-1640 medium and were incubated with 0.1% NBT solution at 37 ℃ for 15 min. Thin smear was prepared, air dried and stained with Leishman stain. A total of 100 macrophages were examined for the presence of dark blue formazone in cells.

2.7.4. Induction of TNF-α, IFN-γ & IL-2 cytokines

Blood was also collected at different time intervals i.e. 2 h, 2 days, 7days & 14 days after *S. typhimurium* infection given to both groups. TNF-α, IFN-γ & IL-2 cytokines were measured and compared by sandwich ELISA according to the manufacturer's instructions.

2.8. Statistical analysis

Results were expressed as mean ± SEM and analyzed using Prism-5.0 version. The statistical difference was calculated using student's *t*-test. *P* value <0.05 was considered statistically significant.

3. Results

3.1. Effect of *O. sanctum* on *S. typhimurium* infection

3.1.1. Protection study

O. sanctum fed rats when infected with *S. typhimurium* showed longer survival rate than control rats. Half of rats in the control group died within 10 days of infection while 10% and 20% death could be recorded in *O. sanctum* fed rats after 10 days and 15 days of infection respectively (P value< 0.001). Thus pretreatment of drug conferred 60% & 80% protection against infection on day 10^{th} and day 15^{th} respectively.

3.1.2. Bacterial load

O. sanctum fed and control group rats were infected with LD_{50} dose of 2.10×10^{9} CFU of *S. typhimurium* and analyzed for bacterial load in blood on day 1, 2 and 3 post infection. *O. sanctum* fed animals showed 10 times reduction in bacterial load on day 1 i.e. 0.11×10^{10}vs 1.20×10^{10} CFU and approximately 30 times reduction on day 2 and day 3 as compared to control i.e 1.1×10^{10} vs 36.0×10^{10} and 0.2×10^{10}vs 28.0×10^{10}CFU, respectively. Thus the bacterial numbers were significantly lower in *O. sanctum* fed rats (P< 0.001).

3.1.3. Phagocytic activity of macrophages

Using NBT assay the number of *S. typhimurium* engulfed phagocytes were found significantly elevated in *O. sanctum* fed rats in response to control group on day 1 post infection(P<0.001) (Figure 1).

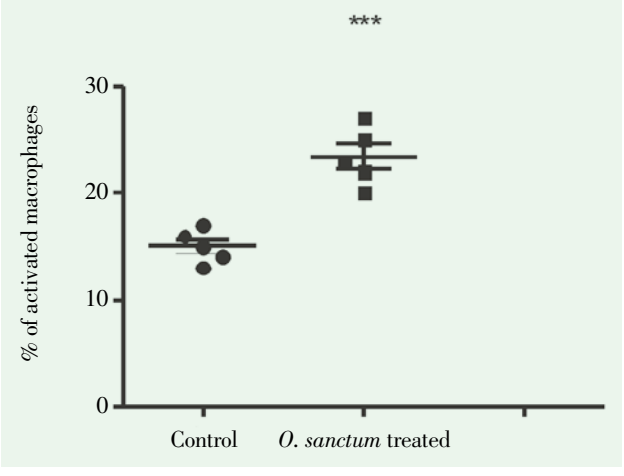

Figure 1. Effect of *O. sanctum* treatment on activation of macrophages isolated from peritoneal fluid of *S.*Typhimurium challenged animals.

3.1.4. TNF-α, IFN-γ and IL-2 concentrations in serum

Induction of TNF-α, IFN-γ & IL-2 was measured at 2 h, 2^{nd} day, 7^{th} day and 14^{th} day post *Salmonella* infection and compared with control group (Figure 2). TNF-α and IL-2 were found to be enhanced at 2 h post infection while IFN-γ was up regulated at later time point i.e. 48 h time interval. The up regulation of TNF-α, IFN-γ & IL-2 was significant in *O. sanctum* fed rats in comparison to control rats. Further it was demonstrated that TNF-α was down regulated and undetectable at 2nd day, 7th day and 14th day post infection while IL-2 concentration was maintained up to 7th day in the serum and become undetectable after 14th day post infection.

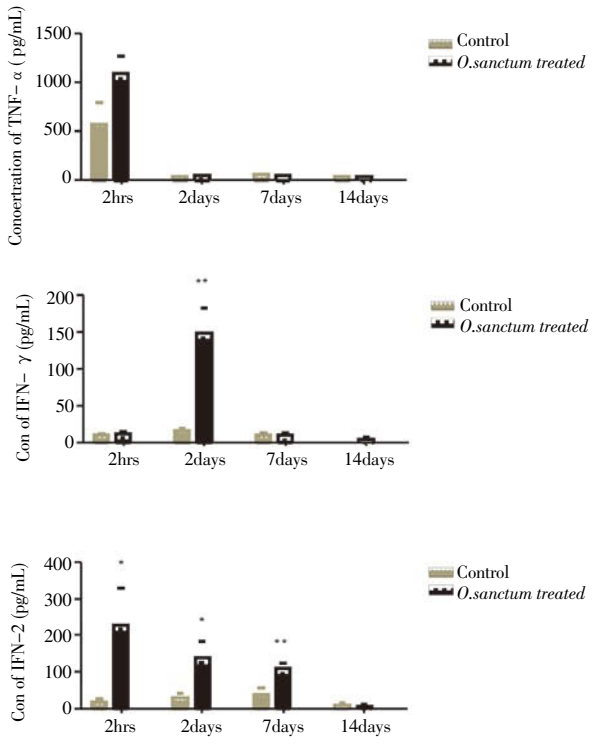

Figure 2. Effect of *O. sanctum* leaves extract on cytokine production following *S. typhimurium* infection.
*: P< 0.01, **: P< 0.001.

Increase in TNF-α in the serum within 2 h clearly demonstrated that serum of *O. sanctum* treated rats contains greater quantity of TNF-α, which plays a role in inhibiting the growth of *S. typhimurium*. Further it has been also demonstrated that serum of *O. sanctum* treated rats contained detectable amount of IL-2 at 2 h while IFN-γ remained undetectable in both the groups. When serum's of both groups were quantitated for these cytokines at 48 hrs, TNF-α, could not be detectable but increased IFN-γ production was observed. IFN-γ showed remarkable up regulation in *O. sanctum* fed rats. This showed that an early response of microbial infection stimulated TNF-α along with IL-2 induction.

4. Discussion

The organism of genus *Salmonella* is the causative agent of a variety of infections in humans, ranging from acute self limiting gastroenteritis to systematic typhoid fever[21]. Following oral infection with virulent *S. typhimurium*, bacteria quickly spread from the gut, probably through the Peyer's patches of the gut associated lymphoid tissue to

organs of the reticuloendothelial system including the liver and spleen. Rate of bacterial growth is rapid causing death of animals within few days with signs of endotoxic shock and high level of bacteria in liver and spleen[22].

Enumeration of bacteria in blood of i.p. challenged *Salmonella* infected & *O. sanctum* treated rats showed that they differ in the number of organisms present on day 1, 2 & 3 of infection with the control rats which were having much higher counts in blood. It appeared that *O. sanctum* has significant protective effect by stimulating different cell types and factors involved in innate and adaptive immune responses.

Macrophages act as first line of defense during infection of *S. typhimurium*[15]. They kill the bacteria by engulfment. Thus, phagocytic activity of macrophages was determined and 55% stimulation in phagocytic activity of *O. sanctum* treated animals was recorded as compared to control animals infected with *S. typhimurium*. It clearly showed that macrophages were more activated due to *O. sanctum* treatment.

Since level of cytokines plays an important role during the protective response in a biological system, it was of interest to investigate the effect of *O. sanctum* on cytokines. Our previous studies[16-18] reported that *O. sanctum* feeding in rats stimulated the *ex vivo* production of TNF-α, IFN-γ & IL-2 cytokines. Thus during *S. typhimurium* infection in vivo stimulation of these cytokines, which are essentially needed for protection against the disease, were estimated.

The controlled production and release of inflammatory mediators like TNF-α play an essential role in the mechanism of phagocytosis and killing of the infectious microorganism thereby protecting the animal[23-25]. TNF-α may be needed for polymorphonuclear cells recruitment to control Salmonellosis at this early time point[14]. The bacterial product most frequently implicated in TNF-α, *in vivo*, is lipopolysaccharide (LPS). Neutralization of TNF-α in vivo can also prevent the host from mounting a protective response and animals succumb to infection[26]. In this study we have found that there was significant increase in TNF-α in control as well as *O. sanctum* treated animals but the augmentation in TNF-α concentration was highly significant in *O. sanctum* treated animals in comparison to control animals, 2 h after infection with *S. typhimurium*. The level of TNF-α was down regulated very soon in both the groups.

In addition to TNF-α, IFN-γ is also essential for the suppression of bacterial growth during sub lethal *Salmonella* infection[27]. A lack of IFN-γ results in increased bacterial replication[28] and host susceptibility[29], but does not influence antibody protection[30]. The main sources of IFN-γ are natural killer (NK) cells & T-cells[12, 31,32]. The response to infected phagocytic cells could be divided into two identifiable stages. First, resting NK cells required IL-2 and/or IL-15 dependent priming. It is well established that murine dendritic cells produce IL-2 and enhances NK cell activation[11, 33]. Second, activated NK cells then can respond directly to infected macrophages by cytotoxicity and IFN-γ production which modulate other components of host immune response[12]. In this study, IFN-γ induction was observed at later time point of 48 h and the induction of IFN-γ is 9.3 fold increased in *O. sanctum* treated animals than control animals. The IL-2 concentration was also much pronounced (12.5 fold) in *O. sanctum* treated animals at 2 h post infection and maintained to a high level in *O. sanctum* treated animals up to 7 days. IL-2 act as a growth factor for Th1 and Th2 lymphocytes and play a direct role in the induction of B-lymphocytes[34,35] which are essential to clear the inter cellular bacteria by producing antibodies. Thus it can be postulated that during *S. typhimurium* infection as soon as bacteria come in contact with macrophages they become activated. Macrophages act as a first line of defense and secrete TNF-α in high concentration. IL-2 with IL-15 and / or IL-18 synergistically activates the resting NK cells and T- cells, the main source of IFN-γ, resulting in IFN-γ production only at late time of infection. Our data showed that augmented TNF-α, IFN-γ & IL-2 levels at different stages in *O.* sanctum fed animals managed to subdue the lethality of LD_{50} dose of *S. typhimurium* infection.

It was also observed that TNF-α production diminished to ground level very soon but the IL-2 was continuously produced and its concentration remained higher up to 7 days. The IFN-γ which was induced at 48 h time point decline very rapidly. Thus it can be emphasized that these cytokine secretions also reflect time dependency which differ according to the type of cytokine.

Thus, the results of the present study indicate significant modulation of different cytokines i.e. TNF-α, IFN-γ & IL-2 secretion by *O. sanctum* extract in *S. typhimurium* infected rats which in turn leads to clearance of bacteria during different time intervals from blood establishing the efficient anti-microbial immune responses and therapeutic potency of *O. sanctum* extract.

Conflict of interest statement

We declare that we have no conflict of interest.

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