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ORIGINAL ARTICLE

IL 6 levels in Periodontitis Induced Rabbits after Treatment with Neem Bark Extract

GUL E NAZISH[1], RAI SADAF NAWAZ[2], AYESHA SHMAKH[1], ARSHADULLAH AFRIDI[3]
[1]*Amna Inayat Medical College, Sheikhupura*
[2]*Fatima Memorial Medical and Dental College, Lahore*
[3]*Shalamar Institute Of Health Science, Lahore*
Correspondence to Dr. Gul e Nazish, Assistant Professor Pharmacology, Email: gulnazish999@gmail.com, Cell: 03324642961

ABSTRACT

Background: IL-6 has crucial role in the response to microbial insults. It acts as pro-inflammatory and anti-inflammatory agent. Hence IL 6 is an important inflammatory marker of periodontal disease. Levels of IL6 in Gingival crevicular fluid strongly indicate presence of periodontal disease which ultimately results in many health hazards.
Aims: To measure levels of IL 6 in periodontitis induced rabbits after treating animals with neem bark extract and amoxicillin at doses of 500mg/kg and 20mg/kg and to see anti-inflammatory effects of neem bark extract.
Methods: Forty eight white New Zealand rabbits were divided in four groups A, B, C and D. Group A was healthy control. Group B was disease group. Group C was neem treated. Group D was amoxicillin treated. Periodontitis was induced and rabbits were given treatment with neem bark extract and amoxicillin for 10 days. Samples were obtained with the help of periopaper absorbent strip and marginal auricular vein and analyzed in laboratory. ELISA test was performed to find levels of IL 6 in all four groups.
Practical implication: To gain the evidence that neem can be used in maintenance of oral hygiene and prevention of oral diseases.
Results: Treatment with neem bark extract and amoxicillin equally reduced IL 6 levels among group c and D after treatment (0.033±0.003) in gingival crevicular fluid when compared with diseased group B (0.089±0.007).P value was less than 0.001.
Conclusion: Neem bark extract has significant effects on reduction of IL 6 levels in gingival crevicular fluid of periodontitis induced rabbits Neem bark extract possess anti-inflammatory and anti-microbial action.
Keywords: IL6, Periodontitis, GCF, Inflammation, Neem

INTRODUCTION

Interleukin-6 (IL-6) is synthesized by different kind of cells in the body like endothelial cells, fibroblasts, and macrophages. It is one of the important inflammatory markers of periodontitis. It has double role as pro-inflammatory and anti-inflammatory marker. It also regulates the expression of other proinflammatory chemokines such as interleukin-1, interleukin-10, and tumor necrosis factor-α. IL-6 affects the composition of the sub gingival bacteria and increases the sensitivity to colonization with periodontitis producing bacteria. It is also a strong stimulator of osteoclast differentiation and bone resorption. It has also been reported that lipopolysaccharide obtained from Porphyromonas gingivalis a periodontal pathogen and IL-1β significantly increase IL-6 production in human fibroblasts.[1]Hence the intensity of interleukin-6 expression is positively correlated with gingival tissue attachment loss and continuous tissue destruction in periodontitis[2]. Although Periodontitis is a localized disease but with the passage of time it can be converted into systemic hyper inflammatory condition affecting the immunity[3]. The gingival crevicular fluid (GCF) is produced in gingival cervices. It contains several cellular and molecular components of the immunological response as well as mediators of inflammation like IL 6 and by-products of tissue destruction formed within the tissues. Therefore, the GCF provides a non-invasive, easily collected fluid to measure the inflammatory mediators like IL 6 released during disease processes[4].

According to the World Health Organization, almost 80% of the people from developing countries depend on ethnomedicines for their initial treatment. People rely on traditional medicines obtained from plant's active ingredients.[5]Neem *(Azadirachta indica)* is a common, evergreen tree, originally native of India. The importance of neem has also been acknowledged in a report entitled *"Neem a tree for solving global problems"*, published in 1992 by U.S. National Academy of Science. Approximately 135 isoprenoid and non-isoprenoid compounds have been obtained from different parts of the neem tree[6]. These compounds are known to exhibit immunomodulatory properties and to activate T cells, B cells and macrophages.[7]Neem extracts are anti-inflammatory, which significantly minimizes the release of proinflammatory cytokines such as TNF-α and IL-6[8]. In this context the study was designed with the aim to see effects of neem bark extract on raised IL6 levels in GCF and serum samples of periodontitis induced rabbits.

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MATERIAL AND METHODS

It was experimental study conducted at Pharmacology Laboratory University of Health Sciences, Lahore. Animal care and experimental procedures were done at experimental research laboratory of university of health science. Sampling was Simple random sampling by balloting method. 48 male New Zealand rabbits approximately 1 year old and weighing between 1 to 1.5 kg, were divided into four groups. Each group comprised of 12 rabbits. All animals were kept in the animal house under standard circumstances of temperature (22-24ºC), humidity (45-65%) and natural day and night cycle. The rabbits were fed on normal rabbit feed and water *ad libitum*. The experimental techniques were followed according to institutional ethical committee animal care guidelines. Group A was healthy animals fed with normal rabbit feed and distilled water. Group B animals were induced with periodontitis and fed with normal rabbit feed and distilled water. Group Canimals were induced with periodontitis and treated with neembark extract at dose of 500mg/kg dissolved in distilled water for 10 days and fed with normal rabbit feed and distilled water. Group D animals group induced with periodontitis and treated with amoxicillin at dose of 20mg/kg dissolved in distilled water and fed with normal rabbit feed and distilled water for 10 days[9].
Neem bark extract Preparation: 1 kilogram of neem bark powder was soaked in 4 liters of distilled water for 24 hours at room temperature with periodic shaking. The water extract was filtered through Whatman qualitative Grade 1 filter paper. Thick brown extract containing approximately 70g of total powder was obtained when aqueous extract was concentrated in water bath. The extract was stored at 4°C in a dark brown bottle to avoid biological degradation[10].
Periodontitis Induction: Periodontits was induced by ligature placement around upper maxillary incisor teeth using 3-0 silk

suture under general anesthesia with 40mg/kg ketamine (Global pharma, Pakistan) IV and 5mg/kg Xylazine (Sigma-Aldrich, USA) injections (IM). *Porphyromonas gingivalis* (10^9 CFU) was mixed with carboxymethylcellulose (Sigma-Aldrich, USA) and this slurry was applied topically to the suture ligated maxillary incisors of Group B, C and D three times a week for total 6-weeks. The sutures were examined at every application. Lost or loose sutures were ligated again.[11]

Sample collection: Sample was drawn with the help of periopaper absorbent strips. Samples stained with blood were discarded. The strips were immediately placed in eppendorf tubes containing 150 µl of phosphate elution buffer with composition (0.05% Tween 20,Nacl 8gram/liter, KCl 0.2gram/liter, Na_2HPO_4 1.44gram/liter, KH_2PO_4 0.24gram/liter), (Merck, USA).Samples were transported to the laboratory, stored at −80°C until analyzed[12]. Blood samples were obtained from the marginal auricular vein on seventh day in serum separating tubes with help of 5 cc syringe

Sample analysis: The IL-6 was analyzed in the GCF samples of all groups by ELISA (Koma- Biotech, Korea)

1. All components were mixed carefully and warmed up room temperature immediately before use.
2. Phosphate buffer saline/tween (washing solution) was diluted at 1:20 in sterile Water.
3. RequiredCoated wells for the assay were marked at Pre-coated ELISA 96 well plate.
4. Standards were diluted in Assay Diluent at 1:2 dilution
5. Allsamples were diluted in proportion to the supposed concentration of the analyte in Assay Diluent.
6. Detection Antibody was diluted to a concentration of 0.25 microgram/milliliter at 1:20 dilution in Assay Diluent
7. Streptavidin-HRP conjugate (Color Development Enzyme)was also diluted at 1:20 in Assay Diluent.
8. 200 microliter of Washing Solution was transferred to each well. Solution was aspirated from wells to remove liquid and plate was washed three times using 300 microliter of washing solution. After washing plate was inverted to remove leftover solution.
9. 100 microliter of standards and sampleswere transferred to each well. PlateIncubated at room temperature for two hours after covering with sealer.
10. Liquid was removed and plate was washed for 4 times.
11. Next 100 microliter of the diluted detection antibody (0.25 microgram/milliliter) was transferred to each well.
12. Plate was again covered with Sealer and Incubated at room temperature for 2 hours.
13. Plate was aspirated and washed 4 times.
14. 100 ul of the diluted Color Development Enzyme (1:20 dilute) was added to each well.
15. Plate was sealed and Incubated for thirty minutes at room temperature.
16. Again plate was aspirated and washed for 4 times
17. Now 100 microliter of color development solution was incorporated into each well and Incubated at room temperature for color development properly. After 5-15 minutes 100 microliter of the stop solution was added to each well to stop the color reaction.
18. By using a micro titer plate reader plate was read at 450 nm wave length.

Statistical analysis: The results were analyzed by using graph pad prism 6.Mean ± Standard deviation (SD) was calculated for quantitative variables. One way *ANOVA* was applied to see the difference in groups. Post hoc Tukey test was applied to observe which groups mean differs from the others. A p-value ≤ 0.05 was considered as statistically significant.

RESULTS

Gingival crevicular levels of IL 6 were increased in group B (0.089±0.007) when compared to group A (0.033±0.001). Treatment with neem bark extract and amoxicillin reduced IL 6 levels equally in group C and D after treatment (0.033±0.003) and(0.031±0.002) respectively when compared with disease group B (0.089±0.007).IL 6 levels in serum samples did not show any significant change among groups A, B, C and D (0.033±0.002, 0.033±0.005, 0.032±0.001, 0.033±0.005).

Fig 1 shows induction and diagnosis of periodontitis (n=36)

Fig 2 shows pink gingiva after neem bark extract treatment (n=12)

Fig 3 shows IL 6 levels in gingival crevicular fluid sample where ### and ***shows P value of less than 0.001. Indicating significant difference between groups (n=12).

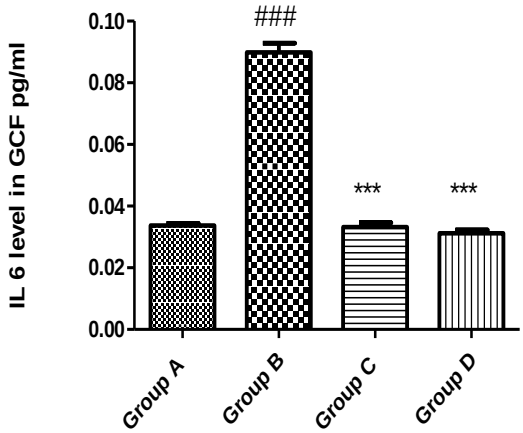

Fig 4 shows IL 6 level in serum showing no difference among all groups (n=12).

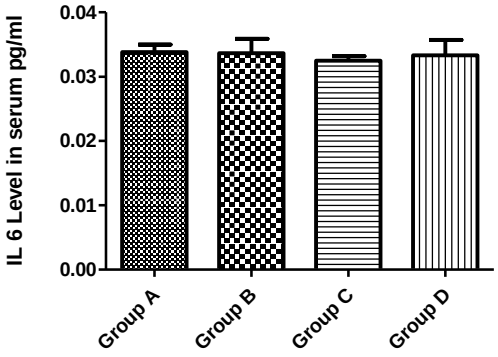

Fig 5 shows raised ESR value in among group B indicating inflammation (n=12)

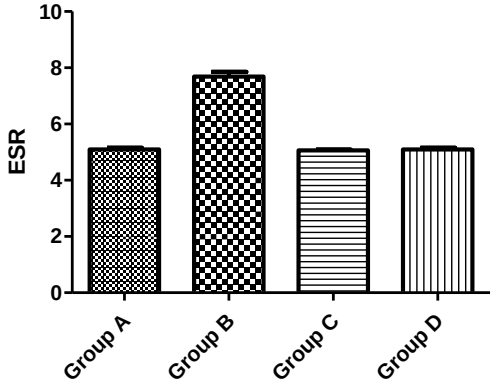

Table1: Mean ± SD of IL 6(pg/ml) level in GCF (n=12)

Parameter	IL 6 pg/ml
Group A	0.033±0.001
Group B	0.089±0.007[a]
Group C	0.033±0.003[b]
Group D	0.031±0.002 [b]

a shows a significant difference with group A
b shows a significant difference with group B

Table 2: Mean ±SD of IL 6(pg/ml) level in serum (n=12)

Parameter	IL 6 pg/ml
Group A	0.033±0.002
Group B	0.033±0.005
Group C	0.032±0.001
Group D	0.033±0.005

Table 3: Mean±SD of Erythrocyte Sedimentation Rate (n = 12)

Parameter	ESRmm/hr
Group A	5.1±0.2
Group B	7.6±o.5[a]
Group C	5±0.09[b]
Group D	5.0±0[b]

[a] shows a significant difference with group A
[b]shows a significant difference with group B

DISCUSSION

Periodontal disease has a complex molecular biology. It involves expression of many cytokines and chemokines responsible for the progression and severity of disease.IL 6 is also known to have a correlation with periodontitis production and progression. Porphyromonas gingivalis bacterium is one of the pathogens causing aggressive type of periodontitis is also strongly linked to the expression of IL 6 in periodontitis. In this study we used porphyromonas gingivalis for induction of periodontitis and to measure the levels of IL 6 in both GCF and serum samples of healthy, diseased and treatment groups.

Role of IL 6 in periodontitis is contradictory. Some researchers have reported no difference in mean IL 6 concentration at diseased and stable sites; some also reported increased IL 6 level in GCF after treatment[13,14]. In our study higher levels of IL 6 in diseased Group B were seen. Our results confirmed a correlation between IL 6 levels and pathogenesis of periodontitis.

IL 6 levels were reduced equally by neem extract and amoxicillin with p value less than 0.001.Role of neem leaf extracts as anti-inflammatory agent and on decreased production of IL 6 has been well supported in literature[15,16]In a previous study we found the same effects of neem bark extract on levels of IL6 with less sample size. [17]However, more investigations with IL 6 mRNA expression from inflamed gingival tissue and from GCF sample at different sampling time are required. Effect of neem bark extract on reduction of IL 6 levels were also compared with amoxicillin treatment an antibiotic because we know IL6 expression is strongly linked with microbial insult. Reduction in IL 6 levels with neem bark treatment was similar to amoxicillin treatment group. This evidence shows that neem possesses both anti-inflammatory and anti-microbial effects[18].

IL 6 levels were not raised in serum levels even after induction of periodontitis with porphyromonas gingivalis. Little data is available in literature in support of systemic manifestation of IL6 levels in periodontal disease in spite of the fact that periodontitis has pronounced systemic presentation.[19]

CONCLUSION

We concluded that aqueous extract of Neem bark extract and amoxicillin at doses of 500mg/kg and 20mg/kg respectively reduced levels of IL 6 in GCF samples of periodontal lesions equally. Neem bark extract possess anti-inflammatory and anti-microbial effects. However based on this pre- clinical data further studies should be carried out to see the effectiveness of Neem bark extract in humans. Toxic effects of neem bark extract at therapeutic doses is also an aspect to be addressed in future studies.

Conflict of interest: All authors have no conflict of interest to declare.

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