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Effect of complete dentures on mucosal cytokine profiles and interleukin levels: a pilot clinical study using quantitative real time PCR

Wpływ protez całkowitych na profil cytokin śluzówkowych i poziom interleukin: pilotażowe badanie kliniczne z wykorzystaniem ilościowej reakcji PCR

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HASŁA INDEKSOWE:
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Summary

Introduction. *Inflammation of the denture-bearing mucosa due to shear stress and functional load transfer from complete dentures is characterized by a complex interaction of pro- and anti-inflammatory mediators. A favourable equilibrium leads to a recovery of homeostasis, while an unfavourable balance adversely affects mucosa and the underlying bone. The present study was an assay to estimate cytokine level and its sequential expression as a biomarker of oral mucosa status after denture use.*

Aim of the study. *To evaluate the cascade of expression of interleukins in patients who have been edentulous for 12-15 months and subsequently treated with complete dentures.*

Material and Methods. *Thirty patients aged 55-65 years, edentulous for 12-15 months without any systemic affliction were selected as study participants. These patients were from remote rural areas and hence could not receive early prosthodontic treatment. They were*

Streszczenie

Wstęp. *Zapalenie błony śluzowej podłoża protetycznego z powodu sił ścinających i przenoszenia obciążenia czynnościowego przez protezy całkowite charakteryzuje się złożoną współzależnością mediatorów pro- i przeciwzapalnych. Utrzymanie korzystnej równowagi prowadzi do przywrócenia homeostazy, podczas gdy niekorzystna równowaga negatywnie wpływa na błonę śluzową i kość, którą pokrywa. Przedstawiona analiza miała na celu ocenę poziomu cytokin i jej sekwencyjną ekspresję jako biomarker stanu błony śluzowej po użytkowaniu protez.*

Cel pracy. *Ocena kaskady ekspresji interleukin u pacjentów bezzębnych od 12-15 miesięcy a następnie leczonych przy pomocy protez całkowitych.*

Materiał i metody. *Trzydziestu pacjentów w wieku 55-65 lat z bezzębiem od 12-15 miesięcy, bez schorzeń ogólnych zostało objętych badaniem. Pacjenci pochodzili z obszarów wiejskich i dlatego nie otrzymali wstępnego leczenia pro-*

approached by the Community Prosthodontic Outreach Initiative team and assessed for oral tissue status prior to any treatment. A biopsy specimen from the mucosa of the buccal shelf area of the mandibular alveolar ridge was taken following the standard surgical protocol and analysed for interleukin levels. Thereafter, these individuals were provided with logistic support to access prosthodontic care and complete denture rehabilitation at our Institution. Subsequent to regular denture wear, biopsy specimens from the same site were again harvested at one month, four months, seven months and eleven months following denture insertion and analysed for interleukin levels.

Results. *Interleukin-1α, -1β and -8 levels were higher than pre-treatment (baseline) levels at all the follow-ups. Expression of interleukin-4 was maximum at one month, declining at four months. Interleukin-6 expression was higher than that at baseline at all follow-ups except at eleventh months. Expression of interleukin-10 level was maximum at one month declining by the fourth month.*

Conclusion. *The limited data indicates that interleukins are produced at a higher rate after patients have been initiated into complete denture wear. mRNA expression of pro-inflammatory cytokines elevates to almost double their level of expression in the initial first few months and gradually decreases till the seventh month to reach a level lower than the initial one.*

tetycznego. Z pacjentami skontaktował się zespół Inicjatywy Protetycznej dla Społeczności, który ocenił stan błony śuzowej przed leczeniem. Pobrano próbki tkanek podczas biopsji z policzków błony śluzowej i wałów dziąsłowych żuchwy zgodnie ze standardowym protokołem chirurgicznym w celu przeanalizowania poziomów interleukin. Następnie, pacjentom ułatwiono dostęp do opieki protetycznej i rehabilitację przy pomocy protez całkowitych w naszym ośrodku. W trakcie użytkowania protez, pobrano próbki po jednym, czterech, siedmiu i jedenastu miesiącach od momentu umieszczenia protezy i przebadano poziom interleukin.

Wyniki. *Poziomy interleukin -1α, -1β i -8 były wyższe niż przed leczeniem, we wszystkich okresach kontrolnych. Ekspresja interleukiny -4 była najwyższa po miesiącu, obniżając sie po czterech miesiącach. Ekspresja interleukiny -6 była wyższa niż w momencie bazowym i we wszystkich okresach kontrolnych z wyjątkiem badania po jedenastym miesiącu. Ekspresja interleukiny -10 była najwyższa po miesiącu obniżając się po czterech miesiącach.*

Wnioski. *Na podstawie otrzymanych ograniczonych danych można wnioskować, że interleukiny są wytwarzane szybciej po tym jak pacjenci zaczęli użytkować protezy całkowite. Ekspresja mRNA prozapalnych cytokin prawie podwajała swój poziom w ciągu pierwszych kilku miesięcy a następnie stopniowo opadała do siódmego miesiąca aby ostatecznie osiągnąć poziom niższy niż bazowy.*

Introduction

Despite a stupendous effort at conservation of teeth, edentulism has been on the rise worldwide. Greater life expectancy and improved living standards have increased the number of patients seeking complete dentures for restoring aesthetics and the masticatory function.[1] Delayed prosthodontic rehabilitation produces profound histological changes in the alveolar structure.[2] According to *Schroeder* et. al.,[3] the adherent fibromucosa of the edentulous ridge has a thick keratinized epithelium that closely mingles with the submucosa, having a dense network of collagen fibers. Prolonged use of dentures renders the fibromucosa thin and hyperplastic with loss of adherence to the periosteum. The occlusal forces transferred to the underlying bone via fibromucosa initiate a micro-anatomical coupling of the osteoblastic and osteoclastic activity mediated by cytokines.

Numerous studies have confirmed the initiation of inflammatory processes in the alveolar mucosa after denture insertion.[4-6]

Inflammation in the mucosal tissue caused by the pressure impingement from the complete denture is characterized by a complex interaction of pro- and anti- inflammatory mediators. A favourable balance leads to a recovery of homeostasis while and an unfavourable balance, associated with an exacerbated release of pro-inflammatory cytokines, leads to deleterious effects on both mucosa and bone.[7,8] Cytokines are biologically active molecules released by specific cells that elicit a particular response on the mucosa of edentulous patients under compressive force.[9] Pro-inflammatory cytokines (IL-1α, IL- 1β, IL-6, IL-8) secreted by phagocytic leukocytes, recruit additional innate cell populations to sites of infection, induce dendritic cell maturation and direct the subsequent specific immune response to the pressure on the mucosa. Anti-inflammatory cytokines (IL-4, IL-10) suppress this process and hence in completely edentate patients there is a balance between pro-inflammatory and anti-inflammatory cytokines.[10-15]

Recent studies[16-18] point to a correlation between inflammatory biomarkers, namely cytokines and bone loss around single tooth implant, implant-supported overdenture and in periodontitis. *Tsuruoka* et al.[19] reported the expression of heat shock protein, vascular endothelial growth factor and cell nuclear antigen in the palatal mucosa of rats upon mechanical loading. However, literature lacks evidence on the enzymatic behaviour of the edentulous ridge mucosa in patients with complete denture prosthesis.

The present study assessed the local production of cytokines and interleukins, known to affect bone remodelling in patients who have been edentulous for an extended period, and then treated with complete dentures. The profile of cytokines produced by mucosal soft tissue covering of the buccal shelf area of the mandibular residual alveolar ridge has been investigated.

Materials and methods

The present study was conducted in the Department of Prosthodontics, Saraswati Dental College & Hospital, Lucknow in collaboration with CSIR-Central Drug Research Institute, Lucknow. Prior approval of the Institutional Ethical Committees (IEC/2019/22) was obtained from both concerned institutions.

To standardize the sample and to avoid bias, stringent selection criteria were followed. The inclusion criteria included: male subjects in the age group of 55-65 years with healthy residual ridges corresponding to class ACP I and ACP II and edentulous for 12 to 15 months. The exclusion criteria included: subjects with history of previously complete denture use, temporomandibular joint disorders, diabetes, autoimmune diseases and poor neuromuscular control.

A Prosthodontic Outreach Program in remote rural areas around our Institution was initiated to identify barriers to prosthodontic treatment of the elderly edentulous patients and to devise a remedial strategy. Epidemiological data on the prevalence of edentulism in remote rural areas were collected and the untreated (control) group was derived from this cohort of patients. Subsequently, these patients were offered free transport and treatment facility for prosthodontic rehabilitation with complete dentures at our institution.

Sixty-six volunteers (42 males, 24 females) from a cluster of twelve villages of a single rural block of Lucknow were provided with detailed information about the Prosthodontic Treatment Outreach project, including the protocol for biopsy collection. It was explained that this was a therapeutic and research initiative, which was directed at improving the immediate and long-term prognosis of complete denture treatment. Of the total: 57 patients were positive responders and were enrolled for the project, 27 patients dropped out of the ongoing project at different

stages, 12 had logistic problems, 13 dropped with the complaint of time constraints while 5 absconded. Thus the remaining 30 patients constituted the experimental cohort. A wedge-shaped biopsy specimen 3 mm deep, 3 mm in length and 1 mm in width[20] was excised from the mucosa of the buccal shelf area of the mandibular edentulous alveolar ridge (primary stress-bearing area)[21] under infiltration anaesthesia from each patient. The sample was secured in an Eppendorf tube, placed in an ice box, stored in liquid nitrogen at -80^{0}C and sent to the Endocrinology laboratory, CSIR (Central Drug research Institute) for evaluation of the pattern of mRNA expression for interleukin level (IL-1α, IL-1β, IL-4, IL-6, IL-8 and IL-10). The same procedure for sample collection was repeated alternately on the right and left buccal slope of the alveolar ridge in the region of mandibular first molar at one month, four months, seven months and eleven months following denture insertion.

Method for complete denture fabrication

A traditional method of denture fabrication was used for all the subjects. The method included a facebow record, horizontal and vertical jaw relation records to mount the dental casts on a semi-adjustable articulator (Hanau Wide-Vue; Whip-Mix Corp.), semi-anatomic polymethyl methacrylate resin teeth (Ruthinium Dental) arranged in a balanced occlusion and compression moulding polymerisation technique. After polymerisation, dentures were remounted on the articulator for occlusal readjustment. Thereafter, dentures were removed from the articulator and polished. The polymerized dentures were immersed in water at 50^{0}C for 1 hour,[22] followed by immersing in water at 37^{0}C for 24 hours.[23] The subjects visited the department on a weekly basis until subjects were free from any discomfort from the dentures. Denture adjustment was performed in a conventional manner. The adjustment of the tissue surface was performed using a pressure indicating paste and a carbide bur (Carbide Bur#7, SHOFU, Japan). Deflective occlusal contacts in centric and eccentric position were detected with 40μ thickness of articulating paper (ARTICULATINGPAPER, GC, Japan), removed with an abrasive bur (Carborundum Point HA#19, SHOFU, Japan). This procedure was repeated until the posterior teeth of maxillary and mandibular dentures evenly contacted each other in the centric occlusal position.

Patients were instructed to mechanically cleanse the dentures three times a day using the denture cleaning kit and to immerse the denture in 0.12% chlorhexidine gluconate solution for 10 minutes once a week.[24]

Assessment of mRNA

The assessment of mRNA for interleukin expression entailed RNA isolation, RNA quantification, cDNA synthesis and amplification by β-actin interleukin primers.

RNA isolation

The mucosa samples were thawed to 4^{0}C and homogenized by adding 1 ml of trizol reagent in polytron homogenizer. 200μl of chloroform per 1 ml of trizol reagent was added to the samples. The samples were vortexed for 15 sec and centrifuged at 15000 rpm for 15 minutes. Following centrifugation, the mixture separated into lower red phenol-chloroform phase, an interphase and colourless upper aqueous phase. The aqueous phase was carefully transferred to another tube without disturbing the interphase. Precipitation of RNA from this aqueous phase was done by mixing 0.5 ml of isopropyl alcohol per 1 ml of trizol reagent used. Samples were incubated at 15 to 30^{0}C for 10 minutes and centrifuged at 15000 rpm for 10 minutes. The RNA precipitated as a gel-like pellet on the side and bottom of the tube. The supernatant liquid

was removed completely. The RNA pellet was washed once with 75% ethanol and air- or vacuum-dried for 5-10 minutes. It is important not to let the RNA pellet dry completely. Partially dissolved RNA samples have an A_{260}/A_{280} ratio < 1.6.

RNA quantification

RNA was quantified by measuring their absorbance of ultraviolet light (OD) at 260 nm. Dilute 1µl of RNA pellet with 39µl of DEPC (Diethyl pyrocarbonate) treated water (1:40). The samples were placed in Nanodrop spectrophotometer using 10µl microcuvette. The A_{260}/A_{280} ratio should be above 1.6. 1 OD (optical density) at 260 nm was equal to 40 µg/ml RNA.

c DNA synthesis

RNA was reverse transcribed in two steps. First step involved addition of 5mM oligoneucleotide (applied biosystems) to 0.5-1mg of total RNA. In the second step, 100 U of superscript reverse transcriptase (life technologies) was added in the presence of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$, 5mM dithiothreitol (DDT) and 0.5 mM dNTPs. The mixture was placed in a thermocycler. After the end of the cycle, the mixture (cDNA) was removed from the thermocycler and stored at 4^0C. For every reaction set, one RNA sample was performed without superscript reverse transcriptase to provide a negative control in the subsequent PCR. For subsequent PCR amplification, maximum 0.5 ml of each cDNA sample was used per 25 ml PCR mixture. The use of larger amount of cDNA in the PCR was avoided, since this may inhibit efficient PCR amplification. The cDNA was diluted with water to the final volume of 100µl.

Amplification by β-actin interleukin primers

Each sample was subjected to an initial amplification by human β-actin specific PCR primers. The primers for individual human interleukin i.e. IL-1α, IL-1β, IL-4, IL-6, IL-8 and IL-10 were ordered from integrated DNA technologies. Based on the amount of amplified β-actin PCR product, an equal amount of reverse-transcribed product was amplified by the following study primer pairs:

IL-1α- 5'GGTTGAGTTTAAGCCAATCCA3' (antisense)
IL-1α- 5'TGCTGACCTAGGCTTGATGA3' (sense)
IL-1β- 5'TACCTGTCCTGCGTGTTGAA3' (antisense)
IL-1β- 5'TCTTTGGGTAATTTTTGGGATCT3' (sense)
IL-4- 5' TCCAACGTACTCTGGTTGG3' (antisense)
IL-4- 5'CAACTTTGTCCACGGACAC3' (sense)
IL-6- 5'GATGAGTACAAAAGTCCTGATCCA3' (antisense)
IL-6- 5'CTGCAGCCACTGGTTCTGT3' (sense)
IL-8- 5'CATCAGTTGCAAATCGTGGA3' (antisense)
IL-8- 5'AGAACTTATGCACCCTCATCTTTT3' (sense)
IL-10- 5'CACCCTCACAAGGCTGCTA3' (antisense)
IL-10- 5'ACTGCAATTCTGCAGCTCAA3' (sense)

PCR was performed in a reaction mixture containing 5jL of cDNA, 200gL of each dNTP, 0.4 gmol/L of each upstream and downstream specific primer, 1.5mmol/L $MgCl_2$ 2.5U of Taq DNA polymerase (Life technologies) in a reaction buffer supplied by the manufacturer. The reaction mixture was placed in a 96 well microplate and real time PCR was performed. PCR products were analysed by electrophoresis through 6% acrylamide Tris borate–EDTA gel followed by autoradiography and quantification by Molecular imager and Molecular Analyst software (Biorad, Hercules, USA). The

T a b l e 1. Interleukins levels at different time intervals

Interleukin	Time interval	Group I (Mean ±SD)	Group II (Mean ±SD)	Statistical Significance	
				't'	'p'
Interleukin- 1α (µg/ml)	Baseline	339.07±14.28	315.87±35.21	2.36	0.025
	1 month	589.13±66.52	318.33±34.15	14.03	<0.001
	4 months	467.07±47.81	317.00±33.34	9.97	<0.001
	7 months	384.67±16.60	319.67±36.14	6.33	<0.001
	11 months	347.40±12.43	319.73±36.22	2.80	0.009
Interleukin- 1β (µg/ml)	Baseline	155.87±9.09	148.33±13.97	1.75	0.091
	1 month	264.73±10.19	149.40±12.73	27.40	<0001
	4 months	210.27±7.16	151.47±13.24	15.13	<0001
	7 months	186.73±9.00	151.67±15.70	7.50	<0.001
	11 months	170.53±9.89	151.60±17.09	3.71	0.001
Interleukin-4 (µg/ml)	Baseline	0.00	0.00	–	–
	1 month	6.08±11.59	0.00	2.03	0.052
	4 months	1.33±3.15	0.00	0.392	0.698
	7 months	0.00	0.00	–	–
	11 months	0.00	0.00	–	–
Interleukin-6 (µg/ml)	Baseline	5.96±1.69	5.92±1.70	0.056	0.956
	1 month	7.42±1.63	5.88±1.71	2.52	0.018
	4 months	6.79±1.64	5.96±1.69	1.37	0.179
	7 months	6.19±1.68	5.97±1.57	0.37	0.713
	11 months	5.79±1.64	5.96±1.69	-0.27	0.788
Interleukin -8 (µg/ml)	Baseline	393.73±17.02	364.47±34.18	2.97	0.006
	1 month	659.00±23.91	366.33±33.22	27.69	<0.001
	4 months	619.20±19.06	364.40±34.08	25.27	<0.001
	7 months	480.20±23.56	365.40±35.12	10.53	<0.001
	11 months	425.73±23.48	359.13±32.69	6.40	<0.001
Interleukin-10 (µg/ml)	Baseline	0.00	0.00	–	–
	1 month	11.16±13.97	0.00	3.095	0.004
	4 months	0.67±1.99	0.00	1.309	0.201
	7 months	0.00	0.00	–	–
	11 months	0.00	0.00	–	–

mRNA data was obtained from software and comparative evaluation was performed.

The data thus obtained was analysed using SPSS (Statistical Package for Social Science) version 23.0. Paired "t" test was used to compare changes in interleukin levels at four different time intervals. A 'p' value of less than 0.05 was considered to be statistically significant.

Results

Interleukin level at different time intervals is shown in Table 1. Interleukin-1α levels were higher than its baseline levels at all the follow-ups. Maximum change in levels of interleukin-1α was found at one month followed by at four months. Minimum change was found at eleven months. All the changes were statistically significant ($p<0.001$).

Interleukin-1β levels were higher than its baseline levels at all the follow-ups. Changes in interleukin-1β from baseline was maximum at one month followed by at four months ($p<0.001$); change was minimum at eleven months ($p=0.001$).

Changes in interleukin-4 was maximum at one month followed by four months. No interleukin-4 was expressed at seven months and thereafter.

Interleukin-6 levels were found to be higher than that at baseline at all the follow-ups except at eleven months. The maximum change was observed at one month while minimum change was observed at eleven months from baseline.

Interleukin-8 levels of subjects were found to be higher than that of group II at all the follow-ups. In Group I change in interleukin-8 was maximum at one month followed by at four months; the change was minimum at eleven months from baseline.

In Group I, change in interleukin-10 level from baseline was maximum at one month followed by thefourth month from baseline.

Discussion

Interleukins are a specific variety of cytokines transmitting signals between leucocytes to dictate responses to stimuli. They are primary markers of tissue inflammation. The IL-1 family (IL-1a and IL-1b) are proteins which induce fever and were called human leucocytic pyrogens. While IL-1a is biologically active, IL-1b is activated through inflammation and Caspase-1. IL-1a and IL-1b exert similar effects by binding to Il-1 type I receptor and are pro-inflammatory agonists. They lead the sequence of expression of interleukins, which is maximal initially (the first month) and wanes over an extended period (eleven months). This is succeeded by the inflammation.IL-expression of IL-2 and IL-4, both members of the common y chain cytokine family which bind to the c receptor (CD132). IL-2 acts as a B cell growth factor, stimulates antibody synthesis and proliferation and differentiation of NK cells to enhance their cytolytic function. IL-4 is produced by basophils, eosinophils, mast cells suppresses type I immunity development, prolongs lifespan of T and B cells and mediates tissue adhesion and inflammation. IL-6 is a multifunction pleiotropic cytokine participating in regulation of immune responses, acute phase responses, haematopoeisis and inflammation produced by endothelial cells, fibroblasts, monocytes and macrophages in response to stimuli (IL-1 and TNF a) during systemic inflammation and activation of acute phase proteins. IL-8 is an interleukin with chemokine activity, regulating neutrophil specific chemotactic factor produced by monocytes, macrophages, neutrophils, lymphocytes, endothelial and epithelial cells after stimulation from IL-1a and b, which leads to activation and recruitment of neutrophils. IL-10 is an anti-inflammatory cytokine produced by monocytes, T-cells, B-cells, macrophages and mediates immune suppression, limits rate of leukocytic infiltration and inflammation.

It inhibits expression of pro-inflammatory cytokines, inhibits T cells, promotes survival and differentiation of B cells and increases production of IgG4. All interleukins were expressed almost immediate to the noxious stimulus i.e. tissue compression, and gradually decreased typically from the first through the fourth, the seventh to the eleventh month, though IL-4 expression ceased at seven months.[12]

There was a sudden increase in the pro-inflammatory cytokines after one month of denture use. A study by *Tsuruoka* et al.[19] has demonstrated the appearance of heat shock protein 70 (HSP 70) in denture supporting tissue (palatal mucosa in rats) under mechanical stress. Studies[13,18] have reported that HSP 70 along with HSP 90 and HSP 60 are capable of inducing the production of proinflammatory cytokines by the monocyte-macrophage system. The rate of proinflammatory cytokines in the mucosal tissue covering the alveolar bone in patients with complete denture increased initially as a result of pressure exerted by denture use. As mucosal tissue adapted to the new denture together with expression of anti-inflammatory cytokines, the values of pro-inflammatory cytokines started to decrease slowly in four months. It decreased further in the seventh and eleventh month. The probable reason for expression of these cytokines was the frictional stimulation to mucosa/residual ridges despite the absence of masticatory function.

Prostaglandin E2 and IL-1β are important mediators in the periodontal inflammation that stimulate bone loss and have inhibitory effect on bone formation.[14] *Schierano* et al.[16] investigated cytokine production in bone and mucosa of patients wearing implant-supported overdenture, and found high level of IL-6, IL-8 and moderate level of IL-1β in the primary culture of gingival fibroblasts of edentulous gingiva that promoted osteoclastogenesis and decreased osteogenesis. The levels of cytokines were the highest at the fourth month and decreased from the eighth month. These results are similar to the results obtained in the present study.

IL-6 synthesis and secretion are stimulated by two major pro-inflammatory cytokines, IL-1β and TNF-α, but the fact that IL-6 remains considerably longer in the plasma makes this molecule a good marker of inflammation.[14] Local production of IL-6 also occurs in inflamed submucosa and mucoperiosteum. Correspondingly, IL-6 in the denture wearer has been found to be higher than that at baseline at all follow-up periods except at the eleventh month.

In a complex network of pro- and anti-inflammatory cytokines acting in the inflamed mucosal tissues, IL-10 is an example of a cytokine with anti-inflammatory effects. IL-10 is a regulatory cytokine which, on the one hand, limits inflammatory responses by inhibiting the expression of pro-inflammatory cytokines and up regulates the recruitment and activation of B cells.[15]On the other, in Group I, the IL-10 expression was maximum at one month antagonizing the action of pro-inflammatory cytokine. No change in IL-10 levels was found at seven and eleven months symbolizing the adaptive nature of the submucosa.

The limitations of this study were a small sample size and single mucosal site for the sample collection. Further study on a larger sample size, with complete profiling of interleukins at different oral mucosal sites, with differential load bearing capacity is warranted.

Conclusion

The present study suggested that inflammation is initiated in the alveolar mucosa and bone following complete denture insertion, with production of pro- and anti-inflammatory cytokines. But, as mucosa adapts to the compressible forces of complete denture, the

expression of interleukin gradually decreases suggesting that rapid loss occurs in the first few months after denture insertion, and slows down thereafter. These findings provide insight into the cascade of events at molecular level following denture placement. A sequential cascade form of interleukin expression in the denture bearing tissue has been shown in this study.

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