[Downloaded free from http://www.jdrr.org on Monday, October 10, 2022, IP: 117.211.126.242]

Original Article

Studies on Cytokine Production in Gutkha and Panmasala Chewers

Meghmala Sheshrao Waghmode, Gaurav Kumalakar Gaikwad, Mayur Anil Ghule, Pravin Jaysingh Indalkar, Neha Nitin Patil

Department of Microbiology, PDEA's Annasaheb Magar Mahavidyalaya, Pune, Maharashtra, India

Abstract

Introduction: Panmasala is one of the products which have been favored by the people of all ages In Indian. It contains areca nut, lime, flavoring agents and catechu. It holds a prominent place in Indian market. It has been reported to affect human health by causing oral cancer and dysfunctioning of vital organs. **Materials and methods:** Current research was carried on testing the effect of saliva of panmasala eaters on cytokine production by peripheral blood mononuclear cells (PBMC's). Panmasala (Vimal) and Gutkha (RMD) were used for the study of cytokine modulation. MACSPlex Cytokine12 assay was used for the estimation of cytokine after treatment of PBMC's with the saliva of panmasala eater. **Results:** Concentration of cytokines (IL 10, IL 12, L 17, IFN α, IFN γ, TNF α, GM CSF,IL 4,IL 6,IL 5,IL 2 and IL 9) was found to be increased in the sample containing PBMC's treated with the saliva of panmasala. **Conclusion:** Based on the findings supported with the statistical analysis, it can be concluded that panmasala and gutkha have negative impact on immune function. There is a strong need to generate social awareness about health hazards of pan masala and gutkha.

Keywords: *Cytokines, gutkha, immunity, oral cancer, panmasala*

Introduction

In India panmasala, betel quid and gutkha are favorite products in rural as well as urban areas.[1] Panmasala is dehydrated product made using catechu, areca nut, slaked lime (calcium oxide and calcium hydroxide), cardamom, artificial perfuming, and flavoring substances.[2] Chewing of betel quid or its variants such as pan masala, gutkha (mitha pan) kiwam and zarda, leads to oral submucous fibrosis (OSMF) which resulted in difficulty in mouth opening.[3] OSMF is the previous stage of oral cancer. Oral cancer is the eleventh most widespread cancer worldwide[4] (WHO 2005). According to the study undertaken in the Department of Oral Pathology, Patna Dental College and Hospital, Patna, where total 50 cases of the patient diagnosed with the OSMF were evaluated to find out the relation between OSMF and chewing habit of areca nut or its products. Based on the histopathological examination of biopsy tissue from oral mucosa, researchers concluded that incidence of OSMF in gutkha chewers is far faster and more rigorous as compared in areca nut products chewers.[3]

Research has been carried out on the studies of malignant transformation of OSMF into oral squamous cell carcinoma (OSCC).[5] A significant finding has been recorded about the correlation between areca and gutkha chewing with the prevalence of OSCC where males are more susceptible.[5]

Oral cancer is often preceded by the disorder known as Oral Potentially Malignant Disorders (OPMDs). The multistep neoplasia "OSCC" has scores of genetic and epigenetic changes allied to cancerous transformation. They are "OPMDs found out to be erythroplakia, oral leukoplakia, and skin rash triggered by the immune system (lichen planus). Studies have been carried out on proinflammatory cytokines in saliva as prospective biomarkers of OPMDs and OSCC.[6-9] Tumor necrosis factor (TNF)-α is a cytokine with diverse effects. The important components in malignant transformation process[10] are inflammation, angiogenesis, programmed cell death, and proliferation. The TNF-TNF receptor system plays a significant role in these malignant transformation process.[10] The TNF-α has been found to damage DNA of cells. This results in malignant transformation due to induction of reactive oxygen species.[11] Moreover, TNF family members contribute to immune

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Waghmode MS, Gaikwad GK, Ghule MA, Indalkar PJ, Patil NN. Studies on cytokine production in gutkha and panmasala chewers. J Dent Res Rev 2021;8:20-4.

Submitted: 26-Aug-2020
Revised: 18-Sep-2020
Accepted: 07-Oct-2020
Published: ***

Address for correspondence:
Dr. Neha Nitin Patil, PDEA's Annasaheb Magar Mahavidyalaya, Hadapsar, Pune - 411 028, Maharashtra, India.
E-mail: nehanitinpatil@gmail.com

Access this article online

Website: www.jdrr.org

DOI: 10.4103/jdrr.jdrr_108_20

Quick Response Code:

[Downloaded free from http://www.jdrr.org on Monday, October 10, 2022, IP: 117.211.126.242]

suppression.[12] The interleukin (IL)-6 and TNF-α can prop up malignant transformation in patients with OSMF.[13]

Research study has been carried out for the timely diagnosis of oral cancer in the people with habit of chewing betel quid and its product via cytokine biomarkers. Recent research has accomplished that chemokines and cytokines would be the salivary biomarkers for the timely diagnosis of oral cancer.[14] The current research was done to study cytokine secretion by peripheral blood mononuclear cells (PBMCs) in saliva sample collected from healthy volunteer after chewing gutkha and pan masala and to predict susceptibility towards OSMF, OSCC and OPMDs.

Materials and Methods

Isolation of peripheral blood mononuclear cells

Venous blood sample 10 mL was drawn from healthy person with his consent. Blood sample was collected into 10 mL ethylenediaminetetraacetic acid tubes, after which PBMCs were isolated as described. In short, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (HiSep LSM LS001) density gradient centrifugation. Cells were washed twice with PBS and re-suspended in RPMI-1640 (Gibco, Invitrogen, Breda, and The Netherlands) (RPMI-1640 Dutch modification supplemented with 10 μg/mL antibiotic antimycotic). The PBMCs were counted using a neubauer chamber and were plated in 96 well round-bottom plates (Moxcare, USA) at a final concentration of 1×10^6/mL, in a total volume of 100 μL. The PBMCs were treated with pan masala saliva and one set was kept as control.[15]

Collection of saliva from panmasala eater

Three healthy volunteers' saliva samples (25 mL) were collected after chewing of panmasala for 15 min using spitting method. After 48 h, same volunteers' saliva samples (25 mL) were collected after chewing of gutkha for 15 min using spitting method. Informed consent was taken from the volunteers. Collected saliva samples were centrifuged at 13,000 rpm and stored at −20°C till its use.

Effect of saliva on cytokine production

For evaluating the influence of panmasala and gutkha on cytokine production by PBMCs 0.5 mL of PBMC (4×10^6/mL) was incubated with 50 μL of saliva (from panmasala and gutkha eaters) and ones set without the treatment of saliva was kept as control. The cultures were maintained for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, cells were removed by centrifugation at 250 g for 10 min, supernatants were collected and kept at −70°C until assayed for cytokines content.

MACSPlex cytokine 12 assay

After 24 h cell-free supernatants were harvested and analyzed using the MACSPlex Cytokine 12 Kit (Miltenyi Biotec GmbH). The study was done on pro-inflammatory (IL-2, IL 6, IL 12p70, IL 17A, interferon [IFN] α, IFN γ and TNF α), growth factor (granulocyte macrophage colony-stimulating factor [GM-CSF]) and anti-inflammatory cytokine (IL 4, IL 5, IL 9, and IL 10). To the unknown samples and to the serial dilutions of the MACSPlex cytokine 12 standard, the MACSPlex cytokine 12 capture beads were added. The cytokines were captured by the MACSPlex Capture beads during a 2-h incubation period. Consequently the mixtures of 12 allophycocyanin (APC)-conjugated anti cytokine antibodies (MACSPlex cytokine 12 detection reagent) were added to form sandwich complexes through a 1-h incubation period. Standard curves of individual 12 cytokines were used for estimation. The MACSPlex Cytokine 12 capture beads for human, consists of 12 bead populations coated with capture antibodies which are specific for the cytokines. Bead population can be differentiated by different fluorescence intensities detected in the Fluorescein isothiocyanate (FTIC) (B1) and PE (B2) channel of the MACSQuant® Analyzer and MACSQunt Analyzer as per instructions given in manual.

Statistical analysis

For statistical analysis, the experimental results were compared with control values using ANOVA with Tukey honestly significant difference (HSD) using statistics kingdom. Spearman's Rho calculator was used to check correlation between cytokines levels and panmasala, gutkha chewing habit.[16] The significant $P < 0.05$ was found out.

Results

Study was done on pro-inflammatory, growth factor and anti-inflammatory cytokine for the selection of cytokine as biomarkers involved in the progression of oral cancer induced by pan masala and gutkha like products.

Cytokine profile was found to be significantly and markedly different due to the effect of panmasala and gutkha as shown in Table 1. As compared to the control, pro-inflammatory cytokine secretion was more by PBMCs treated with the saliva of panmasala and gutkha eater.

Statistical analysis was done using ANOVA with Tukey HSD. Data are given in Table 2, it can be interpreted that secretion of cytokines by PBMC's has significant impact of panmasala and gutkha as compared to the control. Based on the values, it can be concluded that gutkha has more impact on cytokine profile as compared to panmasala.

Nonparametric test Spearman's Rho was used to measure the strength of association between cytokine secretion by PBMC's in control and after treating with the saliva of panmasala eater, PBMC's in control and after treating with the saliva of gutkha eater as well as PBMC's after treating with the saliva of panmasala eater with the cytokine secretion by PBMCs after treating with the saliva

[Downloaded free from http://www.jdrr.org on Monday, October 10, 2022, IP: 117.211.126.242]

Table 1: Estimation of cytokine production by peripheral blood mononuclear cell treated with saliva of panmasala and gutkha chewers

Name of the cytokine	Control (pg/ml)±SEM	Secreted cytokine by PBMCs treated with of saliva after chewing of Panmasala (Vimal) (pg/ml)±SEM	Secreted cytokine by PBMCs treated with of saliva after chewing of Gutkha (RMD) (pg/ml)±SEM
Pro-inflammatory cytokine panel			
IL 2	8.3±0.18	44.76±0.34	417.6±0.65
IL 6	761±0.04	741.73±0.06	915.17±0
IL 12p70	7.2±0.04	34.07±0	260.65±0.1
IL 17A	8.8±0.2	62.7±0.12	289.65±0.06
IFN α	6.83±0.06	35.79±0.09	44.9±0.17
IFN γ	8.49±0.6	40.1±0.1	147.78±0.79
TNF α	683.55±0.78	439.35±0.08	826.49±0.67
Growth factor panel			
GM CSF	121.68±0.56	150.91±0.06	624.26±0.04
Anti -inflammatory cytokine panel			
IL 4	2.19±0.67	7.51±0.78	5.67±0.45
IL 5	5.81±0.06	240.71±0.05	320.69±0.12
IL 9	7.16±0.05	46.91±0.04	526.64±0.05
IL 10	46.62±0.1	40.91± 0.05	299.92±0.04

SEM: Standard error of mean, GM CSF: Granulocyte macrophage colony-stimulating factor, PBMC: Peripheral blood mononuclear cell, TNF: Tumor necrosis factor, IFN: Interferon, IL: Interleukin, RMD: Gutkha Registered Trademark

Table 2: Statistical analysis of cytokine production by peripheral blood mononuclear cells treated with saliva of panmasala and gutkha chewers

Name of the cytokine	*P*	*F* statistic	Tukey HSD interpretation
Pro-inflammatory cytokine panel			
IL 2	3.84670e-12	19143.64183	Significant difference between the pairs
IL 6	0.00000118088	280.826674	Significant difference between the pairs
IL 12p70	0	792997.6352	Significant difference between the pairs
IL 17A	1.34199e-7	582.965381	Significant difference between the pairs
IFN α	0.0000124122	126.570259	Significant difference between the pairs
IFN γ	1.00666e-10	6446.017284	Significant difference between the pairs
TNF α	1.93568e-7	515.612978	Significant difference between the pairs
Growth factor panel			
GM CSF	2.48782e-10	4766.963256	Significant difference between the pairs
Anti-inflammatory cytokine panel			
IL 4	0.0000250748	99.496480	Significant difference between the pairs
IL 5	2.30324e-8	1051.406418	Significant difference between the pairs
IL 9	2.97782e-11	9675.809011	Significant difference between the pairs
IL 10	5.85639e-10	3582.733450	Significant difference between the pairs

HSD: Honestly significant difference, GM CSF: Granulocyte macrophage colony-stimulating factor, TNF: Tumor necrosis factor, IFN: Interferon, IL: Interleukin

of gutkha eater Table 3. Positive correlation was observed between control and test sample 1 for IL6, IL12p70 pro-inflammatory cytokine and IL10 anti-inflammatory cytokine.

Test sample1: Saliva of panmasala eater, Test sample2: Saliva of gutkha eater. Values presented in bold, represent statistically significant data.

Negative correlation was observed between control and test sample 1 for IL2. Whereas positive correlation was observed between control and test sample 2 for IFN α pro-inflammatory cytokine and GM CSF growth factor. Positive and negative correlation was observed between sample 1 and 2 for pro-inflammatory cytokine IL6 and IL 17A, respectively. Significant correlation was observed for proinflammatory cytokine suggesting its possible role in cancer formation in people who are panmasala and gutkha chewers.

Discussion

Immunomodulatory activity of gutkha and panmasala was carried out using multiplex cytokine detection kit. Statistical tests like ANOVA with Tukey HSD and nonparametric

[Downloaded free from http://www.jdrr.org on Monday, October 10, 2022, IP: 117.211.126.242]

Table 3: Spearman's rank coefficient analyses of the association between the cytokine secretion in control and in peripheral blood mononuclear cell's treated with the saliva of Panmasala and Gutkha eater

Name of the cytokine	Spearman's Rho correlation between cytokine secretion in control and test Sample 1		Spearman's Rho correlation between cytokine secretion in control and test Sample 2		Spearman's Rho correlation between cytokine secretion in test Sample 1 and test Sample 2	
	r	*P* (two-tailed)	*r*	*P* (two-tailed)	*r*	*P* (two-tailed)
Pro-inflammatory cytokine panel						
IL 2	**−1**	**0**	0.76073	0.13534	−0.15214	0.80703
IL 6	**0.91667**	**0.02851**	0.25	0.68504	**1**	**0**
IL 12p70	**1**	**0**	0	1	0	1
IL 17 A	−0.36111	0.55042	0.15214	0.80703	**−0.91287**	**0.03047**
IFN α	−0.75	0.14429	**0.91287**	**0.03047**	0	1
IFN γ	−0.30429	0.61863	0.16667	0.78878	0.15214	0.80703
TNF α	0.30429	0.61863	−0.30556	0.6171	0.15214	0.80703
Growth factor panel						
GM CSF	0.58333	0.30186	**1**	**0**	0	1
Anti-inflammatory cytokine panel						
IL 4	0.59235	0.29258	0.30429	0.61863	0.58333	0.30186
IL 5	0.30429	0.61863	−0.47222	0.42191	0.30429	0.61863
IL 9	−0.80556	0.09987	0.08333	0.89402	0.66667	0.2191
IL 10	**0.55042**	**0.36111**	−0.58333	0.30186	−0.08333	0.89402

Test Sample 1: Saliva of panmasala eater, Test Sample 2: Saliva of gutkha eater. Values presented in bold, represent statistically significant data. GM CSF: Granulocyte macrophage colony-stimulating factor, TNF: Tumor necrosis factor, IFN: Interferon, IL: Interleukin

test Spearman's Rho coefficient was employed to find the correlation of cytokine and disease. Cytokine has been utilized as a biomarker for the for tear film cytokine profiling,[17] diagnosis of patients suffering from ocular diseases.[18]

The prevalence of orals squamous cell carcinoma and its rising propensity in the younger population has been tested using saliva as a biomarker.[19]

For the studies on immunocompetence, *in vitro* production of cytokine by PBMCs has been reported to be reliable as it can be the indicator for *in vivo* study also.[20] Salivary levels of Cytokines IL8 and IL1Beta can serve as early markers of OPMD.[21] Cytokine profiling results are on line with previous report where researchers claim about the possible involvement of TNF-α, IL-6, TGF-β, and IL-10 in cancer progression.[22] Tobacco exposure has effect on cytokine IL-12 (pro-inflammatory) and IL-10 (anti-inflammatory) which also affects median survival of prostate cancer patients in India.[23]

Increased level of IL6 causes activation of Janus kinase signal transducer and activator of transcription pathway by which tumor formation occur.[24]

GM-CSF has been reported to have pleiotropic effects on different cell lineages and its therapeutic potential in cancer.[25-27] Further studies are to be performed to diagnose the precise correlation.

The present study confirmed that pathogenesis of OPMDs and OSCC is owing to pro-inflammatory cytokines. The increase in salivary levels of IL-6, IL-12p70, and INF-α could be a useful indicator of malignant transformation process within the oral mucosa.

Conclusion

In India consumption of betel quid and its related products like panmasala and gutkha, is widespread. Research has been carried out on the relation of oral cancer with areca nut and its product. Few studies have been undertaken on testing the effect of panmasala and gutkha on cytokine secretion and its linkage to the oral cancer. Cytokines are low molecular weight proteins concerned with infection, immune responses and inflammation. The changes in cytokine sensitivity have been related with to OSCC. Hence, research studies point to the prospect of employing salivary pro- and anti-inflammatory proteins for screening of oral disorders. The target of the pioneering salivary diagnostics is the recognition of a single or multiple biomarkers that will provide a clinical test for the diagnosis of patients prone to develop OSMF, OSCC and OPMDs.

The studies on the effect of pan masala and gutkha on immunomodulation proved that contents in gutkha have pronounced effect on pro-inflammatory cytokine. Hence, gutkha causes more ill effects on immune function as compared to panmasala. Salivary diagnostics will be useful for studying the progression of oral cancer in people having

[Downloaded free from http://www.jdrr.org on Monday, October 10, 2022, IP: 117.211.126.242]

the habits of chewing betel quid, panmasala, gutkha, and tobacco. Current research will be helpful for the researchers and doctors working on the effect of tobacco and panmasala product. Experiment on the effect of pan masala and gutkha on immunomodulation, proved that contents in gutkha have pronounced effect on pro-inflammatory cytokine. Hence, gutkha causes more ill effects on immune function as compared to panmasala.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Garg A, Chaturvedi P, Mishra A, Datta S. A review on harmful effects of pan masala. Indian J Cancer 2015;52:663-6.
2. Nair U, Bartsch H, Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: A review of agents and causative mechanisms. Mutagenesis 2004;19:251-62.
3. Kumar DT, Rai A, Kumari DM, Rai DS, Kumar DA, Kumar DK. Clinical assessment of occurrence of oral submucous fibrosis in patients from Patna Dental College and Hospital. Int J Med Biomed Stud 2019;3:342-8.
4. World Health Organization. Global Data on Incidence of Oral Cancer, WHO/NMH/CHP/HPR/ORH. World Health Organization; 2005.
5. Rangaswamy S, Chikkalingaiah RG, Sanjeevarayappa PN, Govindraju P. Carcinoma arising in the background of oral submucous fibrosis. Ann Maxillofac Surg 2019;9:247-52.
6. Sahibzada HA, Kurshid D, Khan RS, Naseem M, Siddique KM, Mali M, *et al*. Salivary IL-8, IL-6 and TNF-α as potential diagnostic biomarkers for oral cancer. Diagnostics (Basel) 2017;7:21.
7. Brailo V, Vucicevic-Boras V, Lukac J, Biocina-Lukenda D, Zilic-Alajbeg I, Milenovic A, *et al*. Salivary and serum interleukin 1 beta, interleukin 6 and tumor necrosis factor alpha in patients with leukoplakia and oral cancer. Med Oral Patol Oral Cir Bucal 2012;17:e10-5.
8. Abdel-Haq A, Kusnierz-Cabala B, Darczuk D, Sobuta E, Dumnicka P, Wojas-Pelc A, *et al*. Interleukin-6 and neopterin levels in the serum and saliva of patients with Lichen planus and oral Lichen planus. J Oral Pathol Med 2014;43:734-9.
9. Selvam PN, Sadaksharan J. Salivary interleukin-6 in the detection of oral cancer and precancer. Asia-Pac J Clin Oncol 2015;11:236-41.
10. Krishnan R, Thayalan DK, Padmanaban R, Ramadas R, Annasamy RK, Anandan N. Association of serum and salivary tumor necrosis factor-α with histological grading in oral cancer and its role in differentiating premalignant and malignant oral disease. Asian Pac J Cancer Prev 2014;15:7141-8.
11. Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, *et al*. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res 2006;66:11565-70.
12. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. Carcinogenesis 2009;30:1073-81.
13. Jeng JH, Wang YJ, Chiang BL, Lee PH, Chan CP, Ho YS, *et al*. Roles of keratinocyte inflammation in oral cancer: Regulating the prostaglandin E2, interleukin-6 and TNF-alpfa production of oral epithelial cells by Areca nut extract and arecoline. Carcinogenesis 2003;24:1301-15.
14. Prasad G, McCullough M. Chemokines and cytokines as salivary biomarkers for the early diagnosis of oral cancer. Int J Dent 2013;2013:813756.
15. Mallone R, Mannering SI, Brooks-Worrell BM, Durinovic-Belló I, Cilio CM, Wong FS, *et al*. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: Position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin Exp Immunol 2011;163:33-49.
16. Deshmukh D, Chakrabarti M, Jayasudha R, Hasnat Ali M, Tyagi M, Sharma S, *et al*. Elevated cytokine levels in vitreous as biomarkers of disease severity in infectious endophthalmitis. PLoS One 2018;13:e0205292.
17. Balne PK, Au VB, Tong L, Ghosh A, Agrawal M, Connolly J, *et al*. Bead based multiplex assay for analysis of tear cytokine profiles. J Vis Exp 2017;128:E55993.
18. Le Guezennec X, Quah J, Tong L, Kim N. Human tear analysis with miniaturized multiplex cytokine assay on "wall-less" 96-well plate. Molecular Vision 2015;21:1151-61.
19. Csosz E, Kall´o G, Jakob B, De´ak E, Csutak A, T″ozs´er J. Quantitative body fluid proteomics in medicine A focus on minimal invasiveness. J Proteomics 2016;153:30-43.
20. Friberg D, Bryant J, Shannon W, Whiteside TL. *In vitro* cytokine production by normal human peripheral blood mononuclear cells as a measure of immunocompetence or the state of activation. Clin Diagn Lab Immunol 1994;1:261-8.
21. Aiswarya Lekshmy SU. Study of Interleukin 8 and Interleukin 1B in Saliva of Patients with Oral Squamous Cell Carcinoma and Oral Potentially Malignant Disorders. Master's thesis, Ragas Dental College and Hospital, Chennai; 2013.
22. Landskron G, de la Fuente M, Thuwajit, P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. J Immunol Res 2014:149185.
23. Dwivedi S, Goel A, Mandhan A, Khattri S, Pant KK. Tobacco exposure may enhance inflammation in prostate carcinoma patients: An explorative study in north Indian population. Toxicol Int 2014;19:310-8.
24. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Europ J Cancer 2005;41:2502-12.
25. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 2008;8:533-44.
26. Metcalf D. The colony-stimulating factors and cancer. Nat Rev Cancer 2010;10:425-34.
27. Zhan Y, Vega-Ramos J, Carrington EM, Villadangos JA, Lew AM, Xu Y. The inflammatory cytokine, GM-CSF, alters the developmental outcome of murine dendritic cells. Eur J Immunol 2012;42:2889-900.