Therapeutic and Antigenotoxic Effects of Lycopene in Managing OSMF - A randomized Placebo-Controlled Trial

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ABSTRACT

Introduction/Objective: Lycopene is effective in managing OSMF. Effect of Lycopene on reducing the Micronuclei frequency & correlation of Serum lycopene and BMCF has not been studied in OSMF. This study is designed to assess the effect of lycopene in reducing, clinical symptoms, as assessed by mouth opening (MO), Tongue protrusion (TP), Cheek flexibility (CF), Palpable fibrotic bands (FB), burning sensation (BS), and the genotoxicity in oral submucous fibrosis patients as assessed by buccal micro-nucleated cell frequency (BMCF).

Methods: A randomized, placebo-controlled, triple blinded trial, of 3 months duration. Out of 40 OSMF patients reported during the study period, 36 OSMF patients aged between 17-70 years who fulfilled the inclusion criteria were randomly divided into two groups equally- Group 1 received lycopene 16 mg (Lycored® 4mg 2 BID) per day, and Group 2 received placebo capsules (similar quantity). The outcomes MO, TP, CF, FB, BS, serum lycopene levels and BMCF were recorded at the baseline and the end of 3 months.

Results: In Group 1 compared to Group 2 clinical signs and symptoms MO, TP, BS improved significantly. Serum lycopene levels improved and BMCF reduced significantly. BMCF showed a negative correlation with the serum lycopene levels.

Conclusion: The results of the present randomized controlled study throw more light on the role of lycopene in combination with habit cessation in OSMF, both in the improvement of most of the clinical symptoms and as a potent antigenotoxic agent, by reducing BMCF.

Key Words: Mouth opening, Burning, Micronuclei, Oral submucous fibrosis, Lycopene, BMCF

INTRODUCTION

OSMF a potentially malignant disorder (PMD) has a reported 7–13% malignant transformation.¹Gutkha / areca nut chewing promotes gene damage, early detection of which may help prevent malignant transformation.^{2,3} MN assay is used as a marker of genotoxicity in oral PMD and malignancy.^{2,4}Management of OSMF aims to ameliorate the symptoms, and minimize the malignant transformation.⁵lycopene, a natural antioxidant is a useful therapeutic agent in OSMF.⁶Lycopene has antimutagenic and anti-mitogenic activity. It interferes with free radical chain reaction, cancer cell proliferation. Dietary supplement of lycopene has shown to improve se-

rum lycopene and to reduce the BMCF in PMDs.⁷There are no controlled studies to demonstrate the therapeutic effect of lycopene on disease symptoms and antimutagenic effect on BMCF in OSMF. Hence, the present placebo-controlled study was designed to assess the efficacy of lycopene as a therapeutic and anti-genotoxic agent in OSMF as assessed by BMNC frequency.

METHODS

36 OSMF patients, aged 17-70 years, visiting the Oral Medicine and Radiology department, were enrolled once the in-

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clusion and exclusion criteria were satisfied, using convenience sampling.

Diagnosis of oral submucous fibrosis was based on the classification criteria given by Pindborg JJ.⁸ Inclusion criteria were that patients with a six-month-long habit of chewing areca nut or any other product of areca nut and those who were compliant in quitting the habit following counselling. Patients on antioxidant therapy in recent past six months or who had any other concurrent oral mucosal disease, (e.g., oral cancer, other pre-cancer like leukoplakia, lichen planus) a known history of systemic disease were excluded. Group 1 consisted of 3,6,9 patients in OSMF stage 1, 2, & 3 respectively, Group 2 consisted of 12, & 6 patients in Stage 2 & 3 respectively.

Informed consent of all the participants was obtained. Institutional Ethical Committee gave ethical clearance for the study.

Before the administration of therapy, all the participants were subjected to oral prophylaxis. At every visit, compliance in habit cessation was checked.

Study design:

A randomized, placebo-controlled, triple blinded trial of three months duration.

Randomization:

36 OSMF patients aged between 17-70 were randomly allocated to two groups equally (using a computer-generated allocation series, by Stat Trek's *Random Number Generator*). [Fig 1]

Blinding:

Patients, the investigator (who administered the drugs and measured the outcomes), and the data analyst (person who analyzed the data) were blinded to which group the subject belonged. Allocation and coding were done by a trained clinician, who did not participate in the drug administration or outcome measurements.

Intervention:

Group 1 received lycopene, 16 mg (Lycored[®] 4mg 2 BID p.o) per day, and Group 2 received placebo (similar quantity). LycoRed[™] 4 mg soft gels & the placebo (manufactured by Jagsonpal Pharmaceuticals Ltd., New Delhi, India) were supplied as bottled LycoRed[™] soft gel capsules. Placebo was also dispensed in identical packages. 60 capsules were dispensed at the beginning and every visit (fortnightly). The remaining number of capsules was counted for checking patient compliance. Side effects, if any, were recorded at each follow-up.

Coding:

The packaging bottles contained 60 capsules each. All 36 patients' bottles were coded with the serial numbers, 1 to 36, 1-18 for lycopene, and 19-36 for placebo. Against each serial number, a code of 1 is written for lycopene and 2 for placebo and the patient's group allocation number. (Eg. Code 10(1,10,) is 10th patient in lycopene group, & 28 (2,10) is 10th patient in placebo group). Code was written on six bottles each to suffice for six visits. The codes were noted in a separate sheet against each patient and sealed and preserved for decoding later.

Outcome measurements:

Clinical, cytological and serum assessments were done at the baseline and the end of 3 months. Patients were recalled every 15 days to dispense the medicines, check for compliance and reassurance. Most of the recalls happened within +/-1 of the scheduled day. Clinical parameters recorded included maximum mouth opening, tongue protrusion, cheek flexibility, palpable fibrotic bands, and burning sensation. The distance between the mesio-incisal edges of the maxillary and mandibular right central incisors was measured using a Vernier calliper to record the mouth opening. The distance from the mesio-incisal angle of the upper central incisor to the tip of the extended tongue gave tongue protrusion measurements. The difference ($V_2 - V_1$) in the distance between the two points on a line from the right commissure of mouth to tragus was the Cheek flexibility. The first point was marked at the intersection of this line and the vertical line drawn from the pupil, and the second point was 1cm away toward the tragus. V_1 is the distance between the points at rest, & V_2 is when the patient fully blows his cheeks. The number of the fibrotic bands were recorded by palpation of buccal and labial mucosa. The burning sensation was scored on a Numeric Rating Scale (NRS). Based on the patient's response a marking from 0 to 100 was done, (Score 0: no pain; Score 100: severe pain).

Serum lycopene levels: 4ml fasting venous (from antecubital fossa) blood sample collected in sodium citrate vial, centrifuged for 10 min at 4000 rpm, to separate plasma. Plasma lycopene level was estimated through high-performance liquid chromatography (HPLC) under the chromatographic conditions, including a mobile phase of 47: 47: 06 (Acetonitrile: Methanol: Chloroform), the wavelength of 472 nm using Novak C₁₆ column at a flow rate of 1.5 ml/litre.

BMCF: Scrapings from the middle of buccal mucosa on both sides with a sterile wooden spatula yielded the buccal cells and transferred to slide and fixed with 70% isopropyl alcohol spray. Smears stained using haematoxylin and eosin stain were viewed in a light microscope, screened under ×10 magnification, and the micronuclei were counted using ×40. (Figure. 3)

For Quantification, a total of 1000 cells with intact nuclei and cell boundaries were counted per smear, starting from the left corner using a zigzag method for screening the slides for the presence and number of BMCF as per Tolbert et al.⁹ An oral pathologist and a postgraduate student who made the observations were blinded for the patient's study group. Evaluation for micronuclei was restricted to oral mucosa cells with intact nuclei.

Criteria for scoring included extrachromosomal cytoplasmic micronuclei of two to four picometers in diameter and had the same texture and intensity as the nucleus and were in the same focal plane as the nucleus.

Attrition:

There were two and four dropouts in Group I & 2, respectively. (Fig. 1) An intention to treat outcome analysis assuming the worst-case scenario was used to treat the uneven dropouts.

Statistical analysis:

SPSS Version 16.0 was used for the statistical analysis assuming a significance level of $p < 0.05$. The paired t-test and the independent t-test evaluated the intragroup and intergroup comparisons, Spearman's correlation coefficient the correlation between the serum lycopene levels and BMCF frequency, and Cronbach's alpha tests the inter-observer agreement.

RESULTS

The participants in each group were randomly assigned, received intended treatment, and were analyzed for the primary outcomes. Loss to follow up and the reasons for the losses are given in the flow chart. [Fig 1]

Demographics showed that the two groups had similar values for age, gender & habit index. [Table 1].

Comparison of baseline values of clinical parameters, serum lycopene levels and BMCF between the study groups showed no significant difference. [Table 2]

At the end of three months, both intra and intergroup comparisons were calculated, and the Lycopene group showed statistically significant improvement in the majority of the parameters. (figure 2) The placebo group also showed no significant improvement in all parameters except minimal improvement BS. The intergroup comparison results showed that the lycopene group showed a statistically significant improvement in MO, TP, and BS, serum lycopene levels, and BMCF frequency reduction than the placebo group. Improvement in mucosal bands and cheek flexibility was better in the Lycopene group; however, the difference was not statistically significant. [Table 2; Figure 4].

Group 1 showed a significant negative correlation between serum lycopene levels and oral BMCF frequency (p value=0.004). Adverse effects were nil. (Figure 5)

DISCUSSION

OSMF is a chronic, complex, potentially malignant disorder characterized by juxta-epithelial inflammatory changes of the oral mucosa.¹⁰ Often, reduced mouth opening is a chief complaint among OSMF patients, secondary to the underlying progressive fibrosis and involvement of the submucosal muscles and pterygomandibular raphe. In the present study, mouth opening showed a significant mean improvement of 3.3 ± 2.4 mm in the lycopene group. Similar results were reported in earlier studies (3.4 mm, 2-3 mm, respectively).^{5,11} thereby emphasizing the therapeutic effect of lycopene in addition to habit cessation.⁶

There was a significant increase in tongue protrusion in the lycopene Group (4.14 ± 3.23 mm) compared to placebo. Though no study has quoted the effect of lycopene on tongue protrusion, in this study lycopene has shown to be superior over the reported improvement of 1.7 ± 1.6 mm with pentoxifylline and 2.3 ± 0.4 mm with intralesional steroids combined with hyaluronidase.^{7,12}

There was minimal improvement in cheek flexibility and fibrotic bands in both groups, the difference was not remarkable in the lycopene group compared to the placebo. The results suggest a limited role of lycopene alone in improving cheek flexibility and resolving the fibrous bands in contrast to other therapeutic agents like oral pentoxifylline and intralesional dexamethasone with hyaluronidase injections.^{7,12} Additional mouth exercises may improve the outcome.

Lycopene has an inhibitory effect on the synthesis of collagenase-1, thus inhibiting the synthesis of new collagen and fibrogenesis. Also, lycopene helps in the degradation and remodelling of the already formed collagen fibres by suppressing the elevated levels of tissue inhibitors of matrix metalloproteinases.^{12,13} Previous studies have reported significantly lower levels of serum lycopene in patients with atrophic and erosive lichen planus and oral leukoplakia.^{14,15} Mayne et al. evaluated the association between serum micronutrient levels, including lycopene, and subsequent mortality in a cohort of 259 patients with primary oral/pharyngeal cancer.¹⁶ They found that only plasma lycopene was significantly inversely associated with total mortality. Thus, lycopene has been suggested as part of a treatment regimen in tobacco chewers to prevent the formation, induce remission, or inhibit the premalignant lesion's progression to malignancy.

The mean level of serum lycopene in OSMF patients in the present study was 0.117 ± 0.72 μ mol/L (75 ng/ml). Studies have shown mean serum lycopene levels of 0.57 ± 0.37

$\mu\text{mol/L}$ in healthy individuals, $0.47 \pm 0.38 \mu\text{mol/L}$ in smokers and $0.175 \pm 0.202 \mu\text{mol/L}$ in patients with leukoplakia.^{17,18} The decrease in plasma lycopene levels OSMF as in these conditions could be attributed to the oxidative stress caused by reactive oxygen species generated in substantial amounts following the use of betel quid, qualitative and quantitative differences in their dietary intake, absorption, and metabolism.¹⁹ The dose of 16mg per day is reported to be useful in managing OSMF.⁵ The baseline serum lycopene levels were similar in both the study groups. Patients made no change in the dietary sources of lycopene. At the end of 3 months, Group 1 showed a significant two-fold increase in serum lycopene values, Group 2 showed negligible improvement after the use of placebo. Mohanty et al. reported a similar two-fold increase of serum lycopene level, from 360 to 680 ng/mL in the treatment group.²⁰

BMCF count increases in tobacco chewers and smokers compared to healthy subjects, thereby suggesting cytogenetic damage in the oral mucosa.²¹ A previous study on OSMF subjects reported 0.6-5.3% oral BMCF frequency, similar to the present study with BMCF frequency of 0.4-4.0%.⁴ The significant reduction in BMCF in group 1 (Figure 2) suggests the effectiveness of lycopene in reducing BMCF and its possible role as an antimutagenic agent. Various studies performed on bacteria and rats showed decreased micronuclei count and chromosomal aberrations after the administration of lycopene.^{22,23} Similar studies in oral lichen planus and leukoplakia showed carotenoids' effect in reducing BMCF in the buccal mucosa.^{24,25}

It is reported that Micronuclei may not merely reflect random DNA injury but possibly a mechanism of cell protection. Micronuclei frequency examination has a sensitivity of 94%, a specificity of 100%, and an accuracy of 95%.²⁶

In a study by Benner SE et al., the micronuclei count in oral leukoplakia lesion showed a significant decrease from 1.94 ± 1.80 to 0.71 ± 0.90 following treatment with α -tocopherol. Changes in micronuclei frequency may be more useful in assessing response to a chemopreventive agent. Further research to assess the clinical significance of this small but statistically significant reduction in BMNC may be useful. If epithelial carcinogenesis is a multistep process driven by DNA damage and specific genetic events, it can also be hypothesised that long-term suppression of DNA injury may have a favourable impact on cancer incidence.²⁷

In a systematic review, Neha et al. reported the need for a more standardised controlled study to establish the efficacy of lycopene.²⁸ To the best of our knowledge, the present study is the first of its kind to establish the Therapeutic and antigenotoxic effect of lycopene in OSMF using a controlled study design.

CONCLUSION

Lycopene effectively alleviates the clinical signs and symptoms like MO, BS, and TP of OSMF. It is a potent antigenotoxic agent. The assessment of BMCF is non-invasive and easy. Hence, lycopene is recommended as a treatment modality, supported by habit cessation. The effect size of the small but statistically significant changes observed in the present study needs further research on clinical significance. It may be a suitable adjuvant in treatments like intralesional injections, surgeries, and physiotherapy, which primarily aim to address the improvements in function like mouth opening but fail to address the potentially malignant cellular genotoxic changes occurring in the epithelium. BMCF may be used as a diagnostic and prognostic indicator for the genetic damage of oral mucosa in OSMF

Large-scale randomized trials with stratified samples are needed to support lycopene's antigenotoxic effects in various stages of OSMF patients.

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Figure 1: Flow diagram of the progress of the study.

Figure 2: Pre and post treatment changes in mouth opening in Group 1 patient.

Figure 3: Photomicrograph of buccal smear of OSMF patients showing Micronucleated cells with (H&E 40X).

Figure 4: Stacked graph showing the improvements in Lycopene & Placebo groups.

Figure 5: Correlation between S. Lycopene and BMCF, before and after treatment in Group 1 & Group 2.

Table 1: Showing the age, Sex and habit index distribution among the two groups

Group	Group 1	Group 2	t- test	p- Value
Mean age in yrs	35.38	29.5	1.429	0.162
Gender Male: Female	15:3	17:1		
Mean Habit index	56.139	46.833	.508	.615

Table 2: Comparison of improvement in various parameters in both the groups (Mean±SD) (N=18 in each group)

Parameters	Pre treatment		Post treatment		Improvement		p- Value
	Lycopene	Placebo	Lycopene	Placebo	Lycopene	Placebo	
Mouth opening (mm)	26.8±6.9*	26.7±4.2	30.1±7.9	28.3±5.3	3.3±2.4	1.6±1.8	0.028*
Tongue protrusion (mm)	36.8±7.5	38.5±5.6	41.0±10.0	39.2±5.8	4.1±3.2	0.7±0.8	<0.05*
Cheek flexibility(mm)	0.8±0.3	0.6±0.3	1.0±0.4	0.7±0.4	0.2±0.2	0.14±0.15	0.34
Palpable fibrotic bands	4.28±1.1	3.7±1.1	3.7±1.2	3.5±1.1	0.5±0.9	0.2±0.5	0.39
Burning sensation (NRS)	7.4±2.4	6.1±2.8	2.1±2.2	2.2±1.9	-5.3±2.2	-3.8±1.8	0.036*
Serum lycopene level (ng/ml)	124.1±88.9	111.0±53.8	323.4±250.4	113.5±58.6	119.3±191.2	2.5±12.8	<0.05*
Oral micronucleated cell frequency (%)	1.7±0.9	1.4±0.6	0.9±0.6	1.4±0.6	-0.7±0.7	0.02±0.2	<0.05*