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Clinical Study

A Cytogenetic Study on the Efficacy of Chyawanprash Awaleha as an Antioxidant in Oral Premalignant Cancer

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Background. Chyawanprash awaleha (Cp) is an Ayurvedic rasayana formulation and is used as a genoprotective agent. *Objective*. The present cytogenetic study has been done to investigate the efficacy of Cp against betel quid chewers suffering from oral precancerous lesions through satellite association (SA) assay. *Materials and Methods*. The frequency of SA was analyzed in 21 betel quid chewing oral precancerous lesions patients and then they were divided into 2 groups. Group 1 consisted of 15 patients, advised to quit betel quid chewing and fed with 20 gms of Cp, twice a day for three months. Group 2 consisted of 6 patients, who refused Cp feed but accepted to quit betel quid chewing. At the end of three months, both groups were assessed cytogenetically. *Results*. The frequency of SA was statistically significant in both groups, but an elevated mean difference was observed more in Group 1 than in Group 2. *Conclusion*. The study indicates that betel quid cessation reduces the effect of DNA damage in oral precancerous lesions. But the increased mean difference in SA in Group 1 compared to Group 2 clearly indicates that Cp can further minimize the genotoxic effect caused by mutagenic agents present in betel quid.

1. Introduction

In India, annually 130,000 people die of tobacco related oral cancer taking it to a third position as the most common cancer [1]. Detection, histopathological investigation, genetic tests, creating awareness for tobacco cessation, and treating tobacco related oral cancer patients in their premalignant state are the only hope in lessening the mortality and morbidity associated with the disease. Identifying the patients of their genetic damage through cytogenetic studies and treating them with Chyawanprash Awaleha, an antioxidant, seemed to be a promising strategy and an attractive alternative that reduces toxic effects of tobacco. Chyawanprash Awaleha is an Ayurvedic rasayana formulation, prepared from more than 40 herbs, with *Phyllanthus emblica*, a rich source of antioxidants as its primary content [2]. Chyawanprash Awaleha known for its anti-inflammatory, antioxidant, and anticarcinogenic properties has been used in India as a health food continuously for the past 4000 years [3]. It is almost a house old product in India and is taken as a health food by the young and old people.

The last four decades have witnessed the introduction of a number of relatively rapid genetic tests for detecting the activity of mutagenic and/or carcinogenic chemicals. Among them, satellite chromosomes in associations appear to be one of the most suitable tests to assess the toxic effects of carcinogens like tobacco on chromosomes. Satellite associations (SAs) reflect chromosomal damage and provide a valuable biomarker in detecting carcinogenesis. The phenomena of SA, where satellite chromosomes assume a specific position with their satellites directed towards each other, were first observed in mitotic human chromosomes and were later also found in meiotic chromosomes [4, 5]. The sticky nucleolar material has a tendency to hold the associated chromosomes together through mitosis [6]. Higher frequency of SA was seen in test mothers using oral contraceptives than in control mothers and in Down syndrome, XXY Klinefelter, and XO

Turner syndrome, suggesting that drug intake and chromosomal anomalies predispose to satellite associations [7, 8]. An increased incidence of SA and chromosomal aberrations has been reported in smokers [9]. The frequency of SAs was found to decrease when treated with antioxidants in the diet of the smokers, indicating antioxidants have the capacity to minimize the genotoxicity of the mutagenic agents present in tobacco smoke [2].

The present cytogenetic study in the lymphocyte cultures of betel quid chewing oral precancerous lesion patients was conducted to evaluate the frequency of satellite association and to investigate the efficacy of Chyawanprash Awaleha, paving a way to subsequent disease management.

2. Materials and Methods

2.1. Sample Collection. This observational study included patients who visited a tertiary care hospital, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India, during the years 2012 to 2013. The study group consisted of 21 patients who were in the habit of only betel quid chewing. The "betel quid" ingredients in the study group consisted of betel leaf, areca nut and slaked lime, and sun-dried tobacco. Out of 21 patients, 19 were males and 2 were females, suspected of having oral precancerous lesions on clinical criteria and later by oral tissue biopsy were histopathologically confirmed as premalignant lesions/conditions by the pathologists were included in the study. Patients undergoing radiation treatment, smoking tobacco, and consuming alcoholics and patients with chromosomal anomalies like Klinefelter syndrome, Turner syndromes, and so forth were excluded from the study. Out of the 21 premalignant cancer cases, 11 had leukoplakia (LKP) and 10 submucosal fibrosis (SMF) on clinical criteria and histopathologically out of 11 LKP, 4 showed moderate dysplasia and 7 showed severe dysplasia. Out of 10 SMF, 5 showed intermediate stage and 5 showed advanced stage. The age ranged from 23 to 65 years and the mean age was 41.9 ± 17.7. The duration of their habits was 5–40 years.

2.2. Leukocyte Culture and Slide Preparation. Lymphocyte cultures were set up from heparinized blood, following standard Hungerford method [10]. 0.5 mL of heparinized blood was added to two culture vials for each patient containing 5 mL the complete culture media (Biological Industries, Israel, 01-201-1B). The vials were incubated for sixty-nine and a half hours at 37°C. CO_2 was released each day and the culture vials were shaken 2-3 times daily. At the end of the incubation a drop of 0.01% of colchicine was added to arrest the dividing cells at metaphase and further incubated for 30 mins. The contents of the vials were then transferred to centrifuge tubes and spun at 1500 rpm for 5 mins. The supernatant was discarded and 6 mL of hypotonic (0.075 M KCl) solution was added. Again after incubating for 15 mins, being centrifuged at 1500 rpm, the supernatant was discarded. To the cell pellet 6 mL of freshly prepared fixative (3 : 1, methanol : glacial acetic acid) was added. After an hour, the tubes were again centrifuged and after one more washing a clear white cell pellet was obtained. The cell button was suspended in a small quantity of 7-8 drops of freshly prepared fixative. Two to 4 slides were prepared from each vial by placing a drop of the cell suspension on a previously cooled, clean glass slide and dried in a slide warmer kept at 40°C. The slides were examined under a light microscope (condenser lowered) to see whether the concentration of cells and the spread of the chromosomes enable a detailed examination of metaphase. The slides were then stained in 4% buffered solution of Giemsa for 7–10 minutes, rinsed in running tap water, and air-dried. For each individual, 50 well-spread metaphase plates were analyzed from 2 slides selected at random (one from each vial) for SAs involving a specific arrangement of acrocentric chromosomes of "D" group (chromosomes 13, 14, and 15) and "G" group (chromosomes 21 and 22) with their satellites directed towards each other (Figures 1 and 2). For identifying SAs, the criteria used by Hansson [11] were applied; that is, the satellite ends of the associating chromosomes had to be directed towards each other with their longitudinal axes meeting between their short arms, and the distance between the centromeres of associated chromosomes should not exceed the total length of one "G" chromosome after excluding its satellite. The metaphase cells were digitally imaged with a Cytovision Applied Spectral Imaging System (Applied Spectral Imaging, Israel).

After taking these observations, they were divided into 2 groups. Group 1 consisted of 15 patients (4 moderate dysplasia, 4 severe dysplasia, 3 intermediate stage, and 4 advanced stage), fed with Chyawanprash Awaleha (Cp), 20 gms twice a day for 3 months [2]. A complete cessation of betel quid chewing was ensured during the period of intake

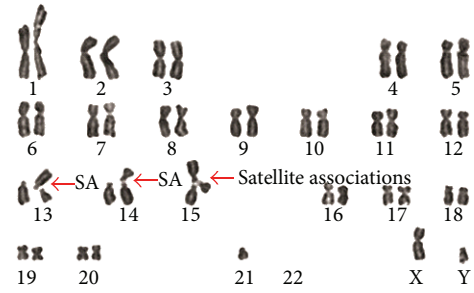

Figure 1: Karyotype from the lymphocyte cultures of oral precancerous lesion patient showing DG and DDG satellite associations of chromosomes.

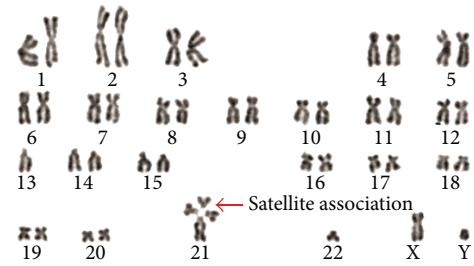

Figure 2: Karyotype from the lymphocyte cultures of oral precancerous lesion patient showing DGGG satellite associations of chromosomes.

Table 1: Paired t-test for Group 1: comparison of the frequency of SA before and after quitting betel quid chewing and Cp feed, respectively.

Group 1	Mean SA	Std deviation	t	P value
Before	58.73	14.90	12.112	0.0001 (S)
After	25.20	6.83		

Table 2: Paired t-test for Group 2: comparison of the frequency of SA before and after quitting betel quid chewing, respectively.

Group 2	Mean SA	Std deviation	t	P value
Before	40.00	8.76	5.031	0.004 (S)
After	31.00	4.732		

of Cp. Group 2 consisted of 6 patients (3 severe dysplasia and 3 intermediate stage), who refused Cp feed but accepted a complete quitting of betel quid chewing, and were also continuously monitored. After 3 months lymphocyte cultures were set up from the heparinized blood from Group 1 and Group 2 patients; SA was again analyzed from 50 well-spread metaphase plates.

2.3. Statistical Analysis. The data obtained were statistically analyzed by the Paired t-test using SPSS statistical software 16 Version. P values less than 0.05 are taken as significant (S).

2.4. Ethical Approval. Informed written consent was taken from all the 21 participants. The study was designed in accordance with the Helsinki II declaration and approved by the Institutional Human Ethical Committee, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India.

3. Results

At the end of three months even though both the groups had completely quit betel quid chewing, the problem of acute burning sensation in the mouth and the clinical presentations of the oral precancerous lesions in Group 1 patients had almost disappeared than Group 2 patients. The frequency of SAs was analyzed from Group 1 patients before quitting betel quid and the same patients SA was analyzed after three months of quitting betel quid and taking Cp feed. SAs were also analyzed on Group 2 patients before and three months after betel quid quitting. The frequency of SA was statistically significant (S) both in Group 1 ($P < 0.0001$) and in Group 2 ($P < 0.004$) as represented in Tables 1 and 2, respectively, with or without Cp feed. But, while comparing the mean differences of Group 1 before and after treatment showed an increase in the mean SA value difference of 33.53 than Group 2 which showed a value 9.00. Histopathological examinations after 3 months revealed a more positive change in Group 1 than Group 2 patients. In Group 1 all the 4 severe dysplasias of LKP patients after three months of treatment showed moderate dysplasia while the 4 moderate dysplasias showed mild dysplasia. Likewise, the 4 advanced stages of SMF showed intermediate stages while from the 3 intermediate stage patients, 2 showed early stages and 1 remained the same. In Group 2, 3 cases of severe SMF dysplasia showed moderate to severe dysplasia while the 3 intermediate stages still showed the same histopathological diagnosis.

4. Discussion

Tobacco related oral cancer is a common and lethal malignancy. The role of betel quid chewing, tobacco chewing, and smoking in the etiology of oral cancer disease has been known for many decades, and any approach aimed at expediting the early detection and treatment of population subgroups at increased risk should be assigned high priority. Therefore, cytogenetic damage in tobacco related premalignant oral cancer patients seems to be an excellent biomarker for determining the effect of exposure to chromosome-damaging agents in betel quid and also to determine the efficacy of the antioxidant property of Cp in such individuals.

The analysis of SAs has gained popularity as an in vitro genotoxicity test and a biomarker assay in humans for genotoxic exposure and effect, as the scoring of SA is relatively simple, requires only a short training and is not very time consuming. A number of studies have been designed to evaluate the potential influence of factors such as gender, age, tobacco chewing, or smoking habit on SA frequency. An increase in the frequencies of SA has been found to increase with cigarette smoke, hookah smoke, and bidi smoke [12–14]. Chromosome-damaging activity of saliva of betel nut and tobacco chewers and its genotoxic association were seen in oral, pharyngeal, and esophageal carcinomas [15]. A cytogenetic study to assess the chromosomal damage among pan chewers demonstrated a more statistically significant increase than the nonconsuming controls [16]. Increased cytogenetic damage has been observed in peripheral blood lymphocytes and exfoliated buccal mucosal cells of pan masala chewers [17].

The paired "t" test from both the groups, namely, frequency of SA before and after quitting betel quid chewing and Cp feed in Group 1 (Table 1) and frequency of SA before and after quitting betel quid chewing only in Group 2 (Table 2), showed a statistically significant P value indicating strongly that whether we take antioxidants as a supplementary diet or not, quitting tobacco should be highly recommended as an excellent interventional tool to prevent the malignant transformation of precancerous lesions and also in long run cure such patients completely without any recurrence of the disease. But in the present investigation when we compared their SAs' mean difference, before and after treatment there was an increased mean value of SA in Group 1 (33.53) than Group 2 (9.00), which could have perhaps been due to the antioxidant property of Cp which has been well established [2, 18].

Cancer is a multistep process involving at least two stages: initiation and promotion. Initiation involves an irreversible alteration of the cellular DNA that permits the carcinogenic transformation of the cell [19]. Promotion

produces conditions that allow the initiated cells to become unstable so that it produces cancer. The super oxide and other oxyradicals are known to be involved in promotion [20]. Since promotion is reversible, there continues to be a hope that the use of antioxidants that control free radical reaction can protect initiated cells against promotion and thus prevent the ultimate development of cancer [21]. Positive histopathological changes were observed in oral SMF patients after treatment with antioxidants, curcumin, and turmeric oil [22]. Chemopreventive role of the so-called antioxidant nutrients, beta-carotene, vitamin C, and vitamin E against oral cavity cancer has also been well documented [23–25]. A major advantage of the antioxidants is that they are generally effective against a wide range of mutagens, both exogenous and endogenous [26]. Chyawanprash was found to possess significant antioxidant property mainly because of its *Phyllanthus emblica* contents, which constitute 90% of its bulk, along with a large number of other secondary plant metabolites [2]. The flavonoid, tannin, and phenolic constituents of Chyawanprash have also been reported to exhibit antioxidant properties. While characterizing the antioxidant activity of amla, Khopde et al. concluded that amla was a more potent antioxidant than vitamin C and also showed that it was much superior antioxidant when combined with other polyphenols than when compared to their equivalent amounts in pure isolated forms [27]. Therefore, with supplementation of diet like Chyawanprash having free radical scavenging capability seems to be a potentially useful approach to reduce genetic damage and to minimize adverse health outcome in betel quid chewing oral precancerous lesion patients as evidently seen in the present study with an increased reduction of SA in Group 1 patients than in Group 2 patients. On examination, more positive histopathological changes observed in Group 1 patients than Group 2 patients also confirm the superior antioxidant capacity of Cp [22]. The anti-inflammatory property of Cp could have brought down the burning sensation in the mouth and diminished appearance of oral cancerous lesions in Group 1 patients than Group 2 patients [2, 27, 28]. Thus even though quitting betel quid chewing remains the treatment of choice for premalignant oral lesions, there is a role for antioxidants in the treatment provided they are used and monitored with caution [29].

5. Conclusion

The study has paved way for genetic counseling, intervention for tobacco cessation, early treatment, and disease management tailored to the individual patient. It is evident from the present study that the antioxidant property present in Chyawanprash Awaleha seemed to have reduced the genetic damage in betel quid chewing oral precancerous lesion patients as clearly observed in the positive histopathological changes and reduction in SAs. The long-term effectiveness of Cp has not been established to date and may require further study. Yet, in a place like India, traditional medication seems to be the need of the hour and Cp may hold a good promise in the treatment of premalignant cancer, providing an early cure for such patients to lead a normal healthy life.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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