A Comparative Evaluation of Subgingival Occurrence of *Candida* Species in Periodontal Pockets of Female Patients Using Hormonal Contraceptives and Non-users – A Clinical and Microbiological Study

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Abstract

Aim: i) To determine the influence of hormonal contraceptive usage on the distribution of *Candida* species in females of child-bearing age, ii) To find associations between hormonal contraceptive use and various periodontal clinical parameters.

Method & Materials: 82 female patients in the 19-45 years age group were divided into 2 groups: Group I-Hormonal contraceptive (HC) users with chronic periodontitis; Group II-Female patients with chronic periodontitis. Periodontal parameters such as mean periodontal pocket depth, mean clinical attachment Loss, mean gingival index and mean plaque index were evaluated. Pooled subgingival sample from the deepest pockets in each quadrant using sterile paper points and sterile curette was obtained from each patient and immediately streaked onto Sabouraud's dextrose agar. Species identification was done by colony colour on CHROM agar medium and Dalmau plate culture technique on corn meal agar.

Results: Among the HC user group and the non-user group, the prevalence of *Candida* species in periodontal pockets was 26.8% and 29.3% respectively. Comparing the prevalence of various *Candida* sub species between the two groups, it was statistically not significant. There was no statistically significant differences between the two groups for mean plaque index, mean gingival index, mean probing depth and mean CAL (P>0.05).

Conclusion: *C. albicans* was the most common Candida species isolated from both groups, followed by *Candida dubliniensis*, *Candida Krusei, Candida tropicalis, Candida glabrata* and *Candida parapsilosis*. No statistically significant difference in the *candida* count or periodontal clinical parameters between the hormonal contraceptive users and non-users group was found.

Key Words: Hormonal contraceptives, Chronic periodontitis, Candida albicans, Candida krusei, Microbiological, Female patients

Introduction

Bacterial plaque has been established as the primary etiological factor in the initiation and progression of periodontal disease [1]. Several hormones have been suggested as important modifying factors that may influence the pathogenesis of periodontal disease [2]. Women on oral contraceptives have shown a higher tendency towards bleeding and inflammation with an increase in gingival crevicular fluid [3-5], loss of attachment [6], and periodontal pocket depth [7]. Injectable contraceptives have also shown a negative impact on gingival health. Additionally alterations in the composition of the subgingival plaque have also been reported toward a marked increase in the proportions of prevotella intermedia [8]. This association between periodontal conditions and sex hormones is evident in the recent periodontal disease classification, which includes the following hormone related categories: puberty associated gingivitis, menstrual cycle associated gingivitis, oral contraceptive- associated gingivitis and pregnancy associated gingivitis [9].

Candida species are yeasts and within the oral cavity, *Candida albicans* is the most frequently isolated. There is clear evidence that *C. albicans* adheres to oral surfaces including acrylic dentures and mucosa. However, *Candida* species have also been isolated from subgingival sites [10-12]. In that milieu, it may act directly or in concert with subgingival bacterial pathogens, or as a cofactor by inducing production of pro-inflammatory cytokines to increase the occurrence of periodontal attachment loss [13]. Recently *Candida* species has been isolated from subgingival sites of women with chronic periodontitis on hormonal contraceptives (HC) [14].

Hence the aim of this study is to evaluate the distribution of Candida species in periodontal pockets in female patients on hormonal contraceptives and non-users. Moreover, a further aim is to find associations between hormonal contraceptive use and the various periodontal clinical parameters.

Materials and Method

A cross sectional study was carried out for a period of 12 months. Patients attending M.S.Ramaiah Dental College and Hospital and M.S. Ramaiah Medical College, Bangalore with history of hormonal contraceptives, i.e., oral and/or injectable contraceptives were included in this study. The sample size was evaluated using N- Master software from the relevant citation [14]. Desired level of significance (1-α) was 95%, for which the required sample size was 41 was arrived at. The sample size was divided into 2 groups as follows: Group I - Hormonal contraceptive users with chronic periodontitis: 41 female patients. Group II – Female patients in same age group with chronic periodontitis: 41 female patients. To be eligible for the study, the patient had to present the following inclusion criteria: Group I – (1) Female patients with age group between 19-45 years, (2) Subjects using hormonal contraceptives either in oral or injectable form for at least a minimum of 8 months, (3) Chronic periodontitis according to Tonetti & Claffey (presence of proximal attachment loss of 5 mm or more in 30% or more of teeth present) [15], Group II – (1) Female patients patients with age group between 19-45 years, (2) chronic periodontitis according to Tonetti & Claffey (presence of proximal attachment loss of 5 mm or more in 30% or more

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of teeth present) [15], (3) Subjects not using any form of hormonal contraceptive medication. The exclusion criteria were as follows: (1) Subjects with metabolic or systemic disorders. E.g. Diabetes, epilepsy, hypertension or metabolic syndrome, (2) Subjects who have had antibiotic therapy in the past 6 months, (3) Subjects who are lactating. The study protocol was approved by the institution's Ethics Committee.

Method of collecting data

A detailed questionnaire was completed by each patient recording a full medical history and hormonal contraceptive usage before clinical examination. Specifically, the type of Hormonal contraceptive and the duration of the medication were recorded. All patients were examined by the same examiner.

Evaluation of clinical parameters

Plaque and gingival inflammation were measured for each site using the indices proposed by Silness and Loe [16] and Loe and Silness [17] respectively. The data for these measurements were presented as the percentage of sites for each patient exhibiting plaque (i.e., plaque index ≥1 and ≥2) and gingival inflammation (gingival index [GI] ≥1 and ≥2). A comprehensive periodontal examination including probing depths (six sites per tooth) and number of teeth was evaluated. Clinical attachment levels were calculated using the cemento-enamel junction or margin of a crown as the reference landmark *(Figure 1a)*. For instance, whenever a gingival recession was present, CAL was measured by adding the probing depth to the distance from the cemento-enamel junction to the free gingival margin.

Specimen collection

Each subject provided a pooled subgingival sample from the deepest pockets in each quadrant using a minimum of four and a maximum of eight sterile paper points per patient. After supra-gingival scaling, paper points were inserted into the gingival sulcus/pockets for 60 seconds and placed thereafter in peptone water for transport. As per Portela [18] Subgingival plaque samples which were obtained using sterile

paper points where inoculated in Sabouraud's Dextrose Agar *(Figure 1b)*. From the same subjects, as per Jabra-Rizk [19], subgingival samples were also collected from periodontal sites with a sterile curette *(Figure 1c)*. Subgingival samples were immediately streaked onto Sabouraud's dextrose agar plates *(Figure 1d)*.

Specimen identification and speciation

Sabouraud's Agar plates were incubated at 35 to 37°C, for 48 to 72 h and checked daily for growth *(Figure 2a)*. *Candida* species were identified by colony character and gram stain. Germ tube test was done to speciate *Candida albicans* and non-albicans species. Species identification was done by colony colour on CHROM agar medium *(Figure 2b)* and Dalmau plate culture technique on corn meal agar. Results were confirmed by carbohydrate fermentation and assimilation tests. Gram staining was done and all the germ tube positives were subjected to growth at 42°C to differentiate between *Candida albicans* and *Candida dubliniensis* as *C. dubliniensis* does not grow at 42°C *(Figure 3a & 3b)*.

Statistical analysis

The means of all the sites were calculated for each individual, for all the outcome variables. The following methods of statistical analysis have been used in this study. The results for each parameter (numbers and percentages) for discrete data and averaged (mean ± standard deviation) for each parameter were calculated.

Proportions were compared using Chi-square test of significance. The student 't' test was used to determine whether there was a statistical difference between the groups in the parameters measured. Statistical analysis was performed per protocol, with the program SPSS for Windows (version 10.0). A level of significance of 5% was used in all the statistical tests.

Figure 2. *Microbiological culture of Candida species. (a) Growth of Candida species on Sabouraud's Agar plates after incubation. (b) Species identification on CHROM agar medium.*
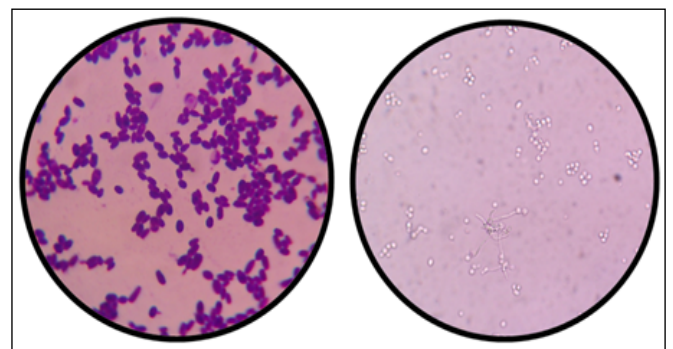
Figure 3. *Microscopical identification of Candida species. (a) Gram staining done. (b) Germ tube test positive.*

Figure 1. *Collection of subgingival sample. (a) Evaluation of clinical parameters. (b) Subgingival plaque samples collection using sterile paper points. (c) Subgingival plaque samples collection using sterile periodontal curette. (d) Samples streaked onto Sabouraud's dextrose agar plates.*

Results

Subjects were recruited from June 2012 to May 2013. The mean age group of patients in the hormonal contraceptive group and non-user group was 30.12 and 36.71 respectively. Among the hormonal contraceptive users, 73.2% of the subjects were using Injectable form of hormonal contraception and 26.8% of the subjects were using Oral form of hormonal contraception. The duration of hormonal contraceptive use among users is ranged between 8 months and 48 months.

Among the hormonal contraceptive group and the non-user group, the prevalence of *Candida* species in periodontal pockets was 26.8% and 29.3% respectively, while 73.2% in the Hormonal contraceptive group and 70.7% in the non-user group were culture negative *(Figure 4)*. The distribution of Candida sub species in both the groups was compared and it was found to be statistically non-significant (P>0.05) *(Table 1)*.

Mean plaque index, Mean gingival index, Mean probing depth and Mean CAL among hormonal contraceptive users were 1.73 ± 0.44, 1.76 ± 0.44, 5.47 ± 1.05 mm and 5.03 ± 1.51 mm respectively. Mean plaque index, Mean gingival index, Mean probing depth and Mean CAL among non-users were 1.75 ± 0.47, 1.76 ± 0.46, 5.74 ± 1.14 mm and 5.50 ± 1.55 respectively. It was found to be statistically non-significant (P>0.05). Correlating the presence of *Candida* species with the severity of chronic periodontitis among the hormonal contraceptive and non-user group was done by comparing the Mean plaque index, Mean gingival index, Mean probing depth and Mean CAL between the two groups. It was found to be statistically non- significant (P>0.05) *(Figure 5-6)*.

Discussion

In this study, the authors have observed no significant differences in the periodontal parameters such as clinical attachment loss, periodontal probing depth, gingival and plaque indices. This is in accordance with the NHANES survey done by Taichman [20]**.** In NHANES III it was anticipated that women using the modern low dose OC pills which were available during that period would exhibit an increased, yet less marked, prevalence of gingivitis and periodontitis than those not using OCs. It was concluded that there was no statistically significant differences between plaque scores, gingival scores or loss of attachment in individuals taking OCs were seen when compared to controls.
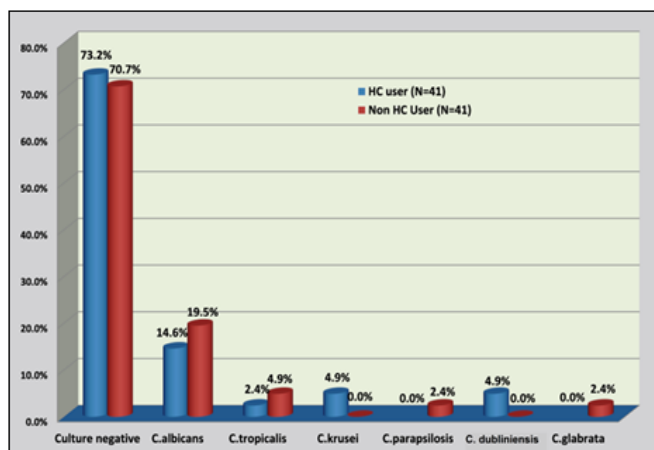

Figure 4. *Prevalence of Candida species in periodontal pockets.*

Another study demonstrated similar results to this study. Subgingival plaque samples of healthy women were investigated bacteriologically before subjects took a contraceptive and 10 and 20 days after subjects started the medication. It was observed that there were no changes in clinical parameters of the teeth investigated during 3 weeks of the study. On the other hand, the periodontopathogenic species Prevotella intermedia was found in plaque samples of 22 women [21].

A review of a recent clinical study investigating low-dose OCs lends support to our findings. The study demonstrated, using a prospective, split-mouth, experimental gingivitis model in 30 premenopausal women that low-dose OC formulations failed to intensify gingival disease after evaluating mean plaque index, gingival index, or gingival crevicular fluid volumes. These findings, in keeping with ours, resulted in the authors concluding that low-dose OC formulations do not influence the inflammatory response of the gingival tissues to dental plaque [5].

Conversely, in a clinical study which examined the effect of OCs and injectable progestin-only contraceptives on periodontal conditions, found that women who used hormonal contraceptives for <2 years showed higher mean GI scores than non-users [6]. However, the study did not differentiate between participants using OCs or injectable progestin-only hormones. As Preshaw & Mariotti point out [5], this is of potential importance because the daily dose of progestin from the progestin-only injectable method is estimated to be 10 times higher than the dose of progestin found in the OC formulation used in the Tilakaratne et al study [6].

The lack of an association between hormonal contraceptives use and periodontitis shown in our analysis supports a previous study [22], which detected no change in the periodontal attachment levels in women taking oral contraceptives compared to controls. In contrast, another study reported a significant loss of attachment in women using oral contraceptives for over 18 months, although there were no differences in gingival inflammation between OC users and non-users [23].

The usage of injectable contraceptives in this study did not show significant association with the periodontal parameters. This is in contrary to a study**,** which is the only study published in literature with regards to Injectable Depot medroxyprogesterone (DMPA) use and periodontal health. The study suggests that DMPA use may be associated with periodontal diseases. The prevalence of gingivitis was 53.9% for females who reported having used DMPA compared with 46.1% for DMPA never-users [24].

Also in contrary to this study, a study showed that OC use may increase the risk of severe periodontitis. OC users, particularly smokers showed a statistically significant increase in the prevalence of severe periodontitis. OC users had deeper probing depths (≥ 5 mm) than non-users. Moreover, OC users had higher gingival index scores and clinical attachment loss, ≥ 2 and ≥ 5 mm, respectively, than non-users [14].

In the oral cavity, yeasts commonly colonize the tongue, palate, and buccal mucosa [25]. It has also occurred in the subgingival plaque of adults with severe periodontitis [11]. Yeasts, especially *C. albicans* have been recovered from periodontal pockets in a large number of patients with chronic

Table 1. *Distribution of various candida species among candida positive subjects - hormonal contraceptive users versus non users. *P<0.05= Statistically significant, P>0.05=Not significant.*

	ORGANISM						Total
	C. albicans	*C. tropicalis*	*C. krusei*	*C. parapsilosis*	*C. dubliensis*	*C. glabrata*	
HC user	6	1	2	0	2	0	11
	54.50%	9.10%	18.20%	0.00%	18.20%	0.00%	100.00%
Non HC User	8	2	0	1	0	1	12
	66.70%	16.70%	0.00%	8.30%	0.00%	8.30%	100.00%
Total	14	3	2	1	2	1	23
	60.90%	13.00%	8.70%	4.30%	8.70%	4.30%	100.00%
P value	0.253						
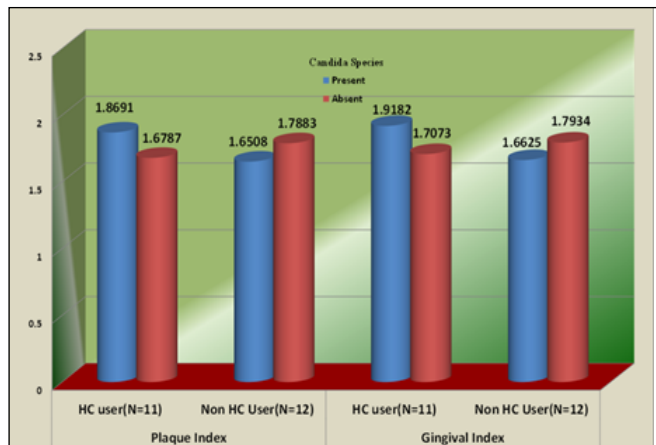
Figure 5. *Correlating the presence of candida species with clinical parameters (PI, GI) among the groups.*
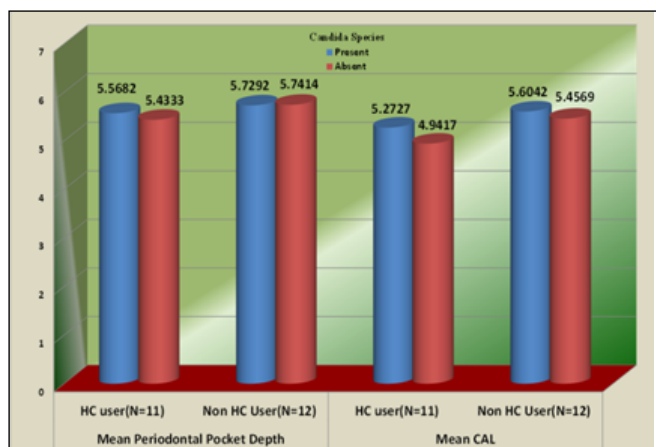
Figure 6. *Correlating the presence of candida species with clinical parameters (Mean PPD, Mean CAL) among the groups.*

periodontitis demonstrating prevalence rates of 7.1% to 44.4% [14]. In this study we have shown a prevalence rate of 28.0% which is in accordance with various studies published in the literature.

A recent study is in accordance with our study showing a prevalence rate of 30%. Twelve patients (30%) with CP presented yeasts in the subgingival biofilm while only three patients (15%) in the healthy subjects group were positive for these microorganisms. Although several yeast species were found such as *C. parapsilosis*, *Rhodotorula sp.*, *C. dubliniensis* and *C. tropicalis*, only *C. albicans* was present in all the patients with yeast-positive CP [26]. Another study showed a prevalence rate of 34.3% from the periodontal pockets of marginal periodontitis patients and 42.2% of the healthy subjects. *C. albicans*, *C. dubliniensis* and *C. parapsilosis* and 19 biotypes were identified from the marginal periodontitis

patients [27].

A recent study evaluated the prevalence of *Candida* species in subgingival sites of women using oral contraceptives. The prevalence of *Candida* colonization was 95.1% in the OC group and 78.4% in the control group. The reason for the high percentage of *Candida* colonization which the authors quoted was unknown but it may be linked to gender and dietary factors [14]. This high prevalence rate is in contrary to our study.

Several yeast species have been isolated from periodontal pockets in patients with periodontitis. *C. albicans* was the species most frequently isolated but other species including *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lipolytica* and *C. guilliermondii* were isolated. Other species such as Saccharomyces cerevisiae, Trichosporon mucoides and Rhodotorula spp. have also been identified [10-12,14,18,19].

Although in this study the number of *Candida* species in the group of patients taking hormonal contraceptives versus the control group was very similar, some species were more frequently isolated in one of the two groups. Only one study has demonstrated the presence of *Candida krusei* isolated from periodontal pockets previously in the literature [14]. To our knowledge, this is the second study demonstrating *Candida krusei* from periodontal pockets. The point to be noted is that the isolates of *Candida krusei* were obtained only from female patients on hormonal contraceptives and none from the control group.

Candida dubliniensis is a recently discovered yeast species principally associated with carriage and disease in the oral cavities of human immunodeficiency virus (HIV)-infected individuals. To date the majority of isolates of this species have been identified in Europe and North America [28]. The authors showed for the first time the presence of *C. dubliniensis* in the periodontal pockets of women taking hormonal contraceptives. This is in contrary to the study done by Brusca et al., which failed to isolate *C. dubliniensis* from periodontal pockets of patients on OCs [14].

The present findings suggest that hormonal contraceptive use may cause a subgingival selection for certain *Candida* species, such as *Candida krusei* and *Candida dubliniensis*. This is in contrary to Brusca et al. that demonstrated OC users having a subgingival selection of certain species such as such as *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* [14].

The association between *Candida* and periodontitis is controversial. Although different *Candida* species have been isolated from periodontal pockets of patients with periodontitis and hyphae have been found to invade the periodontal

connective tissue [29], the absolute proof implicating *Candida* in the pathogenesis of periodontitis is still lacking. A recent study demonstrated that in most cases *Candida* isolates were accompanied by well-known periodontopathogens, such as *P. intermedia*, *P. gingivalis*, and *A. actinomycetemcomitans*. In those cases, it seems likely that the bacteria co-isolated with Candida were the putative agents responsible for the development of periodontitis. No such association regarding the severity of periodontitis and the association of any Candida species was noted in our study [14].

Conclusion

We found that *C. albicans* was the most common Candida species isolated from both groups, followed by *Candida dubliniensis*, *Candida krusei*, *Candida tropicalis*, *Candida glabrata* and *Candida parapsilosis*. Hormonal contraceptive use seems to cause a selection of certain *Candida* species in periodontal pockets, such as *Candida krusei* and *Candida dubliniensis*. *C. glabrata* and *C. parapsilosis* seemed to be negatively affected because it was not isolated in the periodontal pockets of hormonal contraceptive users. No statistically significant difference in the candida count between the hormonal contraceptive users and non-users group was found.

The results of our analysis did not substantiate the previous theory that hormonal contraceptive use is associated with gingivitis or periodontitis, which can be attributed to the modern low dose OC pills and injection forms available in the recent times. Furthermore, the present findings suggest that there may be no detrimental association between Hormonal contraceptive and periodontal diseases for the majority of women who use these products. The limitation of the study is that we have considered the sample collection at cross sectional level and hence further studies should be conducted with a larger sample size and longitudinal study design to conclude definitively. While it is premature to make definitive statements regarding a protective cause-and-effect relationship between hormonal contraceptive use and periodontal conditions due to the cross-sectional nature of the data, these findings suggest an important re-examination of the perceived relationship between hormonal contraceptive use and periodontal diseases. Further studies are needed to clarify the role of hormonal contraceptives in periodontal disease and to determine the extent to which oral health behaviors affect the initiation or progression of periodontal disease.

Acknowledgments

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