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The efficacy and safety of ADP-1 (*Lactobacillus paracasei* GMNL-33) for periodontal pathogens, a placebo-controlled trial

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Abstract

Aims: The inhibitory effect of an oral administration of a new strain of ADP-1 (*Lactobacillus paracasei* GMNL-33) on the growth of periodontal pathogens was evaluated in this study by a double blind test.

Methods: Forty volunteers with periodontitis were randomized into two groups, one for the ADP-1 (*Lactobacillus paracasei* GMNL-33) (10^9 cells per day) treatment and the other for placebo. Putative periodontal pathogens counts (*Porphyromonas gingivalis*, and *Prevotella intermedia*) were measured at baseline, 4 weeks, and 8 weeks.

Results: Results showed that total bacteria counts decreased significantly in the test group, compared to the placebo control. The growth of selected periodontal pathogens was almost completely inhibited. The improvement of probing pocket depth (PPD) was significantly ($p<0.05$).

Conclusions: The data indicate that an oral administration of ADP-1 (*Lactobacillus paracasei* GMNL-33) possesses an antimicrobial effect.

Key words:

heat treated lactobacillus, periodontal pathogen, a placebo-controlled trial

Introduction

A unique microbiota was found in the inflamed periodontal pocket which probably was associated with human periodontal disease. Some strains of lactic acid bacteria (LAB) can produce hydrogen peroxide (oxidizing agent) and/or bacteriocins (proteinaceous compounds) that are antimicrobial substances. Therefore LAB can be used as bioprotective agents to control infections in intestine, vagina or oral cavity. Previous studies demonstrated that children taking milk containing probiotic *Lactobacillus rhamnosus* GG suffered less dental caries. Short-term consumption of yogurt containing bifidobacteria would affect the salivary levels of *Streptococcus mutans* and lactobacilli in young adults (Nase et al. 2001; Ahola et al. 2002). However, several studies showed that some species of lactobacilli are thought to be associated with the development of dental caries. *Lactobacillus paracasei* subsp. *paracasei* was once isolated from healthy oral cavity of Thai volunteers and showed good antimicrobial activity to many oral pathogens (Sookkhee et al. 2001). To avoid acid cariogenic capacity of LAB and keep bacteriocin-like inhibiting substance (BLIS) (Ishihara et al. 1985; Balakrishnan et al. 2001), we used ADP-1 (*Lactobacillus paracasei* GMNL-33) to treat human periodontitis. Therefore the aim of present study was to examine whether oral administration of ADP-1 (*Lactobacillus paracasei* GMNL-33) can suppress the growth of oral pathogens and decrease halitosis without lowering the pH values of oral cavity (Badet et al. 2001).

METHODS AND MATERIALS

Isolation of ADP-1 (*Lactobacillus paracasei* GMNL-33)

The ADP-1 (*Lactobacillus paracasei* GMNL-33) was isolated from human gastrointestinal tract (GenMont Biotech, Tainan, Taiwan) and identified on the basis of morphological examination and biochemical profiles according to the API 50CHL test kit (bioMerirux Vitek Inc., Hazel Wood, MO, USA) (Gardiner et al. 1998; De Angelis et al. 2001).

ADP-1 (*Lactobacillus paracasei* GMNL-33) was inoculated into de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD, USA) under anaerobic condition (BBL GasPak System) at 37°C for 16 to 24 h and adjusted to 10^9 cells per ml and 7.3 log cells per ml according to MacFarland Calibration standard Set (MacFarland No.1 = 8.48 log cell per ml), spectrophotometer (OD_{600nm}, SmartSpec™3000, Bio-Rad) and spreading.

Preparation of ADP-1 (*Lactobacillus paracasei* GMNL-33) and placebo tablets

Each ADP-1 (*Lactobacillus paracasei* GMNL-33) tablets containing 8.52 log cells, palatinit, sucralose, emulsifier (sugar fatty acid surfactant) and flavour (powder type). Placebo tablets contained palatinit, sucralose, emulsifier (sugar fatty acid surfactant) and flavour (powder type). All tablets were manufactured by Ezaki Glico Co., Ltd., in Japan.

Participants and study design

This clinical trial was a randomized, double blind, placebo-controlled study conducted at the Department of Periodontology of the Stomatology Research Center of the Chung Shan Medical University Hospital. Forty patients (mean age 47.7 ± 9.8 years)

who had not previously received any type of periodontal therapy were enrolled. Bleeding on probing, probing depth, and clinical attachment level measurements were assessed by the same examiner through the whole course. The consents were signed by all of the participants and the ethical approval for this study was given by Chung Shan Medical University Hospital (CSMUH No. CS05065). The volunteers were randomized into either placebo or treatment groups. According to the groups, patients in each groups received 3 tablets containing ADP-1 (*Lactobacillus paracasei* GMNL-33) or none per day for consecutive 8 weeks, and the tablet was taken within 30 minutes after each meals. Clinical measurement including oral flora total count, periodontal pathogens count (*Porphy. gingivalis* and *Prev. intermedia*) and periodontal probing depth (PPD) were examined at the beginning of test, 4 weeks and 8 weeks after enrollment (Sookkhee et al. 2001).

Collection and processing of plaque samples

Subgingival plaque samples were collected with two paper points from the periodontal pocket of lesion site then placed in 1 ml of reduced transport fluid RTF (Koll-Klais et al. 2005). The samples were transferred immediately to an anaerobic environment, where they were vortex mixed and homogenized. Part of each sample was serially diluted and plated in enriched blood agar, Brucella Agar (BBL Microbiology system, Cockeysville, MD, US) enriched 5% defibrinated sheep blood, 0.1% Hemin, and 0.1% Vitamin K under anaerobic condition at 37°C for 48 - 96 h. The total bacterial counts were indicated by their morphology under dark-field microscopy were also determined, long wave UV light fluorescence test (Blak-Ray Lamp, Model UVL-56, Long wave U.V.-366nm, Penn State University, USA). And

the colonies with different morphology were gram stained. *Porphy. gingivalis* was white colony without color under UV light. The *Prev. intermedia* was black colony, lacerate morphology under UV light (Moore et al. 1982; Overman 2000; Frias et al. 2001; Gafan et al. 2004).

Periodontal probing depth (PPD)

One of the commonly used clinical measures of periodontal disease progression and restoration of oral health is probing depth (PD, sometimes referred to as probing pocket depth). This measure was made with specially marked periodontal probes held parallel to the tooth and inserted under the free gingival margin and gently "walked" to the base of the sulcus (i.e., pocket). The blunt periodontal probes (P2N, HU-FRIEDY) were typically marked with rings or bands that measured distance in millimeters. The mean PPD across three teeth of one patient formed the periodontal probing depth as estimate. The mean PPD < 5 mm were defined as disease improvement. The PPD was generally measured as the distance from the base of the sulcus to the top of the free gingival margin (Hefti 1997).

Statistical analysis

The primary outcome measure was the change of positive culture rate of putative periodontal pathogens in the subgingival plagues from baseline following treatment. The secondary outcome measure was the change of halitosis after treatment. The sample size of 40 patients provided sufficient power (90%) to detect a difference of 20% between treatment and placebo groups ($\alpha = 0.05$). A p-value of less than 0.05 was considered statistically significant. The SPSS 10.0 software was used for data management and statistical analysis. Data were presented as mean ± S.D. The

Mann-Whitney U Test and Fisher's Exact Test were used to analyze the inter-group difference between two groups, respectively. The Wilcoxon Signed Rank Test and McNemar Test were used to analyze the intra-group difference among baseline, 4-week visit and 8-week visit, respectively. All lost rates, missing values and reasons for drop out or premature termination of the study were recorded.

RESULTS

Inhibition of the periodontal pathogen growth

Twenty patients, aged at 47.00 ± 10.75 years, were allocated to the placebo group and the other twenty patients, at aged 48.50 ± 8.85 years, to the treatment group (Table 1). There was no significant difference between these two groups in terms of demographic data. Total bacteria counts decreased significantly at 4-week and 8-week visits in the treatment group, whereas no significant change was found in the placebo group. In the final visit, the inter-group comparison showed statistical difference between treatment and placebo groups regarding to the total bacteria count in the oral cavity (Table 2). Further analysis demonstrated that ninety-five percent of patients showed no pathogenic growth in the subgingival plague samples after 4 weeks of the ADP-1 (*Lactobacillus paracasei* GMNL-33) treatment. This effectiveness could last to the final visits, compared to the baseline visit ($P = 0.016$). In contrast, there was no significant change in the negative culture rate of oral periodontal pathogens from subgingival plague samples. These results showed that heat-treated ADP-1 (*Lactobacillus paracasei* GMNL-33) has significant antimicrobial activity to

periodontal pathogens (Table 3). Compared to the placebo group, the antimicrobial activity of ADP-1 (*Lactobacillus paracasei* GMNL-33) also showed statistical significance between groups at 4-week and 8-week visits ($P < 0.01$) (Table 3).

Improvement of PPD

The probing pocket depth less than 5 mm was considered as clinical significance in the periodontal disease progression (Wennstrom and Lindhe 2002; Rupf et al. 2005; Heden and Wennstrom 2006). After taking ADP-1 (*Lactobacillus paracasei* GMNL-33) tablets for 8 weeks, the probing pocket depth was significantly improved, compared to the placebo group ($P < 0.05$) (Table 4).

Table 1 Distribution of demographic variable

Demographic variable		Placebo N=20	Treatment N=20	*P* value
Sex*	Female	6(30.0%)	9(45.0%)	0.327
	Male	14(70.0%)	11(55.0%)	
Age†		47.00 ± 10.75	48.50 ± 8.85	0.429

*The data was analyzed by Fisher's Exact test.

†The data was analyzed by Mann-Whitney U Test.

Table 2 The distribution of oral microorganism total count (log cells)

Time	Placebo N=20, Mean ± SD (Range, Median)	Treatment N=20, Mean ± SD (Range, Median)	*P* value*
Baseline	5.73 ± 0.74 (4.72~7.43, 5.49)	5.94 ± 0.70 (4.76~6.90, 6.14)	0.277
4 weeks	5.80 ± 0.92 (4.23~7.51, 5.80)	5.32 ± 0.97 (3.41~6.83, 5.33)	0.165
8 weeks	5.77 ± 0.89 (3.83~7.04, 5.92)	5.22 ± 0.82 (3.23~6.40, 5.36)	0.043§
P value†	0.999	0.005§	
P value‡	0.970	0.001§	

*Difference between placebo and treatment group, analyzed by Mann-Whitney U Test

†Difference between baseline and 4 weeks analyzed by Wilcoxon Signed Rank Test

‡Difference between baseline and 8 weeks, analyzed by Wilcoxon Signed Rank Test

§$P < 0.05$

Table 3 The inhibition of periodontal pathogens

Time		Placebo, N=20 N (%)	Treatment, N=20 N (%)	*P* Value*
Baseline	Positive	10(50.0)	8(40.0)	0.404
	Negative	10(50.0)	12(60.0)	
4 weeks	Positive	9(45.0)	1(5.0)	0.003§
	Negative	11(55.0)	19(95.0)	
8 weeks	Positive	9(45.0)	1(5.0)	0.003§
	Negative	11(55.0)	19(95.0)	
	P value†	0.999	0.016§	
	P value‡	1.000	0.016	

*Difference between placebo and treatment group, analyzed by Fisher's Exact Test

†Difference between baseline and 4 weeks, analyzed by McNemar Test

‡Difference between baseline and 8 weeks, analyzed by McNemar Test

§$P < 0.05$

Table 4 The improvement of probing pocket depth (PPD)

Time	Score (mm)	Placebo, N=20 N (%)	Treatment, N=20 N (%)	*P* value*
Baseline	< 5.0	0(0.0)	1(5.0)	0.311
	≥ 5.0	20(100.0)	19(95.0)	
4 weeks	< 5.0	5(25.0)	6(30.0)	0.723
	≥ 5.0	15(75.0)	14(70.0)	
8 weeks	< 5.0	10(50.0)	16(80.0)	0.047§
	≥ 5.0	10(50.0)	4(20.0)	
	P value†	0.063	0.063	
	P value‡	0.004**	0.002**	

*Difference between placebo and treatment group, analyzed by Fisher's Exact Test

†Difference between baseline and 4 weeks, analyzed by McNemar Test

‡Difference between baseline and 8 weeks, analyzed by McNemar Test

§$P < 0.05$

**$P < 0.01$

DISSCUSSION

Epidemiologic studies have shown that most adults present some mild form of periodontal diseases, and that 15 - 20% suffered from severe periodontitis. Prevention of periodontal diseases is mainly based on plaque control. Although such approaches have been proven to be effective in controlling these diseases, periodontal diseases still remain as the major public health problem in the general population, with substantial economic implications. Mechanical debridement is initiated when lesions (presence of periodontal pockets, loss of attachment, radiographic evidence of bone loss, bleeding) are observed (Baehni and Guggenheim 1996). The alternative would be to emphasize that prevention, and treatment should be targeted at controlling the etiological agent(s). *Porphy. gingivalis* and *Prev. intermedia* are the major periodontal bacteria species in most of the progressive periodontitis forms. New methods such as probiotic approach to eliminate pathogenic members of the microbiota can be investigated. Bacteriotherapy is an alternative and promising way to prevent infections by using harmless bacteria to displace pathogenic microorganisms (Koll-Klais et al. 2005). Our studies demonstrated that oral administration of ADP-1 (*Lactobacillus paracasei* GMNL-33；10^9 cells per day for 4 weeks) could successfully inhibit the growth of subgingival pathogens without any obvious side-effects. Most importantly, the probing pocket depth in the clinical measures of periodontal disease progression improved significantly, compared to the placebo group after 8-week non-invasive treatment.

Lactobacilli are used in the prevention and treatment of several diseases, but they are also known to play a role in the pathogenesis of dental caries (Hatakka et al. 2001;

Ishikawa et al. 2003; Matsuoka et al. 2004; Montalto et al. 2004).After 8-week therapy, the pH value of oral cavity still remained the same. In addition, previous studies showed that oral cavity can be a natural reservoir for intestinal lactobacilli. Frequent supplement of probiotic could be beneficial to our innate immunity, producing more biogenic effects. We therefore concluded that oral administration of ADP-1 (*Lactobacillus paracasei* GMNL-33) could provide a safe, non-invasive and effective way to prevent and treat periodontal diseases.

Subjects reported no severe adverse effects such as fever, abdominal pain, or diarrhea during the study period.

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