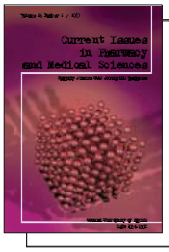

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Effect of supernatants from *Lactobacillus acidophilus* culture on ATP levels in human gingival fibroblasts

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ABSTRACT

Bacteria of *Lactobacillus* genus comprise around 1% of physiological flora in oral cavity. Despite numerous studies on *Lactobacillus* bacteria, their interaction with cells of host's oral cavity has not been fully recognized.

Studies were performed on effects of supernatants obtained from bacterial cultures of *Lactobacillus acidophilus* strains on ATP levels in human gingival fibroblasts (HGF-1) and on their viability. ATP levels were evaluated using luminescence test and cell viability was estimated using a fluorescence test.

Mean levels of ATP in cultures of control fibroblasts, HGF-1, supplemented with 10% PBS amounted to 4.90 ± 0.32 mln of RLU (relative light units). In turn, mean level of ATP in cultures of HGF-1 fibroblasts supplemented with supernatants of H_2O_2-producing *L. acidophilus* cultures amounted to 5.94 ± 0.31 mln of RLU, and in the cultures supplemented with supernatants of *L. acidophilus* producing no H_2O_2 it amounted to 5.88 ± 0.28 mln of RLU. The levels of ATP obtained in HGF-1 cultures with supernatants of *L. acidophilus* were significantly higher than those in control cultures. On the other hand, ATP levels in HGF-1 cultures with supernatants of H_2O_2-producing *L. acidophilus* cultures and with supernatants of H_2O_2-not producing *L. acidophilus* cultures showed no significant differences.

The presented for the first time in this study increase in ATP synthesis in gingival fibroblasts under effect of extracellular products of *L. acidophilus* cultures may represent an important protective mechanism in which oral lactobacilli influence human gingival fibroblasts.

Keywords: *Lactobacillus acidophilus*; ATP; human gingival fibroblasts; microbial products

INTRODUCTION

Periodontitis represents a chronic periodontal disease, which leads to destruction of periodontal tissues. Chronic periodontitis may develop in various age groups but it most frequently affects adults, manifesting moderate or severe clinical course [20]. In etiopathogenesis of periodontitis the main role is played by periodontopathogens [5,11]. The clinical important periopathogens include, i.a., *Porphyromonas gingivalis* and *Prevotella intermedia*, representing obligatory anaerobes and facultative anaerobe *Aggregatibacter actinomycetemcomitans* [9,27,29]. Around 1% of physiological oral cavity flora represent oral lactobacilli, which are detected in saliva in numbers exceeding 10^6 CFU/ml [26], comprising mostly *L. acidophilus* [4].

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Lactobacillus bacteria produce substances of antibacterial activity, including lactic acid, hydrogen peroxide (H_2O_2) [18,22,23] and bacteriocins [13,25]. *Lactobacillus* spp. producing H_2O_2 were demonstrated to have the potential to prevent progression of chronic diseases in periodontium [30]. Bacteria of *Lactobacillus* genus manifest also immunoregulatory and inflammation-modulating properties, representing a significant element of the local antibacterial resistance [6]. At the same time, effects of *Lactobacillus* bacteria on intracellular processes and proliferation of host's oral cavity cells have not been fully recognized.

Taking the above into account in this study, we analysed the effect of supernatants isolated from cultures of clinical strains of oral lactobacilli, on the levels of adenosine triphosphate (ATP) in human gingival fibroblasts and on their viability.

MATERIALS AND METHODS

Patients. The studies were performed on bacterial strains selected from 10 patients (30-50 years of age, mean 38.2±4.5 years) with moderate chronic periodontitis.

Bacterial cultures. Bacteria of *Lactobacillus* genus were cultured on Rogosa agar and the cultured isolates obtained in anaerobic conditions were identified using API 50 CHL (bioMerieux). The capacity of hydrogen peroxide production among *Lactobacillus* strains was defined in culture of the obtained isolates in presence of 5% CO_2 at the temperature of 37°C for 48 hours in a differentiating medium, TMB-Plus agar, prepared according to Rabe and Hillier [21]. Development of an altered colour of the growing colonies (appearance of a blue colour) indicated production of hydrogen peroxide.

Production of supernatants. Cultures of individual bacteria provided material to obtain 0.5 McF bacterial suspension in 2 ml physiological saline. Two ml of RPMI-1640 (Sigma) were supplemented with 0.1 ml bacterial suspension and cultured for 24 hours in anaerobic conditions. Subsequently the cultures were passed through sieves to obtain supernatants.

Cell cultures. Gingival fibroblasts, HGF-1 (CRL-2014, ATCC) were cultured in T-25 culture vessels (Nunc), in an incubator at the temperature of 37°C, in atmosphere of 5% CO2. The culture medium involved DMEM (ATCC) solution enriched with 10% foetal bovine serum (FBS, Sigma), supplemented with penicillin 100 U/ml and streptomycin 20 mg/ml (Sigma).

Evaluation of ATP levels. Evaluation of ATP levels in cultures of gingival fibroblasts HGF-1 was performed using a luminescence test (CellTiter-Glo Luminescent Cell Viability Assay, Promega). The tests were performed in presence of a buffered physiological saline, PBS (15 µl /10^5 HGF-1 cells/135 µl medium) and in presence of supernatants obtained from cultures of individual bacteria (15 µl /10^5 HGF-1 cells/135 µl medium). Estimations of ATP level in HGF-1 gingival fibroblasts were performed in presence of a supernatant isolated from every isolated bacterial strain, in three repetitions. The medium involved DMEM (ATCC) solution enriched with 10% foetal bovine serum (FBS, Sigma). The prepared cells were incubated for 24 hours in an incubator, at 37°C in presence of 5% CO_2. Then, the cells were rinsed with the culture medium and tested for presence of ATP. Each of the samples was supplemented with 150 µl of the prepared reagent (substrate plus buffer), mixed for 2 minutes and incubated for 10 minutes at room temperature. The results were read using a luminometer (GloMax, Promega). The light emitted in presence of ATP was quantitated in relative light units (RLU). The intensity of emitted light quants was directly related to ATP content in the tested sample.

Testing of viability in gingival fibroblasts. The viability testing in HGF-1 gingival fibroblasts took advantage of the Live/Dead Viability/Cytotoxicity Kit (Invitrogen, USA) fluorescence test. The studies were conducted in Lab-Tek Chamber Slide (Nunc) culture chambers in presence of a buffered physiological saline (PBS, 50 µl /0.5x10^6 HGF-1 cells/450 µl medium) or in presence of a supernatant obtained from cultures of individual bacterial strains (50 µl /0.5x 10^6 HGF-1 cells/450 µl medium). Viability of HGF-1 gingival fibroblasts was tested in presence of every supernatant, isolated from individual bacterial strains, in three repetitions. The medium involved DMEM (ATCC) solution, enriched with 10% foetal bovine serum (FBS, Sigma). The prepared cells were incubated for 24 hours in an incubator at the temperature of 37°C in presence of 5% CO_2. Subsequently, the cells were washed in the culture medium and tested for their viability. The readouts were made at zero time and following 6, 12, 18 and 24 hours, using the fluorescence microscope, Nikon Eclipse E200 (magnification of 1000x).

Statistical analysis. Results obtained in the studies were analysed using the computer software STATISTICA 8 for Windows. In the comparative analysis related to ATP level in studied groups the one-way ANOVA with Tukey-Kramer test were applied. In the analysis of gingival fibroblast viability the non-parametric Kruskal--Wallis test was applied. In every test, hypotheses were verified at the significance level of $p=0.05$.

RESULTS

In the presented experiments there were isolated 10 strains of *Lactobacillus acidophilus* (with 4 strains producing H_2O_2 and 6 strains unable to produce H_2O_2) from the patients.Changes in ATP level in HGF-1 gingival fibroblasts, taking place under effect of supernatants of every culture containing the isolated strains of bacteria were presented in means of the effects. Mean levels of ATP in cultures of control HGF-1 fibroblast cultures, supplemented with 10% PBS amounted to 4.90 ± 0.32 mln of RLU. Mean level of ATP in cultures of HGF-1 fibroblasts with supernatants of H_2O_2–producing *L. acidophilus* amounted to 5.94 ± 0.31 mln of RLU, and in HGF-1 cultures with supernatants of *L. acidophilus* producing no H_2O_2 it reached 5.88 ± 0.28 mln of RLU. Levels of ATP obtained in HGF-1 cultures with supernatants of *L. acidophilus* were significantly higher than those in control cultures ($p<0.0001$). On the other hand, the levels of ATP in cultures of HGF-1 with supernatants of *L. acidophilus* which produced H_2O_2 and with supernatants of *L. acidophilus* producing no H_2O_2 manifested no significant differences ($p = 0.7620$). The studies documented, that the supernatants of *Lactobacillus acidophilus* strains induced a significant increase in ATP level in HGF-1 gingival fibroblasts (Fig. 1).

In studies on viability of HGF-1 gingival fibroblasts following any duration of incubation, i.e. at zero time or following 6, 12, 18 or 24 hours viability of gingival fibroblasts ranged between 94% and 98%. No significant

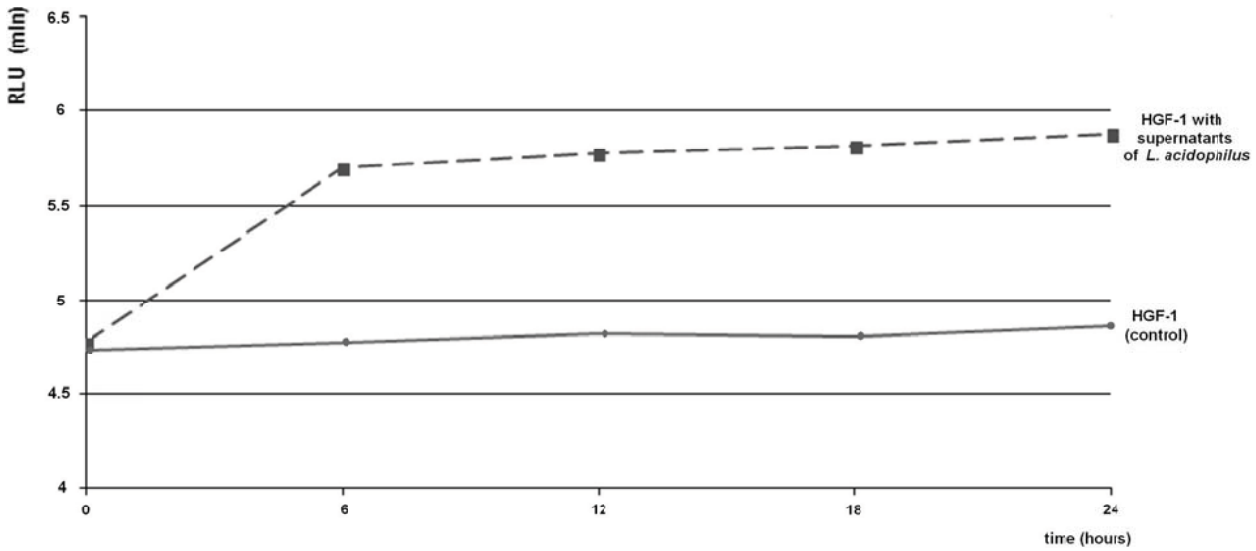

Fig. 1. Mean levels of ATP (luminescence in millions of RLU) in cultures of HGF-1 gingival fibroblasts at zero time and following 6, 12, 18 or 24 h incubation with PBS (control) and with supernatants obtained from cultures of *Lactobacillus acidophilus*

differences were disclosed between control samples and samples containing supernatants isolated from cultures of *Lactobacillus acidophilus* strains.

DISCUSSION

Bacteria of *Lactobacillus genus*, through production of substances with antimicrobial activity, may restrict or prevent colonization of pathological microflora, including periopathogens [1,31]. Oral lactobacilli may restrict development of inflammatory lesions in chronic periodontitis. It has been shown that H2O2-producing oral lactobacilli may prevent the progress of chronic periodontitis, most probably restricting secretory activity of Th17 cells and growth of periodontopathogens [28].

The results presented in this study, have demonstrated that supernatants originating from 24 hour cultures of *L. acidophilus* strains interact with human gingival fibroblasts, significantly increasing their production of ATP. The increase in ATP levels could not be related to H_2O_2 produced by the *L. acidophilus* strains. Moreover, we have shown that supernatants originating from cultures of *L. acidophilus* strains exert no inhibitory effect on viability of HGF-1 gingival fibroblasts.

The presented in this study ATP levels have been estimated in cultures of human gingival fibroblasts, representing the prevailing connective tissue cells in periodontium [14]. Adenosine triphosphate (ATP) is the molecule that transfers the energy captured during respiration to the many sites that use energy in the cell. The released energy is required for metabolic activity of cells. ATP is used in many cellular processes, respiration, biosynthetic reactions, motility, and cell division [12]. A decrease in cellular ATP production provides metabolic conditions leading to cell death by different mechanisms, including apoptosis, autophagy or necrosis [16,24]. Recent studies have shown that intra- and extracellular factors may cause an increase in ATP level in cells. One of such factors involves selenium, which stimulates proliferation of chondrogenic cell ATDC5 through acceleration of cell cycle progression, accompanied by cyclin D1 induction due to enhancement of intracellular ATP content [32]. Chen et al. demonstrated, using the test with MTT that ATP (0.1–100 mM) increased human cardiac fibroblasts proliferation in a concentration-dependent manner. ATP stimulates the proliferation of cardiac fibroblasts by promoting the progression of cells from the G0/G1 phase to the S phase. ATP was demonstrated also to participate in the regulation of cell cycle progression by modulating the expression of cyclin D1 and cyclin E proteins in cardiac fibroblasts [3]. The increase in proliferation under effect of ATP was detected also in the case of pulmonary artery adventitial fibroblasts [7] and mouse embryonic cells [10].

In studies presented heretofore, the increase in ATP levels in gingival fibroblasts could not be related to H_2O_2 produced by the *L. acidophilus strains*. Thus, the effect on ATP-production by human gingival fibroblasts has to be exerted by another factor, linked to *Lactobacillus*. The conclusion is supported by studies proving that lactobacilli, including *L. acidophilus,* may release high amounts of exopolysaccharides (EPS) [2,15]. Recently it has also been noted that EPS may exert a protective effect on human endothelium cells, manifesting a significant capacity in scavenging of free radicals [19]. Halper et al. demonstrated *in vivo* a stimulatory effect of *L. acidophilus* supernatants on proliferation of fibroblasts [8]. On the other hand, Li et al. found that *Lactobacillus acidophilus* cultures affected growth and development of embryonic cells *in vitro* [17].

The presented for the first time in this study increase in ATP synthesis in gingival fibroblasts under effect of extracellular products contained in cultures of *L. acido-*

philus may represent an important mechanism through which oral lactobacilli protect human gingival fibroblasts.

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