Effects of Cyclic Tensile Stress on Human Periodontal Ligament Fibroblasts Apoptosis*

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ABSTRACT Objective: To investigate the effect of cyclic stretch on Human periodontal ligament fibroblasts apoptosis and PI3k/Akt signaling pathway involved. Methods: In vitro culture -tensile stimulate models of HPDLFs were established by using a multi-passage load adding system. Cyclic stretch was applied on the fibroblasts in different groups for 1, 6, 12, and 24 h, respectively.The loading was set for 15% surface elongation, with frequency 1/6 Hz. Meanwhile, the blank normal control group and negative control group, 0h+LY294002 was established by static group. The cell apoptosis was determined by Hoechst 33258 staining. The expression of Bcl-2 and Bax mRNA was detected by RT-PCR. Results: Hoechst 33258 staining showed that after treatment with loading, the cell took the typical appearance of apoptosis with chromatin condensation and apoptotic bodies. RT-PCR displayed that the rate of Bcl-2 / Bax mRNA expression decreased in loaded HPDLFs group compared with that in unloaded HPDLFs($P < 0.01$). The rate decreased to the lowest level at 12h following loading, and then enhanced gradually. Compared with that in the loading group, the HPDLFs apoptosis increased at corresponding time points in the LY294002 group ($P <0.05$). Conclusions: The cyclic stretch can promote the apoptosis of HPDLFs in a time-dependent manner, then the HPDLFs apoptosis was inhibited PI3K/AKT signaling pathway may participate in the regulation of apoptosis of HPDLFs induced by cyclic stretch.

Key words: Human periodontal ligament fibroblasts; Apoptosis; Cyclic tensile stress
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Introduction

Periodontitis is a complex etiology, which is caused by the interaction of many factors of destructive periodontal disease, and regulated by the body defense mechanisms and a variety of systemic and local factors. Bite force, as one of the local factors, plays a very important role in the whole development of periodontitis. Many findings suggest that an appropriate mechanical loading is necessary for periodontal tissue to maintain homeostasis, otherwise periodontal tissue damage occurs when it is subjected to over-mechanical load. Human periodontal ligament fibroblasts (HPDLFs), which are the main components of the periodontal ligament, is playing the significant role in the normal metabolism, reparative regeneration, and physical health maintenance of the periodontal tissues [1-3]. Mechanical stimulations have significant impact on cell growth and migration, while little is known on the mechanism of HPDLFs behavior alteration under cyclic strain application.

Phosphoinositide 3-kinases (PI3K) represent a family of intracellular signaling proteins, which control a variety of essential cellular behaviors such as proliferation, survival and apoptosis. Cell apoptosis was strongly associated with the reconstruction of tissues from many regions. PI3K activation enhances cell survival and antagonizes apoptosis via Akt/PKB in many cell types including cardiomyocytes [4-6], cardiac fibroblast [7], and VSMCs [8] . However, little was known on whether PI3K/Akt pathway is involved in the mechanical strain-induced apoptosis of HPDLFs. In this present study, HPDLFs were subjected to cyclic strain with different loading duration to investigate the apoptosis of HPDLFs. Therefore, it could be helpful to understand the pathogenic mechanism of occlusal trauma and periodontitis .

1 Materials and methods

1.1 Materials

HPDLFs (ScienCell USA), DMEM (HyClone USA), fetal bovine serum (HyClone USA) USA LY294002 (sigma USA), Flexcell Strain Unint-5000T(Co-made by Doctor Yuan Xiao from the Department of Stomatology, Qingdao Municipal Hospital (Oral) and Harbin Institute of Technology), Six-well BioFlex flexible cell Petri Dish (Flexcell International, USA), PrimeScript RT-PCR Kit (TaKaRa Biomedical).

1.2 Experimental methods

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1.2.1 Cell culture The HPDLFs were maintained in Dulbecco's Modified Eagle Media (DMEM)supplemented with 10% fetal bovine serum (FBS),100 μ/mL penicillin and 100μ/mL streptomycin and cultured at 37℃ with 5% CO_2, and the media were changed every 48h. With the cells reaching 80% confluence, they were harvested for cyclic strain loading experiments.

1.2.2 Establishment of mechanical stimulation models HPDLFs were seeded into collagen I-coated 6-well Bioflex plates at a density of 1× 10^5 cells, 2 mL/well. The medium was replaced every 48h. Cells achieving 80% confluent were serumstarved in DMEM for 24 h, and cell synchronization was made. The cells were randomly divided into seven group: 0h, 0h+LY2940021, 6h, 12h 12h+LY294002, and 24 hours, and then were subjected to cyclic strain by using the FX-5000T with15%-elongation magnitude at the same frequency of 10 cycles per minute, each cycle including 3 seconds for tension and 3 seconds for relaxation .In the LY294002 group, HPDLFs were pre-incubated with LY294002(20 mmol/L), a specific PI3K/Akt inhibitor, for 1 h before cyclic strain application. Control cells were cultured on similar plates and were maintained in the same incubator without cyclic strain.

1.2.3 Hoechst 33258 fluorescent staining After loading of cyclic strain, HPDLFs were fixed using Hoechst fixative at room temperature for 30 min, washed with cold phosphate buffered saline at 4℃ twice, stained with 0.5 mL Hoechst33258 dye for 5 min, and washed with PBS. After the instillation of anti-fluorescence quenching sealed solid-liquid were added, we observed the HPDLFs with fluorescence microscope.

1.2.4 Analysis of Bcl−2and Bax mRNA levels Cells from each group were collected. Total RNA was isolated using the TRIZOL reagent (Invitrogen, USA), and an aliquot (1 μg of purified total RNA) was subjected to RT-PCR analysis using PrimeScript RT-PCR Kit for RT-PCR (Invitrogen). The resulting cDNAs were used as a template for PCR amplification to generate products corresponding to the mRNAs encoding various gene products. Target gene expression in fibroblasts was detected with fluorescence semiquantitative polymerase chain reaction.

Primers were shown in Table 1. Amplification condition was as follows: an initial denaturation at 95℃ for 5min, denaturation at 98℃ for 10s, annealing at 58℃ for 30s, extension at 72℃ for 30s and a final elongation step of 10 min at 72℃ (35 cycles for Bax) and 95℃ for 5 min, 98℃ for 10s, 60℃ for 20s, 72℃ for 30s and a final elongation step of 10 min at 72℃ (30 cycles for glyceraldehyde-3-phosphate dehy- drogenase (GAPDH)). And 95℃ for 5 min, 98℃ for 10s, 64℃ for 30s, 72℃ for 30s and a final elongation step of 7 min at 72℃ (36 cycles for Bcl-2). Then PCR products were identified by 2% agarose gel electrophoresis (110 V, 40min).Finally, the ratio of target bands and GAPDH,and bands were calculated by Gel-Proanalyzer .

Table 1 Expression of Bax，Bcl-2 and gapdh mRNA

Primer*	Sequence(5'-3')	Size(bp)
Bax	Forward:AGTGGCAGCTGACATGTTTT	152bp
	Reverse:GGAGGAAGTCCAATGTCCAG	
Bcl-2	Forward:ATGTGTGTGGAGAGCGTCAACC	196bp
	Reverse:TGAGCAGAGTCTTCAGAGACAGCC	
GADPH	Forward:TCATGGGTGTGAACCATGAGAA	146bp
	Reverse:GGCATGGACTGTGGTCATGAG	

1.3 Statistics

The data in quantitative PCR analysis were presented as mean ± SD are from at least three independent experiments in each condition and analyzed using SPSS 17.0 software. One-way ANOVA was used for intragroup comparison. A p-value less than 0.05 was considered significant.

2 Results and analysis

2.1 Hoechst 33258 fluorescent staining results

Hoechst 33258 staining showed that the nucleus in the normal control group presented with dispersed uniformly round or oval-shaped fluorescent. Compared with the static, the HPDLFs after 15% of cyclic strain application at 1/6Hz-frequency took the typical appearance of apoptosis, including chromatin condensation, crescent or ring fluorescence and apoptotic bodies. An increase in the number of shrunken rounded cells was observed at12h, and the speed of apoptosis in the LY294002 group was quicker than the control group (Fig. 1).

2.2 RT−PCR results

It is demonstrated that there were significant differences in the Bcl-2/Bax between the six stess loading groups and control groups ($P < 0.05$).The Bcl-2/Bax levels of HPDLFs began to decrease after loading, reached to the minimum at l2 h ($P < 0.05$)and began to arise at 24 h after loading. Furthermore, the Bcl-2/Bax value in 12h+LY294002 group decreased to a more remarkable level than the 12 h group, but there were no significant difference between them. It suggested that cyclic strain induced HPDLFs apoptosis. The rate of apoptosis increased as the loading time prolonged (Fig. 2-3) .

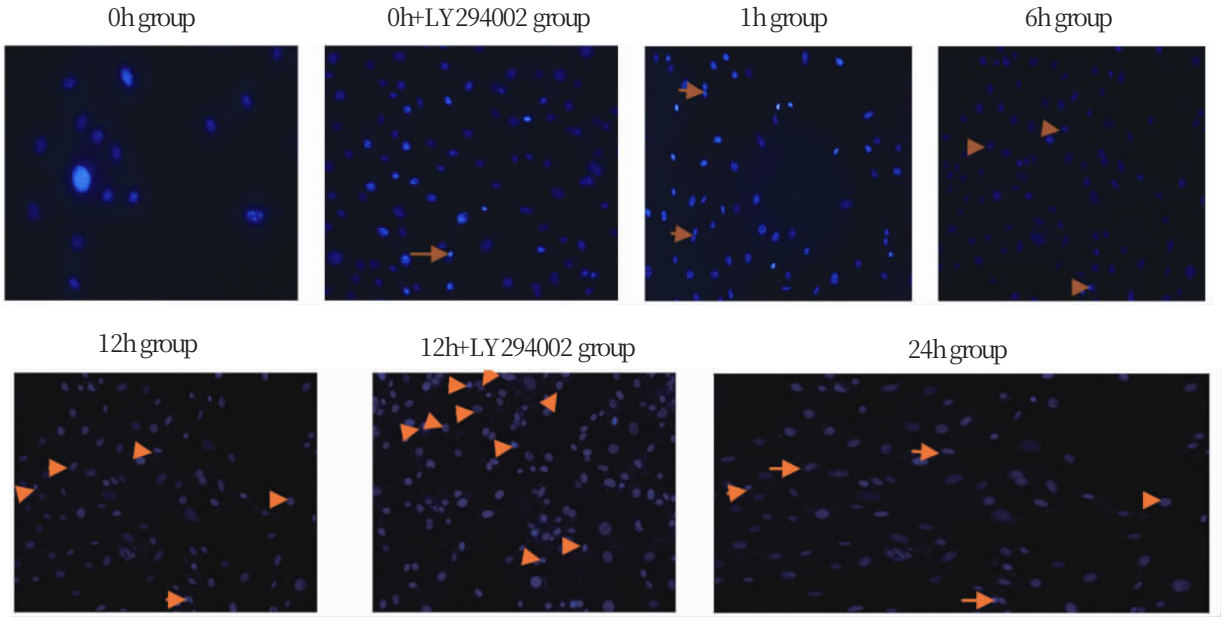

Fig. 1 Hochest 33258 of HPDLFs (× 200)

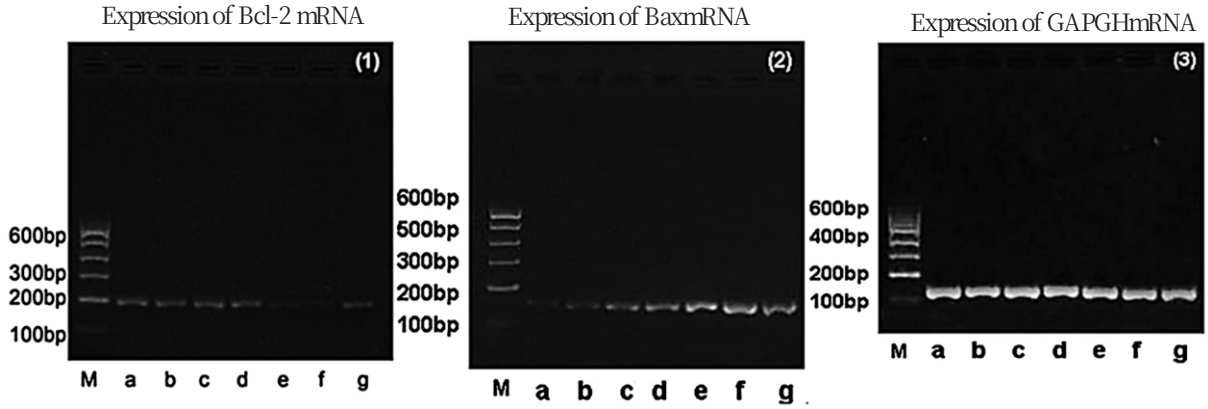

图 2 Bcl-2、Bax **和** GAPDH mRNA **产物的电泳图**

Fig.2 Expression of Bcl-2，Bax，GAPDH mRNA under different strength time

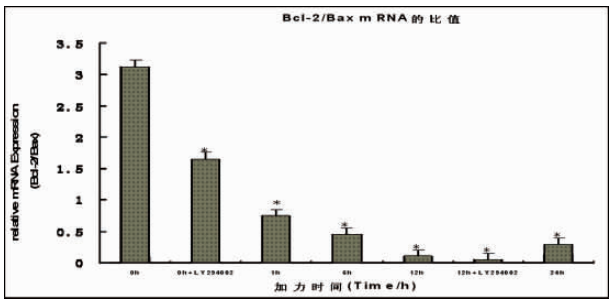

图 3 **不同加力时间内** Bcl-2/Bax mRNA **表达的比值**(P < 0. 05)

Fig. 3 Expression of Bcl-2/Bax mRNA under different strength time(P < 0. 05)

3 Discussion

Apoptosis is a multi-step, multi-pathway and initiative cell-death programme that is inherent in every cell of the body[9], and plays a key role in the remodeling of periodontal tissues [10]. In addition,mechanical stimulation is also an important factor of tissue remodeling. Human periodontal ligament fibroblasts in vivo, as one of load-sensitive cells, can sense the mechanical load and subsequently change their diverse cellular functions such as cell proliferation and apoptosis, and these changes result in alteration of structure, composition, and function of periodontal tissues[11]. In this study, after loading, cells gradually exhibited cell rounding and shrinkage, vacuolization and even detached from bottom in a time-dependent manner, which showed the significant characteristics of cell apoptosis [12]. Meanwhile, increased chromatin condensation, nuclear fragmentation and apoptotic bodies over time were observed by Hoechst 33258 staining.

Cyclic strain has been shown to influence many aspects of cellular metabolism that may affect subsequent biological behaviors of cells, including proliferation, migration, apoptosis, and the expressions of gene and protein [13]. As we know that the Bax and Bcl-2, which are important members of the Bcl-2 family, act as pro-apoptotic and anti-apoptotic regulators that are involved in a wide variety of cellular activities[14]. The rate of Bcl-2/Bax will decide the cell apoptosis[15]. This study indicated that the apoptosis of

HPDLFs subjected to 15% of cyclic strain which was based on numerical data derived from a finite element model [16] changed in a time- dependent manner apparently, and peaked at 12 h, and then apoptosis decreased at 24 h. But previous studies showed that the cyclic stretch (15%, 24 h) induced a significant increase in the apoptosis of AF cells [17]. Zhong pointed out another peak of apoptosis of hPDLCs (6 h, 20%)[18]. The variances may result from the difference of stretch in amplitude, duration and species. Anyway, it can be proved that the apoptosis of HPDLFs can be induced by cyclic strain although the apoptosis peak appears at different moment after stress loading.

Several studies have shown that PI3K/Akt signaling pathway play essential roles in mechanical strain-mediated migration and proliferation of HPDLCs[19], which could regulate apoptosis with a reduced expression of Bax and an increased expression of Bcl-2[20]. In this present research, the Bcl-2 /Bax levels of the HPDLFs with the PI3K inhibitor LY294002, compared with that in the control group, was obviously reduced at corresponding time points. Interestingly, there was no significant difference on HPDLFs apoptosis between 12 h and 12 h in the inhibitor. This possible mechanism involved is that the apoptosis of HPDLFs in response to stretching strain are co-regulated by various signaling pathways such as p38 MAPK[21], JNK[22] , ERK-mediated signaling[23]. Furthermore, it is considered a possibility that PI3K/Akt signal pathway may involve in stress-induced apoptosis process, while the apoptosis process was regulated by a series of enzyme and controlled by various genes.

In summary, the Apoptosis of HPDLFs in the early stage increased in a time- dependent manner in response to stretching strain. PI3K/AKTsignaling pathway may participate in the process of apoptosis of HPDLFs induced by cyclic stretch.This finding will contribute to a better understanding of the mechanism of mechanical stretch-induced remodeling of the periodontal tissues during the periodontitis therapy.

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周期性张应力对牙周膜成纤维细胞凋亡的影响*

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摘要 目的:在体外条件下,探讨周期张应力作用对人牙周膜成纤维细胞凋亡的影响及 PI3k/Akt 信号通路在细胞凋亡中的作用。**方法**:应用多通道细胞牵张应力加载系统,以 HPDLFs(人牙周膜成纤维细胞)为对象构建细胞体外培养-力学刺激模型,对照组为 0h,0h+LY294002,加力组 1 h,6 h,12 h,12 h+LY294002,24 h,力值定为 15%,频率为 1/6HZ,即 10 循环/分钟。采用 Hoechst33258 染色检测细胞形态和凋亡情况,应用 RT-PCR 技术检测 Bcl-2、Bax 的表达情况。**结果**:Hoechst 33258 细胞染色结果显示,对照组的细胞核为弥散均匀的圆形或椭圆形荧光,实验组的细胞核或细胞质内出现可见致密浓染的颗粒、新月体或环状荧,RT-PCR 结果显示 Bcl-2 与 Bax 基因表达均呈现时间依赖性。12 h HPDLFs 的细胞凋亡数达最高峰值(P<0.01),24 h 细胞凋亡峰值开始下降,但仍高于未加力组(P<0.05)。与对照组相比加入 LY294002 后,Bcl-2/Bax 比值较加载相同时间的加力组小(P<0.05)。**结论**:一定的时间范围内,周期性张应力能促进 HPDLFs 凋亡,随着时间的延长(24h),细胞凋亡受到抑制;PI3K/Akt 信号传导通路可能参与在周期性张应力介导的 HPDLFs 的凋亡。

关键词:人牙周膜成纤维细胞;周期性张应力;PI3K/Akt;细胞凋亡
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