Changes of Proliferation and Secretion of Human Gingival Fibroblasts in Different Glucose Concentrations: An *in vitro* Study

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Abstract: *Background:* To investigate the changes of the proliferation of human gingival fibroblasts (HGFs) and the secretions of Type I collagen (COL1), Type III collagen (COL3) and fibronectin (FN) in different glucose concentrations. *Methods:* HGFs were cultured in the cell culture medium with different glucose concentrations (5.5 mmol/L (group A), 8.8 mmol/L (group B), 10 mmol/L (group C), and 15 mmol/L (group D)) for 7 days. The proliferation of HGFs was measured using an MTT method, and COL1, COL3, and FN were tested with ELISA. The results and their correlations were statistically analyzed. *Results:* As the glucose concentrations increased, the quantities of HGFs proliferation and various proteins secreted decreased. HGFs proliferation in groups C and D was significantly less than that in groups A and B (P<0.05). The proliferation in group D were significantly less than that in group C (P<0.05). The amount of COL1, COL3, and FN secreted by HGFs in groups C and D were significantly less than those in groups A and B (P<0.05). The secretions in group D were significantly less than that in group C (P<0.05). The positive correlations were shown between HGFs proliferation quantities and secretions of COL1 (r=0.686), COL3 (r=0.693) and FN (r=0.474) (P<0.05). *Conclusions:* Proliferation of HGFs was inhibited with increasing glucose concentration, and the secretion function of HGFs was reflected in the variation of HGFs proliferation.

Keywords: Gingival Fibroblast; High Glucose; Cell Proliferation; Collagen; Fibronectin

DOI: 10.57237/j.cmf.2024.01.002

1 Introduction

Implant restoration is the first choice for patients suffering from teeth loss. The implant's long-term stability requires a good gingival tissue interface at the implant neck [1]. The implant-gingival tissue interface has an epithelial attachment similar to the junctional epithelium of natural teeth, which is a biological barrier that forms a tight epithelial junction. Typically, this barrier is mainly formed by the adhesion of junctional epithelium and gingival fibers to the implant surface [2]. Human gingival fibroblasts (HGFs) are the main cells of human gingival tissue, which participate in the formation and regeneration of periodontal tissue and maintain the

Funding: This work was supported by Scientific Research Funding of Science and technology Commission of Shanxi Province (No. 20210302124359)

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Li-Li Fan and Zhen-Qiang Li are co-first authors.

Received: August 15, 2022; Accepted: September 30, 2022; Published Online: January 20, 2024 http://www.stomatol.org

integrity and function of periodontal tissue. Type I and Type III collagen and fibronectin secreted by HGFs are important adhesion proteins of gingival soft tissue attached to the implant surface [3].

Diabetes is one of the risk factors affecting the success of implant restoration. Many studies have reported the effect of diabetes on bone cells and osseointegration [4], and those on the healing of gingival tissue at the implant neck are yet lacking. In our previous study, it was observed that the attachment and proliferation of HGFs on pure titanium surfaces were inhibited by increases in glucose concentrations (5.5 mmol/L, 8.8 mmol/L, 10 mmol/L, and 15 mmol/L) [5]. In this study, HGFs were cultured *in vitro* in high glucose conditions. The changes of the proliferation of HGFs and secretions of Type I collagen, Type III collagen and fibronectin in different glucose concentrations was explored by MTT and ELISA methods, which further clarified the pathological mechanism underlying the effect of high glucose conditions on the attachment of gingival cells on titanium surface.

2 Materials and Methods

2.1 Main Reagents and Instruments

Following main reagents and instruments were used: human gingival fibroblast cell line (Shanghai Jiahe Biotech Co., Ltd), fetal bovine serum (Gibco, USA), DMEM medium (Gibco, USA), 0.25% Trypsin (Gibco, USA), MTT cell proliferation and cytotoxicity detection kit (Nanjing KeyGen Biotech Co., Ltd), Type I human collagen (COL1) enzyme-linked immunosorbent assay (ELISA) kit (E-EL-H0130c, Elabscience Biotech Co., Ltd), Type III human collagen (COL3) ELISA Kit (E-EL-H0774c, Elabscience Biotech Co., Ltd), human fibronectin (FN) ELISA Kit (E-EL-H0179c, Elabscience Biotech Co., Ltd), CO_2 constant temperature incubator (MCO-15AC, SANYO), electrothermal constant temperature incubator (ICV-45O, ASONE, Japan), Micro high-speed centrifuge (C2500-R-230V, Labnet, USA), inverted microscope (IX51, OLYMPUS), low-speed centrifuge (5702R, Eppendorf), enzyme-labeled instrument (MULTISKAN MK3, Thermo), clean bench (SW-CJ-1FD, Suzhou AirTech Co., Ltd), and micro pipette (Beijing DLab company).

2.2 Preparation of Glucose Culture Medium in Different Concentrations

To prepare the high-glucose culture mediums, ordinary DMEM medium was added with a certain amount of L-glucose. The glucose concentrations for the culture mediums were as follows: 5.5 mmol/L (Group A), 8.8 mmol/L (Group B), 10 mmol/L (Group C), and 15 mmol/L (Group D). The osmotic pressure was 274-302 mOsm/kg dihydrogen monoxide (H_2O).

2.3 Inoculation and Culture of Cell Line

The HGF cell lines were cultured in DMEM supplemented with 10% FBS for 48 hours (with 1 fluid change), and sub-cultured until generation 5. The cells were prepared to 4.0×10^4 piece/mL cell suspension for the experiments. The cells were inoculated in equal amounts of cell suspensions in four different sets of glucose medium, and after continued incubation for 7 days. All cells were treated with trypsin to a uniform cell suspension for use. Ten wells were set up at each concentration.

2.4 MTT Assay

After 7 days of culture, cell suspensions of the four groups were plated in 96-well plates (100 mL/well) and incubated at 37 °C. Then, 10 μL of sterile MTT (5 mg/mL) was added to each well and incubated for another 4 h. Subsequently, 150 μL DMSO was added to each well to stop the reaction. Proliferating changes of HGFs were observed with the inverted microscope. The absorbance was measured at 568 nm on a microplate reader. The mean absorbance value of each group was calculated for statistical analysis.

2.5 ELISA Assay

The cells were cultured for 7 days, after which the cell suspension was taken, and the supernatant was discarded. Type I and Type III collagen and human fibronectin protease ELISA kits were used, respectively, and the subject matter was put into the ELISA plate. A biotinylated antibody, HRP Conjugate working solution, and substrate solution were successively added, and the termination solution was added to terminate the reaction. The amount of COL1, COL3, and FN secreted by the cells in each group was determined.

2.6 Statistical Analysis

Statistical Package for Social Sciences (SPSS) software version 13.0 (SPSS Inc., Chicago, IL, USA) was used for all the statistical analyses. The absorbance value (A value) of cell proliferation measured by MTT and the amount of COL1, COL3, and FN secreted by HGFs measured by ELISA in each group were tested by one-way ANOVA. The correlation between A value of cell proliferation and the amount of COL1, COL3, and FN secreted by cells were analyzed by Pearson correlation coefficient. $P<0.05$ represented statistically significant differences.

3 Results

3.1 Effects of Glucose Concentration on HGFs Proliferation

A value of the proliferation of HGFs measured by the MTT method was directly proportional to the number of cells. There was no significant difference in A values between group A and group B (P>0.05), and there was a significant difference in A values between group C and group D (P<0.05). A values of group C and group D were significantly less than those of group A and group B (P<0.05). The results showed that when the glucose concentration was higher than 10 mol/L, the proliferation of HGFs was inhibited. With the increase of the concentration, the number of HGFs gradually decreased (see Table 1 and Figure 1).

3.2 Effects of Glucose Concentration on HGFs Secretions

The changes in secretions of three proteins with the increase of glucose concentration were revealed in Figure 2. There was no significant difference in the amount of COL1, COL3, and FN secreted by HGFs between group A and group B (P>0.05). The secretion of group C was significantly more than that of group D (P<0.05). The secretions of group C and group D were significantly less than those of group A and group B (P<0.05; see Table 1).

Table 1 Comparison of cell proliferation quantity and secretions of various proteins after 7 days of culture

	A value of cell proliferation	**COL1 concentration (ng/ml)**	**COL3 concentration (ng/ml)**	**FN concentration (ng/ml)**
A: 5.5 mmol/L	2.184 ±0.010	23.021±1.142	7.886±0.752	63±4.1
B: 8.8 mmol/L	2.180 ±0.008	22.460±1.764	7.506±0.557	60±3.5
C: 10 mmol/L	1.996 ±0.045 [a]	19.390±1.769 [a]	6.172±0.807 [a]	51±4.0 [a]
D: 15 mmol/L	1.832 ±0.008 [b]	16.173±1.820 [b]	5.743±0.312 [b]	44±2.1 [b]
F	57.375	134.570	99.103	101.359
P	0.000	0.000	0.000	0.000

Note: [a]: Compared with group A and group B, $P<0.05$; [b]: Compared with the other three groups, $P<0.05$.

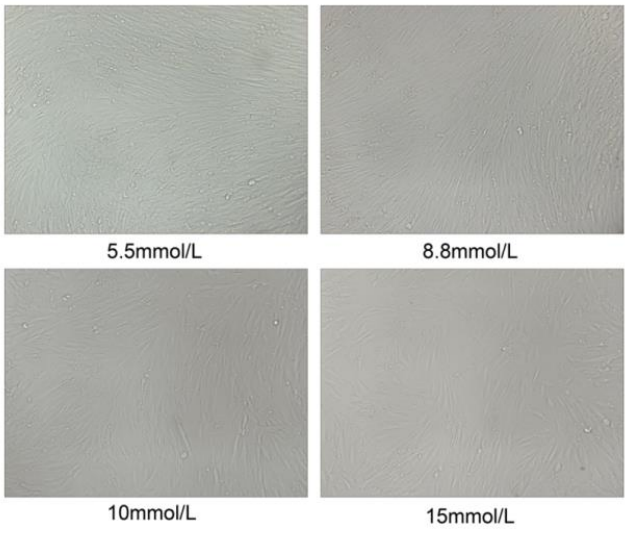

Figure 1 Proliferating changes of HGFs cultured in different glucose concentrations for 7 days. HGFs, human gingival fibroblasts

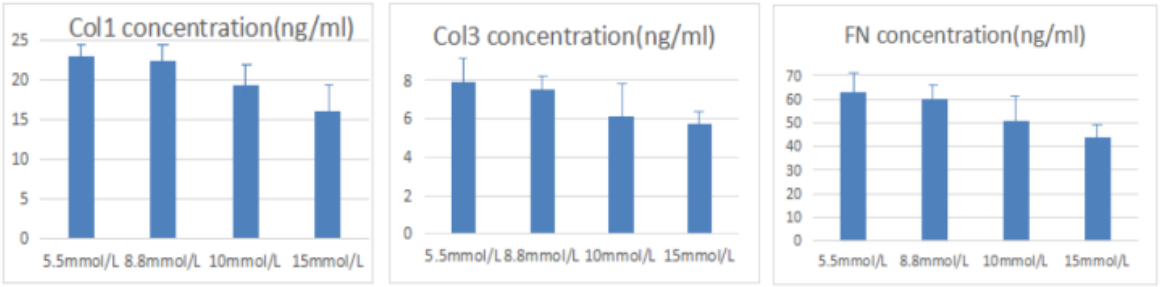

Figure 2 Changes of COL1, COL3, and FN secreted by HGFs cultured in different glucose concentrations for 7 days

3.3 Correlation Between a Value and Changes of Protein Secretions

There was a positive correlation between A value and the change of COL1 secretion (Y=31.70＋0.68X, r=0.686, P<0.05). There was a positive correlation between A value and the change of COL3 secretion (Y=24.12＋0.94X, r=0.693, P<0.05). There was a positive correlation between A value and the change of FN secretion (Y=15.71＋0.53X, r=0.474, P<0.05).

4 Discussion

The chronic complications of diabetes have become a public problem posing a serious threat to human health [6]. The relationship between diabetes and oral diseases has received considerable attention in the past few decades [7]. The mechanisms of hyperglycemia-induced diabetic complications have been deeply and continuously studied. Since the 1960s, it has been found that the increased activity of the polyol pathway, the formation of advanced glycation end products (AGE), the activation of protein kinase C (PKC), and the increased activity of the hexosamine pathway are involved in the pathogenesis of hyperglycemia-induced periodontal injury [6]. Epidemiological studies have demonstrated that the severity and incidence of periodontal disease in diabetic patients are higher than in the non-diabetic population. Liao *et al.* investigated the periodontal condition in a community in Beijing urban area and concluded that the periodontal condition in type 2 diabetic patients was worse than that in the non-diabetic population [8]. Moreover, the Third National Health and Nutrition Examination Survey results showed that the incidence of periodontitis in the diabetic population was significantly higher than that in the non-diabetic population (17.3%/9%) [9].

Peri-implantitis is the one of the most important causes of implant restoration failure, and its pathogenesis is similar to periodontitis [3]. Fibroblasts are the main cell types of gingival connective tissue and periodontal ligament. Type I collagen, Type III collagen and fibronectin secreted by fibroblasts have an important role in the formation and regeneration of periodontal tissue, maintaining integrity, and implementing the function. In this study, HGFs were seeded in the cell culture medium with different glucose concentrations for 7 days. MTT method was used to determine the HGFs proliferation quantities. It was found that when the glucose concentration was higher than 10 mol/L, the proliferation of fibroblasts gradually decreased with the increase of glucose concentration, indicating that hyperglycemia could inhibit the proliferation of HGFs. Similar studies showed that diabetes could enhance the apoptosis of fibroblasts in the periodontal ligament and reduce the number of cells in periodontal disease, and the biological function of fibroblasts was affected at the same time [10, 11]. In this study, when the glucose concentration was higher than 8.8 mmol/L, the secretions of Type I collagen, Type III collagen, and fibronectin were decreased.

The results presented above had implications for our follow-up study on the effect of AGE on HGFs. Immunohistochemical studies using anti-AGE antibodies demonstrated that periodontal cells were damaged by intracellular AGE through three mechanisms: (1) the function of intracellular proteins modified by AGEs precursors changes; (2) extracellular matrix components modified by AGEs precursors interact with other matrix components and extracellular matrix receptors; (3) plasma proteins modified by AGEs precursors bind to RAGE in

endothelial cells, mesangial cells, and macrophages to induce the production of inflammatory factors [12]. In addition, the role of advanced oxidative protein products (AOPP) was also mentioned. AOPP is a general term for stable products produced by protein oxidation during oxidative stress and has some similarities with AGE in structure, function, and biological effects. Deng et al. found that AOPP could inhibit cells proliferation and secretion of Type I collagen and fibronectin and increase the synthesis of matrix metalloproteinase-1 which could promote collagen dissolution and accelerate the process of periodontal disease [13]. The studies above suggested that the decreases in the secretions of Type I collagen, Type III collagen, and fibronectin may be related to intracellular changes in high glucose condition.

5 Conclusion

In this study, the changes of proliferation quantities and secretion function of gingival tissue cells in different glucose concentrations was analyzed and discussed, thus providing a theoretical basis to investigate the pathological changes of gingiva at the implant neck in the diabetics with implantation.

Author Contributions

Conceptualization: Nan Zhang

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Investigation: Wen-Xiao Chang

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Project Administration: Zhen-Qiang Li, Yan-Yan Liu, Lin-Lin Gao

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Writing – Review & Editing: Nan Zhang, Zhen-Qiang Li

Conflict of Interest

All the authors do not have any possible conflicts of interest.

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Acknowledgments

Thanks to Scientific Research Funding of Science and technology Commission of Shanxi Province for its financial support.

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