Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

The Effect of Phenytoin on the Proliferative Ability of Periodontal and Gingival Ligament Fibroblasts in Cell Culture Medium

Li Zhang[1#*], Qi Gao[2#], Xuezhen Peng[1], Shuman Deng[1], Li Zhang[1]

[1] Department of Stomatology, Shenzhen Children's Hospital, Shenzhen, Guangdong518026, China

[2] Department of Oralfacial Surgery, Shenzhen Second People's Hospital, Shenzhen, Guangdong518029, China

#They contributed equally to this work.

ARTICLE INFO

Original paper

Article history:
Received: January 21, 2022
Accepted: March 17, 2022
Published: May 31, 2022

Keywords:
Periodontal Regeneration, Gingiva, Phenytoin, Fibroblast, Periodontal Ligament

ABSTRACT

Periodontal ligament fibroblasts (PDLFs) play a vital role in the period of periodontal regeneration. In addition, studies show that diphenylhydantoin (phenytoin) increases the growth of gingival fibroblasts. If this effect is also present in the periodontal ligament (PDL) fibroblasts, it may be used to regenerate periodontal tissues. Accordingly, this study aimed to compare the effect of phenytoin on the growth rate of gingival fibroblast cells and PDL in the cell culture medium. In this regard, 10 Wistar rats were selected. The gingival specimen was obtained from the area between the upper teeth, and the PDL specimen was obtained from the middle third of the lower teeth root. After transferring the samples to a suitable culture medium for culturing PDL and gingival fibroblasts, each sample was divided into two experimental and control groups. In the experimental group, 20 mg/ml phenytoin dissolved in sodium hydroxide was added to Dulbecco's modified Eagle's medium (DMEM). After 48 hours, fibroblast cell proliferation was assessed through a 1-WST cell proliferation kit by ELISA. The proliferation of gingival fibroblast cells and PDL in both test and control groups were statistically analyzed by the independent t-test. The results showed that the effect of phenytoin on the proliferation of gingival fibroblast cells and PDL fibroblast cells is significant. Also, the proliferation of PDL cells was significantly different from gingival cells in the experimental group ($P < 0.001$) and was higher in PDL cells. In general, in this study, it was found that phenytoin *in vitro*, like *in vivo*, is able to increase the proliferation of gingival fibroblast cells, and this phenytoin effect is also present in PDL fibroblast cells.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.5.19

Introduction

The ultimate goal of periodontal treatments is to stop tissue destruction and regenerate lost tissue (1). Different cell lines can be involved in the process of regenerating tooth-supporting tissues. These categories include gingival epithelial cells, gingival connective tissue cells, periodontal ligament connective tissue (PDL) cells, and bone cells. According to Melcher's theory, cells of a specific origin can cause a particular type of repair (2).

The most desirable condition is when the cells accumulated in the periodontal wound originate from the periodontal ligament connective tissue. In this case, complete periodontal regeneration will be achieved as new connective tissue attachment (3). Guided tissue regeneration (GTR) presented by Nyman is based on this and prevents the movement of epithelial cells and gingival connective tissue to the periodontal wound and causes the accumulation of PDL connective tissue cells (4).

The current standard methods for regenerative therapies have many limitations and lack predictable results (5). Therefore, to improve the results of these processes, researchers have presented various materials and methods based on growth stimulation, proliferation, and cell differentiation. Phenytoin (sodium diphenylhydantoin) was first introduced in 1938 as an antiepileptic drug. One of the side effects of this substance is gingival hyperplasia, which is caused by the increased proliferation of gingival fibroblasts and increased activity of some subgroups of fibroblasts (overproduction of collagen). Therefore, this phenytoin property has been used to promote wound healing in medicine (6). The effect of phenytoin on the proliferation and function of gingival fibroblasts *in vitro* has also been proven (7).

Given the above, if phenytoin can also increase the proliferation of PDL fibroblasts, it can be used to

*Corresponding author. E-mail: zhl_ada@163.com

stimulate, reproduce, and accumulate periodontal ligament fibroblast cells in the GTR process. This study aimed to evaluate the effect of phenytoin in a cell culture medium on gingival fibroblasts and PDL.

Materials and methods

Preparing gingival and PDL samples

Samples were taken from 10 4-week-old Wistar rats (weighing 150 g). The animal was first anesthetized by ether inhalation and immediately transferred to a sterile hood after removing the animal's head. The mouth and teeth were then rinsed with chlorhexidine solution, and the tissue was rinsed with chlorhexidine rinsing serum. Then, with a sterile razor blade, the interdental gums were cut between the incisors and transferred to the primary medium. The incisors and the alveolar bone were removed from the surrounding hard and soft tissues. The alveoli and teeth were rinsed with serum and then gently removed from the corresponding alveoli with a scalpel. Immediately, from the middle third of the root, a biopsy of PDL was removed. Gingival and PDL samples were transferred to the medium.

Culture medium

The primary medium included DMEM (Dulbecco's Modified Eagle Medium), gentamicin sulfate 50 mg/ml, L-glutamate, penicillin 100 Units, and streptomycin 100 mg/ml, and Fetal calf serum (FCS) 10%. The prepared samples were crushed under a sterile hood and placed in unique flasks in an incubator containing CO_2 at 37°C. After 24 h, the cultured cells were placed in secondary medium (including DMEM + antibiotic + 10% FCS). The samples were regularly monitored for infection and cell growth in the following days, and their medium was changed if necessary. As soon as the fibroblasts spread on the surface of the culture flask and filled them, the cells were separated from the culture surface by trypsinization (dilution of 1.3 trypsin 2.5%). After several passages and good cell growth, the samples were trypsinized again and counted in a certain volume to determine the cells in the NeoBar high volume.

Experimental studies

Two thousand cells were cultured in 96 wells plates based on cell count. After 72 hours, cultivation houses were divided into control and experimental groups. In the plates of the experimental group, phenytoin was added at a rate of 20 mg/ml (soluble in sodium hydroxide). The exact amount of sodium hydroxide was added to the plates of the control group. This was done in both PDL and gingival specimens. After 48 hours of drug incubation, cells were eluted from the drug, and cell proliferation in each subgroup was measured by ELISA using Wst-1 cell proliferation kit. It should be noted that several PDL-related specimens were lost in this experiment due to the inherent limitations of primary cell culture. Ten gingival specimens and 6 PDL specimens remained at the end of the investigation.

Statistical review and analysis

This study first tested the numbers obtained from ELISA measurement using the Kolmogorov-Smirnov test for gingival cell population and PDL. Then according to the normality of these data, the rate of cell proliferation in the control group with control using analysis - independent t-test was compared and tested. Values of p less than 0.05 were considered significant.

Results and discussion

The proliferation rate of gingival fibroblast cells was significantly increased by adding phenytoin solution to the culture medium (Table 1). Periodontal ligament fibroblasts also had a significant increase in their proliferation in the presence of phenytoin in the cell culture medium (Table 1).

Table 1. Comparison of the proliferation of gingival fibroblast and PDL in experimental and control groups (mean ± SD)

Cell Type	Control Group	Experimental Group	P-value
Gingival Fibroblasts	0.13945 ± 0.0111	0.1282 ± 0.0113	<0.01
PDL Fibroblasts	0.1659 ± 0.0331	0.17883 ± 0.0754	<0.05

The effect of phenytoin on PDL cell proliferation is more than gingiva ($p < 0.001$). Therefore, an equal amount of phenytoin was added to the culture medium under the same conditions in cell culture media. PDL cell proliferation rate was significantly higher than gingival cells (Figure 1).

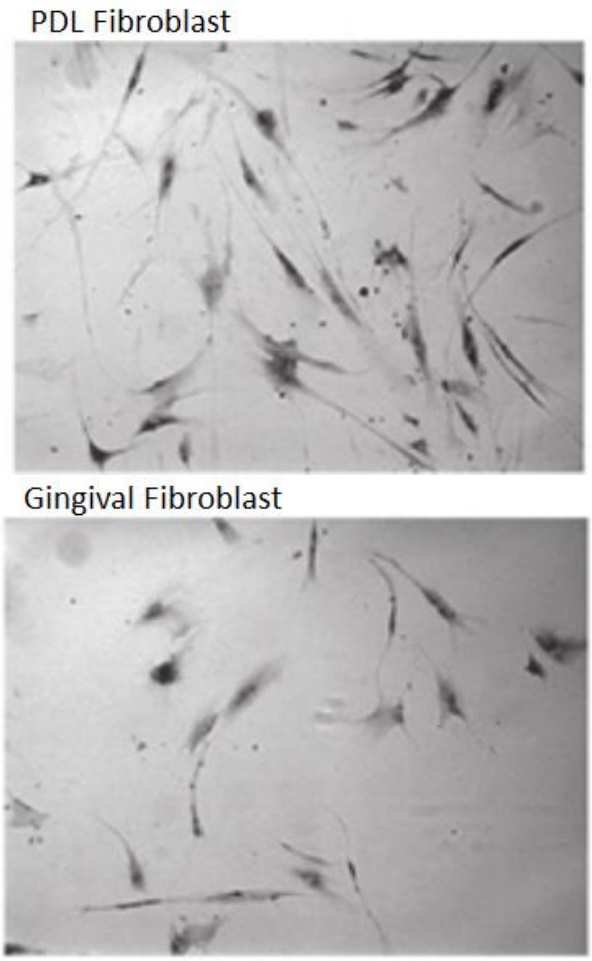

Figure 1. PDL and gingival cell proliferation on Dulbecco's modified Eagle's medium (DMEM)

This study indicates that phenytoin increases the proliferation of gingival fibroblasts in a cell culture medium and the proliferation rate of gingival fibroblasts exposed to phenytoin for 48 hours (experimental group) was significantly different from the control group. Showed ($P < 0.05$). This finding is consistent with the study findings by Alubaidy *et al*. (8). These researchers examined the effect of phenytoin on the mitotic activity of human gingival fibroblasts. They showed that the mitotic index in the phenytoin group (PHT) is higher than in the control group.

Morimoto *et al*. (9) also showed that phenytoin stimulated the growth of feline gingival fibroblasts *in vitro*. However, some researchers have concluded that phenytoin does not stimulate the growth of gingival fibroblasts. For example, Candotto *et al*. (10) added phenytoin (5-10 mg/ml) and nifedipine (100-200 mg/ml) to fibroblasts. They showed that these substances have a special effect on further reducing total protein and collagen. Also, fibroblasts obtained from patients with increased volume due to phenytoin consumption show an increase in the synthesis of sulfated glycosaminoglycan *in vitro* (11).

Another study has shown that the use of phenytoin reduces the breakdown of collagen, which is due to the production of an inactive fibroblastic collagenase (12).

The addition of phenytoin to gingival fibroblasts increases the level of translatable collagen mRNA (13). Also, the extracellular matrix produced by increased gingival fibroblasts (due to phenytoin consumption) facilitates fibroblast proliferation, which is a prerequisite for cell growth (14). In addition, the mechanism of phenytoin effect on gingival fibroblasts in an *in vitro* study has shown that it inhibits the uptake of Ca^{2+} by these cells and is related to the rate of fibroblast proliferation (15).

This study found that the effect of phenytoin on PDL cell proliferation was significant ($p = 0.05$). Since no other research has been reported on the impact of phenytoin on PDL fibroblasts, either *in vitro* or *in vivo*, the mechanism of action on PDL fibroblasts is unclear. However, this study showed that phenytoin increased PDL cell proliferation *in vitro*. In addition, due to the effect of phenytoin on the inhibition of hepatic Ca^{2+} uptake by gingival fibroblasts, it can be inferred that this also applies to PDL fibroblasts, and phenytoin has an effect on the cell cycle and calcium channels on PDL fibroblasts.

However, the proliferation of PDL fibroblasts has been more rapid than that of the gums in various studies. Chen *et al*. (16) compared the cultures of human gingival fibroblasts *in vitro* and showed that human PDL cells (HPDL) were faster than human gingival cells (HGF). Studying PDL fibroblasts in humans showed that PDL fibroblasts reach confluent faster. According to them, this is due to the more giant gingival cells than PDL cells due to the higher DNA and protein content of gingival fibroblasts than PDL fibroblasts (13).

The rat was used in this study. Although some researchers believe that this animal is resistant to the increase in gingival volume caused by phenytoin consumption, many of these animals have been used to study the metabolic activity and tissue binding of this drug (17). In this study, phenytoin also intensified the proliferation of gingival fibroblasts in animals,

indicating that Rat gingival fibroblasts are not resistant to phenytoin. In addition, it was found that phenytoin can increase the growth of fibroblasts not only *in vivo* but also *in vitro*.

In the regeneration process of any tissue in the body, it is necessary for the cells in that tissue to grow and differentiate and make extracellular proteins. In this study, only one aspect of regeneration, namely cell proliferation, has been addressed, and the issue of cell differentiation and production of extracellular material has not been investigated. Some studies have shown that phenytoin indirectly increases collagen by reducing the ability of the extracellular matrix to degrade (especially by cathepsin L) and by producing a type of inactive fibroblastic collagen, thereby increasing tissue volume (11, 18). This aspect of regeneration should also be considered to determine the effect of phenytoin on the extracellular matrix of extracellular PDL fibroblasts (19).

It should be mentioned that PDL fibroblasts, unlike connective tissue fibroblasts, are connective to other parts of the body and include different types with distinct phenotypes (20). One of these types of fibroblasts was classic, soft tissue similar to the types found in the skin and gums, and one type is an osteoblast-like cell with alkaline phosphatase content (21). This type of PDL fibroblasts is critical in periodontal regeneration due to its participation in mineralized tissue formation and its ability to differentiate into cementoblasts (22). Given the importance of this issue, it is necessary to examine the effect of phenytoin on various subspecies and phenotypes of PDL fibroblasts to determine whether the resulting proliferation was only related to the increase in growth of normal fibroblasts in PDL or included the type. It also becomes osteoblast-like. The use of phenytoin as an adjunct in the wound healing process should be given special attention because today, phenytoin is used topically for wound healing (23). Na *et al*. (13) have shown that phenytoin alters wound healing by increasing fibroblast infiltration and neovascularization.

In general, applying phenytoin 20 mg/ml for 48 hours in a PDL fibroblast cell culture medium can increase the intensity of their proliferation. Therefore, this substance can be introduced as an adjuvant in stimulating periodontal regeneration in the GTR process by conducting similar studies.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

References

1. Heta S, Robo I. The side effects of the most commonly used group of antibiotics in periodontal treatments. Med Sci 2018; 6(1): 6.
2. Tomokiyo A, Yoshida S, Hamano S, Hasegawa D, Sugii H, Maeda H. Detection, characterization, and clinical application of mesenchymal stem cells in periodontal ligament tissue. Stem Cells Int 2018; 2018.
3. Wu Z, Chen S, He Y et al. Connective tissue growth factor promotes cell-to-cell communication in human periodontal ligament stem cells via MAPK and PI3K pathway. J Periodontol 2022; 93(3): e60-e72.
4. Hirashima S, Kanazawa T, Ohta K, Nakamura K-i. Three-dimensional ultrastructural imaging and quantitative analysis of the periodontal ligament. Anat Sci Int 2020; 95(1): 1-11.
5. Liu J, Zhao Z, Ruan J et al. Stem cells in the periodontal ligament differentiated into osteogenic, fibrogenic and cementogenic lineages for the regeneration of the periodontal complex. J Dent 2020; 92: 103259.
6. Militi A, Cutroneo G, Favaloro A et al. An immunofluorescence study on VEGF and extracellular matrix proteins in human periodontal ligament during tooth movement. Heliyon 2019; 5(10): e02572.
7. Komatsu K, Ideno H, Shibata T, Nakashima K, Nifuji A. Platelet-derived growth factor-BB regenerates functional periodontal ligament in the tooth replantation. Sci Rep 2022; 12(1): 1-16.
8. Al-Ubaidy SS, Al-Janabi NY, Al-Tai SA. Effect of phenytoin on mitotic activity of gingival tissue and cultured fibroblasts. J Periodontol 1981; 52(12): 747-749.
9. Morimoto C, Takedachi M, Kawasaki K et al. Hypoxia stimulates collagen hydroxylation in gingival fibroblasts and periodontal ligament cells. J Periodontol 2021; 92(11): 1635-1645.
10. Candotto V, Pezzetti F, Baj A et al. Phenytoin and

gingival mucosa: A molecular investigation. Int J Immunopathol Pharmacol 2019; 33: 2058738419828259.
11. Farook FF, M Nizam MN, Alshammari A. An update on the mechanisms of phenytoin induced gingival overgrowth. Open Dent J 2019; 13(1).
12. Kang Y, Yang R, Wei Z et al. Phenytoin sodium-ameliorated gingival fibroblast aging is associated with autophagy. J Periodontal Res 2020; 55(5): 642-650.
13. Na J, Zheng L, Wang L et al. Phenytoin Regulates Migration and Osteogenic Differentiation by MAPK Pathway in Human Periodontal Ligament Cells. Cell Mol Bioeng 2022; 15(1): 151-160.
14. Kanno CM, Oliveira JAd, Ervolino E, Soubhia AMP. Effects of cyclosporin, nifedipine and phenytoin on gingival myofibroblast transdifferentiation in monkeys. J Appl Oral Sci 2018; 27.
15. Djais AI, Adam AM, Thahir H et al. Management of Gingival Enlargement Caused by Phenytoin Used in Epilepsy Patients: A Case Report. J Int Dent Med Res 2019; 12(2): 705-708.
16. Chen J-T, Wang C-Y, Chen M-H. Curcumin inhibits TGF-β1-induced connective tissue growth factor expression through the interruption of Smad2 signaling in human gingival fibroblasts. J Formos Med Assoc 2018; 117(12): 1115-1123.
17. Kim SS, Nikoloudaki G, Darling M, Rieder MJ, Hamilton DW. Phenytoin activates Smad3 phosphorylation and periostin expression in drug-induced gingival enlargement. Histol Histopathol 2018; 33(12): 1287.
18. Takeuchi R, Matsumoto H, Taguchi C et al. Mechanism of Drug-induced Gingival Overgrowth: Phenytoin Inhibits the Apoptosis of Human Gingival Fibroblasts. Int J Oral Med Sci 2021; 20(2): 109-116.
19. Janjic Rankovic M, Docheva D, Wichelhaus A, Baumert U. Effect of static compressive force on in vitro cultured PDL fibroblasts: monitoring of viability and gene expression over 6 days. Clin Oral Invest 2020; 24(7): 2497-2511.
20. Blawat K, Mayr A, Hardt M et al. Regulation of autophagic signaling by mechanical loading and inflammation in human PDL fibroblasts. Int J Mol Sci 2020; 21(24): 9446.
21. Nazet U, Schröder A, Spanier G, Wolf M, Proff P, Kirschneck C. Simplified method for applying static isotropic tensile strain in cell culture experiments with identification of valid RT-qPCR reference genes for PDL fibroblasts. Eur J Orthod 2020; 42(4): 359-370.
22. Ullrich N, Schröder A, Jantsch J, Spanier G, Proff P, Kirschneck C. The role of mechanotransduction versus hypoxia during simulated orthodontic compressive strain—an in vitro study of human periodontal ligament fibroblasts. Int J Oral Sci 2019; 11(4): 1-10.
23. Fleissig O, Reichenberg E, Tal M, Redlich M, Barkana I, Palmon A. Morphologic and gene expression analysis of periodontal ligament fibroblasts subjected to pressure. Am J Orthod Dentofacial Orthop 2018; 154(5): 664-676.