Phenytoin Effects on Inflammatory Mediator's Production by Gingival Fibroblasts, A Comparative Study in Children and Adult

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Abstract

Background: About 30 to 50% of patients taking Phenytoin develop significant gingival alternations especially in buccal anterior part of oral cavity. This study was done to compare the synthesis of its inflammatory mediators and related gingival overgrowth in different ages.

Methods: Samples were collected from biopsy of a healthy gingival of four adults in 35-42 years old through crown lengthening surgery and four children in 4-11 years old through impact tooth surgery, after local anesthesia and from the keratinized soft tissues around the teeth. Gingival biopsies were transferred to a medium which containing DMEM and cultured on specific plates 25 cm^2 and put on incubator containing CO_2 with temperature of 37ºC. MTT was used to compare the Proliferation rate of fibroblasts. Supernatant of culture medium of test and control sinks were collected by sampler and concentration of IL1β, PGE2, IL6, TGFβ, TNFα and IL8 were analyzed by ELISA.

Results: Different proliferation rate of Phenytoin induced gingival fibroblasts in adults (0.073 ± 0.177) as compared to children (0.056 ± 0.028) was not significant. Production of PGE2, TGFβ and IL6 by Phenytoin induced gingival fibroblasts in children was increased as compared to adults ($p<0.05$). Production of IL8 by Phenytoin induced gingival fibroblasts in children was decreased compared to adults ($P=0.02$)**.**

Conclusion: Phenytoin induced gingival fibroblasts of children produce more amounts of IL1β, PGE2, IL6, TGFβ and IL8 as compared to adults' fibroblasts. More Comprehensive studies with well-documented designs using other methods are recommended to verify these results.

Introduction

It is well-known that the anticonvulsant drug, Phenytoin (PHT), induces Gingival Overgrowth (GO) as a side effect. The mechanism of Phenytoin-induced GO, however, is not well understood. Phenytoin induced gingival enlargement is a usual term for all gingival lesions related to hyperplasia [1]. Extra Cellular Matrix (ECM) is accumulated by collagenous components of gingival connective tissues in the presence of inflammations and gingival overgrowth is among the most prevalent side effects of Phenytoin [2]. Phenytoin is the drug of choice for treatment for grand mal, temporal lobe, and psychomotor seizures since was first introduced in the 1930s [3]. It is estimated that about 30% to50% of patients taking Phenytoin develop significant gingival alternations [4].

Phenytoin induced gingival overgrowth was first reported in 1930 and buccal surface of both upper and lower jaws have been seen to have greater amounts of GO [5,6]. Microscopic analysis of biopsies which have been taken from individuals with Phenytoin –induced GO reveals a redundant tissue of apparently regular composition or with an increased amount of collagen and number of fibroblasts [7]. Some studies demonstrated that Phenytoin is able to inhibit production of Extra Cellular Matrix (ECM) by gingival fibroblasts [8,9]. However, there are some other reports showing that accumulation of proteins in ECM can occur because of an imbalance between the synthesis and degradation of ECM and it can be a possible cause of GO [10].

A decrease in the Ca^{2+} influx by Phenytoin has been reported which reduces the uptake of folic acid and limit the production of active collagenase [11]. The drug decreases collagen endocytosis through induction of a lower expression of α2_ β1-integrin by fibroblasts. Phenytoin can stimulate the myofibroblasts. Other cytokines are also involved directly in Phenytoin-Induced Gingival Overgrowth (PIGO). Fibroblasts which have been activated by Phenytoin produces large amounts of IL-1, 6 and 8, these are capable of activating the proliferation of T cells, recruitments of Neutrophils to the involved gingiva. This interaction seems to be highly associated with fibrotic diseases [12]. Some cytokines and growth factors were found in higher levels in gingival overgrown tissues, including Interleukin- 6 (IL-6), IL-1. IL- 13 induces the formation of latent TGFβ in higher levels in GO [11,12]. Experimental studies in animals also have demonstrated a role for Th2-immune responses and cytokines IL-4, IL-13, IL-5, and IL-21 in fibrotic processes [12]. Phenytoin has showed complex effects on the immune systems including an induction of Th 2 response. Increased production of IL-9 and decreased levels of IL-1α and Interferon-γ (IFN-γ) have been reported in Phenytoin treated mice [13]. Phenytoin increases Il-6 and IL-8 production of fibroblasts [9]. Il-6 can activate the proliferation of Lymphocytes T and B which have been related with fibrosis in various organs. Cycooxygenase-2 pathway seems to be involved by increasing amounts of IL-6 [14]. Chemokine factor of PMNs (IL-8) has been associated with fibrosis in the liver and kidney as well [15-17]. Phenytoin increases the number of PMN and T cells in gingival tissues, so, it can activate them by the Upregulation of IL-6, 8 [4].

Due to lack of enough information regarding the background mechanism of Phenytoin effects especially in synthesis of

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inflammatory mediators, this study was performed to compare the proliferation rate of fibroblasts and production of IL-1 beta, IL-6, IL-8, TGF-β, TNF-alpha and PGE2 induced by Phenytoin on fibroblasts taken from patients on two groups of adults and children in Shahid Beheshti University of Medical sciences, 2012.

Materials and Methods

Gingival sampling of adults and children, extraction and culturing of fibroblasts in laboratory

In this experimental study, four adults (2 men and 2 women) with the age of 35-42 years old and four children (3 boys and one girl) in 6-11 years old in a good systemic and general condition without any systemic diseases or use of any drugs; were selected. Excluding criteria were systemic diseases, use of any drug and pregnancy in adult women. Persons who are bearing any systematic diseases or clinical symptoms of periodontitis were excluded as well. They were asked to brush their teeth and rinse Chlorhexidine for 30 seconds before the surgeries. Samples were collected as biopsies through crown lengthening and teeth exposure surgeries from the keratinized soft tissues around the impacted canines for the adults and children respectively. Children had been referred by orthodontics and pediatrics departments. Experimental design was approved by the Ethics Committee in Shahid Beheshti Medical University. Following informed consent of donors, samples were removed from excess tissue during the surgery under local anesthesia. (We emphase the word "excess tissue" which means it was supposed to be removed in a routine surgery was used for the study and there was no interference with the surgeries).

Gingival biopsies were transferred to a medium containing DMEM (Biochrom AG, Germany), (Dulbecco's Modified Eagle Medium), streptomycin (50 u/ml), penicillin (50 u/ml), (Sigma, Missouri & America) & Fetal, FBS (10% Bovine Serum), (Sigma, Missouri & America), prepared samples were crushed under sterile hood by a jaw crusher to about 1 cm in size, manually adapted for that and cultured on specific plates 25 cm^2 and put on incubator containing CO_2 with temperature of 37ºC in a humidified atmosphere for 48 hours. Samples were regularly controlled and their medium was changed if required. The medium for experimental period was the same used at initial phase of the cell culture. Upon spreading of fibroblasts over culture plate and filling it, cells were separated from culture plane through traipsing operation and transferred to next flasks (Nunc, Copenhagen & Finland) toward performing passage operation [18-20].

Pharmaceutical counting, dividing & incubation methods of fibroblasts in children and adults

After four times passage & observing fine growth of the cells, samples were separated again from the flask plane by 25% Tripsinase enzyme and put on 2 ml volume culture medium. Neobar slides were used for determining of cell numbers. Based on cell counting, gingival fibroblast with density of 60×10^3 of cell were cultured in each sink of 24-cell plates (Nunc, Copenhagen & Finland). After 48 hours, culture sinks were divided into two control & test groups which in Phenytoin test cells (Sigma, Missouri & America) were added in 20 micrograms/ml and control group cells were remained without any drugs. The above mentioned process was performed similarly on samples of adults and children. There were 3 wells for the cells of each person as a test and 3 wells for the cells of each person as control group. It means there were totally 6 for each person to evaluate the Interleukin productions and 6 wells for the MTT (Methyl-tritiated-thymidine) tests. The same process was performed for the adults and children separately. Then, samples were put in incubator containing CO_2 with temperature of 37ºc and 95% humidity for 48 hours. It is noteworthy that, regarding limitations exist in primary cell culture, some of fibroblasts relevant to children were lost, in such a way that the remaining were 24 samples of adults and 18 samples of children.

Pilot study

According to the above mentioned methods, a similar study was performed on a biopsy sample of a healthy gingival of an adult in 36 years old through crown lengthening surgery. In this study, set up of the process for separation of fibroblasts from the sample, possibility of counting number of cells, cells incubation with Phenytoin, evaluation possibility of IL1β & PGE2 with available commercial kits and accuracy of ELISA system available in the Faculty of Dentistry with probable errors therein were studied.

Evaluation of IL1β & PGE2, IL6, TGFβ, TNFα and IL8 production amount by ELISA

To compare the production amounts of inflammatory mediators resulted from either with or without drug induced gingival fibroblasts reaction, superficial liquid (supernatant) of culture medium in test and control sinks were collected by sampler. Following 48 hours of incubation, the media of cell cultures was changed and according to pilot study and some of the published articles, 20 microgram/ml of Phenytoin (Sigma-Aldrich, St. Louise, MO, USA) was added to experimental wells while only complete media was added to control wells. We used Duo Set® IC, ELISA Development Systems (R&D Systems, Minneapolis, MN 55413, USA) to compare the concentrations of IL1β & PGE2 by using kits relevant to R & D Company according to their standard protocol (Prepared by Hormoz Pazhouhan Company, Tehran, Iran).

Study of fibroblasts proliferation and their comparison in adults and children through MTT method

Methyl-Tritiated-Thymidine (MTT) test is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. MTT is able to show the number of viable cells or cell proliferation with measuring cellular metabolic activity through NAD (P) H-dependent cellular oxidoreductase enzymes. MTT confirmed the mitochondrial activity in cells and it had direct relation with cell growth and its survival which was performed according to MTT coloring protocol.

Gingival fibroblasts resulted from fourth passage were cultured in a 96-cell plate (Nunc, Copenhagen & Finland) with density of 5000 cells in each sink together with 200 cc volume culture medium and after 48 hours were divided into two control and test groups.

20 microgram/milliliter Phenytoin (Sigma, Missouri, USA) was added to test group and control cells were remained without any drugs. After 48 hours incubation with drug, culture medium of sinks were changed and replaced by

100 cc new medium containing MTT salt (Darmstadt, Merck, Germany) with density of 0.5 mg/ml color in Phosphate Buffered Saline (PBS) (Sigma). Cells were put in incubator containing CO_2 with temperature of 37ºC and 95% humidity for 48 hours. Insoluble crystals of formason produced by living cells were dissolved by acidic Isopropanol alcohol (Merck, Germany, DMSO) and were transferred to a specific ELISA reading plate and Optic Density (OD) amount thereof was achieved by ELISA (STATFAX, Florida & America) in wavelength of 570 nm. In this method, absorption intensity has direct relation with number of living cells.

Sampling method and sample size calculation

Sample size was determined regarding the previous studies [9,15], making the confounding factors including temperature, humidity and other laboratory process, as similar as possible and design of this study. Finally, four adults' and four children's gingival samples were verified by statistical counseling department. It is noteworthy that, regarding that the study is in vitro, amount of cultured fibroblasts were formed the real samples of the study. Creating information charts and forms for each measured variable such as generation speed of fibroblasts, amount of IL1β & PGE2 in culture medium which histological information and their evaluations were registered by ELISA through specific kit in these forms.

Data analysis methods

Achieved numbers from ELISA measurement were tested by normal distribution amount of adults and children gingival cells population through Kolmogorov Smirnov. Regarding normal distribution of these data, cellular proliferation in test and control groups was compared and tested using T-Test analyses. ANOVA analysis was used to evaluate IL1β & PGE2 production amounts. According to counseling with department of statistics, Repeated Measurements Analysis was used to evaluate the correlation among cellular samples and gingival biopsy of each person separately. Alpha error level was set at 0.05.

Results

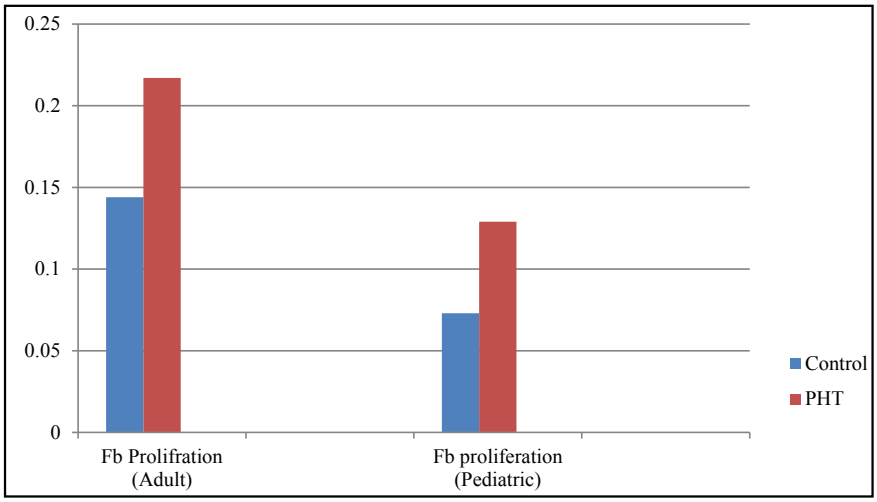
Proliferation of fibroblasts (MTT)

The Optical Density (OD) values of proliferation rate of adult fibroblasts with the presence of Phenytoin was changed from 0.144 ± 0.125 in control group to 0.215 ± 0.37 in test group, and that was not statistically significant (P=0.362) (*Figure 1*). The Optical Density (OD) values of proliferation rate of fibroblasts in children was increased in with the presence of Phenytoin in control group from 0.073 ± 0.065 to 0.129 ± 0.037 in test group, and that was not statistically significant (P=0.128) (*Figure 1*).

Different proliferation rate of Phenytoin induced gingival fibroblasts in adults (0.073 ± 0.177) as compared to children (0.056 ± 0.028) was not significant (P < 0.05).

Production of IL1β by gingival fibroblasts in adults and children (ELISA)

Synthesis of IL1β in gingival fibroblasts in adults was changed from 4.8 ± 0.29 to 4.88 ± 0.213 in the presence of Phenytoin, this difference was not statistically significant (P=1.00) (*Figure 2*). Synthesis of IL1β in gingival fibroblasts in children was increased from 4.6 ± 0.216 to 5.61 ± 0.194 in the presence of Phenytoin, so this difference was statistically significant (P=0.00) (*Figure 2*). Production of IL1β by Phenytoin induced gingival fibroblasts in children (1.01 ± 0.68) was changed as compared to adults (0.083 ± 1816) and



Proliferation rate of fibroblasts in children with phenytoin treatment: from 0.07 ± 0.07 in control group to 0.13 ± 0.037 in test group, P=0.128 Different proliferation rate of Phenytoin induced gingival fibroblasts in adults (0.07 ± 0.18) as compared to children (0.06 ± 0.0) was not significant (P<0.05).

***Figure 1**. Proliferation rate of fibroblasts in adults with phenytoin treatment: from 0.14 ± 0.13 in control group to 0.22 ± 0.37 in test group, P>0.05.*

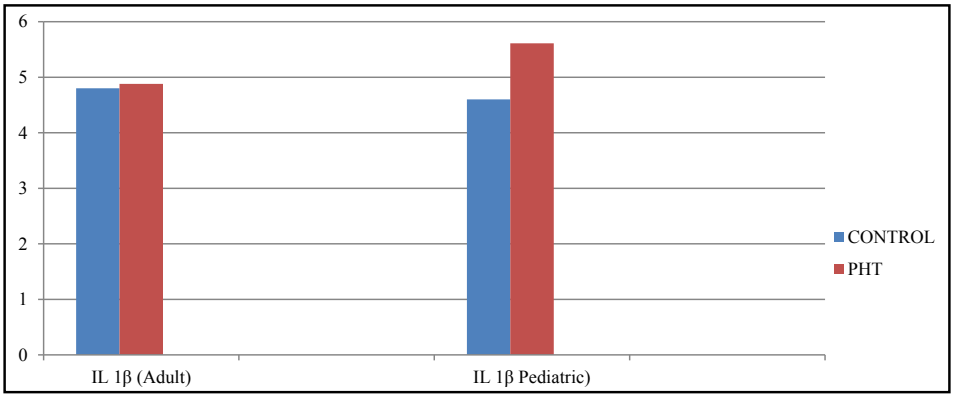


***Figure 2**. IL1 beta production with or without Phenytoin treatment, all were non-significant, P>0.05.*

that was not statistically significant (P=0.2).

Production of PGE2 by gingival fibroblasts in adults and children (ELISA)

Synthesis of PGE2 in gingival fibroblasts in adults was decreased from 559.87 ± 37.80 to 493.27 ± 18.16 in the presence of Phenytoin, this difference was not statistically significant (P=0.075) (*Figure 3*). Synthesis of PGE2 of gingival fibroblasts in children was increased from 559.87 ± 5.26 to 650.89 ± 4.4 in the presence of Phenytoin, this difference was significant statistically (P=0.05) (*Figure 3*).

Production of PGE2 by Phenytoin induced gingival fibroblasts in children was increased from 91.02 ± 0.1 as compared to adults 66.2 ± 29.6, that was statistically

Production of IL6 by gingival fibroblasts in adults and children (ELISA)

Synthesis of IL6 in gingival fibroblasts in adults was decreased from 95.73 ± 14.77 to 123.90 ± 14.83 in the presence of Phenytoin, this difference was statistically significant (P<0.05) (*Figure 4*). Synthesis of IL6 in gingival fibroblasts in children was increased from 196.87 ± 2.04 to 408.93 ± 1.94 in the presence of Phenytoin, this difference was statistically significant (P<0.05) (*Figure 4*). Production of IL6 by Phenytoin induced gingival fibroblasts in children was increased as compared to adults, that was statistically significant (P=0.00).

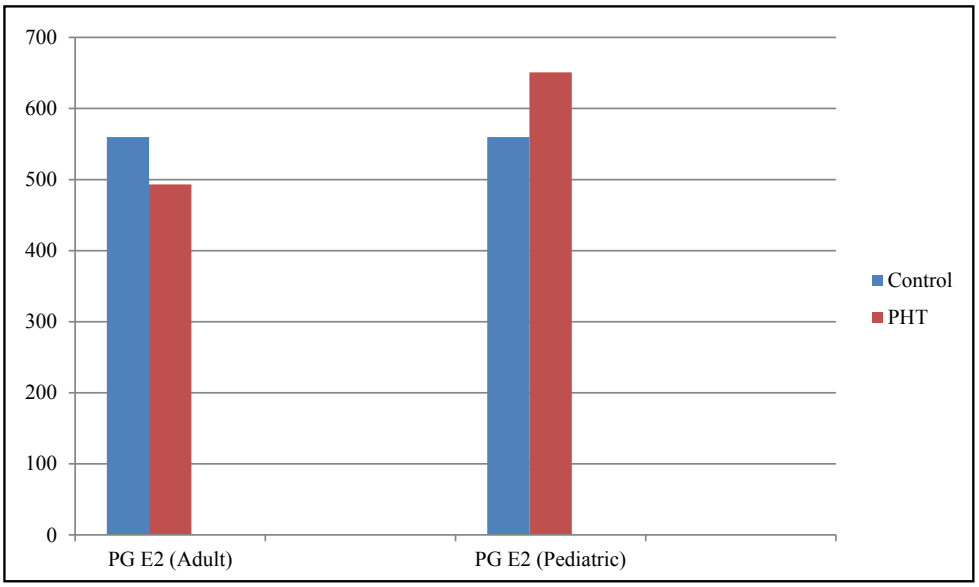
Production of TGFβ by gingival fibroblasts in adults and children (ELISA)

Synthesis of TGFβ in gingival fibroblasts in adults was increased from 9.23 ± 7.52 to 37.84 ± 8.38 in the presence of Phenytoin, this difference was statistically significant (P=0.000) (*Figure 5*). Synthesis of TGFβ in gingival fibroblasts in children was increased from 17.87 ± 2.83 to 31.50 ± 2.92 in the presence of Phenytoin, this difference was statistically significant (P=0.000) (*Figure 5*).

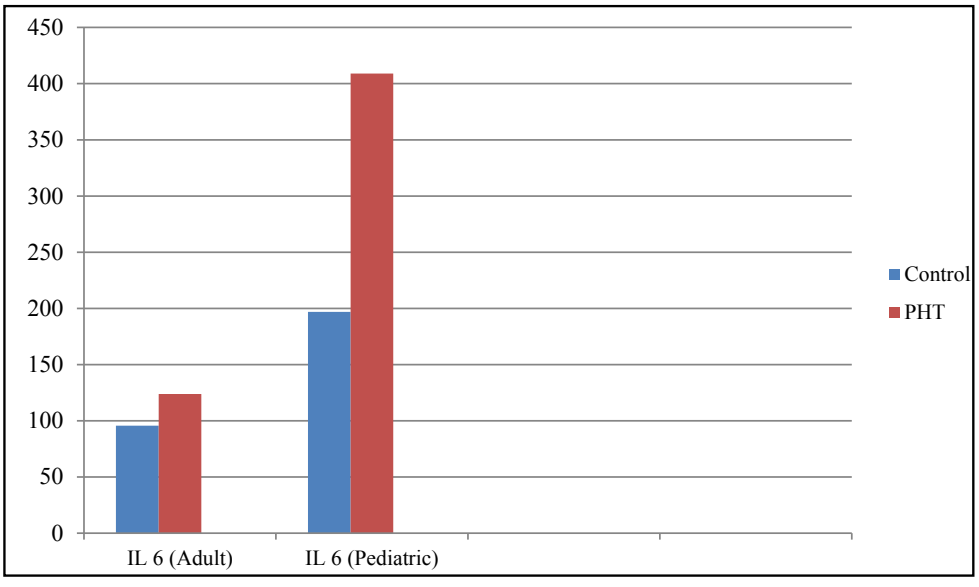
Production of TGFβ by Phenytoin induced gingival fibroblasts in children was increased as compared to adults, so this variation was statistically significant (P=0.025).

Production of TNFα by gingival fibroblasts in adults and children (ELISA)

Synthesis of TNFα in gingival fibroblasts in adults was changed from 17.80 ± 0.57 to 18.05 ± 2.43 in the presence of Phenytoin, this difference was not statistically significant (P=0.8) (*Figure 6*). Synthesis of TNFα in gingival fibroblasts in children was decreased from 17.75 ± 0.35 to 17.63 ± 0.71 in the presence of Phenytoin, this difference was not statistically significant (P=0.6) (*Figure 6*).



Adults: NS, P=0.075
Children: P<0.05
***Figure 3**. PG E2 production with or without Phenytoin treatment.*



Production of IL6 by Phenytoin induced gingival fibroblasts in children was increased as compared to adults, that was statistically significant (P=0.00).
***Figure 4.** IL 6 production with or without Phenytoin treatment, both were statistically Significant, P<0.05.*

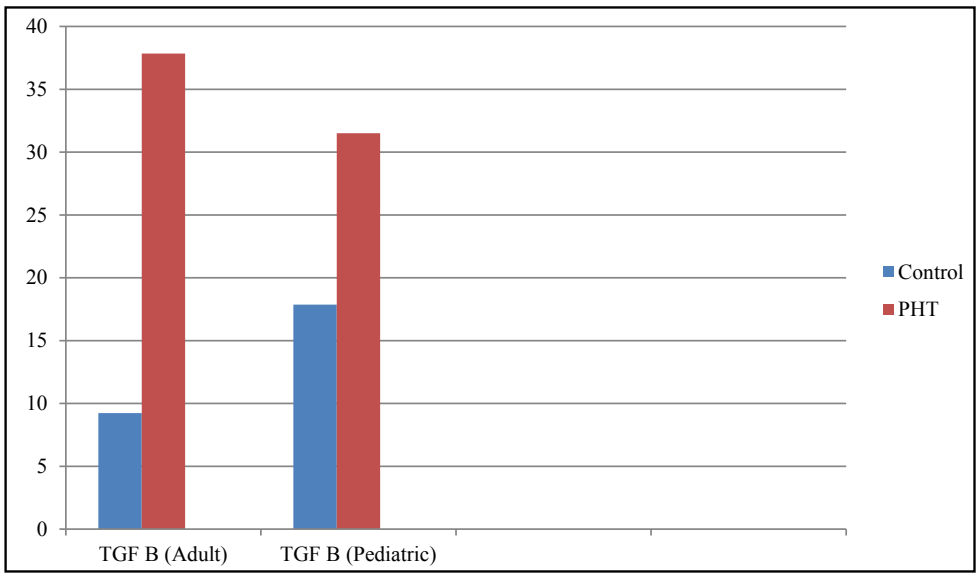


Figure 5. *TGF beta production with or without Phenytoin treatment, All differences were significant, P<0.05.*

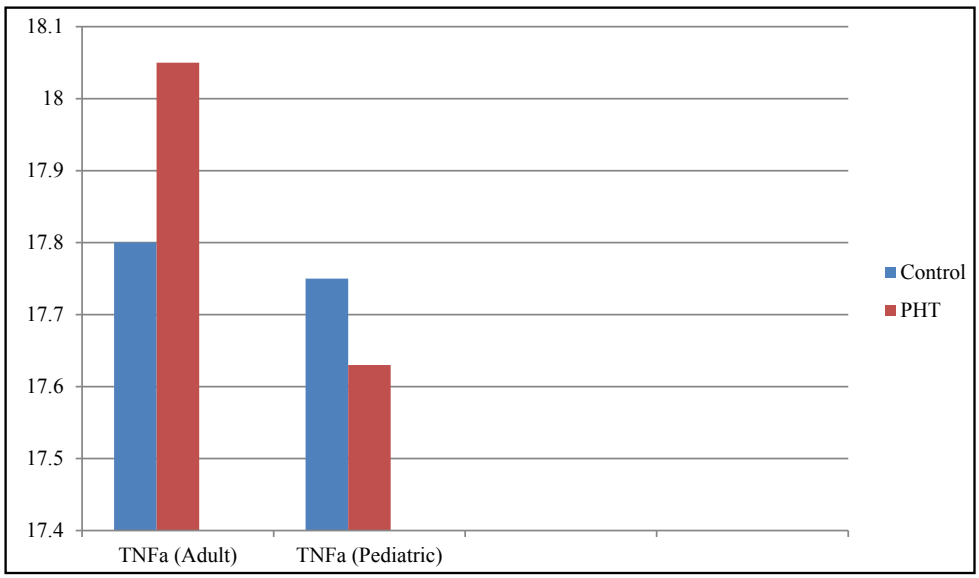


Figure 6. *TNF alpha production with or without Phenytoin treatment. All differences were non-significant, P<0.05.*

Production of TNFα by Phenytoin induced gingival fibroblasts in children was decreased as compared to adults, this variation was not statistically significant (P=0.8).

Production of IL8 by gingival fibroblasts in adults and children (ELISA)

Synthesis of IL8 in fibroblasts of adults was increased from 114.24 ± 12.09 to 178.23 ± 36.76 in the presence of Phenytoin, this difference was statistically significant (P=0.002) (*Figure 7*). Synthesis of IL8 in fibroblasts of children was increased from 609.71 ± 343.71 to 2098.01 ± 846.91 in the presence of Phenytoin, so this difference was statistically significant (P=0.005) (*Figure 7*). Production of IL8 by Phenytoin induced gingival fibroblasts in children was decreased as compared to adults, this difference was statistically significant (P=0.02).

Discussion

The speed of multiplication in Phenytoin induced gingival fibroblasts in children and adults hadn't significant difference as compared to the group without Phenytoin. These results were in agreement Hassel et al. [21], Yamada et al. [22], Modeér et al. [23] and Le et al. [24] articles.

Al-ubaidy et al. [25], Brunius et al. [18] and Modéer et al. [26] introduced some articles that disagreed to our study. Hassell et al. [21] and Yamada et al. [22] showed that after application of Phenytoin, there was no significant increase in multiplication of fibroblasts.

Modéer performed an experimental study on fibroblasts of gum in children, and he observed some increase in PGE2 in invivo and invitro that was similar to our results [26].

In another study on TNF, LE and Weinstein showed that this mediator have some stimulating effects on fibroblasts, however, this study was done on fibroblasts of the skin. In addition, they did not report any stimulating effects on the fibroblasts of lung [24]. In our study, increase of TNFα was observed by fibroblasts of gum in the presence of Phenytoin that was not significant, but we showed some reductions in production of TNFα in children in the presence of Phenytoin which was not significant as well.

Al-ubaidy showed that in the presence of Phenytoin, fibroblasts had some remarkable multiplications [25], and this was completely different from our study showing that there is no significant different in the speed of multiplication Phenytoin induced gingival fibroblasts. Brunius reported that in culture media of children fibroblasts, TNFα alone did increase IL 1b and PGE 2 production while phenytoin did not. However, the two together had an enhanced effect. It means

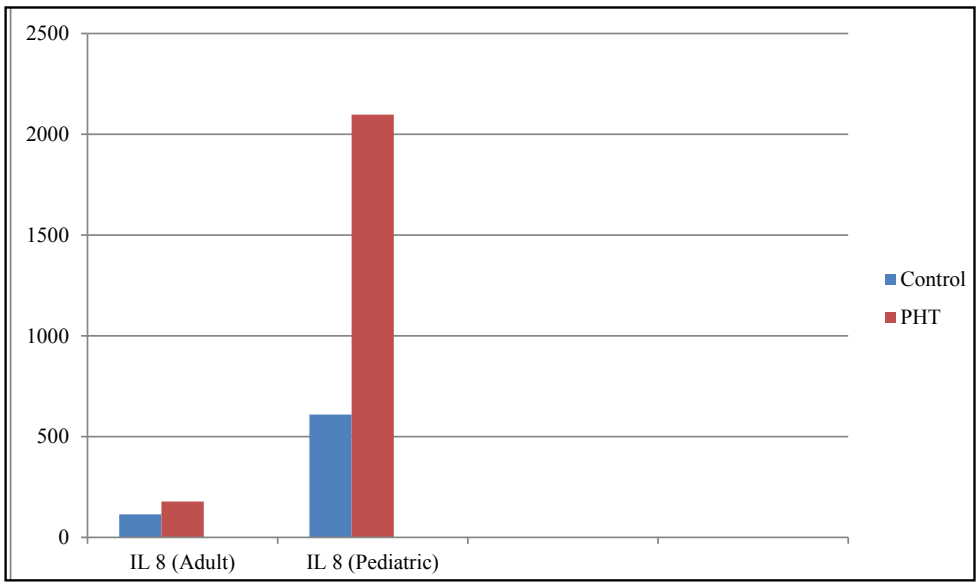


***Figure 7**. IL 8 production with or without Phenytoin treatment. All differences were significant P<0.05.*

the application of Phenytoin and TNFα simultaneously, increases the production of IL1β and PGE2 in the culture environment [18]. Above result was a little different with the results in our study. Synthesis of IL1β showed no significant increase in Phenytoin induced gingival fibroblasts of children and adults in current study, while the frequency of synthesis of PGE2 had merely significant increase in fibroblasts of gum in children in the presence of Phenytoin, but it was decreased in adults.

Modeér in another study expressed that Phenytoin causes the increase in the production of IL8 and IL6 in fibroblasts of human gum [26]. This result was in agreement with ours since the synthesis of IL6 and IL8 in Phenytoin induced gingival fibroblasts in adults had a significant increase.

Brunius showed that Phenytoin is necessary beside TNFα to stimulate the production of IL1β and the production of IL1β by Phenytoin itself is impossible. TNFα cannot stimulate the formation of PGE2 in fibroblasts of gum as well and this is intensified in the presence of Phenytoin [24,25,27].

In another study by Modeér et al, they used the serum-free medium including Albumin serum 0.1% [26], while in current study; we used the culture environment including DMEM, Streptomycin and Penicillin.

One of the distinguishing characteristics of this study was the analysis on the 6-11 years old children. Most of the previous studies have evaluate these mediators just in adults while epidemiologic studies shows more prevalent GO in children as compared to adults. As we know, the increase of the volume of gum in children who suffer from epilepsy while they use Phenytoin is 67%; same percent for the adults is 50% [28].

Since Phenytoin induced gingival fibroblasts seem to produce greater amounts of inflammatory cytokines including IL1-beta, IL6 and IL8 as compared to those in adults, this study implies the key role of inflammation to induce hyperplasia and to justify the different prevalence of gingival hyperplasia in different ages. According to the high prevalence of GO especially in children, the broader studies are necessary. However, there are some limitations in the analysis on the samples of children including the difficulty of taking samples in children and cooperation of the child for surgery, also the intended tissue should not have any systemic disease, gum inflammation or any kind of periodontitis.

This study was done on some limited samples. Comprehensive studies with well-documented design are highly recommended. In addition, the present study was done on inflammatory mediators by ELISA. Although ELISA system is accurate, but probably it will be possible to do these kind of studies from the genetic point of view with PCR method for example to find the RNA chain to look for the expression of the cytokines genes at the same time.

Acknowledgement

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