Effect of Phenytoin and Age on Gingival Fibroblast Enzymes

Surena Vahabi1, Bahareh Nazemisalman2, Mojtaba Vahid Golpaigani3, Anahid Ahmadi4

1Assistant Professor of Periodontics Department, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2Assistant Professor of Pedodontics Department, Dental School, Zanjan University of Medical Sciences, Zanjan, Iran
3Associate Professor of Pedodontics Department, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4Dentist

Abstract
Objective: The alteration of cytokine balance is stated to exert greater influence on gingival overgrowth compared to the direct effect of the drug on the regulation of extracellular matrix metabolism. The current study evaluated the effect of phenytoin on the regulation of collagen, lysyl oxidase and elastin in gingival fibroblasts.

Materials and Methods: Normal human gingival fibroblasts (HGFs) were obtained from 4 healthy children and 4 adults. Samples were cultured with phenytoin. MTT test was used to evaluate the proliferation and ELISA was performed to determine the level of IL1β and PGE2 production by HGFs. Total RNA of gingival fibroblasts was extracted and RT-PCR was performed on samples. Mann-Whitney U test was used to analyze the data with an alpha error level less than 0.05.

Results: There was a significant difference in the expression of elastin between the controls and treated samples in both adult and pediatric groups and also in the lysyl oxidase expression of adult controls and treated adults. No significant difference was found between collagen expression in adults.

Conclusion: The significant difference in elastin and lysyl oxidase expression between adult and pediatric samples indicates the significant effect of age on their production.

Keywords: Phenytoin; Fibroblasts; Lysyl oxidase; Elastin; Collagen1; RT-PCR

INTRODUCTION
Phenytoin is a common antiepileptic drug; which acts on voltage-gated sodium channels in neural cells [1]. It has been in the limelight of research for its effect on clinically detectable gingival overgrowth with a quoted prevalence figure of 50% [2]. Despite similar clinical manifestation of gingival overgrowth induced by antiepileptic (phenytoin) and calcium channel blockers (nifedipine), various degrees of inflammation exist between them [3].
The severity of overgrowth has been correlated with the drug concentration in the gingival crevicular fluid (GCF), degree of protein binding and bioavailability of the drug [4, 5]. However, not all patients on phenytoin develop gingival overgrowth. Existence of various types of fibroblasts in gingival and other tissues explains their heterogeneity due to factors such as protein synthesis, collagen production, glycosaminoglycan accumulation, replicative life span in culture, proliferation rate, cell size distribution and response to exogenously added substances [6, 7]. Resultantly, phenytoin may either stimulate or inhibit synthetic or proliferative activity in the aforementioned subpopulations [8-12]. As clearly stated in previous studies, extracellular collagen degradation is controlled by collagenase, a known matrix metalloproteinase (MMP), responsible for pathologic turnover of connective tissue [4, 13-15]. Its intracellular degradation, however, is controlled by fibroblast phagocytes known as normal turnover. Phenytoin’s inhibitory effect on collagen phagocytosis by fibroblasts is shown to lead to the decrease of collagen degradation [16]. This indicates that overgrowth is not solely attributed to the increase of collagen synthesis but also to the decrease in its degradation. This collagen is resistant to the tissue MMP of inflammatory bacterial collagenase [17, 18]. Another study has demonstrated up regulation of collagen 1, 2 and glycosaminoglycans [19].

The alteration of cytokine balance is suggested to exert greater influence on gingival overgrowth compared to the direct effect of the drug on the regulation of extracellular matrix metabolism or proliferation of gingival overgrowth [20]. Decreased Laminin 5 and discontinuous expression of collagen IV were also reported in the disrupted basal membrane of overgrown gingival tissue [21]. On the other hand, TGFB1 was shown to decrease E-Cadherin and increase the expression of MMP2, MMP9 and MMP 13 [22]. As for the role of lysyl oxidase in early passage of fibroblasts, a TGF-B stimulated CTGF was shown to contribute to biosynthesis of lysyl oxidase and collagen [23, 24]. Lysyl oxidase is a copper dependant enzyme; which plays an important role in connective tissues [25]. It finalizes the ECM production by cross-linking elastin-collagen [26]. Recently revealed functions of lysyl oxidase include regulation of cellular and gene transcription due to its ability of oxidizing substrates and adjustment of cell function, hypothetically by modulation of growth factors [27].

Formation and maturation of collagen and elastin are vastly dependent on post-translational modification/alteration. Alteration of lysyl oxidase leads to the changes in the ratio of synthesis of elastin and collagen [28]. Epidemiologic studies have revealed drug induced gingival overgrowth to be more dominant in male adolescents and children [29]. One study has introduced age as an important risk factor for drug induced gingival overgrowth [30]. Alteration of fibroblasts, decrease of collagen and non-collagenous protein, change in the fibroblast size, and their mitotic activity with age could be contributory [31-33].

There is continuous debate in the literature regarding the difference of mechanism of action and cellular effect of phenytoin between fibroblasts of children and adults. Also despite today’s broad insight into the molecular basis of gingival overgrowth, the exact relationship between cytokine levels and extracellular matrix, elastin biosynthesis and mRNA levels remains yet unexplained. There has only been an assumption of age’s effect; thus, further studies are indeed needed. In this study we aimed to assess the effect of phenytoin on the regulation of connective tissue proteins such as lysyl oxidase, elastin and collagen1 genes in fibroblasts of adults and children using RT-PCR.
MATERIALS AND METHODS

Cell Culture:
Pediatric samples were obtained from four 4-11 year-old healthy patients while performing impacted tooth extraction. Adult fibroblast samples were derived from four 34-42 year-old healthy adults who underwent crown lengthening surgery. Following informed consent of donors, samples were removed from excess tissue during the surgery under local anesthesia. The experimental protocol was approved by the ethics committee of our university. Cells were plated in Dulbecco’s Modified Eagle’s Medium (DMEM; Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (50 U/ml), and penicillin (50 U/ml). Samples were then cultured in 25cm² plates, and incubated at 37°C containing 5% CO2. When necessary, samples were fed with fresh medium at their regular control intervals for contamination and cell growth. Fibroblasts were trypsinized when out grown from the explants. Then, they were transferred to succeeding flasks (Nunc, Copenhagen, Denmark) for secondary culture. The fourth passages of the cells were used for the experiments.

Enzyme-Linked Immunosorbent Assay (ELISA):
24 well plates (Nunc, Copenhagen, Denmark) were divided equally into control and experimental groups. Gingival fibroblasts were seeded into wells at a density of 60x–10³ cells/well. After 48 hours, phenytoin (Sigma-Aldrich, St. Louis, MO, USA) (20 μg/ml) was added to the experimental wells. Samples were then incubated at 37°C in 5% CO2. When necessary, samples were fed with fresh medium at their regular control intervals for contamination and cell growth. Fibroblasts were trypsinized when out grown from the explants. Then, they were transferred to succeeding flasks (Nunc, Copenhagen, Denmark) for secondary culture. The fourth passages of the cells were used for the experiments.

RT-PCR:
Total RNA of gingival fibroblasts was extracted with R Neasy Minikit (Qiagen, USA). RT reaction was performed in a mixture containing 1mM MgCl2(Fermentas, Lithuania), 0.2mM dNTP, 1X RT buffer , 0.5μg oligo dT primer , 200U MMuLV reverse transcriptase and DEPC water up to 20 µl final volume reaction. The primers’ sequences and RT-PCR program are respectively listed in Tables 1 and 2. PCR reactions were carried out in a final volume of 30 µl consisting of 5µl cDNA, 0.2mM of dNTPs mix, 1pmol of each primer (lysyl oxidase, elastin and collagen) and 1.25U Taq DNA polymerase (Fermentas, Lithuania).

MTT Assay: The MTT assay was performed according to the manufacturer’s instructions. Gingival fibroblasts were seeded into 96-well plates (Nunc, Copenhagen, Denmark) at a density of 5X10⁴ cells/well and were cultured in a 200cc medium. Samples were divided into control and experimental groups following 48 hours of incubation. Phenytoin was then added to the experimental wells. After 48 hours of incubation, the medium was substituted with 100 cc of a fresh medium containing a 0.5 mg/ml solution of tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Incubation was carried out at 37°C in 95% humidified atmosphere containing CO2 for 4 hours. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) was used to dissolve the crystals of formazan produced by the living cells. The quantity of formazan was assessed at a wavelength of 570 nm with ELISA plate reader (Stat Fax, Florida, USA) according to the manufacturer’s instructions.
Table 1. The Oligonucleotide primers for RT-PCR

| Primer          | Sequence                                           | Product Base Pairs |
|-----------------|----------------------------------------------------|--------------------|
| COLLAGENLAGEN1  | F 5’acttctggac gcgggacgctg cag3’                  | 300bp              |
|                 | R 5’aacctctagttcctggttgaccag3’                    |                    |
| Lysyl oxidase   | F 5’atccaatggagaacaacgggagcggcgg c3’              |                    |
|                 | R 5’gecccaacatgcgctcgctagcgtg c3’                 |                    |
| Elastin         | 5’TGGAGCCCTGGGATATCAAG                              | 170bp              |
|                 | GAAGCACCAACATGTAGCAC 3’                            |                    |

| Table 2. PCR thermal program |
|-----------------------------|
| Genes | Program | Repeats | |
| COLLAGENLAGEN1 | Denaturation 94°C 30s | 35 |
| Lysyl oxidase | Denaturation 94°C 30s | 35 |
| Elastin | Denaturation 94°C 30s | 35 |
| PCR program is like the others | PCR program is like the others |
| PCR product 170bp | PCR product 170bp |

Gene expression

PCR products quantification which showing mRNA levels
Results were analyzed via 2% agarose gel electrophoresis and SYBR® Green staining. The quantification of PCR bands was performed with Work-Lab software. Protein regulation in treatment groups was performed using Mann Whitney U test. The mean average of the elastin, lysyl oxidase and collagen expression in control groups of both pediatric and adult samples is shown in Table 3. Kruskal-Wallis test was used to determine the difference between expression of elastin, lysyl oxidase and collagen in the control and treated samples in both pediatric and adult groups. For the study of protein regulation in treatment groups statistical analysis was performed using Mann Whitney U test which was applied to determine the difference between expression of elastin, lysyl oxidase and collagen in the control and treated samples in both pediatric and adult groups. P values were set less than 0.05 according to the Table 4. Data were entered and statistically analyzed using SPSS 16 for Windows.

RESULTS
The mean average of the elastin, lysyl oxidase and collagen protein expression in control groups of both pediatric and adult samples are shown in Table 1. Significant differences were found in the expression of elastin between controls and treated samples in both adult and pediatric groups and also in the lysyl oxidase expression of controls and treated adults (P<0.05).

No significant difference was found between collagen expression in adult controls and treated adults. Similarly, there was no difference in lysyl oxidase and collagen gene expression in the control and treated groups of pediatric samples. Assessment of the effect of age on each of the treatment groups was performed by Mann Whitney U test. Comparison of protein expression between adult/ pediatric samples of control group and adult/pediatric samples of treated group, for the effect of age, was assessed via the Mann Whitney U test (Table 2).

Table 3. Means of the Elastin, Lysyl oxidase and Collagen Protein expression in control groups of both pediatric and adult samples

| Protein | Elastin | Lysyl Oxidase | Collagen  |
|---------|---------|---------------|----------|
| Control Group |        |               |          |
| Pediatric    | 219.3±28.6 | 43.6±22.6     | 84.90±61.9 |
| Adult        | 161.0±74.8 | 60.0±7.1      | 90.63±34.8 |

Table 4. Difference between expression of Elastin, Lysyl oxidase and Collagen Proteins in the control and treated samples in both Pediatric and adult groups.

| Protein            | Elastin | Lysyl Oxidase | Collagen  |
|--------------------|---------|---------------|----------|
| Sample Group       |        |               |          |
| Pediatric control and PHENYTOIN treated | P=0.042 | P=0.253 | P=0.253 |
| Adult control and PHENYTOIN treated     | P=0.042 | P=0.002 | P=0.470 |
The only significant difference was in the elastin and lysyl oxidase expression between adult and pediatric samples of control group and also between the adult and pediatric samples of phenytoin treated groups. This indicates that age had a significant effect on the expression of elastin and lysyl oxidase in both control and phenytoin treated samples.

**DISCUSSION**

The present study analyzed the effect of phenytoin on the regulation of connective tissue proteins such as lysyl oxidase, collagen and elastin. It also investigated the effect of age on the regulation of aforementioned proteins. In a previous study on the cytokine regulation of collagen, lysyl oxidase and elastin in gingival fibroblasts, no investigation was done on the effect of age [34].

In the control group of our study, collagen and lysyl oxidase were expressed more in adult fibroblasts compared to pediatric samples; whereas, elastin was expressed more in pediatric fibroblasts. The pattern was similar in the phenytoin treated group.

Phenytoin significantly down-regulated elastin expression of adult and pediatric samples. It had an up-regulatory effect on the lysyl oxidase and collagen expression of adult and pediatric fibroblasts, the latter being insignificant. It has already been shown that the drug induced gingival overgrowth is partially due to cytokines such as bFGF, TGF-B CTGF, IL-1B and IL-6 [35-40]. Receptors of bFGF and TGF-B1 were detected in the lamina propria of gingival overgrowth assuming their collagen elaborative effect on gingival overgrowth [3]. CTGF and bFGF were reported to have a regulatory effect on lysyl oxidase and collagen [39, 40]. In contrast to collagen, elastin and elastic fibers are estimated to have a small proportion of 5% in the gingival tissue [41, 42]. Study of gingival overgrowth showed an increased amount of ECM while the increase of collagen and elastic fibers was witnessed in some lesions [43-45]. The increase of lysyl oxidase has been reported in diseases such as oral submucous fibrosis and lung and liver fibrosis [46, 47]; this highlights the role of lysyl oxidase in the balance of ECM metabolism.

**Table 5.** The mean of Protein expression in pediatric and adult samples of control groups

| PHENYTOIN Groups | Protein | Pediatric | Adult   |
|------------------|---------|-----------|---------|
| Elastin          | 243.84±24.3 | 122.47±58.5 |
| Lysyl oxidase    | 49.83±22.4  | 83.55±22.4  |
| Collagen         | 104.14±77.0 | 107.60±21.8 |

**Table 6.** Comparison of protein expression between adult/pediatric samples of control group and adult/pediatric samples of treated group, indicating the effect of age, was assessed via Mann-Whitney test

| Studied Groups | Control Groups of Pediatric and Adult Samples | Phenytoin Groups of Pediatric and Adult Samples |
|----------------|-----------------------------------------------|-----------------------------------------------|
| Protein        | P=0.114                                       | P=0.000                                       |
| Elastin        |                                               |                                               |
| Lysyl oxidase  | P=0.253                                       | p=0.012                                       |
| Collagen       | P=0.253                                       | P=0.114                                       |
Elastin and collagen are mainly formed and matured by lysyl oxidase in the post-translational modification. Their cross-linking and fixation into ECM are done by lysyl oxidase [48]. Therefore, increase of lysyl oxidase may alter the ratio of elastin to collagen synthesis [49]. TGF-B has an up-regulatory effect on the lysyl oxidase and collagen mRNAs. It also has been shown to induce CTGF; which in turn increases lysyl oxidase activity via increase of insoluble collagen [39]. It has been shown that regulation of elastin mRNA levels and elastin synthesis in connective tissue cells is implicated by mRNA stability. TGF-B can affect this mRNA stability as well as mRNA stability of some other connective tissue genes such as collagen1 [49]. The effect of TGF-B on elastin mRNA of skin fibroblasts leads to an increase of elastin mRNA concentration and elastin synthesis [49-53]. In our study, lysyl oxidase was increased in both treated groups; however, the increase of lysyl oxidase coincided with the increase of elastin in pediatric samples but decrease of elastin in adult samples. This outcome may be explained by fibroblast heterogeneity or the difference of connective tissues between adults and pediatrics. TGF-βs and BMPs have an up-regulatory effect on collagen [54-58]. A decrease of mRNA for type 1 collagen after 1-2 day incubation with phenytoin was reported while no change was observed in type IV collagen mRNA [59]. Kato et al, who studied the effect of TNFα and phenytoin on collagen metabolism demonstrated that these factors lead to collagen accumulation by impairing collagen metabolism in gingival fibroblasts. Both these factors inhibit collagen endocytosis. On the other hand, phenytoin increases the effect of TNF-α. As previously stated, collagen synthesis is regulated by MMPs and its antagonist TIMPS. In their study, phenytoin decreased mRNA expression of MMP1 and 2 and increased TIMP-1 mRNA expression leading to the suppression of collagen degradation. They concluded that phenytoin enhances collagen accumulation in gingival fibroblasts exposed to low levels of TNF-α. Since this factor exists in inflammation, it explains why overgrowth is worsened in cases of chronic gingival inflammation [55-57]; this is in agreement with our study where collagen was insignificantly increased in the phenytoin treated samples of both adults and pediatrics.

Increase of TIMP and TGF-B and decrease of MMP1 were observed in both adult and pediatric samples. It should be pointed out that the changes in pediatric samples were significant; whereas, those in adults where insignificant [54]. In our study, there was a significant difference in the regulatory effect of phenytoin on elastin and lysyl oxidase between adult and pediatric treated fibroblasts. This indicates the effect of age on the regulation of these proteins. No significant difference was reported in the expression of collagen between phenytoin treated fibroblasts of adults and pediatrics indicating that age did not affect on the regulation of collagen. To explain this, one should look deeper into the effect of age on the homeostasis of connective tissue; all body tissues undergo alterations with age and gingival tissue is no exception. Previous studies documented decrease of collagen and non-collagenous proteins’ metabolism with age as well as modification in the size of fibroblasts and mitotic activity of both fibroblasts and epithelial cells [59-63].

Gagliano et al. reported a decrease in the collagen gene expression and TIMP-1 with no change in MMP-1 and TGF β1 mRNA levels in adult fibroblasts compared to young ones [64]. In drug treated samples, there were higher levels of collagen-1 in (CsA) treated young fibroblasts. Studies have reported down-regulation of MMP-1 protein levels in young fibroblasts treated with CsA [64, 65]. In their study, similar collagen content was reported in young and aging fibroblasts. An unchanged level of MMP-1 and TIMP-1 mRNA was reported.
They documented similar MMP-1 and MMP-2 protein levels assuming that gingival collagen content is not regulated at its degradation level. There was also no alteration in TGF-1 gene expression in aging fibroblasts. This assumes similar tone of this factor influencing collagen turnover. Likewise, in our study no significant change was observed in the MMP-1, 2, TIMP and TGF-B levels in adult and pediatric fibroblasts. As previously stated, TGF-B does have an up-regulatory effect on collagen. However, the up-regulatory effect of the drug on collagen content in our study might be due to different drugs used in our study compared to Gagliano’s report [64]. It is suggested that collagen regulation in young and aged gingiva could partly depend on maturation pathways and post-translational modifications such as collagen cross-linking. This study is in agreement with the result of our study which may implicate the pathways of collagen turnover might be similarly affected by drug treatment in young and aging fibroblasts. Thus, young and aged fibroblasts may respond similarly to drugs in terms of collagen regulation. This suggests that phenytoin treatment affects collagen turnover pathways to a similar extent in young and aging gingival fibroblasts.

As for lysyl oxidase, its enzyme activity is reported to be stimulated by addition of 1 to 50 ng/ml CTGF to gingival fibroblasts. TGF-beta 1 is also reported to increase lysyl oxidase activity and its mRNA levels [67]. This effect was shown to be dose- and time-dependent. But, no such effect was observed on elastin. This is in agreement with our study where increase of TGF-B and consequently lysyl oxidase led to the increase of elastin in pediatric samples but decreased in adult samples. The highest stimulatory effect of TGF-beta1 on lysyl oxidase mRNA activity occurred after 48 hours of fibroblast treatment with 500 pM of TGF-beta1. A study on lysyl oxidase and its mRNA level, quantified by real-time reverse transcriptase-polymerase chain reaction, showed their decrease in adult skin fibroblasts when compared with fibroblasts from children [68]. The elastin mRNA level in contrast remained stable at all ages. The inconsistency of this finding with our result might be due to the difference between skin and gingival fibroblasts and also the heterogeneity within gingival fibroblasts. Observation of rat aorta showed high lysyl oxidase expression in early development with significant reduction in adulthood [69]. In our study lysyl oxidase and collagen content was more in adults. The outcome disagrees with the aforementioned data; the reason might be sample differences. While aging is defined by decreased elastin/collagen ratio and decreased expression of lysyl oxidase tropocollagen and collagen 1, this could vary in different cell types and also in different samples especially when exposed to drugs [69]. These are incompatible with our study where collagen and lysyl oxidase levels were shown to be less in pediatric fibroblasts compared to adult fibroblasts both in control and treated samples. This might be due to the type of studied fibroblasts. Apart from the differences between skin and gingival fibroblasts, the heterogeneity within gingival fibroblasts should also be taken into account. Phenytoin stimulated pediatric fibroblasts to produce more levels of IL1β while there was no change in the adult group. Also, the mild decrease of T cells and the decrease of TGF-B with age should be regarded when the difference of adult and pediatric connective tissue components is considered. It is obvious that the increased mass of gingival tissue consists of increased amount of substance; it is said that glycosaminoglycans also influence the rate and quality of fibril formation by its interaction with collagen. This emphasizes the different reaction to drugs of the responder cell type compared to its morphologically identical gingival counterpart [70].

**CONCLUSION**

Pediatric fibroblasts of all 3 proteins increased by phenytoin. However, in adult samples, the
increase of collagen and lysyl oxidase was parallel with the decrease of elastin.
The protein expression between adult and pediatric samples indicated that age had a significant effect on the expression of elastin and lysyl oxidase in both control and phenytoin treated samples.

REFERENCES
1- Segal MM, Douglas AF. Late sodium channel openings underlying Epileptiform activity is preferentially diminished by the anticonvulsant phenytoin. J Clin Nephrology 1977; 77: 3021-34.
2- Angelopoulos AP, Goaz PW. Incidence of diphenylhydantoin gingival hyperplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod1972; 34: 898-906.
3- Seymour RA, Heasman PA. Drugs and the periodontium. J Clin Periodontol 1988; 15:1-16.
4- Seymour RA, Thomason JM, Ellis JS. The pathogenesis of drug induced gingival overgrowth: J Clin Periodontol 1996; 23:165-175
5- Seymour Ravels JS, Thomason JM. Risk factors for drug-induced gingival overgrowth. J Clin Periodontol 2000; 27: 217-223.
6- Benveniste K, Bitar M. Effects of phenytoin on cultured human gingival fibroblasts, phenytoin-induced teratology and gingival pathology. Edited by T Hassell, M Johnston, Dudley K. New York, Raven Press 1980; 199-214.
7- Hassell TM. Stimulation and inhibition of fibroblast subpopulations by phenytoin and phenytoin metabolites: Pathogenetic role in gingival enlargement. Pediatr Dent 1981; 3:137-153.
8- Kasai S, Hachimine K. Effect of 5, 5-diphenylhydantoin sodium on the synthesis of collagen by some fibroblastic cell lines including gingiva derived cells. Bull Tokyo Dent Coll 1974; 15: 53-62.
9- Houck JC, Cheng RF, Waters MD. The effect of Dilantin upon fibroblast proliferation. Proc Soc Exp Biol Med 1972; 139: 969-971.
10- Kasai S, Yoshizumi T. Effect of diphenyl hydantoin sodium on the proliferation of cultured cells in vitro. Bull Tokyo Dent Coll 1971; 12: 223-234.
11- Blumenkrantz N, Asboe-Hansen G. Effect of diphenyl hydantoin on connective tissue. Acta Neurol Scand 1974; 50: 302-306.
12- Liu TZ, Bhatnagar RS. Inhibition of procollagen proline hydroxylase by Dilantin. Proc Soc Exp Biol Med 1973; 142: 253-255.
13- Chang C, Werb Z. The many faces of metalloproteases; cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol 2001; 11: 37-343.
14- Shikata H, Utsumi N, Shimojima T, Oda y. Okada Y. Increased expression of type VI collagen genes in drug induced gingival enlargement. FEBS Lett 1993; 334: 65-68.
15- Romanosn GE, Strub JR, Bernimoulin JP. Immunohistochemical distribution of extracellular matrix proteins as a diagnostic parameter in healthy and diseased gingival. J Periodontol 1993; 64: 110-119.
16. McCulloch C A, Knowles G. Deficiencies in collagen phagocytosis by human fibroblasts in vitro: a mechanism for fibrosis. J Cell Physiol 1993; 155, 461-471.
17- Goutchkin J, Shoshan S. Inhibition of collagen breakdown by diphenylhydantoin. Biochem Biophys Acta 1980; 631:188-191.
18- Aikaterini D, Mina M, Effie I. Gingival overgrowth in children: epidemiology, pathogenesis and complication: A literature Review. J Periodontol 2005; 76: 3-10.
19- González OA, González JM. Morphological and phenotypic differences in fibroblasts obtained from gingival overgrowth secondary to phenytoin: A pilot study. Revista Odontologica Mexicana 2009; 13: 17-23.
20- Trackman PC, Kantarci A. Connective tissue metabolism and gingival overgrowth. Crit Rev Oral Biol Med 2004; 15: 165-175.
21- Kantarci A, Nseir Z, Kim YS, Sume SS, Trackman PC. Loss of basement membrane integrity in human gingival overgrowth. J Dent Res 2011; 90: 887-93.
22- Sume SS, Kantarci A, Lee A, Hastert H,
Trackman PC. Epithelial to mesenchymal transition in gingival overgrowth. Am J Pathol 2010; 177: 208-218.

23- Hong HH, Uzel MI, Duan C, Sheff MC, Trackman PC. Regulation of lysyl oxidase, collagen and connective tissue growth factor by TGF-B1 and detection in human gingival. Lab Invest 1999; 79: 1655-1667.

24- Uzel MI, Kantarci A, Hong HH et al. Connective tissue growth factor in phenoitoin – induced gingival overgrowth. J Periodontol 2001; 72: 921-931.

25- Lucero HA, Kagan HM. Lysyl oxidase: an oxidative enzyme and effector of cell function. Cell Mol Life Sci 2006; 63: 2304-2316.

26- Hong HH, Uzel MI, Duan C, Sheff MC, Trackman PC. Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF-beta1 and detection in human gingival. Lab Invest 1999; 79: 1655-67.

27- Atsawasuwan P, Mochida Y, Katafuchi M, Kaku M, Fong KS, Csiszar K, et al. Lysyl oxidase binds transforming growth factor-β and regulates its signaling via amine oxidase activity. J Biol Chem 2008; 283: 34229-40.

28- Aril JA. Phenoitoin-induced depression of salivary IgA and gingival hyperplasia. Epilepsia 2007; 17: 283-291.

29- Aikaterini D, Mina M, Effie I. Gingival overgrowth in children: epidemiology, pathogenesis and complication: A literature Review. J Periodontol 2005; 76: 3-10.

30- Daley TD, Wysocki GP. Cyclosporine and its significance to the periodontist. J Periodontol 1984; 55:708.

31- Johnson BD, Page RC, Narayanay S, Pieters HP. Effects of donor age on protein and collagen synthesis “in vitro” by human diploid fibroblast. Lab Invest 1986; 55: 490-6.

32- Karring T, Loe H. The effect of age on mitotic activity in rat oral epithelium. J Periodontal Res 1973; 8: 164-70.

33- Celenligil-Nazliel H, Ayhan A, Ruacan S. The effect of age on proliferating cell nuclear antigen expression in oral gingival epithelium of healthy and inflamed human gingiva. J Periodontol 2000; 71: 1567-74.

34- Hsiang-Hsi Hong, Philip C, Trackman. Cytokine regulation of gingival fibroblasts lysyl oxidase, collagen and elastin. J Periodontal 2002; 73: 145-152.

35- Dill RE, Miller EK, Weil T, Lesley S, Farmer GR, Iacopino AM. Phenoitoin increases gene expression for platelet-derived growth factor B chain in macrophages and monocytes. J Periodontol 1993; 64:169-173.

36- Iacopino AM, Doxey D, Cutler CW, Nares S, Stoever K, Fojt J, et al. Phenoitoin and cyclosporine A specifically regulate macrophage phenotype and expression of platelet-derived growth factor and interleukin-1 in vitro and in vivo: possible molecular mechanism of drug-induced gingival hyperplasia. J Periodontol 1997 Jan;68(1):73-83.

37- Willamson MS, Miller EK, Plemons J, Ress T, Iacopino AM. Cyclosporine A upregulates interleukin 6 gene expression in human gingiva: possible mechanism of gingival overgrowth. J Periodontol 1994; 65: 895-903.

38- Satio k, Mori S, Iwkura M, Sakamoto S. Immunohistochemical localization of transforming growth factor b, basic fibroblasts growth factor and heparin sulphate glycosaminoglycans in gingival hyperplasia induced by nifedipine and phenoitoin. J Periodontal Res 1996; 31: 545-555.

39- Hong HH, Uzel MI, Duan C, Sheff MC, Trackman PC. Regulation of lysyl oxidase, collagen and connective tissue growth factor by TGF-B1 and detection in human gingival. Lab Invest 1999; 79: 1655-1667.

40- Uzel MI, Kantarci A, Hong HH, Uygur C, Sheff MC, Firatli E, et al.. Connective tissue growth factor in phenoitoin-induced gingival overgrowth. J Periodontol 2001; 72: 921-931.

41- Chavrier C, Hartmann DJ, Couble ML, Herbage D. Distribution and organization of the elastic system fibers in healthy human gingival: Ultrastructural and immuno histochemical study. Histochem 1988; 89: 47-52.
42- Bourke KA, Haase H, Li H, Daley T, Bartold PM. Distribution and synthesis of elastin in porcine gingival and alveolar mucosa. J Periodontal Res 2000; 35: 361-368.
43- Hassel TM, Page RC, Narayanan AS, Cooper CG. Diphenhydantoin (Dilantin) gingival hyperplasia: drug induced abnormality of connective tissue. Proc Natl Acad Sci USA 1976; 73: 2909-2912.
44- Narayanan AS, Hassell TM. Characterization of collagens in phenytoin–enlarged human gingiva. Coll Relate Res 1985; 5: 513-518.
45- Tipton DA, Fry HR, Dabbous MK. Altered collagen metabolism in Nifedipine-induced gingival overgrowth. J Periodontal Res 1994; 29: 401-409.
46- Kagan HM. Lysyl oxidase: Mechanism, regulation and relationship to liver fibrosis. Pathol Res Pract 1994; 190: 910-919
47- Ma RH, Tsai CC, Shieh TY. Increased lysyl oxidase activity in fibroblasts cultured from oral submucous fibrosis and squamous cell carcinoma. J Oral Pathol Med 1999; 28:246-251
48- Kagan H. Characterization and regulation of lysyl oxidase. In: Mecham RP, ed. Biology and Regulation of Extracellular Matrix: Regulation of Matrix Accumulation. Orlando, FL: Academic Press; 1986: 321–398.
49- Hew Y, Grzelczak Z, Lau C, Keeley FW. Identification of a large region of secondary structure in the 3′-untranslated region of chicken elastin mRNA with implications for the regulation of mRNA stability. J Biological Chem 1991; 274: 14415–14421.
50- Kahari VM, Olsen DR, Rhudy RW, Carrillo P, Chen YQ, Uitto J. Transforming growth factor-beta up-regulates elastin gene expression in human skin fibroblasts: evidence for post-transcriptional modulation. Lab Invest 1992; 66: 580–588.
51- Kucich U, Rosenbloom JC, Abrams WR, Bashir MM, Rosenbloom J. Stabilization of elastin mRNA by TGF-beta: initial characteri-

zation of signalling pathway. Am J Respir Cell Mol Biol 1997; 17: 10–16.
52- McGowan S, Jackson SK, Olson PJ, Parekh T, Gold LI. Exogenous and endogenous transforming growth factors-beta influence elastin gene expression in cultured lung fibroblasts. Am J Respir Cell Mol Biol 1997; 17: 25–35.
53- Penttinen RP, Kobayashi S, Bornstein P. Transforming growth factor beta increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. Proc Natl Acad Sci U S A 1988 Feb;85(4):1105-8.
54- Janssens K, Ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta1 to the bone. Endocr Rev 2005; 26: 743–774.
55- Yamane K, Suzuki H, Ihn H, Kato M, Yoshikawa H, Tamaki K. Cell type-specific regulation of the TGF-beta-responsive alpha2 (I) collagen gene by CpG methylation. J Cell Physiol 2005; 202: 822–830.
56- Kahai S, Vary CP, Gao Y, Seth A. Collagen type V, alpha1 (COL5A1) is regulated by TGF-beta in osteoblasts. Matrix Biol 2004; 23: 445–455.
57- Takuwa Y, Ohse C, Wang EA, Wozney JM, Yamashita K. Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. Biochem Biophys Res Commun 1991; 174: 96–101.
58- Luppen CA, Leclerc N, Noh T, Barski A, Khokhar A, Boskey AL, et al. Brief bone morphogenetic protein 2 treatments of glucocorticoid-inhibited MC3T3-E1 osteoblasts rescues commitment-associated cell cycle and mineralization without alteration of Runx2. J Biol Chem 2003; 278: 44995–45003.
59- Salo T, Oikarinen KS, Oikarinen A. Effect of phenytoin and nifedipine on collagen gene expression in human gingival fibroblasts. J Oral Pathol Med 1990; 19: 404-7.
60- Johnson BD, Page RC, Narayanan S, Piers HP. Effects of donor age on protein and
collagen synthesis “in vitro” by human diploid fibroblast. Lab Invest 1986; 55: 490-6.
61- Amstad-Jossi M, Schroeder HE. Age related alterations of periodontal structure around the cemento-enamel junction and of the gingival connective tissue composition in germ-free rats. J Periodontal Res 1987; 13: 76-9.
62- Karring T, Loe H. The effect of age on mitotic activity in rat oral epithelium. J Periodontal Res 1973; 8: 164-70.
63- Celenligil-Nazliel H, Ayhan A, Ruacan S. The effect of age on proliferating cell nuclear antigen expression in oral gingival epithelium of healthy and inflamed human gingiva. J Periodontol 2000; 71: 1567-74.
64- Gagliano N, Costa F, Tartaglia GM, Pettinari L, Grizzi F, Sforza C et al. Effects of aging and cyclosporine A on collagen turnover in human gingiva. Open Dent J. 2009; 3: 219–226.
65- Kinane DF. Regulators of tissue destruction and homeostasis as diagnostic aids in periodontology. Periodontol 2000; 24: 215-25.
66- Border WA, Ruoslahti E. Transforming growth factor in disease: the dark side of tissue repair. J Clinical Invest 1992; 90: 1-7.
67- Hong HH, Uzel MI, Duan C, Sheff MC, Trackman PC. Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF-beta1 and detection in human gingiva. Lab Invest 1999; 79: 1655-67.
68- Cenizo V, André V, Reyermier C, Sommer P, Damour O, Perrier E. LOXL as a target to increase the elastin content in adult skin: a dill extract induces the LOXL gene expression. Exp Dermatol 2006; 15: 574-81.
69- Behmoaras J, Slove S, Seve S, Vranckx R, Sommer P, Jacob MP. Differential expression of lysyl oxidases LOXL1 and LOX during growth and aging suggests specific roles in elastin and collagen fiber remodeling in rat aorta. Rejuvenation res 2008; 11: 883-9.
70. Kantor ML, Hassell TM. Increased accumulation of sulphated glycosaminoglycans in cultures of human fibroblasts from phenytoin-induced gingival overgrowth. J Dental Res 1983. 62: 383.