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THE INFLUENCE OF NIFEDIPINE INDUCTION TO GINGIVAL EPITHELIAL CELL PROLIFERATION

(*in vivo* study in rat)

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Abstract

It is well known that nifedipine administration in hypertensive patients results in gingival hyperplasia. The aim of this study was to study the pattern of nifedipine-induced gingival hyperplasia, based on morphometric and histogical changes as well as on PCNA (Proliferating Cell Nuclear Antigen) expression in the gingival epithelium. In total, 36 male Sprague Dawley rats at the age of 6 – 8 weeks were divided into nine experimental groups and three control groups. Each animal received daily DMSO (dimethyl sulfoxide) *via* oral intubation at a dosage of 0 (for control groups), 15, 30 or 60 mg/ kg (experimental groups) of body weight for 7, 21 or 42 days. After the animals were sacrificed, impression of the lower gingival tissue was taken to measure mesio-distal distance, labio-lingual distance and papilla height. The number of blood vessels and the thickness of gingival epithelium were assessed from hematoxylin and eosin stained sections. Proliferative activity of the epithelial cells was determined by immunohistochemical analysis using PCNA monoclonal antibody. Significant increase in the mesio-distal and labio-lingual distance of the lower gingival tissue was detected morphometrically ($p < 0.05$). There were more blood vessels in the experimental groups than in the control groups, however there was no specific pattern based on the dosage or duration of nifedipine administration. On the other hand, significant differences were found in the gingival epithelial thickness and proliferative activity between the experimental and the control groups. PCNA-positive cells were observed in basal and suprabasal layers, but nearly none in lamina propria.

Introduction

Nifedipine, a calcium channel blocker is widely used for long period of time in the clinical management of hypertension or myocardial disease[1,2,3,4]. One of side effects of nifedipine utilization was gingival enlargement particularly on labial surface of anterior lower gingiva and of anterior upper gingival[1,2,5]. However, this enlargement did not occur either on edentulous area or on lingual surface of anterior lower gingival. Clinically, the gingival enlargement was similar to that of verapamil and phenytoin usage[4], however the occurrence of the gingival enlargement is still controversy[6]. Local factors such as stimulus or dental plaque may exaggerate the occurrence of gingival enlargement[7]. Although drug-induced gingival enlargement is not attributed to tumorigenesis, however some cases identified as squamous cell carcinoma resulting from gingival hyperplasia induced by cyclosporin and phenytoin [8].

Gingival enlargement prevalency varies from 10–50% because of dilantin utilization [9], from 13–50% because of phenytoin administration and 15– 83% because of nifedipine usage[10]. Gingival enlargement is influenced by dosage and duration of drug induction. Dosage of 250 μg/g in diet will lead to gingival enlargement after 20 days administration. However on the fortieth day the graph of enlargement will be flat[2]. Drug dosage is not a determinant factor in the occurrence of gingival enlargement[6]. However nifedipine concentration in the gingival tissue or in its metabolite is more to be an essential variable the gingival enlargement manifestation[11]. Drug combination, dosage and duration of administration do not give rise any significantly difference in gingival enlargement[12]. In human, gingival enlargement is detected after 1–2 month usage of the drug, in dog it occurs after 7 weeks and in rat happens after 8–9 months [13].

Mechanism of gingival enlargement induced by nifedipine has been studying intensively. In patient with gingival enlargement, concentration of nifedipine in gingival cervicular fluid is higher compared to that in the plasma [4]. In normal condition, fibroblast growth might be controlled and its production is constant because of coordination among transcriptional mechanism, post translation mechanism and its intracellular degradation[14]. Collagen synthesis is influenced by hormonal factor and epidermal growth factor (EGF). Phenytoin could stimulate testoteron metabolism and increase 5α dihydrotestoterone level which stimulates fibroblast growth, leads to the proliferation of cellular components. Increased EGF level causes the more responsive cell to EGF and the more stimulation to DNA synthesis[2]. The calcium channel blocker could decrease Ca^{++} influx through cell membrane by decreasing the membrane permeability and by blocking the removal of intracellular Ca^{++} as well. Hence, these give rise to decreasing or blocking of cellular secretion function [10]. Nifedipine is assumed to influence directly in gingival fibroblast [15] by forming the connective tissue matrix. Homeostasis disturbances may produce many locus in which medication could interfere in synthesis and degradation of collagen [16]. Collagenolytic sign of inflammatory cells in fibroblast and metaloproteinase are phenomena that depend on calcium. Calcium antagonist might interfere Ca^{++} transportation leading to collagen resorbtion disturbances. On the other hand, *in vitro* study regarding the effect of nifedipine to normal gingival fibroblast result in decreasing of collagen and protein synthesis [5]. It is also happened on enlargement of gingival fibroblast after induced with verapamil[4].

Histologically, nifedipine-induced gingival enlargement inclined to be an enlargement with normal composition, it was neither hypertrophy nor hyperplasia[2]. In corium area, hyperplasia of fibroblast was detected however inflammatory cells were not found[13]. Hyperplastic epithelial cells, hyperplastic fibroblast and increasing collagen fibers were detected in connective tissue [1,17,18]. On the other hand, it was found elongation and proliferation features in rete pegs, increasing the number of capillary vessels and infiltration of inflammatory cells[19]. Increasing of collagen fiber indicates that there is an abnormal collagen metabolism and fibroblast enlargement indicates that biosynthesis activity grows up. The gingival enlargement has a relationship with extracellular matrix[18]. Cell proliferation could be detected by immunohistochemistry analysis using a specific marker such as PCNA since PCNA is a very important DNA replication protein in signal transduction pathway of keratinocyte division [20].

From above description it is essential to study the pattern of gingival hyperplasia based on the morphometric, histogical changes and PCNA expression of gingival

epithel resulted from nifedipine induction in different dosage and duration.

Material and Methods

Thirty six male Sprague Dawley rats aged 6 – 8 weeks were used in this study. The gingival tissue of the lower jaw after induced with nifedipine in DMSO at a dosage of 0 mg, 15 mg, 30 mg or 60 mg/kg body weight was observed on the 7th, 21st and 42nd days. After the animal was sacrificed, the lower jaw was taken impression for the measurements of mesio-distal, labio-lingual gingival and papilla height. For the mesio-distal gingival measurement : the widest gingival distance from the right second incisor to the left one was measured; for the labio-lingual gingival measurement : the widest gingival distance from labial to lingual surface of interdental space of first upper incisors was measured; for the height of central papilla was obtained by measuring the distance from the peak of papilla to the most concave surface of margin gingiva of the first incisor. Then, the jaw was fixed in buffered formalin 10% for ± 24 hours and the labial gingival was excised to be embedded in paraffin. Five micrometer sections were provided and stained with Hematoxylin&Eosin for blood vessels counting and measuring the epithelial thickness. Some other sections were immunohistochemically stained with PCNA to observe the number of proliferative cells.

Results

The measurement of the lower jaw of rat after induced with nifedipine according to the dosage and duration of induction shown in Table 1.

Table 1. Mean (X) and standard deviation (SD) of the mesio-distal (X_1), labio-lingual (X_2), and the height of central papilla (X_3) of the rat's gingival according to dosage and duration of nifedipine induction

Dosage		Duration					
		7th day		21st day		42nd day	
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
15 mg	X_1	3.767	0.237	3.989	0.040	4.006	0.032
	X_2	3.342	0.087	3.506	0.122	3.574	0.102
	X_3	2.160	0.690	2.001	0.252	1.992	0.112
30 mg	X_1	3.962	0.242	4.332	0.066	4.556	0.096
	X_2	3.528	0.132	3.751	0.406	3.777	0.294
	X_3	2.032	0.039	2.041	0.020	2.328	0.050
60 mg	X_1	4.417	0.136	4.494	0.115	5.103	0.181
	X_2	3.797	0.055	3.814	0.099	3.828	0.066
	X_3	2.032	0.237	2.163	0.166	2.339	0.037
Control	X_1	3.706	0.251	3.999	0.028	4.013	0.007
	X_2	3.286	0.035	3.529	0.142	3.584	0.080
	X_3	1.032	0.119	2.099	0.212	1.861	0.220

From the Table 1 was shown a tendency of increasing mesio-distal or labio-lingual measurement, however there was no tendency of increasing papilla height measurement. The result of ANOVA for gingival morphometric was conducted based on the dosage and duration of nifedipine induction as shown in Table 2.

From the Table 2 above demonstrated that significance difference was found ($p < 0.05$) regarding the increasing mesio-distal and labio-lingual gingival measurement because of nifedipine induction based on the dosage and duration. However no significant difference was perform ed when the gingival measurement was attributed to the duration of induction and the height of papilla.

Table 2. ANOVA of gingival morphometric according to the dosage and duration of nifedipine induction

		F	p
Inter-dosage	x_1	44.722	0.000
	x_2	20.547	0.000
	x_3	12.107	0.000
Inter-duration	x_1	17.232	0.000
	x_2	13.338	0.000
	x_3	2.598	0.078
Dosage-duration	x_1	49.040	0.000
	x_2	12.612	0.000
	x_3	9.609	0.000

Fig. 1 : Rat's gingival epithel with high rete pegs (arrow) (H&E, X400).

Fig. 2 : Immunohistochemistry staining using PCNA monoclonal antibody in rat's gingival epithel (X1000). Positive stainings were shown in nuclei appeared as dark brown stainings (arrows) especially in basal and parabasal layers accompanied with loose connective tissue.

Table 3. Mean and standard deviation of the number of blood vessel and epithelial thickness measurement based on the dosage and duration of nifedipine induction.

Dosage (mg) / Duration (day)	Blood vessel	Epithelial thickness (mm)
15/7	1.440 ± 0.191	0.124 ± 0.038
15/21	2.107 ± 0.387	0.146 ± 0.016
15/42	1.217 ± 0.510	0.129 ± 0.005
30/7	1.993 ± 0.577	0.143 ± 0.020
30/21	1.330 ± 0.33	0.129 ± 0.045
30/42	1.773 ± 0.836	0.159 ± 0.009
60/7	1.443 ± 0.709	0.159 ± 0.008
60/21	1.773 ± 0.196	0.147 ± 0.013
60/42	1.550 ± 0.191	0.183 ± 0.023
Control	1.663 ± 0.57	0.110 ± 0.011

The result of blood vessels enumeration and epithelial thickness measurement are shown in Table 3.

From the table above shown that the number of blood vessel in experimental group was higher compared to the control group. However the increasing of that did not describe any particular pattern either according to the dosage or duration of nifedipine induction. From the ANOVA demonstrated no significant difference ($p>0.05$).

It was clear that the epithel of the experimental group was thicker than that of the control group and it was also demonstrated in ANOVA based on the dosage and duration of nifedipine induction ($p< 0.05$). The result of ANOVA from the enumeration of blood vessel and the epithelial thickness appear in Table 4.

Proliferative activity of epithelial cell was determined by counting the number of positive staining cell analysed by immunohistochemistry using PCNA monoclonal antibody from 100 gingival epithelial cells for 1000X magnification and it was repeated for three times per section. The result are demonstrated in Table 5.

From the table above seen that the higher dosage and the longer of nifedipine induction gave the more positive PCNA-stained cells. The most positive cell was found at the dosage of 60 mg for 7 days. By using ANOVA the proliferative index of epithelial cell are given in the table below.

From the table above shown that among the dosage, duration and interaction of both gave significant difference regarding the proliferative activity of gingival epithelial cells.

Table 4. ANOVA of the enumeration of blood vessel and the epithelial thickness according to the dosage and duration of nifedipine induction

	F	P
Inter - dosage		
- Blood vessel	0.070	0.975
- Epithelial thickness	6.752	0.005
Inter - duration		
- Blood vessel	0.207	0.890
- Epithelial thickness	4.197	0.026
Dosage - duration		
- Blood vessel		
- Epithelial thickness	0.579	0.794
	1.764	0.164

Table 5. Mean and standard deviation of proliferative activity of gingival epithelial cells using PCNA - staining according to the dosage and duration of nifedipine induction

Dosage (mg/day)	Mean ± SD
15/7	41.67 ± 8.50
15/21	23.00 ± 1.73
15/42	33.67 ± 3.51
30/7	47.67 ± 6.65
30/21	57.33 ± 4.93
30/42	57.00 ± 10.58
60/7	59.77 ± 3.42
60/21	54.00 ± 2.00
60/42	54.67 ± 3.78
control	44.67 ± 2.88

Table 6. ANOVA of epithelial cell proliferative index according to the dosage and duration of nifedipine induction

	F	p
Inter - dosage	34.421	0.000
Inter - duration	3.750	0.036
Dosage - duration	3.053	0.030

Discussion

From the result of this study, it was apparent that morphological gingival size significantly increased especially for mesiodistal and labio-ligual gingival distances. Histologically, the connective tissue became loose, proliferation was dominantly performed in basal and suprabasal cells as detected immunohistologically. The number of blood vessel did not significantly increase yet, but the epithelial thickness increased significantly.

If there is an excessive or continuous stimulation, a cell will adjust to maintain homeostasis by increasing metabolic activity characterized by accumulation of intra and extra-cellular matrix that clinically manifestated as hyperplasia, hypertrophy, atrophy or metaplasia [21].

Gingival hyperplasia because of nifedipine induction is caused by involvement of alteration of Na^+ and Ca^{++} flow, of folic acid, of collagenase activation and local factor such as bacterial plaque. Nifedipine results in decreasing of Na^+ flow either direct or indirect depends on Na^+ and Ca^{++} exchange. This causes decreasing of folic acid that results in intracellular folic acid deficiency. In turn, production of *trypsin-like collagenase activator* will be limited, so stock of collagenase will be limited, as well [17].

Fibroblast and endothelial cell of vascular tissue are classified as stabil cells because of their low replication level, however these cells might replicate rapidly if appropriate stimulation exists [21]. The statement above could be used as a reason why there was no significant difference of the number of blood vessels between the experimental and the control groups. Nifedipine action in bothering the Ca^{++} influx into cell was assumed as a stimulation to fibroblast in extracellular matrix formation. Because of limited collagenase product it results in enlargement of tissue morphologically [18].

Oral epithelial cell denotes labil cell and has high proliferative capacity[21]. Nifedipine induction will stimulate basal and parabasal cells of gingival epithel to become more active in proliferation that resuted in increasing gingival epithelial thickness. It has been reported that *c-myc* expression could be detected in nucleus of basal and parabasal cells of the nifedipine/phenytoin-induced gingival hyperplasia [22]. It is reasonable since *myc* is the first nuclear gene involved in the kinase cascade of oral epithelial cell division [20].

From the histological finding in this study demonstrated loose connective tissue, probably as a result of Ca^{++} influx disturbances that is clinically seen as tissue's oedema. On the other hand, indeed, there was increasing number of blood vessels in the experimental group, however it was clear that no specific pattern appeared according to the dosage or duration of nifedipine induction. This result suggested that no obvious inflammatory process involved in this nifedipine-induced gingival enlargement. The latter result supported a finding that histologically nifedipine induction inclined to be an enlargement with normal composition neither hypertrophic or hyperplastic process[2]. This tenet also confirmed a result of study that in nifedipine induced tissue did not find any inflammatory cell [13]. Increasing number of the new blood vessels as a result of new capillary opening that in

The latter result supported a finding that histologically nifedipine induction inclined to be an enlargement with normal composition neither hypertrophic or hyperplastic process[2]. This tenet also confirmed a result of study that in nifedipine-induced tissue did not find any inflammatory cell [13]. Increasing number of the new blood vessels as a result of new capillary opening that in normal condition those capillaries are closed [21]. The opening of the capillary is considered as the additional capillary as the result of rete pegs elongation since there was an increasing metabolic activity because of nifedipine induction.

Conclusions

Nifedipine induction resulted in increasing gingival size particularly on labial surface area of the anterior lower jaw. On the other hand, cellular proliferative rate of gingival epithel increased significantly especially in basal and suprabasal layer. However, the enlargement neither involved inflammatory or hypertrophic/ hyperplastic processes, it tended to be an enlargement with normal composition.

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