IMMUNOHISTOCHEMICAL ASSESSMENT OF THE ROLE OF FOLIC ACID IN MANAGEMENT OF GINGIVAL HYPERTROPHY INDUCED BY NIFEDIPINE IN RABBITS

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ABSTRACT

Aim of the study: this study is aimed to evaluate the effect of folic acid as oral supplement ion in management the hypertrophy of gingivae that occur due to Nifedipine.

Materials and method: the study involve twenty eight male newzeland rabbit classified randomly into fourteen rabbit (control group) receive Nifedipine for about fourteen days for induction of gingival enlargement and the other fourteen rabbit (experimental group) receive both Nifedipine and oral folic acid to evaluate the effect of folic acid on reduction of gingival enlargement.

Result: statistical analysis show significant differences between two group with P-value ≤ 0.05, in clinical examination there is high gingival enlargement in control group but less elevation in experimental group.

In immunohistochemistry analysis there is mild positive reaction of matrix metalloproteinase (MMP) in control group with strong positive reaction in experimental group.

Conclusion: oral folic acid is most important supplement that should taken for good health of oral mucosa and it show higher efficacy in reduction gingival enlargement in rabbit.

Key words: Nifedipine, anti-hypertensive, folic acid, gingival enlargement, MMP1.

I. INTRODUCTION

The gingival sulcus is a region of potential space between a tooth and the surrounding gingival tissue and is lined by sulcular epithelium. The depth of the sulcus is bounded by two structure: apically by the gingival fibers of the connective tissue attachment and coronally by the free gingival margin. A healthy sulcular depth is (3) millimeters or less, which is readily self-cleansable with a properly used toothbrush or the supplemental use of other oral hygiene aids. (1)

Gingival diseases including gingival overgrowth can be categorized as ‘plaque-induced’ and non plaque-induced; however, often a more specific primary etiology can be identified. (2) There are several conditions which are not reliant on plaque induction, being genetic, systemic or infective in nature. In these conditions, associated plaque accumulation may exacerbate the clinical presentation. Gingival enlargement is the pathologic enlargement of the gingiva having multiple etiologies among which drug-induced enlargement is a common reason (3,4). Drugs such as immunosuppressant, antihypertensive, and antiepileptic are chief groups causing the hypertrophy (5). Nifedipine is one of the common drugs used to treat hypertension, and gingival enlargement occurs in around 45% of cases. These drugs which affect intracellular calcium metabolism or transport may in some case stimulate gingival fibroblasts to cause increased deposition of extracellular matrix components, such as glycosaminoglycan (6,7). Clinical features of Drug Induced Gingival Overgrowth(DIGO), include appearance and localization, are similar
for all inducing drugs. In the initial stages, it appears as a localized nodular enlargement of the interdental papilla (horizontal growth) and, with further progression, extends to the dental crown (vertical growth). The inducing drug (calcium channel blockers, CCBs) decrease folate cellular uptake in gingival fibroblast cells. The secondary effect of decreased cellular folate is that decrease the synthesis of active MMP which necessary to convert the inactive collagenase to active collagenase within the gingiva; therefore, there is an insufficient amount of active collagenase necessary to breakdown excess gingival connective tissues (built up secondary to inflammation) resulting in the side effect of DIGO.

II. MATERIAL AND METHOD

The study carried out in the department of dental Basic Science, College of Dentistry, University of Mosul; with approved of Scientific Committee, A.L.39/21, the period was from 29/9/2020 to 1/5/2021 the study involve twenty eight adult male newzeland rabbits weighting between (900g-1.25kg) were included in this study. All animals with a good health to be used through the study Diagnoses were established according to the clinical findings of gingival enlargement. Reasrch grouping classified as:

Control group (14 rabbit) receive oral Nifedipine drug 40mg/kg/day tablet (adalat)® up to fourteen days for induction of gingival enlargement then examined for clinical measurement and euthanized for histological examination and immunohistochemistry.

Experimental group (14 rabbit) taking oral folic acid 15mg/kg /day tablet (folicum)® and Nifedipine 40mg /kg/day for 14 days then taking for clinical examination and euthanized for histological examination and immunohistochemistry.

Clinical evaluation and measurement:
All Rabbits are undergo to clinical examination before treatment and after 14 days of drug administration, we measured gingival sulcus with periodontal probe for each rabbit of the control and experimental group to ensure the differentiation in two group.

Histological examination: The biopsy obtained from buccal side of two anterior teeth and separate the gingivae from bone by using periostium elevator carefully to preserve any gingival tracing, then put in the solution of 10% neutral buffered formalin (NBF) for 24 hours and place in graduated increase of the alcohol concentration for 10 hours the concentration 70%, 80%, 90%, and 100% respectively for dehydration of spacemen. Xylol, used for 6 hours to remove the remaining alcohol, the biopsy embedded in paraffin wax to obtained block of wax ready for sliced to section of five microns in series were cut by using microtome then stained by eosin and hematoxyllin to prepare it for light microscopical examination.

Immunohistochemistry (IHC) evaluation: after preparation of gingival tissue for IHC assay the following protocol will worked for evaluation MMP1 concentration by using (mmp1 IHC kit for Rabbit) (abcam)® the tissue prepared by Deparaffinization of the sample and bring to distal water then Incubate with Hydrogen peroxidase for 10 minutes and Wash with PBS after that Incubate with sodium citrate ph6 ---30minutes in 55cº then again Wash with PBS and Incubate with protein block for 10 minutes at room temperature then Wash with PBS(phosphate bacteria species) and Incubate with primary anti body (AB) ---overnight in refrigerator. (moisten chamber ) then Wash with PBS for about 4 times then Add biotinylated goat anti-polyvalent---10minutes at room temp and Wash 4 times with PBS after that Apply streptavidin peroxidase –10 minutes at room temp and Rinse 4 times in PBS then Add 30µl (1drop) (3,3' -Diaminobenzidine)DAB chromogen to 1.5 ml(50drops) of DAB substrate. Mix by swirling and apply to tissue incubate for 1-10 minutes then Rinse 4 times in PBS then Counterstain with gill Hematoxyline, Dehydrate, xylene, coverslip.

Statistical analysis
The data were expressed as mean ± SD, difference between control and experimental groups were statistically analyzed by using independed t-test and freedman test . The level of significance at P ≤ 0.05 and the test used foe IHC according to man Whitney rank test and the result show significant at P ≤ 0.05.

Result
1. Clinical finding of gingival sulcus depth (G.S.D)
The statically analysis of clinical finding obtained by using independed sample T test show a mean of (G.S.D) in control group is about(4.92 + 0.73), where the mean of treated group is about (1.35+ 0.49 ) this result give significant relation between control and experimental group with (P ≤ 0.05).(table 1)

(Table1) statistical analysis of clinical examination of gingival sulcus depth

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean +_ SD</th>
<th>Sig. (2- tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.92+_0.73</td>
<td>0.000*</td>
</tr>
<tr>
<td>Experimental</td>
<td>1.35+_0.49</td>
<td></td>
</tr>
</tbody>
</table>

Chart of clinical finding of G.S.D that show the higher elevation of free gingivae in control group and less gingival overgrowth in experimental group as showed in (figure 1)

(Figure 1) Descriptive analysis of clinical Finding of gingival sulcus depth

2. Histological finding

2.1: histological finding of gingival sulcus depth (G.S.D)

The statistical analysis of this independed sample t- test, show obvious overgrowth of G.S.D In control group with mean (12.9+ 1.49) While in experimental group there is an obvious reduction in G.S.D with mean about( 8.6+ 1.3) this result show significant differences between two group with P- value ≤0.05.(table 2)

Table (2) Histological examination of gingival sulcus depth

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean +_ SD</th>
<th>Sig. (2 tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
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<td>0.000*</td>
</tr>
<tr>
<td>Experimental</td>
<td>8.6+_1.3</td>
<td></td>
</tr>
</tbody>
</table>

Chart that analyse the histological finding of G.S.D show gingival enlargement in control group and little enlargement in experimental group as in (figure 2)

The histological examination under the microscope show higher elevation in free gingivae ubove the normal with elevation extend to 4mm in control group(figure4), the free gingivae elevation in treated group reach about 2mm(figure5)
2.2- Histological finding of inflammatory cell infiltration (I.C.I)

The statistical analysis of two group with freedman test and according to criteria of I.C.I. show a mean of control group is (2.2+0.8) compare to a mean of experimental group that equal to (0.6+0.4) with significant differences (P-value ≤ 0.05). (Table 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean +_SD</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2+0.8</td>
<td>0.001*</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.6+0.4</td>
<td></td>
</tr>
</tbody>
</table>

Inflammatory cell (neutrophil cell) higher number present in control group with little inflammatory cell in treated group as shown in (figure 3)

The histological examination of tissue section of rabbit gingivae in control group under microscopical lenses show higher number of inflammatory cell with increase fibroblast cell and high density connective tissue in basement membrane (figure 6)

While in experimental group show mild number of inflammatory cell with decrease fibroblast cell and less density connective tissue()
(Figure 4) digital imaging of gingival sulcus depth in control group.

(Figure 5) digital imaging of gingival sulcus depth in experimental group.
3. **Immunohistochemistry assay MMP1:**

According to man Whitney rank test show low amount of MMP1 concentration in control group with mean about 7.17.

But the concentration of MMP1 in experimental group is higher when use folic acid treatment with mean about 17.83.

Result is significant at P value ≤ 0.05.
Table (4) statistical analysis of matrix metalloproteinase (MMP1)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean rank</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.17</td>
<td>0.000*</td>
</tr>
<tr>
<td>experimental</td>
<td>17.83</td>
<td></td>
</tr>
</tbody>
</table>

Descriptive analysis that show this increases in the density of connective tissue that explain the concentration of MMP1 can show in (figure8)

The immunohistochemistry of Rabbit gingivae in control group under light microscope show less staining of fibroblast and connective tissue (figure9)

In experimental group we show positive reaction with greater staining of fibroblast cell and connective tissue (figure 10)

![Figure (8) descriptive analysis of MMP1 concentration.](image)

![Figure 9: photomicrograph of rabbit oral mucosa of control group shows negative reaction (-) of MMP1 expression to immunohistochemistry in the cytoplasm of fibroblasts (→) and collagen fibers (↔) of connective tissue of submucosa. IHC for MMP1, 400X.](image)
III. DISCUSSION

The aim of our study was to explain the effect of drug in the induced gingival enlargement, this effect revealed the benefits of use supplement of folic acid for reduced the effect of Nifedipine side effect on gingivae in Rabbit also should take a care especially if there is folic acid deficiency. (11)

Gingival enlargement also called gingival hyperplasia or gingival overgrowth all these name for the same clinical disease which occur due to multiple causes like genetic or sikell cell anemia or plaque induced and also due to side effect of many drugs like anticonvulsant , antihypertensive ,and some of hormonal contraceptive. (12)

Nifedipine is one of most effective drug that use in the treatment of hypertension but one of the most causes of side effect than other anti-hypertensive in the same class that more show in dental clinic is gingival over growth and one of the most explanation to this effect is that Nifedipine may cause a decrease in sodium along with calcium flux and in cellular folic acid uptake. This can produce a systemic response as well as a localized folate deficiency in the gingival tissue. (13) The folate deficiency induced by Nifedipine can cause degenerative changes in sulcular epithelium and also exacerbate inflammation as seen through the study. (14, 15)

More ever we know the higher efficacy of folic acid in treatment of many disease so we use it in the trial for management of gingival enlargement ,this case is in agreement with Barbe et al 2020 (16) who show clinically and histologically that the deficiency of folic acid is one of most causes to induced gingival enlargement in which there is most of plasma cell infiltration in the free gingivae when measure the tissue histological by hematoxylin and eosin as with our case not only this also show higher differecnces when measure immunohistochemistry and show dense collagen fiber in the affected area all this show in agreement with this study on the effect of folic acid in reducing the gingival enlargement.

Djais et al 2019 (17) found that anticonvulsant drugs can inhibit intracellular calcium ion entry. Tungare and paranjipe 2019 (18) also suggest that phenytoin may regulate cytokine changes in gingival tissue, thereby causing dysregulation of connective tissue and matrix components found in gingival enlargement. Gupta et al 2017 (19) showed a reduction in the expression of the genes encoding collagen types I and III in combination with a higher density of these fibers in gingival overgrowth. Candoto et al 2019 (20) demonstrated that connective growth factors were elevated in phenytioin-induced gingival overgrowth, which characterizes a more fibrotic tissue.

Our data suggest that Nifedipine-induced GE is produced by accumulation of extracellular matrix components, including collagen, by a mechanism dependent on reduction of concentration of MMP1 which lead to accumulation.
of collagen fiber this show in agreement González et al 2017(21) who show that drug-induced GE that invokes the complex interaction among gingival fibroblasts activity, connective tissue renewal, and an inflammatory process that leads to an increase of matrix extracellular components in gingival tissue through blocking of TRAP1 channel in the oral gingivae.

IV. CONCLUSION:

Nifedipine induced gingival enlargement remain the most common causes for gingival over growth and can reduced their side effect on oral mucosa by administration of oral supplement folic acid due to their higher effect on reduction this case of enlargement.

REFERENCES: