

RESEARCH ARTICLE

RADIOGRAPHIC AND IMMUNO HISTOCHEMICAL DIAGNOSTIC STUDY OF STRONTIUM RANELATE AND METAL-SUBSTITUTED HYDROXYAPATITE BONE GRAFT MATERIALS IN DIABETES MELLITUS WITH CHRONIC PERIODONTITIS

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Abstract

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Key words:-

Strontium Ranelate, Metal Substituted Hydroxyapatite, Digital Radiograph, Immunohistochemical, Matrix Metalloproteinases2 **Objective:** The aim of the present study is to assess the radiographic

Objective: The aim of the present study is to assess the radiographic and immunohistochemical effect of strontium ranelate (SR) and metal substituted hydroxyapatite (MSHA) on the treatment of chronic periodontitis among diabetic rats

Materials and Methods: The study involved ten adult male and female rice rats (1-month-old) weighting (250- 300g). After a 24-hour fast, a single intraperitoneal dose of freshly prepared alloxan was injected to induce diabetes. A month after the injection of alloxan, the rats were randomly assigned to one of the two treatment modalities: SR with gengigel or MSHA with gengigel. Digital periapical radiography was taken at baseline, a month after the injection of alloxan to see resorbed alveolar bone and after 3months post-operative surgery for radiographic assessment. The diabetic rats were sacrificed using an overdose of anesthesia, and gingival tissue samples were collected. The specimens were processed for hematoxylin and eosin (H & E) staining and immune stain for expression of matrix metalloproteinases 2 (MMP-2).

Results: Digital periapical radiography showed an increase of nearly 0.37 mm in the height of the alveolar bone in the side of the SR group while the other side in the MSHA group increased by nearly 0.12 mm. A statistically significant reduction in the expression of MMP2 in the SR group as compared to the MSHA group was found upon comparing the immunohistochemical results of the 2 groups.

Conclusion: Radiographic and immunohistochemical results showed that SR was a promising material in the treatment of periodontal diseases.

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Introduction:-

Supporting structures of teeth include the periodontal ligament, alveolar bone, and gingival tissues. [1] Periodontal disease is an infectious pathology caused by a limited number of specific bacteria; however, host factors are the major determinants for the disease occurrence and progression rather than the presence of these bacteria. [2] Many factors influence the periodontal disease progression including social and behavioral, systemic, genetic, and dental factors. [1] Also, the presence of diabetes influences the periodontal tissues by inducing vascular abnormalities, altering neutrophil function and collagen synthesis. [3]

The chronic untreated loss of periodontal tissues: gingiva, alveolar bone, periodontal ligament and cementum, ultimately results in tooth loss leading to functional and aesthetic repercussions. Various treatment modalities (surgical and non-surgical) have been investigated to try repair/regenerate damaged periodontal tissues. In an attempt to achieve periodontal regeneration, soft and hard tissue replacement grafts, guided tissue/bone regeneration (GTR/GBR), root surface biomodifications, and delivery of growth factors have been developed.[4]

A new material has been used for replacement of bone loss. This material is called strontium ranelate. Strontium ranelate (SR), a new active drug shown to reduce osteoporosis (OP), can stimulate osteoblast proliferation and inhibit osteoclast resorption, which allows prompt bone formation while decreasing bone resorption. Strontium ranelate has a good application prospect and was designed to contribute to the dental implant in postmenopausal women with OP. There was a growing number of studies investigated the effect of SR for treating OP, and the result showed that it could increase the bone weight of vertebra, humerus, and femur of animals. [5,6]

Nano sized hydroxyapatite (HAP) is the main component of mineral bone. Living bone constantly undergoes a coupled resorptive formative process known as bone remodeling. The process involves simultaneous bone removal and replacement through the respective activities of osteoblasts and osteoclasts, with the accompanying vascular supply and a network of canaliculi and lacunae. Magnetic hydroxyapatites containing iron (Fe) or manganese (Mn) or both were previously studied to form composite materials.Nanoparticles are incorporated into different materials such as polymers, noble metals, metal oxides and silica. [7]

The use of rat models in the evaluation of periodontal pathogenesis has been applied because of the similarity of the structure of the periodontal tissue between rats and humans.[8] There is substantial information on potential mechanistic pathways that support a close association between diabetes and periodontitis, but there is a real need for longitudinal clinical studies using larger patient groups together with animal model and cell/tissue in vitrostudies. [9]

Conventional radiographs generate 2D images in which the roots are superimposed on the bone and as a result the bone changes such as furcation involvement and buccal and lingual bone changes are difficult to be observed. [10]Matrix Metalloproteinase 2 (MMP-2) is implicated in organ growth, endometrial cycling, wound healing, bone remodelling, tumour invasion, and metastasis.[11] This enzyme functions through the degradation of components of the basement membrane, including type IV collagen, fibronectin, elastin, laminin, aggrecan, and fibrillin.[12]

Indeed, the purpose of this study was to compare between SR and metal substituted hydroxyapatite (MSHA) bone graft materials on treatment of chronic periodontitis among diabetic mellitus.

Subjects and Methods:-

The experimental Study:

Animal selection:

Ten adult male and female rice rats (1month old) weighting (250- 300g) from the Laboratory, animal care, Mansoura University were used throughout the experiment. The experimental protocols were carried out following ethical principles for laboratory animal studies and approved by the Institutional Animal ResearchCommittee.

Diabetes Induction:

Ten rats, that were pre-investigated for normal glycaemic condition using BLOOD GLUCOSE METER IN ANIMAL LAB, were injected, after a 24-hour fast, with a single intraperitoneal dose of freshly prepared alloxan [alloxan monohydrate (1H, 3H) Pirimidintotrone, 150 mg/kg body weight, i.p.] [13]diluted at 0.2% in a 0.05 M citrate buffer, pH 4.5 (Sigma Aldrich, St. Louis, MO USA) to induce diabetes. Six hours after alloxan injection, 10% glucose

solution was offered for 24 hours to avoid fatal hypoglycemia as a consequence of massive insulin liberation that occurs after ß cells destruction. Glucose concentrations (expressed as mg/dL) were measured at the seventh day after alloxan injection with a commercial Accu-Check Active[14] glucometer (Roche Diagnostics GmbH, 68298 Mannheim, Germany) with blood obtained by puncture of the tail vein. The diagnosis of diabetes was based on hyperglycemia (blood glucose (non-fasting) more than 300 mg/dl) [15] using blood glucose meter. The diagnosed diabetic rats received 1-2 IU/g/day of subcutaneous insulin NPH (Insulatard HM) from Novo Nordisk, Bagsvaerd [16] Figure 1.



Figure 1:- a, Photograph showing intraperitoneal injection of Alloxan, b, Photograph showing blood glucose level of diabetic rat 7 days after Injection of alloxan, C, Photograph showing subcutaneous injection of insulin NPH (Insulatard HM).

Radiological examination:

After diabetic induction, the mandible of rats underwent radiographic examination and then was prepared for X-ray whichwas taken by CCX digital, computer controlled x-ray timer by Trophy Trex Group, France, and were received on RVG sensor by visiodent, France.

Technique:

The right side and the left side of the mandible were placed on the RVG sensor, and the X-ray cone was perpendicular on both the sensor and the mandible. The cone was 10 cm away from the sensor, and the x- rays were taken with exposure dose of 0.10 and exposure time of 0.08 seconds. This radiographic examination was taken again after 3 months.

Radiographic analysis:

- 1. Preoperative bone defect (at baseline) was measured as the distance from cementoenamel junction (CEJ) to the base of the bone defect. This measurement was taken a month after the injection of alloxan and radiographed by digital periapical radiography
- 2. Postoperative bone defect (after 3 months) was measured as the distance from cementoenamel junction (CEJ) to the base of the bone formation.
- 3. Postoperative bone fill (bone gain after 3 months) was deducted by subtracting the preoperative bone defect from the postoperative bone defect.

Surgical procedure:

Experimental rats groups:

A month after the injection of alloxan, a randomized split-mouth study was used in the selected diabetic rats to receive one of the two proposed treatments in one side and the other treatment in the contralateral side. This randomization was achieved by computer generated table.

First proposed treatment (Group I):

was applied in 10 randomized sides and included a surgical flap with a mix of strontium ranelate [made in Arab Republic of Egypt (A.R.E) and approved by Solicitors Regulation Authority (SRA)] with gengigel [manufactured by Ricerfarma and made in european union (eu)]

Second proposed treatment (Group II):

was applied in the 10 contralateral sides and included a surgical flap with a mix of metal substituted hydroxyapatite bone graft materials [Pure Hydroxyapatite (HAP) nanoparticles doped with Mn2+ and Fe3+ ions]. It was manufactured in the Department of Physics, Faculty of Science Mansoura University]with gengigel

The surgical phase:

Animals were anesthetized with intramuscular injection of diazepam (0.5mg\kg) and ketamine hydrochloride (20mg/kg). [17]Then, a clean cut horizontal incision was created along the outer surface of the surgical side mandible using a surgical blade no 15 mounted on scalpel handle no 3. A mucoperiosteal elevator was then used to reflect the skin and gingival tissue to expose the bone of the surgical side of the mandible. The area of surgery was irrigated by normal saline. Then, according to the selected treatment modality either SR (Osteostatine) or MSHA was mixed with 2-4 drops of hyaluronic acid (Gengigel) to make a putty form that was carried by a spoon like instrument to be placed into the defect site until filled, and then condensed gently with a sterile smooth amalgam condenser. The two edges of the skin were approximated and sutured using 3/0 silk mounted on half circle needle 3/0 (Figure 2).



Figure 2:- a, Photograph showing incision followed by elevation of skin and gingiva to expose the bone of the right side of the mandible, b, The mix of strontium ranelate and gengigel placed into the defect site until filled, c, The mix of Metal substituted hydroxyapatite and gengigelPlaced into the defect site until filled, d, Photograph showing repositioning of the flap and suturing with 3/0 black silk mounted half circle needle 3/0.

Post-surgical care:

Amoxacillin 25 mg was prescribed twice daily for 3 days

Scarification of the animals:

Animals were sacrificed 3 months after the surgery. Rats were euthanized by overdose of diethyl ether and then the mandible was taken for radiological analysis, and gingival specimens were taken for immunohistochemical diagnostic study.

Sample collection:

Gingival tissue samples were collected using the following technique. Firstly, longitudinal surgical incision aimed at sulcular epithelium was done to obtain 3X3 mm gingival specimens. 10 specimens were obtained from each group. Soft tissues were, immediately, kept in 10% formalin in order to be sent to the pathology laboratory. After paraffin embedding, specimenswere cut into 4µm sections which were then dewaxed in xylol. Then, xylol was replaced with alcohol and then rehydrated in descending grades of alcohol ending with distilled water. Finally, slides were stained

with heamatoxylin for 15 min, and then 1% Eosin stain for 10 minutes.Excess of each stain was washed by rinsing in tap-water for 5 min after each stain.

Immunehistochemical Method:

Immuestaining for MMP2 was performed by SPlink HRP Detection Bulk Kit- for Mouse and rabbit antibodies(GBI LabsTM, WA, USA).

Primary antibody:

Rabbit Polyclonal antibody against MMP2, diluted at (1:50) (GeneTex Inc- CL, USA) was used. The positivity of the immune stain signals of MMP-2 expression has been classified as: pale yellow, buff, or brown color confined to the cytoplasm of the expressing cells. The intensities of the expression were evaluated and assessed as following: (0) for no staining, (1) for mild stain, (2) for moderate stain, (3) for intense or strong stain. Image analyzing was performed to evaluate the proportion of cells that express the immune stain and a percentage was given.

Statistical Analysis:

Data were charted, decoded then analyzed by using the computerized program (SPSS), version 17.

Results:-

Digital periapical radiography results: comparing baseline and after 3 months measurements showed increase of nearly 0.37 mm in the height of the alveolar bone in the side of the group I (Strontium Ranelate) while the other side in the group II (Metal Substituted Hydreoxy Apatite) increased nearly 0.12mm in the height of the alveolar bone (Figure 3,4, Table 1).



Figure 3:- a, Digital periapical radiography for measurement of the bone level of the right site defect of diabetic rice rat.b, Digital periapical radiography for measurement of the bone level of the right site defect of diabetic rice rat after 3 months treated by strontium ranelate bone graft material.



Figure 4:- a, Digital periapical radiography for measurement of the bone level of the left site defect of diabetic rice rat, b, Digital periapical radiography for measurement of the bone level of the left site defect of diabetic rice rat after 3 months treated by metal substituted hydroxy apatite.

| | Baseline | 3 months | | bone gain |
|--|------------------------------|-----------------|-------------------------------|---------------------------|
| The study groups | Mean± SD | Mean± SD | Paired samples t-test P | (BG) Mean± SD |
| Group I (n=10) | 1.25±0.33 | 0.69±0.14 | 6.258*** 0.000 | 0.55±0.27 |
| Group II (n=10) | 1.22±0.37 | 0.93±0.29 | 3.911** 0.004 | 0.30±0.23 |
| Independent samples t-test P value | 0.156 ^{ns} 0.878 | 2.269 0.036* | Paired samples t-test P | 2.278 0.036* |

Table (1):- Mean values of vertical bone loss (VBL) among the study groups at base-line and 3 months post-operatively and bone gain (BG) after 3 months post-operatively.

Histopathological evaluation:

The histological aspect of cases included in the present research has various trends that depend on the presence or absence of the aimed periodontal diseases. Therefore, a H&E stain procedure was performed on biopsies collected from all groups; group I and group II. Specimens from group I that were examined 3 months after grafting with strontium ranelate bone graft material exhibited a significant decrease in fibroblasts ,endothelial cells and chronic inflammatory cells. Although some cases showed mild inflammatory cells in very deep layers of the sub-epithelial connective tissues, a signs of healing were observed in sulcular epithelium. There were significantly different histological features among specimens from this group in terms of disease severity. Some cases revealed hyperkeratinization of gingival epithelium (Figure 5).

Specimens from group II (Gingival samples of diabetic rats treated with metal substituted hydroxy apatite bone graft material) that were examined 3 months after grafting with metal substituted hydroxy apatite bone graft material showed fibroblasts ,endothelial cells and diffuse infiltration by chronic inflammatory cells mainly lymphocytes. These cells were confined to the reticular and papillary area. We also found a various degrees of ulceration related to sulcular epithelium. The severity of the ulceration varied according to severity of the disease (Figure 6).



Figure 5:a,b, photomicrograph of groupI (Gingival specimen of diabetic rats treated with strontium ranelate bone graft material) shows orthokeratinized stratified squamous epithelium (OKSSE)with elongated rete pegs (RP) and exhibits few fibroblasts (F), blood vessels (BV) and mild chronic inflammatory cells (IC) infiltration in connective tissue (H&E X 400).



Figure 6:- a, photomicrograph of groupII (Gingival specimen of diabetic rats treated with metal substituted hydroxy apatite bone graft material) shows non keratinized epithelium (NKE) covered by atrophic-ulcerative stratified squamous epithelium and exhibit proliferative fibroblasts , blood capillaries and moderate to intense chronic inflammatory cell (IC) infiltration in connective tissue (H&EX 200), b, photomicrograph of groupII shows exhibit proliferative fibroblasts , blood capillaries and moderate to inflammatory cell infiltration in connective tissue. (H&EX 400).

Immunohistochemical Result to MMP-2:

Positive reaction to Gelatinase A (MMP-2) was detected in group I and group II. The reaction in the epithelial layer was confined to the basal and supra-basal layers. Positive intense reaction was also found in the sub-epithelial connective tissue. An intense immune reactivity for MMP2 was more observed in the group II compared to group I.

At cellular level, we observed a strong reaction related to native cells of the connective tissue mainly fibroblasts, endothelial cells and chronic inflammatory cells mainly lymphocytes. Among these cells, fibroblasts and endothelial cells expressed the greatest immune reaction for MMP-2. There were variations in the immune reactivity between group I and group II specimens and high significant difference was observed between the two groups (Table 2, Figure 7).

Table(2):- Immunohistochemistry for MMP-2 stain parameters in group I and group II among the rice rats at 3 months post-operatively.

| Immunohisto chemical Expreession | Mild | Moderate | Strong |
|-------------------------------------|--------|----------|--------|
| group I (10) | 7(70%) | 2(20%) | 1(10%) |
| group II (10) | 1(10%) | 3(30%) | 6(60%) |



Figure 7:- a, Photomicrograph shows mild immunoreactivity for MMP2in specimen of group I confined to the epithelium and scattered in connective tissue with mild immunoreactivity in fibroblasts, endothelial cells and inflammatory cells (PAP-DAB X400). b, photomicrograph show strong cytoplasmic immunoreactivity of MMP2in

specimen of group II presented in fibroblasts, endothelial cells and chronic inflammatory cells mainly lymphocytes.(PAP-DAP X 400)

Immunohistochemical statistical analysis:

Independent samples t-test shows a highly significant difference between readings of immune stain of the included groups (t-test=7.398). There was a significant decrease in immune stain for MMP-2 in Group I (1.20 ± 0.42) as compared to group II (2.70 ± 0.48) (P < 0.001) (Table 3).

Table (3):- Immunohistochemistry for MMP-2 stain parameters in group I and group II (Mean±SD) among the rice rats at 3 months post-operatively.

| | Group I (n=10) | Group II (n=10) | Independent samples t-test |
|------------------|----------------------|-----------------------|-------------------------------|
| | Mean± SD | Mean± SD | Pvalue |
| Immunohisto | 1.20 ± 0.42 | 2.70 ± 0.48 | 7.398 |
| chemical stains- | | | 0.000*** |

Group I = Gingival samples of diabetic rats treated with strontium ranelate bone graft material. Group II = Gingival samples of diabetic rats treated with metal substituted hydroxy apatite bone graft material

Discussion:-

Animal models have an important role in the generation of new knowledge in medical sciences, including periodontology. These experimental models have distinct advantages because they can illustrate in vivo cellular characteristics and reactions that occur in humans. Animal models in periodontal disease are particularly important in the development of the scientific basis for understanding the pathological processes.[18]

This study compared between the SR and MSHA in diabetic- induced rats with induced bone defects. Digital radiograph was performed to estimate bone level before and after treatment in those rats. Histological and Immunohistochemical of MMP-2 evaluation in the gingival tissue components was performed in gingival' rats samples. In the strontium ranelate group, the reduction of gingival inflammation wasin agreement with that reported by Nunes et al., [19] and Pelletier et al., [20] who found that SR has anti-inflammatory effect by reducing the release of cytokines, tumour necrosis factor alpha (TNF- α) and interleukin 1 beta(IL-1 β). Also, this result can be attributed to the anti-inflammatory, anti-edematous, and anti-bacterial effects hyaluronic acid. Hyaluronic acid has been employed in the treatment of gingivitis, and periodontal pockets. [21]

This study was in agreement with Bhardwaj et al., who evaluated the efficacy of an indigenously prepared zinc incorporated nanohydroxyapatite (ZINH) bone graft in the treatment of intrabony defects. A split- mouth study, which consists of 11 systemically healthy subjects with 45 sites, were randomly treated with ZINH or with nanoHA alone. Statistically significant improvements in all clinical parameters were seen in the test sites at 12 months. They concluded that ZINH bone graft can be considered as a prospective bone regenerative material. [22]

Li et al., who concluded that Mn-substituted hydroxyapatites exhibited a remarkably beneficial effect on bone cells, and it has been proved that a relatively high Mn2+ doping concentration promotes the osteocalcin production. Also, Mn-substituted hydroxyapatites were able to support human osteoblast differentiation, proliferation and metabolism activation. [23] Hence, the adhesion of osteoblast to HA coating is a crucial step for subsequent osteoblast functions. Mn2+ ions increase ligand binding affinity of integrate and activate cell adhesion [24] Wu et al. reported that iron (Fe2+) substituted HA nanoparticles were super paramagnetic and showed good biocompatibility. It has been also reported that iron compounds are able to promote the nucleation of apatites, adsorbing salivary calcium and phosphate ions, and, thus, to favor the replacement of minerals. [25]

Using image analysis program for digital periapical radiography after 3 months of treatment showed that there was statistical increase in BG in strontium ranelate group (0.37 mm bone gain) as compared to metal substituted hydroxy

apatite (0.12 mm bone gain).Radiographic findings showed that SR treated group showed more bone gain than metal substituted hydroxyapatite group These findings are in agree with Elgendy & Shoukheba, [26] who concluded that strontium ranelate 2% gel appears to be safe and may support periodontal wound healing/regeneration in intrabony periodontal defects induced in dogs without complications. Bone biomechanical and structural characteristics such as mineral density can be increased by SR. This tremendous regeneration potential of strontium may be related to reduction of osteoclast number and regulating the production of osteoprotegerin (OPG) as well as Receptor Activator of Nuclear Factor K-B Ligand (RANKL). Low doses of SrRan were found to increase OPG expression and production and to decrease RANKL expression by osteoblasts in vitro [27].

Our study represents a contribution aimed at better characterization of the hyper-inflammatory response associated with localized sites in gingival tissue of site of strontium ranelate and other gingival tissue of site of metal substituted with hydroxyl apatite. Independent samples t-test shows a highly significant difference between readings of immune stain of the included groups (t-test=7.398). There was a significant decrease in immune stain for MMP-2 in Group I (Gingival specimen of diabetic rats treated with strontium ranelate bone graft material) (1.20 ± 0.42) as compared to group II (Gingival specimen of diabetic rats treated with metal substituted hydroxy apatite bone graft material) (2.70 ± 0.48) (P < 0.001).

The present study expose significant differences regarding the levels of MMP-2 expression in specimen group I and group II; however, it suggested that gingival fibroblast cells are the major source of this MMP-2.

Immunohistochemistry for MMP-2 showed intensive cytoplasmic staining of the cells especially basal layer of the gingival epithelium and underlying connective tissue in DM group II. MMP-2 presented a continuous increase up to 7 days of inflammation. Since macrophage recruitment increased MMP-2 content, higher expression of MMP-2 in the gingival epithelium of the DM group II is the outcome of the inflammation. Our study shows mild immune-reactivity for MMP2 in specimen of group I confined to the epithelium and scattered in connective tissue with mild immune-reactivity in fibroblasts, endothelial cells and inflammatory cells. It also shows strong cytoplasmic immune-reactivity of MMP2 in specimen of group II presented in fibroblasts, endothelial cells and chronic inflammatory cells mainly lymphocytes

The incorporation of strontium into mesoporous bioactive glass scaffolds was shown to be a viable way to stimulate the biological response of periodontal ligament cells as well as stimulate bone formation in bone defects in an osteoporotic rat model. [28]The histologic evaluation of the 2.5 and 5 mg groups showed more attempts at bone formation with less inflammatory reaction as compared to the control group. This could be attributed to the anti-inflammatory effect of SR that has been proposed due to the antagonising effect of SR to NF- κ B activation. Furthermore, in unpublished data by the same group, they proposed that SR may act as TNF inhibitor which could explain its anti-inflammatory properties. [29]

In the present study, strontium ranelate has anti-inflammatory and anti-microbial effect on the soft tissue associated with periodontal diseases.Local application has demonstrated anti-inflammatory effects as well as anabolic effects on bone and a stimulatory effect on vasculogenesis. This was attributed to the increase in the expression of MMP-2 and vascular endothelial growth factor [30] which may explain the results of the present study. Our findings are also in line with a former research that demonstrated the ability of local simvastatin application to enhance healing of the bone defects even in the diabetic rat models. [31]

In the present study MSHA bone graft material has no anti-inflammatory and anti-microbial effect on the soft tissue associated with periodontal diseases. This result does not agree with Ernie Setia watie, who showed that HA participates in tissue repair and wound healing and is used topically as anti-inflammatory and anti-oedematous agent. The anti-inflammatory effect may be due to action of exogenous hyaluronan acting as a scavenger draining prostaglandins, metalloproteinases and other bioactive molecules. This increases proliferation, metabolism and cell migration, thereby accelerates bone healing. [32]

Conclusions:-

Strontium ranelate and MSHA bone graft material have shown promising result as well as an ability to augment the bone improving promoting defect fill. However, it should be noted that the effect of MSHA is less than the strontium ranelate bone graft material.Digital periapical image provides high resolution images that can be used to gather diagnostic tools for assessing periodontal bone health. Immunohistochemical results revealed anti-

inflammatory effect of strontium ranelate on the soft tissue with periodontal diseases. However, this feature is not present in Metal Substituted Hydroxyapatite bone graft material

Recommendations:-

Further studies are required on patients taking soft tissue biopsies to evaluate the efficiency of strontium ranelate bone graft on the soft tissues as they were used on experimental animals.

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