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RESEARCH ARTICLE

EFFECT OF ADJUNCTIVE USE OF LOCAL SIMVASTATIN AND CHITOSAN BASED SIMVASTATIN ON GCF LEVELS OF NETRIN-1 AND ITS RECEPTOR UNC5B IN PERIODONTITIS

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Abstract

Background: Netrin was believed to have a regulating role on the inflammatory reaction via its receptor UNC5B. Thus it takes apart in bone destructive diseases. Periodontitis represent a localized inflammatory bone destructive disease, so Netrin-1could be involved in anti-inflammatory regulation of periodontitis. However, to the best of our knowledge, few studies were available in the literature assessing the role of Netrin-1 in periodontal health and disease.

Aim: The present study was designed to assesssGCF Netrin-1and its receptor UNC5B levels in health and disease of periodontal tissues and evaluate effect of using simvastatin gel 1.2% as well as chitosan based simvastatin gel 1.2% as an adjunct to SRP on their levels.

Patients and methods: The study design included 10 periodontally healthy subjects in addition to 45 systemically healthy stage II periodontitis patients divided equally into three groups andtreated by;SRP, SRP+simvastatingel 1.2%and SRP+chitosanbasedsimvastatingel 1.2% (positive control & two study groups respectively). At baseline periodontalparameters were recorded and GCFsampling was performed for all participants and after treatment for periodontitis patients.Netrin-1 and and its receptorUNC5B levels were assessed by enzyme-linked immunosorbent assay.

Results: Periodontal indicesat baseline inperiodontitis groupsshowed significant improvement after treatment comparedto its values at baseline. In both study groups, all periodontal parameters were significantly higher versus positive control group. At baseline, GCF levels of netrin-1 and its receptor UNC5B were significantly lower in

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periodontitis patients versus healthy controlsbaselineandincreasedsignificantly after receiving different treatment modalities.
Conclusion: Localsimvastatingel 1.2%orchitosan basedsimvastatingel 1.2% asadjunct with SRPis effective in management of stage IIperiodontitis. Netrin-1 promotes inflammation resolution in periodontitis.

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Introduction:-

The pathogenesis of periodontitis is inflammatory,[1]originated by bacteria of the oral biofilm.[2]The damage of periodontal tissue is deteriorated by persistent inflammation and dysbiosis.[3]The therapeuticscaling and root planing(SRP) success is limited in cases involvingsevere periodontitis and inaccessible areas.[4] Thus, various supplementarypharmaceuticaltherapy such as anti-inflammatory drugs, antibiotics, bisphosphonatesandstatinsenhances the therapeuticoutcome.[5-10]

Statins are lipid-lowering drugs. This pharmacologic groupalso possess pleiotropic effects due to theirantibacterial, anti-inflammatory,immunomodulatoryand antioxidative properties.[11]This groupalso have anabolic effects on the bone by enhancing bone morphogenetic protein-2 expression, thus promotingthe differentiation and activation of osteoblasts.[15]Therefore regarding their advantageous properties, statins are presented as helpfulagents for enhancement of periodontal therapy outcome.[16,17]

Chitosan is the deacetylated form of chitin. It is a linear polymer of N-acetyl-D-glucosamine and deacetylated glucosamine that shares some properties of hyaluronic acid and glycosaminoglycan.[18]Chitosan interfereswithdental biofilm by interactions betweenits positive charged free amino groups and theanionic parts of the bacterial cell wall, thus inhibiting bacterial aggregation.[19,20]

Gingival crevicular fluid is considered acritical diagnostic measure in periodontitis. Its significancearise from its proteins and peptides contents which arise from the host inflamed tissues. The GCF biomarkers incorporate host derived enzymes such aselastase,matrix metalloproteinases,aspartateaminotransferaseand beta-glucuronidase.Inflammatory mediators and products such as prostaglandin E2,interferon-α, substance-P,tumor necrosis factor-α, cytokines and monocyte chemoattractant protein are present in GCF. It also, includes bone turnover markers such as alkaline phosphatase, cathepsin B, osteocalcin and collagen.[21,22]

Netrins described in human kindare a class of extracellular, laminin-like proteins,[23]includingnetrin-1, netrin-3, and netrin-4, and dual glycosylphosphatidylinositol-attached membrane peptides (Netrin G1 and G2).[24]Netrin-1 is specified as a neuroimmune guidance cue throughout developing the nervous system.[25]Thismolecule performs a criticaltaskthroughguiding axons to its objectivethrough engagement with one of its main receptor uncoordinated (UNC) 5 family members: UNC5A, UNC5B, UNC5C, and UNC5D.[26] Over the past decade, netrin-1 has been involved in various physiological responses and additional functions ranging from angiogenesis to inflammation and UNC5B is the only netrin-1 receptor which is mainly expressed in the vascular system such as, human monocytes, granulocytes, and lymphocytes .[27]

The participation of the nervous system to inflammation is more than vasodilatation and immune-cell recruitment. Some neuropeptides are synthesized and released from inflammatory cells in cases of pathological conditions,[28] where they act on a local levelby means of receptors on other neurons or immune cells.[29]The action, differentiation and survival of nerve cells can be regulated bycytokines and other products of immune cells.[30]Existence ofcommunication between theneurological and immune systems is suggested bythe identification of GCF neuropeptide and its receptors on immune cells, which may lead toregulation of the inflammatory response.[31,32]Thus the nervous system is considered as anessentialregulatorof inflammation in periodontal diseases .[33]Therefore the aim of this study was to evaluate of the effect of local gel application (simvastatin gel 1.2% and chitosan based simvastatin gel 1.2%) on the level of GCF netrin-1 and UNC5Bin treatment of stage II periodontitis.

Patients and Methods:-

This research was approved by the Dental Research Ethics Committee, Faculty of dentistry, Mansoura University (A14071221). Verbal information and written consents were performed for all participants in the present study.Fifty-five systemically healthy individuals, were selected from the Periodontologyclinic, Faculty of Dentistry, Mansoura University after comprehensive medical and dental histories.

The study individualsincluded 45 patients with periodontitis (mean age 37 ±7.9) and 10 periodontally clinically healthy controls (mean age 42.5 ±9.3). The patients were selected according tostage and extent of periodontitis following the new classification scheme.[34,35]Stage IIgeneralized periodontitis patients were included in the study; interdental CAL=3-4 mm at site of greatest loss; PPD ≤ 5 mm and radiographic bone loss involving the coronal third of root (15-33 %). Periodontally healthy individuals had no pockets, no radiographic bone loss, good andgingival index<1.

Included patients were over 20 yearsage and had at least 20 natural teeth (excluding third molar). The patients were excluded if they were smokers or with history of smoking[36]; had any diagnosed medical[37] illness that could affect the periodontal condition; subjected to antibiotics,[38] nonsteroidal anti-inflammatoryor any other drugs; pregnant or lactating[39], having serum CRP >5 mg/L, received periodontal treatmentor requiring endodontic or orthodontic therapy.

Study Design and treatment phases:

The study design included four groups consisting of two control and two study groups. The control groups were classifiedinto Group I,comprised 10 periodontally healthy individuals as a negative control, and Group II comprised 15 periodontitis patientsmanaged by SRP only as a positive control. Whereas, the study groups were Group III comprised15periodontitis patients treated bySRP followed by local delivery of simvastatin gel1.2 %,[40] and Group IV comprised 15periodontitis patients treated by SRP followed by local delivery of chitosan based simvastatin gel1.2%. All periodontitis patients were not informed about which group they belong to as it was performed by another masked examiner.

Clinical indices; including PI, [41] GI,[42] PPD[43] and CAL[44] were recorded for the fourgroups at baseline and six weeks after treatment for the three periodontitis groups by the same examiner. Concerning PI and GI, the mean score of each tooth was obtained by summation of the four scores per tooth and calculating one-fourth. The index score for each subject was obtained by summation of the index scores per selected teeth (stage II periodontitis)and division of their sum by the number of teeth examined. For both PPD and CAL, measured at six sites per tooth using a manual periodontal probe (Unc15, Hu-Friedy).The mean score of each tooth was obtained by summation of the six scores per tooth and calculating one-sixth. The index score of the person was obtained by adding scores per teethand division of the sum by the number of examined teeth.

The nonsurgical periodontal treatment, for periodontitis patients in groups II, III and IV, included meticulous full mouth SRP using ultrasonic scaler (Cavitronselect, Dentsply) and hand specific gracey curettes (Gracey curettes, Hu-Friedy). This step was repeated weekly if indicated and good oral hygiene measures were clarified to the patients throughout the study period.

Additionally, the selected periodontal pockets in patients in group III were additionally injected with simvastatin gel 1.2% using plastic syringe with a needle of a flexible large gauge to facilitate easy and effective gel application. The injection started from the base of the pocket top word slowly to ensure that the gel reached the pocket depth. Whereas, in group IVpatients,pockets were injected with chitosan based simvastatin gel 1.2% in the same manner. Patients in these groups received the same oral hygiene measures instructions starting from 24 hrs after gel application which was repeated once weekly for six weeks. The gel was prepared in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University in September 2020 using Carbopol-940, hydroxypropyl methylcellulose, simvastatin, chitosan and triethanol amine (Sigma –Aldrich, St. Louis, USA) in addition to propylene glycoland sodium benzoate (ELNasr company, Egypt).

Collection of Gingival crevicular fluid (GCF)

Prior to sample collection, the supragingival plaque was carefully removed and teeth cleaned from any debris or blood by washing with water spray.The selected sites to be sampled were isolated with cotton rolls and gently air dried. OneGCF sample wasobtained from all participants at baseline. Another GCF sample was obtained after

treatment in periodontitis patients.GCF samples contaminated with blood were discarded. GCF samples were then placed into sterile Eppendorf tubes containing 100 µl phosphate buffer pH 7.4 and stored immediately at -80°C till time of analysis.[45]Netrin 1 and Netrin-1 receptor UNC5Bwere assayed by sandwich ELISA supplied by Bioassay technology laboratory (China) according to manufacturer instructions.

Results:-

The current study was a randomized clinical trial carried out on fifty-five individuals (38 females and 17 males). Study subjects were age and gender matched. The age of the selected individuals ranged from 35 to 50 years with mean age 37±7.9, 41.5±6, 42.5±9.3 and 42.5±10.6 in groups I, II, III and IV, respectively. No untoward side effects were recorded by patients, from using either simvastatin gel1.2%chitosan basedsimvastatin gel1.2%, during the whole period of the study.

Table (1):- Comparison of the clinical parameters in the studied periodontitis groups at baseline and after six weeks:

ClinicalParameters		Group II	Group III	Group IV	P valueT0
PI	**T0**	2.49±0.18	2.53±0.23	2.32±0.22	0.036
	T1	0.83±0.12	0.79±0.14	0.78±0.12	
Comparison of Groups at T1		P1=0.808	P2=0.714	P3=0.984	
GI	**T0**	1.76±0.18	1.79±0.15	1.78±0.14	0.884
	T1	1.16±0.20	0.37±0.07	0.36±0.06	
Comparison of Groups at T1		P1<0.001*	P2<0.001*	P3=0.979	
PPD	**T0**	3.54±0.24	3.5±0.25	3.42±0.23	0.455
	T1	2.51±0.20	1.71±0.20	1.69±0.20	
Comparison of Groups at T1		P1<0.001*	P2<0.001*	P3=0.983	
CAL	**T0**	2.67±0.23	2.65±0.32	2.73±0.21	0.713
	T1	2.27±0.20	1.48±0.23	1.45±0.19	
Comparison of Groups at T1		P1<0.001*	P2<0.001*	P3=0.910	

PI: Plaque Index, GI: Gingival Index, PD: Probing Pocket Depth, CAL: Clinical Attachment Level.
T0: baseline, T1: after six weeks.P: p value
P1: comparison of group II with group III, P2: comparison of group II with group IV, P3: comparison of group III with group IV.
*: Statistically significant difference at $P < 0.05$

Regarding the clinical parameters, the mean values and standard deviations of these parameters in the treated groups of periodontitis patients were shown in **table (1**). There were no statistically significant differences in these parameters between the treated groups of periodontitis patients at baseline, except in the PI.

Additionally, there was no statistically significant difference in the PI between the different treated groups six weeks later. On the other hand, inter-groups comparisons of GI, PPD and CAL showed statistically significant differences between group II and each of group III (P1) and group IV (P2) at P values < 0.001. However, there were no statistically significant differences in GI, PPD and CAL between groups III and IV after treatment.

Table (2):- GCF levels of Netrin-1 in all studied groups at baseline and after six weeks.

Netrin-1		**Group I**	**Group II**	**Group III**	**Group IV**	
Mean±SD						
T0		28.5±1.05	12.3±0.65	12.3±0.67	12.3±0.79	
T1		28.5±1.05	21.6±0.72	23.4±0.84	26.4±1.27	
P value						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**
T0	<0.001*	<0.001*	<0.001*	1	1	1

T1	<0.001*	<0.001*	0.001*	0.004*	<0.001*	<0.001*

SD, standard deviation, ANOVA test is used.
T0: baseline, T1: after six weeks.
P1: comparison of control group (I) with group II, P2: comparison of control group (I) with group III, P3: comparison of control group (I) with group IV, P4: comparison of group II with group III, P5: comparison of group II with group IV, P6: comparison of group III with group IV.
*: Statistically significant difference at $P < 0.05$

The mean values and standard deviations of the GCF levels of netrin-1 in all studied groups at baseline and after six weeks were shown in **table (2)**. The levelof netrin-1 was higher in group I of healthy subjects than other groups of periodontitis patients at the baseline. In those patients, the GCF concentrations of Netrin-1 were increased after receiving different treatment modalities according to their groups, however, its level was significantly less than those of the healthy subjects.

Inter-group comparisons before and after treatments were also shown in table (1). There were statistically significant differences between group I and each of group II (P1), group III (P2), and group IV (P3) at P value ≤ 0.001 before and after treatments. Regarding the groups of periodontitis patients, there were no statistical significant differences between patients of group II and those of either group III (P4) or group IV (P5) at baseline (P value = 1). Moreover, there was no statistically significant difference was present between patients of group III and those of group IV (P6 = 1) before the treatment.

However, there was a statistically significant difference between patients of group II treated by SRP and those of group III who received adjunctive local1.2% simvastatin gel at P4 = 0.004. Additionally, the concentrations of GCF Netrin-1 was statistically higher in patients of group IV who received chitosan based 1.2% simvastatin gel than those of group II (P5) and group III (P6) at $P < 0.001$.

Table (3):- GCF levels of Netrin-1R in the studied groups at baseline and after six weeks.

Netrin-1R		**Group I**	**Group II**	**Group III**	**Group IV**	
Mean±SD						
T0		20.8±7.4	11.6±1.76	11.7±3.43	12.3±2.79	
T1		20.8±7.4	13.1±1.96	14.5.4±4.01	19.7±4.32	
P value						
	P1	**P2**	**P3**	**P4**	**P5**	**P6**
T0	0.001*	<0.001*	0.001*	1	0.977	0.981
T1	0.018*	0.046*	0.961	0.915	0.017*	0.047*

SD, standard deviation, ANNOVA test is used.
T0: baseline, T1: after six weeks.
P1: comparison of control group (I) with group II, P2: comparison of control group (I) with group III, P3: comparison of control group (I) with group IV, P4: comparison of group II with group III, P5: comparison of group II with group IV, P6: comparison of group III with group IV.
*: Statistically significant difference at $P < 0.05$

Considering Netrin-1 receptor UNC5B, healthy subjects had significantly higher GCF levels than the other groups of periodontitis patients before treatment at P value ≤ 0.001, as shown in table (3).Furthering, no significant difference was found between the groups of periodontitis patients at the base line.

After six weeks of treatment, the level of UNC5B was increased in all treated groups regardless of the treatment received.Group I level was significant higher versus both group II (P1 = 0.018) and group III (P2 = 0.046).Meanwhile, no significant difference was present between group II (SRP) and group III (SRP + simvastatin gel1.2%)at P4 = 0.915. Group IV (SRP +chitosan basedsimvastatin gel1.2%) was significantlyhigherversus each of group II (P5 = 0.017) and group III (P6 = 0.047).On the other hand, no significant difference was present between the healthy subjects of group I and patients of group IV (P3 = 0.961).

Statistically significant differenceswas found regardingintra-group comparisons of the Netrin-1 and Netrin-1 receptor UNC5B concentrations and also all the clinical parameters in each of group II, III and IV at the base line and after six weeks of treatment (data not shown).

There was no significant correlation between the levels ofnetrin-1 and netrin-1 receptor with all the clinical parameters before and after treatment in group II. However, there was a significant positive correlation between the total concentration of netrin-1 and PPD at base line in group III. Regarding group IV, there were a significant negative correlation between netrin-1 receptor amount and PI before treatment and a significant positive correlation between netrin-1 concentration and GI after treatment. Furthermore, there were significant negative correlations between levels ofnetrin-1 and each of the GI, PPD and CAL after treatment and also between levels ofnetrin-1 receptor and the GI only six weeks later (data not shown).

Discussion:-

Initial periodontal therapy, including subgingival debridement is considered an important clinical impact.[46] However, SRP is not free of limitations.[47] Therefore, localandsystemicadjunct therapies were tested.[48] However, better compliance, less side effects andhazard of developing bacterial resistance to medications were reported with local treatment in comparison with drugs used systemically.[49]

Regarding the values of PI, GI, PPD and CAL at baseline, no significant difference was found between positive control (SRP) and study groups(SRP+1.2%simvastatin&SRP+chitosan based 1.2%simvastatin)except in the PI.After six weeks of SRP, positive control and both study groups showed significant improvement in all periodontal parameters, comparedto its values at baseline while there was no statistically significant difference in the PI between the different treated groups with repeated oral hygiene measures motivation. Meanwhile,all periodontal parameters of both study groups were improved significantly versus positive control groupwhile there while no significant difference was found between both study groups.

Furthermore, our results were in consistence with the study performed by Colombo et al.[50]They reported that SRP with repeated oral hygiene measures motivation, resulted in significant improvement of periodiodontal parameters. Moreover, it came on the same line withthemeta-analysis and systematic review performed by Ambrósio et al.,[51] Shah el al.[52],Bertl el al.[53]and Khoderel al.[54]They reportedbetterenhancement in periodiodontalparametersof study group after locally applied statins as adjunct to SRP versus control group treated by SRP only.

Plaque control and SRP, result in an enormousdepletion of periodontal burden and reestablishment of a healthy compatible microbiotawhich clinically reflected by improvement in the clinical periodontal parameters.Furthermore, the superior results of both study groups could be illustrated by the fact that statinsinfluences the severity of periodontitis. It suppressadherence and extravasation of leukocytes ininflammedlocationresulting in declining co-stimulation of T cells and reducing IL-1β, IL-6 and TNF-α.[55–58]It also has anti-inflammatory effect and inhibit major histocompatability complex class II (MHC-II) molecules, which are involved in the activation of T-lymphocytes and antigen presentation.[59,60]

Despite the non-significant difference, the superiority in the therapeutic outcomes noted among group IV than those of group III could be explained by the additional benefit obtained from using chitosan as base for SMV whichexhibits excellent tissue wetting properties,[61]biocompatibility, biodegradability,[62] hemostatic activity[63]in addition to theantimicrobial action, especially toward*Porphyromonasgingivalis* and *Aggregatibacteractinomycetemcomitans.*[64,65]Itintensifies the recovery of connective tissue, angiogenesis, and bone formation.[66,67] Also it promotes the productivity ofplatelet derived growth factor and transforming growth factor β1 and inhibits the overproduction of prostaglandin E2.[68-70]

Regarding netrin-1and netrin-1 receptor UNC5Bin GCF at baseline, there was a noticed reduction in periodontitis patients compared to controls,where no statistically significant difference was present between the three periodontitis groups.However, the level of netrin-1and netrin-1 receptor UNC5Bin GCF were significantly increased after treatment in all treated periodontitis groups, but still significantly less than those of healthy subjects.Meanwhile, Netrin-1level was statistically higher in group IV (SRP+chitosanbasedsimvastatin gel1.2%) than both group III(SRP+simvastatin gel1.2%) and group II(SRP) and it was statistically higher in group III than group II. Regarding Netrin-1 receptor UNC5B after treatment, its level was significantly higher in group I (healthy) and group IV (SRP+chitosanbasedsimvastatin gel1.2%) versus both of group II (SRP) and group III

(SRP+simvastatin gel1.2%). On the other hand, no statistically significant difference was present between groups I & IV and between groups II &III.The present study results confirmed a previous study results performed by some research team members (in press).

Tadagavadi et al.and Ly et al., explained these results be by the ability of netrin-1 to suppress the leukocytes migration through UNC5Breceptor mediated increase in cAMP and inhibition of chemotaxis in vivo andin vitro. [71,72]Their results were also in agreement withRosenberger et al., Mirakaj et al. and Mirakaj et al.[73-75]They reported that netrin-1 administration to mice suppressedinflammation by suppression of the production of inflammatory cytokines and chemokines. Besides, a studyperformed by Ranganathan et al.also reportedthat,netrin-1 regulate the inflammatory response of neutrophils & macrophages through suppression of COX2 mediated PGE2 production.[76]

The results of the present study were in contrary to Gunpinar et al. results regarding the GCF levels of netrin-1;they assessed samples of serum, whole salivaand GCF taken from systemically healthy, nonsmoking 20 periodontitis and 20 periodontally healthy subjects at baseline and four weeks after non-surgical periodontal therapy.[77]Meanwhile came in agreement with their results regarding the serum and saliva levels of netrin-1 at both baseline as well as post treatment. However, the lack of agreement in both GCF sample results could be due to the difference in the laboratory techniques used, sampling sizes collected, study populations status and severity and the short time of follow up, while the agreement between their results regarding serum sample and our GCF sample could be theoreticallyexplained by the fact that mainserum is the source of GCF.

However, to the best of our knowledge, few studies assessing the role of netrin-1 and its receptors in periodontitis populations were available, so the conflict between Gunpinar et al.[77] results and our results require more controlled clinical trialsto clarify the pathway that is regulated by Netrin-1/Unc5b axis and elucidate its potential for diagnosis and treatment of periodontal disease.

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