

The Study On The Effect of Nanoemulsion For The Prevention And Treatment Of Gingival Inflammation

Chang-Hoon Chae, Jun-Woo Park*

Institute of Nano-Bio Fusion Technology, NanoCureTech Inc,

**Dept. of Oral and Maxillofacial Surgery, College of Medicine, Hallym University*

Abstract

We have found out the relationship of nanoemulsion containing nano vitamin C, E and propolis and gingival disease. We've confirmed effect of nanoemulsion through the experiment of *in vivo* and *in vitro*. We tested cell viability of gingival fibroblast cells by MTT assay and mRNA appearance of interleukin-1 β , using mouse that was guided inflammation. Anti-microbacterial activity for Antibacterial effect's experiment was carried out by using *S.aureus* and *E.coli*. In addition, inflammation tissue has been observed with scanning electrical microscopy. In this study, expression of interleukin-1 β was decreased after adding nanoemulsion containing nanovitamin C, E and propolis. We've also obtained good results from the test of Antibacterial effect against *S.aureus* and *E.coli*. Also, swelling of inflammation tissues observed by scanning electrical microscopy has gone down.

In conclusion, we have gained confidence that nanoemulsion containing nano vitamin C, E and propolis has very high Antibacterial effect against bacteria in oral. And it made us guess that inflammation of gingival reduces after decreasing interleukin-1 β .

Thus, we expect that nanoemulsion containing nano vitamin C, E and propolis gives good effects to patient having gingival disease.

Key words

Inflammation, Nano-vitamin C, E, Propolis, Interleukin-1 β , Gingival fibroblast cell

INTRODUCTION

In the mouth, due to many causes oral diseases arise and gingival and periodontal diseases are the most common. Oral diseases such as cancers are often caused by external causes such as smoking and environmental factors. Especially because of stress and westernized eating patterns, gingival diseases are continually increasing and it threatens the high quality of life by weakening the alveolar bone and eventually leading to loss of teeth. To these days NSAIDs are commonly used to treat gingival diseases and they are proven to be effective in gingival

and periodontal illness. However, research on drugs that act only on the periodontal and gingival tissues are actively going on in many countries in the world.

When the gingiva undergoes inflammation, human immunity plays an important role. There are many cause of inflammation. Inflammation is a immunologic response involving blood vessels and blood cells to local injury, infection, hypersensitivity, physical reaction, chemical reaction, necrosis of tissues and emotional stress.

Especially in a moist environment like mouth, toxins produced by plague and calculus infiltrate into the gingiva causing gingivitis. Also, after the 40s, human immunity and healing capacity of the tissues are lowered which activates microorganisms. Therefore as the population ages, the rate of periodontal diseases increases. Periodontal diseases aggravate without any symptoms until extraction of the tooth is the only possible treat-

* Corresponding author

Jun-Woo Park

Dept. of OMFS, Kang Dong Sacred Heart Hospital, Hallym Univ.
445 Gil-dong, Gangdong-gu, Seoul, 134-010, Korea
Tel: 82-2-483-9647

ment.

The purpose of the study is to find out the effect of the nanovitamin C, E Propolis complex which is biocompatible to the gingival and periodontal diseases. *In vitro* experiments of periodontal cells, *in vivo* experiments of mice with inflammation, and electromicroscopic and antimicrobial test of staphylococcus were performed.

MATERIAL AND METHODS

1. *In vitro* Human Gingival Fibroblast experiments

Human Gingival Fibroblast used in this experiments were purchased from ATCC(ATCC, Manassas, VA, USA). It was cultured using DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum under the condition of 37°C 5% CO₂. In 80% confluency 0.01% trypsin-EDTA was used for serial culture. Hemocytometa was used to count the cells and interleukin-1 β (Santacruz, CA, USA) was purchased from the company. sodium nitroprusside (SNP, Simga-Aldrich, Louis, MI, USA) was mixed with sterile water to be used in the experiment. SNP is known as a strong material to cause acidic destruction. In each cell, the final concentration was 100uM, and cell reaction was observed one hour after administering SNP.

2. Cell viability assay using MTT assay

MTT celltiter Aqueous one solution cell proliferation assay kit was used to test cell activity and cell survival rate. The culture incubated in 96well plate and to the grown culture was added 100uM SNP. The culture were incubated for 1 hour at 37°C CO₂ incubator. The culture were incubated for 24 hours, and the 96well plate was quantified spectrophotometrically by measuring absorbance at 540nm using a ELISA reader(Molecular Device, sunnyvale, CA, USA). The absorbance value was calculated by using SigmaPlot 4.0(SPSS Inc., CA USA).

3. *In vivo* inflammation induced mouse experiment

6 weeks old mice under 20 grams were purchased and the temperature was maintained as 22 \pm 2°C and humidity as 50 \pm 5%. The mice were used after adaptation for one week of 12 hours of light frequency. TRI Reagent Total RNA Extraction Kit(Promega, CA, USA), chloroform, isopropanol alcohol, Ethanol, Actone (MERC

Inc., HA, Germany), Oligonucleotide(Genotech, DaeJeon, Korea), 4% paraformaldehyde(Sigma-Aldrich, CA, U.S.A), Diethyl Pyrocarbonate(Sigma, CA, USA), Maxime RT-PCR PreMix kit(iNtRON BIOTECHNOLOGY, Seoul, Korea), agarose(BIO-RAD, CA, USA), TAE buffer(Tris-Acetic-EDTA buffer), DNFB(2,4-DINITROFLUORO BENZENE. St. Louis, MO, USA) were used. DNFB is known to arouse inflammation. Electrophoresis kit was used to see gene expression by using UV illuminate(Bio-Rad, CA, USA). Spectrophotometer(BIO-RAD, USA) was used at 260um to observe the concentration.

4. RNA extraction and RT-PCR

Ear tissue of a mouse after cervial bone dislocation was frozen in liquified nitrogen, made into powder, and stored in a tube. TRI Reagent 800ul was added and it was vortexed with chloroform 100ul. It was centrifuged and the upper layer was removed. It was inverted by isopropanol and stored in room temperature for 10 minutes. After 10 minutes, it was centrifuged at 10000 rpm and the top layer was removed. 70% ethanol was added and washed for 10 minutes. After drying 100 ul DEPC water is added to melt the RNA pellets. Spectrometer at 260um was used to see the concentration of the RNA. Add template RNA and IL-1b primer into the RT-PCR PreMix tubes. Add RNase-free water into the RT-PCR tubes to a total volume of 24ul. Dissolve the blue pellet by pipetting. Perform RT-PCR reaction of smplex as following using PCR machine(Hybaid, CA USA). Load samples on 1.5% agarose gel without a loading-dye buffer and perform electrophoresis. The concentration of DNA expression was confirmed by UV Illumination lamp.

5. Antimicrobial test

Staphylococcus aureus, ATCC 6538 was used and they were cultured at 37 degrees and 50% of humidity for 24 hours. Then the number of microorganism was set to 200000. Staphylococcus aureus was smeared on an agar mixed petri dish and incubated at 37 degrees for one hour. Diameter of 7mm and width of 3.5mm zone was created in the incubator and stored for 16 hours to see the antimicrobial activity.

6. Electron microscope

An electron microscope observation was done. Tissue

coated cover glass were put on cooling holder and incubated with 200ul 4% PFA at room temperature for 1 hour. Samples were observed in reduced vacuum. For observation under low vacuum, samples were further sputter coated with evaporated gold for 2 min using the Quick Ion Coater(Sanyu Denshi Co, Ltd., Tokyo, Japan). The accelerating voltage was 15kV low vacuum mode imaging. Specimens were placed 14mm from the base detector. Electron micrographs were obtained at magnifications of x1.0K.

RESULT

1. MTT assay analysis

Cell survival rate test was used to see the effect of SNP on the HGF cells. The cell survival rate is highest in 540nm. As in Fig 1, the group in which the nanoemulsion was applied in addition to the SNP, showed higher cell

survival rate. Therefore nanoemulsion inhibited the decrease of cell count in the presence of SNP, a toxic material.

2. Analysis of mRNA of Interleukin-1 β

Interleukin-1 β is a substance that causes inflammation and the drug effect could be observed by RT-PCR experiment that expresses mRNA of Interleukin-1 β . For seven days experiments on mice were done. Number 1 mouse was a control which had no Interleukin-1 β . Number 2 mouse was a mouse with inflammation which had a high level of Interleukin-1 β . Number 3 mouse was positive control which had inflammation and increased level of Interleukin-1 β . Number 4 was treated with the nanoemulsion which had 50% of the Interleukin-1 β . The level of Interleukin-1 β was almost the same as the number 1 mouse which was normal without any treatment.

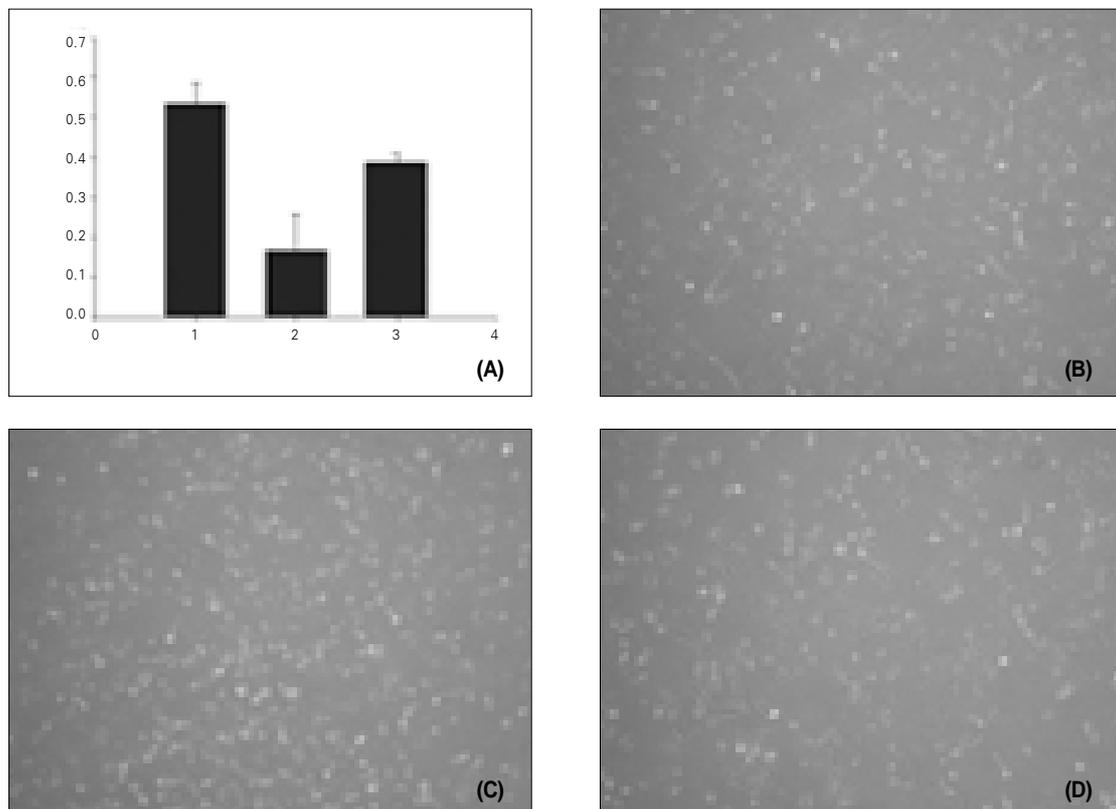


Fig. 1. The results of MTT assay showed a little decreased in viable cell number in No 3, whereas No 2 is significant decreased in viable cell number. (A): 1. control, 2. SNP treated, 3. Nano emulsion. (B),(C),(D): The morphology of cultured cells by phase contrast microscopy. (B) control of HGF fibroblast cell, (C) HGF fibroblast cell treated SNP (D) HGF fibroblast cell was treated by SNP and nano emulsion.

3. Antimicrobial test

In order to observe an effect of the microbial effect of the nanoemulsion, An experiment was performed with *Staphylococcus aureus* and *E. coli*. The bacteria were smeared on an agar culture medium and incubated in 37 degree for one hour. As in Fig. 3, greater concentration of nanoemulsion was associated with greater antimicrobial capacity. Especially in 200um of nanoemulsion the antimicrobial capacity was shown to be significantly high in *Staphylococcus aureus* test.

4. Electron microscope observation

Each tissue specimen under inflammation showed swelling, and this swelling of the tissue was observed with an electron microscope. A loop-like structure is a connective tissue which looks swollen (Fig. 4). Fig 4a is normal tissue and 4b is inflamed tissue. b and c are show swelling and d shows decreased swelling. Fig 4 indicates that the nanoemulsion has an effect of relieving the swelling of tissues.

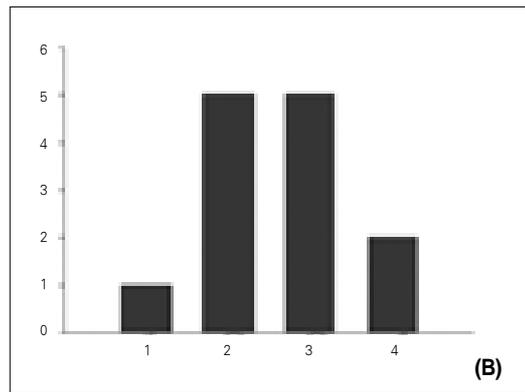
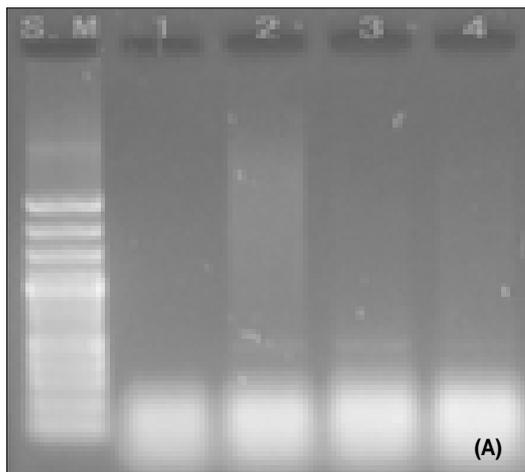


Fig. 2. A: The expression of Interleukin-1 β DNA pattern, B: The grape of Interleukin -1 β pattern, S.M: size marker, 1. control 2. treated by DNFB 3. treated by Vaseline 4. treated by Nano emulsion. Nano emulsion was decreased in DNA expression level. But Vaseline and positive control were increased in expression level.

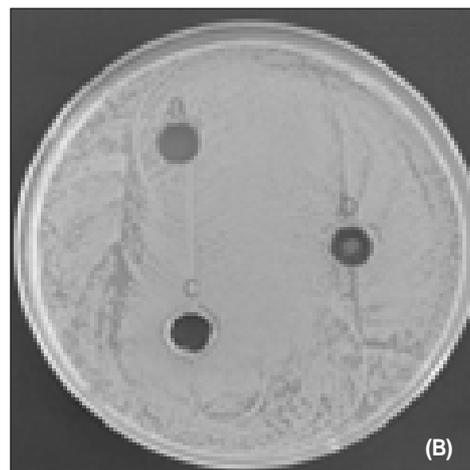
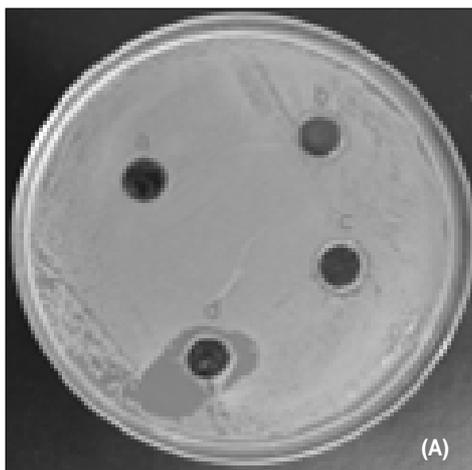


Fig. 3. Antibacterial effect of *S.aureus* and *E.coli*. (A): *S.aureus*, a. control, b. nano emulsion 50ul, c. nano emulsion 100ul d. nano emulsion 200ul, (B) *E.coli*. a. control, b. nano emulsion 50ul c. nano emulsion 100ul.

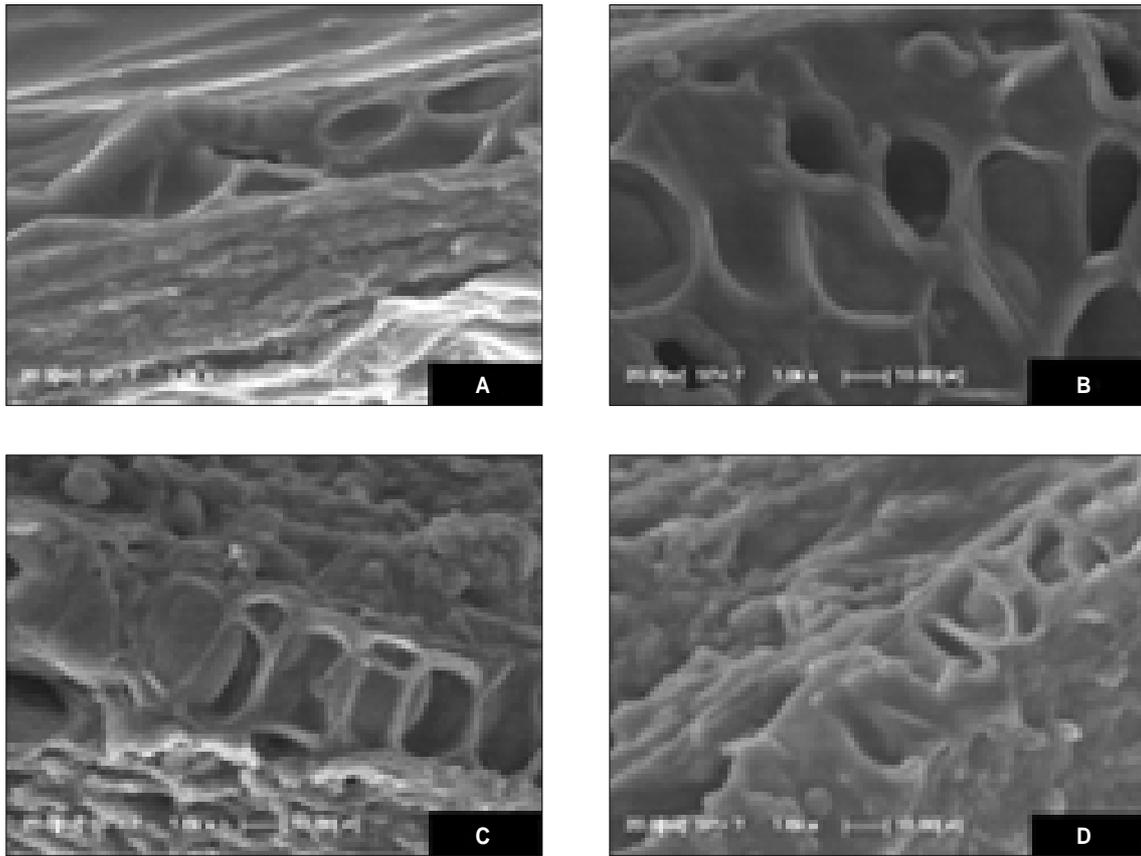


Fig. 4. This pictures shows SEM. ear's skin was a little bit swelled but it was recovered to normal condition after being treated nano emulsion. A. control, B. treated by DNFB, C. treated by Vaseline, D. treated by nano emulsion.

DISCUSSION AND CONCLUSION

It is difficult to treat gingival and periodontal diseases due to a unique environment. The most important issue in treatment of gingival diseases is rapid transportation of the drug material and stable retention. In order to fit into these characteristics there have been numerous reseraches on nano materials in the field of medicine. Nano-sized particles could be rapidly absorbed into the target tissues. However for medical use, the material must be biocompatible. These days researches on nano-biotechnology are very active and one of the examples is gene chip that is used in diagnosis of cancer and genetic diseases. Also nano-biotechnology is used in drug delivery system in coating of the drug. There are numerous products that are based on the nano-biotechnology and yet most of them are in their trial stage and side effects of them are not reported. Nanovitamin C and E are already biocompatible, and they are known to act in immunity and antioxidation. Rapid resorption of these materials

seem to have a positive effect on the tissues.

Based on this study, nanaoemulsion significantly reduced the cytokines which is an inflammation-induced material, and this could be alluded to activation of the nano-sized vitamins. Also, nanoemulsion showed antimicrobial effect dependent on the concentration, and reduced the edema of the ear of the mice. This material may relieve gingivitis, and have an antimicrobial action as well.

In conclusion, in the experiment of HGF cell and mice with inflammation, it was shown that the nanoemulsion had a statistically significant anti-inflammatory action. Through the experiment of expression of mRNA, it was also seen that the nanoemulsion significantly reduced the amount of Interleukin-1 β . Further research is needed for the exact anti-inflammatory mechanism but it seems that the nanoemulsion inhibits the expression of inflammation-related cytokines. Also, it showed to be effective against the bacteria which is one of the causes of gingival diseases. These results altogether indicate that the

nanoemulsion is effective in protection of gingiva and treatment of gingival diseases.

1. It significantly reduced the edema of the ears of mice that had inflammation.
2. From the result that the mRNAs of Interleukin-1 β significantly decreased, it was confirmed that the nanoemulsion inhibited the release of inflammatory cytokines.
3. It proved to inhibit the destruction of human gingival fibroblast in placement of SNP, which is toxic chemical. This showed that the nanoemulsion has an antioxidant effect.
4. As a result of experiments with Staphylococcus and E. coli, it showed that it was antimicrobial with significant association with concentration.
5. Subsided edema of mice that had inflammation was also confirmed by electron microscope.
6. Further research is needed to find out more about the mechanism of this nanoemulsion material.

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