Synergistic Test of Chitosan-Ozonated Olive Oil and Chitosan-Coenzyme Q10 as Bone Graft Materials

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Abstract

Chitosan contains two reactive groups (amino and hydroxyl), which can be chemically or physically modified, thereby chitosan has a high potential in tissue engineering applications. Chitosan is an ingredient that is often used as a graft material, however, it is less osteoconductive when not combined with other materials. Other ingredient that possess high antioxidants and can be used routinely, both topically and systemically is coenzyme Q10 in the treatment of periodontal conditions. Ozonated olive oil is also used as a therapeutic agent in inflammation to stimulate and accelerate the healing of soft tissues and bones. In the present study, the synergy of chitosan-ozonated olive oil combination and coenzyme Q10-chitosan gel combination on the increase of bone formation was examined. A purely laboratory experimental study was carried out in this study. Synergy measurement of chitosan combination was delivered by FTIR spectrum test and MTT assay. The results revealed that between the combination of chitosan-ozonated olive oil and chitosan-coenzyme Q10 gel had the same spectrum graph as pure chitosan. This data showed that the combination of both materials did not form a new reaction. Furthermore, the MTT assay showed that the combination of chitosan-ozonated olive oil and chitosan-coenzyme Q10 gel were not toxic and could increase the number of fibroblast cells. In conclusion, the combination of chitosan-ozonated olive oil and gel chitosan-coenzyme Q10 indicated a synergistic relationship without mutual influence and non-toxicity.

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Introduction

The occurrence of periodontal pockets due to chronic inflammation of the periodontal tissue will be followed by resorption of alveolar bone [1], hence flap surgery is required so that the debridement process can be performed more optimally for healing of periodontal tissue [2]. According to Kaushal *et al.*[3] flap surgery alone is still not optimal in regenerating periodontal tissue quickly and properly [3]. The addition of *bone graft* to flap surgery has been proved to be better in stimulating the periodontal tissue, including the alveolar bone regeneration [4].

Bone graft is a material that can stimulate bone healing through the *osteoconduction*, *osteoinduction* and *osteogenesis* processes. So far, *autograft* and *allograft are* known as the *gold standard for* treating bone defects, but they still often cause infections in the body [5,6]. Currently, there are a lot of studies on nature originated alternative materials because they are considered simpler and provide less harmful side effects. One of the natural materials that has begun to be developed for biomedical applications is chitosan, a chitin derivative with the formula of *N-acetyl-Dglucosamine* which is obtained from the exoskeleton of crustaceans such as shrimp, crabs or clams. Chitosan has high *osteoconductivity* properties, easy application and gradual biodegradation, making chitosan has good potential to regenerate bone [7], however the scaffold of chitosan derived from a single material is still not optimal for new bone formation [8].

The combination of chitosan with other biomaterials can have a synergistic effect on cell

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adhesion, cell differentiation and extracellular matrix formation thereby increasing tissue regeneration [9]. Various substances can be added to increase the effectiveness of chitosan, one of which is *ozonated* olive oil. Ozone is used as a therapeutic agent in inflammation to stimulate and accelerate soft tissue and bone healing [10,11]. Another material that can also be added to optimize the effectiveness of chitosan is Coenzyme Q10, a high antioxidant material that can be used both topically and systemically in the treatment of periodontal conditions [12].

Ozone therapy is one of the most popular methods in medicine and dentistry as it has a high degree of biocompatibility with fibroblasts, cementoblasts, and epithelial cells. A study report showed that ozone can improve periodontal disease [13]. *Ozonated* olive oil has a function as an anti-bacterial, anti-inflammatory, and stimulates the *growth factor* namely. *Vascular Endothelial Growth Factor* (VEGF), *Platelet-Derived Growth Factor* (PDGF), and *transforming growth factor* β (TGF-β), which stimulates the regeneration of the periodontal tissues [14]. The growth factors will stimulate osteoprogenitor cells to differentiate into osteoblast cells. Osteoblast cells require a scaffold and mediators induction in bone remodeling. Scaffold serves as a medium for stem cells and osteoblasts to be attached to, live and breed well in bone defects thereby forming new bone [15]. Chitosan combined with *ozonated* olive oil is the latest, potent, and safe synergistic combination 16.

Coenzyme Q10 (CoQ10) is a high antioxidant that participates in the process of aerobic cellular respiration, able to clean the cellular *Reactive Oxygen Species* (ROS) and protects the skin from aging induced by UVA [17]. Its properties play an important role in cellular metabolism, participate as electron carriers in mitochondria and *extramitochondrial* membranes, and also protect membranes and lipoproteins from protein oxidation and lipid peroxidation (ubiquinol, CoQ10H2). [18].

Chitosan material is modified with addition of coenzyme Q10 has a function as an antimicrobial agent and produces good antioxidant compounds [12]. Adequate antioxidants supply to cells can increase the efficiency of energy production, tissue oxygenation and periodontal tissue repair [19].

Fibroblasts are the main connective tissue cells located in the lamina propria of the oral mucosa, including the gingiva. Observations on the fibroblast cells viability in culture can be used as an indicator to determine the effect of concentration and exposure time of a substance, including its cytotoxicity effect. The viability of fibroblast cells before and after exposure to cytotoxins expressed as a percentage of cell death is a parameter that can be measured using several cytotoxicity test methods, thereby the cytotoxic effect of a substance can be determined [20].

Toxicity test is part of the dental materials evaluation required in standard screening procedures. The purpose of the cytotoxic test is to determine the toxic effect of a substance directly on cell culture. One of the common method used for toxicity test of MTT assay method is a calorimetric test based on the breakdown of a yellow *tetrazolium* salt (MTT) and dissolve in water into *formazan* blue crystals which are insoluble in water [21]. The MTT assay method has the advantage that it is relatively fast, sensitive and accurate, and can be used to measure large samples and the results can predict the cytotoxic properties of a material [22].
This study aims to determine the synergistic effect between the combination of chitosan-coenzyme Q10 and chitosan-ozone as bone graft material by measuring the viability of gingival fibroblast cells.

Materials and methods

This study is a pure experimental study that has received approval for research ethics with No.00155 / KKEP / FKG-UGM / EC / 2019 from the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta.

Preparation of Chitosan gel

Lactic acid solution at 1% is made by diluting 90% lactic acid (Merck) with distilled water, i.e. 1 ml 90% lactic acid was added with distilled water until it reaches a volume of 90 ml. 0.1 gram of pharmaceutical grade chitosan powder (Himedia) was dissolved in 10 ml of 1% lactic acid solution using a *magnetic stirrer*. The gel was put into a *syringe* equipped with a blunt needle [23]. The gel was made using asepsis technique to maintain the sterility of the gel used. Perio-QTM gel is a Coenzym Q10 mixed with *vegetable glycerin* in a 1:9 ratio, in the form of a gel produced by PerioQ Inc., Manchester, USA.

Measurement of the synergism of the combination of chitosan with FTIR

The substance to be measured is identified, in the form of atoms or molecules. The infrared ray which acts as the light source is divided into two beams, one is passed through the sample and the other through the comparator. Then successively pass through the *chopper*. After passing through a prism or grating, the beam will fall on the detector and be converted into an electrical signal which is then recorded by a recorder. An amplifier is required when the resulting signal is very weak. The standard used is ASTM E1252, which can be easily tested by FTIR, including polymer pellets, parts, opaque samples, fibers, powders, wire coatings and liquids. A typical infrared scan is produced in the mid-infrared region of the light spectrum. The mid-infrared region is 400-4000 cm^{-1} wavenumbers, which equals a wavelength of 2.5 to 25 microns (10^{-3}mm).

Gingival Fibroblast Cell Incubation Process

Fibroblast cell culture in the form of a cell line was planted in a roux bottle for 4 days and then incubated. The fibroblast cells were then planted on a 96-well microplate with Rosewell Park Memorial Institute 1640 (RPMI-1640) medium to obtain a density of $1x10^4$ cells/well and incubated for 24 hours to obtain good growth. The wells were randomly selected to ensure the presence of fibroblasts, it was observed with an inverted microscope. The Rosewell Park Memorial Institute 1640 (RPMI-1640) medium in the wells was removed with a micropipette and replaced with a new one, then added 25µL of the gel solution to be tested in various concentrations. 50µL of DMSO was incubated for 24 hours at 37°C. Each tested gel was put into 25 wells.

MTT assay test of combination of chitosan-*ozonated* olive oil and chitosan-coenzyme Q10.

Gingival fibroblast primary cells were cultured in 100-well microplates. The number of cells was counted with trypan blue before being planted in microplate wells in different cell numbers. After being incubated overnight, and it is assumed that the cell has attached to the bottom of the well. MTT assay was performed and the absorbance was measured [33]. As a solvent of *formazan*, 50pl of 20% SDS was used which was put into each well and allowed to stand overnight. After the primary cells of gingival fibroblasts grew confluent at the bottom of the well, the medium was replaced and a combination of chitosan-*ozonated* oil as well chitosan-coenzyme Q10 was added. Each test material was repeated 5 times. After the absorbance value was known, the number of viability of living cells was counted. Furthermore, viability was expressed by comparing the absorbance value of the treatment group exposed to the test material with the control group (sample without test material) using the formula from in vitro technologies, namely:

$$\text{Total cell viability (\%)} = \frac{\text{Testing absorbance} - \text{medium absorbance}}{\text{Control cell absorbance} - \text{medium absorbance}} \times 100\%$$

Results

This study used 2 kinds of tests, the FTIR Spectrum test and the MTT assay test. The data collected in this study were the test results of the combination of chitosan-*ozonated* olive oil and chitosan-coenzyme Q10 characterized using infrared spectroscopy (FTIR) and the percentage results of living fibroblast cells from the combination of chitosan-*ozonated* olive oil and chitosan-coenzyme Q10.

A.1. Result of The FTIR Test on Chitosan-*Ozonated* Olive Oil and Chitosan-Coenzyme Q10 Combinations

	Wave Number	Intensity
1	3795.91	20.82
2	2916.37	8.73
3	1982.82	13.69
4	1728.22	8.94
5	1450.47	9.40
6	1242.16	9.31
7	1165.00	9.34
8	663.51	12.06
9	547.78	12.04
10	347.19	6.75

A.1.1. Table of FTIR Spectrum Wave Number of Chitosan-Ozonated Olive Oil.

No	Wave Number	Intensity
1	3988	16.9
2	3934	17.2
3	3240	6.06
4	2947	6.76
5	2885	6.98
6	2083	11.86
7	1643	6.97
8	1334	7.46
9	1234	7.59
10	1111	6.92

A.1.2. Table of FTIR Spectrum Wave Number of Chitosan-Coenzyme Q10.

FTIR Spectrum	Significance Level (p)		Description
	Chitosan+Q10	Chitosan + *Ozonated* olive oil	
Wavelength	*0.230*	*0.328*	*Normal*

A.1.3. Table Results of the *Shapiro-Wilk* Normality Test on the Wavelength of Chitosan-Coenzyme Q10 and Chitosan-*Ozonated* Olive Oil (unit cm^{-1})

Result of normality test with *Shapiro-Wilk test* in table A.1.3 showed a significance level of $p > 0.05$ in the chitosan-coenzyme Q10 group and the chitosan-*ozonated* olive oil group. This means that there is a normal data distribution, then the Independent t-test parametric test is carried out to determine whether there is a significant difference between the treatment groups.

FTIR Spectrum	*Independent t-test*		Description
	t-count	Significance level (p)	
Wavelength	1,750	0.097	Not significant

Table A.1.4. Results of Independent t-test on the Wavelength of Chitosan-Coenzyme Q10 and Chitosan-*Ozonated* Olive Oil (unit cm^{-1})

Results of Independent t-test on wavelengths in both groups showed significant value of $p>0.05$, which means that there is no statistically significant difference between chitosan-coenzyme Q10 group and chitosan-*ozonated* olive oil group. When viewed from the mean value, the chitosan-*ozonated* olive oil number is greater than chitosan-coenzyme Q10 number.

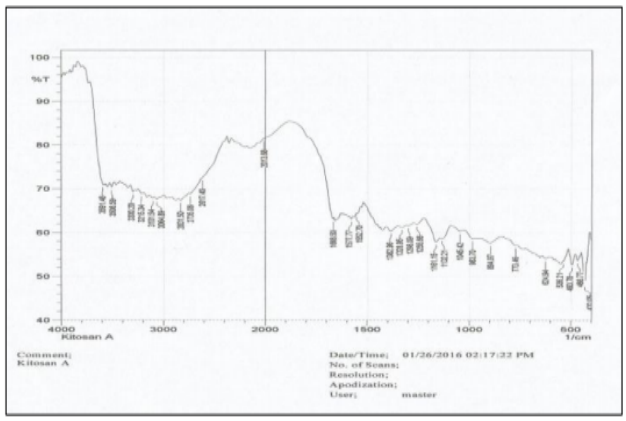

Figure 1. FTIR Graph of Standard Chitosan.

The infrared spectrum of standard chitosan shows twelve main peaks in the range of 897.41; 1026.63; 1077.93; 1154.64; 1259.54; 1422.73; 1587.94; 1660.55; 2361.41; 2922.85; 2922.85 and 3377.95 cm^{-1}. The stretching vibration at wave number 1660.55 cm^{-1} is the absorption band of the C=O bond group which indicates the presence of a secondary amide group, while the absorption band of 3377.95 cm^{-1} is the bending vibration of the OH group and the NH_2 group.

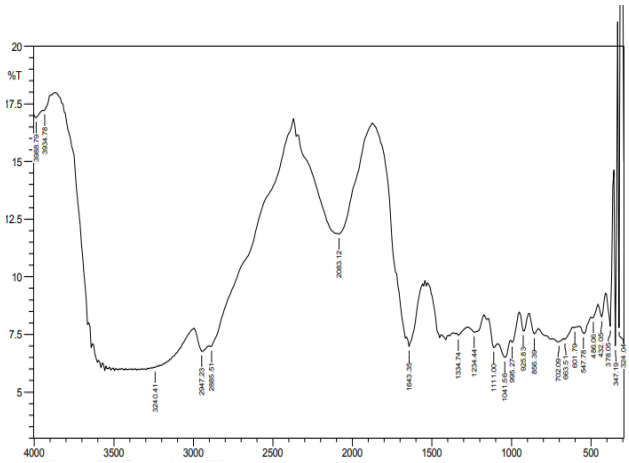

Figure 1. FTIR Spectrum Graph of Chitosan-Coenzyme Q10 Combination.

Based on observations, the functional groups identified in the sample (Chitosan-Coenzyme Q10) include functional groups of N-H, C-H, Amide I and Amide II which are characteristics of chitosan. The absorption map of the sample showed overlapping in several wave numbers. The IR spectrum in the sample showed FTIR spectra which looks wider in the wavenumber region of 2083cm^{-1}. Analysis of

FTIR result of the sample showed the identified group of Coenzyme Q10 Hydrogen isoprene at a wavelength of 3000 cm^{-1} to 1500 cm^{-1}.

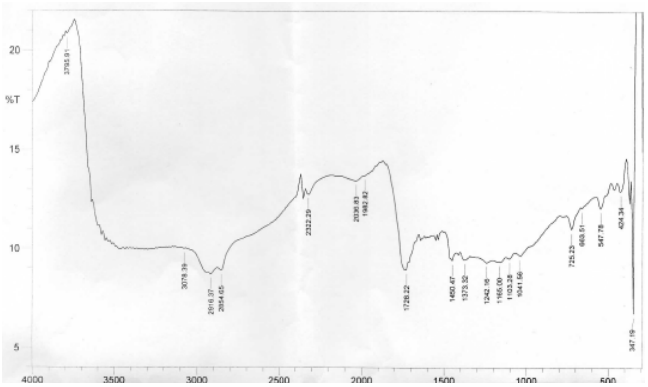

Figure 1. FTIR Spectrum Graph of Chitosan-*Ozonated* Olive Oil Combination.

The results of the FTIR analysis of *chitosan-ozonated* olive oil obtained spectrogram peaks showing functional groups consisting of OH, CH, NH, amide and carbonyl groups at wave numbers 3795.91 cm^{-1} , 2916.37 cm^{-1} , 1982, 82 cm^{-1} , 1728.22 cm^{-1} and 1165 cm^{-1} are presented in Table 2. At the peak wave of the 3795.91 cm^{-1}, the chitosan-*ozonated* olive oil reaction was strengthened, while the weakest was at a wave number of 347.19 cm^{-1}.

A.2. Viability of Fibroblast Cells of the Combination of Chitosan-Coenzyme Q10 and Chitosan-*Ozonated* Olive Oil Groups

Chitosan-Coenzyme Q10 (%)	Day-7	Day-14
1	54.1	63.7
2	60.1	62.9
3	53.7	63.8
4	60.0	63.7
5	61.3	65.6
6	57.3	65.4
7	59.1	67.0
8	60.9	63.1
9	62.2	61.3
10	59.6	62.4
11	61.6	61.9
12	61.5	64.9

A.2.1. Table of the Living Fibroblast Cells Percentage in the Combination of Chitosan-Coenzyme Q10.

Chitosan-*Ozonated* Olive Oil (%)	Day-7	Day-14
1	52.7	60.0
2	58.9	68.6
3	57.6	62.0
4	61.5	61.9
5	62.1	67.2
6	61.4	65.2
7	57.5	66.8
8	61.9	65.0
9	63.3	64.2
10	62.7	67.9
11	62.4	64.7
12	59.8	65.7

A.2.2. Table of The Living Fibroblast Cells Percentage in the Combination of Chitosan-*Ozonated* Olive Oil Group

Cell Viability	n	Chitosan + Q10	Chitosan + *Ozonated* olive oil
Living fibroblast cell count	24	62.18 ± 1.08	63.09 ± 0.95

Table 3. Mean and Standard Deviation of Fibroblasts Cell Viability in Chitosan-Coenzyme Q10 and Kitosan-*Ozonated* Olive Oil Groups (unit%)

Table 3 showed the mean number of living fibroblasts in the chitosan + *ozonated* olive oil group was higher with a mean and standard deviation of 63.09 ± 0.95% compared to the number of living fibroblasts in the chitosan + coenzyme Q10 group with a mean and standard deviation of 62.18 ± 1.08%.

Percentage of Fibroblast Cell Viability	*Mann Whitney*		Description
	t-count	Significance level (p)	
Day-7 to day-14	0.823	0.415	Not Significant

Table 4. Results of *Independent t-test of* Fibroblast Cell Viability of Chitosan-Coenzyme Q10 and Chitosan-*Ozonated* olive oil *Groups.*

The results of the *Independent t-test* on Fibroblast Cell Viability in both groups showed a significance value of p>0.05, which means that there is no statistically significant difference between the chitosan-coenzyme Q10 group and chitosan-*ozonated* olive oil group.

Discussion

1. FTIR of Chitosan-Coenzyme Q10 Combination

The coenzyme Q10 system with chitosan has a similar FTIR spectrum profile. This condition showed that there is no difference in the interaction of several components when forming the system. This condition indicates that there is a possibility of intermolecular hydrogen bonds of average strength with the hydroxyl groups of some components. In addition, the results of the interaction of the two materials showed the absence of a new peak as a characteristic that appeared in the FTIR spectrum of coenzyme Q10 and Chitosan. Based on the results of the FTIR spectrum, it can be seen that there were no residual compounds resulting from chemical interactions between Coenzyme Q10 and the constituent components of the Chitosan system. This indicates that there is a physical reaction in the formation of the system. The formation of the Coenzyme Q10 system with Chitosan matrix can be identified from the characteristic peaks in the spectral band area.

2. FTIR of Chitosan – *Ozonated* Olive Oil Combination

Based on the characteristic analysis of the infrared spectrum compared to the standard chitosan spectrum, it showed that there was no significant difference between the two spectra. FT-IR on chitosan-*ozonated* olive oil as shown in Figure 2 showed that there was no clear difference between the materials compared to the pure chitosan spectrum. Its peak at 3520 cm^{-1} corresponds to the O-H stretching wave that extends and shifts to 3795 cm^{-1}. Increased wave intensity of 1982 cm^{-1} which corresponds to the C-H wave. A new band appears at 1450 cm^{-1} which corresponds to the carboxyl group. The FTIR spectrum also did not show the formation of new polymers. This showed that *ozonated* olive oil did not damage the structure of chitosan and can be combined into a synergistic material.

3. Fibroblast Cells Viability of Combination of Chitosan-Coenzyme Q10 and Chitosan-*Ozonated* olive oil

Viability test of fibroblast cells in this study used MTT assay method. MTT assay was used to evaluate the cytotoxicity of the combination of chitosan on fibroblast cell viability. MTT is a cytotoxicity test that uses a simple method of calculating the number of cells for both cytotoxicity and cell proliferation purposes, with standard absorption readings on microplates without the need for cell transfer. The MTT method is based on the conversion of *tetrazolium* salt (MTT) to *formazan* in the mitochondria of fibroblast cells. Yellow MTT is absorbed into fibroblast cells and broken down through a reduction reaction by the mitochondrial succinate dehydrogenase enzyme. This enzyme is found in the mitochondrial matrix and small particles in the cristae. It is this enzyme that converts MTT into blue *formazan* crystals which indicate that the cell is alive. The parameter that is often used to determine cytotoxicity in vitro is Inhibition Concentration 50% (IC 50). In general, IC 50 is the concentration of a substance capable of inhibiting cell proliferation up to 50% of the population [24].

Chitosan is known to have non-toxic, biocompatible, biodegradable and *mucoadhesive* properties so it is often used in the pharmaceutical field as a carrier system for drugs, hormones, proteins, enzymes and genes. Chitosan is positively charged in an acidic medium due to the presence of protonated amino groups that increase the solubility level. Biodegradation properties are properties that affect short and long-term toxicity, where materials that are difficult to degrade will accumulate in organs and cells [25]. Chitosan has *biodegradable* and *biocompatible* properties and also forms a scaffold so that it can repair bone damage. The results of the research by Soeroso *et al*. showed that chitosan increased osteogenesis and can be used as a scaffold to steer bone development [26]. However, a single dose of chitosan has disadvantages when used as a support in tissue regeneration due to its low mechanical resistance [27]. According to Vazquez *et al.* chitosan requires a *cross-linking agent* that can optimize the elasticity and resistance of chitosan in order to obtain good biocompatibility [28]. The combination of chitosan with other biomaterials can have a synergistic effect on cell adhesion, cell differentiation and extracellular matrix formation thereby enhancing tissue regeneration [9].

This study showed an increase in the number of fibroblast cells in both groups. Howling *et al.* reported that there was a relationship

between the degree of *deacetylation* and the fibroblasts proliferation, chitosan with a high degree of *deacetylation* (approximately 89%) can stimulate fibroblast activity and decrease its activity when the degree of *deacetylation* is low. This study used chitosan with a degree of *deacetylation* of 85% which was sufficient to increase the proliferation of fibroblasts which have an important role in producing collagen thereby increasing the percentage of collagen density [29].

The combination of chitosan-*ozonated* olive oil group showed a greater increase in the number of fibroblast living cells than the combination of chitosan-coenzyme Q10 group, even though it was not significant. *Ozonated* olive oil increased fibroblast migration and the epithelial–mesenchymal transition (EMT) process via PI3K/Akt/mTOR signaling pathway *in vitro* [30]. *Ozonated* olive oil increases the expression of *growth factors,* namely *Vascular Endothelial Growth Factor* (VEGF), *Platelet-Derived Growth Factor* (PDGF), and *Transforming Growth Factor* (TGF-β) which stimulate the growth of the number of fibroblasts. PDGF and TGF- stimulate cell proliferation, TGF-β also induces osteogenic differentiation of mesenchymal stem cells. Osteoblast differentiation was controlled by IGF and bone morphogenetic proteins. VEGF is also very important in stimulating angiogenesis which is necessary for bone formation and remodeling [31]. This shows that the combination of chitosan-ozonated olive oil can be used as a bone graft material to increase tissue regeneration [15]. The increase in the number of fibroblast living cells in the combination of chitosan-coenzyme Q10 group was due to the amino content in chitosan being able to prolong drug release so that the anti-inflammatory effect of coenzyme Q10 lasted longer and helped tissue regeneration including periodontal tissue [32]. From the table of results, it can be seen that the living human gingival fibroblasts cells in the chitosan-coenzyme Q10 group and the chitosan-*ozonated* olive oil group showed a value of more than 50%. Based on the IC50 parameter, it can be interpreted that both groups are not cytotoxic to human gingival fibroblasts.

Based on the discussion above, it can be seen that the combination of chitosan-coenzyme Q10 and chitosan-*ozonated* olive oil are a synergistic combination and does not show cytotoxicity against fibroblast cells. This is the basis for further research, so that later the combination of chitosan-coenzyme Q10 and chitosan-*ozonated* olive oil can be applied clinically as a support for periodontal therapy.

Conclusions

The combination of chitosan-*ozonated* olive oil and chitosan gel-coenzyme Q10 is a synergistic combination and is non-toxic. The combination of these two ingredients can increase the number of fibroblast living cells so that it can help increase tissue regeneration.

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Authors Contributions

All authors have made equal contributions.

Declaration of Interest

The authors report no conflict of interest.

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