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Thamar A. Bossiela
Dentist at Ministry of Health B.D.S. (2000) Cairo University, Cairo, Egypt., thamarahmed8@gmail.com

Naglaa Shawki Elkilani
Professor and Head of Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology Department Faculty of Dental Medicine for Girls' Al-Azhar University, Cairo, Egypt.

Eman M. Kandeel
Professor and Head of Organic and Applied Chemistry Department, Faculty of Science for Girls' Al-Azhar University, Cairo, Egypt.

Amal A. Ibrahim
Lecturer of Oral Medicine, Periodontology, Oral Diagnosis and Radiology, Faculty of Dental Medicine for Girls Al-Azhar University.

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ORIGINAL STUDY

Evaluation of the Effect of Different Concentrations of Nanocurcumin on Periodontal Fibroblastic Cell Culture Isolated from Periodontitis Patient

Thamar A. Bossiela [a,*], Naglaa S. El-Kilani [b], Eman M. Kandeel [c], Amal A. Ibrahim [d]

[a] Ministry of Health B.D.S. (2000), Cairo University, Cairo, Egypt
[b] Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology Department, Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt
[c] Organic and Applied Chemistry Department, Faculty of Science for Girls, Al-Azhar University, Cairo, Egypt
[d] Oral Medicine, Periodontology, Oral Diagnosis and Radiology, Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt

Abstract

Purpose: The goal of this study was to see how varying concentrations of nanocurcumin (NC) affected the culture of periodontal ligament fibroblastic cells (PDLFCs). **Materials and methods:** Nanocurcumin was prepared at concentrations (10 μmol/mL, 3 μmol/mL, 1.5 μmol/mL, 0.7 μmol/mL, and 0.3 μmol/mL). Periodontal ligament fibroblastic cells were extracted, characterized using Flowcytometry and cultured in Dulbecco's Modified Eagle Medium (DMEM). The healthy PDLFCs served as the positive control group; however, the PDLFCs of periodontitis patients were divided into two subgroups. 1st subgroup: PDLFCs treated with NC different concentrations, counted by hemocytometer and Cell proliferation assay was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 24, 48, and 72 h for calculation of NC half maximal effective concentration (EC50) at each interval then detection of cell apoptosis when treated with the resultant NC EC50. 2nd subgroup: untreated cells were used to compare with each result. **Results:** There was a statistically significant difference in the mean Log10 cell count, cell viability %, and optical density between the 10 μmol/mL nanocurcumin and all concentrations of nanocurcumin, and the overall *P* value for intergroup comparison was highly statistically significant. **Conclusion:** Nanocurcumin had no in vitro cytotoxicity to PDLFCs at any concentrations used by MTT assay. Nanocurcumin may be a promising medicine for periodontitis treatment and periodontal regeneration.

Keywords: Apoptosis, Nanocurcumin, Periodontal ligament fibroblastic cells

1. Introduction

Periodontal disease is classified as a chronic immunoinflammatory disease as a result of a complex interplay between periodontal bacteria, their byproducts, and the host response, which results in periodontal ligament and alveolar bone destruction, as well as clinical attachment loss [1].

Periodontal disease initiation and progression are linked to particular putative periodontal pathogens, usually gram-negative, anaerobic bacteria found in biofilm. Host reactions are primarily responsible for the microbial challenge of antigens, lipopolysaccharide, and destruction; hence therapeutic techniques to modify the host response should be considered alongside antibacterial therapies in the management of periodontitis [2].

Periodontal ligament fibroblastic cells, the main cells around teeth, perform a wide range of functions, including tissue homeostasis, the formation of a collagenous extracellular matrix (ECM) by secreting structural proteins, and regeneration because they can produce multiple cytokines in response to a bacterial insult, thus playing an important role in the local pathogenesis of periodontal disease, as well as regulatory functions in innate immune system defense reaction [3].

Because of its safety, phytotherapy has recently gained popularity in medicine and dentistry around

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* Corresponding author at: Al-Azhar University, 11754, Egypt.
E-mail address: thamarahmed8@gmail.com (T.A. Bossiela).

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the world. Turmeric, a popular home remedy, is currently receiving greater attention in medical studies. Curcumin is a lipophilic polyphenol derived from the root of *Curcuma longa*, and it is the active element in turmeric. Curcumin enhances wound healing by acting as an anti-inflammatory, antibacterial, antioxidant, and immune modulator [4].

Curcumin controls NF following TLR4 activation by LPS in periodontal disease. Curcumin inhibits a number of cytokines and inhibits the activity of many enzymes, including inducible nitric oxide synthase, cyclooxygenase 2 (COX2), and lipoxygenase. Curcumin suppresses the growth of a variety of periodontal pathogens in a dose-dependent way [5].

However, regardless of such biological activities of CUR, it is hydrophobic, has a low rate of absorption in the gastrointestinal tract, and has poor bioavailability and stability. As a result of the known properties of nanoparticles; Nano delivery systems can improve the hydrophilicity, chemical stability, and bioavailability of curcumin in final products when compared to its free form. Several studies have been published with the biological molecule curcumin in the form of nanocurcumin to improve its bioavailability and stability [6].

Only a few studies examining the effect of varying concentrations of nanocurcumin on fibroblast cytotoxicity and wound healing are available in the literature, to the best of the authors' knowledge. The purpose of this study was to determine the impact of different concentrations of nanocurcumin on periodontal ligament fibroblastic cell [7].

2. Materials and methods

Curcumin was supplied as 95% extra pure powder (Alfa Chemical, India) and then Nanocurcumin was prepared in the Department of Applied and Organic Chemistry, Faculty of Science for Girls, Al-Azhar University [8,9].

2.1. Fibroblast donors

Periodontal ligament fibroblasts were obtained from the extraction of premolar teeth of periodontitis patients and healthy children presented for orthodontic treatment. All the subjects were informed about the study and signed consents were taken from each patient who participated. This study was reviewed by the Research Ethics Committee (REC) of the Faculty of Dental Medicine for Girls, Al-Azhar University, code (REC-ME-22-03).

2.2. Cell culture

2.2.1. Isolation of periodontal ligament fibroblasts cells (PDLFC)

The received sample was suspended in PBS (pH:7.4), a cocktail of antibiotics and antimycotic, in addition to 1% DEMSO as preservative media. The periodontal ligaments tissues were scraped from the middle third of the root then PDLFCs were isolated from the periodontal tissue using an enzyme digestion method by mincing the tissues into small pieces in a Petri dish containing PBS (pH 7.4) and an antibiotics cocktail. The tissues were digested into collagenase type I and dispase solution then tissue clumps were passed through a cell strainer.

2.2.2. Characterization of fibroblasts by assessment of FGF gene expression using RT-PCR

Total RNA was obtained using the RNeasy extraction kit (Qiagen, Hilden, Germany), which was carried out according to the manufacturer's instructions. Gene-specific Quanti Tect primer assay Gene-specific for FGF and ACTB primers were purchased from Qiagen, Germany. Also, the spindle shape of fibroblasts was assessed by electron microscope (Fig. 1).

Fibroblastic cells were cultured and analyzed in two subgroups as follows:

Periodontal ligament fibroblastic cells were isolated from two 'healthy' and 'diseased' individuals

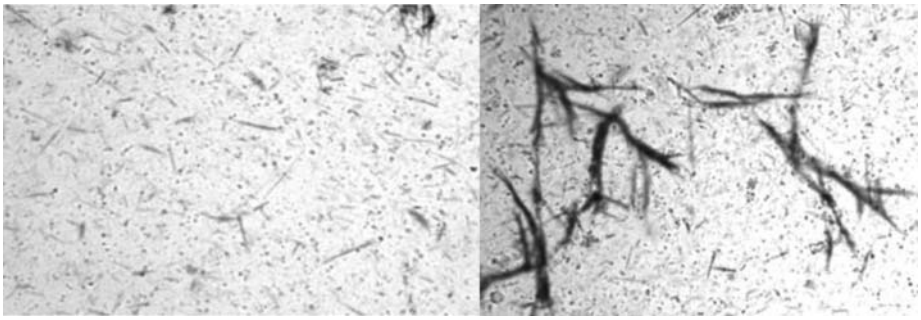

Fig. 1. Images of cultured gingival fibroblast cells by Electron microscope.

and cultured in two 75 cm^2 flasks in Gibco Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Germany) containing 10% foetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Germany) and 1% penicillin G sodium (10 000 UI), streptomycin (10 mg), and ampho (Gibco, Thermo Fisher Scientific, Germany). Flasks were incubated at 37 °C in a 5% CO_2 environment. Cells from the third passage were used in this experiment. After 7 days of incubation, the PDLFCs that were isolated from healthy individuals were counted by Trypan blue to consider as a positive control group. The harvested cells were cryopreserved in 1% DEMSO and FBS, and for subsequent analysis, the samples were kept at −80 °C. On the other hand, the PDLFCs isolated from a patient with periodontal disease were analyzed in two subgroups as follows:

First subgroup: PDLFCs fibroblastic cells are cultured with different concentrations of nanocurcumin at three time intervals '24, 48, and 72 h', in order to identify the EC50 of nanocurcumin with the highest proliferation potential of dental fibroblasts.

Second subgroup: untreated PDLFCs, which serve as the negative control group.

2.2.3. Calculation of EC50 of nanocurcumin in the proliferation of fibroblasts cells at three time intervals

Fibroblast cells treated for 24, 48, and 72 h: cell count by Trypan blue, cell proliferation assay (MTT), and calculation of EC50 of nanocurcumin of fibroblasts cells treated with different concentrations of nanocurcumin at 24, 48, and 72 h.

Detection of apoptosis of fibroblast cells treated with the EC50 of nanocurcumin at three time (24, 48, and 72) intervals using Annexin/Propidium iodide staining, and the percentage of apoptotic cells was compared to cells that had not been treated.

FITCAnnexinV and Propidium iodide (PI) staining were conducted after trypsinization. To distinguish apoptotic cells, the Dead Cell Apoptosis Kit with Annexin V FITC and PI for Flow Cytometry (Invitrogen, cat no: V13242) was employed (early). The red fluorescence of propidium iodide marks necrotic cells. Apoptotic cells glow green, dead cells fluoresce red and green, and viable cells fluoresce little or not at all after treatment with both probes.

2.3. Statistical analysis

The One-Way Analysis Of Variance (ANOVA) test was used to analyze the results, followed by a post hoc test, and Levene's test was used to test the normality of the data, with a P value of 0.05 regarded statistically significant (95% significance level). To normalize the variance distribution, every cell count data was collated and logarithmically converted in SPSS. Data were analyzed using the statistical software SPSS (version 25, IBM Co. USA).

3. Results

3.1. Calculation of EC50 of nanocurcumin in proliferation of fibroblasts cells at three time intervals

For the 2nd subgroup, the cells were expressing FGF (30.71) and for the actin gene was 39.76 (Housekeeper gene). The nanocurcumin EC50 of Fibroblasts proliferation at 24, 48, and 72 h were (3.9 μmol/mL, 3.5 μmol/mL and 3 μmol/mL), respectively.

3.2. Effect of nanocurcumin concentration on the fibroblast cells at different time intervals (intragroup comparison)

Table 1, Fig. 2.

3.2.1. Viability

There was a directly proportional relationship between the percentage of cell viability and nanocurcumin concentration at the three time intervals.

3.2.2. Cell proliferation

There was a statistically significant difference in the mean OD between the 0 μmol/mL nanocurcumin and all concentration of nanocurcumin at the three time intervals and the overall P value for intergroup comparison was highly statistically significant at $P \leq P$ value ≤ 0.001 (Tukey post hoc test).

Table 1. Nanocurcumin after the three time intervals.

Nanocurcumin concentration	24 h	48 h	72 h
0 μmol/mL	43.00%^{A}	54.89%^{A}	65.66%^{A}
0.3 μmol/mL	50.87%^{B}	61.68%^{B}	75.17%^{B}
0.7 μmol/mL	66.02%^{C}	75.33%^{C}	82.20%^{C}
1.5 μmol/mL	76.10%^{D}	82.60%^{D}	86.60%^{D}
3 μmol/mL	91.29%^{E}	93.25%^{E}	96.83%^{E}
10 μmol/mL	99.92%^{F}	99.35%^{F}	100.00%^{F}
P value intergroup comparison	0.000*	0.000*	0.000*

P value $\leq$ 0.05 was considered statistically significant, and P value $\leq$ 0.001 was considered highly statistically significant.
* = Highly significant ($P \leq 0.001$).
Capital letters for intergroup comparison and the means with different superscripts are statistically significantly different at $P \leq 0.05$ (Tukey post hoc test).

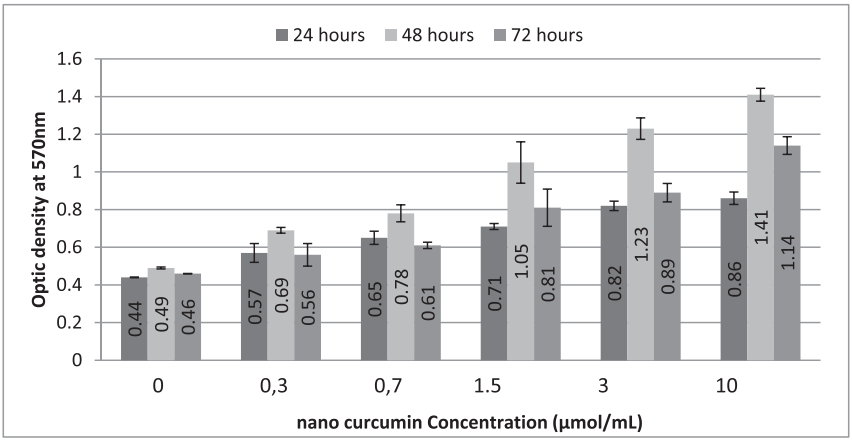

Fig. 2. Mean and SD of cell proliferation at different concentrations of nanocurcumin after the three time intervals.

3.3. Detection of apoptosis of fibroblast cells treated with the EC50 of nanocurcumin at the three time intervals

Fibroblast cells were treated with the calculated EC50 at three time intervals (24, 48, and 72 h) and untreated apoptotic cells percentage (Baseline) was 38.8%, and apoptotic cells percentage at 4 µmol/mL nanocurcumin after 24 h incubation was 31.2%, these percentage decreased to 20.2% at 3.5 µmol/mL nanocurcumin for 48 h incubation, and the lowest percentage (9.1%) achieved at nanocurcumin concentration 3 µmol/mL for 72 h incubation.

4. Discussion

Curcumin's antibacterial, anti-inflammatory, immunomodulatory, and wound-healing activities have recently been confirmed in numerous researches. In the literature, there are few research testing the effect of nanocurcumin at various concentrations on fibroblast cytotoxicity and wound-healing properties [4].

Curcumin is bacteriostatic and bactericidal against a wide spectrum of harmful bacteria. Its antibacterial properties are due to its chemical nature as a lipophilic polyphenol that can penetrate the bacterial cell wall's lipid bilayer, increasing cell permeability. It also has a dose-dependent inhibitory effect on *Porphyromonas gingivalis* proteases (RGP and KGP) [5].

Even though curcumin is a naturally occurring polyphenol, it is found to be highly cytotoxic and causes cell apoptosis at high concentrations, a property that leads to its use as an anticancer drug. Therefore, our study attempted to evaluate the effect of different concentrations of nanocurcumin on PDFLCs of periodontitis patients and EC50 of nanocurcumin at three time intervals, as well as the EC50 of the used nanocurcumin different concentrations [10].

Periodontal tissue's principal architect building cells are fibroblasts. By modifying tissues and repopulating wounds, they help to maintain periodontal tissue homeostasis [1].

When antimicrobial drugs are used in conjunction with mechanical periodontal therapy, their impact on PDLFCs growth, survival, migration, and proliferation must be thoroughly assessed. In order to evaluate the biocompatibility of biomaterials, it is necessary to assess cytotoxicity using a variety of cytotoxicity testing methodologies. Nanocurcumin's cytotoxicity against human PDL fibroblasts was examined in vitro in this work. At any of the concentrations employed in the MTT experiment, no cytotoxicity was identified (fibroblast cells remained alive) [11].

There are a few drawbacks to this study that should be mentioned: The MTT assay is a 'relative test', in which the OD values of a treated cell population are compared to the OD values of an untreated cell population, which is arbitrarily scaled at 100%. The cell viability results in this investigation were consistently above 100% for the majority of the nanocurcumin test doses. This could be due to the effect of nanocurcumin alone, or it could be due to its color, which may have contributed to the OD values obtained. That is why in the current study Trypan blue was used to count cells and determine vitality [11].

In experimentally produced inflammation, there was a decrease in fibroblastoid cells after treatment with nanocurcumin [12]. Because enhanced fibroblast proliferation is linked to inflammation, it was suggested that the decrease in fibroblastic cells may be due to nanocurcumin's anti-inflammatory impact [13]. However, they hypothesized that the general decrease in p38 MAPK and NF-κB activity found in

gingival tissues could be linked to a change in the phenotype of gingival fibroblasts, as these cells express inflammatory cytokines and metalloproteinases in inflamed microenvironment [12].

Curcumin has been demonstrated to have potent wound-healing effects. It aids in the production of granulation tissue, collagen deposition, and wound closure. When compared to untreated wounds, curcumin-treated wounds have more fibroblasts, as well as more infiltrating macrophages and neutrophils [11].

4.1. Conclusions

Curcumin has a broad biological range and could be used to treat chronic periodontitis as an immunoinflammatory disease as an alternative anti-inflammatory, antibacterial, and immunomodulatory drug. Within the constraints of the current investigation, it can be stated that 10 μmol of nanocurcumin causes the most fibroblast vitality and proliferation, whereas 3 μmol causes the least fibroblast cytotoxicity. Nanocurcumin has the potential to be a more proliferative and less cytotoxic agent, a periodontal regeneration enhancer as well.

4.2. Recommendations

Further studies of using nanocurcumin as a therapy for periodontal disease, especially in vivo studies.

Conflicts of interest

The authors declare that they have no conflict of interest.

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