Journal of Dental Sciences (2018) **13**, 103–108

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

Original Article

Effects of coenzyme Q_{10} encapsulated in nanoliposomes on wound healing processes after tooth extraction

Kosta Todorovic [a], Goran Jovanovic [a], Ana Todorovic [b], Aleksandar Mitic [c], Nenad Stojiljkovic [d], Sonja Ilic [d*], Nikola Stojanovic [e], Slavica Stojnev [f]

[a] *Department of Oral Surgery, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*
[b] *Department of Stomatology, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*
[c] *Department of Restorative Dentistry and Endodontics, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*
[d] *Department of Physiology, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*
[e] *Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*
[f] *Department of Pathology, Faculty of Medicine, University of Niš, Zorana Đinđića 81, 18000 Niš, Serbia*

Received 24 August 2017; Final revision received 29 September 2017
Available online 6 December 2017

KEYWORDS
coenzyme Q_{10};
inflammation;
nanoliposomes;
tooth extraction;
wound healing

Abstract *Background/purpose:* Tooth extraction is often followed by a number of different complications that demand additional treatment. In order to accelerate healing processes and decrease the complication occurrence various agents, growth factors, natural and synthetic antioxidants (e.g coenzyme Q_{10}-CoQ_{10}), are applied. Due to the partially known health-promoting effects of CoQ_{10} we decided to assess potential of it's encapsulated in nanoliposomes form on wound healing process following tooth extraction.
Materials and methods: Effects of free and encapsulated form of CoQ_{10} on wound healing processes after tooth extraction in rats, 3 and 7 days following surgical procedure, was studied by means of tissue biochemical (myeloperoxidase activity and nitric oxide (NO) concentrations) and pathohistological analysis.
Results: The obtained results indicate that the encapsulated form of CoQ_{10} compared to control and CoQ_{10} treated animals statistically significantly decreases inflammatory process estimated through myeloperoxidase activity and NO concentrations, as well as based on histopathological analysis 3 and 7 days following surgery.

* Corresponding author. Fax: +381 18 423 8770.
E-mail address: sonjaili@yahoo.com (S. Ilic).

https://doi.org/10.1016/j.jds.2017.10.004
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Conclusion: The results of this study unequivocally prove that the encapsulation of CoQ_{10} in nanoliposomes enhances CoQ_{10} activity by accelerating wound healing process after tooth extraction.
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Introduction

Tooth extraction is a common dental procedure that is followed by numerous complications. Depending on a treatment protocol complications can be classified as: (a) minor (severe pain, bleeding, swelling, trismus) – treated conservatively and (b) major (hemorrhage, abscess formation, osteomyelitis, fistula) which requires hospital admission and possible surgical intervention.[1] Tooth socket healing process, as any other wound healing, involves inflammation, cell proliferation, matrix deposition and tissue remodelling.[2] Neutrophils, the first recruited cells in healing process, are responsible for reactive oxygen species (ROS) production thus ROS overproduction during the wound healing process might be impaired. Additionally, macrophage (activated ones) infiltration into the periodontal tissues increases nitric oxide (NO) concentration.[3,4] Nitric oxide is a diatomic free radical produced by activated phagocytic leukocytes (neutrophils, macrophages) that has both harmful and beneficial properties on the tissues pathophysiological processes.[5]

Wound healing acceleration and possible complications reduction is of great importance for everyday dental practice.[6] Numerous agents such as PDGF, IGF, EGF growth factors,[3,7,8] copaiba oil,[9] caffeic acid phenethyl ester,[10] ellagic acid[11] and coenzyme Q_{10}[6] are known to reduce inflammation and promote alveolar socket healing after tooth extraction. However, the main disadvantage of these agents include rapid degradation (short lifetime), uncontrolled release and low diffusion potential. One of such agents is coenzyme Q_{10} (CoQ_{10}), an endogenous essential molecule for every cell in the body,[12] which is involved in energy production, and plays key role in mitochondria function.[13] Coenzyme Q_{10} is a vitamin-like, oil-soluble molecule[6] with poor bioavailability and delivery properties due to its insignificant water solubility.[14] Two mayor activities of CoQ_{10} have been reported: (a) a mitochondrial electron-transport activity involved in the efficient production of high-energy phosphates necessary for muscle contraction and other cellular functions, and (b) an antioxidant activity.[15] Also, it was proven that in isolated mitochondria CoQ_{10} can protect mitochondrial membrane proteins and DNA from ROS damage.[16]

In order to achieve target-specific delivery systems, minimize off-target effects of therapeutic agents and overcome their shortcomings in topical application effectiveness, researchers have focused to develop nanotechnology-based delivery systems.[17] Liposomes (spherical vesicles with lipid bilayer) are recognized as an advanced drug carrier for the administration of nutrients, pharmaceuticals, and gene delivery.[18] Various molecules used as potential therapeutics could be encapsulated into liposomal systems in order to improve their wound healing activities.[19]

Although there are several studies pointing to the beneficial effects of CoQ_{10} on cutaneous wound healing,[15] corneal ulcers treatment[20] and many other medical fields, there is a limited amount of information about its utilization in dentistry. Thus we aimed to evaluate the effects of topical application CoQ_{10} encapsulated in nanoliposomes on wound healing after tooth extraction that has not yet been investigated.

Material and methods

Animal housing

Male and female Wistar rats ($n = 48$), weighting from 200 to 250 g, were kept in plastic cages, received tap water and food *ad libitum*, under standard laboratory (temperature – 22 ± 2 °C, relative humidity – 55 ± 5) conditions with equal duration of light/dark cycle. All experiments were conducted at the Institute of Biomedical Research, Medical Faculty, Niš, Serbia and are in accordance with all ethical regulations of European Union (EU Directive of 2010; 2010/63/EU) and Republic of Serbia (323-07-00073/2017-05/2).

Nanoliposomes encapsulation with coenzyme Q_{10} and encapsulation efficacy determination

Phospholipid nanoparticles solution (10%), in a form of nanospheres, was purchased from Nattermann Phospholipids (Germany). The encapsulation by CoQ_{10} (Sigma-Aldrich St. Louis, USA) at the concentration of 6 mg/ml, isolated after centrifugation at 6500 g for 30 min at 4 °C, was performed based on the method previously described.[17] The efficacy of encapsulation was determined in a mixture of CoQ_{10}-loaded liposomes and ethanol (3 ml) that was further vortexed for 3 min and centrifugated (2000 rpm, 5 min). The upper layer was separated and the absorbance was measured at 275 nm (V-1800 Shimadzu spectrophotometer). The encapsulation efficacy (%) was calculated as (amount of incorporated CoQ_{10})/(initial amount of added CoQ_{10}) × 100.

Experimental procedure

All animals were randomly divided into four groups each containing 12 rats. The surgical procedures were performed under intramuscular general anesthesia induced by 10% ketamine (Richter Pharma AG, Wels, Austria). Maxillary

incisors were extracted in all rats with a dental explorer and extraction forceps. After hemostasis, extraction wound was treated with topical application using a cotton ball, according to the following schedule:

Control (C) group—without treatment;
Free nanoliposomes (NL) group treated with 10% solution of empty nanoliposomes;
Coenzyme Q_{10} (Q) group treated with coenzyme Q_{10} dissolved in soybean oil (6 mg/ml);
Encapsulated nanoliposomes (NLQ) group treated with coenzyme Q_{10} encapsulated in nanoliposomes (6 mg/ml).

On the days 3 and 7 after extraction 6 rats from each group were sacrificed under general anaesthesia (ketamine) and tissue samples were collected for biochemical and pathohistological analysis.

Tissue homogenization

Tissue samples were collected after animal sacrification 3 and 7 days following tooth extraction. Wound tissue homogenates (10%) were prepared in ice cold distilled water and were centrifugeted at 12,000 rpm for 15 min (at 4 °C) in order to obtain clear supernatant that was further used for analysis. The amount of proteins was determined using a standard Lowry's method.[21]

Myeloperoxidase activity determination

Tissue myeloperoxidase (MPO) activity was determined based on the method previously described.[22] Briefly, MPO activity was measured through the amount of oxidized o-phenylenediamine in the presence of tissue homogenate supernatant and H_2O_2. The reaction was stopped by the addition of sulfuric acid and the absorbance was measured at 540 nm using a Multiscan Ascent (Labsystems, Finland). The MPO activity was expressed as optical density (OD)/mg of proteins.

Nitrate concentration determination

The concentration of nitrates present in tissue homogenates was measured using a Griess reagent.[23] The mixture consisted of tissue homogenate and Griess reagent was incubated at room temperature for 10 min and the absorbance of each sample was measured at 540 nm using a microplate reader. The nitrate concentrations were calculated using a standard curve of sodium nitrate.

Pathohistological and morphometric analysis

After sacrificing, the frontal maxillary segment of the head of each experimental animal was dissected and the samples were immersed in a 10% buffered formalin solution. The decalcification was carried out in 0.1 M EDTA solution for 21 days. Appropriate tissue samples were processed using routine histological methods and moulded into paraffin, from which 5 μm thick histological cross-sections were obtained. The tissue sections were deparaffinized and stained using standard protocol with haematoxylin and eosin (HE).

The pathohistological analysis was performed on the light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) and digital photographs obtained using the imaging system (Olympus cellSens platform standard, Olympus Corporation, Tokyo, Japan). Morphometric analysis was done with computerized image analysis system ImageJ. The number of polymorphonuclear leukocytes was measured under the objective lens magnification 40× at three sites of each section. Each area was 100 μm square.

Statistical analysis

Results expressed as the mean ± SD were compared using One-Way Analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons (Graphpad Prism version 5.03, San Diego, CA, USA). Probability values (p) less than 0.05 were considered statistically significant.

Results

Encapsulation efficacy

The efficacy of encapsulation of CoQ_{10} in nanoliposomes was determined to be 81%.

Tissue biochemical parameters

Tissue NO concentration and MPO activity were found to be highest 3 days after tooth extraction in both C and NL groups of animals and decline during the next week (Fig. 1). The application of CoQ_{10}, both free and encapsulated, statistically significantly reduced MPO activity and NO concentration at both investigated time points (Fig. 1), where the encapsulated Q_{10} was found to be more potent than the free form.

Pathohistological and morphometric analysis

At day 3 in alveolar sockets the presence of inflammatory infiltrate and granulation tissue in the gingival and periodontal connective tissue could be seen. The infiltration of inflammatory cells was generally most prominent in the C (Fig. 2A) and NL group (Fig. 2B). The inflammatory infiltrate was comprised of macrophages and numerous polymorphonuclear leukocytes, with the presence of extravasated erythrocytes and a small number of lymphocytes (Fig. 2A—D). The number of polymorphonuclear leukocytes was statistically significantly decreased in Q and NLQ groups compared to C and NL groups of rats ($p < 0.001$) (Table 1). The inflammatory infiltration was somewhat less pronounced in groups administered with CoQ_{10} (Fig. 2C, Table 1), while significantly less pronounced inflammatory infiltrate was observed in group in which CoQ_{10} encapsulated in liposomes was administered (Fig. 2D, Table 1). The intensive process of angiogenesis (neovascularization), with budding of the existing blood vessels and proliferation of the endothelial cells from the margins of the damaged

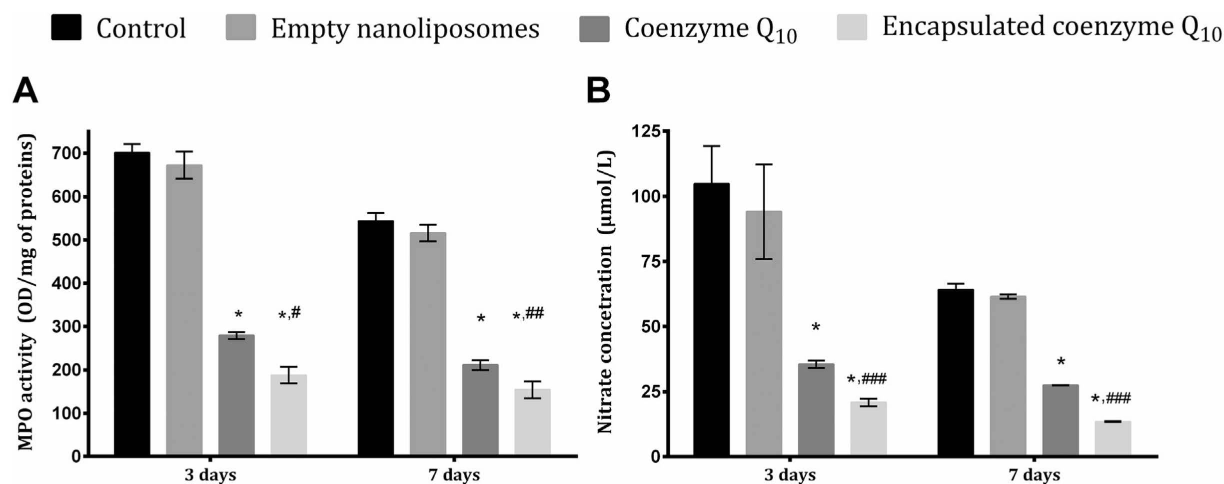

Figure 1 NO concentration (A) and MPO activity (B) in healing tissue from the extraction wounds in rats from the C, Q, NL and coenzyme NLQ groups. Tissue specimens were taken after 3 and 7 days. The data are presented as mean ± SD ($n = 6$), statistical significance was calculated by One Way ANOVA followed by Tukey test. $^{*}p < 0.001$ vs. C; $^{\#}p < 0.001$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.05$ vs. Q.

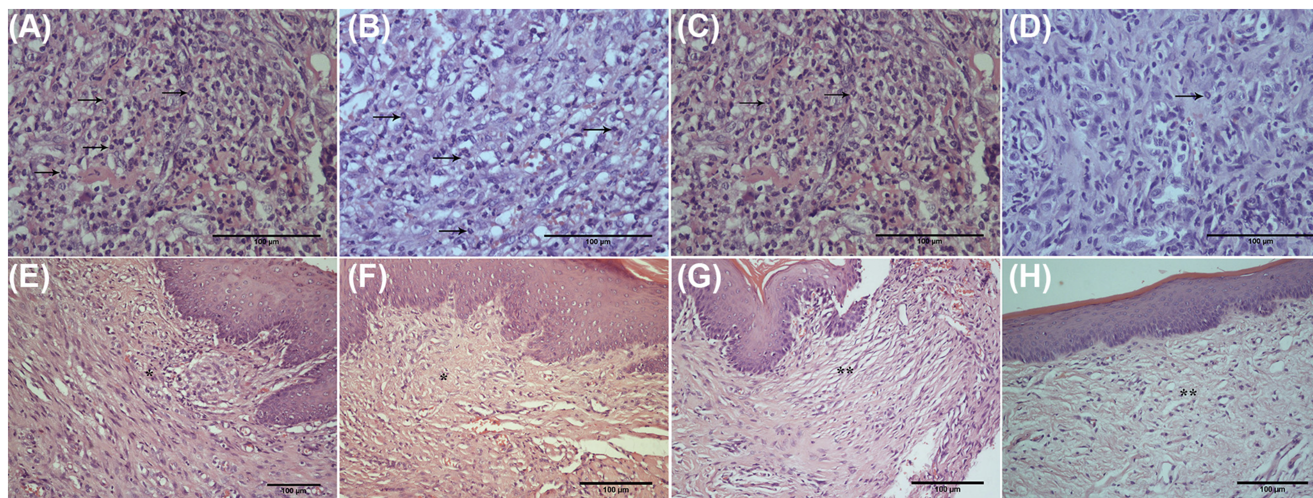

Figure 2 Soft tissue healing after tooth extraction. The first row shows histological samples of gingival structures at the level of the place of extraction obtained on the 3rd day in (A) C, (B) NL, (C) Q and (D) NLQ group (arrow shows polymorphonuclear leukocytes) (Haematoxylin-eosin staining, original magnification ×400). The second row shows samples obtained on the 7th day in (E) C, (F) NL, (G) Q and (H) NLQ group (*fibrovascular granulation tissue; ** mature fibrous granulation tissue) (Haematoxylin-eosin staining, original magnification ×200).

Table 1 Number of polymorphonuclear leukocytes found in the experimental groups of animals.

Days/Groups	C	NL	Q	NLQ
3rd day	75.0 ± 5.0	73.3 ± 2.9	38.3 ± 5.8[a,b]	28.3 ± 3.7[a,b]
7th day	30.0 ± 4.9	28.3 ± 6.7	6.7 ± 2.2[a,b]	3.3 ± 1.4[a,b]

Data are presented as mean ± SD.
[a] $p < 0.001$ vs. C group.
[b] $p < 0.001$ vs. NL group.

blood vessels, was evident 3 days after the surgery in all experimental groups (Fig. 2A–D).

Seven days after tooth extraction the micromorphological analysis revealed a histological progress of the post-extraction wound repair process. The squamous epithelium in maturation covered the entire extraction wound in all examined animals, i.e. the epithelial closure of the defect was complete and the inflammatory infiltration was significantly reduced (Fig. 2E–H). A reduced number of polymorphonuclear leukocytes was most noticeable, present either individually or in clusters of several cells

along the margin of the alveolar sac (Table 1). Neutrophils were considerably more numerous in samples from C and NL group (Fig. 2E and F) than in the groups treated with both forms of CoQ_{10} (Fig. 2G and H). Almost complete disappearance of polymorphonuclears from the inflammatory infiltrate was registered in the NLQ group (Fig. 2H, Table 1). The presence of the predominant fibrovascular granulation tissue was reported in C group (Fig. 2E). Contrary to this, the presence of the mixture of the fibrovascular and mature, fibrous granulation tissue with clearly defined blood vessels, discrete inflammatory infiltrate and thick, dense collagen beams was confirmed in the samples from the NLQ group (Fig. 2H).

Discussion

This is the first study that examined the effects of topical application CoQ_{10} encapsulated in nanoliposomes on alveolar socket healing after tooth extraction in rats. Although, a previous study investigated the effects of CoQ_{10} on wound healing,[6] present study evaluated the activity of both free and encapsulated form of CoQ_{10} since it is well documented that the encapsulation of active compound increases its activity.[7,8,17]

The healing of the extraction wound goes through three stages which are successively linked to each other and whose strict chronological definition is quite difficult since micromorphological images intertwine with each other. The first phase of wound socket reparation is characterised by the development of the inflammatory reaction and removal of debris, and the initial proliferative stage, with the development of the granulation tissue.[2] Neutrophils release an arsenal of proteolytic enzymes which digest damaged tissue elements and ROS and numerous other inflammatory mediators. The phagocytic activity of inflammatory cells removes necrotic cell debris and coagulum.[2] In animals treated with CoQ_{10}, in the free form or encapsulated in liposomes, faster regeneration rate of gingival and periodontal soft tissue structures was visible. The acceleration of healing process was more pronounced in group treated with nanoliposomes encapsulated with CoQ_{10} (Fig. 2D and H) than in group treated with free CoQ_{10}. Similar results, a reduction in inflammatory reaction and increase in collagen deposition 3 days following surgical procedure, were previously obtained in animals where free CoQ_{10} was applied in a form of ointment.[6] Also, the expression of IL-1β, TNF-α, NF-κB and HO-1, cytokines involved in inflammation and oxidative tissue damage, were significantly suppressed by CoQ_{10} application for 3 days following surgical procedure,[6] contributing to the theory where the decrease in inflammation increases healing processes.[24] The results of the study conducted by Yoneda et al.[6] where CoQ_{10} was applied indicate that 3-day period is sufficient for socket to heal. After 7 days the maturation of the granulation tissue, increased collagen fibres deposition, reduction of the inflammatory infiltrate and complete epithelisation could be seen. In our study, the groups where two forms of CoQ_{10} were applied, especially the one with the encapsulated from of CoQ_{10}, earlier epithelisation of the gingival defect, less pronounced inflammatory infiltration, earlier deposition of collagen fibres and more intense maturation of connective tissue structures were present.

Usually, an increase in ROS production coexists with a decrease in the antioxidant defense system and the imbalance between the prooxidant and antioxidant systems may lead to further oxidative damage of periodontal tissues.[6,8] Myeloperoxidase is an antimicrobial leukocyte-derived enzyme found in high concentrations in the primary granules of leukocytes that catalyzes the formation of a number of ROS. Also, MPO-derived oxidants significantly contribute to tissue damage during inflammation. There is a clear connection between MPO activity and NO concentration, where the NO can directly influence MPO activity and on the other side MPO can convert nitrite and peroxides into a nitrating agent that cause protein and lipid damage.[4,25] Inflammation is consider to be a significant component in tissue repair, however excessive/exacerbation of inflammation may delay/alter tissue repair process.[26] Previously MPO activity was found to be suppressed in wound tissue of animals treated orally with CoQ_{10},[15] thus the results related to the MPO activity in our study are somewhat expected. Interestingly the MPO activity in wound tissue of animals treated with encapsulated CoQ_{10} was proven to be statistically significantly lower compared to both control and CoQ_{10} treated groups (Fig. 1). Also, the results concerning NO concentration in wound tissue are in good correlation with MPO activity and pathohistological analysis. Where the reduction in NO concentration (Fig. 1) leads to less pronounced inflammatory infiltrate, visible in groups treated with both forms of CoQ_{10} (Fig. 2). In organism NO acts as eider host defense system activator, regulator of tissue homeostasis and/or different tissue structures developmental regulator.[27] The influence of NO on wound healing is debatable, where on one hand it is suggested that it promotes healing, while on the other that it acts as a proinflamatory molecule causing tissue destruction and delaying healing processes.[26,28,29] Also, besides the inducible nitric oxide synthase (iNOS), tissue arginase activity significantly influences tissue NO concentration (decrease it) by competing with iNOS for their substrate L-arginine.[30] The present results concerning the tissue NO concentrations in groups treated with both forms of CoQ_{10} contribute to the theories where the reduction in inflammatory response, enables damaged tissue to readily enter the later stages of healing which involve cell proliferation and tissue remodeling.[26]

The results of this study unequivocally confirm the hypothesis that the application of CoQ_{10} decreases the levels of MPO and NO compared to control group, and that CoQ_{10} encapsulation into nanoliposomes statistically significantly increases the activity of CoQ_{10} directly contributing and accelerating wound healing process in our experimental model. The encapsulation in nanoliposomes could possibly increase CoQ_{10} solubility, concentration and delivery in target tissue after its application. These results were also supported by the histopathological findings of the extraction socket wound from animals treated with encapsulated form of CoQ_{10}. Also, it was suggested the increase in CoQ_{10} activity could be achieved by its direct injection in socket.[6] However, this rather invasive procedure could possibly be

substituted by encapsulation of CoQ_{10} in liposomes which could enable CoQ_{10} to reach to the deeper parts of the socket and exert its activity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

This work was funded by the Project of the Faculty of Medicine, University of Niš (Project No. INT-MFN-13).

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