Immunohistochemical determination of osteopontin expression in tooth extraction wound tissue inflammatory cells – the effects of coenzyme Q10

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ABSTRACT
Tooth extraction wound complications occur in everyday clinical practice and their management can sometime be challenging. The process is followed by the characteristic pathohistological changes occurring in the soft tissue surrounding extraction wound that is known to follow several precisely regulated phases. Coenzyme Q10, omnipresent molecule, was found to potentially influence the wound healing process, however, its usage is limited due to its poor availability after application. This study evaluates for the first time the effects of coenzyme Q10 (free and encapsulated in nanoliposomes) treatment regime on osteopontin expression in the inflammatory cells surrounding rat tooth extraction wound and correlates these findings with the oxidative stress (lipid peroxidation and protein oxidative damage) and inflammation (myeloperoxidase and nitric oxide) related biochemical changes in the same tissue. Our results revealed that the encapsulated coenzyme Q10 is statistically significantly ($p<0.001$) more potent in preventing tissue inflammation and oxidative damage, as well as to inhibit osteopontin expression. Correlation analysis pointed to the fact the coenzyme Q10 activity is not only related to its impact on inflammatory cells activity but to other types of cells as well. The obtained results suggest the use of coenzyme Q10 in dental practice and as a food supplement should be recommended due to its ability to significantly enhance the wound healing process. This is especially true for the encapsulated form of the coenzyme Q10, which exerted the same extent of healing potential almost two times faster than the free form.

Keywords: Osteopontin, coenzyme Q10, tooth extraction wound, inflammation.

Determinación Inmunohistoquímica de la Expresión de Osteopontina en las Células inflamatorias del tejido lesionado por extracción dental – Efectos de la Coenzima Q10

RESUMEN
Las complicaciones en las lesiones por extracción dental frecuentan la práctica clínica, y su tratamiento puede ser desafiante. El proceso consiste en un seguimiento de cambios característicos histopatológicos que ocurren en el tejido blando que rodea la herida de extracción, y se reconoce por varias etapas precisamente reguladas. La coenzima Q10, una molécula omnipresente, se usó para influenciar potencialmente el proceso de cicatrización, pero su utilidad se restringe a su limitada disposición pos-aplicación. Este estudio evalúa por primera vez los efectos de la Coenzima Q10 (libre y encapsulada en nanoliposomas), su régimen de tratamiento en la expresión de osteopontina de las células inflamatorias del tejido lesionado por extracción dental en roedores, y correlaciona estos resultados con el estrés oxidativo (peroxidación de lípidos y daño oxidativo de proteínas) y la inflamación (mieloperoxidasa y óxido nítrico) con respecto a los cambios biológicos de este mismo tejido afectado. Nuestros resultados revelaron que la Coenzima Q10 encapsulada es, significativamente de forma estadística ($p<0.001$), más potente para prevenir tanto la inflamación del tejido y el daño oxidativo, como para inhibir la expresión de osteopontina. El análisis de correlación señala la actividad de la Coenzima Q10, no solo relaciona su impacto en la actividad de las células inflamatorias, sino también, en otros tipos de células. Estos resultados sugieren recomendar la Coenzima Q10 para la práctica odontológica y como suplemento alimenticio, por su capacidad para mejorar significativamente los procesos de cicatrización. En especial, se recomienda la Coenzima Q10 encapsulada, ya que cuenta con el mismo potencial de alcance de curación, casi el doble de rápido que la no encapsulada.

Palabras claves: Osteopontina, coenzima Q10, lesión de extracción dental, inflamación.

INTRODUCTION

Regardless of the wound etiology, each organism is attempting to maintain tissue homeostasis by preventing hemorrhage and infection. Some conditions affecting the human organism, such as diabetes, cachexia, vitamin deficiency, exposure to radiation, etc., can significantly affect the process of wound healing due to the disturbances they are causing within organism/cell functioning [1].

The tooth extraction in rats is a common model for the evaluation of the impact of various substances on the closure and healing processes [2-4]. After tooth extraction wound tissue is going through several phases which include cell proliferation, migration, and inflammation [1]. The inflammatory processes are guided by various inflammation molecules such as nitric oxide (NO) and myeloperoxidase (MPO) [5]. Also, during the process different cells act as a source of reactive oxygen species (ROS) production, thus, ROS overproduction might impair the wound healing process. Among the molecules acting at the site of inflammation the role of osteopontin, a phosphorylated glycoprotein, as signal of cell migration, adhesion, and survival should not be neglected [6].

Coenzyme Q10 (CoQ10) or ubiquinone-10 is an endogenous molecule that serves as a cofactor in the mitochondrial electron transport system [7]. Also, it acts as a potent antioxidant of lipid membranes and a modulator of different pro-inflammatory gene expression such as IL-6 and TNF-α [8]. The concentrations of this molecule can vary with age, gender, and general health8 and when is present in sufficient quantities CoQ10 is readily absorbed by all types of cells [9].

The deficit can be the consequence of insufficient ingestion, disturbed biosynthesis, and/or increased consumption within the body [10]. A decrease in CoQ10 quantities is directly connected with a diminished ATP synthesis and cell energetic disbalance [9], as well as with an increase in reactive oxygen species (ROS) production and DNA damage [10]. The importance of CoQ10 in dentistry arises from the fact that in gingival biopsies obtained from patients with damaged periodontal tissue CoQ10 levels are decreased [11].

During the last couple of decades, great attempts are made to design an adequate drug carrier system (liposomes) which would enable target-specific delivery and minimalize off-target effects of the applied agent [12,1]. On several previous occasions encapsulated CoQ10 was investigated as a treatment option for cutaneous [12] and mucosal [1] wounds. The rationale behind these studies is the ability of CoQ10 to modulate the inflammatory response and to prevent ROS derived tissue macromolecules damage.In a model of the cutaneous wound healing process, a 3-day application of the encapsulated CoQ10 was found to decrease the expression of IL-1β, TNF-α, NF-κB, and HO-1 and to increase the collagen deposition [12]. On the other hand, in a model of mucosal wound closure, the encapsulated CoQ10 was found to be more potent in decreasing the number of inflammatory cells and to decrease the wound tissue NO concentrations and MPO activity [1].

Prompted by the above mentioned, this study aimed to evaluate, compare, and correlate the results of the application of free and nanoliposome encapsulated CoQ10 on wound tissue healing. During a 10-day study, three-time points were chosen for the soft tissue sample collection and analysis. Using both biochemical and pathohistological analysis of the wound soft tissue, our study deems to prove the greater benefits arriving from the application of CoQ10 encapsulated in nanoliposomes. Also, for the first time, osteopontin expression in the inflammatory cells surrounding the tooth extraction wound, and the impact of CoQ10 will be examined.

MATERIALS AND METHODS

Animal housing

Male and female Wistar rats, weighting from 200 to 250 g were kept under standard laboratory (temperature: 22 ± 2 °C, relative humidity: 55 ± 5) conditions with an equal duration of light/dark cycle and with water and food available *ad*

libitum. All experiments were conducted at the Institute of Biomedical Research, Medical Faculty, Niš, Serbia and are in accordance with all ethical regulations of the European Union (EU Directive of 2010; 2010/63/EU) and those of Republic of Serbia (323-07-00073/2017-05/2).

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

Phospholipid nanoparticle 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. The encapsulation efficacy of CoQ_{10}-loaded liposomes was determined at 275 nm (UV-1800 Shimadzu spectrophotometer) after ethanol extraction following the standard protocol [2]. The encapsulation efficacy (%) was calculated as:

$$\text{Efficacy (\%)} = \frac{\text{incorporated CoQ10}}{\text{initial CoQ10}} \times 100$$

EXPERIMENTAL PROCEDURE

All animals were randomly divided into four groups each containing 18 rats. The surgical procedures were performed under 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, the extraction wound was treated with topical application using a cotton ball (2,4), according to the following schedule: (I) Control (C) group–without treatment; (II) Free nanoliposomes (NL) group treated with 10% solution of empty nanoliposomes; (III) Coenzyme Q_{10} (Q10) group treated with coenzyme Q_{10} dissolved in soybean oil (6mg/ml); (IV) Encapsulated nanoliposomes (ENLQ10) group treated with coenzyme Q_{10} encapsulated in nanoliposomes (6mg/ml).

On the days 3, 5, and 10 following tooth extraction 6 rats randomly selected from each group were sacrificed under ketamine general anesthesia and the tissue samples were collected for biochemical and pathohistological analysis.

Pathohistological, immunohistochemical 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. Further tissue processing was performed as described previously [4]. Initially cut 5 μm sections of the soft tissue surrounding wound were stained with standard hematoxylin and eosin staining technique and examined. Five micrometers thick tissue sections were deparaffinized before antigen retrieval (pH 6.0) in a microwave for 20 min. Afterwards, the sections were stained with anti-Osteopontin antibody (1:200 dilution, ab 6856, Abcam, SAD) for 1 h at 23 °C. The detection of the primary antibody was done using a rabbit specific HRP/DAB(ABC) detection Kit (ab64261, Abcam, SAD) following the manufacturer's protocol.

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 a computerized image analysis system Image J. An average number of osteopontin positive inflammatory cells per high power field (HPF) (lens magnification x400) were counted on at least 5 different fields per tissue sample.

Tissue oxidative and inflammatory status determination

After homogenization of the soft tissue surrounding the extraction wound (1/10 *w*/*v* in ice-cold PBS) homogenates were centrifuged (900 ×g, 10 min) and clear supernatants were isolated for further biochemical parameters determination. The number of proteins was determined using Lowry's method as described previously [13]. The concentration of the advanced oxidized protein

products (AOPP) in tissue homogenates was determined by a spectrophotometric method based on the reaction of AOPP with KI in an acidic medium [14]. The concentrations of AOPP were expressed as micromole per milligram (μmol/mg) of tissue proteins. The extent of lipid peroxidation in the soft tissue surrounding the extraction wound was determined based on the levels of malondialdehyde (MDA) in a reaction between thiobarbituric acid and modified lipids [15]. The concentration of MDA was calculated using a standard curve and the results are expressed as mmol of MDA per mg of tissue proteins. The activity of the antioxidant enzyme catalase (CAT) was determined by the spectrophotometric method which is based on the ability of catalase to dissolve the substrate (H_2O_2) [16]. The intensity of a colour reaction was measured at 405 nm and the enzyme activity was expressed as unity per gram (U/g) of tissue proteins.

The concentration of nitrates present in tissue homogenates was measured using a standard Griess protocol with slight modification [17]. The intensity of the formed colour was measured at 540 nm and nitrate and nitrite concentrations (further termed as NO), expressed as millimole per milligram (mmol/mg) of tissue proteins, were calculated using a standard curve of sodium nitrate. The activity of myeloperoxidase (MPO) in the wound tissue homogenates was measured following a reaction between o-phenylenediamine which in the presence of enzyme and H_2O_2 give a coloured product [18]. 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 per milligram of proteins (OD/mg).

Statistical analysis

Results expressed as the mean ± SD (Standard Deviation) 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. The correlation coefficients between the number of osteopontin positive inflammatory cells and biochemical parameters were estimated for the values obtained at the period of 3-, 5- and 10-days following tooth extraction. The magnitudes of Pearson correlation coefficients were treated as follows: very weak (0.0-0.19), weak (0.2-0.39), moderate (0.4-0.59), strong (0.6-0.79), and very strong (>0.8) [19].

RESULTS

Disturbance in post-extraction wound soft tissue biochemical parameters reflecting oxidative status and inflammatory signaling was modulated by both free and encapsulated Coenzyme Q10

Application of both free and encapsulated CoQ10, during a 10-days of the experiment, led to a statistically significant diminution in the studied oxidative stress-related biomarkers (MDA and AOPP), compared to the control animals (Fig. 1A,B), except for the last studied time period at day 10. Interestingly, the application of the encapsulated CoQ10 was found to be more potent in preventing AOPP formation than the free CoQ10, while the same was not noted in the case of MDA (Fig. 1A,C).

A statistically significant decrease in CAT activity found in the control group of animals was prevented by the application of both forms of CoQ10 during the experiment (Fig. 1B). More pronounced, statistically significant, the effect of the encapsulated CoQ10 on CAT activity was noted only on the last examination period, ie. 10-days after tooth extraction (Fig. 1B). Our results indicate that the nitric oxide concentrations (expressed as NO^{2-}/NO^{3-}) and MPO activity are increased in the soft tissue surrounding the extraction wound (Fig. 1D,E).

Local application of both forms of CoQ10 decreased NO concentration and MPO activity (except on day 10) in wound tissue, whereby the activity of the encapsulated form was statistically significantly greater than the one of the free CoQ10 (Fig. 1D,E).

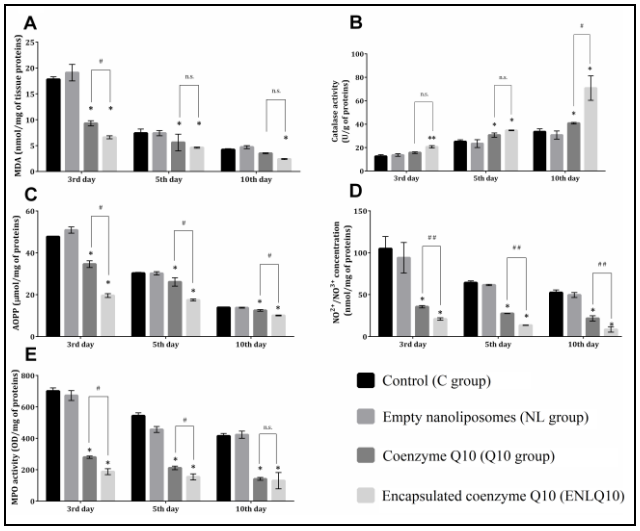

Fig. 1. Soft tissue surrounding tooth extraction wound biochemical parameters obtained from different experimental groups. Biochemical parameters: (A) MDA, (B) CAT, (C) AOPP, (D) NO^{2+}/NO^{3+}, (E) MPO. Data are given as mean ± SD (for each evaluated time point number of animals was 6). *$p<0.001$ vs C group; #$p<0.001$, ##$p<0.01$ vs Coenzyme Q10 group, n.s. (no statistical difference).

Both free and encapsulated coenzyme Q10 application decreases the inflammatory cell infiltration and osteopontin expression in the soft tissue surrounding the extraction wound

Osteopontin immunopositivity 3-days after tooth extraction in the soft tissue surrounding the wound was primarily detected in inflammatory cells, macrophages, and to a lesser extent in neutrophils (Fig. 2A-D). In the control groups (C and NL) as well as in animals treated with CoQ10 the counted number of osteopontin positive cells almost identical (Table1). Animals treated with encapsulated CoQ10 (ENLQ10 group) were found to have a significantly lower number of osteopontin positive cells compared to both the control and Coenzyme Q10 group.

The number of osteopontin positive cells in the C and NL group on the 7th day was decreased compared to the 3rd day, however, the staining intensity was almost identical to the one on the first examination period. In the experimental groups (CoQ10 and ENLQ10) the number of osteopontin positive inflammatory cells was found to be significantly decreased compared to the C group. Here again, the number of positive cells was significantly lower in tissue sections obtained from animals belonging to ENLQ10 compared to ones from the CoQ10 group.

In the tissue samples obtained from the animals in the last investigated time period (10 days post-extraction), osteopontin positivity was only present in non-inflammatory cells, except in some rare cases in the C and NL group.

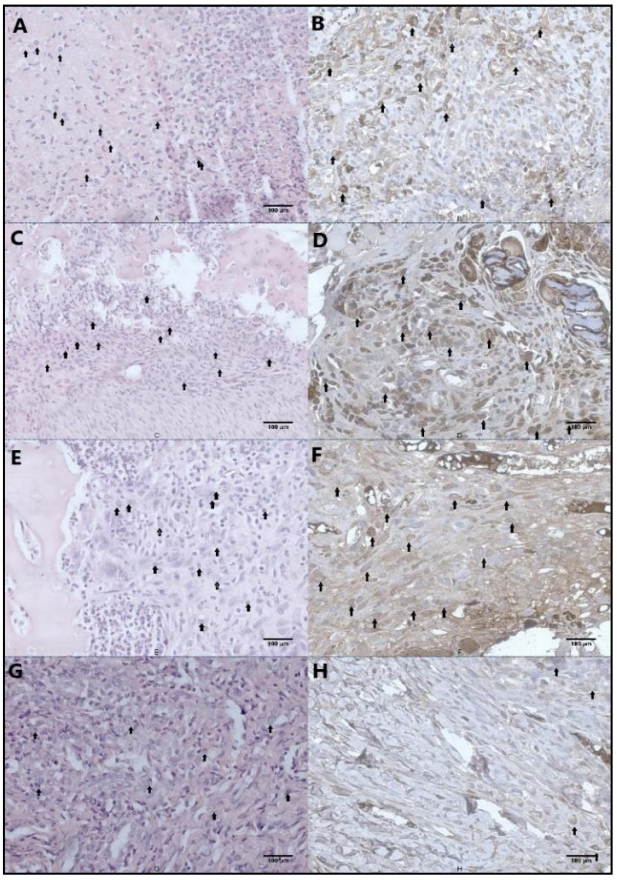

Fig. 2. Micromorphological images of the soft tissue surrounding tooth extraction wound (5th day) stained with H&E and osteopontin obtained from either control (A,B), empty nanoliposomes (C,D), coenzyme Q10 (E,F), and encapsulated coenzyme Q10 (G,H) group. Inflammatory cells on images are designated with arrows. Given magnification is 200X.

Table 1. Number of osteopontin positive inflammatory cells per high power field in the soft tissue surrounding tooth extraction wound obtained from animals belonging to different experimental groups.

DAY	GROUP			
	C	***NL***	***Q10***	***ENQ10***
3rd	82 ± 7.2	77.9 ± 4.3	75.5 ± 8.2	31.8 ± 10.6*,#
5th	48.2 ± 10.1	52.3 ± 7.1	33.9 ± 8.8**	11.1 ± 5.9*,#
10th	2.5 ± 4.3	3 ± 3.8	*NA*	*NA*

NA – not applicable.

Osteopontin expression statistically significantly correlates with inflammatory parameters, but not with oxidative stress, in the soft tissue surrounding extraction wound only 3 days after the procedure

Correlation analysis of the control group animal data obtained from the biochemical and morphometric analysis revealed that the two inflammatory parameters (NO concentrations and MPO activity) have a statistically significant strong correlation with osteopontin expression (Table 2) only in the first examination period (3rd day post-extraction). All other parameters were found to correlate to a different extent with osteopontin expression within inflammatory cells during 3 evaluated time periods; however, the correlation was not statistically significant.

Table 2. Correlation coefficients (R square) and p values from the correlation matrices obtained for osteopontin expression and tissue biochemical parameters obtained from control animals.

PARAMETERS	NO_2/NO_3	MPO	MDA	AOPP	CAT
Osteopontin expression 3rd	0.9; < 0.05	0.9; < 0.01	0.5; > 0.05	0.8; > 0.05	0.1; > 0.05
Osteopontin expression 5th	0.9; > 0.05	0.03; > 0.05	0.53; > 0.05	0.8; > 0.05	0.08; > 0.05
Osteopontin expression 10th	0.3; > 0.05	0.6; > 0.05	0.8; > 0.05	0.2; > 0.05	0.6; > 0.05

In CoQ10 treated rats osteopontin expression statistically significantly correlates with both inflammatory and oxidative stress-related parameters

The analysis of the correlation matrices obtained for the studied biochemical parameters and quantified osteopontin expression within polymorphonuclear cells are presented in Table 3.

Table 3. Correlation coefficients (R square) and p values for the osteopontin expression and tissue biochemical parameters obtained from CoQ10 treated animals.

PARAMETERS	NO_2/NO_3	MPO	MDA	AOPP	CAT
Osteopontin expression 3rd	0.9; < 0.001	0.9; < 0.001	0.9; < 0.001	0.9; < 0.001	0.9; < 0.001
Osteopontin expression 5th	0.9; < 0.001	0.9; < 0.001	0.8; < 0.001	0.9; < 0.001	0.8; < 0.001
Osteopontin expression 10th	*NA*	*NA*	*NA*	*NA*	*NA*

NA – not applicable.

The data clearly indicate that all studied biochemical parameters in the CoQ10 treated animals are statistically significantly associated with the number of osteopontin positive cells both 3 and 5 days after the treatment. Since there were no osteopontin positive inflammatory cells to be counted in the two studied groups on the 10th day of the treatment the correlation analysis could not be performed (Table 3). Data are given as mean values ± SD (n=6 for each time point). The comparison was done using a One-Way ANOVA followed by Tukey's hoc test. *p<0.001, **p<0.05 vs. C group; #p<0.001 vs. Coenzyme Q10 group, NA (not applicable).

DISCUSSION

Treatment with both free and encapsulated CoQ10 statically prevented an increase in the soft tissue surrounding extraction wound oxidative stress-related biomarkers (MDA, AOPP) and a decrease in the studied antioxidant enzyme (CAT). On the other hand, a treatment regime with both forms of CoQ10 was found to significantly prevent excessive inflammatory response in the wound tissue (Fig. 1). The activity of CoQ10 is directly connected with its function in a mitochondrial respiratory chain for adenosine triphosphate synthesis, as well as to its ability to protect cell lipids and proteins from ROS [20]. Also, the ability of CoQ10 to inhibit cyclooxygenase-2 and nuclear factor-κB, which are directly associated with the occurrence of oxidative stress, most certainly contributes to its activity [21].

Moreover, the encapsulation of CoQ10 leads to the enhancement in its antioxidant and anti-inflammatory potential which is evident both at early and late examination period, 3 and 10 days, respectively. This is not the first study showing that the encapsulation of CoQ10 in nanoliposomes increases its biological potential [12,2], nor that the encapsulation process itself is a very good option for substances whose target delivery is hampered [22,23].

In this study, for the first time, we found that the expression of osteopontin in the inflammatory cells

surrounding the extraction wound was diminished by the applied CoQ10, with the more pronounced effect noted in animals treated with the encapsulated form of this antioxidant (Table 1, Fig. 2G,H). The inhibition of this inflammation-related biomarker and generally a decrease in the number of inflammatory cells could be achieved by several mechanisms associated with the CoQ10 mode of action [20,21]. Since the majority, if not all, osteopontin positive inflammatory cells arrive via circulation through the newly formed granulation tissue [24] the ability of CoQ10 to increase the collagen deposition and to cause faster wound tissue closure and maturation [2], this is just one of the mechanisms of action explaining the present results.

Performed correlation analysis for the data obtained from the biochemical and morphological investigation revealed that inflammation-related parameters (NO concentration and MPO activity) correlate well with the number of osteopontin positive cells, enabling their activation and adhesion [25], in both control and experimental groups (Table 2 and 3).

These findings can be potentially explained by the presence of generated pro-inflammatory cytokines (TNF-α, IL-1β, NO, etc.) in the surrounding wound tissue which up regulate osteopontin expression in macrophages [26]. Having this in mind one of the proposed mechanisms of action of CoQ10 might involve its ability to inhibit inducible nitric oxide synthesis [21]. Altogether, these data point to the fact that osteopontin might be used as a tissue inflammatory biomarker. More recent studies also point to the significance of osteopontin as a potential pro-inflammatory serum marker for insulin resistance since its quantity correlates well with serum MDA and IL-1β levels [27].

Studied polymorphonuclear/macrophage-derived enzyme MPO was found to decrease actin dynamics, cell spreading, and to cause cell cycle arrest, all leading to an impaired mucosal integrity restoration [6]. These effects of MPO could be related to the results of our study where an increase in osteopontin positive cells on the 3rd day, consisting mainly of macrophages and polymorphonuclear cells (Fig. 2B,D), is highly correlating with MPO activity. This time point overlaps with the period when the post-extraction wound is not completely closed in either control or NL group. Also, the correlation existing between osteopontin positive cells on the 3rd day and the NO concentrations is in accordance with current knowledge and understanding of NO as an inflammatory mediator and its involvement in the wound healing process [28,29].

The results of this correlation analysis are not completely in accordance with the findings related to the expression of proinflammatory cytokines, which could induce osteopontin expression, through the 8-day study [12]. Namely, the significant association between osteopontin present in the inflammatory cells and NO/MPO was only evident on day 3 of the experiment and not at the later investigation periods (Table 2). These results imply that after the initial stimulation of osteopontin expression, within the inflammatory cells, its further destiny is not completely dependent on the same cytokines that lead to its gene up regulation. The absence of a statistically significant correlation between the extent of osteopontin expression and the studied oxidative stress-related parameters in control and NL groups are suggesting that the tissue oxidative damage is not primarily/exclusively arriving from the infiltrative inflammatory cells. Generated ROS which inhibits respiration (ATP generation), and causes macromolecule (proteins and lipids) damage can originate from non-osteopontin expressing cells such as endothelial cells, fibroblasts, etc. [1].

On the other hand, the good correlation between the results of osteopontin expression and tissue oxidative stress in the experimental groups (Table 3) might be explained through the numerous beneficial anti-ROS generating properties of CoQ10 which affect different cells simultaneously [1,21].

CONCLUSIONS

The present study investigated, for the first time, the impact of CoQ10 application in its free and nanoliposome encapsulated form on the soft tissue surrounding tooth extraction wound oxidative stress-related biochemical parameters and osteopontin expression. The results of the study revealed that CoQ10 application statistically significantly decreased tissue oxidative damage and inflammation which is followed by a drastic decrease in osteopontin positive inflammatory cells. The obtained results suggest that the usage of CoQ10 in dental practice and as a food supplement should be recommended due to its ability to significantly enhance the wound healing process. This is especially true for the encapsulated form of the CoQ10, which exerted the same extent of healing potential almost two times faster than the free form.

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