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Article

Curcumin nanoliposomes mitigate wound tissue inflammatory response caused by tooth extraction

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Curcumin is a plant-derived compound with polypharmacological properties that are hampered by its poor solubility, fast degradation, etc. Wound closure complications that follow tooth extraction are numerous, and relatively frequently additional treatment is needed to prevent unwanted process chronification. The present study aims to compare the effects of free and the nanoliposome-encapsulated curcumin on tooth extraction wound closure. The experiments were performed on Wistar rats where both forms of curcumin were applied topically on a tooth extraction wound for seven days. Changes in tissue oxidative stress (malondialdehyde and oxidized proteins concentrations, and catalase activity) and inflammation (nitric oxide levels and myeloperoxidase activity) related parameters were studied three and seven days following the tooth extraction. Also, the extent of pathohistological changes and osteopontin immunohistochemical expression were studied. The obtained results indicate that both forms of curcumin prevent an increase in oxidative stress and inflammation-related parameters in the studied samples at 3- and 7-day time points. Additionally, we found that curcumin diminished tissue inflammatory response and osteopontin expression, while at the same time it caused faster granulation tissue maturation. The encapsulation of curcumin in nanoliposomes proved to be better in improving the extraction wound healing process than the free curcumin, giving this formulation a potential in the pharmaceutical industry.

Keywords: Curcumin. Nanoliposomes. Wound healing. Inflammation. Oxidative stress.

INTRODUCTION

Tooth extraction represents one of the most common dental procedures that is often followed by different complications mainly associated with the disturbances in the wound healing process (Todorović *et al*., 2018). The healing process of tooth socket involves highly programmed processes of inflammation, cell proliferation, matrix deposition, and tissue remodeling. Thus, the change occurring in any of the phases, prolongation or absence, can cause the disturbance in wound healing e.g. different complications and/or chronic wound occurrence (Delavary *et al*., 2011). The transition between the phases largely depends on the function and maturation of mastocytes, fibroblasts, keratinocytes and macrophages (Rodero, Khosrotehrani, 2010).The specific localization of the tooth extraction wound, *i.e*. oral cavity and the presence of microorganisms, saliva and the constant pressure on the wound can all be the cause of alterations in the process and could lead to complications (Farina, Trombelli, 2012). The enhancement of the wound closure process and the prevention of complications

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can be achieved by either oral or local application of different antioxidants, especially those of natural origin (Akbik *et al*., 2014; Farina, Trombelli, 2012).

The wound healing process is initially under the influence of neutrophils, the first cells attracted to the wound surrounding tissue, followed by macrophages, which are additionally activated by reactive oxygen species (ROS) deriving from neutrophils (Todorović *et al*., 2018). The production of ROS in the initial step of the wound healing process can be considered beneficial since it prevents wound infection and induces an immune response (Akbik *et al*., 2014). However, a prolonged and intense ROS production can lead to oxidative tissue damage and a significant prolongation of this process (Guo, Dipietro, 2010). Also, excess ROS production is followed by release of different pro-inflammatory molecules/enzymes, such as nitric oxide (NO), tumor necrosis factor alfa (TNF-α), interleukin 1 beta (IL-1β), myeloperoxidase (MPO), which additionally contribute to the extension of the mentioned process (Todorović *et al*., 2018).

Curcumin (CUR) is a compound originating from turmeric plant (Anamika, 2012) which is known to possess numerous beneficial properties such as anti-cancer, anti-nociceptive, anti-inflammatory, anti aging and wound healing (Mitić *et al*., 2017). The wound healing potential of CUR is believed to be based on its effect on the inflammatory process (Mohanty, Das, Sahoo, 2012), as well as on the production and deposition of collagen fibers (Mitić *et al*., 2017). Additionally, CUR is known to enhance epithelial regeneration and to cause an increase in fibroblast proliferation and vascular density (Akbik *et al*., 2014). However, the application of CUR is highly limited due to its poor absorption after oral application, fast first-passage metabolism, high lipophilicity and sensitivity to light (Anand *et al*., 2007). During the past years, numerous researchers have incorporated CUR into different pharmaceutical formulations (chitosan–alginate sponges, polymeric bandages, etc.), though none of the processes increased the potential of CUR in the wound healing process (Pietra *et al*., 2017; Akbik *et al*., 2014). The suggestion, based on some other studies evaluating the activity of CUR as an anti-cancer agent, is that its healing potential might be increased by incorporating this curcuminoid into nano-vesicles (Akbik *et al*., 2014).

Bearing in mind all the previously mentioned facts we aimed to evaluate whether the encapsulation of CUR into nanoliposomes would increase its healing potential in the tooth extraction wound healing model during the 7-day period. The potential of free and encapsulated form of CUR would be studied through the changes in tissue oxidative damage and inflammatory biochemical parameters. Also, the process of wound healing will be studied on the microscopic level using standard histological and immunohistochemical techniques.

MATERIAL AND METHODS

Drugs and chemicals

Phospholipid nanoparticles solution (10%), in the form of nanospheres, was purchased from Nattermann Phospholipids (Germany), while ketamine (10%), a general anesthetic, was purchased from Richter Pharma AG (Wels, Austria). Curcumin (CUR) and all other used chemicals were obtained from either Sigma-Aldrich (St. Louis, USA) or CarlRoth (Karlsruhe, Germany).

Liposomes preparation and encapsulation efficacy evaluation

The encapsulation procedure and the determination of its efficacy were performed based on the previously standardized methods (Stojiljković *et al*., 2019; Todorović *et al*., 2018; Stojiljković *et al*., 2018).The solution of nanoparticles was mixed with CUR in order to achieve the final concertation of 4 mg/ml and after the overnight incubation, the isolation of encapsulated CUR was done by centrifugation (6500xg, 30 min at 4°C). The efficacy of encapsulation was determined in a mixture of CUR-loaded liposomes and ethanol, where after the separation the absorbance was measured at 275 nm (V-1800 Shimadzu spectrophotometer). The encapsulation efficacy (%) was calculated as (amount of incorporated CUR)/(initial amount of added CUR) x 100.

Animals and housing

Male and female Wistar rats (200-250 g) were kept in standard laboratory conditions (temperature 22±2 °C, relative humidity 55±5, with an equal duration of light/dark cycle), received tapwater and food ad libitum during the entire experiment. All experiments were conducted at the Institute of Biomedical Research, Medical Faculty, Niš, Serbia and are in accordance with all the ethical regulations of the European Union (EU Directive of 2010; 2010/63/EU) and Republic of Serbia (Ethical Committee approval Nº. 323-07-00073/2017-05/2 given in February 2017).

Experimental procedure

On the day of the experiment, the animals were allocated into four experimental groups, each consisting of 12 animals. Maxillary incisors were extracted from the anesthetized animals (ketamine, 10%) using dental explorer and extraction forceps. The first group served as a control (**C**), the second served as a control for empty nanoliposomes (**NL**), while the remaining two were used for the treatment with either free CUR (**FCUR**) or encapsulated CUR (**NLCUR**). After hemostasis, the extraction wound was treated topically daily for 7 days with empty **NL** and with free or encapsulated CUR (4 mg/mL) using a cotton ball, as previously described (Todorović *et al*., 2018; Mitic*et al*., 2017).The animals from the **C** group were not treated after tooth extraction and hemostasis. Three and seven days following the tooth extraction, 6 rats from **C**, **NL**, **FCUR,** and **NLCUR** groups were euthanized by means of general anesthesia and tissue samples were collected for biochemical analysis and pathohistological examinations.

Tissue homogenates preparation and protein concentration determination

Tissue samples from all animal groups were collected after animal euthanasia, 3 and 7 days following the tooth extraction. Wound tissue homogenates (10%, w/v) were prepared in ice-cold distilled water and were centrifuged at 12000 rpm for 15min (at 4 °C) in order to obtain the clear supernatant that was further used for the determination of different biochemical parameters. The amount of proteins was determined using the standard Lowry's method and the values were calculated from the standard curve constructed using bovine serum albumin (Lowry *et al*., 1951).

Biochemical analysis

Determination of oxidative stress-related parameters

The extent of lipid peroxidation was determined based on the levels of malondialdehyde (MDA) using a reaction with thiobarbituric acid under acidic conditions. The intensity of the colored product that was developed with heating was measured at 532 nm using Multiscan Ascent (Labsystems, Finland)(Stojiljković *et al*., 2019). The concentration of MDA in different tissue homogenates was calculated using a standard curve and the results are expressed as μmol of MDA per g of tissue proteins.

The concentration of advanced oxidized proteins products (AOPP) in tissue homogenates was determined by the spectrophotometric method that is based on the reaction of modified proteins with potassium iodide (Stojiljković *et al*., 2019). The intensity of the reaction was measured immediately at 340 nm and the concentrations of AOPP were expressed as μmol/mg of proteins.

Catalase (CAT) activity was measured spectrophotometrically at the wavelength of 405 nm, according to the method previously described (Stojiljković *et al*., 2018). The enzyme activity was evaluated by quantifying the formed yellow complex of ammonium molybdate and the remaining H_2O_2. The activity of CAT was expressed in IU/mg proteins.

Determination of inflammatory parameters

Tissue myeloperoxidase (MPO) activity was determined based on the previously described method (Radulović *et al*., 2017). Briefly, the enzyme activity was measured according to the amount of end product of oxidized o-phenylenediamine whose absorbance was measured at 540 nm. The MPO activity was expressed as optical density (OD) per mg of proteins.

Wound tissue NO concentrations were determined using the standard Griess protocol (Stojiljković *et al*., 2018). The absorbance of the mixture consisting of the tissue homogenate supernatant and Griess reagent was measured at 540 nm using a microplate reader. The nitrite concentrations (inμmol/l) were calculated using a standard curve of sodium nitrite and the results are expressed as μmol/mg of proteins.

Pathohistological analysis

After the euthanization, the removed tissue samples were fixed in 10% buffered formalin, processed routinely and molded into paraffin. Initially, the extracted and the decalcified sample was cut from the center of the extraction socket in the sagittal plane towards the lateral parts in order to obtain 3 samples. The first sample was the one nearest to the sagittal plane, while the second and third ones were 0.3 and 0.6 cm lateral of the center of the extraction socket, respectively. From each sample three 4-5 μm tick tissue sections were made and stained using standard hematoxylin and eosin (HE) technique. Tissue examinations were performed using a lightmicroscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) and digital photographs were obtained using the imaging system (Olympus cell Sens platform standard, Olympus Corporation,Tokyo, Japan) at 200x magnification. The extent of tissue inflammation, the amount of granulation tissue formation and the degree of angiogenesis was scored as follows: 0 – parameter expression is poor; 1 – the evaluated parameter expression is moderate; 2 – parameter expression is strong (Mitić *et al*., 2017).

Osteopontin immunohistochemical staining and expression quantification

Heat-induced antigen retrieval procedure and exogenous peroxidase blockage were employed to tissue sections prior to the addition of polyclonal rabbit anti-osteopontin antibody (ab 6856, Abcam, SAD). After the exposition to primary antibody (1:200 dilution) for 60 min at 23°C, the slides were washed and a standard detection kit consisting of HRP/DAB system was applied (Rabbit specific HRP/DAB(ABC) Detection IHC Kit, ab64261, Abcam, SAD) and counterstained with Mayer's hematoxylin. The expression of osteopontin was afterward quantified based on the brown color precipitates on the places where the antigen-antibody reaction occurred (Mori, Shaw, Martin, 2008; Ivanovski *et al*., 2000).

Statistical analysis

The results obtained from the biochemical analyses were presented as mean values ± standard deviation (SD) and were mutually compared using One Way ANOVA followed by Tukey's post hoc test (Graphpad Prism version 5.03, San Diego, CA, USA). Probability values (p) less than 0.05 were considered statistically significant.

RESULTS

The values of the studied lipid and protein oxidative damage related parameters (MDA and AOPP) were found to be similar in the tissue of animals from **C** and **NL** groups at both studied time points (Table I). However, a spontaneous decrease in MDA and AOPP tissue content was clearly visible when one compares the data obtained from the animals sacrificed 3 and 7 days following the tooth extraction. The application of CUR significantly prevented MDA and AOPP formation, while its encapsulated form proved to be significantly more efficient than the free one at both studied time periods (Table I).

TABLE I - Effect of free and encapsulated CUR on oxidative stress and inflammatory parameters in the soft tissue surrounding tooth extraction wound

Parameter		Group I (C group)	Group II (NL group)	Group III (FCUR group)	Group IV (NLCUR group)
Oxidative stress-related parameters					
MDA (µmol/mg of proteins)	3-days	17.8±0.5	19.1±1.6	9±0.2$^{*,\#}$	6.9±0.4$^{*,\#,\P}$
AOPP (µmol/mg of proteins)		47.7±1.9	50.9±2.1	32.5±2.9$^{*,\#}$	18.5±0.$^{5*,\#,\P}$
CAT activity (IU/mg of proteins)		12.5±1.5	13.6± .1	16.8±0.9$^{*,\#}$	19.6±0.1$^{*,\#,\P}$
MDA (µmol/mg of proteins)	7-days	7.4±0.8	7.7±0.4	5.7±0.1$^{*,\#}$	4.4±0.1$^{*,\#,\P}$
AOPP (µmol/mg of proteins)		30.2±0.4	31.3±0.8	25.4±0.3$^{*,\#}$	17.1±0.3$^{*,\#,\P}$
CAT activity (IU/mg of proteins)		25.1±1.7	23.4±3.4	31.4±2.7$^{*,\#}$	36.7±3.2$^{*,\#,\P\P}$
Inflammation related parameters					
NO (µmol/ mg of proteins)	3-days	51.2±11.3	52.9±7.6	35.4±2.6$^{**,\#\#}$	16.6±7.1$^{*,\#,\P\P}$
MPO (OD/mg of proteins)		520.7±50.6	539.2±62.9	254.5±25.6$^{*,\#}$	195.6±10.5$^{*,\#}$
NO (µmol/ mg of proteins)	7-days	36.5±13.1	29.4±2.2	15.5±4.5$^{*,\#}$	9.8±4.7$^{*,\#}$
MPO (OD/mg of proteins)		206.8±13.9	197.5±16.3	152.2±8.5$^{*,\#}$	114.7±4.9$^{*,\#,\P}$

The data are presented as mean ± SD (n = 6), statistical significance was calculated by one way ANOVA followed by Tukey's post hoc test. * $p<0.001$, ** $p<0.01$ vs. **C** group; $^{\#}p<0.001$, $^{\#}$ $p<0.01$ vs. **NL** group; ¶ $p<0.001$, ¶¶ $p<0.05$ vs. **FCUR** group.

The activity of CAT in the soft tissue surrounding the tooth extraction wound did not differ between **C** and **NL** groups at both studied time points (Table I). Local application of the CUR, both free and encapsulated one, maintained the CAT levels significantly higher than in **C** and **NL** groups. This difference was especially prominent 7 days following the tooth extraction (Table I). Also, it is worth mentioning that the encapsulation of CUR (**NLCUR** group) was found to be statistically significantly more potent in maintaining CAT activity than the free CUR (**FCUR** group).

Here studied inflammatory parameters, NO concentration and MPO activity, were found to be almost identical in **C** and **NL** groups both 3 and 7 days following the tooth extraction (Table I). The application of CUR (**FCUR** and **NLCUR** groups) had a significant impact on NO concentrations, as well as on MPO activity and led to their decrease compared to the control groups (Table I). In the case when the encapsulated form of CUR (**NLCUR** group) was applied, more pronounced effects on NO concentrations, compared to the one of the free CUR, were noted in the first investigated period. On the other hand, the activity of MPO 7 days after the tooth extraction was more affected by the encapsulated than the free CUR (Table I).

Pathohistological findings

Three days after tooth extraction

In the tissue samples obtained from the animals 3 days after the tooth extraction a massive inflammatory reaction with severe tissue edema could be noted (Figure 1A-D). The inflammatory infiltrate, comprised of macrophages and polymorphonuclear leucocytes, was the most pronounced in **C** and **NL** groups, and occasionally around the areas with micro abscesses (Figure 1A and B, Table II). The application of CUR in either of the two formulations (**FCUR** and **NLCUR** groups) had a similar effect on the extent of inflammation in the soft tissue

around wound 3 days after the tooth extraction (Figure 1C and D, Table II).

On all analyzed tissue samples, the formation of granulation tissue with an abundance of newly formed blood vessels was evident (Figure 1). The formed blood vessels appeared small, comprised of angioblast, immature endothelial cells with oval nuclei that protrude in the vessel lumen, surrounded with amorph mass of extracellular matrix. Using the semiquantitative scoring system, the extent of granulation and the degree of angiogenesis was quantified, however, there was no substantial difference between the experimental groups, although in the groups treated with CUR a slight decrease in the two processes could be seen (Table II).

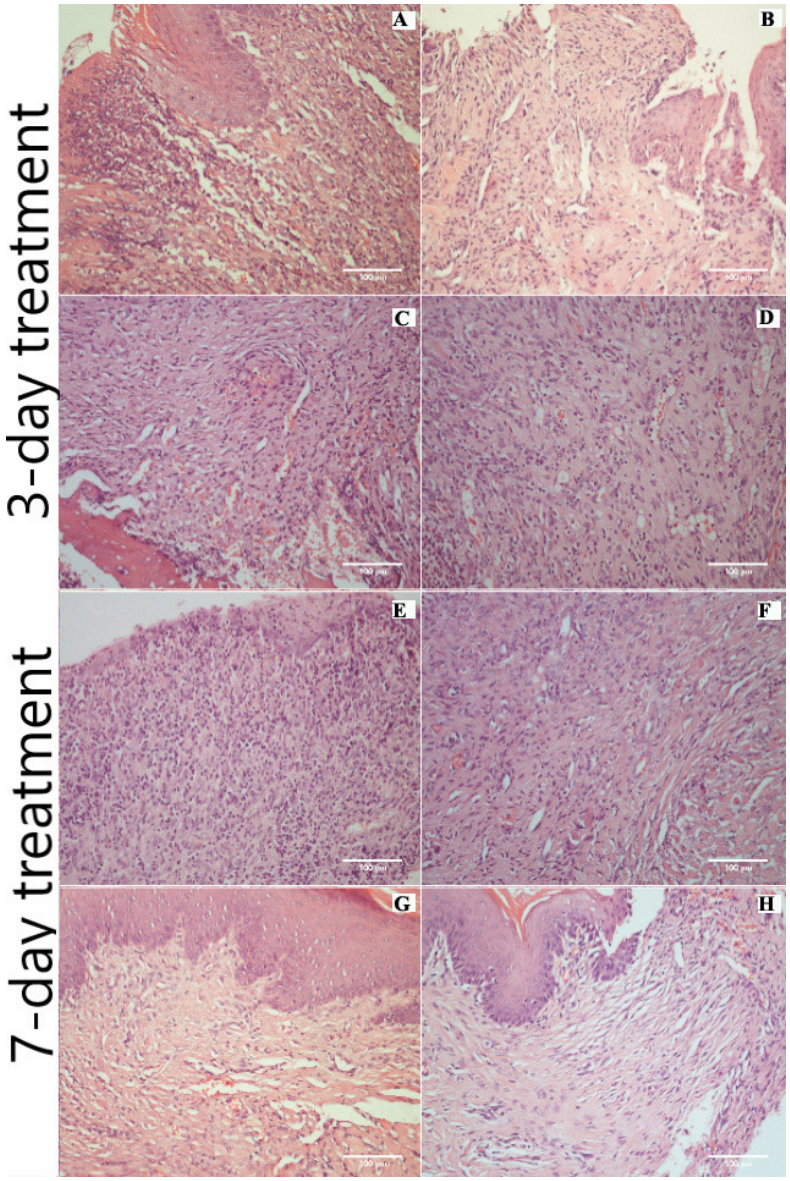

FIGURE 1 - Histopathological findings obtained for different experimental groups 3 and 7 days following the tooth extraction: (A) and (B) – **C** and **NL** groups tissue samples 3 days after the tooth extraction with numerous inflammatory cells and significant tissue edema present; (C) and (D) – **FCUR** and **NLCUR** groups of rats with a 3-day treatment where moderate inflammatory infiltrate and tissue granulation is visible; (E) and (F) – **C** and **NL** groups tissue samples 7 days after the tooth extraction with mononuclear and polymorphonuclear cells, as well as granulation tissue and angiogenesis processes still present; (G) and (H) – **FCUR** and **NLCUR** groups of rats with a 7-day treatment where the almost complete absence of inflammation and significant maturation of soft tissue is visible (HE staining, x200 magnification).

TABLE II - The sum of semiquantitative scores obtained for histopathological parameters from different experimental groups

Parameter		Group I (C group)	Group II (NL group)	Group III (FCUR group)	Group IV (NLCUR group)
Inflammatory infiltrate	3-days	6	5.5	4	3.5
Granulation tissue		6	6	5	4.5
Angiogenesis		4.5	5	5	4.5
Inflammatory infiltrate	7-days	4	4	2.5	1.5
Granulation tissue		5	4.5	4	3.5
Angiogenesis		5	5	2	2

Seven days after tooth extraction

On all studied samples 7 days after the tooth extraction the wound tissue was covered with a layer of epithelial cells and there was an evident turnover in the type of inflammatory cells from polymorphonuclear to mononuclear cells (Figure 1E-H). The present mononuclear cells, lymphocytes and macrophages, were forming a boundary towards the healthy tissue. In the two groups of untreated animals, **C** and **NL** group, a high number of polymorphonuclear cells was still present. The treatment with encapsulated CUR (**NLCUR** group) had a more pronounced effect on the extent of tissue inflammation compared to its free form (Figure 1G and H; Table II).

The tissue samples previously comprised mainly of granulation tissue with numerous immature blood vessels, now 7 days after the tooth extraction, the tissue was mainly compact with much less pronounced features than 3 days after the tooth extraction (Figure 1E-H; Table II). This tissue was comprised of myofibroblasts or fibroblast surrounded by still not completely organized collagen fibers, and with almost mature blood vessels (Figure 1E-H). Such features were most prominent in the group of animals treated with encapsulated CUR (**NLCUR** group, Figure 1H; Table II), where the mature fibrous and vascular tissue surrounded with thick collagen fibers could be seen.

Immunohistochemical findings

Positive immunohistochemical osteopontin reaction was detected in inflammatory cells, fibroblast and keratinocytes present in the tissue surrounding wound, as well as in the newly formed granulation tissue on the samples obtained both 3 and 7 days following the tooth extraction (Figure2).

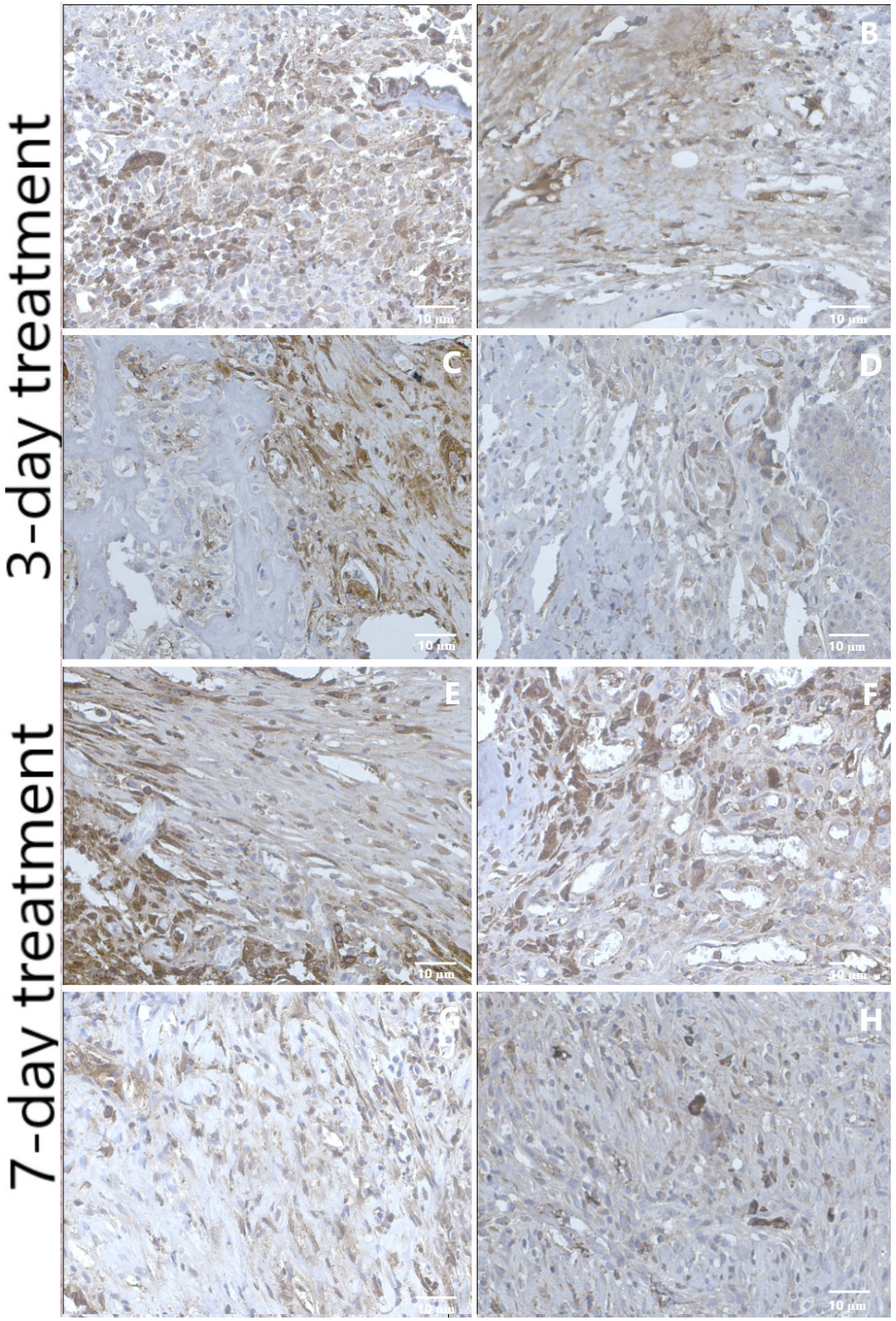

FIGURE 2 - Immunohistochemical staining of osteopontin in the soft tissue surrounding wound of all experimental groups: (A) and (B) – **C** and **NL** groups tissue samples 3 days after the tooth extraction where the most intense immunohistochemical reaction was noted mainly in the inflammatory cells; (C) and (D) – **FCUR** and **NLCUR** groups of rats with a 3-day treatment where a certain degree of reduction in the immunohistochemical reaction was noted; (E) and (F) – **C** and **NL** groups tissue samples 7 days after the tooth extraction with osteopontin expression in keratinocytes and fibroblast-like cells and rarely in the inflammatory cells; (G) and (H) – **FCUR** and **NLCUR** groups of rats with a 7-day treatment almost absent or located in occasional cells (Anti-osteopontin immunohistochemical staining, x400 magnification).

Three days after tooth extraction

Most inflammatory cells stained with osteopontin were macrophages, oval cells with abundant cytoplasm and euchromatic nuclei, and to a less extent neutrophilic granulocytes. Also, osteopontin expression was present in most of the mesenchymal cells, ovoid or spindle-like shaped, as well as in endothelial cells of the newly formed capillary network. The intensity of osteopontin expression, quantified based on the color reaction intensity, substantially varies between the experimental groups (Figure 2). Three days after the tooth extraction in the soft tissue surrounding wound the most intense immunohistochemical reaction was noted in **C** and **NL** groups, which is linked with cell density and the areas of tissue affected by the inflammatory reaction (Figure 2A and B). A certain degree of reduction in the immunohistochemical reaction was noted in tissue samples obtained from the **FCUR** group (Figure 2C), while the most prominent reduction was noted in the **NLCUR** group where only histiocyte- or fibroblast-like cells were slightly positive to osteopontin (Figure 2D).

Seven days after tooth extraction

Seven days after the tooth extraction in the wound tissue osteopontin was expressed predominantly in keratinocytes and fibroblast-like cells, while osteopontin expression in the inflammatory cells was rarely detectable. The most pronounced reaction was observed in **C** and **NL** groups (Figure 2E and F), while the immunoreactivity appeared to be decreased in the **FCUR** group (Figure 2G), and almost absent or located in some cells in **NLCUR** group (Figure 2H).

DISCUSSION

The purpose of this study was to determine to what extent the encapsulation of CUR in nanoliposomes would improve its wound-healing potential. The employed method of encapsulation gave satisfactory results, with the determined encapsulation efficacy of 74%. The two-membrane system of nanoliposomes represents an almost ideal method for the targeted drug delivery. The encapsulation of substance prevents its early enzymatic degradation, as well as its chemical and immunological inactivation (Bozzuto, Molinari, 2015). It is found that CUR takes a trans-bilayer orientation within the phospholipid bilayer of nanoliposomes, anchored by hydrogen bonding to the phosphate group of lipids in a manner analogous to cholesterol (Barry *et al*., 2009). Through the several known mechanisms, liposomes are merged with cells (Bozzuto, Molinari, 2015), and in the present case this is very important since the inflammatory cells such as neutrophils and macrophages can be considered as liposome "cleaners", due to their ability to engulf these nanoparticles.

As mentioned, the inflammatory cells play one of the crucial roles in the wound healing process (Rodero, Khosrotehrani, 2010) and by targeting these cells wound closure can be significantly altered. The loss of tissue integrity, such as seen after tooth extraction, almost immediately leads to an inflammatory response characterized by the cell infiltration and the release of pro-inflammatory molecules (Joe, Vijaykumar, Lokesh, 2004). Our results revealed a similar process in the soft tissue surrounding the tooth extraction wound, at both studied time periods, with a significant increase in osteopontin positive inflammatory cells (Figure 1 and 2, Table II), MPO activity and NO concentrations (Table I). The proposed mechanism of action of CUR on tissue inflammatory response involves the inhibition of NF-(κ) B (nuclear factor kappa-light-chain-enhancer of activated B cells) activity, which in turn downregulates pro-inflammatory cytokines transcription (Akbik *et al*., 2014). Having in mind this mechanism, one can anticipate the substantial positive influence of CUR after its application on the wound tissue and such results were not a surprise. However, after the application of encapsulated CUR (**NLCUR** group), more prominent beneficial effects were seen, especially at the later time period (7 days following the tooth extraction). These effects could be recognized based on the scattered inflammatory cells that were found during the investigation of the soft tissue (Table II and Figure 1H and 2H).

In the present study, we evaluated the expression of osteopontin, a molecule with the ability to attract inflammatory cells and cause the interaction between

the cells and the extracellular matrix in the wound tissue (Chimento *et al*., 2017; Denhardt *et al*., 2001). At both time periods in the untreated groups of animals, numerous cells were found to be positive to osteopontin (Figure 2A, B, E, and F), while in the groups treated with CUR the expression of this molecule was significantly diminished. These effects were especially prominent in the **NLCUR** group after 7 days of treatment, where only rare cells expressed osteopontin (Figure 2H). The activity, or to be more precise the amount of osteopontin, directly depends on the amount of pro-inflammatory cytokines (e.g., TNF-α, IL-1β) secreted by the inflammatory cells (Chimento *et al*., 2017). Bearing in mind that CUR inhibits the activity of NF-(κ)B(Akbik *et al*., 2014), we can possibly interpret the ability of CUR to inhibit inflammatory cell infiltration through the inhibition of osteopontin activity as well.

The prolonged accumulation of ROS leads to the oxidative stress which causes tissue macromolecules, lipids and proteins and DNA damage (Mohanty, Das, Sahoo, 2012), leading to a decrease in both enzymatic and non-enzymatic antioxidant capacities (Cano Sanchez *et al*., 2018). Such an increase in ROS concentrations has a direct cytotoxic and pro-degenerative potential, which at the end reflects the process of wound closure (Cano Sanchez *et al*., 2018). The wound tissue at early stages is predominantly susceptible to ROS associated damage (Cano Sanchez *et al*., 2018), and an increase in the amount of the two studied oxidative stress-related parameters confirms this.

In our study, the application of encapsulated CUR significantly prevented the increase in these two parameters, even more than the free CUR both 3 and 7 days following the tooth extraction (Table I). There are several proposed mechanisms of action of CUR, elaborated in *in vitro* or *in vivo* models where this compound was topically applied, which could explain the observed activity (Akbik *et al*., 2014). Namely, CUR could act as a direct scavenger of H_2O_2 (Gopinath *et al*., 2004) and $O_2^{\bullet-}$(Ghoneim *et al*., 2002) radicals, thus decreasing the generation of highly reactive OH^-radicals responsible for lipid and protein oxidative damage (El-Bahr, 2013)**.** Also, local application of CUR on cutaneous excised wounds caused a significant change in enzymatic antioxidant enzymes thus leading to the enchantment of wound healing (Mohanty, Das, Sahoo, 2012; Panchatcharam *et al*., 2006). Additionally, the encapsulation of naturally occurring antioxidant in the here used type of nanovesicles more effectively prevented the formation of MDA and AOPP in the wound tissue than the free one.

Wound tissue antioxidant capacity was studied through the change in CAT activity (Table I), which is frequently decreased when ROS based tissue damage occurs (Stojiljković *et al*., 2019). The application of both formulations of CUR leads to a significant increase in CAT activity in the wound tissue compared to the untreated animals (Table I). These effects were more pronounced at the later study period (7 days following the tooth extraction), especially in the group treated with encapsulated CUR (**NLCUR** group). Such results could possibly be explained through the potential of CUR to increase the transcription of the antioxidant enzymes including CAT (Kant *et al*., 2014). Thus, by increasing the amounts of available CUR in wound tissue, through its better delivery in nanovesicles, the process of antioxidant enzyme synthesis would be more intense.

CONCLUSIONS

Due to the necessity to increase the absorption and to decrease the metabolism and high lipophilicity of CUR, we employed the method of its encapsulation in nanoliposomes. The improvement of the wound-healing process by the applied treatment with encapsulated CUR was more effective than when the CUR was applied in its free form. These claims are based on the results from the oxidative tissue damage and inflammation-related parameters, as well as on the pathohistological and immunohistochemical analysis of the tissue samples. In the end, we can say that the process of CUR encapsulation should be applied for some future pharmaceutical formulations that are to be used for the treatment of both skin and mucosal wounds.

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