Curcumin effect on nitro-oxidative stress in ligature-induced rat periodontitis

Received for publication, April 21, 2015
Accepted, May 27, 2015

ADINA BIANCA BOȘCA1,*, ELENA DINTE2, HORĂŢIU COLOSI1, ARANKA ILEA3, RADU-SEPTIMIU CĂMPIAN3, ANA UIFĂLEAN1, ALINA ELENA PÂRVU1
1 Faculty of Medicine, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 2 Faculty of Pharmacy, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 3 Faculty of Medical Dentistry, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania
Corresponding author: Tel. 0740248923, email: biancabosca@yahoo.com

Abstract
The study aimed to assess the effect of curcumin on nitro-oxidative stress in ligature-induced rat periodontitis. Periodontitis was induced in male Wistar-Bratislava albino rats with a silk ligature around the inferior incisors. Curcumin was administered alone or with piperine. Rats were randomly assigned to five groups (n=5): 1. PER - periodontitis; 2. OC – oral curcumin; 3. OCP - oral curcumin and piperine; 4. OCPLC – oral curcumin and piperine, and local curcumin; 5. LC – local curcumin. Oral curcumin (1g/kg b.w.) and piperine (5mg /kg b.w.) were administered daily by gavage. Local treatment with curcumin was performed with a 2% muco-adhesive gel. Blood was collected and serum nitro-oxidative stress was evaluated through total oxidative status (TOS), total antioxidant capacity (TAC), total nitrites and nitrates (NOx) and oxidative stress index (OSI). The results demonstrated that orally administered curcumin, either alone or associated with piperine significantly reduced the serum NOx, TOS and OSI. Oral curcumin alone increased TAC. Piperine association did not significantly reduce systemic nitro-oxidative stress compared with curcumin alone. Local curcumin did not significantly influence the serum parameters. In conclusion, in rat ligature-induced periodontitis, oral administration of curcumin was effective in reducing the systemic nitro-oxidative stress, whereas local delivery showed no effect.

Key words: curcumin, nitro-oxidative stress, ligature-induced periodontitis, rat

Introduction
Nowadays it is well known that the complex pathogenesis of periodontitis implicates both the presence of the microbial plaque and the host immune-inflammatory response. Hence, more and more emphasis is laid on the development of host modulatory therapies as adjuncts to the antimicrobial treatment [1]. Agents that modulate host response include systemically or locally delivered pharmaceuticals and herbal extracts capable to restore the biological balance by controlling the release of pro-inflammatory cytokines, by blocking the activity of enzymes or by neutralizing the free radicals [2].

Antioxidants scavenge free radicals and prevent collateral tissue damage caused by oxidative stress, thus emerging as prophylactic and therapeutic agents [3]. Current trends in healthcare focus on herbal products, which symbolize safety, in contrast to synthetic drugs considered potentially harmful for humans and the environment [4].

Turmeric, the yellow spice obtained from the rhizomes of Curcuma longa, a perennial member of the Zingiberaceae family cultivated in Southeast Asia, has been used in traditional
Ayurvedic medicine even since the 19th century BC [5]. Two centuries ago, in 1910, curcumin was first isolated from turmeric and its structure was described as diferuloylmethane; it is a polyphenol, the primary component of curcuminoids, which also include curcumin co-purified derivate: demethoxycurcumin and bisdemethoxycurcumin [6,7].

Within the last six decades, numerous clinical, experimental and in vitro studies have been conducted in order to investigate the pharmacological, pharmacokinetic and pharmacodynamic activities of curcumin [8]. Increasing evidence has assigned to curcumin anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and anticancer properties [8,9,10] and proved its efficiency in various pathologies, including periodontitis [11,12].

The aims of this study were to assess the antioxidant effect of curcumin and to compare the efficiency of various therapeutic approaches in experimental periodontitis in rats. We evaluated and compared the nitro-oxidative stress parameters related to the different routes of curcumin administration.

Material and methods

Chemicals
Curcumin was purchased from Organika Health Products Inc. 11871 Hammersmith way Richmond, BC V7A 5E5 Canada. Each capsule contained 500mg powder of Curcuma longa rhizome, with a content of 95% curcuminoids.

Piperine was purchased from Provita Nutrition, Celex Canadian Laboratories. Each capsule contained piperine powder of Piperum nigrum 9.92mg and biotine 80µg.

Analytical grade chemicals were used exclusively. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), xylenol orange [o-cresosulphonphthalein-3,3-bis(sodium methyliminodiacetate)], ortho dianisidine, vanadium (III) chloride (VCl₃), hydrogen peroxide (H₂O₂), methanol, diethyl ether sulphanilamide (SULF) and ferrous ammonium sulphate were purchased from Sigma-Aldrich (Germany) and Merck (Germany).

Curcumin muco-adhesive gel preparation
The muco-adhesive gel was prepared by the incorporation of the curcumin powder into a gel matrix to obtain a concentration of 2% curcuminoids; the matrix exhibited rheological and in vitro adhesive properties that were adequate for the application on the gingiva and into the gingival sulcus. Evaluation of the gel viscosity was performed using a Brookfield DVIII Ultra viscometer. Assessment of the in vitro muco-adhesive properties was performed by the mucosal detachment method, using an experimental device [13].

Experimental design
The experiment was performed on twenty five adult male Wistar-Bratislava albino rats, weighing between 200 and 250 g that were bred in the Animal Facility of “Iuliu Hatieganu” University of Medicine and Pharmacy. The rats were kept in a room with controlled temperature (21±1°C) and humidity (50-55%) and a 12h light-12h dark cycle. Animals were fed with standard pellet (Cantacuzino Institute, Bucharest, Romania) basal diet and water ad libitum.

Periodontitis was induced in rats by placing a silk ligature around the inferior incisors. The experiment was performed on twenty five adult male Wistar-Bratislava albino rats, weighing between 200 and 250 g that were bred in the Animal Facility of “Iuliu Hatieganu” University of Medicine and Pharmacy. The rats were kept in a room with controlled temperature (21±1°C) and humidity (50-55%) and a 12h light-12h dark cycle. Animals were fed with standard pellet (Cantacuzino Institute, Bucharest, Romania) basal diet and water ad libitum.

Periodontitis was induced in rats by placing a silk ligature around the inferior incisors. The rats were anesthetized by intramuscular injection of 50 mg/kg body weight (b.w.) ketamine and 20 mg/kg b.w. xylazine. Silk threads were tied around the inferior incisors and were fixed by suture to the gingiva. Ligatures were maintained in place for fourteen days and the progression of the periodontal inflammation was monitored daily, and whenever the ligature was lost, it was immediately replaced.
After periodontitis induction, the ligatures were removed and then the rats were randomly assigned to five groups (comprising five animals each), according to the treatment they received: 1. periodontitis group (PER) – no treatment; 2. OC - PER and curcumin administered orally; 3. OCP - PER plus curcumin and piperine administered orally; 4. OCPLC - PER plus curcumin and Piperine administered orally, associated with curcumin administered locally; 5. LC – PER plus curcumin administered locally. Curcumin and piperine were administered daily, by intragastric gavage in doses of 1g curcumin/kg b.w. and 5mg piperine/kg b.w. The solutions for the general administration were prepared using sunflower seeds oil as a vehicle. Curcumin was also administered locally; the muco-adhesive gel (0.1ml) was applied daily on the gingiva.

After completing the ten days treatment, blood samples were harvested for serum nitro-oxidative stress tests.

The experimental protocol was approved by the Institutional Animal Ethical Committee of the “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca (approval No. 107/06.03.2015).

**Oxidative stress evaluation**

Serum samples were filtered through 10-kDd filters (Sartorius AG, Goettingen, Germany) and contaminant proteins were extracted with a 3:1 (v:v) solution of methanol/diethyl ether.

NO synthesis (NOx) was indirectly determined using the Griess reaction. Nitrate was reduced to nitrite by combining 100 μL of filtered and extracted serum supernatant with 100 μL of 8 mg/mL VCl3; then, the Griess reagents were added, 50 μL of SULF (2%) and 50 μL of NEDD (0.1%). The sample was incubated 30 minutes at 37°C and the absorbance was read at 540 nm. Serum NOx concentration (expressed as nitrite μmol/L) was determined using a sodium nitrite-based curve [14].

The serum total oxidative status (TOS) was measured using a colorimetric assay that measured the oxidation of ferrous ion to ferric ion in the presence of various reactive oxygen species in an acidic medium [15]. Then, ferric ions were detected by reaction with xylenol orange. Assay measurements were standardized with hydrogen peroxide (H2O2) used as the oxidative species, and the results are expressed in μmol H2O2 Equiv./L.

The serum total antioxidant capacity (TAC) was measured using a colorimetric assay that monitored the rate of hydroxyl radical production by the Fenton reaction the changes in the absorbance of coloured dihainisidyl radicals [16]. Upon addition of a serum sample, the antioxidant present in the serum suppressed the oxidative reactions initiated by hydroxyl radicals. Inhibition of dihainisidyl oxidation prevented the subsequent colour change, thus measuring the serum TAC. This assay was calibrated using trolox and results were expressed as mmol trolox Equiv/L.

The oxidative stress index (OSI) was calculated as the ratio of the TOS to the TAC: OSI (Arbitrary Unit) = TOS (μmol H2O2 Equiv/L) / TAC (mmol trolox Equiv/L) [17].

The spectroscopic measurements were performed using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Tokyo, Japan).

**Statistical methods**

All results were expressed as mean ± standard deviation (SD). Normal distribution was assessed using Shapiro-Wilk test. Statistical comparisons between the groups were made using one-way ANOVA, followed by Tukey's post hoc test. A p-values < 0.05 were regarded as statistically significant. Pearson’s and Spearman’s correlation tests were performed in order to evaluate statistical correlation. Data was analyzed using R 3.0.2 - software environment for statistical computing and graphics.
Results

We assessed the serum levels of NOx, TOS, TAC and OSI after the treatment (Table 1) and we compared the PER group with the other groups, and we also compared the groups treated with curcumin.

Table 1. Effects of Curcumin on nitro-oxidative stress parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PER</th>
<th>OC</th>
<th>OCP</th>
<th>OCPLC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx (µmol/L)</td>
<td>111.64±10.463</td>
<td>76.677±6.374</td>
<td>88.223±15.773</td>
<td>78.474±12.71</td>
<td>99.24 ±13.073</td>
</tr>
<tr>
<td>TOS (µmol Equiv H₂O₂/L)</td>
<td>43.825±7.682</td>
<td>20.983±4.588</td>
<td>27.919±10.372</td>
<td>35.376±4.301</td>
<td>32.299±3.1</td>
</tr>
<tr>
<td>TAC (mmol Equiv TROLOX/L)</td>
<td>1.089±0.001</td>
<td>1.092±0.001</td>
<td>1.09±0.003</td>
<td>1.091±0.001</td>
<td>1.09±0.001</td>
</tr>
<tr>
<td>OSI</td>
<td>0.402±0.07</td>
<td>0.192±0.041</td>
<td>0.255±0.094</td>
<td>0.324±0.039</td>
<td>0.296±0.028</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± standard deviation. NOx = total nitrites and nitrates; TOS = total oxidative status; TAC = total antioxidant capacity; OSI = oxidative stress index; PER = periodontitis group; OC = curcumin administrated orally; OCP = curcumin and piperine administrated orally; OCPLC = curcumin and piperine administrated orally associated with curcumin locally; LC = curcumin locally; values are expressed as mean ± SD of five determinations.

NOx serum levels were significantly lower in the curcumin-treated groups, OC (p<0.01), OCP (p<0.001) and OCPLC group (p<0.001), compared with PER group. Moreover, there was no significant difference between PER group and LC group (p>0.05), and between groups treated with curcumin (p>0.05) (Figure 1).

TOS serum levels were significantly lower in rats that received oral curcumin alone - the OC group (p<0.001), and oral curcumin associated with piperine - OCP group (p<0.01) compared with PER group. Furthermore, TOS was significantly higher in rats treated with oral curcumin associated with oral piperine and local curcumin compared with oral curcumin alone (p<0.05) (Figure 2).
TAC serum levels were significantly higher in rats treated with oral curcumin compared with PER group (p<0.01) and the LC group (p<0.05) (Figure 3).

OSI was significantly lower in rats treated with oral curcumin alone (p<0.001) and oral curcumin associated with piperine (p<0.01) compared with PER group. Additionally, OSI was significantly higher in rats that received oral curcumin with piperine and local curcumin compared with rats treated with curcumin alone (p<0.05) (Figure 4).

In PER group, OSI was highly correlated with TOS (r = 0.99) and TAC (r = 0.80), TOS was correlated with TAC (r = 0.81) and NOx was negatively correlated with OSI (r = −0.78), TOS (r = −0.79) and TAC (r = −0.83).

In OC group, OSI was highly correlated with TOS (r = 0.99), and was less correlated with TAC (r = 0.53) and TOS (r = 0.53).
In OCP group, there was a high correlation of OSI with TOS \((r = 0.99)\), NOx \((r = 0.70)\) and TAC \((r = 0.81)\). Additionally, NOx was correlated with TOS \((r = 0.69)\).

In OCPLC group, OSI was correlated with TOS \((r = 0.99)\) and TAC \((r = 0.60)\). Moreover, TOS was correlated with TAC \((r = 0.60)\).

In LC group, OSI was correlated with TOS \((r = 0.99)\) and was negatively correlated with NOx \((r = -0.69)\).

**Discussions**

Our study was the first to assess nitro-oxidative stress parameters as indicators of the therapeutic value of curcumin in experimental rat ligature-induced periodontitis.

During the experiment, curcumin did not affect animals’ behaviour compared with the PER group, did not have adverse effects and no animal died. These results were in agreement with other studies using doses ranging from 30 mg or 100 mg/kg b.w. [12,18] up to 500 mg curcumin/kg b.w. by gavage [19] for the treatment of periodontitis experimentally induced in rats [20,21]. Orally administered curcumin has a low bioavailability due to the very low gastrointestinal absorption, intense metabolism and increased elimination [22, 23]. Initial findings of Wahlstrom and Blennow, who studied the administration, systemic distribution and excretion of curcumin in rats, suggested that one of the reasons for the low bioavailability of oral curcumin was the high percentage (75%) of curcumin eliminated by feces, while urine concentration was negligible [24]. Taking into consideration the data provided by numerous experimental studies focusing on curcumin metabolic biotransformation [19, 25, 26], we administered a higher dose of curcumin. However, Huang et al., Ireson et al., Shoba et al. suggested that the poor absorption is partially caused by the hydrophobic status of curcumin molecule [27, 28, 29]. In other experiments, either corn oil was used as a vehicle [12], or more complex formulations were tested as an attempt to obtain the suitable viscosity and stability of curcumin suspension. Sharma et al. used a mixture of glycerol formol: cremophore: water (5:2:2), in which curcumin was suspended and partially dissolved [19]. In order to overcome this shortcoming, we used sunflower oil vehicle for the oral administration of curcumin.

Periodontitis is a chronic inflammatory process associated with local and systemic nitro-oxidative stress. Local nitro-oxidative stress is one of the mechanisms responsible for the periodontal destructions and bone resorption that might lead to teeth loss. Systemically, nitro-oxidative stress may affect other tissues and organs, afar from the periodontal inflammatory site [30]. Therefore, over the last years, the term “periodontal disease” is used in order to suggest the bi-directional relationship between periodontitis and disorders such as rheumatoid arthritis, chronic kidney dysfunction, ischemic heart disease, premature births and other [31].

Nitro-oxidative stress is induced by an excessive production of reactive oxygen species (ROS) [32], associated with increased synthesis of NO by the inducible nitric oxide synthase (iNOS) [33]. The increased production of reactive species might be associated with a decrease in antioxidant capacity. This is why the nitro-oxidative stress should be evaluated by measuring ROS, NO, and antioxidant capacity [34].

In the present study, in order to evaluate curcumin therapeutic effects in experimental ligature-induced periodontitis in rats, we employed global tests. NO synthesis was measured indirectly by the quantification of serum nitrites and nitrates [35], ROS were measured by the assessment of TOS, and antioxidant capacity was assessed by TAC [13-16,34,36]. Since the final result depends on the proportion between ROS and the anti-oxidant mechanisms, determination of OSI was necessary [16].
Serum levels of NOx, TOS, TAC and OSI are considered to be markers of the nitro-oxidative stress [37,38] and may be used to evaluate the antioxidant effect of curcumin, according to the dose or the route of administration [39]. Our results indicated that oral curcumin in doses of 1g/kg b.w. was effective in improving the nitro-oxidative stress parameters. Curcumin used as a single therapeutic agent exhibited a significant antioxidant effect by reducing NOx, TOS and OSI and by increasing TAC compared with the PER group.

Nitro-oxidative stress reduction must be appropriate, because an excessive decrease might cause a deficient immune response. This might be the consequence of the inhibition of ROS and NO formation, or the result of high doses of anti-oxidants [31]. Thus, the decrease in OSI without normalization is a positive result of the curcumin therapy.

Recent studies have demonstrated that diets rich in fruit and vegetables exert protective properties against numerous diseases not only by the effect of individual antioxidants (such as pro-vitamin A, vitamin C and E), but also by the presence of low-molecular antioxidants (such as polyphenols and anthocyanins) [33].

On the other hand, a large body of evidence demonstrated that after oral administration, curcumin undergoes extensive conjugation and reduction in the liver and gastrointestinal tract [28] and the resulting metabolites exert no pharmacological activity. [40]. Sharma et al. identified several such chemical species, including curcumin glucuronide, curcumin sulfate, hexahydrocurcumin, tetrahydrocurcumin, and dihydrocurcumin, which were found in intestinal and hepatic microsomes, and cytosol, respectively [28,41,42]. Therefore, one of the major challenges of latest experimental studies was to increase curcumin absorption and systemic bioavailability. Various curcumin-drug vehicle combinations, curcumin derivatives, and curcumin analogues were explored [23]. Shoba et al. showed that the association of various adjuvants such as piperine increased the serum concentration, extent of absorption and bioavailability of oral curcumin in rats due to the inhibitory effect of piperine on curcumin glucuronidation [29].

In our study, the combination of curcumin and piperine significantly reduced NOx, TOS and OSI compared with PER group, but failed to significantly increase TAC. Moreover, there was no significant difference between rats treated with curcumin alone and rats treated with curcumin combined with piperine. Therefore, we concluded that curcumin associated with piperine exerted no higher benefit in reducing the nitro-oxidative stress.

These results were not consistent with other studies. A possible limitation of our study was that the dose of piperine was too low to exert a significant pharmacological effect. Shoba et al. reported a significant increase in curcumin serum levels (154%) by using comparable curcumin doses (2g/kg b.w.), but higher piperine doses (20mg/kg b.w.) [29].

The correlations between the parameters revealed that in PER group, the increased nitro-oxidative stress is the result of excessive ROS and NO synthesis, associated with a deficiency in TAC. Oral curcumin induced a correlated decrease in OSI and TOS. Curcumin associated with piperine significantly improved OSI correlation with TOS, NO and TAC. This result suggested that piperine association to curcumin is recommended even if there were no significant differences between NOx, TOS and OSI of OC and OCP groups.

For the local delivery, we used curcumin included in a muco-adhesive gel that was adherent to the oral mucosa and maintained for a prolonged period, thus enabling an increased release of active ingredients into the gingival tissues. Other experimental studies focusing on locally delivered curcumin assessed the therapeutic efficiency by clinical parameters. Hosadurga et al. used a 2% curcumin gel and reported a reduction of the gingival index and probing pocket depth, but no effect on the alveolar bone loss [43]. Our local curcumin treatment had no important inhibitory effect on the nitro-oxidative stress parameters.
Curcumin effect on nitro-oxidative stress in ligature-induced rat periodontitis

compared with the PER group. In OCPLC, association of local treatment did not improve nitro-oxidative stress tests correlation. Furthermore, we associated the local delivery with the oral administration of curcumin and piperine. That combined therapy did not improve OCP effects. These findings indicated that the limitations of this formulation may reside in the low amount of curcuma powder incorporated in the muco-adhesive gel, the hydrophobic properties of curcumin and the viscosity of the gel matrix.

Further research is needed in order to assess the systemic effect of higher oral doses of curcumin and piperine. Efficiency of local delivery could be improved by using formulations with higher curcumin content, multiple daily applications and prolonged treatment interval.

Conclusions
Orally administrated curcumin, either alone or associated with piperine significantly reduced the systemic nitro-oxidative stress in rat experimentally induced periodontitis. Curcumin alone had a better antioxidant effect than the combined therapy, curcumin and piperine. Local curcumin treatment did not influence the serum nitro-oxidative stress parameters and showed no supplementary antioxidant effect when added to oral curcumin.

Abbreviations: DPPH - 1,1-diphenyl-2-picrylhydrazyl; NEDD - N-(1-Naphthyl) ethylenediamine dihydrochloride; NOx - total nitrates and nitrates; OSI - oxidative stress index; ROS - reactive oxygen species; SULF - diethyl ether sulphanilamide; TAC - total antioxidant capacity; TOS - total oxidative status.

Acknowledgement
This paper was published under the frame of European Social Found Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/138776.

References


Curcumin effect on nitro-oxidative stress
in ligature-induced rat periodontitis


