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Research Paper

Effects of verbascoside, biotechnologically purified by *Syringa vulgaris* plant cell cultures, in a rodent model of periodontitis

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Abstract

Objectives Verbascoside has previously been characterized as an effective scavenger of active free radicals and an inhibitor of lipid peroxidation. In the present study, we have investigated the effects of verbascoside from *Syringa vulgaris* in a rat model of ligature-induced periodontitis.

Methods Male Sprague–Dawley rats were lightly anaesthetized with pentobarbitone (35 mg/kg). Sterile, 2-0 black braided silk thread was placed around the cervix of the lower left first molar and knotted medially. Animals received vebascoside 2 mg/kg orally, daily for 8 days.

Key findings On the eighth day after placement of the ligature, we evaluated several markers of inflammation: (i) myeloperoxidase activity, (ii) thiobarbituric acid-reactant substance measurements, (iii) NF-κB expression, (iv) iNOS expression, (v) the nitration of tyrosine residues, (vi) activation of the nuclear enzyme poly(ADP-ribose) polymerase, (vii) Bax and Bcl-2 expression and (viii) a degree of gingivomucosal tissue injury. Oral administration of verbascoside (2 mg/kg daily for 8 days) significantly decreased all of the parameters of inflammation as described above.

Conclusions These results demonstrate that verbascoside exerts an anti-inflammatory role during experimental periodontitis and is able to ameliorate the tissue damage associated with ligature-induced periodontitis.

Keywords alveolar bone loss; apoptosis; periodontal diseases; reactive oxygen species; verbascoside

Introduction

Human periodontal diseases are inflammatory disorders that give rise to damage in the surrounding cells and connective tissue structures, including the alveolar bone where it causes tooth loss.[1] It has been demonstrated that the most frequent cause of periodontitis are bacteria.[2] The toxins, enzymes and metabolites of the bacteria present in the dental plaque play a key role in the initiation and in a further enhancement of the inflammatory process.[3] The inflammatory process is characterized by several events such as recruitment of leukocytes into the tissues and the gingival crevice, the release of oxidizing enzyme such as myeloperoxidase, an indicator of polymorphonuclear leukocyte (PMN) accumulation and release of reactive oxygen species (ROS) from activated leukocytes.

The progression of chronic periodontitis is a continuous process that undergoes periods of acute exacerbation.[4] The established lesion is dominated by plasma cells, which are situated primarily in the coronal connective tissues as well as around vessels. Collagen loss continues in both lateral and apical directions as the inflammatory cell infiltrate expands, resulting in collagens extending spaces deeper into the tissues, which are then available for leukocyte infiltration.[5] In recent years, more attention has been focused on the role of reactive oxygen species, lipid peroxidation products and antioxidant systems in the pathology of periodontitis. Recent medical and dental research in this area is geared towards the

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prevention of free-radical-mediated diseases by using specific nutrient antioxidants.[6] Elevated lipid peroxidation and disturbed antioxidant status have been reported in experimental periodontitis.[7]

Phenylpropanoid glycosides (PPGs; also synonymous of phenylethanoid glycosides) are water-soluble derivatives of phenylpropanoids (PPs), a large group of natural polyphenols widely distributed in the plant kingdom.[8] There is growing evidence that PPGs, like other plant polyphenols in general and PPs in particular, are powerful antioxidants, either by direct scavenging of reactive oxygen and nitrogen species or by acting as chain-breaking peroxyl radical scavengers.[9] Recently, PPs have been reported to be effective in the chemoprevention of tumours[8] as well as to have anti-thrombotic, wound healing and cardio-protective actions.[10] Verbascoside (VB) belongs to the PPG group and is structurally characterized by caffeic acid (phenylpropanoid moiety) and 4,5 hydroxyphenylethanol (phenylethanoid moiety) bound to a β-(D)-glucopyranoside. VB contains a rhamnose unit bound to glucose, which acts as a bridge, and it belongs to the extensive family of phenylpropanoids, natural molecules that are water soluble and widely found in the plant kingdom and which have been mainly isolated from medicinal plants. Recently it has been demonstrated that VB promotes skin repair and ameliorates skin inflammation due to their ROS-scavenging, antioxidant, iron-chelating and glutatione-S-transferasi (GST)-inducing properties.[11]

There is now growing interest in the biotechnological approach to produce plant-derived active substances using non-genetically modified plant cell cultures.[12] However, the industrial development and utilization of PPGs for medicinal use is limited because the chemical synthesis of these compounds is extremely complex and expensive. Their extraction from mature plants has a very low yield, the final PPG-containing products are poorly standardized due to unavoidable variations in the plant growth conditions and they are often contaminated with environmental pollutants. On the other hand, plant cell cultures derived from medicinal plants are perfect sources of PPGs, biosynthesis of which could be specifically induced and directed to a certain compound depending on the nature of plant cells and a stimulus used.

The aim of the present study was to evaluate the anti-inflammatory and antiapoptotic mechanisms of action of the VB biotechnologically produced by *Syringa vulgaris* plant cell cultures, in an experimental model of ligature-induced periodontitis. In the present study, we evaluated several markers of inflammation such as the degree of gingivomucosal tissue injury (histology) in rats subjected to ligature-induced periodontitis, NF-κB expression, iNOS expression and expression of proteins that regulate programmed cell death and apoptosis such as Bax and Bcl-2. Our results demonstrate that in a well-characterized experimental model of periodontitis in rats, VB ameliorates the inflammatory process associated with periodontal disease.

Materials and Methods

Plant cell line

The *Syringa vulgaris* plant utilized by Istituto Ricerche Biotecnologiche (IRB) to originate the cell line derives from the Botanical Garden of the University of Bologna, Bologna, Italy.

The stabilized and highly selected cell line was obtained from dissected young *Syringa vulgaris* leaves sterilized by NaOCl and Tween 20. The stabilized and selected cell line was deposited at the Plant Cell Bank (DSMZ, Deutsche Sammlung Von Mikroorganismen und Zellkulturen, Braunschweig, Germany) coded internally IRBSV25/B and internationally DSM 16857. The IRBSV25/B plant cell line was used for the industrial culture fermenters.

Verbascoside containing extract preparation

Syringa vulgaris IRBSV25/B cell cultures obtained at the end of the fermentation process were collected and mechanically homogenized by Ultraturrax. The solid residue, mainly cellular debris, was then separated from the aqueous phase containing verbascoside by centrifugation at 1000*g* for 10 min. The yield of verbascoside was approximately 3 g/l of the plant cell culture liquid suspension. The verbascoside in the supernatant was recovered by solid phase extraction on XAD4 resin, followed by elution with 80/20 ethanol/water (v/v) mixture. Then, the eluted verbascoside was concentrated under reduced pressure and liophylized. The final extract, a pale yellow powder, contained verbascoside in an proportion over 80% (w/w), together with minor admixture (>10% w/w) of other caffeic acid derivatives. The further purification of verbascoside was performed by repeated column chromatography on C18 silica gel and Sephadex LH20 and subsequent crystallization, obtaining a final product with verbascoside content above 97% (w/w). The standard raw extract with a purity of 50 ± 1% (w/w) was obtained from the 80% (w/w) cell extract by addition of maltodextrins.

HPLC analysis

The analysis was performed using an HPLC system (Agilent, series 1100 DAD, Hewlett-Packard) consisting of an auto sampler, high-pressure mixing pump and with a C18 (2) Phenomenex 4.6 × 150 mm column. The gradient system was Phase A water/0.01 N phosphoric acid, Phase B acetonitryl/0.01 N phosphoric acid. The flow rate was 1 ml/min. UV maximum absorbance of the DAD was at 330 nm. The retention time for verbascoside was 5.493 min.

The reference product used to set up the quantitative method was internally prepared by IRB. The chemical structure of the product was determined and confirmed by means of MS and NMR at the Department of Organic Chemistry at the University of Milan. The NMR spectra were recorded in CD3OD solvent on a Bruker Avance 400 instrument. The calibration curve of the verbascoside was in good linearity over the range 20–150 μg/ml ($r = 0.9997$) and the average recoveries of verbascoside was 99.2% ($n = 5$, RSD 0.23%).

Animals

Male Sprague–Dawley rats (280–400 g, Harlan Nossan, Milan, Italy) were used for all studies. Rats were housed in individual cages (five for each group) and maintained under a 12 : 12 light–dark cycle at 21 ± 1°C and 50 ± 5% humidity. The animals were acclimated to their environment for 1 week and had ad-libitum access to tap water and standard rodent standard diet. All animal experiments complied with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1

12/18/1986) and the USA (Animal Welfare Assurance No. A5594-01, Department of Health and Human Services, USA). All behavioral testing was conducted in compliance with the NHI laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission # 92-256 to SC). The study was approved by the University of Messina Review Board for the care of animals.

Surgical procedure

Male Sprague–Dawley rats (280–400 g) were lightly anaesthetized with surgical doses of sodium pentobarbitone (35 mg/kg). Sterile, 2-0 black braided silk thread was placed around the cervix of the lower left first molar and knotted medially as previously described.[7] After the rats had recovered from the anaesthetic they were allowed to eat commercial laboratory food and drink tap water *ad libitum*.

Experimental groups

Rats were randomly allocated into the following groups: *Ligature + vehicle group* rats were subjected to ligature-induced periodontitis and animals received vehicle orally (1 h after the ligature placement and daily treatment for eight days); *Ligature + vebascoside group:* rats were subjected to ligature-induced periodontitis and animals received vebascoside (2 mg/kg orally, 1 h after the ligature placement and daily for 8 days).

At 8 days after induction via ligature of periodontitis the rats ($n = 10$ from each group for each parameter) were sacrificed in order to assess the effects of the compound on an acute lesion. The right side, which was not subject to ligature, was used as control. The dose of verbascoside (2 mg/kg orally) was chosen on the basis of previous studies.[13]

Measurement of vascular permeability by Evans blue extravasations

Vascular permeability was determinate as previously described.[14] Briefly, animals received Evans blue (2.5% dissolved in physiological saline) at a dose of 50 mg/kg via a femoral venous catheter. Extravasated Evans blue in the excised gingivomucosal tissue samples was extracted with 1 ml formamide for 48 h at room temperature for spectrophotometric determination at 620 nm. Results were expressed as micrograms per gram of gingivomucosal tissue.

Measurement of alveolar bone loss

In the same set of experiments, the distance from the cementoenamel junction of first lower molars to the alveolar crest was measured with a modification of the method by Crawford *et al*.[15] Recordings were made along the median axis of the lingual surface of the mesial and mediolingual roots of the lower first left and right molars as previously described.[7] These measurements were performed by an independent investigator who was unaware of the treatment regimens. The alveolar bone loss induced by the ligature was expressed as a difference between the left and the right side.

Histological examination

For histopathological examination, biopsies of gingival and mucosal tissue from the buccal and lingual aspect of the teeth were taken 8 days after the induction of periodontitis by ligature. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin and sectioned. The sections, orientated longitudinally from the teeth crowns, were stained with trichrome and haematoxylin eosin stains. The total number of infiltrating leukocytes (e.g. neutrophils and mononuclear cells) in cortical interstitial spaces from gingival and mucosa tissues were assessed quantitatively by counting the number of polymorphonuclear cells in 20 high-power fields.

Radiography

Mandibles were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and legated mandibles was performed by X-ray (Philips X12 Germany) with a 40 kW exposure for 0.01 s. A radiographic examination at 8 days after ligature placement revealed bone matrix resorption in the lower first left molar as previously described.[7]

Malondialdehyde measurement

Malondialdehyde (MDA) levels in the gingivomucosal tissue were determined as an indicator of lipid peroxidation as previously described.[16] Gingivomucosal tissue, collected at the specified time, was homogenized in 1.15% (w v1) KCl solution. A 100 µl aliquot of the homogenate was added to a reaction mixture containing 200 µl of 8.1% (w v1) sodium dodecyl sulphate, 1.5 ml of 20% (v v1) acetic acid (pH 3.5), 1.5 ml of 0.8% (w v1) thiobarbituric acid and 700 µl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000*g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm.

Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described.[17] Gingivomucosal tissue, collected at the specified time, was homogenized in a solution containing 0.5% hexadecyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20 000*g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol/min of peroxide at 37°C and was expressed in milliunits per gram of wet tissue.

Immunohistochemical localization of iNOS, nitrotyrosine and ADP-ribose

Immunohistochemical localization of iNOS, nitrotyrosine and ADP-ribose (PAR) in the gingivomucosal tissue was determined as previously described.[7]

Western blot analysis for IkB-α, NF-κB p65, iNOS, bax and Bcl-2

In brief, gingivomucosal tissues from each rat were suspended in extraction buffer A containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 μM pepstatin A, 20 μM leupeptin and 1 μM sodium orthovanadate, homogenized at the highest setting for 2 min and centrifuged at 1000*g* for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets containing enriched nuclei were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 M leupeptin and 0.2 mM sodium orthovanadate. After centrifugation at 15 000*g* for 30 min at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. Protein concentration was determined with the Bradford-based kits (Bio-Rad, Milan, Italy). The levels of IkB-α, iNOS, Bax and Bcl-2 were quantified in cytosolic fraction, while NF-κB p65 levels were quantified in nuclear fractions. Proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred on PVDF membranes (Hybond-P, Amershan Biosciences, UK). The membranes were blocked with 1 × PBS and 5% (w/v) non-fat dried milk for 40 min at room temperature, and subsequently probed with specific antibodies IkB-α (1 : 1000; Santa Cruz Biotechnology, Inc. DBA s.r.l. Milan, Italy), anti-iNOS (1 : 500; Santa Cruz Biotechnology, Inc. DBA s.r.l. Milan, Italy), anti-Bax (1 : 500), anti-Bcl-2 (1 : 500 Santa Cruz Biotechnology, Inc. DBA s.r.l. Milan, Italy) or anti-NF-κB p65 (1 : 1000; Santa Cruz Biotechnology, Inc. DBA s.r.l. Milan, Italy) in 1 × PBS, 5% (w/v) non-fat dried milk and 0.1% Tween 20 at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine antimouse IgG secondary antibody or peroxidase-conjugated goat antirabbit IgG (1 : 2000; Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. The relative expression of the protein bands was quantified by densitometric scanning of the X-ray films with GS-700 imaging densitometer (GS-700; Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst, IBM, Milan, Italy).

Materials

Primary anti-nitrotyrosine (Upstate Biotech cod 06-284 DBA s.r.l. Milan, Italy), anti-iNOS (sc-8310, Santa Cruz DBA s.r.l. Milan, Italy), anti-PAR (sc-1561 Santa Cruz DBA s.r.l. Milan, Italy), IkB-alpha (sc-371 Santa Cruz DBA s.r.l. Milan, Italy), NF-kBp65 (sc-8008, Santa Cruz DBA s.r.l. Milan, Italy), Bax (sc-526, Santa Cruz DBA s.r.l. Milan, Italy), Bcl-2 (sc-492, Santa Cruz DBA s.r.l. Milan, Italy), iNOS (#610432 BD Transudction Laboratories DBA s.r.l. Milan, Italy) was obtained from (DBA, Milan, Italy). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of *n* observations, where *n* represents the number of animals studied. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Bonferroni or Student's unpaired *t*-test. A *P* value less than 0.05 was considered significant.

Results

Effects of verbascoside treatment on NF-κB activation in periodontitis

A basal level of IκB-α was detected in the gingivomucosal tissues from the contralateral side, whereas in the gingivomucosal tissues from ligature-operated rats IκB-α levels were substantially reduced (Figure 1a, a1). VB treatment prevented the ligature-induced IκB-α degradation in the gingivomucosal tissue at 8 days following ligation (Figure 1a, a1).

Furthermore, ligature caused a significant increase of NF-κB p65 subunits in the nuclear fractions from gingivomucosal tissues from ligature-operated rats compared to the gingivomucosal tissues from the contralateral side (Figure 1b, b1). A significant reduction of the NF-κB p65 nuclear levels was observed in the tissues from VB-treated rats (Figure 1b, b1).

Effects of verbascoside on iNOS expression in periodontitis

Sections of gingivomucosal tissues from the contralateral side from VB-treated rats did not reveal any immunoreactivity for iNOS (Figure 2a see densitometry D) within the normal architecture. At 8 days following ligation, a positive staining for iNOS (Figure 2b see densitometry D) was found in the gingivomucosal tissues from ligature-operated rats. VB abolished the staining for iNOS (Figure 2c see densitometry D). No positive staining for iNOS was found in gingivomucosal tissues from the contralateral side from vehicle-treated rats (data not shown). Furthermore, ligature caused a significant increase of iNOS expression, as assayed by Western blot analysis, in the gingivomucosal tissues from ligature-operated rats compared to the gingivomucosal tissues from the contralateral side (Figure 2e, e1). A significant reduction of the iNOS levels was observed in the tissues from VB-treated rats (Figure 2e, e1).

Effects of verbascoside on nitrotyrosine formation, lipid peroxidation and poly (ADP-ribose) polymerase activation in periodontitis

Nitrotyrosine, a specific marker of NO-dependent oxidative stress, was measured by immunohistochemical analysis in the gingivomucosal tissue sections to determine the localization of 'peroxynitrite formation' and/or other reactive nitrogen derivatives produced during experimental periodontitis. Sections of gingivomucosal tissues from the contralateral side from VB-treated rats did not reveal any immunoreactivity for nitrotyrosine (Figure 3a see densitometry H) within the normal architecture. At 8 days following ligation, a positive staining for nitrotyrosine was found in the gingivomucosal tissues from ligature-operated rats (Figure 3b see densitometry H). VB treatment abolished the staining for nitrotyrosine (Figure 3c see densitometry H). No positive staining for nitrotyrosine was found in gingivomucosal tissues from the contralateral side from vehicle-treated rats (data not shown). In

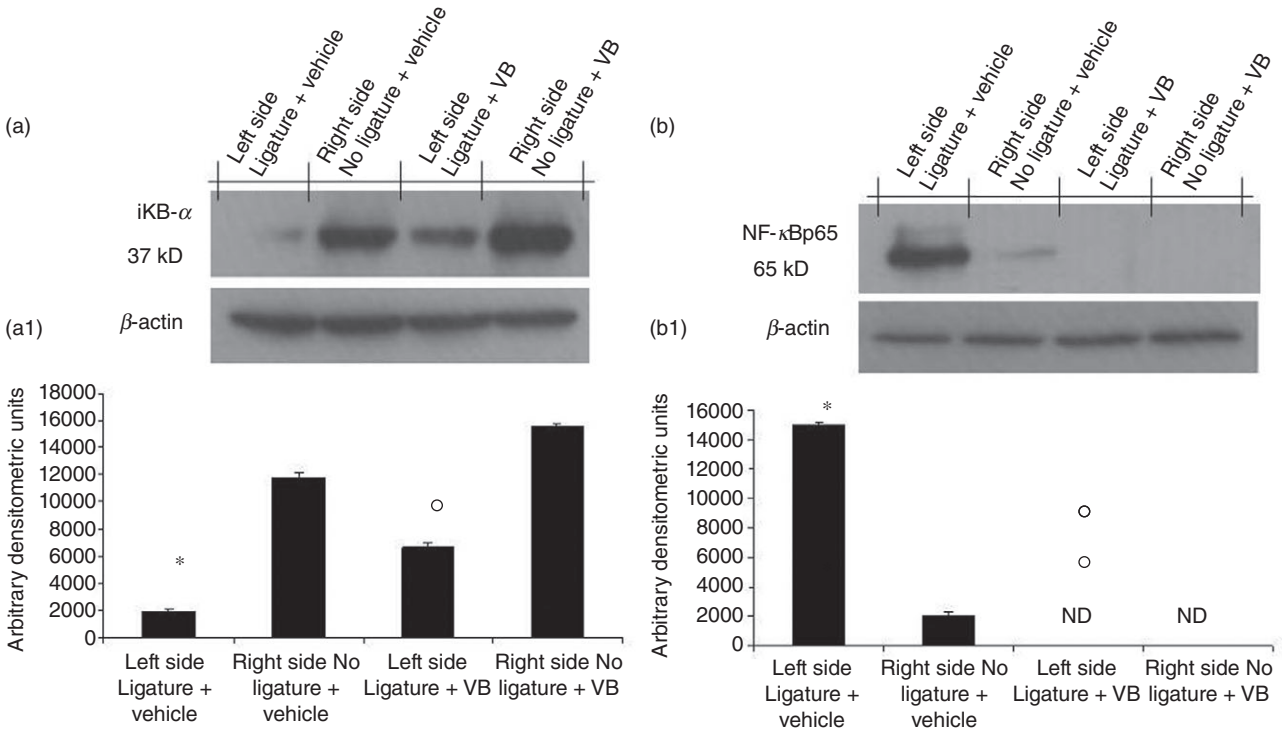

Figure 1 Effects of VB treatment on NF-κB activation. Representative Western blots showing the effects of VB on (a and a1), IκB-α degradation and (b and b1) NF-κB p65 subunit in the nuclear fractions in the gingivomucosal tissues from ligature-operated rats. A representative blot of lysates (panels a and b) obtained from five animals per group is shown and densitometry analysis of all animals is reported. The results in panels a1 and b1 are expressed as mean ± SEM from $n = 5/6$ gingivomucosal tissues for each group. *$P < 0.01$ vs non-ligated. °$P < 0.01$ vs ligated.

addition, at 8 days following ligation a significant increase in levels of MDA, a marker for oxidative stress, was observed.

Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress and lipid peroxidation in cells. MDA levels observed in the gingivomucosal tissues (Figure 3d), when compared with levels in the gingivomucosal tissues from the contralateral side, (Figure 3d) were significantly attenuated by the daily treatment with VB.

We also evaluated the formation of polymer of PAR, which is synthesized by PAR polymerases (PARPs) from NAD(+). PAR is an indicator of in-vivo PARP activation and regulates cell survival and cell-death programmes. Immunohistochemistry for PAR revealed the occurrence of positive staining for PAR in the gingivomucosal tissues from ligature-operated rats (Figure 3f see densitometry H). VB treatment reduced the degree of positive staining for PAR (Figure 3g see densitometry H) in the gingivomucosal tissues. Note that no positive staining for PAR was found in gingivomucosal tissue sections from the contralateral side from VB-treated rats (Figure 3e see densitometry H) and from vehicle-treated rats (data not shown).

Effects of verbascoside on plasma extravasation and neutrophils infiltration in periodontitis

Before the measurement of Evans blue extravasation, the mean arterial pressure of vehicle-treated and VB-treated animals was recorded. VB treatment did not affect the mean arterial blood pressure (vehicle-treated 126 + 6 mmHg, $n = 10$; VB-treated 122 + 4 mmHg, $n = 10$). Ligation significantly increased Evans blue extravasation in gingivomucosal tissue compared to the contralateral side (Figure 4b). VB treatment prevented this increase in Evans blue extravasation, but did not change the Evans blue content of the contralateral side (Figure 4b). Myeloperoxidase activity was significantly elevated ($P < 0.001$) at 8 days after ligation (Figure 4a) and VB-treatment significantly reduced these levels (Figure 4a). No significant change in myeloperoxidase activity was observed in the gingivomucosal tissues from the controlateral side (Figure 4a).

Effect of verbascoside on tissue damage

When compared to gingivomucosal tissue sections taken from the controlateral side from VB-treated rats (Figure 4c), histological examination of gingivomucosal tissue sections of ligature-operated rats showed oedema, tissue injury, as well as infiltration of the tissue with inflammatory cells (Figure 4d). VB treatment reduced the degree of gingivomucosal tissue injury (Figure 4e). Quantification of infiltrating polymorphonuclear cells into the gingivomucosal tissue showed that there were only a minimal number of polymorphonuclear cells in tissue from the controlateral side from VB-treated rats (Figure 4f). No pathological signs were observed in gingivomucosal tissue sections from the controlateral side from

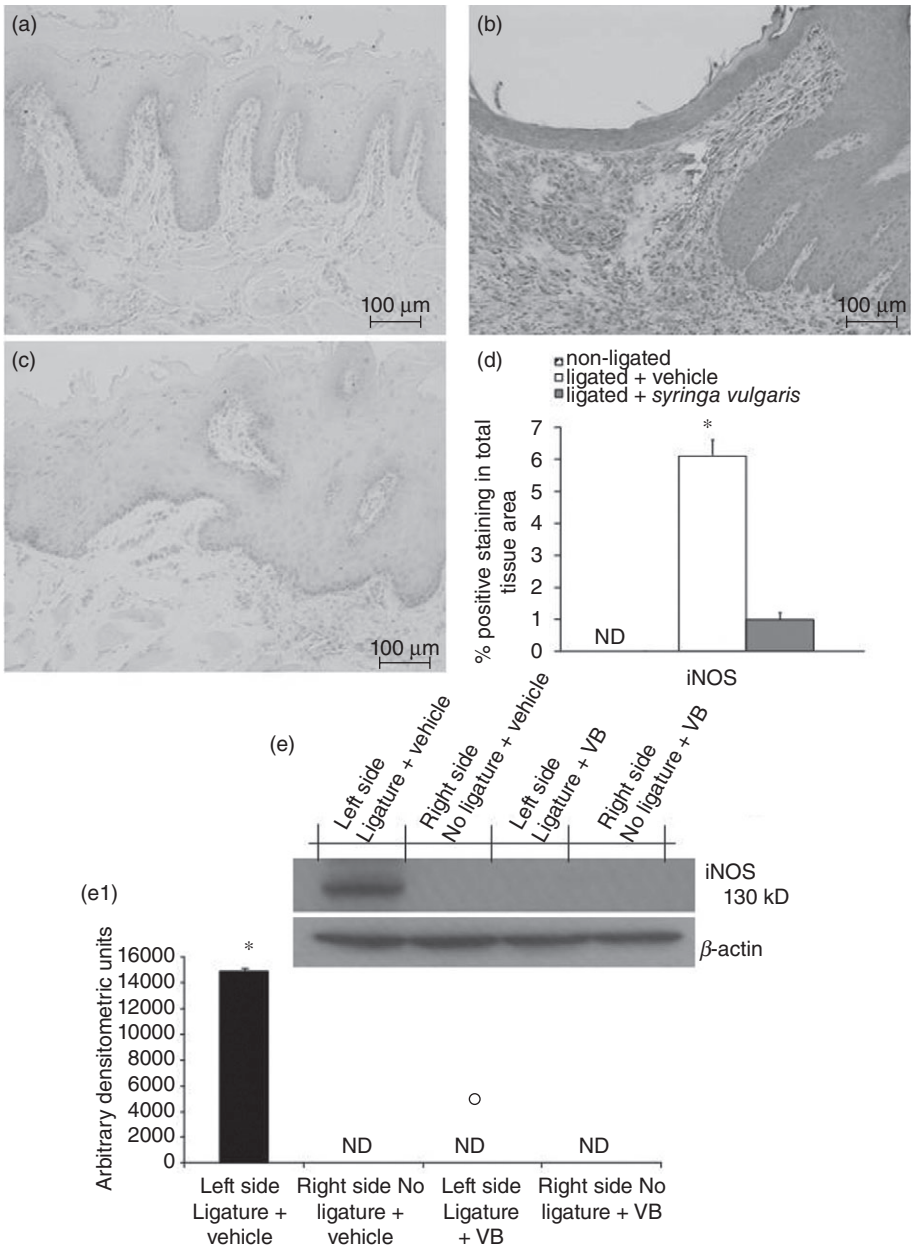

Figure 2 Effects of VB on iNOS expression. Sections of gingivomucosal tissues from the contralateral side of VB-treated rats did not reveal any immunoreactivity for iNOS (a). Positive staining for iNOS (b) was observed in gingivomucosal tissue after ligature. In gingivomucosal tissue of VB treated rats no positive staining was observed for iNOS (c). Densitometry analysis of immunocytochemistry photographs (d; $n = 5$ photos from each samples collected from all rats in each experimental group) for iNOS from gingivomucosal tissue was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Densitometry data are expressed as a percentage of total tissue area. Moreover, a significant increase in iNOS expression (e, e1), assayed by Western blot analysis, was detected in gingivomucosal tissue after ligature. Treatment with VB significantly attenuated iNOS (e, e1) expression in gingivomucosal tissue after ligature. A representative blot of lysates (e, e1) obtained from five animals per group is shown and densitometry analysis of all animals is reported. The figure is representative of at least three experiments performed on different experimental days. *$P < 0.01$ vs non-ligated. °$P < 0.01$ vs ligated.

vehicle-treated rats (data not shown). However, a large number of infiltrating polymorphonuclear cells were observed in the gingivomucosal tissue of ligated rats (Figure 4f). VB administration significantly reduced the numbers of polymorphonuclear cells infiltrating into the gingivomucosal tissue (Figure 4f).

Effect of verbascoside on bone destruction

A radiographic examination of the mandibles, at 8 days after ligature placement, revealed bone matrix resorption in the lower left first molar region after ligation (Figure 5a). There was no evidence of pathology in right first molar (data not

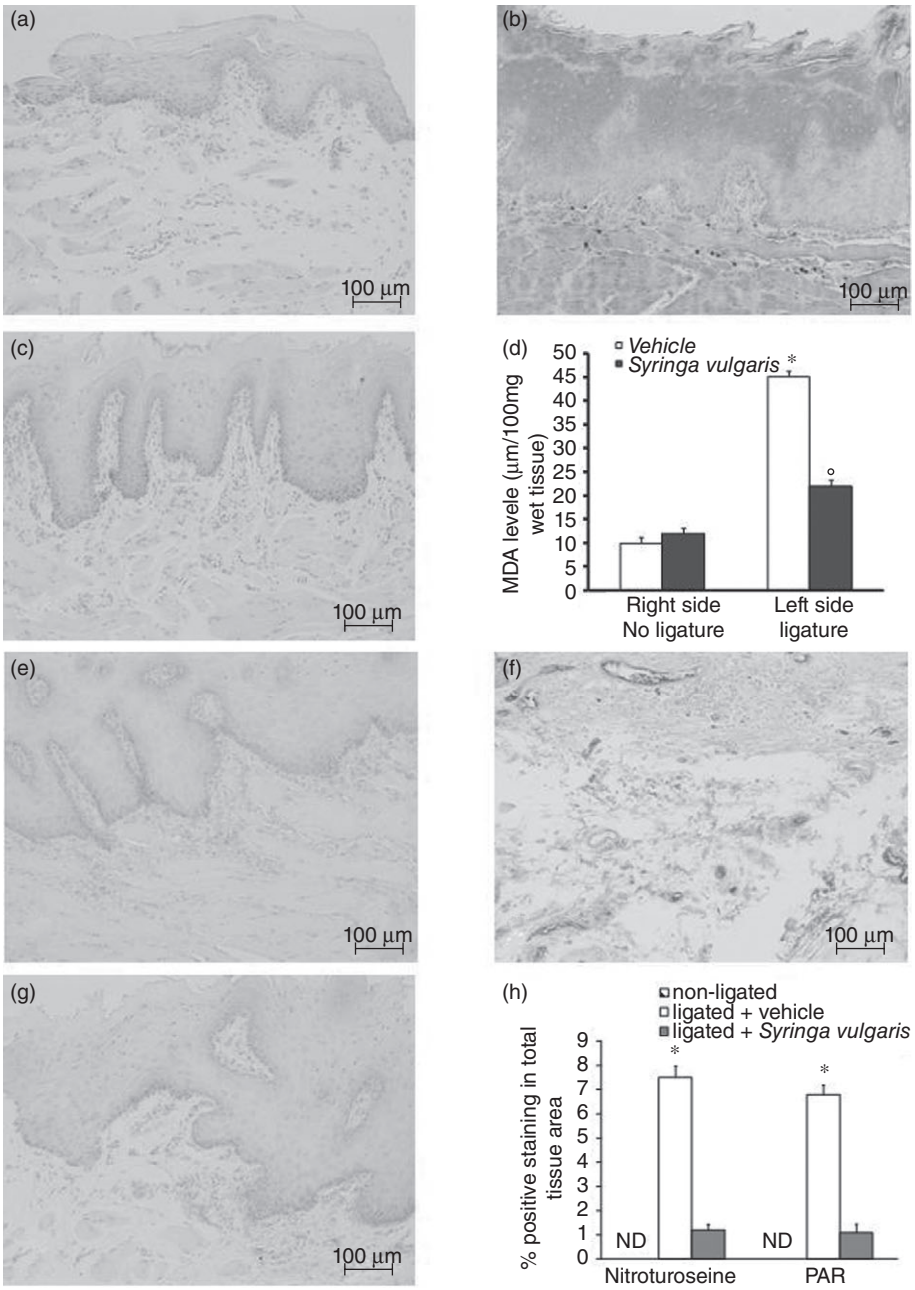

Figure 3 Effect of VB on ligature-induced nitrotyrosine formation, lipid peroxidation and PARP activation. No positive staining for nitrotyrosine was observed in gingivomucosal tissues from the contralateral side from VB-treated rats (a). Positive staining for nitrotyrosine (b) was observed in gingivomucosal tissue after ligature. In gingivomucosal tissue of VB treated rats no positive nitrotyrosine staining was observed (c). In addition, malondialdehyde (MDA) levels, an index of lipid peroxidation, were significantly increased in gingivomucosal tissue after ligature lung (d). VB significantly reduced the ligature-induced elevation of MDA tissues levels (d). In addition, immunohistochemistry for PAR, an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in gingivomucosal tissue after ligature (f). VB treatment reduced the degree of positive staining for PAR (g) in the gingivomucosal tissue. No positive staining for PAR was observed in gingivomucosal tissues from the contralateral side from VB-treated rats (e). Densitometry analysis of immunocytochemistry photographs (h; $n = 5$ photos from each samples collected from all rats in each experimental group) for nitrotyrosine and PAR from gingivomucosal tissue was performed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Densitometry data are expressed as percentage of total tissue area. The figure is representative of at least three experiments performed on different experimental days. $^{*}P < 0.01$ vs non-ligated. $^{\circ}P < 0.01$ vs ligated.

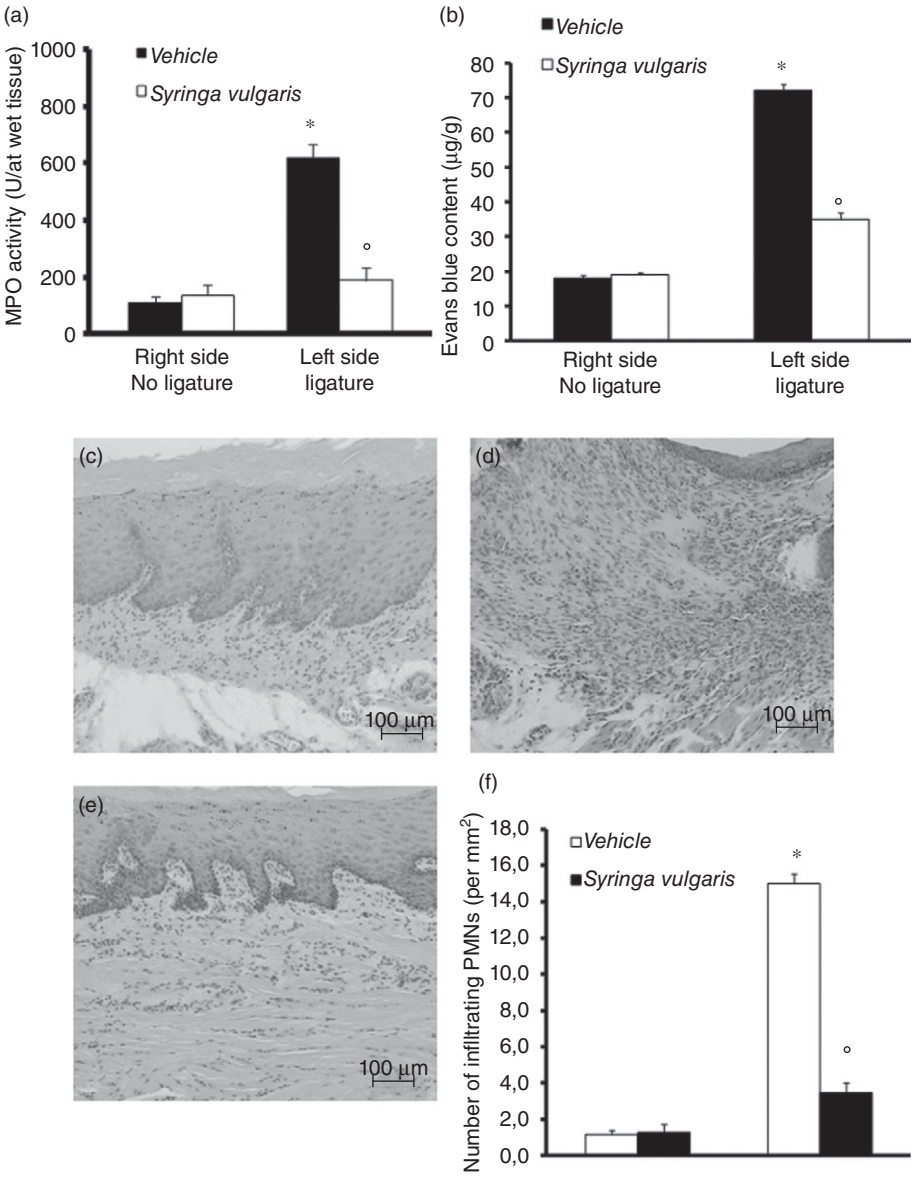

Figure 4 Effects of VB on plasma extravasation, neutrophils infiltration and tissue damage. Myeloperoxidase activity (a) and Evans blue content (b) in gingivomucosal tissue were significantly increased by ligature compared to the contralateral side. VB significantly reduced Evans blue content and myeloperoxidase activity levels. Moreover, the gingivomucosal section from the contralateral side obtained from VB-treated rats (c) demonstrated no tissue damage. Inflammatory cell infiltration and oedema were observed in gingivomucosal sections from ligature-treated rats (d). Significantly less oedema and inflammatory cell infiltration were observed in gingivomucosal sections from ligature-treated rats that had been treated with VB (e). The total number of infiltrating leukocytes (e.g. neutrophils and mononuclear cells) in gingivomucosal tissue was assessed quantitatively with thricromic stain by counting the number of polymorphonuclear cells in 20 high-power fields (f). The figure is representative of at least three experiments performed on different experimental days. Data represent the mean ± SEM for 20 counts obtained from the gingivomucosal tissue of each treatment group. *$P < 0.01$ vs non-ligated. °$P < 0.01$ vs ligated.

shown). VB markedly reduced the degree of bone resorption in the lower left first molar region after ligation (Figure 5b). In addition, a significant alveolar bone loss, between the lower first left and the right first molars induced by the left side ligature, was observed in vehicle treated rats (Figure 5c). VB treatment resulted in a significant inhibition of alveolar bone loss after ligation (Figure 5c).

Effects of verbascoside on Bax and Bcl-2 expression

A basal level of Bax was detected in the gingivomucosal tissues from the contralateral side (Figure 5d, d1). Bax levels were substantially increased in the gingivomucosal tissues at 8 days following ligation (Figure 5d, d1). On the other hand, VB

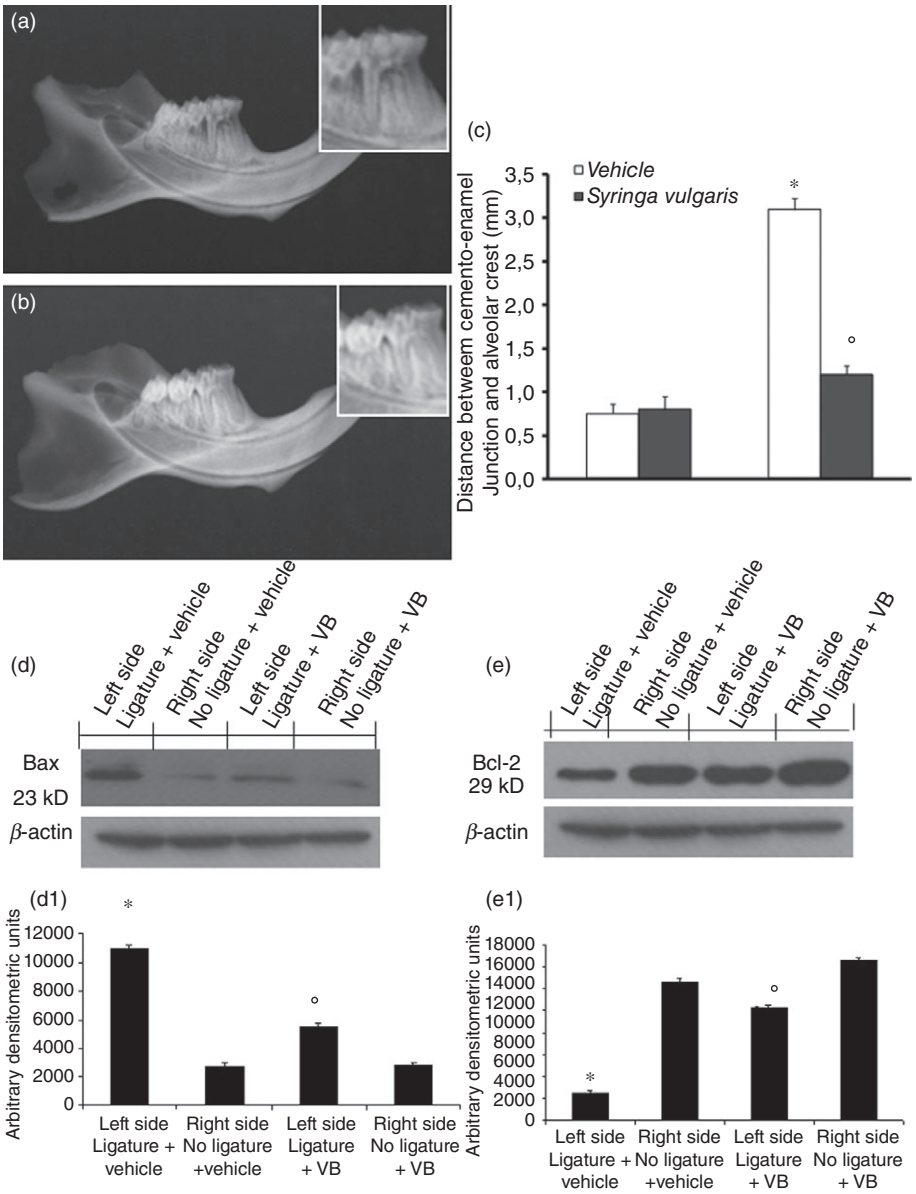

Figure 5 Effect of VB on bone destruction and on apoptosis. The alveolar bone from ligated (8 days) rats demonstrated alveolar bone resorption (a). VB treatment suppressed alveolar pathology in the rat alveolar bone (b). A significant increase in the distance between cementoenamel injunction and alveolar crest at mediolingual root of the first molar was observed in ligature-treated rats. VB treatment significantly reduced the increase in the distance between the cemento-enamel injunction and alveolar crest. The radiographic figure is representative of at least three experiments performed on different experimental days. Representative Western blots showing the effects of VB on Bax (d, d1) and Bcl-2 (e, e1) expression in the gingivomucosal tissues from ligature-operated rats are also shown. A representative blot of lysates (panel d and e) obtained from five animals per group is shown and densitometry analysis of all animals is reported. The results in panels d1 and e1 are expressed as mean ± SEM from $n = 5/6$ in the gingivomucosal for each group. *$P < 0.01$ vs sham group. *$P < 0.01$ vs non-ligated. °$P < 0.01$ vs ligated. Data represent the data from 20 counts obtained from the gingivomucosal tissue of each treatment group. *$P < 0.01$ vs non-ligated. °$P < 0.01$ vs ligated.

treatment prevented the ligation-induced Bax expression (Figure 5d, d1). To detect Bcl-2 expression, whole extracts from gingivomucosal tissues of rats were also analysed by Western blot analysis. A basal level of Bcl-2 expression was detected in the gingivomucosal tissues from the contralateral side (Figure 5e, e1). At 8 days following ligation, Bcl-2 expression was significantly reduced (Figure 5e, e1). Treatment of rats with VB (mg/kg) significantly attenuated ligation-induced inhibition of Bcl-2 expression (Figure 5e, e1).

Discussion

In the present study, we demonstrate that VB reduces (i) the development of ligature-induced periodontitis, (ii) the

infiltration of the gingivomucosal tissues by polymorphonuclear cells, (iii) NF-κB expression, (iv) iNOS expression, (v) the nitration of tyrosine residues, (vi) PARP-1 activation, (vii) Bax and Bcl-2 expression, and (viii) the degree of gingivomucosal tissue injury (histology) in rats subjected to ligature-induced periodontitis. All of these findings support the view that VB attenuates the degree of experimental periodontitis in the rat.

Our results demonstrate that VB exerts a significant inhibitory effect on plasma extravasation during periodontitis. Our study also confirms earlier findings that one of the characteristic signs of inflammation, Evans blue extravasation, is higher on the ligated side on the eighth day than on the opposite side.[14]

Although radiography is not a perfect diagnostic tool, partly because the X-rays are two-dimensional representations of three-dimensional structures, in part because particular clinical and biological characteristics do not translate into changes in radiographic features, a thorough radiographic examination, performed with the same contrast, density and angle, allowed us to determine that ligature induces significant alveolar bone resorption, as measured at 8 days, an effect which was blocked by VB treatment.

In addition, we also report in the present study that ligature-induced peridontitis in the rat results in a significant infiltration of inflammatory cells into the gingivomucosal tissues and we also demonstrated that treatment with VB reduces this inflammatory cell infiltration as assessed by myeloperoxidase, with moderation of the tissue damage as evaluated by histological examination. Neutrophils are recruited into the tissue and can then contribute to tissue destruction by the production of reactive oxygen metabolites that further amplify the inflammatory response by their effects on macrophages and lymphocytes.[18] A possible mechanism by which VB attenuates polymorphonuclear cell infiltration is by down-regulating adhesion molecules ICAM-1 and P-selectin, as previously demonstrated.[13] These findings are in accordance with those of Berglundh and Lindhe,[19] who also found a significant increase in inflammatory cell infiltration in inflamed gingiva as compared to a healthy sample.

Several cellular mechanisms, including the mode of gene regulation and signal transduction, may account for the anti-inflammatory effect of VB. Recent evidence suggests that the activation of NF-κB may also be under the control of the oxidant/antioxidant balance.[20] NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation.

We report here that ligation causes a significant increase in the NF-κB p65 subunit in the nuclear fractions from gingivomucosal tissues at 8 days, whereas treatment with VB significantly reduces this increase. Moreover, we also demonstrate that VB inhibits IκB-α degradation. The exact mechanisms by which VB suppresses NF-κB activation in inflammation are not known. However, there are various studies that have clearly demonstrated that potent antioxidant properties prevent NF-κB activation.[21]

NF-κB has been shown to activate, via transcription, the genes encoding pro-inflammatory cytokines, iNOS. In an earlier study using aminoguanidine, an inhibitor of iNOS, we demonstrated that in periodontitis inducible nitric oxide synthase expression has detrimental effects, such as a cytotoxic action toward the host tissues and alveolar bone resorption due to the stimulating effect of nitric oxide on the activity of the osteoclasts.[7] In this study we determined the expression and thus the formation of iNOS through the technique of immunohistochemistry. The same technique was used to determine the localisation of iNOS in biopsies from patients with periodontitis.[22] Our results demonstrate that VB attenuates the expression of iNOS in periodontal tissue, in accordance with our recent demonstration that VB inhibits the expression of iNOS, another model of inflammation.[13]

Furthermore, we found that the tissue damage induced by ligature in vehicle-treated rats is associated with high levels of tissue malondialdehyde, as well as nitrotyrosine formation.[7,23] In the present study we confirmed that ligation induces an intense immunostaining for nitrotyrosine formation, suggesting that a structural alteration of gingivomucosal tissues occurs, most probably due to the formation of highly reactive nitrogen derivatives. It has been demonstrated that several chemical reactions involving nitrite, peroxynitrite, hypochlorous acid and peroxidases can induce tyrosine nitration and may contribute to tissue damage, including that of gingivomucosal tissue.[24,25] In addition to nitric oxide, peroxynitrite is also generated in ligature-induced periodontitis.[25]

Therefore, in this study we clearly demonstrated that VB treatment prevents the release of NO and the formation of peroxynitrite. ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA that trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme PARP, resulting in the activation of the 'PARP suicide hypothesis'. There is recent evidence that the activation of PARP may also play an important role in experimental periodontitis.[7,26] We demonstrate here that VB attenuates the increase in PARP activity in the periodontal tissue.

Triggering of apoptosis has been implicated in the pathophysiology of periodontitis.[27] There are numerous reports on the role of ROS and PARP-1 in the regulation of apoptosis.[28,29] It has been shown that ROS mediate apoptosis in many different cell types and that Bcl-2 overexpression can sometimes protect cells from apoptosis mediated by ROS. However, the mechanism by which Bcl-2 prevents ROS-induced apoptosis is unknown. An alternative but not mutually exclusive hypothesis is that ROS act to down-regulate endogenous Bcl-2 levels within cells. Because levels of Bcl-2 within cells are critical to antiapoptotic activity, decreasing Bcl-2 could be a mechanism to sensitize cells to apoptosis. By detoxifying ROS, antioxidants such as verbascoside may therefore reverse the ROS-induced decline in Bcl-2 and prevent apoptosis.[30]

We confirm in this study that ligation-induced periodontitis leads to a substantial activation of apoptotic cell death, which probably contributes to the evolution of tissue injury. In fact, while in ligature-operated rats, we are witnessing an increase in the levels of Bax (pro-apoptotic protein) and a down-regulation of BCL-2 (anti-apoptotic protein), for treatment with VB, obtaining adverse effects, there is in fact a down-regulation for BAX and up-regulation by BCL-2.

Conclusions

This study provides the first evidence that VB causes a substantial reduction of ligature-induced periodontitis in the rat. Our findings suggest that interventions that reduce the generation or the effects of ROS may be useful in conditions associated with local or systemic inflammation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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