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EFFECT OF CIMETIDINE ON NITRO-OXIDATIVE STRESS IN A RAT MODEL OF PERIODONTITIS

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Abstract

Background and aims. *Periodontitis is a chronic inflammation that involves nitro-oxidative stress with damaging periodontal structural effects. We aimed to evaluate the consequences of low-dose cimetidine on nitro-oxidative stress in periodontitis.*

Methods. *A rat model of ligature-induced periodontitis was used. After two weeks, the periodontitis groups were treated with cimetidine, aminoguanidine, N-nitro-L-arginine methyl ester and trolox for one week. On day 21, blood was drawn and the serum analyzed for measurement of total nitrites and nitrates, total oxidative status, total antioxidant response, and oxidative stress index.*

Results. *Cimetidine had an inhibitory effect on the synthesis of nitric oxide (p=0.001), total oxidative status (p=0.01) and oxidative stress index (p=0.01). Total antioxidant reactivity was increased by cimetidine (p=0.01). The effects of cimetidine were almost like those of aminoguanidine, NG-nitro-L-arginine methyl ester, and trolox.*

Conclusions. *Low-dose cimetidine can be used as adjunctive host modulatory therapy in chronic periodontitis because it reduces nitro-oxidative stress.*

Keywords: periodontitis, nitric oxide, oxidative stress, cimetidine

Introduction

Periodontitis is a chronic inflammatory disease resulting from the complex interactions between subgingival bacteria and the defense mechanisms of the periodontium. Periodontitis causes the formation of periodontal pockets, alveolar bone resorption, damage to the structures supporting the teeth and, finally, tooth loss [1–3]. In periodontitis, everyday activities can result in the release of bacteria with transient low bacteremia that may cause infections at distant sites and a systemic increase of the levels of various proinflammatory mediators. These mechanisms make periodontitis an important risk factor for several systemic diseases: rheumatoid arthritis, chronic asthma, multiple sclerosis, diabetes mellitus, coronary heart disease, and cancer [4,5].

Most conventional treatments aim to remove bacteria from the periodontium. Based on pathogenetic mechanisms, the therapeutic approaches have changed towards the pharmacologic modulation of exaggerated host responses (host modulatory therapy (HMT)) in addition to microbial elimination [1,6,7,8].

In chronic inflammation, high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced to defend against pathogens. ROS are formed from superoxide (O_2^-) and hydrogen peroxide (H_2O_2)

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through a series of reactions (Haber–Wiess chemistry) [4]. Excess ROS target susceptible biomolecules such as proteins, lipids and DNA, thereby causing oxidative stress.

RNS are formed from nitric oxide (NO) [9]. NO is generated from L-arginine by NO synthase (NOS), and NO is a highly reactive molecule [10]. During inflammatory processes, large amounts of NO are generated by inducible nitric oxide (iNOS) [11]. iNOS is generated in macrophages by various stimuli: bacterial lipopolysaccharide, tumor necrosis factor-α, interleukin-1β, and interferon-γ. iNOS in macrophages can produce NO and O_2^- under immunostimulation, as well as low concentrations of L-arginine [12]. iNOS-mediated NO production can promote pathological inflammation because excess RNS tend to cause nitro-oxidative stress [13]. Oxidative chemistry induced by RNS is mediated primarily by peroxynitrite ($ONOO^-$) and nitroxyl (NO^-). Peroxynitrite originates from the reaction between NO and O_2^-, whereas NO^- can result from various chemical pathways. Nitrosative stress occurs if intermediates are produced from nitrosated thiol, hydroxy and amine groups. Therefore, reduction of ROS and RNS has been established as a HMT approach for periodontitis [14].

Cimetidine (CIM) is a powerful H_2 receptor antagonist. It is known to have pleiotropic immunomodulatory activities. It enhances T helper cells, inhibits suppressor T cells, induces the production of anti-tumor cytokines, and has pro-apoptotic effects [15]. CIM also eliminates the effects of histamine on chemotaxis and O_2^- production by phagocytes [16]. CIM is an antagonist of cytochrome P-450 (CYP)-mediated reactions. iNOS contains a CYP and CYP reductase domain. Because of the similarity of structure of iNOS and CYP, CIM may block the inflammation-generated production of NO catalyzed by iNOS [17]. We aimed to evaluate the effect of low-dose CIM on nitro-oxidative stress in a model of periodontitis in rats.

Materials and Methods

Ethical approval of the study protocol

The study protocol was approved by the Animal Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy of Cluj-Napoca (Cluj-Napoca, Romania) (58/09.12.2008).

Ligature-induced periodontitis

Periodontitis was induced in male Wistar rats (200–300 g) anesthetized with ketamine and xylazine (90 and 15 mg/kg, respectively, i.p.). A cotton ligature was placed around the cervices of the right side of mandibular first molars. It was knotted on the vestibular side so that it remained subgingival on the palatinal side. The ligature was removed immediately after the procedure in sham-operated rats [18,19].

After 14 days, rats were allocated randomly to six treatment groups of ten rats: I –negative control of sham-operated rats (control) + physiological (0.9%) saline (0.5 mL, i.p.); II – ligature-induced periodontitis (PER) + saline (0.5 mL, i.p.); III – PER + CIM (100 mg/kg/day, p.o.) [20]; IV – PER + aminoguanidine (AG) (60 mg/kg/day, i.p.) [21] (AG is a selective NOS2 inhibitor); V – PER + N-nitro-L-arginine methyl ester (NAME) (20 mg/kg/day, i.p.) [22] (NAME is a non-selective NOS inhibitor); VI – PER + trolox (50 mg/kg/day, p.o.) [23] (trolox is an antioxidant).

Treatments were administered daily for 7 days. Rats were housed in a germ-free facility (Experimental laboratory, Pathophysiology Department, Iuliu Hatieganu University of Medicine and Pharmacy) and fed a hard-pellet diet for the duration of the study. Upon completion of the study (21 days), blood was drawn by retro-orbital puncture. Serum was analyzed for measurement of total nitrites and nitrates (NO_x), total oxidative status (TOS), total antioxidant response (TAR) and oxidative stress index (OSI). Experiments were carried out in triplicate. After experiments, rats were killed by cervical dislocation.

Evaluation of NO synthesis

NO synthesis was evaluated indirectly by measuring serum levels of nitrites and nitrates. First, serum samples were passed through 10-kDa filters (Sartorius AG, Goettingen, Germany) and contaminant proteins removed by extraction with a 3:1 (v:v) solution of methanol/diethyl ether. The methanol/diethyl ether ratio in samples was 1:9 (v:v) [24]. The Griess reaction was used to determine the levels of nitrites and nitrates (NO_x) indirectly. In brief, 100 µL of 8 mg/mL VCl3 was added to 100 µL of filtered and extracted serum supernatant to reduce nitrates to nitrites, followed by addition of Griess reagents, 50 µL of sulfanilamide (2%) and 50 µL of N-(L-naphthyl) ethylenediamine dihydrochloride (0.1%). After 30-min incubation at 37°C, absorbance in the sample was read at 540 nm. The NO_x concentration in serum was determined using a sodium nitrite-based curve, and expressed as µmol/L of nitrite [25].

Evaluation of oxidative stress

TOS of the serum was measured using a colorimetric assay [26]. This assay measured oxidation of the ferrous ion to the ferric ion in the presence of various ROS in an acidic medium. The ferric ion was detected by its reaction with xylenol orange. Assay measurements were standardized using H_2O_2 as the oxidative species. Assay results are expressed in µmol H_2O_2 equiv/L.

TAR in serum was measured using a colorimetric assay [27]. This assay measured the rate of production of hydroxyl radicals by the Fenton reaction, which was monitored by following changes in the absorbance of colored dianisidyl radicals. Upon addition of a serum sample, hydroxyl radical-initiated oxidative reactions were suppressed by antioxidants in the serum. Inhibition of

dianisidyl oxidation prevented the subsequent color change, thereby enabling measurement of the total antioxidant capacity of the serum. This assay was calibrated using trolox, and results expressed as mmol trolox equiv/L.

The ratio of TOS to TAR represents OSI (an indicator of the degree of oxidative stress) [24] and is given by the formula:

OSI (arbitrary units) = TOS (μmol H_2O_2 equiv/L) / TAR (mmol trolox equiv/L).

All chemicals were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Taufkirchen, Germany) and were of ultra-pure grade.

Statistical analyses

Values are the mean and standard deviation (SD). Otherwise, the median and quartiles are reported (Q1 = first quartile; Q3 = third quartile). For multiple group comparisons, one-way ANOVA was used, as appropriate. If significant differences were determined with ANOVA, *post hoc* analyses were conducted using the Tukey test to determine differences between individual groups. The Mann–Whitney test was used for non-parametric data. Pearson's and Spearman's correlation analyses were used to calculate relationships between parameters. $P<0.05$ was considered significant. Analyses were conducted using SPSS v16.0 (SPSS, Chicago, Il, USA).

Results

CIM had a significant inhibitory effect on NO synthesis compared with PER (p=0.001). This was better than the effects of the iNOS inhibitor AG (p=0.008), the non-specific NOS inhibitor NAME (p=0.003), and the antioxidant trolox (p=0.05). CIM reduced NO_x almost to that seen in the SHAM group (p=0.91) (Table I).

Compared with the PER group, CIM treatment induced a small decrease in TOS (p=0.026) but did not reduce NO_x to the level seen in the SHAM group (p=0.039). Comparison of CIM treatment to treatments with NOS inhibitors revealed AG to have a lower inhibitory effect (p=0.009) and NAME to have a comparable effect (p=0.106). Only trolox induced a more significant decrease in TOS than CIM (p=0.015). Furthermore, TOS reduction after CIM treatment was correlated with NO_x reduction (r=0.70) (Table I).

Antioxidant mechanisms were assessed by measuring TAR. Compared with the PER group, TAR was increased significantly by CIM treatment (p=0.0029). The NOS inhibitors AG (p=0.0001) and NAME (p=0.0001), and trolox ($p=1x10^{-4}$) had better antioxidant effects than CIM. The effect of CIM on TAR was correlated significantly with the effect on NO_x (r=–0.95) (Table I).

CIM reduced OSI in the PER group (p=0.002), and the effect was comparable with that of AG (p=0.99) and NAME (p=0.367) but did not reach the level seen in the SHAM group (p=0.004). Trolox had a significantly better inhibitory effect (p=0.002) on OSI. In the CIM group, OSI was correlated with NO_x (r=0.71) and TOS (r=0.83) (Table I).

Discussion

The present study demonstrated CIM to have an important inhibitory effect on periodontitis-induced nitro-oxidative stress in rats, which is in accordance with other studies [28].

Nitro-oxidative stress is an important mechanism of tissue damage in chronic inflammation. NO synthesis can be evaluated indirectly by measuring the end products of NO oxidation: nitrite and nitrate anions. Nitrite can be reduced to NO by hypoxia, tissue acidosis, or by enzymes. These phenomena make serum levels of nitrates indicators of NO production *in vivo* and important complementary reservoirs of NO in physiological conditions [29].

CIM is used to treat and prevent gastric ulceration. It binds to the heme-iron portion of CYP to inhibit CYP activity. Because of the similarity of structure of iNOS and CYP as well as the post-translational role of CYPIIIA in cytokine-mediated NO synthesis, CIM may block the inflammation-generated production of NO catalyzed by iNOS. In periodontitis, oral rinse solutions of CIM have been shown to enhance the antibacterial functions of crevicular neutrophils [16,17,20].

Non-steroidal antiinflammatory drugs in association with CIM increase anti-inflammatory activities because

Table I. Parameters of nitro-oxidative stress in the study groups

		SHAM	PER	AG	NAME	TROLOX	CIM
TOS	Mean	60.80	182.60	166.43	170.39	53.46	120.00
(μM equiv H_2O_2/L)	SD	12.07	58.50	27.25	26.38	14.70	6.49
TAR	Mean	42.72	20.23	39.24	38.94	43.34	27.00
(mM equiv trolox/L)	SD	2.82	0.48	2.73	2.98	2.05	1.73
OSI	Mean	1.43	9.02	4.25	4.38	1.24	4.50
	SD	0.33	2.85	0.73	0.55	0.77	0.17
NO_x	Mean	33.00	67.10	53.50	43.70	44.20	35.00
(μM/L)	SD	1.83	8.02	13.61	8.26	6.62	3.77

TOS = total oxidative status; TAR = total antioxidant reactivity; NO_x = total nitrites and nitrates; PER = periodontitis; AG = aminoguanidine; NAME = N-nitro-L-arginine methyl ester.

CIM also has other immunomodulatory effects: stimulation of lymphocyte proliferation; reduction of T-cell activity; inhibition of the antigen–antibody reaction [30]. In the present study, a low dose of CIM could reduce NO_x at a comparable level with that seen with NOS inhibitors. Hence, modifying the destructive effect of NO using low-dose CIM might enable periodontal breakdown to be decreased and the periodontium stabilized.

Conclusion

The present study provided evidence for the hypothesis that low-dose CIM has anti-inflammatory activity in a model of periodontitis in rats by reducing nitro-oxidativestress. Our findings suggest that CIM may be a useful adjunctive HMT in conditions associated with periodontitis.

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