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IMPACTO DO ESTRESSE NA DOENÇA PERIODONTAL

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de doutor em Clínica Odontológica, Área de Periodontia.

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Faço minhas as palavras de Gonzaguinha, para brindar a beleza da vida e dizer que somos eternos aprendizes....

...Viver e não ter a vergonha de ser feliz,
Cantar, e cantar, e cantar,
A beleza de ser um eterno aprendiz.
Ah, meu Deus! Eu sei
Que a vida devia ser bem melhor e será,
Mas isso não impede que eu repita:
É bonita, é bonita e é bonita!
E a vida? E a vida o que é, diga lá, meu irmão?
Ela é a batida de um coração?
Ela é uma doce ilusão?
Mas e a vida? Ela é maravilha ou é sofrimento?
Ela é alegria ou lamento?
O que é? O que é, meu irmão?
Há quem fale que a vida da gente é um nada no mundo,
É uma gota, é um tempo
Que nem dá um segundo,
Há quem fale que é um divino mistério profundo,
É o sopro do criador numa atitude repleta de amor.
Você diz que é luta e prazer,
Ele diz que a vida é viver,
Ela diz que melhor é morrer
Pois amada não é, e o verbo é sofrer.
Eu só sei que confio na moça
E na moça eu ponho a força da fé,
Somos nós que fazemos a vida
Como der, ou puder, ou quiser,
Sempre desejada por mais que esteja errada,
Ninguém quer a morte, só saúde e sorte,
E a pergunta roda, e a cabeça agita.
Fico com a pureza das respostas das crianças:
É a vida! É bonita e é bonita!
É a vida! É bonita e é bonita!

RESUMO

Estudos em animais e epidemiológicos têm sugerido que o estresse pode alterar o estabelecimento e a progressão da doença periodontal (DP). Entretanto, dados relacionados ao efeito do estresse e seus mecanismos envolvidos na DP ainda são limitados. Os objetivos deste estudos foram: i) revisar sistematicamente a literatura sobre a influência do estresse crônico (EC) na DP; ii) analisar o impacto do EC induzido em ratos na progressão da DP e na regulação de genes relacionados à progressão da doença, bem como na alteração dos biomarcadores do estresse (catecolaminas e corticoesterona); iii) avaliar a viabilidade do uso da droga metirapone (MT), como um modelo experimental em ratos, para inibir a produção de glicocorticóides (GC), determinando, assim, o efeito do EC nos tecidos periodontais. Para a revisão sistemática, foi realizada uma busca na literatura e os dados dos estudos foram extraídos e avaliados por dois revisores independentes. Para os trabalhos em animais, foram realizados experimentos em ratos machos, Wistar, divididos em grupos com 20 animais cada: controle, DP induzida por ligadura, DP + EC (restrição de movimento e isolamento, 12h/dia) e DP + EC + administração de MT (3 doses/dia de 50mg/Kg). Após 30 dias todos os animais foram sacrificados. Amostras de sangue foram coletadas para mensurar os biomarcadores do EC, o tecido marginal, ao redor dos sítios com e sem ligaduras foi coletado para avaliar a expressão de genes por meio de PCRq (Reação de Polimerase em Cadeia quantitativa) e as mandíbulas foram removidas e fixadas para mensuração histométrica da perda óssea interradicular (POI). Análise dos dados demonstrou que: i) a maioria dos estudos analisados apresentaram um desfecho positivo entre EC e DP; ii) os biomarcadores do estresse, na presença de EC, podem localmente modular a DP por meio de um aumento local nas proporções dos genes pró-inflamatórios e pró-reabsorção, favorecendo, assim a destruição óssea periodontal; e, iii) a administração de MT resultou num importante efeito na redução dos níveis sistêmicos de GC, entretanto, pode-se observar que a administração da droga alterou a expressão de fatores importantes na modulação da DP e conseqüentemente refletiu nos níveis de POI. Dentro dos limites deste estudo, pode-se concluir que o EC significativamente apresenta uma relação com a DP e o aumento local de fatores pró-inflamatórios e pró-reabsorção pode ser o mecanismo envolvido na progressão da doença. Além disso, a administração de MT é capaz de reduzir os níveis sistêmicos de GC, entretanto, modula a expressão de fatores relacionados à progressão da DP, resultando em POI.

Palavras – chave: revisão sistemática, estresse crônico, doença periodontal, metirapone.

ABSTRACT

Animal and epidemiological studies have suggested that stress may modify the establishment and progression of periodontal disease (PD). However, data regarding the effect of stress and the mechanisms involved in PD are limited. The aim of this study was: i) to review systematically the literature about the influence of chronic stress (CS) on PD ii)to evaluate the impact of CS, induced in rats, in the progression of PD and regulation of genes related to the disease progression, as well as the variations of stress biomarkers (cathecolamines e corticoesterone); iii) to evaluate the feasibility of the use of metyrapone (MT) as an experimental model to inhibit glucocorticoid (GC) production and, therefore, as a method to determine the effect of CS on periodontal tissues. A systematic literature search was performed and the data of the studies were independently extracted and evaluated by two reviewers. The animal studies were carried out on male Wistar rats assigned to 3 groups with 20 animals each: control, PD induced by ligature; PD associated with CS (restraint stress and isolation, 12 h/day) and PD + CS + MT administration (3 daily doses, 50mg/Kg). After 30 days, all animals were sacrificed. Blood samples were obtained and the concentrations of corticosterone and catecholamines measured as biomarkers of CS, marginal tissues around ligated and non-ligated teeth were harvested and gene expression assessed by qPCR (quantitative Polymerase Chain Reaction) and the jaws were removed and fixed to histometrically determine the interradicular bone loss (IBL). Data analysis demonstrated that: i) the majority of the studies showed a positive outcome between CS and PD; ii) the stress biomarkers may locally modulate PD by an increase of the local ratio of pro-inflammatory and pro-resorptive genes, thus favoring tissue destruction; and, iii) MT administration resulted in an important lowering effect of GC systemic levels, however, it could be observed that MT administration modified the expression of important factors which modulate PD, and consequently reflected the IBL. Within the limits of this study, it may be speculated that CS has a significant relationship with PD and the local increase in pro-inflammatory and pro-resorptive factors can be the mechanisms involved in disease progression. Moreover, MT administration is able to lower systemic levels of GC, however, it modulates the expression of factors related to periodontitis progression, resulting in IBL.

Key words: systematic review, chronic stress, periodontal disease, metyrapone.

PREFÁCIO

Esta tese está baseada nos seguintes artigos científicos:

1. Peruzzo DC, Benatti BB, Ambrosano GMB, Nogueira-Filho GR, Sallum EA, Casati MZ, Nociti Jr. FH. A systematic review on stress and psychological factors as possible risk factors for periodontal disease. *J Periodontol* 2007; 78: 1491-1504.
2. Peruzzo DC, Benatti BB, Antunes IB, Andersen ML, Sallum EA, Casati MZ, Nociti Jr. FH, Nogueira-Filho GR. Chronic stress may modulate periodontal disease. A study in rats. *J Periodontol* 2007 (accepted).
3. Peruzzo DC, Benatti BB, Andersen ML, Tufik S, Casati MZ, Nociti Jr. FH. Evidence that metyrapone can act as a modulator of periodontal breakdown. Short communication. *J Periodonto Res* (submitted).

SUMÁRIO

INTRODUÇÃO GERAL

As doenças periodontais inflamatórias são lesões que afetam os tecidos que circundam e sustentam os dentes (Löe, 1993) e têm o biofilme bacteriano como fator etiológico primário (Löe et al., 1965). As alterações que ocorrem na transição de uma condição de saúde para doença periodontal passam por sucessões de eventos celulares e moleculares, coordenados pelo sistema imune do hospedeiro, com a intenção de protegê-lo do desafio microbiano. Em condições de saúde, vários mecanismos de defesa atuam contra a presença de bactérias como: substâncias da saliva e do fluido crevicular gengival (glicoproteínas, mucinas, imunoglobulinas – IgA, IgG), opsonização e aglutinação por anticorpos específicos e fagocitose por leucócitos polimorfonucleares, entre outros (Gibbons, 1984). Contudo, após o desafio crônico, os tecidos periodontais ficam continuamente expostos a componentes bacterianos que alteram a homeostasia local. O tecido fica povoado por linfócitos T e por macrófagos os quais produzem uma variedade de citocinas (Kornman et al., 1997), estas podem estimular a produção de enzimas proteolíticas, as chamadas metaloproteinases (MMPs), as quais degradam os componentes da matriz e estão relacionadas à reabsorção óssea (Birkedal-Hansen et al.,1993). Com a alteração da homeostasia local, a eficiência da migração dos neutrófilos fica reduzida e é provável que maior quantidade de neutrófilos sejam ativados dentro do tecido, liberando metaloproteinases e derivados reativos do oxigênio, comprometendo o equilíbrio dos tecidos.

Embora seja indiscutível o papel do biofilme bacteriano na etiologia das doenças periodontais, a severidade e a progressão destas doenças são, também, determinadas por fatores relacionados à resposta do hospedeiro (Seymour, 1991). Exemplo clássico é o fato de que mesmo a gengivite sendo uma doença altamente prevalente, estudos epidemiológicos têm demonstrado que somente 5-20% da população sofrem de formas severas de periodontite, indicando que alguns fatores podem estar modulando a suscetibilidade ou a resistência à evolução da doença periodontal (Genco, 1996). Desta forma, existiriam condições de natureza biológica, comportamental ou ambiental que

poderiam influenciar no estabelecimento e na progressão da doença, os chamados fatores de risco (Clarke & Hirsch, 1995; Albandar, 2002). Atualmente, os fatores de risco aceitos e comprovados em estudos epidemiológicos e longitudinais, são o tabagismo, o diabetes mellitus e associação de alguns tipos de microorganismos (Albandar, 2002). Entretanto existem os indicadores ou potenciais fatores de risco que podem influenciar na prevalência e na severidade das periodontites e dentre estas condições relacionadas a um possível maior risco à doença periodontal, está o estresse (Genco, 1996).

A relação entre enfermidades periodontais e fatores psicossocias (como, por exemplo, estresse, depressão e ansiedade) está relativamente bem estabelecida, principalmente nos casos de gengivite ulcerativa necrosante (GUN) (Cohen-Cole et al., 1983; Monteiro da Silva et al., 1995). Fatores como gengivite pré-existente, fumo e estresse psicológico agudo estão relacionados à ocorrência de GUN. Esta associação tem sido comprovada em estudos que demonstraram a elevada incidência de GUN em pessoas que passam por situações estressantes como, por exemplo, estudantes em períodos de exames acadêmicos, em pessoas submetidas a regimes de estresse agudo e que não conseguem controlar ou resolver seus problemas ao longo do tempo, bem como em indivíduos severamente deprimidos (Cohen-Cole et al., 1983; Monteiro da Silva et al., 1995). Além dos estudos de GUN, estudos em humanos, utilizando questionários e análises de comportamento frente a condições estressantes também encontraram relação entre estresse e severidade da periodontite crônica (Green et al., 1986; Monteiro da Silva et al., 1996; Moss et al., 1996; Genco et al., 1999). Pacientes com maior número de experiências psicológicas desagradáveis apresentaram maior acúmulo de biofilme bacteriano (Croucher et al., 1997), além de desenvolver mais periodontite crônica (Green et al., 1986). Um estudo realizado por Axtelius (1998) demonstrou maior índice de periodontite crônica em pacientes que apresentavam dificuldade para dormir associado à ansiedade e perfil psicológico mais vulnerável. Adicionalmente, a suscetibilidade à doença periodontal pode estar relacionada a fatores psicológicos, especificamente à personalidade do indivíduo, a qual afeta a reação do indivíduo aos eventos estressantes ao longo da vida, incluindo aqueles vivenciados no ambiente de trabalho, segundo Freeman & Goss (1993). No

entanto, a determinação da magnitude e da causalidade da relação entre estresse e doença periodontal ainda necessitam de melhor comprovação.

A resposta ao estresse parece estar relacionada a um mecanismo mediador entre condições psicológicas desfavoráveis e doença periodontal inflamatória (Gaspersic et al., 2002). O estresse pode estar relacionado à doença periodontal basicamente por meio de dois modelos: modelo comportamental – em que ocorre aumento no consumo de nicotina, higiene oral menos efetiva, mudanças nos hábitos nutricionais – ou modelo biológico, através da redução do fluxo salivar, alteração da circulação gengival e alterações na resposta imune-inflamatória (Monteiro da Silva et al., 1995).

O sistema imune produz dois tipos de reações específicas: as mediadas por substâncias químicas ou humoral e as mediadas por células (O'Leary, 1990). A imunidade humoral é mediada por linfócitos B que produzem anticorpos (Ac) sistemicamente circulantes. A imunidade celular, por sua vez, é mediada por linfócitos T que agem diretamente sobre um antígeno (Ag). De acordo com a presença de fatores de diferenciação na superfície dos linfócitos T, eles são subdivididos em T *helper* (Th), T supressor (Ts), T citotóxico (Tc) e células *natural killer* (NK) (Calabrese et al., 1987). Recentes trabalhos (Elenkov & Chrousos, 1999; LeResche & Dworkin, 2002; Gemmell & Seymour, 2004) caracterizam duas subclasses de células Th, as quais secretam distintas citocinas que podem guiar a resposta imune em direções qualitativamente diferentes. As células Th1 secretam primariamente interferon gama (IFN-γ) e interleucina – 2 (IL-2), as quais estão envolvidas na reação imune mediada por células e na ativação dos linfócitos Tc. Por outro lado, as células Th2 secretam IL-4, IL-5, IL-6 e IL-10, as quais ativam a formação de anticorpos e estão associadas a reações alérgicas imediatas. De uma maneira geral, há um equilíbrio entre as respostas mediadas pelas células Th1 e Th2, quando as células Th1 são ativadas, as citocinas secretadas por essas células (IFN-γ) inibem a secreção de citocinas pelas células Th2. Em contraste, a IL-10, interleucina secretada pelas células Th2 ativadas, inibem a resposta mediada por células Th1 (Reed, 1995). Estudos em animais (Sadik et al., 1986) e em humanos (Salgame et al., 1991) demonstraram que uma resposta mediada por células Th2 era dominante quando associada à doença progressiva severa, enquanto uma resposta associada a células Th1 era predominante quando associada a doenças estáveis.

Estudos experimentais sugerem que estresse psicossocial está relacionado a doenças alérgicas e inflamatórias, como a doença periodontal, e que glicocorticóides podem ser responsáveis pela modulação da resposta Th1/Th2, descrita acima (Rook et al., 1994; Breivik et al., 2000). Em resposta a um agente estressor, algumas mudanças fisiológicas decorrem da ativação do eixo simpático do Sistema Nervoso Autônomo (SNA), enquanto outras, da ativação do eixo Hipotálamo-Pituitário-Adrenal (HPA) (Dhabhar & McEwen, 2001), como demonstrado na figura 1 (em anexo). Estudos realizados em animais e em humanos (Bronsschot et al., 1994; Ader et al., 1995; Dhabhar 1995) demonstraram que o estresse agudo induz a um aumento inicial, seguido por redução drástica do número de leucócitos circulantes. A ativação do SNA resulta na estimulação da medula adrenal e secreção de catecolaminas como epinefrina (E) e norepinefrina (NE), aumentando a liberação de prostaglandinas e proteases (Genco et al., 1998). Essa é uma situação que ocorre no início da resposta ao estresse (reação aguda), com duração de alguns minutos ou poucas horas. Por outro lado, em resposta tardia ao estresse ou durante um período de estresse crônico, a ativação do eixo HPA resulta na produção de hormônio liberador de corticotropina (CRH) e de arginina vasopressina (AVP) pelo hipotálamo (Ader et al., 1995). Esses hormônios vão estimular a hipófise a produzir o hormônio adrenocorticotrófico (ACTH) que, por sua vez, vai agir sobre o córtex da glândula adrenal, responsável pela produção de glicocorticóides (GC), dos quais o cortisol é secretado em humanos e a corticoesterona em ratos (Breivik, 2000). Os GC regulam uma série de funções corporais, incluindo efeitos supressivos através de mecanismos altamente específicos (Genco et al., 1998). *In vivo*, os GC reduzem o número de linfócitos circulantes, monócitos e eosinófilos, além de inibir o acúmulo destas células nos sítios inflamatórios (Cupps & Fauci, 1982). Ao nível molecular (Figura 2, em anexo), os GCs agem sobre células inflamatórias incluindo macrófagos, neutrófilos, eosinófilos e mastócitos inibindo importante funções como quimiotaxia, secreção e degranulação (Schleimer et al., 1989). Os GC suprimem também a cascata da resposta imuno-inflamatória através da inibição da apresentação de antígeno feita pelo macrófago, inibição da proliferação de linfócitos e da diferenciação em células efetoras como linfócitos *helper*, linfócitos citotóxicos, células NK, e células B formadoras de anticorpos (Snyder & Unanue, 1982). Os GC também possuem

efeito inibidor sobre a produção de citocinas incluindo IL-1, IL-2, IL-3 e IL-6, fator de necrose tumoral (TNF-α), interferon-gama (IFN-γ) e mediadores inflamatórios derivados do ácido aracdônico como as prostaglandinas e leucotrienos (Schleimer et al., 1989).

Como descrito acima, evidências demonstram que o CRH, as catecolaminas e os glicocorticóides, além de outros elementos relacionados à cascata desencadeada por um agente estressor, podem modular o sistema imune em ambas as direções, seja nos níveis de repouso ou na elevação dos níveis associados ao estresse (Chrousos & Gold, 1992). Desta forma, o estresse pode exercer localmente um efeito pró ou anti-inflamatório, que será regulado nos tecidos por fatores específicos envolvidos, ou também pela presença ou ausência de subtipos de receptores de células imunes (por exemplo: variações nas concentrações de receptores β-adrenérgicos versus receptores α-adrenérgicos). Uma vez que o estresse pode modular a expressão de certas interleucinas capazes de proteger o organismo da destruição, esse fator pode alterar o equilíbrio do sistema imune, predispondo a uma maior progressão e severidade da doença periodontal (Genco, 1992). Breivik et al. (2001) demonstraram que a atividade do eixo HPA influenciou na velocidade de progressão da periodontite crônica induzida em ratos. Os autores verificaram uma exacerbada resposta ao estresse, ou seja, após aumento sistêmico dos níveis de GC, a resposta pró-inflamatória, mediada por células Th1, ficou diminuída, enquanto a resposta imune foi direcionada para a produção de anticorpos e maior liberação de citocinas pelas células Th2. Os animais com hiperatividade do eixo HPA responderam ao acúmulo de biofilme com uma resposta imune mediada por anticorpos e apresentaram maior destruição tecidual, em comparação aos animais com padrão de resposta mediado por células Th1 e com menor atividade do eixo.

Apesar da existência de estudos em animais (Breivik et al., 2000/2001; Gaspersic et al., 2002; Benatti et al., 2003; Takada et al., 2004), estudos observacionais (Croucher et al., 1997; Axtelius et al., 1998; Wimmer et al., 2002) e estudos intervencionistas que tentam relacionar estresse e doença periodontal (Wimmer et al., 2005; Vettore et al., 2005) são poucas as evidências dos mecanismos envolvidos sobre a influência do estresse crônico na modulação da resposta imune-inflamatória local à doença periodontal.

PROPOSIÇÕES GERAIS

Os objetivos deste estudo foram:

1- Revisar sistematicamente evidências sobre a influência do estresse na doença periodontal (periodontite crônica);

2- Analisar o impacto do estresse crônico induzido em ratos na:

a) progressão da doença periodontal induzida por ligadura;

b) liberação de catecolaminas e corticoesterona circulantes;

b) regulação de genes pró-inflamatórios e anti-inflamatórios;

c) regulação de genes relacionados à reabsorção óssea;

3- Avaliar a viabilidade do uso da droga metirapone, como um modelo experimental para inibir a produção de glicocorticóides e determinar o efeito do estresse crônico nos tecidos periodontais.

CAPÍTULO 1

A SYSTEMATIC REVIEW ON STRESS AND PSYCHOLOGICAL FACTORS AS POSSIBLE RISK FACTORS FOR PERIODONTAL DISEASE

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ONE SENTENCE SUMARY:

Data analysis of the included studies showed that the majority of studies reported a positive relationship between stress and psychological factors and periodontal disease.

ABSTRACT

Background: Clinical observations and epidemiological studies suggest that some negative life events and psychological factors may contribute to increase the susceptibility to periodontal disease. The aim of the present study was to systematically review the evidence from case-control, cross-sectional, and prospective clinical trials studies reporting on the influence of stress and psychological factors on periodontal disease. The focused question addressed in this systematic review was whether the scientific evidence demonstrated so far is enough to consider stress and psychological factors as a risk factor for periodontal disease.

Search strategy: A literature search was conducted using two databases (MEDLINE and the Cochrane Oral Health Group specialist trials register) from 1990 to 2006, in addition to searching reference lists of original and review articles. The search strategy used was the combination of the terms: "stress", "periodontal disease" and "psychosocial disorders".

Selection criteria: Studies were selected if they were published in dental journals between 1990/01/01 – 2006/04/01, only human studies, and studies with adults and middle aged subjects. Suitable variables included were: control for the potential effect of confounding factors, adequate criteria to define periodontal disease, adequate criteria for establishing stress and methodological quality. Only English language articles were considered and unpublished data were not sought.

Data collection and analysis: Two reviewers independently extracted information regarding quality and study characteristics, in duplicate. The studies were assessed regarding their methodological characteristics, statistical analysis, characteristics of the periodontal outcome measures and psychological measurements.

Results: Of the 58 articles identified in the search, 10 were excluded because they were reviews and 34 did not comply with the selection criteria. Finally, 14 articles were included in the analysis and their quality and main study characteristics were assessed, according to the criteria pre-established in the protocol of the study. The following studies were found; 7 case-control studies, 6 cross-sectional studies, and one prospective clinical trial. With regard to the results of the studies, 57.1% found a positive outcome between psychosocial

factors/stress and periodontal disease, 28.5% observed a positive outcome for some characteristics and a negative outcome for others, and 14.2% found a negative outcome.
Conclusion: Within the limitations of this systematic review it could be concluded that the majority of studies showed a positive relationship between stress and psychological factors and periodontal disease. However, future well designed and more representative studies should be considered to confirm these factors as a risk for periodontal disease.

Key words: Stress, psychological factors, periodontal diseases

INTRODUCTION

Epidemiological studies have demonstrated that periodontitis does not affect all subjects in the population in a similar manner. Studies from many parts of the world, as reviewed by Johnson et al.,[1] indicate that only a subpopulation of 7% to 15% of the dentate adult population are affected by destructive periodontal disease. Although bacteria are well established as the etiological agents of periodontal disease, the fact that its presence alone is not capable of producing advanced tissue destruction in all individuals suggests that there is an individual response and adaptation ability to a certain amount of bacterial biofilm without progression of the disease. Environmental risk factors such as smoking and diabetes mellitus may modify the host response and hence modify disease progression, severity and outcome.[2] Other factors, such as stress, depression and anxiety are not yet confirmed as absolute risk conditions, but have been identified in some observational studies as potential factors which may affect periodontal disease. [3,4]

The biological plausibility for such an association is supported by studies showing that psychosocial conditions, such as depression and exposure to stressor agents, may affect the host immune response, making the individual more susceptible to develop unhealthy conditions, and also affecting the periodontal health.[5,6] There are a number of studies that show that psychological stress can downregulate the cellular immune response by at least three different manners.[7,8,9,10] First, stress-induced response is transmitted to the hypothalamo-pituitary-adrenal (HPA) axis, and promotes the release of corticotropic-

releasing hormone (ACTH) from the pituitary gland, and glucorticoid hormones from the adrenal cortex. Glucocorticoids released into the cortex of the supra-renals have been shown to decrease pro-inflammatory cytokines production (interleukins, prostaglandins and tumour necrosis factor). Second, exposure to stressor agents can induce the sympathetic nervous system (SNS) to release adrenaline and noradrenaline from the adrenal medulla and, therefore, can exert an immunosuppressive effect,[11] which can indirectly provoke periodontal tissue breakdown.[12] Third, stress can induce the release of neuropeptides from sensory nerve fibers (neurogenic inflammation), and the presence of neuropeptides has been implicated as a neurogenic promoter in various inflammatory processes modulating the activity of the immune system and the release of cytokines.[13] In conclusion, the impact of stress on the immune system has been widely documented, and a possible influence on chronic inflammatory periodontal disease is likely to occur. In addition, the modification of patients' health behavior has also been reported to be a possible mechanism behind the influence of stress and psychosocial factors on periodontal conditions. Thus, individuals with high stress levels tend to adopt habits that are harmful to periodontal health, such as negligent oral hygiene, intensification of nicotine consumption, or changes in eating habits with negative effects for the immunologic system.[14]

Cross-sectional studies revealed a clear-cut correlation between the progressive course of periodontal disease and the psychosocial stress status of patients.[9,10,15] This concept is not new in the field, as stress has been known for more than 40 years to be an important predisposing factor in the development of necrotizing ulcerative gingivitis (NUG) (for a review see Johnson & Egel[16]). The periodontal literature contains numerous studies, with different methodological designs, examining the relationship between physical and psychosocial stressors and destructive periodontal changes mediated by the stress system.[15,17,18,19] Animal and human studies have been reported under experimental conditions and observational research methods; the latter predominately seek an association between the existence of self-reported stressors and indicators of periodontal disease. The several studies that point to a relationship between such factors and clinical characteristics of periodontal disease[3,4,7] used different criteria to classify periodontal disease. The variety of methodologies applied in these studies, as well as the absence of a control group and

lack of control for confounding variables for periodontal disease, makes it difficult to conclude on the real effects of psychosocial factors over periodontal pathogenesis.[20,21] To date, there has been no conclusive data on the impact of stressor agents as a risk factor on the periodontal tissues. Therefore, the aim of the present study was to systematically review the evidence from case-control, cross-sectional and prospective clinical trials reporting on the influence of stress and psychological factors on periodontal disease. The focused question addressed in this systematic review was: in patients with periodontal disease, is the scientific evidence demonstrated so far enough to consider stress and psychological factors a risk factor for periodontal disease?

MATERIAL and METHODS

Development of a protocol

In order to answer the selected focused question, a protocol was developed. The protocol included all aspects of the review methods: search strategy, inclusion criteria for studies, screening methods, data abstraction, quality assessment by two independent reviewers, and data synthesis.

Criteria for including studies in this review

Search Strategy

A literature search was conducted using two databases (MEDLINE and the Cochrane Oral Health Group specialist trials register) from 1990 to 2006, in addition to searching reference lists of original and review articles. The search strategy used was the combination of the terms: “stress”, “periodontal disease” and “psychosocial disorders”. The limits established were: date (1990/01/01 – 2006/04/01), human studies, dental journals and studies with adults (19 to 44 years old) and middle aged subjects (45 to 64 years old). Only English language articles were considered and unpublished data were not sought. Hand searching of journals for missed trials was not carried out.

Types of studies

This review included the following studies: case-control, cross-sectional, and prospective clinical trials.

Selection process

After the search, all review articles and animal studies were excluded and 58 suitable articles were selected. Their titles and abstracts were screened independently by two reviewers. Selection agreement was evaluated by Kappa score on a 3 x 3 contingency table (included, excluded, unclear). Disagreement regarding inclusion was resolved by discussion between the reviewers. The full texts of 21 articles were obtained and their quality and main study characteristics were assessed by two reviewers. Of the 21 articles, only 14 could be evaluated, while the other 44 articles did not present the suitable variables according to the main criteria pre-established in the protocol of the study. Suitable variables included were: control for the potential effect of confounding factors, adequate criteria to define periodontal disease, adequate criteria for establishing stress and methodological quality (see next section for more detail). The agreement between the reviewers was determined by Kappa scores in contingency tables for each topic. Any disagreement was discussed and resolved. Contact with some authors was necessary to resolve some doubts and to request missing information. Finally, data from the 14 selected articles were extracted and appraised by two independent reviewers (D.C.P. and B.B.B.), and verified by the Scientific Advisor (F.H.N.J.). The other 44 studies are listed in the Appendix 1, together with the main reason for exclusion.

Evaluation process

Quality of individual components was assessed independently. The quality assessment was derived from two guidelines for systematic reviews: Cochrane Reviewers` Handbook[22] and NHS Centre for Reviews and Dissemination.[23]

To be included in this systematic review, the studies needed to present:

- Control for the potential effect of confounding factors that could interfere in the course of periodontal disease (by: randomization, restriction, matching, stratification and/or statistical modeling);

- Adequate criteria to define periodontal disease (e.g. probing depth, attachment loss, alveolar bone loss, bleeding on probing, recession level, remaining periodontal support, missing teeth);
- Adequate criteria for establishing stress (e.g. psychometric instrument);
- Methodological quality (described in details bellow);

It was decided that the methodological quality for the included studies would be assessed with a predetermined appraisal form focusing on the following issues:

- Randomization: designated as present or absent. When present, it was classified as adequate, inadequate, or unclear. Adequate if generated by random number table, tossed coin, and shuffled cards. Unclear if the study refers to randomization but either does not adequately explain the method or no method was reported. Inadequate randomization methods include alternate assignments, hospital numbers, and odd/even birth date.
- Calibration: designated as present or absent. When present, it was classified as adequate, or unclear. Adequate if the method of calibration was referred and the value for calibration was reported. Unclear if the study refers to calibration, but either does not adequately explain the method or no calibration value was reported.
- Matching: classified as present or absent.
- Stratification: classified as present or absent.
- Restriction: classified as present or absent.
- Statistical adjustment: classified as present or absent.

In addition, the presence of other characteristics was also evaluated:

a. Study design;
b. Type of sample, inclusion and exclusion criteria,
c. Allocation concealment;
d. The outcomes measured regarding psychosocial factors and periodontal disease;

RESULTS

The screening of titles and abstracts had a selection agreement defined by a Kappa score of 0.93 (standard error, SE=0.039) between reviewers quality of the papers. Of the 58 articles identified in the search, 44 were excluded because they did not comply with the selection criteria (see Appendix 1). Finally, 14 articles were included in the analysis.

Study design

The following studies were identified: seven case-control studies,[3,24,25,26,27,28,33] six cross-sectional studies[4,15,29,30,31,32] and one prospective clinical trial,[34] as shown in Table 1.

Population description

In most studies, the patients belonged to universities or hospitals or were referred for specialized treatment.[4,24,25,26,27,30,31,33,34] Four studies involved patients from epidemiological studies[3,15,29,32] and one study involved patients from both the Department of Prosthetic Dentistry and Periodontology and from a private dental surgery.[28] From these studies, only one referred a randomization form in the sample selection[29] (Table 2).

Characteristics of the outcome measures

Most studies evaluated the changes in clinical attachment level (CAL) and in periodontal pocket depth (PPD) by the use of standard manual probes, although electronic probe was also employed[3] (Table 2). Eight studies used the CAL as the criteria for established periodontal disease, the others applied different criteria, such as PPD and alveolar bone loss (ABL) in addition with recession level (Re), remaining periodontal support (RPS), bleeding on probing (BOP) and missing teeth (MT). Some type of intra and/or inter-examiner calibration was observed in eight studies, the remaining six studies did not report calibration (Table 2). Of the 14 studies, just two performed periodontal therapy: Wimmer et al.[34] used the CAL as the clinical parameter to evaluate the periodontal condition after 2 years of regular maintenance and Vettore et al.[33] recorded CAL at baseline and 3 months after non-surgical periodontal treatment.

Psychological measurements

Different psychological factors were evaluated in the studies: stress, distress, anxiety, depression, loneliness, daily strain. For this purpose, all studies used some type of questionnaire as a psychometric instrument. In these studies, only five (35.7%) reported questionnaire adaptation for the sample analyzed.[26,27,30,32,33]

Control for the confounding variables.

Since some characteristics, such as age, gender, smoking, race, oral hygiene may affect both psychological and dental variables to be assessed, it is necessary to apply methods to adjust these confounding factors. Confounding could be dealt with at the design stage of an investigation by: randomization, restriction, matching, stratification and/or statistical modeling, such as regression model analyses. As such, one study[29] randomized the sample, four studies performed matching between the groups: Moss et al.[3] and Croucher et al.[24] for age, race and gender; Axtelius et al.[25] performed matching for initial disease extent, age and gender; and Monteiro da Silva et al.[4] for race and gender (Table 2). Furthermore, stratification of the sample was performed in three studies: Moss et al.[3] (age, gender and race), Axtelius et al[25] (smoking habits and oral hygiene) and Genco et al.[15] (high and low emotional focused copers). Restriction was achieved by exclusion criteria in all papers evaluated.

Considering the statistical analyses used in the selected papers, most of the studies (9 or 64.3%) used regression model analysis,[3,15,24,25,26,27,29,30,31,32] while the other five (35.7%) used different types of statistical analyses,[4,26,28,33,34] including: analysis of covariance, Varimax rotation with Kaiser-normalization and Pearson's correlation. The statistical adjustments were reported in thirteen studies (92.8%) (Table 1 and 2) for different combinations of variables (age, gender, smoking, oral hygiene, socioeconomic data). Statistical adjustment was not performed only in one study[25], however, the authors described that the matching and initial stratification of the groups was efficient to control any confounding factors.

Odds Ratio

After the statistical analysis, six studies revealed their results by Odds Ratio (OR) followed by their respective Confidence Interval (CI=95%). The following studies found a relationship between psychosocial factors and some characteristics related to periodontal disease (Table 1, Figure 1). Moss et al.[3] found association between Immunoglobulin G (IgG) for *Tannerela forsythia* (Tf) and periodontal disease among individuals who had higher scores for depression: OR= 6.75 (1.25 – 36.5). OR for case status and role strain scores of 2.27 or more: 2.84 (1.08-7.46). Genco et al.[15], in a logistic regression analysis indicated that, of all the daily strains investigated, only financial strain (FS) was significantly associated with greater attachment and bone loss (OR= 1.70, CI= 1.09 – 2.65 and OR= 1.68, CI= 1.20 – 2.37, respectively) after adjustment for age, gender and cigarette smoking. When coping behaviors were evaluated, it was found that those with greater financial strain with inadequate coping had a higher risk for severe attachment loss (OR = 2.24, CI= 1.15 – 3.17), and alveolar bone loss (OR = 1.91, CI= 1.15 – 3.17) than those with low levels of financial strain within the same coping group. Hugosson et al.[29] observed that age (OR= 1.04, CI= 1.01 – 1.06), plaque index (OR= 1.01, CI= 1.01 – 1.02), smoking (OR= 3.27, CI= 1.89 – 5.66), internal versus external locus of control (OR= 1.08, CI= 1.02 – 1.15) and marital status (widow/widower) (OR= 2.69, CI= 1.28 – 5.64) were variables that significantly increased the risk of severe periodontal disease. Hilgert et al.[32], based on logistic regression, found that cortisol levels were positively associated with: means of CAL $\geq$ 4mm (OR= 5.1, CI= 1.2 – 20.7), 30% of the sites with CAL $\geq$ 5mm (OR= 6.9, CI= 1.7 – 27.1), and 26% of sites with PD $\geq$ 5mm (OR= 10.7, CI= 1.9 – 54.1), after adjustment for confounding variables.

On the other hand, two recent studies did not demonstrate that psychosocial factors increase the risk for periodontal disease. Bivariate analysis, in the study by Castro et al.,[27] did not find significant differences in life events, anxiety symptoms, trait or state of anxiety, or depression symptoms between patients suffering from periodontal disease and patients with no history of periodontitis. Multivariate logistic regression, controlled for confounding factors, demonstrated no significant association between psychosocial factors and periodontal disease. Finally, results of the ordinal logistic regression analysis model

that included age, plaque index, smoking and psychosocial factors showed that patients with psychiatric symptoms (OR=1.24, CI= 0.33-4.78), depression symptoms (OR=0.57, CI= 0.15-2.21) and with hopelessness (OR=0.70, CI= 0.13-3.84) were not at a greater risk of developing established periodontitis.[30]

Results of the studies

With regard to the results of the studies, 57.1% (8 studies) found a positive outcome between psychosocial factors/stress and periodontal disease, 28.5% (4 studies) observed a positive outcome for some characteristics and a negative outcome for others, and 14.2% (2 studies) found a negative outcome. The main conclusions of all studies are summarized in Table 1.

DISCUSSION

Systematic reviews have become widely used for evaluating scientific evidence across all fields of biomedicine. A systematic review can be defined as a review of a clearly formulated question that attempts to minimize bias using systematic and explicit methods to identify, select, critically appraise and summarize relevant research with the aim to provide a comprehensive and contemporary appraisal of research. In essence, research methodology is employed in the conduct of the review. Such reviews are therefore fundamentally different from traditional "narrative" review articles in their purpose and in their potential to aid clinical decision-making.[35] Therefore, the aim of the present study was to systematically review the evidence from selected studies on the influence of stress and psychological factors as a risk for periodontal disease. Several components of quality assurance were included in this review, since the possibility of reviewer bias must be minimized. Thus, screening of abstracts/titles and full-text articles was always performed in duplicate and independently. The same approach was taken for assessment of study quality. Thus, the possibility of bias from one reviewer was reduced. In general, in the present study, data analysis demonstrated a positive association between stress/psychological factors and periodontal disease. This relationship is derived mainly from cross-

sectional[4,15,29,31,32] and case-control studies.[3,24,25,26,33] One prospective clinical trial[34] study also found a positive association between these factors and periodontal disease. However, Solis et al.,[30] in a cross-sectional study, and Castro et al.,[27] in a case-control study, were not able to reproduce these findings.

Observational studies with different designs can assess risk factors for developing periodontal disease and can be considered according to their capability of producing credible scientific evidence. Hypothesis generating studies concerning psychosocial variables include the case reports (not included in this systematic review) by Moulton et al.[36] and De Marco.[37] In these studies it would be possible to observe a presumed primary relationship between these variables and periodontitis. Cross-sectional and case-control studies supply stronger evidence for this correlation. Studies that suggest a strong evidence for risk factors are cohort studies. In this systematic review, no cohort study was found that could be included. However, when we performed a Medline search, we found two studies that followed patients for a specified period of time, the first within 12 months of follow-up and the second, within 5.5 years of follow-up.[8,38] Both studies emphasized the relationship between occupational stress and periodontal disease, finding significant associations. However, these studies did not report the criteria for considering periodontal disease and neither performed controls for confounding variables.

The methodological approaches used in these fourteen studies are quite different; these differences may be responsible for the conflicting results between some studies. Such differences involve the type of periodontal disease investigated, the parameters used for periodontal status evaluation, the type of psychosocial variable analyzed, the questionnaire used for its assessment, as well as adequate controls for potential confounders (i.e., age, gender, tobacco, diabetes, oral hygiene, educational level, socioeconomic level, sample size, allocation concealment, blindness, calibration).

Confounding is a term that describes the situation where an estimate of the association between an exposure and the disease is mixed up with the real effect of another exposure on the same disease, the two exposures being correlated.[39] The control of potential confounders is a challenge when dealing with a disease of high prevalence, such as periodontitis. One way of controlling non-paired variables is at the design stage of an

investigation by randomization, restriction and/or matching.[39] In this systematic review, a small number of studies performed matching[3,4,24,25] or randomization[29] of the sample. Restriction was performed by exclusion criteria in all the articles. Nevertheless, another way of controlling non-paired variables is by the use of statistical modeling. Thirteen studies performed statistical adjustment, from which nine used some type of regression model analysis, while the other five used different types of statistical methods to control the confounding variables.

There is a great difficulty, in the literature, regarding the establishment of clear criteria to define either health or periodontal disease[2,40] and different levels of periodontal attachment loss and inflammation are essential in order to guarantee a clear cut difference in exposure among groups. In the evaluated studies, several criteria were applied to characterize periodontal disease: clinical attachment loss, periodontal pocket depth, alveolar bone loss, remaining periodontal support, bleeding on probing and missing teeth. As an example of diversity, Croucher et al.[24] used as criteria the presence of at least one site with a PD of 5.5 mm or more; Wimmer et al.[28] divided chronic periodontitis into slight or moderate (1 or 2 mm CAL/3 or 4 mm, respectively) and severe (≥ 5mm); Solis et al.[30] formed groups based on the criteria proposed by Machtei et al.,[41] considering subjects with two or more interproximal sites from different teeth with a CAL of 6 mm or greater and at least one additional site with a PD of 5mm or greater, excluding 3rd molars. Castro et al.[27] established its own criteria for the definition of periodontal disease and health, forming groups with distinct characteristics between them regarding PPD, CAL and BOP: individuals should present at least 20 teeth, and have advanced periodontal disease, characterized as CAL ≥ 4mm and BOP in at least 10 teeth, and PPD ≥ 6mm in at least five teeth. This heterogeneity between the studies, makes the comparison of the results of the studies difficult, as well as not allowing the performance of sensitivity analyses in a meta-analysis.[22,23]

Another important aspect of the methodology was the definition of the instruments of psychological aspects. To date, there are no biological markers available or any other measurable means to define safely most psychiatric disturbances.[42] The psychological variables are usually measured by self-reported scales and do not allow an assessment of

the subjective and behavioral aspects of individuals. When this type of instrument is used in research, one should bear in mind that the informers may supply incorrect information and that situation bias may also take place, that is, the condition of instability of the clinical phenomenon being evaluated.[30] Stress is not the same experience for everyone. It depends on how much social support, if any, is available from family and friends, which could lessen the potential stress. Furthermore, more important than the presence of stressful agents is how a person handles or copes with them.[43] Discrepancies in the results found in the literature may also be explained by differences concerning the employed psychometric instruments and diversity of the psychological factors variables examined. Another possible explanation is that the patient's stress responses may reflect recent symptoms, while periodontal disease is a chronic event. Nevertheless, Genco et al.[15] using measures of chronic stress showed differences for stress among patients with different levels of periodontal disease. It is also important that the questionnaire must be validated for the sample analyzed. In this systematic review, only five studies mentioned questionnaire adaptation as a methodological approach.[26,27,30,32,33]

Psychosocial factors lead to changes in the oral habits and in behavioral responses of the host, such as poor hygiene and smoking, and the host's responses to environmental determinants such as stress.[44] Stress can be best understood as part of a complex and dynamic system of transaction between individuals and their environment. Stress is a part of the human condition which is universally present but to varying degrees and with different effects on individuals.[45] Stress is compatible with good health, which is necessary to cope with the challenges of every life. Problems start when the stress response is inappropriate to the size of the challenge, producing neuroendocrine and biochemical changes, resulting in significant adverse effects on the proper functioning of the immune system.[46,47]

The cellular and molecular basis for the interaction between stress and periodontal disease can be explained by the HPA axis, such as the promotion of the release of corticotropic-releasing hormone (CRH) from the hypothalamus and glucocorticoids from the adrenal cortex.[14] Glucocorticoids exert major suppressive effects through highly specific mechanisms at multiple levels. For example, they reduce the number of circulating

lymphocytes, monocytes and eosinophils and also inhibit the accumulation of macrophages, eosinophils and neutrophils at inflammatory sites.[48,49] Glucocorticoids also inhibit the cascade of the immune response,[50] the production of cytokines,[51] and the secretion of IgA and IgG.[14] Secretory IgA antibodies may protect by reducing initial colonization of periodontal pathogens. IgG antibodies may exert protection by opsonizing periodontal organisms for phagocytosis and killing by neutrophils. Hence, these changes have major suppressive actions on the immune and inflammatory responses and give rise to increased susceptibility, which leads to establishment of periodontal infection, which in turn results in destructive periodontitis. In addition, stress can also result in responses being transmitted to the autonomic nervous system, resulting in secretion of cathecolamines which affect the release of prostaglandin and proteases[52,53] which in turn could enhance periodontal destruction. Finally, the effect of psychosocial stress on periodontal disease has been hypothesized to occur through the influence of stress on behavioral changes which affect at-risk health behaviors such as smoking, poor oral hygiene, and poor compliance with dental care.[14] Further prospective studies and randomized clinical trials are needed to understand the role of stress and psychological factors as putative risk factors for periodontal disease. Studies using biochemistry markers, psychological assessment and multiple measurements of variables may be more indicated to clarify the role of psychosocial factors and their mechanisms of action in the periodontal tissues.

In conclusion, within the limitations of this systematic review (that limited the search strategy in studies from 1990 to 2006, human studies, dental journals, studies with adults and middle aged subjects, and only English language articles were considered), the majority of studies showed a positive relationship between stress/psychological factors and periodontal disease. However, caution should be used to interpret this review because the different methodologies found in the included studies (e.g. design of the studies, adjusting for confounding factors, randomization, blindness, calibration), may have an impact on the results of the reports. In addition, the inherited difficulties in isolating the variable stress along with the lack of a reliable standardized psychological analysis to quantify and define most psychiatric disturbances (e.g. depression, anxiety, loneliness), the individual ability of patients to cope with negative life events, and finally the different types (aggressive,

chronic periodontitis) and clinical parameters (PPD, CAL and ABL) utilized to determine periodontal tissue breakdown, may act as confounding biases and cause result distortions at several stages. Although a positive relationship trend was observed between stress and periodontal disease, further representative research is needed to determine the impact of the stress and psychological factors as a risk factor for periodontal disease.

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APPENDIX 1: Excluded studies and main reason for exclusion.

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TABLES

Table 1. Aim, experimental design, statistical model and adjustment, Odds Ratio (Confidence Interval – CI 95%), outcome for periodontal disease and psychological factors and main conclusion of the selected studies.

Authors, years	Aim	Experimental Design	Statistical Model and Adjustment	OR (CI 95%)	Outcome	Main Conclusions
Moss et al., 1996[3]	To explore the role of psychosocial factors including social strain, depression, and coping as host factors that influence adult periodontitis with the use of self-reported questionnaire data.	Case-control	Bivariate logistic regression. For age, sex and current smokers status.	IgG Tf and periodontal disease among individuals who had higher scores for depression: 6.75 (1.25-36.5). Case status and role strain scores of 2.27 or more: 2.84 (1.08-7.46).	Positive/ negative	Elevated Depression may play a role in immune function during periods of social strain. These environmental factors in adult periodontitis exploratory analysis have served to identify specific lines of inquiry concerning psychosocial measures as important environmental factors in adult periodontitis.
Croucher et al., 1997[24]	To evaluate whether a comprehensive range of life-events are associated with an objective measure of periodontitis in adults.	Case-control	Multiple logistic regression. For tobacco smoking, dental plaque levels, employment status, education, marital status and number of missing teeth.	No	Positive	Psychosocial factors, here represented by impact of life events, employment and marital status, as well as dental plaque levels and tobacco smoking cluster together as important correlates of periodontitis and

						these factors may be important determinants of periodontitis.
Monteiro da Silva et al., 1996[4]	To investigate more formally possible associations between a number of relevant psychosocial factors and rapidly progressive periodontitis	Cross-sectional	Multivariate analysis of covariance. For smoking status, drinking habits, oral hygiene, educational background and age.	No	Positive/ negative	The rapidly progressive periodontitis (RPP) group presented significantly increased depression and loneliness compared to the routine chronic adult periodontitis (RCAP) and control groups.
Axtelius et al., 1998[25]	To investigate the perspective of a stress system disorder in pathogenesis of therapy-resistant periodontitis. The goal was to find indications that the stress-behaviour-immune system model holds as an explanatory mode for the understanding of periodontal disease.	Case-control	Logistic multivariate regression. Without adjustment.	No	Positive	The report highlights the possible contribution of stress factors in the context of therapy resistant periodontal disease.
Genco et al., 1999[15]	To investigate the relationship of periodontal disease to stress, distress, and a coping behaviors in a large population-based sample of adults.	Cross-sectional	Logistic multivariate regression. For age, gender and smoking.	Financial strain with greater CAL and ABL: OR= 1.70 (1.09 - 2.65) and OR= 1.68 (1.20 - 2.37); Financial strain + inadequate coping and risk for more	Positive	Psychosocial measures of stress, associated with financial strain and distress, manifest as depression and are significant risk indicators for

				severe AL: OR = 2.24 (1.15 - 3.17), and for ABL: OR = 1.91 (1.15 - 3.17)		more severe periodontal disease in adults in age -adjusted model.
Hugoson et al., 2002[29]	To investigate the prevalence of some negative life events and psychological factors and their relation to periodontal disease.	Cross-sectional	Univariate logistic regression. For age, gender, plaque index, and smoking.	Age: OR= 1.04 (1.01 – 1.06), plaque index: OR= 1.01 (1.01 – 1.02), smoking: OR= 3.27 (1.89 – 5.66), internal versus external locus of control: OR= 1.08 (1.02 – 1.15) and marital status (widow/widower): OR= 2.69 (1.28 – 5.64) were variables that significantly increased the risk of severe periodontal disease.	Positive	Traumatic life events, such as the loss of a spouse, may increase the risk for periodontal disease. An individual's ability to cope with stressful stimuli (coping behaviour), as measured by the beliefs of locus of control of reinforcements may play a role in the progression of periodontal disease.
Wimmer et al., 2002[28]	To study, with the help of a questionnaire, the stress coping patterns used by patients with periodontal disease to react to specific stressful situations, to determine whether patients with periodontitis have specific stress coping strategies, and whether they differ from those of periodontally healthy controls.	Case-control	Varimax rotation with Kaiser-normalization and Pearson's correlation. For smoking, age and education.	No	Positive/ negative	Periodontitis patients with inadequate stress behavior strategies are at greater risk of severe periodontal disease.

Vettore et al., 2003[26]	To investigate the relationship of stress and anxiety with periodontal clinical characteristics.	Case-control	Univariate analysis of covariance. For socioeconomic data and cigarette consumption.	No	Positive/ negative	Individuals with high levels of trait anxiety appeared to be more prone to periodontal disease.
Solis et al., 2004[30]	To evaluate the association between periodontal clinical parameters and anxiety, depression and psychiatric symptoms.	Cross-sectional	Ordinal logistic regression. For age, plaque index and smoking..	Psychiatric Symptoms: OR=1.24 (0.33-4.78); Depression Symptoms: OR=0.57 (0.15-2.21); Hopelessness: OR=0,70 (0.13-3.84).	Negative	No evidence was found for an association between depression, hopelessness, psychiatric symptoms and established periodontitis.
Wimmer et al., 2005[34]	To investigate the influence of individual coping behavior on a non-surgical periodontal therapy and on the subsequent course of disease.	24 month prospective clinical trial.	ANOVA and Pearson's correlation. For gender, smoking habits and education level.	No	Positive	Passive coping strategies were more pronounced in advanced disease as well as cases of poor response to a non-surgical periodontal treatment, whereas patients with active coping modes had milder disease and a more favorable course of treatment.
Vettore et al., 2005[33]	To evaluate the influence of stress and anxiety on the response to non-surgical periodontal treatment (NPT) in patients with chronic periodontitis.	Case-control,	Univariate analysis of covariance. For dental plaque and number of cigarettes.	No	Positive	The data suggest an influence of trait of anxiety and stress on the response to NPT.

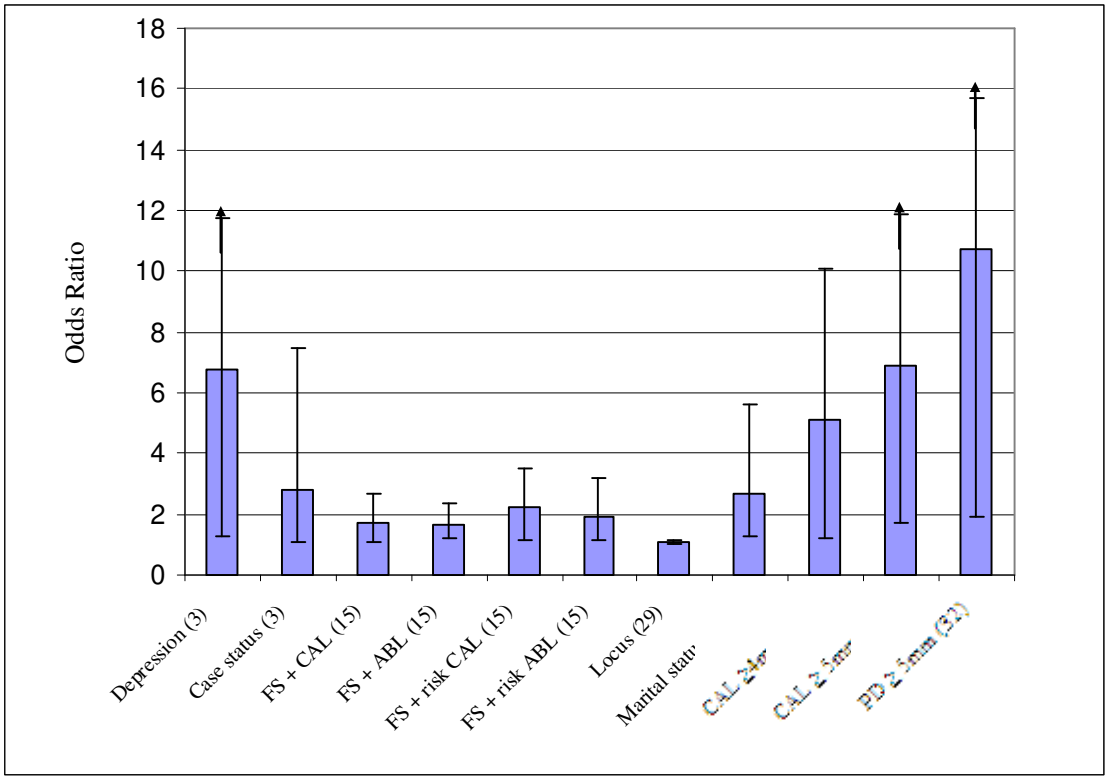
Dolic et al., 2005[31]	To investigate the associations between psychological and periodontal parameters.	Cross-sectional	Stepwise multiple regression analyses. For age, sex, and smoking (pack years).	No	Positive	The results provide evidence for associations of psychosocial factors and periodontal disease. Some environmental traits seem to be related to more favorable periodontal status.
Castro et al., 2006[27]	To investigated the association between life events, anxiety, and depression with periodontitis.	Case-control	Bivariate logistic regression. For smoking.	Positive association of periodontitis with age: OR=1.15 (1.06-1.24); male gender: OR=2.71 (1.13-6-49); smoking : OR=6.05 (1.67-21.94) and educational level: OR= 6.49 (1.14-36.95)	Negative	There was no significant association between periodontitis and the psychosocial factors analyzed.
Hilgert et al., 2006[32]	To evaluate the extent and severity of chronic periodontitis and its association with the levels of cortisol and the scores of an inventory of stress symptoms in a population age 50 years or older.	Cross-sectional	Multivariate logistic regression. For demographic and socio-economic variables, diabetes, smoking, oral hygiene habits, time since the last visit to the dentist, previous periodontal treatment, VPI, GBI, BOP, stress phases and scores and caregiving status.	Cortisol levels were positively associated with: CAL ≥ 4mm= 5.1 (1.2 - 20.7), 30% of the sites with CAL ≥ 5mm= 6.9 (1.7 - 27.1), 26% of sites with PD ≥ 5mm= 10.7 (1.9 - 54.1).	Positive	Cortisol levels were positively associated with the extent and severity of periodontitis.

Table 2. Quality assessment of the selected studies.

Autors, years	Randomization	Calibration	Stratification	Matching	Statistical Adjustment	Main criteria for periodontal disease
Moss et al., 1996[3]	Absent	Present	Present	Present	Present	CAL
Croucher et al., 1997[24]	Absent	Absent	Absent	Present	Present	PPD
Monteiro da Silva et al., 1996[4]	Absent	Absent	Absent	Present	Present	ABL
Axtelius et al., 1998[25]	Absent	Absent	Present	Present	Absent	PPD
Genco et al., 1999[15]	Absent	Present	Present	Absent	Present	CAL
Hugosson et al., 2002[29]	Present	Present	Absent	Absent	Present	ABL
Wimmer et al., 2002[28]	Absent	Absent	Absent	Absent	Present	CAL
Vettore et al., 2003[26]	Absent	Present	Absent	Absent	Present	PPD
Solis et al., 2004[30]	Absent	Present	Absent	Absent	Present	CAL/PPD
Wimmer et al., 2005[34]	Absent	Absent	Absent	Absent	Present	CAL
Vettore et al., 2005[33]	Absent	Present	Absent	Absent	Present	PPD
Dolic et al., 2005[31]	Absent	Absent	Absent	Absent	Present	CAL/PPD
Castro et al., 2006[27]	Absent	Present	Absent	Absent	Present	CAL
Hilgert et al., 2006[32]	Absent	Present	Absent	Absent	Present	CAL/PPD

FIGURE

Figure 1. Odds Ratio and Confidence Interval (95%) for relationship between periodontal disease and psychological factors.



The arrows indicate CI ≥ 5.

CAPÍTULO 2

Chronic stress may modulate periodontal disease. A study in rats.

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ONE SENTENCE SUMARY: Chronic stress might significantly increase periodontitis progression and, locally, an increase in pro-inflammatory and pro-resorptive factors may be involved.

Running title: Chronic stress and periodontal disease

Number of tables and figures: 4

ABSTRACT

Background: The present study aimed to i) evaluate whether chronic stress (CS) affects ligature-induced periodontal disease (PD), and ii) investigate the impact of CS on the mRNA levels of interleukin (IL)-1β, IL-1ra, IL-6, IL-10, interferon (INF)-γ; receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in the gingival tissues of rats.

Methods: Sixty male Wistar rats were randomly assigned into three groups: G1 – control (non-ligated sites); G2 – PD; and G3 – PD associated with restraint stress for 12 hours/day for the entire study. After 30 days, all animals were killed by decapitation. Blood samples were taken and the concentrations of corticosterone and catecholamines were measured as biomarkers of CS. Marginal tissues around ligated and non-ligated teeth were harvested and gene expression assessed by quantitative polymerase chain reaction technique (qPCR). Moreover, the area of bone loss (ABL) was histometrically determined.

Results: Data analysis showed that: i) CS increased serum levels of stress biomarkers ($p<0.05$); ii) ligature placement resulted in a significant ABL, as compared to non-ligated sites; iii) CS significantly increased the amount of ABL in inflammed sites ($p<0.001$); and iv) CS significantly increased mRNA levels of pro-inflammatory (IL-1β, IL-6 and INF-γ) and anti-inflammatory (IL-10) cytokines and pro-resorptive factor (RANKL) in ligated sites ($p<0.05$).

Conclusion: Within the limits of this study, it may be concluded that CS significantly increased bone loss resulting from ligature-induced periodontitis by a local increase in pro-inflammatory and -resorptive factors.

KEY WORDS: Chronic stress, periodontal disease, inflammation

INTRODUCTION

Epidemiological studies have demonstrated that periodontitis does not affect all subjects in the population in a similar manner. Studies from many parts of the world, as reviewed by Johnson et al.,[1] indicate that only a subpopulation of 7% to 15% of the dentate adult population are affected by destructive periodontal disease. Although bacteria are well established as the etiological agents of periodontal disease, the fact that its presence alone is not capable of producing advanced tissue destruction in all individuals suggests that there is an individual response and adaptation ability to a certain amount of bacterial biofilm without progression of the disease. Environmental risk factors, such as smoking and diabetes mellitus may modify the host response and hence modify disease progression, severity and outcome.[2] Other factors, such as stress, depression and anxiety are not yet confirmed as absolute risk conditions, but have been identified in some observational studies as potential factors that may affect periodontal disease.[3]

Stress has been linked to periodontal disease since the middle of the last century; and most reports comprise necrotizing forms of periodontal disease.[4] In the past decade, more evidence has emerged from epidemiological studies relating periodontitis to stress, depression and negative life events.[3,5] Animal studies conducted in the 1960s have demonstrated a possible detrimental role of stress in periodontal tissues.[6,7] More recently, Gaspersic et al.[8] found less attachment and more alveolar bone loss after exposing the experimental animals to restraint stress. In a series of studies with rats, Breivik et al.[9] demonstrated that periodontal disease susceptibility and progression could be explained, at least partly, by brain-neuroendocrine-immune regulatory mechanisms. Genetically determined hypothalamus-pituitary-adrenal (HPA) reactivity seems to play an important role and a possible feedback is likely to occur from periodontal disease.[9] To date, several cross-sectional studies[5,10,11] and case-control study[12] found that HPA axis hyperactivation may increase the odds ratio for periodontal disease. In addition, a systematic review[13] strongly suggest that stress, distress and inadequate coping behaviors[5] are important risk indicators for periodontal disease.

The biological plausibility for such an association is supported by studies showing that psychosocial conditions, such as depression and exposure to stress agents, may affect the host immune response, making the individual more susceptible to the development of unhealthy conditions, and also affecting periodontal health.[14] It has been suggested that acute stress, defined by the effect of a short-term psychological stress agent,[15] may produce an increase in the innate immunity, antigen presentation, and antibody and cytokine production.[15] Whereas chronic stress, defined by the effect of a long-time or persistent stress agent, may usually lead to a diminished immune response including a depress in the immunoglobulin-A (IgA), immunoglobulin-G (IgG), neutrophils functions and cytokine production, all of which may be important in protection against infection by periodontal organisms.[16] In summary, although several animal[17,18] and human studies[3,6,11,16] have suggested the influence of stress in the susceptibility and progression of periodontal disease, there is very limited information on how stress locally modulates periodontal disease through its effect on the immuno-inflammatory system. Therefore, based on the clinical relevance of this subject and the lack of information in the literature, the present study aimed at investigating the effect of chronic stress (CS) on the bone loss resulting from ligature-induced periodontitis in rats and assessing the impact of CS on the mRNA levels of key factors that regulate the inflammatory and resorptive processes in the gingival tissues.

MATERIAL AND METHODS

Animals

Sixty male Wistar rats, of 4 weeks of age, were used in the present study. The sample size was estimated using data from previous studies published by our group and calculated using a power of 0.8 and an alpha of 0.05. [17] The animals were acclimatized to the housing conditions during the course of 4 weeks, and a 12 hour light and dark cycle was applied. The animals were housed five per cage at a permanent temperature of 21ºC. Standard rat chow pellets and water *ad libitum* were available, except during times when restraint stress was applied. After weight stratification, the animals were randomly assigned

into three experimental groups of 20 rats each: G1 – control; G2 – periodontal disease (PD) induced by cotton ligature; and G3 - PD associated with restraint stress. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee (#789-1/2005).

Experimental procedures

General anesthesia was obtained by intra-muscular administration of ketamine (0.5ml/kg). As a parallel single blinded study, both mandibular first molars of each animal received cotton ligatures in the dental-gingival area to induce experimental PD, except for the animals in the control group which were submitted to a sham procedure that consisted of the same procedures used for ligature placement, e.g. animal handling and anesthesia administration, except for ligature placement. The ligatures were kept in place during the entire experimental period and served as a retention device for oral microorganisms. Restraint stress was daily applied from 8 p.m. to 8 a.m. (12 hours) for the entire experimental period (30 days) in G3 with the use of a flexible plastic mesh (30x30cm) and the animals housed in plastic pipes (10x30cm) full of holes for restrain and isolation purposes. As nocturnal animals, it has been shown that the effect of the stressor agent may be significantly enhanced if applied at this time.[18] The animals from G3 were, therefore, unable to move and had no access to food or water during the period of restraint. During this time, animals from G1 and G2 also received limited access to food and water as an attempt to control such variables (pair feeding). At the end of the daily 12-hour cycle, all animals were returned to their cages. Additional care was taken, during handling of the animals, at the time of sacrifice (30 days after ligature placement) to avoid additional unwanted stress with consequent alterations of its biomarkers, and decapitation took place within 2 minutes of the last release from the restrainer, for G3, and from the cage for G1 and G2.

Sample Collection

Immediately after sacrifice, trunk blood was collected in precooled plastic vials, containing 0.2 ml of EDTA solution (10%) and sodium heparin for corticosterone and catecholamines determination, respectively. Blood samples were centrifuged at 2,300 rpm for 15 minutes at 4ºC. Plasma was extracted, stored in plastic tubes and frozen at -20ºC to

assess corticosterone and catecholamines levels. In addition, the marginal gingival tissues around the mandibular first molars were harvested (approximately 100mg), rinsed with cold sterile saline solution and stored[#] at -70°C for future mRNA level analysis. Finally, the jaws were removed and fixed in 4% neutral formalin for 48h for histological processing in order to determine the area of bone loss in ligated and non-ligated teeth for all the experimental groups.

Laboratory Assays

Corticosterone concentration was assayed by a double-antibody RIA method, using a commercial kit** and following the manufacturer's recommendations. The sensitivity of the assay was 0.25µg/dl. Catecholaminess (adrenaline and noradrenaline) concentrations were assayed by HPLC. Duplicate aliquots were used for both analyses.

Histological procedures

The fixed jaws were subsequently demineralized in an EDTA buffered solution (10% - pH=7.0) for 45 days. Paraffin serial sections (6µm) were obtained in a mesial-distal direction, and stained with hematoxylin and eosin. After excluding the first and the last sections in which the furcation region was evident, ten equally distant sections of each tooth were selected for histometric analysis. Using an image analysis system[††], the area of bone loss in the furcation region (ABL) or the area of periodontal ligament (PLA) was histometrically determined by a blinded and calibrated examiner (Intra Class Correlation = 0.92). For histometric analysis the area of the interradicular ABL/PLA was defined by the area of connective tissue limited by the tooth and bone crest surfaces on ligated and non-ligated teeth.

RNA extraction

Total RNA from gingival biopsies was extracted using the TRIZOL reagent[‡‡] according to the manufacturer's recommendation. The RNA pellet was resuspended in diethylpyrocarbonate-treated water and stored at -70°C. The RNA concentration was determined from the optical density using the Biophotometer[§§]. Samples were quantitatively assessed for the expression of the following genes: IL-1β, IL-1ra, IL-6, IL-10, IFN-γ, RANKL and osteoprotegerin (OPG) for all groups. In addition, in order to determine whether periodontal tissues express the main receptors for the biomarkers of stress used in

the present study, e.g. corticosterone and cathecolamines, gingival tissues harvested from periodontally healthy animals (G1) were assessed for the expression of glucocorticoid receptor (Gr) and adrenergic receptor β2 (Ar- β2).

Real-time PCR reactions

Reverse transcription: Total RNA was DNase‖‖ treated and 1μg was used for cDNA synthesis. The reaction was carried out using the first-strand cDNA synthesis kit,¶¶ following the manufacturer's recommendations.

Primer design: Primers were designed using probe design software¶¶ (Table 1).

qPCR reactions: qPCR was performed¶¶ using the sybergreen system.¶¶ The reaction product was quantified using a software,¶¶ with glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as the reference gene. Experiments were run twice with comparable results and water was used as the negative control.

Statistical Analysis

Body weight analysis was performed using one-way ANOVA at day zero and at the end of experimental period. If statistical difference was detected, a pair-wise multiple comparison test (Bonferroni t-test) was used to identify the difference among the groups. Mean values and standard deviation of ABL/PLA, corticosterone and catecholamines and mRNA data were determined for each group and compared statistically using the one-way ANOVA (α=0.05). If statistical difference was detected, a pair-wise multiple comparison test (Bonferroni t-test) was used to identify the difference among the groups. Furthermore, an intergroup analysis was performed by the non-parametric Kruskal-Wallis test (α=0.05), in order to statistically compare RANKL/OPG, IL-1β/IL-1ra and IL-6/IL-10 ratios, followed by a pair-wise multiple comparison performed by Dunn's test in the cases where difference was detected.

RESULTS

Body weight

The mean body weight of all rats was approximately 180g at the beginning of the study and did not show statistically significant differences among the groups ($p>0.05$). At the end of experimental period, there was a significant increase in the mean body weight for the animals in the three experimental groups with a significant difference among them ($p<0.05$). G1 gained significantly more weight during the study than G2 and G3. The final body weight was 381.64 ± 32.99g, 300.78 ± 83.84g and 238.45 ± 18.46g, for G1, G2 and G3, respectively.

Systemic Biomarkers of Stress

Thirty days after the beginning of the experimental period, intergroup analysis demonstrated that both corticosterone and catecholamines plasmatic levels, used as biomarkers of CS, were significantly increased in G3, as compared to G1 and G2 (Table 2).

Histometric findings

Data analysis demonstrated a significant difference in the ABL in the furcation region ($p<0.001$) between G2 and G3 and the PLA in G1. Ligature placement (G2) resulted in a significant bone loss in the interradicular area of the first mandibular molars ($p<0.001$), as compared to non-ligated sites. In addition, CS significantly increased the rate of periodontitis progression in ligated sites (G3), as compared to ligated sites not exposed to CS (G2) (Table 2). Figure 1 illustrates the histological aspects of the interradicular bone loss for G2 and G3, and PLA G1.

Gene expression analysis

The results for the mRNA levels in the gingival tissues around ligated and non-ligated teeth, in G1, G2 and G3, are presented relative to Gapdh values. At first, it was observed that Ar-β2 and Gr, the main receptors for catecholamines and corticosterone, respectively, are constitutively expressed in the periodontal tissues (data not shown). Gene expression analysis further demonstrated that IL-1β, INF-γ, IL-10 and RANKL mRNA levels were significantly increased by inflammation produced by ligature placement, as compared to the non-ligated sites (G1 versus G2 - $p<0.05$), whereas mRNA levels for OPG

were similar between G1 and G2 ($p>0.05$). Additionally, gene expression data demonstrated that the association of inflammation (ligated sites) with CS significantly increased the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and INF-γ ($p<0.05$), the anti-inflammatory cytokine IL-10 ($p<0.05$) and the pro-resorptive factor RANKL ($p<0.05$); whereas this association significantly decreased mRNA levels of the anti-inflammatory IL-1ra and had no effect on OPG mRNA levels ($p>0.05$), as compared to G1 and G2. Finally, significantly increased RANKL/OPG, IL-1β/IL-1ra and IL-6/IL-10 ratios were found in the stress group (G3) as compared to both control (G1) and PD (G2) groups ($p<0.05$), while G1 and G2 presented similar ratio values ($p>0.05$). Figure 2 illustrates gene expression data.

DISCUSSION

Stress may be defined as a response state of the organism to forces acting simultaneously on the body, which, if excessive – that is, straining the capacity of adaptive processes beyond their limits – lead to diseases of adaptation and eventually to diseases of exhaustion and death.[19] In this way, stress is an important factor in the etiology and maintenance of many inflammatory diseases, including periodontal disease.[20] In the present study, histometric analysis re-edited previous findings[8,9,18,21] showing that CS may significantly affect the amount of bone loss in sites with periodontal disease. Moreover, the effect of CS on the expression of important factors involved in the pathogenesis of periodontal disease was assessed in the gingival tissues. For the first time, it was shown that CS may modulate the expression of pro- and anti-inflammatory and pro-resorptive factors, including IL-1β, IL-6, IFN-γ, IL-10, IL-1ra and RANKL, in sites with periodontitis.

The cells of the immune system are widely distributed throughout the body, when an infection occurs it is the inflammatory response that allows marshalling of immune system elements to specific sites. Early events in the inflammatory reaction to infection are typically clinically undetectable. As the infectious process becomes more chronic, clinically evident inflammation occurs, generating high levels of cytokines and other mediators of inflammation associated with activation of the stress system.[16,20] Data of the present study showed that inflammation, produced by ligature placement, significantly increased local

mRNA levels for IL-1β, INF-γ, IL-10 and RANKL, when compared to the non-inflammed sites, whereas mRNA levels for OPG were similar between inflammed and non-inflammed sites. Our findings, therefore, confirm a number of previous reports indicating that elevated levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and INF-γ, and the pro-resorptive factor RANKL are associated with sites showing periodontal destruction.[22]

Cytokines play an important role in the activation of inflammatory and pro-resorptive cells. On the other hand, stress-induced elevations in glucocorticoid levels have been shown to alter the carefully regulated dynamic system that controls the development of the pro/anti-inflammatory response.[23] In the present observations on the kinetics of the local inflammatory response, it could be observed that the association of inflammation with CS significantly increased the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and INF-γ. IL-1β is one of the most potent and most multifunctional cell activators. The biological activity for IL-1β is extremely diverse, with the focus on the activation of acute-phase proteins, prostaglandins, other cytokines, the induction of collagen and collagenase synthesis, and calcium resorption in the bone.[24] IL-6 is known as a Th2 cytokine that stimulate humoral immunity and with broader effects in inflammatory immunity,[25] such as regulation of antibody profiles, B cell differentiation, stimulation of IgG secretion and promotion of the expansion of locally activated T cells.[26] Besides its involvement in regulating humoral immunity, one of the main activity of IL-6 in bone is its effect on osteoclastogenesis and bone resorption.[27] Further, as has been demonstrate,[28] IL-6 stimulates bone resorption either by increasing RANKL or by directly inducing osteoclast formation by a RANKL-independent mechanism. Moreover, IL-6 production was recently shown to be positively correlated with severity of diseased sites and, therefore, it has been suggested that IL-6 may play a role in modulating the inflammatory cascade of chronic periodontitis.[29]

The role of INF-γ in periodontal tissue destruction is controversial. INF-γ is produced by Th1 cells and may induce IL-1α, TNF-α and prostaglandin E2 (PGE2) production by macrophages, indicating that it is primarily pro-inflammatory in nature.[30] Evidence has suggested that INF-γ^+ Th1 cells are strongly associated with enhanced alveolar bone loss during periodontal infections, and that high absolute levels of pro-

inflammatory cytokines, including INF-γ, are closely associated with periodontal disease severity.[30] Furthermore, significantly higher proportions of gingival mononuclear cells from periodontitis patients expressed INF-γ mRNA than did those from healthy subjects.[31] In contrast, it has also been shown that INF-γ may directly inhibit RANKL-mediate osteoclastogenesis.[32] Consequently, INF-γ is concluded to have a biphasic character, being both destructive and protective. Additionally, in the present study, data analysis demonstrated that CS significantly increased mRNA levels of the anti-inflammatory cytokine IL-10 and the pro-resorptive factor RANKL in inflammed sites, whereas significantly decreased mRNA levels of the anti-inflammatory IL-1ra and had no effect on OPG mRNA levels, when compared to G1 and G2. In periodontal disease, the anti-inflammatory cytokine IL-10 has been reported to have a significant impact on modulating bone resorption.[33] Moreover, studies suggest the involvement of RANKL and OPG in the pathogenesis of periodontitis, demonstrating that osteoclast formation from precursor cells, as well as osteoclast activation, requires the molecule RANKL.[34] OPG, a secreted glycoprotein, is a decoy for RANKL that inhibits osteoclast formation and differentiation.[35] As a consequence of such modulation increased RANKL/OPG, IL-1β/IL-1ra and IL-6/IL-10 ratios were found in the present investigation, favoring, therefore, a pro-inflammatory and pro-resorptive response. In contrast to the findings of the present study where CS was found to increase mRNA levels of pro-inflammatory cytokines locally in the gingival tissues, as reviewed by Boyapati & Wang,[36] stress-associated decreased levels of IL-1α and TNF have been reported to delay wound healing. Furthermore, reductions in IL-1 and IL-8 have also been found in the fluid from blister wounds in patients exhibiting higher stress scores, compared with controls.[37] Finally, studies in rodents have demonstrated that restrain stress significantly decreased wound healing due to an impaired production of IL-1 and TNF.[38,39]

Numerous interdisciplinary psychoneuroimmunological studies have provided evidence that glucocorticoids modulate the stress response at a molecular external stimuli, generating emotional stress responses that may influence and modulate the immune system via nervous and neuroendocrine system.[23,40] Glucocorticoids may modulate immune responses in numerous ways, including gene expression, transcription, translation, post-

translation processing, protein secretion, and cell progenitor proliferation and differentiation.[23] Emotional stress results in the release of catecholamines from the sympathetic nerve fibers of the autonomic nervous system that innervate the tissues of the immune system and in the hormonal secretion of catecholamines from cells of the adrenal medulla.[16] In addition, during a stress response the activation of HPA-axis release ACTH (adrenocorticotrophic hormone) into the circulation. ACTH then acts on the adrenal cortex and causes production and release of glucocorticoid (GC) hormones. Thus, GC levels can be used to monitor this activation.[21] In the present study, data analysis demonstrated that both corticosterone and catecholamines levels, used as biomarkers of CS, were significantly increased, after 30 days of daily stress, in the restraint group (G3), as compared to non-restraint groups (G1 and G2) and, therefore, these findings confirm the efficacy of the method used in the present investigation to cause stress. Since corticosterone and catecholamines are biologically active stress products that bind to specific receptors to regulate genes involved in the stress system, the expression of these receptors is of fundamental importance for both corticosterone and catecholamines to produce their biological effect.[23] Therefore, an additional aim of the present study was to determine whether periodontal tissues express the main adrenergic receptor- β2 and GC receptor (Ar-β2 and Gr) receptors for catecholamines and corticosterone, respectively. Data analysis demonstrated that both receptors are constitutively expressed in healthy periodontal tissues and, therefore, one can assume a direct local effect of both factors on the periodontium. Although, it was initially believed that stress, especially as mediated through the HPA axis, was immunosuppressive,[23,41] accumulated evidence makes it clear that corticotropin releasing hormone, catecholamines and other elements of the stress system may in fact influence the immune system in both directions, whether at resting (baseline) or at elevated levels associated with stress.[40]

Taken together, the results of the present study indicate that although the presence of GC may produce a systemic immunosuppressive effect, as related in previous studies,[23,41] locally when stress is associated with PD it may modulate bone destruction in PD by involving local increased levels of pro-inflammatory factors (IL-1β, Il-6 and INF-γ), decreased levels of anti-inflammatory cytokines (IL-1ra) and increased levels of a pro-

resorptive factor (RANKL). Such an effect may result in increased IL-1β/IL-1ra, IL-6/ IL-10 and RANKL/OPG ratios favoring, therefore, tissue destruction. To the authors‘ knowledge, this is the first study that suggests that CS may promote a local imbalance in the pro/anti-inflammatory and pro/anti-resorptive system in the periodontal tissues. However, additional studies are necessary to further explore these findings, and also to evaluate the role of other important factors, such as other cytokines, inflammatory factors and psychoneuroimmunologics products involved in the pathogenesis of stress modulation of periodontal breakdown.

FOOTNOTES

RNAlater,® Ambion Inc., Austin, TX, USA

** ICN Biomedicals, Costa Mesa, CA, USA

†† Image-Pro,® Media Cybernetics, Silver Spring, MD, USA

‡‡ Gibco BRL, Life Technologies, Rockville, MD, USA

§§ Eppendorff AG, Hamburg, Germany

‖‖ Turbo DNA-free,® Ambion Inc., Austin, TX, USA

¶¶ LightCycler,® Roche Diagnostics Co., Indianapolis, IN, USA

FastStart DNA Master^{plus} SYBR Green Kit, Roche Diagnostics Co., Indianapolis, IN, USA

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TABLES

Table 1: Primer sequences for each gene, annealing temperature, and amplicon length.

Gene	Sequence (5´ → 3´)	Amplification profile [temperature (°C)/time (sec)]	Amplicon (bp)
GAPDH	GCCTTCTCTTGTGACAAAGTG	95/0; 51/9; 72/3	163
	TGGTGATGGGTTTCCCG		
IL-1β	TGTGGATCCCAAACAATACCC	95/0; 52/12; 72/3	151
	TAGGAAGACAGGTCTGTGCTC		
IL-1ra	CCTGCAAGATGCAAGCC	95/0; 55/6; 72/20	152
	GAACACATTCCGAAAGTCAATAGG		
IL-6	GAGAAGTTAGAGTCACAGAAGGAG	95/0; 55/7; 72/20	191
	ATTTAGATACCCATCGACAGGATATATT		
IL-10	CCTCTGGATACAGCTGCGA	95/0; 55/6; 72/20	166
	TGTCACGTAGGCTTCTATGC		
AR-β2	TTTACATCCTCCTTAACTGGTTGG	95/0; 55/3;72/6	154
	CTGTTGCTAGAGTAGCCGT		
GR	CTGAGCAGATTAACCGTCCT	95/0; 55/5;72/6	169
	TACGGGCTTGGTTGCTAT		
IFN-γ	AATACTTGAGAGCCAGATTATCTCTTTCTA	95/0; 55/5;72/6	196
	TTTGTGCTGGATCTGTGGG		
RANKL	AGCGCTTCTCAGGAGTT	95/0; 51/9; 72/3	151
	TACCAAGAGGACAGACTGACTTTA		
OPG	AGTGAAGATAAGCTGCTTATAGTTAGG	95/0; 55/7; 72/20	151
	GCTGGAGGATCTTCATTCCC		

Gapdh: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; IFN-γ: Interferon gamma; AR-β2: adrenergic receptor β2, GR: glucocorticoid receptor; RANKL: Receptor activator of nuclear factor-kappaB ligand; OPG: Osteoprotegerin

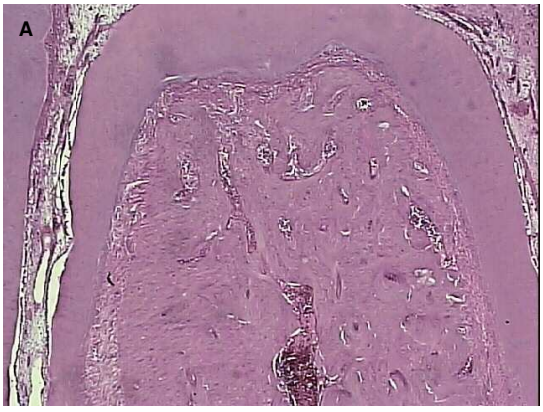
Table 2. Mean values and standard deviation for the area of interradicular bone loss (ABL) in G2 (DP) and G3 (DP + stress) and area of periodontal ligament (PLA) for G1 (control) and concentrations of corticosterone and catecholamines (noradrenaline and adrenaline) for all the experimental groups.

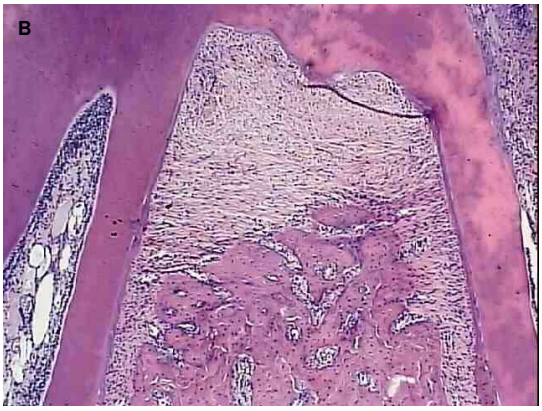
	G1	G2	G3	P value
ABL/PLA (μm^2)	0.07 ± 0.01^C	0.31 ± 0.08^B	0.88 ± 0.09^A	$p<0.001$
Corticosterone (pg/ml)	68.07 ± 51.37^B	60.55 ± 32.34^B	380.06 ± 129.20^A	$p<0.05$
Noradrenaline (pg/dl)	133.85 ± 42.93^B	148.55 ± 32.73^B	300.25 ± 95.43^A	$p<0.05$
Adrenaline (pg/dl)	123.71 ± 36.33^B	129.86 ± 31.78^B	261.84 ± 103.15^A	$p<0.05$

Mean and standard deviation followed by distinct letters in line differ statistically (ANOVA and Bonferroni's test)

FIGURES

Figure 1A-C. Photomicrograph illustrating bone loss in the furcation region of the mandibular molars, which was quantitatively assessed. A, B and C are representatives of control (G1), periodontal disease (G2) and stress/ periodontal disease (G3) groups, respectively. Abbreviations: ABL: area of bone loss; APL: area of periodontal ligament; IAB: interradicular alveolar bone; T: tooth. Original magnification 12.5x – H&E staining.





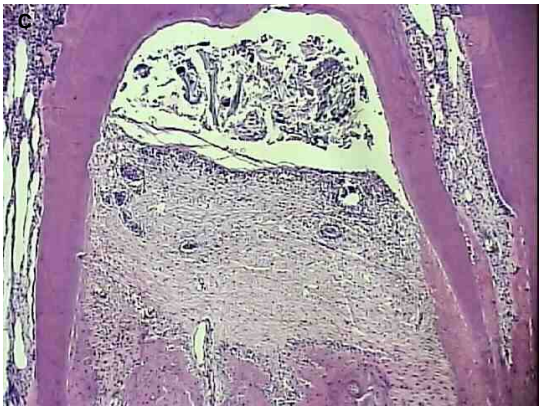
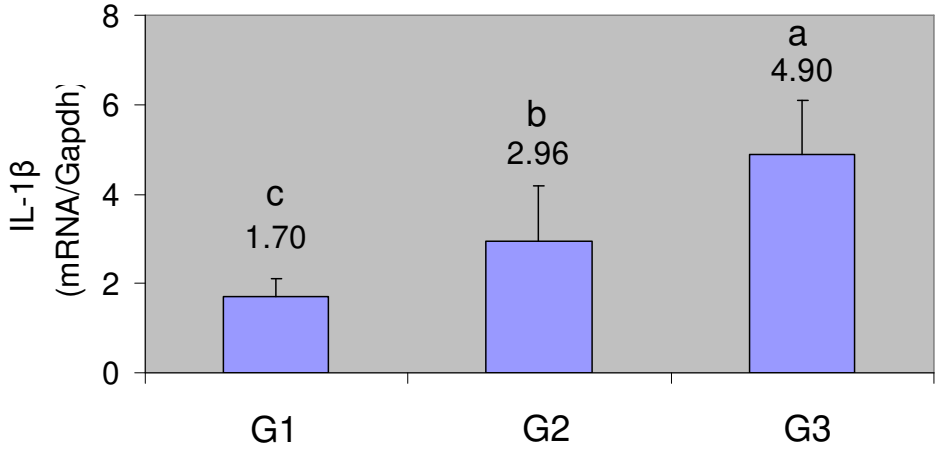
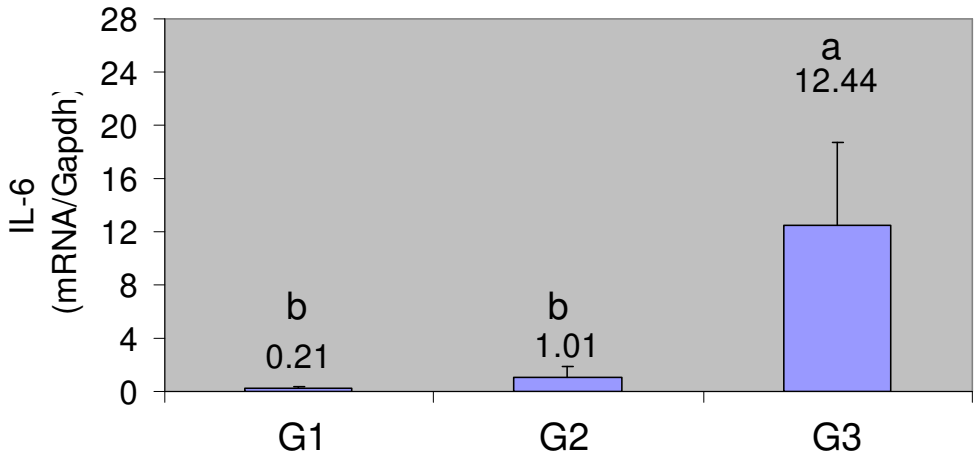


Figure 2A-J. Mean and standard deviation mRNA levels (mRNA/GAPDH) for the pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ), the anti-inflammatory cytokines (IL-1ra and IL-10), the pro- and anti-resorptive factors (RANKL and OPG, respectively); and IL-1β/IL-1ra, IL-6/IL-10 and RANKL/OPG ratios in the gingival tissues harvested from the control sites (G1), sites with periodontal disease (G2), and from sites with periodontal disease associated with chronic stress (G3). Mean values followed by different letters at the top of each bar indicate statistical differences determined by an intergroup analysis (α=0.05) (Kruskal-Wallis and Dunn's test for ratio; ANOVA and Bonferroni t-test for other analysis).

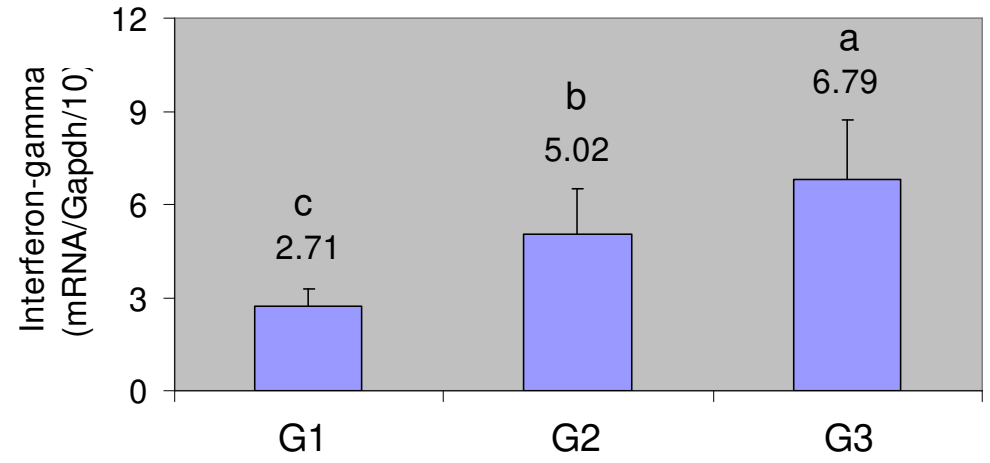
A



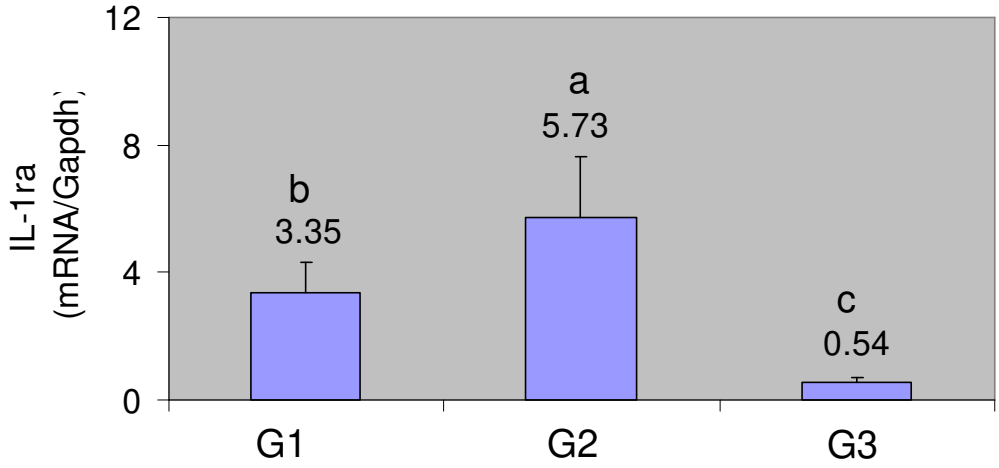
B



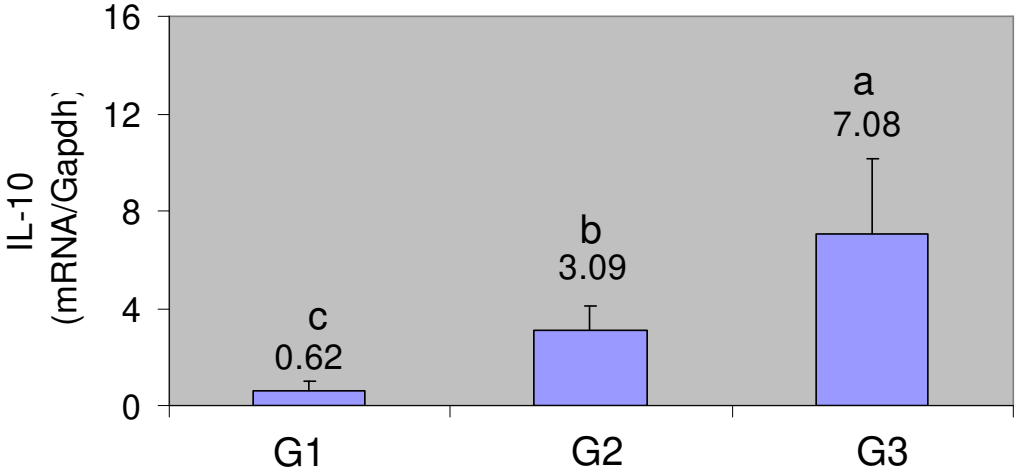
C


Interferon-gamma
(mRNA/Gapdh/10)
12
9
6
3
0
c
2.71
b
5.02
a
6.79
G1
G2
G3

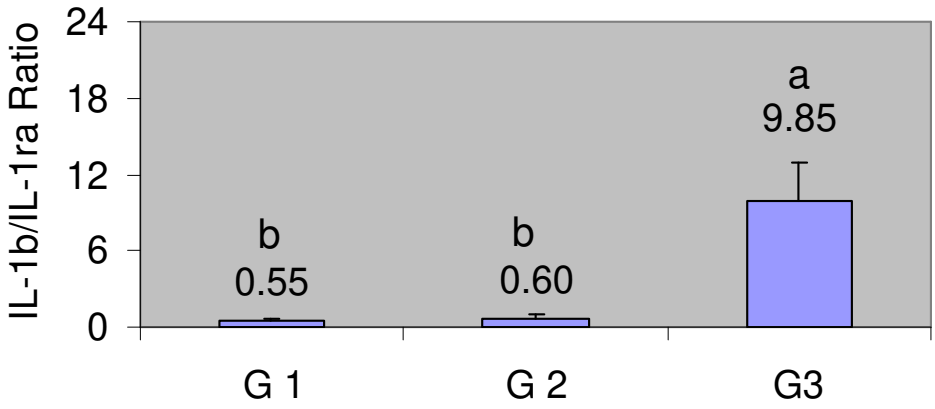
D


IL-1ra
(mRNA/Gapdh)
12
8
4
0
b
3.35
a
5.73
c
0.54
G1
G2
G3

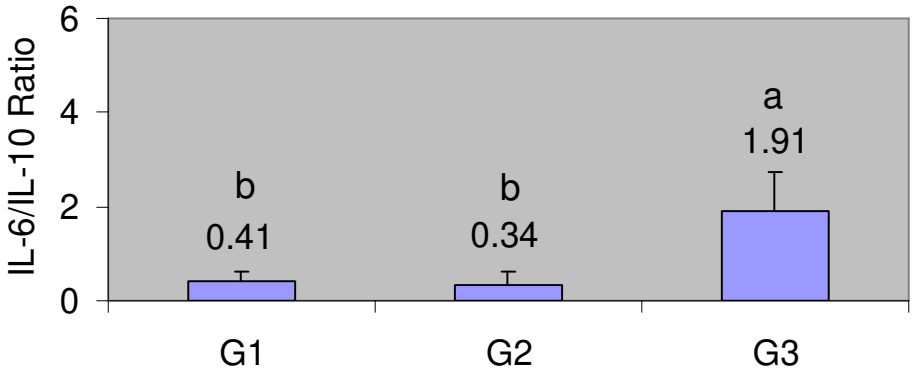
E


IL-10
(mRNA/Gapdh)
16
12
8
4
0
c
0.62
b
3.09
a
7.08
G1
G2
G3

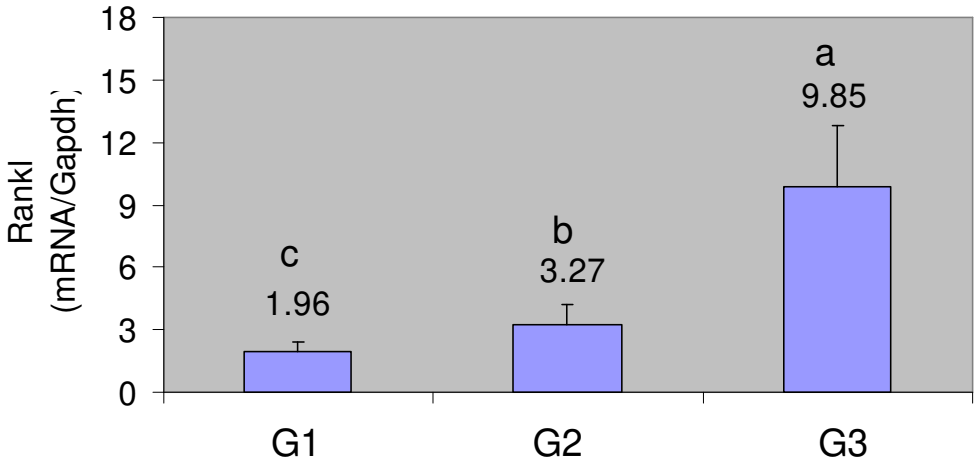
F


IL-1b/IL-1ra Ratio
24
18
12
6
0
b
0.55
b
0.60
a
9.85
G 1
G 2
G3

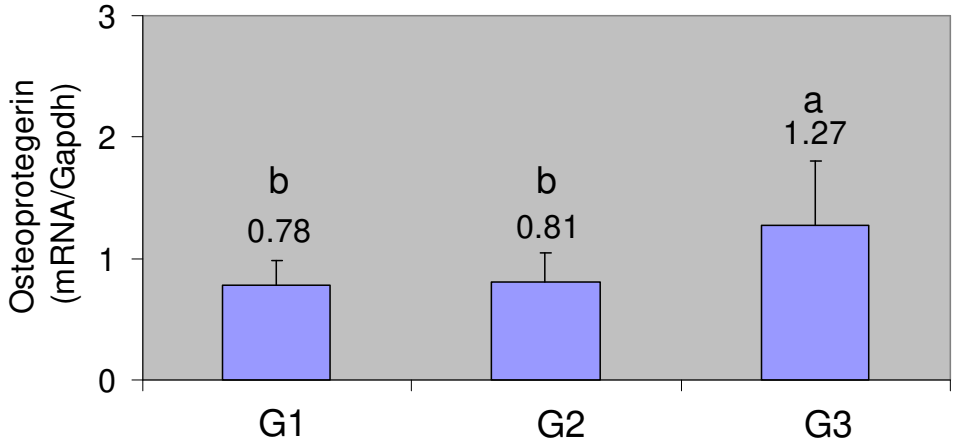
G


IL-6/IL-10 Ratio
6
4
2
0
b
0.41
b
0.34
a
1.91
G1
G2
G3

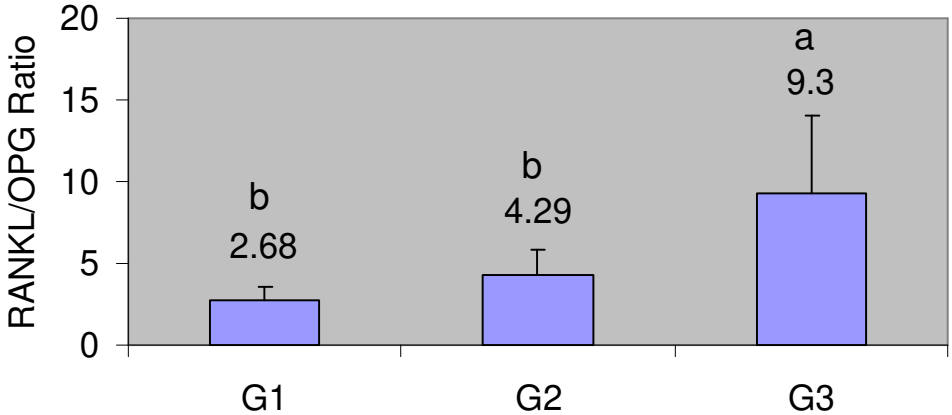
H


Rankl
(mRNA/Gapdh)
18
15
12
9
6
3
0
c
1.96
b
3.27
a
9.85
G1
G2
G3

I


Osteoprotegerin
(mRNA/Gapdh)
3
2
1
0
b
0.78
b
0.81
a
1.27
G1
G2
G3

J


RANKL/OPG Ratio
20
15
10
5
0
b
2.68
b
4.29
a
9.3
G1
G2
G3

CAPÍTULO 3

Evidence that metyrapone can act as a modulator of periodontal breakdown. Short Comunication

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Running title: Metyrapone can modulate periodontal bone loss.

ABSTRACT

Objective: The aim of this study was to evaluate the viability of the use of metyrapone (MT) as an experimental model to inhibit glucocorticoid (GC) production and, therefore, as a method to determine the effect of stress-induced GC overproduction on periodontal tissues.

Material and methods: Sixty rats were assigned into three groups: G1-control; G2-periodontal disease (PD) induced by cotton ligature, associated with restraint stress (RS); and G3-PD + RS + 3 daily doses of MT. After 30 days, all animals were killed and blood samples were taken to determine the concentrations of corticosterone serum levels. Marginal tissues around teeth were harvested and gene expression was assessed by quantitative polymerase chain reaction (qPCR). The area of bone loss (ABL) was also histometrically determined.

Results: Intergroup analysis showed that: PD + RS significantly ($p<0.001$) increased the ABL in ligated sites, as compared to non-ligated sites (G1); furthermore, MT significantly ($p<0.05$) decreased the production of GC in G3. However, MT also increased ABL and the mRNA levels of all the pro-inflammatory factors assessed (INF-γ, TNF-α, IL-1β and IL-6), when compared to G1 and G2.

Conclusion: Within the limits of this study, it may be concluded that MT can modulate periodontal disease and, therefore, is not a viable approach to assess the role of stress-induced increased levels of GC on periodontitis progression.

INTRODUCTION

Clinical observations and epidemiological studies suggest that stress may contribute to increase the susceptibility to periodontal disease,[1] possibly through the production and release of GC hormones[2]. Methods used to inhibit the stress-induced release of GC for the study of the putative physiological role of GC include adrenalectomy[3] and metyrapone administration[4]. Metyrapone, an 11-β steroid hydroxylation inhibitor, is a powerful and

selective inhibitor of corticosteroid (CT) synthesis in animals and humans.[4] It affects the conversion of the CT precursor (deoxycorticosterone) in the rat adrenal cortex and, thus, prevents the synthesis and subsequent release of CT, which is the predominant GC into the bloodstream.[3] Thus, the aim of this study was to evaluate the viability of the use of MT as an experimental model to inhibit GC production and, therefore, as a method for use in determining the effect of stress-induced GC overproduction on periodontal tissues.

MATERIALS AND METHODS

Sixty four-week-old male Wistar rats were acclimatized to the housing conditions one week before the beginning of the experiments and a 12-hour light and dark cycle was applied (Animal Care and Use Committee approval #789-1/2005). After weight stratification, the animals were randomly assigned to: G1-control (n=20); G2- PD induced by cotton ligature associated with RS for 12 hours/day (n=20); and G3-PD + RS + 3 daily doses of MT (50mg/Kg/3x3hours) (n=20). RS consisted of the use of a flexible plastic mesh (30x30cm) in which the animals were housed in plastic pipes (10x30cm) for restraint and isolation purposes, and applied daily from 8 p.m. to 8 a.m. (12 hours) for the entire experimental period (30 days), in G2 and G3. After 30 days, animals were killed by decapitation and trunk blood was collected for CT determination (ICN Biomedicals, Costa Mesa, CA, USA). Gingival biopsies were assessed by qPCR, as previously described[5], for the following genes: TNF-α, INF-γ, IL-1β and IL-6. Finally, the area of bone loss in the furcation region (ABL) for ligated sites, and the area of periodontal ligament (APL) for unligated sites was histometrically determined by a blinded and calibrated examiner (Intra-class correlation = 0.92). Statistical analysis was performed by one-way ANOVA (α=0.05), followed by the Bonferroni t-test.

RESULTS AND DISCUSSION

Intergroup analysis showed that: i) RS increased serum levels of GC in G2, when compared to G1 and G3 (Table 1); ii) PD + RS (G2) significantly ($p<0.001$) increased the ABL in ligated sites, as compared to non-ligated sites (G1); iii) MT was ($p<0.05$) able to significantly decrease the release of GC in G3, however it increased the amount of ABL and the mRNA levels of all the pro-inflammatory factors assessed (INF-γ, TNF-α, IL-1β and IL-6), when compared with G1 and G2 (Figure 1). Although a stress-induced increase in GC production through the hypothalamic-pituitary-adrenal (HPA) axis has been largely used as the main hypothesis to explain the effect of stress on periodontal disease, the role of GC in such a process remains to be further investigated. Possible approaches to determine the impact of GC on any biological process include blocking the interaction between GC and its receptors or altering GC production. MT has been considered as an alternative method to adrenalectomy[6] for the inhibition of systemic GC production, since animals will eventually die after adrenalectomy and, therefore, studies of chronic processes such as periodontal disease are not possible. In the present study, the viability of the use of MT as a model to determine the impact of a stress-induced increase of GC on the periodontal tissues was tested. Data analysis showed that, although MT administration had an important effect on the reduction of systemic GC levels, MT administration additionally produced an unexpected local effect on the mRNA levels of important pro-inflammatory cytokines involved in the pathogeneses of periodontal disease, leading to an increased alveolar bone loss resulting from ligature-induced periodontitis. Although MT administration has been reported to alter the expression of genes including vasopressin and corticottopin-releasing factor[4], this is the first study to report that MT may locally potentiate the effects of periodontal disease. In summary, data from the present study indicate that MT can act as a modulator of periodontal bone breakdown resulting from an inflammatory process, making the use of MT unviable in studies aiming to determine the role of stress-induced increased levels of GC on periodontitis progression.

ACKNOWLEGDEMENTS

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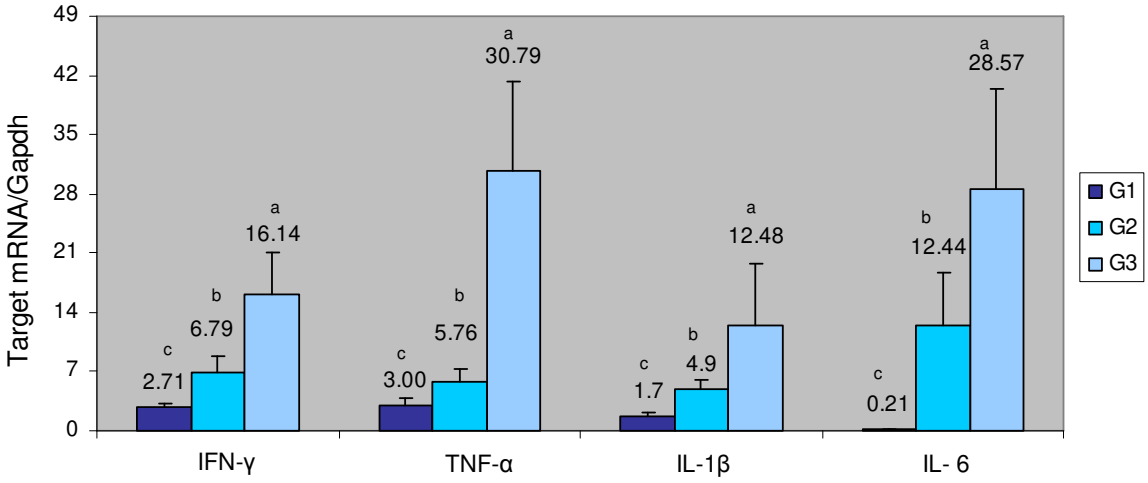
Table 1. Mean values and standard deviation for interradicular bone loss (BL) in G2 and G3 and the area of periodontal ligament (PLA) for G1 and corticosterone serum levels for all the experimental groups.

	G1	G2	G3	p value
ABL/APL (μm^2)	0.07 ± 0.01^C	0.88 ± 0.09^B	2.18 ± 0.66^A	$p<0.001$
Corticosterone (pg/ml)	68.07 ± 51.37^B	380.06 ± 129.2^A	133.72 ± 42.89^B	$p<0.05$

Mean and standard deviation followed by distinct letters in the same line differ statistically (ANOVA and Bonferroni's test).

Figure 1. Mean and standard deviations of mRNA levels (mRNA/Gapdh) for the pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6,) in the gingival tissues harvested from G1, G2 and G3. Mean values followed by different letters at the top of each bar indicate statistical differences, determined by an intergroup analysis (α=0.05) (ANOVA and Bonferroni t-test).



Mean and standard deviation followed by distinct letters in line differ statistically (ANOVA and Bonferroni's test).

CONSIDERAÇÕES GERAIS

Diversos autores têm demonstrado interesse em estudar os efeitos dos fatores psicossociais na etiologia das doenças periodontais (Moss et al., 1996; Genco et al., 1998; Vettore et al., 2003; Solis et al., 2004; Castro et al., 2006). Este interesse ocorre devido a duas razões principais: o fato de que o estresse e/ou a depressão afetam uma grande parcela da população e, por outro lado, o fato de que as condições mentais e emocionais afetam a resposta imune dos indivíduos, predispondo-os para o surgimento de diversas patologias, entre elas, a doença periodontal (Marucha et al., 1998; Ader et al., 2001).

É bem estabelecido o fato de que bactérias, organizadas em um biofilme dental, são os agentes etiológicos das doenças periodontais (Löe et al., 1965), no entanto, a presença das bactérias por si só, não é capaz de produzir avançada destruição em todos os indivíduos de uma maneira semelhante. Isto significa que há uma resposta individual e uma capacidade de adaptação a uma certa quantidade de biofilme dental bacteriano, sem que haja progressão da doença. Fatores ambientais, biológicos e comportamentais tais como hábito de fumar, diabetes, higiene oral e estresse, associados a fatores econômicos e sócio-culturais podem alterar a resposta individual e o equilíbrio saúde-doença, favorecendo o estabelecimento da doença periodontal (Genco et al., 1996, Kornman et al., 1997, Albandar et al., 2002).

Um dos possíveis mecanismos pelo qual o estresse pode modificar a extensão e a severidade das periodontites está baseado na interação neuro-imune-endócrina que ocorre pela ação de hormônios e mediadores químicos produzidos pelo organismo em situações de estresse (Genco et al., 1998; LeResche & Dworkin, 2002). Outro mecanismo da influência do estresse e dos fatores psicosociais nas condições periodontais é a modificação do comportamento do paciente. Indivíduos com altos níveis de estresse tendem a piorar seus hábitos, de modo a influenciar negativamente na saúde periodontal, tais como: negligencia da higiene oral, aumento do consumo de cigarros e ingestão de bebidas alcoólicas, descontrole do diabetes, mudança na dieta, o que resulta em desequilíbrio das funções do

sistema imune (Genco et al., 1998). Baseados nessas informações, teve-se como objetivo analisar criteriosamente a literatura pré-existente, a fim de avaliar se há evidências suficientes para considerar o estresse e os fatores psicossocias associados, como fatores de risco para a doença periodontal. Fator de risco pode ser definido como como um fator ambiental, comportamental ou biológico confirmado por seqüência temporal, ou seja, estudos longitudinais, que, quando presente, aumenta a probabilidade da ocorrência da doença e, se se ausente ou removido, reduz essa probabilidade (AAP, 1996). Os fatores de risco estão relacionados com o estabelecimento da doença: ou fazem parte do processo de causa, ou expõem o hospedeiro ao processo (Genco et al., 1998). Dos 58 estudos em humanos obtidos, somente 14 preencheram os critérios para serem incluidos na revisão sistemática. Destes, 57,1% (8 estudos) encontraram um desfecho positivo entre estresse/fatores psicossociais e doenças periodontais e 28,5% dos estudos encontraram desfecho positivo para algumas características psicossocias e negativa para outras. Cabe aqui ressaltar a dificuldade em se comparar estes estudos, devido às diferenças inerentes do desenho de cada estudo como: tipos de doença periodontal investigada, parâmetros clínicos utilizados para classificar as doenças, tipo de variável psicossocial analisada (estresse, depressão, ansiedade) e controles metodológicos para os fatores de confusão (idade, gênero, fumo, diabetes, higiene oral, tamanho da amostra, cegamento e calibração dos pesquisadores). Além disso, faltam estudos longitudinais que possam melhor caracterizar o impacto do estresse no desenvolvimento da doença periodontal. Desta forma, ficou evidente que há uma importante inter-relação entre os fatores psicossocias e a periodontite crônica. Entretanto a determinação da magnitude, dos mecanismos envolvidos e da causalidade ainda necessitam de melhor comprovação, a fim de poder definir o estresse como um fator de risco para a periodontite.

Com o objetivo de investigar a influência do estresse crônico na modulação da resposta imune-inflamatória local e sistêmica frente à doença periodontal, bem como alguns dos mecanismos envolvidos, foi utilizado um modelo experimental animal. Após 30 dias de estresse crônico, pôde-se observar que as análises histométricas reeditaram os achados de estudos prévios (Breivik et al., 1996; Breivik et al., 2000; Gasperisic et al., 2002; Takada et al., 2004), demonstrando que o estresse crônico pode significativamente aumentar a

quantidade de perda óssea nos sítios com doença periodontal. Pôde-se observar também que o estresse crônico foi capaz de alterar a expressão local de importantes fatores envolvidos na patogênese da doença periodontal. Os resultados desse estudo demonstraram que o estresse crônico modulou a expressão de fatores pró e anti-inflamatórios e fatores pró-reabsorção, nos sítios periodontais.

As células do sistema immune são amplamente distribuidas pelo corpo, quando uma infecção ocorre a resposta inflamatória permite a mobilização dos elementos do sistema imune para sítios específicos. Eventos precoces das reações inflamatórias são clinicamente indetectáveis, mas à medida que o processo evolui, ocorre um aumento nos níveis de citocinas e outros mediadores inflamatórios, que também estão associados com a ativação do sistema do estresse, permitindo que a presença da inflamação nos sítios periodontais, seja clinicamente detectável (Genco et al., 1998; LeResche & Dworkin, 2002; Ellis & Beaman, 2004). Dados do presente estudo demonstraram que a inflamação, produzida pela ligadura, aumentou significativamente os níveis locais de RNAm para IL-1β, INF-γ, IL-10 e RANKL, quando comparados a sítios não inflamados. Nossos achados, portanto, estão de acordo com prévios estudos, indicando que elevados níveis de citocinas pró-inflamatórias, tais como as IL-1β, IL-6 e INF-γ, e o fator pró-reabsorção RANKL estão associados com sítios que sofrem destruição periodontal (Ellis & Beaman, 2004).

As citocinas, de um modo geral, possuem um importante papel na ativação sistêmica de células inflamatórias e de fatores pró-reabsorção. Por outro lado, a elevação dos níveis de glicocorticóides, produzidos pelo estresse, tem demonstrado alterar o sistema dinâmico de regulação que controla o desenvolvimento da resposta pró/anti-inflamatória (O'Connor et al., 2000). No presente trabalho, pode-se observar que a associação da inflamação com o estresse crônico aumentou significativamente os níveis locais das citocinas pró-inflamatórias IL-1β, IL-6 e INF-γ. No presente estudo, também foi observado um aumentou significativo nos níveis de RNAm para a citocina anti-inflamatória IL-10 e para o fator pró-reabsorção RANKL e anti-reabsorção OPG, em sítios inflamados e submetidos ao estresse, entretanto houve uma redução significativamente nos níveis de RNAm da citocina anti-inflamatória IL-1ra, quando comparados aos grupos controle a ao grupo doença periodontal. Como conseqüência desta modulação, um aumento nas

proporções locais de RANKL/OPG, IL-1β/IL-1ra e IL-6/IL-10, como foi encontrado, favorece a resposta pró-inflamatória e pró-reabsorção, resultando na destruição periodontal.

Numerosos estudos na área de psiconeuroimunologia têm provido evidências de que os glicocorticóides (GC) modulam sistemicamente a resposta ao estresse a nível molecular, gerando uma resposta emocional que pode influenciar e modular o sistema immune, via sistema nervoso e neuroendócrino (Chrousos & Gold, 1992; O'Connor et al., 2000). Os GC podem modular a resposta imune de diferentes maneiras, incluindo o processo de expressão gênica, transcrição, tradução, pós-tradução, secreção de proteínas e diferenciação e proliferação de células progenitoras (O'Connor et al., 2000). Estresse emocional resulta na liberação de neuropeptídeos do sistema nervoso autônomo, que inerva os tecidos do sistema imune, e na secreção hormonal de catecolaminas nas células da medula adrenal (Genco et al., 1998). Além disso, durante uma resposta mediada pelo estresse, ocorre a ativação do eixo HPA e liberação de ACTH (hormônio adrenocorticotrófico) na circulação. O ACTH age no córtex adrenal, causando produção e liberação de hormônios GC. Assim, níveis de GC e catecolaminas podem ser utilizados na monitoração da ativação deste eixo (Breivik et al., 1996). No presente estudo, a análise dos dados demonstrou que os níveis, tanto de corticosterona como de catecolaminas, usados como biomarcadores do estresse crônico, estavam significativamente aumentados, após 30 dias de indução diária de estresse no grupo G3, quando comparado aos grupos G1 e G2 (capítulo 2). Desta forma, esses achados confirmam a eficâcia do método utilizado com o objetivo de causar estresse. Uma vez que corticosterona e catecolamina são produtos biologicamente ativos do estresse, os quais se unem a receptores específicos, para regular a expressão de genes envolvidos no sistema do estresse, a expressão desses receptores é de fundamental importância para que corticosterona e catecolamina possam produzir seus efeitos biológicos (O'Connor et al., 2000). Sendo assim, um objetivo adicional do presente estudo foi determinar se os tecidos periodontais expressam os principais receptores para corticosterona e catecolamina (Ar-β2 e Gr). A análise dos dados demonstrou que ambos receptores são expressos nos tecidos periodontais saudáveis, podendo assim assumir que há um efeito local direto, de ambos os fatores, no periodonto (dados não demonstrados). Embora acreditava-se inicicialmente que o estresse apresentava um efeito imunosupressivo (O'Connor et al., 2000; Seiffert et al., 2002),

acumuladas evidências tornaram claro que os glicocorticóides, catecolaminas e outros elementos relacionados ao sistema do estresse, podem de fato influenciar o sistema imune em ambas as direções: seja reduzindo ou elevando a resposta imunológica (Chrousos & Gold, 1992).

Sendo assim, os resultados do presente estudo indicam, de forma inédita na literatura, que embora a presença de GC possa produzir um efeito sistêmico imunossupressor, como relatado em estudos prévios (O'Connor et al., 2000; Seiffert et al., 2002), localmente, quando o estresse está associado à doença periodontal ocorre a modulação da destruição óssea devido a um aumento local dos níves de fatores pró-inflamatórios, diminuição dos níveis de citocinas anti-inflamatórias e aumento dos níveis de fatores pró-reabsorção. Tal efeito pode ser o resultado do aumento das proporções de IL-1β/IL-1ra, IL-6/ IL-10 e RANKL/OPG, favorecendo assim a destruição óssea.

Como pode ser observado em diversos estudos epidemiológicos (Croucher et al., 1997; Axtelius et al., 1998; Wimmer et al., 2002), o estresse crônico pode aumentar a severidade e a suscetibilidade à periodontite crônica, possivelmente devido ao aumento na produção e liberação de glicocorticóides (Genco et al., 1999). Possíveis abordagens para determinar o impacto do aumento da liberação de GC nos tecidos periodontais, resultante do estresse crônico, incluiria o bloqueio da interação entre GC e seus receptores presentes nos tecidos periodontais ou alteração na produção destes GC. Sendo assim, o bloqueio da liberação de GC tem como objetivo inibir os efeitos danosos dessas substâncias, causados no periodonto. Os métodos utilizados para conseguir esse bloqueio incluem a adrenalectomia (Breivik et al., 2000) e a administração de metirapone (Roozendal et al., 1996). A adrenalectomia consiste na excisão total da glândula adrenal, causando supressão da produção de outras substâncias importantes para a homeostasia do organismo, além dos GC (Gordan & Flögel, 2000). A droga metirapone (*11-β steroid hydroxylation inhibitor*), é um potente e seletivo inibidor da síntese de GC, pois inibe a conversão do precursor de corticosterona (deoxicorticosterona) no córtex adrenal, prevenindo assim a síntese e subseqüente liberação de GC (Young, 2000). Ou seja, metirapone bloqueia as elevações de costicosterona durante eventos estressantes, sem afetar os níveis basais dessas substâncias (Freo et al., 1992). O presente estudo (capítulo 3) teve como objetivo utilizar a

administração de metirapone (MT) como um método alternativo à adrenalectomia para controlar a liberação de glicocorticóides, pois o método da adrenalectomia reduz a expectativa de vida do animal, tornando inviável o experimento com duração de 30 dias. Diferentemente do nosso trabalho, Breivik et al. (2000), que avaliaram os efeitos da reatividade do eixo HPA na destruição periodontal em ratos, utilizaram o modelo da adrenalectomia em ratos, porém por um período experimental de 15 dias.

No presente estudo, a análise dos resultados demonstrou que MT foi capaz de reduzir significativamente a liberação de GC, nos grupos submetidos ao estresse e medicados, no entanto, produziu um aumento na quantidade de perda óssea, medida histometricamente, e na expressão dos níveis de RNAm para os genes das citocinas pró-inflamatórias (IL-1β, IL-6, INF-γ, TNF-α). Em linhas gerais, nossos resultados indicam que MT pode agir como um modulador para destruição óssea periodontal, resultante de um processo inflamatório, tornando assim o uso desta medicação inviável, quando se objetiva determinar o papel dos níveis aumentados de GC, liberados a partir do estresse, na progressão da periodontite. Porém mais estudos devem ser realizados, a fim de investigar o complexo mecanismo existente no sistema neuro-imune-endócrino.

CONCLUSÃO

Dentro dos limites dos estudos apresentados, pode-se concluir que:

1. Os estudos em humanos analisados na revisão sistemática, demonstraram, em maioria (57,1%), um desfecho positivo entre estresse/fatores psicológicos e doença periodontal. Sendo assim, pode ser observado que há uma importante inter-relação entre os fatores psicossocias e as doenças periodontais. No entanto faltam estudos longitudinais que possam melhor caracterizar o estresse como um verdadeiro fator de risco para o desenvolvimento da doença periodontal.
2. A presença do estresse crônico, pode localmente modular a doença periodontal por meio de um aumento local nas proporções de IL-1β/IL-1ra, IL-6/ IL-10 e RANKL/OPG, favorecendo, assim a destruição óssea periodontal.
3. A administração de três doses diárias de metirapone apresentou um importante efeito na redução dos níveis sistêmicos de glicocorticóides, entretanto, pode-se observar que a administração da droga alterou a expressão de fatores importantes na modulação da doença periodontal e conseqüentemente refletiu nos níveis de perda óssea interradicular. Desta forma, uma vez que a administração da droga, independente da presença do estresse, foi capaz de modular a progressão da doença, a utilização dessa droga torna-se não recomendável em estudos em que se objetiva bloquear o efeito dos glicocorticóides a fim de determinar o papel do estresse na progressão da doença periodontal.

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ANEXOS

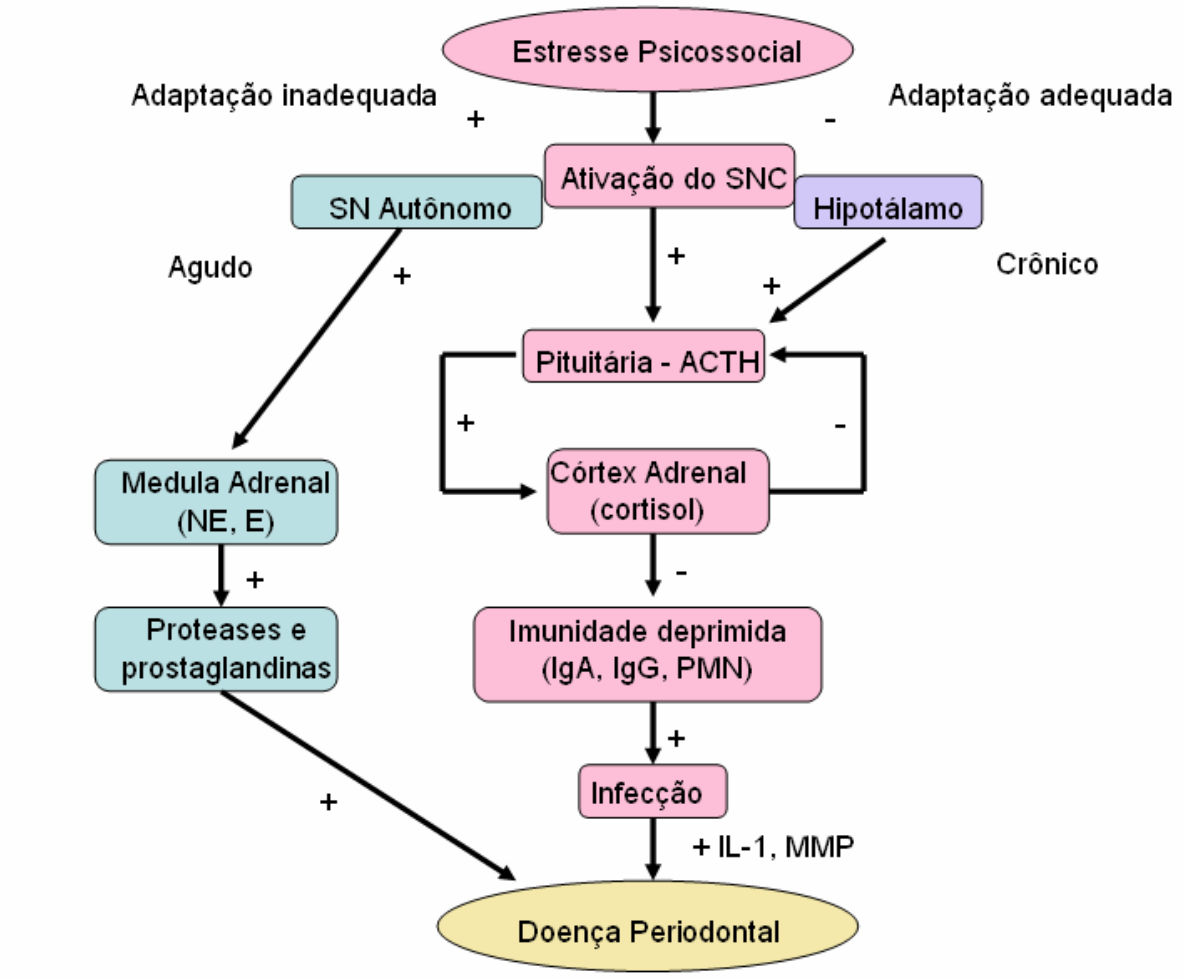

Figura 1. Modelo fisiológico para os efeitos do estresse sobre a doença periodontal. Adaptado de Genco et al. 1998. Abreviaturas: +/-, ativação/inibição; SNC, Sistema Nervoso Central; ACTH, hormônio adrenocorticotrófico; NE, noradrenalina; E, epinefrina; PMN, polimorfosnucleares neutrófilos; IgA/IgG, Imunoglobulina A/G.

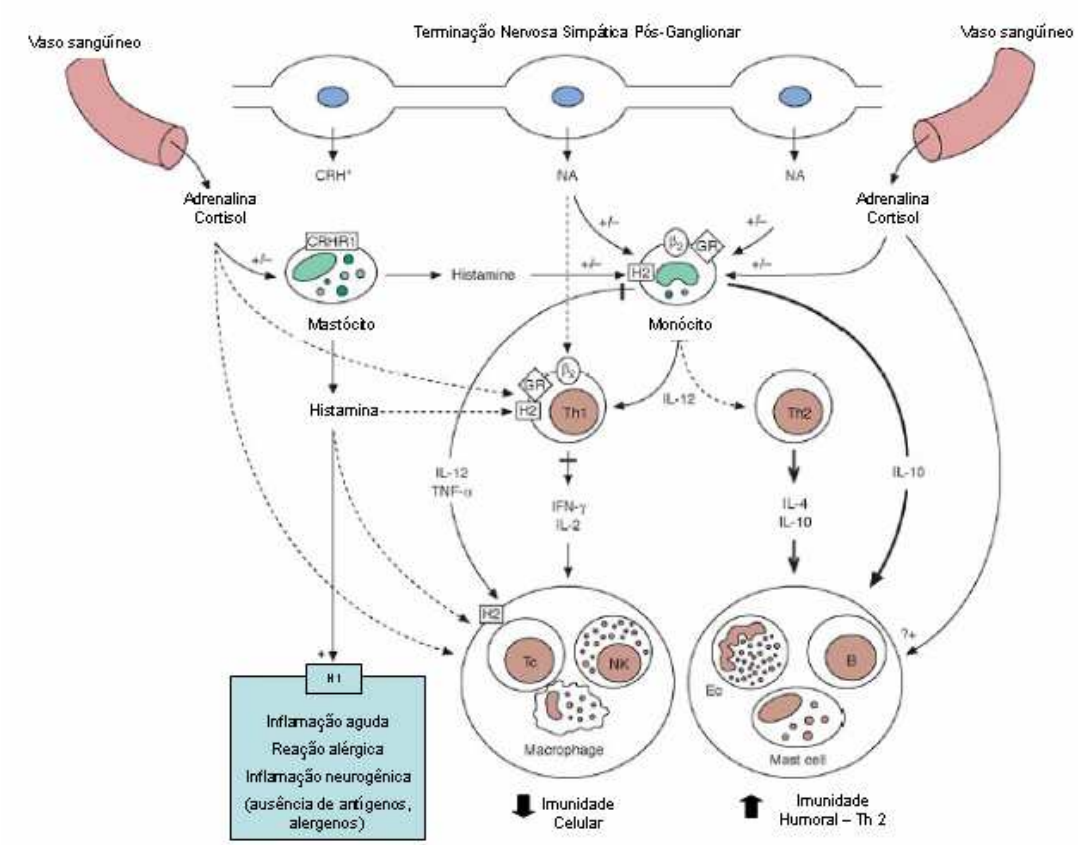

Figura 2. Influência do estresse e do hormônio liberador de corticotropina na resposta imune-inflamatória e alérgica através da estimulação de glicocorticóides. Linhas sólidas representam estimulação, linhas em negrito representam aumento da estimulação e linhas pontilhadas representam inibição. Abreviaturas: β2, adrenoreceptor -β2; +/-, estimulação/inibição; B, células B; CRHR1, receptor 1 de CRH; Eo, eosinófilo; GR, receptor de glicocorticóide; H1/H2, receptor de histamina 1 e 2; IFN-γ, interferon-γ; IL, interleucina; NA, noradrenalina; NK, células *natural killer*; Th1 e Th2, células *helper* tipo 1 e 2; TNF-α, fator de necrose tumoral α; adaptado de LeResche e Dworkin, 2002