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KARINA ANTUNES NEVES

PERDA ÓSSEA ASSOCIADA À CICLOSPORINA A: AVALIAÇÃO DENSITOMÉTRICA, BIOQUÍMICA E IMUNOLÓGICA.

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do grau de Doutora em Estomatopatologia. Área de Patologia.

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Orientador: Prof. Dr. Luís Carlos Spolidorio

Banca examinadora:

Prof. Dr. Carlos Benatti Neto

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DEDICATÓRIA

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

25(OH)D3	- Calcitriol sérico
ALP	- Fosfatase alcalina
BGP	- Osteocalcina sérica
BMD	- Densidade Mineral Óssea (Bone Mineral Density)
CsA	- Ciclosporina A
eNOS	- Óxido nítrico sintase endotelial
FDA	- Food and Drug Administration
FGF	- Fator de crescimento de fibroblastos
GM-CSF	- Fator estimulador de colônia granulócitos-macrófagos (granulocyte-macrophage colony-stimulating factor)
IFNγ	- Interferon gama
IL	- Interleucina (1, 2, 3, 4, 6, 7, 8, 10 e 13)
iNOS	- Óxido nítrico sintase induzida
LPS	- lipopolisacarídio de E. coli
MTT	- [3-(4,5-dimetiltiazol-2-yl)-(2,5- difeniltertrazolim brometo]
NFκ-(RANK-L)	- Ligante do fator nuclear κB (nuclear factor kappa B ligand)
NF-AT	- Fator nuclear de células T ativadas (nuclear factor of activated T cells)
nNOS	- Óxido nítrico sintase neuronal
NO	- Óxido nítrico (nitric oxide)
NOS	- Óxido nítrico sintase (nitric oxide synthase)
OPG	- Osteoprotegerina
OVX	- Ovariectomia
PGE_2	.Prostaglandina E_2
PTH	- Hormônio da paratireóide
TNFα	- Fator de necrose tumoral alfa
TNFβ	- Fator de necrose tumoral beta
TGFβ	- Fator de crescimento transformador beta

RESUMO

Vários relatos na literatura sugerem que a Ciclosporina A (CsA) aumenta a remodelação óssea com a reabsorção excedendo a formação, resultando em perda óssea. Os mecanismos da perda óssea associada à CsA são complexos e envolvem marcadores ósseos celulares e produtos dos osteoblastos e osteoclastos. Dentre os mediadores imunológicos, a CsA parece ter efeitos na produção de óxido nítrico (NO), o qual exerce efeitos bifásicos sobre o osso. O objetivo do presente trabalho foi avaliar os efeitos da CsA sobre o osso através da mensuração da densidade mineral óssea (BMD), análise bioquímica (fosfatase alcalina total, cálcio sérico e fósforo) e relacionar com a expressão de NO e TNFα pelos macrófagos peritoniais de camundongos.

Sessenta camundongos foram divididos em 4 grupos e receberam injeções subcutâneas diárias de CsA 50mg/kg peso corporal, durante os períodos de 7, 14, 28 e 60 dias (n=10) e o controle recebeu solução salina (n=5). Amostras sanguíneas foram obtidas através de punção cardíaca e foram analisados: cálcio sérico, fósforo e fosfatase alcalina. Os macrófagos peritoniais foram obtidos de cada camundongo, 3 dias após a injeção intraperitonial de tioglicolato de sódio. As células foram incubadas com lipopolossacarídeo (LPS) e os sobrenadantes foram usados para o ensaio de produção de NO e TNFα. As radiografias digitais dos fêmures foram obtidas através de um sistema de imagem digital, CDR^{R}. A densidade mineral óssea (BMD) foi avaliada com o auxílio do software Photoshop 7,0 (Microsoft, 2003).

A concentração dos marcadores séricos ósseos variou com o tempo de administração da CsA. Houve diminuição de cálcio após 28 dias e fósforo e ALP após 7 dias. A BMD diminuiu significativamente na epífise distal dos fêmures, a partir de 14 dias e na epífise proximal, somente após 60 dias, nos camundongos submetidos ao tratamento com CsA. A produção de NO e TNF-α aumentou significativamente nos camundongos tratados com CsA (período de 7 e 14 dias). A partir dos resultados do presente trabalho confirmamos que a CsA induziu perda óssea. Houve um sinergismo entre a perda óssea e a diminuição de ALP, cálcio e fósforo sérico, assim como o aumento dos níveis de NO e TNF-α.

ABSTRACT

Several reports suggest that Cyclosporin A (CsA) causes increased bone turnover and remodeling with resorption exceeding formation, resulting in bone loss. The mechanism of CsA-induced bone loss is complex and can involve cellular bone markers and osteoblasts and osteoclasts products. CsA seems to have effects on nitric oxide (NO) production, which has a biphasic effect on osteoclastic bone resorption. The aim of the present study was to evaluate the effects of the CsA on bone by Bone Mineral Density (BMD), biochemical analysis (Total Alkaline Phosphatase – ALP and serum calcium) and to relate them with the expression of NO and Tumor necrosis factor alpha (TNFα) by peritoneal macrophage of mice.

Sixty male mice were distributed into 4 groups and CsA (Sandimmun Novartis) was injected subcutaneously in a daily dose of 50mg/kg body weight during the periods of 7, 14, 28 and 60 days (n=10), whereas control mice received saline solution (n=5). Blood samples were obtained by direct cardiac puncture. Serum calcium and total alkaline phosphatase levels were obtained. Macrophages were obtained from the peritoneal cavities of treated and control mice 3 days after intraperitoneal administration of sodium thyoglycollate. Cells were incubated with lipopolysaccharide (LPS). After incubation, the culture supernatants were used for NO and TNFα production assays. Digital radiographs of femurs were obtained with the use of a computerized imaging system, CDR^{R}. Bone mineral density (BMD) measurements were made with the help of the software Photoshop 7,0 (Microsoft, 2003).

The concentration of serum bone markers varied with the period of CsA administration. There was a significant decrease in the calcium levels after 28 days and ALP after 7 days. The BMD decreased significantly on distal epiphysis of femurs after 14 days and on proximal epiphysis after 60 days, in the mice submitted to CsA therapy. NO and TNF-α production had a significant increase in mice treated with CsA (7 and 14 days). Based on the results of the present study we confirmed that CsA induces bone loss *in vivo*. There was a synergism between the bone loss and the decrease of alkaline phosphatase, and calcium, and the increase of NO and TNF-α.

INTRODUÇÃO

Os avanços na compreensão dos eventos que controlam o sistema imune, aliados à terapia imunossupressora e ao aprimoramento de técnicas cirúrgicas propiciam, de forma incontestável, avanços significativos no sucesso da inibição da rejeição dos transplantes.

A Ciclosporina A (CsA) é uma potente droga imunossupressora, inicialmente isolada do fungo *Tolypocladium inflatum Gams* (Borel & Kis, 1991). O uso da CsA como agente imunossupressor no início de 1980 contribuiu para o avanço dos transplantes de órgãos. Atualmente, é amplamente usada na terapia de pacientes que receberam transplantes de órgãos evitando a rejeição aguda do enxerto e no tratamento de algumas doenças autoimunes (Epstein, 1996).

A CsA liga-se ao receptor citoplasmático ciclofilina, que inibe a atividade da calcineurina, um importante fator limitante na ativação das células T (Jorgensen et al, 2003). A calcineurina exerce um papel importante regulando o fator de transcrição NF-AT (nuclear factor of activated T-cells) durante a ativação e formação do linfócito T citotóxico (Hemenway & Heitman, 1999; Stepkowski, 2000).

O NF-AT em condições fisiológicas regula genes como o da interleucina 2 (IL-2), interleucina 4 (IL-4) e interferon gama (IFNγ), causando a transcrição dos mesmos e a secreção das referidas citocinas. O bloqueio da calcineurina e, conseqüentemente, do NF-AT é considerado, portanto, o principal efeito da CsA, causando uma supressão da expressão normal destes mediadores e seus receptores (Garcia et al, 2004). Desta forma, a resposta imune celular e humoral são reduzidas inibindo a rejeição do enxerto (Mascarell & Truffabachi, 2003). Estes efeitos iniciais levam à vários efeitos secundários em macrófagos e outras células dependentes de citocinas e linfocinas, produzidas por células T ativadas (Tannirandorn et al, 2000).

A terapia com CsA está amplamente relacionada com diversos efeitos secundários indesejáveis como nefrotoxicidade (Lynn et al., 2001; Grynio & Cruzado, 2004), hepatotoxicidade, neurotoxicidade (Gijtenbeek et al., 1999), hipertensão (Koomans & Ligtemberg, 2001) e alterações gengivais (Doufexi et al, 2005). Pacientes transplantados

tratados com CsA, geralmente apresentam perda óssea com altas taxas de fratura (Ramsey-Goldman et al, 1999; Cohen & Shane, 2003).

Desta forma, a proposta do presente trabalho foi:

- Avaliar a perda óssea induzida pela CsA em camundongos através da determinação da concentração de cálcio, fósforo e fosfatase alcalina e da densidade mineral óssea.
- Determinar a produção do óxido nítrico e fator de necrose tumoral alfa (TNF-α) em cultura de macrófagos peritoniais nos camundongos tratados com CsA.

Capítulo I

REVISÃO DA LITERATURA

Artigo enviado para publicação no periódico Oral Diseases.

Mechanisms of Cyclosporin A –induced bone loss: the role of nitric oxide and NF-AT receptor.

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Running title: Mechanisms of Cyclosporin A –induced bone loss: the role of nitric oxide and NF-AT receptor.

Key words: Cyclosporin A, nitric oxide, NF-AT receptor, bone loss

The use of the immunosuppressive drug Cyclosporin A (CsA) has facilitated organ transplantation, which has become the management of choice for many patients with chronic and life-threatening illnesses (Epstein, 1996). Some immune diseases have been treated with CsA (Ponticelli, 2005)

The molecular mechanisms whereby CsA inhibit T cell activation are well understood (Stepkowski, 2000; Shibasaki et al, 2002). CsA binds to its immunophilin, cyclophilin at the cytoplasm to form complexes, which in turn inhibit the phosphatase activity of calcineurin, an important limiting step in the activation of T cells (Stepkowski, 2000; Jorgensen et al, 2003). Calcineurin, a serine-threonine specific calcium-calmodulin-activated phosphatase, plays a key role in regulating the potent nuclear factor of activated T-cells (NF-AT) during T-cell activation (Hemenway & Heitman, 1999). By blocking the calcineurin activity, CsA prevents the induction of genes encoding for cytokines as interleukin-2 (IL-2), which is necessary for the proliferation and maturation of T cells, and interferon-gamma (IFN-γ), which, in turn, is critical for the activation of macrophages. Thus, humoral and cellular immune responses are reduced enabling allograft acceptance (Mascarell & Truffabachi, 2003) (Fig. 1).

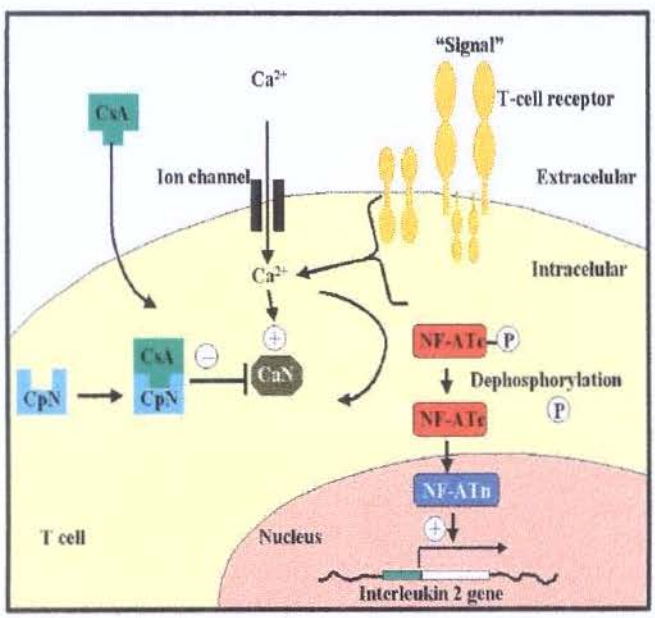

Fig.1 - Mechanism of action of cyclosporin A

In the cytoplasm, CsA binds to its immunophilin, cyclophylin (CpN), forming a complex. The CsA–CpN complex binds and blocks the function of the enzyme calcineurin (CaN), which has a serine/threonine phosphatase activity, that fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc), and thereby the transport of NF-ATc to the nucleus. Physiologically, the NF-ATc–NF-ATn complex binds to the promoter of the interleukin 2 (IL-2) gene and initiates IL-2 production. Consequently, with CsA, T cells do not produce IL-2, which is necessary for full T-cell activation. (Adapted from (Hemenway & Heitman (1999) and modified y the authors.)

CsA therapy is associated with a range of agent-specific side effects: nephropathy (Lynn et al., 2001; Grynio & Cruzado, 2004), hypertension (Koomans & Ligtenberg, 2001), hepatic dysfunction, neurological disturbances (Gijtenbeek et al., 1999) and gingival overgrowth (Doufexi et al, 2005).

There is much controversy in the literature about the effect of CsA on bone metabolism. Treatment with CsA following solid organ transplantations generally results in bone loss (Marcen et al, 2005). Bone loss following transplantation is usually rapid in the early phase with stabilization after one year (mean lumbar spine BMD decreases rapidly, especially during the first 6 months); although, concealed within this general statement is the reality of wide variability between apparently similar patients and also between the different categories of organ recipient (Ramsey-Goldman et al, 1999). Fractures rates are variable and often extremely high. Compared with expected rates in the normal population, fracture incidence was five times higher in male kidney recipients aged 25 to 64 years. In women the incidences were 18- and 34-fold higher in kidney recipients aged 25-44 years and 45-64 years, respectively (Ramsey-Goldman et al, 1999). The high fracture rate observed in dialysis patients increases even further after success full kidney transplantation, especially in diabetes (Casez et al, 2002). Bone loss rate following cardiac, liver, and lung transplantation is strikingly high during the first 6 months with fracture rates to match, ranging from 22% to 36%, 24% to 65%, and as high as 73%, respectively (Cohen & Shane, 2003; Shane et al, 1996). The possible mechanisms of CsA over bone metabolism will be discussed in this review.

Normal Bone Remodeling

Bone is constantly undergoing the process of remodeling. In bone remodeling, there is a constant resorption on a particular bony surface, followed by a phase of bone formation. There is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts (Roodman, 1999). The current concept of bone remodeling is based on the hypothesis that osteoclastic precursors become active and differentiated into osteoclasts, and thus the process of bone resorption begins. This stage is followed by a bone formation phase. The termination of bone resorption and the initiation

of bone formation in the resorption lacuna occur through a coupling mechanism. The coupling process ensures that the amount of bone removed is equivalent to the amount of bone laid down during the subsequent bone formation phase. The nature of the activation and coupling mechanism involves various lymphokines, fibroblast growth factor (FGF), transforming growth factor beta (TGF-β) and prostaglandins.

Osteoclastogenesis is an important factor of the coupling process. Osteoclastogenesis is under the control of osteoblasts: factors such as the parathyroid hormone (PTH), 1,25 dihydroxyvitamin D_3, and prostaglandin E_2 (PGE_2), which promote resorption, work initially on osteoblasts and marrow stromal cells to up-regulate the expression of a cell surface molecule called receptor activator of NF-κ-(RANK-L) (receptor activator of nuclear factor kappa B ligand). NF-κ-(RANK-L) protein belongs to the tumor necrosis factor (TNF) family (Teitelbaum, 2000). Once expressed, RANK-L binds to RANK, a receptor on osteoclast precursor mononuclear cells of the monocyte/macrophage family, establishing cell-to-cell contact (Roodman, 1999). These precursor cells fuse to form large multinucleated osteoclasts, which are the cell ultimately responsible for bone destruction. (Wiebe et al, 1996; Ross, 2000; Teitelbaum, 2000). Thus, regulation of RANK-L expression is the key to the pathogenesis of many osteopenic disorders (Wei et al, 2005). In fact, the ratio of RANK-L to its soluble, antiosteoclastogenic decoy receptor - osteoprotegerin (OPG), is a reasonable indicator of the magnitude of systemic bone loss in these pathological conditions (Wei et al, 2005).

The resorption phase typically proceeds for about ten days and is then followed by several months of bone replacement (Baker, 2000). Osteoclasts disappear and are replaced by osteoblasts. Osteoblasts are derived from mesenchymal precursors cells (Puzas, 1993) that are attracted chemotactically. Then, bone cells mitogens, including TGF-β, platelet-derived growth factor (PDGF), bone morphogenetic protein, FGF and insulin-like growth factors-I and –II, induce the proliferation and differentiation of osteoblasts (Baker, 2000). Osteoblasts secrete bone matrix proteins, including type-I collagen, proteoglycans, osteocalcin, osteopontin and the previously mentioned growth factors, and later stimulate their mineralization (Mundy, 1991-A)

Normal Bone Regulatory Factors

Normal bone remodeling is regulated by several systemic hormones as well as by local factors such as NO, prostaglandins, growth factors and cytokines. The intimate connections between the immune system and bone remodeling are becoming apparent (Baker, 2000).

Immune cells are present in the bone microenvironment and secrete many of the factors that regulate bone remodeling. Some of these factors promote bone resorption and others inhibit bone resorption or actively induce bone formation. The majority of these, even the factors that induce resorption, act on the osteoblast. The osteoblast in turn signals the activation of the osteoclast through the RANK-L described earlier (Baker, 2000)

The cytokines considered most frequently as active promoters of resorption in normal bone are: interleukin-1 (IL-1), TNF (including TNF-α and TNF-β) and interleukin-6 (IL-6). All except IL-1 can be secreted by T lymphocytes; IL-1 and TNF-α are also produced by macrophages (Baker, 2000).

On the other hand, TNF is probably the dominant cytokine extant in inflammatory osteolysis (Feldmann & Maini, 2001; Gravallese, 2002; Romas et al, 2002; Redlich et al, 2002; Ritchlin et al, 2003), since TNF-induced osteoclastogenesis requires at least constitutive levels of RANK-L. This dependency of TNF-mediated osteoclastogenesis on attendant RANK-L is underscored by the absence of meaningful osteoclast recruitment or bone destruction in the face of experimental inflammatory arthritis in mice in which RANK-L receptor has been deleted (Li et al, 2004). In addition, Wei et al (2005) have shown that TNF induces RANK-L expression by marrow stromal cells.

Like TNF, IL-1, another major osteoclastogenic cytokine, promotes RANK-L expression by marrow stromal cells and osteoblasts (Hofbauer et al, 1999). Recent studies have shown that IL-1 mediates TNF-induced RANK-L expression and the consequent osteoclastogenesis (Wei et al, 2005).

Other important resorptive factors released by stimulated macrophages include PGE2, and nitric oxide (NO) (Baker, 2000). Interleukin 8 (IL-8) is chemotactic for neutrophils, and can also induce neutrophils to secrete the resorptive cytokines IL-1β and TNF-α. Other T-cell factors inducing resorption are interleukin 3 (IL-3) and IFNγ. IFNγ

actually induces bone formation *in vitro*, but bone resorption *in vivo*, demonstrating that the outcome of cytokine induction is difficult to predict, likely due to the complexity of the cytokine/bone network (Baker, 2000)

Other anti-inflammatory cytokines blunt osteoclastogenesis by selectively inhibiting RANK-L signaling in osteoclast precursors (Wei et al, 2002). Interleukin 4 (IL-4) is an abundant anti-inflammatory cytokine, produced mainly by activated T lymphocytes, that inhibits TNF and IL-1 induced RANK-L expression.

Osteoporosis and Cyclosporin A

Osteoporosis is characterized by low bone mass accompanied by abnormalities of bone architecture. Osteopenia describes a state of reduced bone mass. The National Institute of Health (NIH) defines osteoporosis as "a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. Bone strength reflects the integration of two main features – bone density and bone quality." Thus, osteoporosis is generally measured in transplanted patients implying the diagnosis of bone mineral density (BMD) (Cunningham, 2005).

It is generally accepted that CsA causes bone loss in humans (Josephson et al, 2004; Cueto-Manzano et al, 1999) contributing to post-transplantation bone disease. CsA has complex and incompletely understood actions on bone (Epstein, 1996). Several clinical studies in transplant patients indicate that CsA therapy induces bone loss. However, since it is difficult to be certain, since glucocorticoids and CsA are used almost universally as a combined therapy of glucocorticoids (Tannirandorn & Epstein, 2000). Treatment with CsA following solid organ transplantations, such as heart or liver, generally results in bone loss. The pathogenesis of post-transplantation bone disease is only partially understood. Clinically, in organ transplant recipients, bone loss occurs rapidly within the first year after transplantation (mean lumbar spine BMD decreases rapidly, especially during the first 6 months) (Shane et al, 1993; Sambrook et al, 1994; Cunninghan, 2005) and continues, particularly in the femoral neck (Sambrook et al, 1994). Clinical investigation with bone marrow transplanted patients that received CsA treatment showed an osteoclast stimulation

and osteoblast suppression with marked retardation of mineral apposition and low bone formation rates (Lee et al, 2002).

There are convincing evidences, with histological observations, that CsA causes increased bone turnover and remodeling with resorption exceeding formation, resulting in bone loss in animal experiments. *In vivo* experiments have shown that CsA produces severe osteoporosis in the rat (Epstein et al, 1990; Movsowitz et al, 1990; Epstein, 1996) that is dependent on dose and duration (Movsowitz et al, 1988). This high turn over osteopenia is characterized by a rapid remodeling with an increased serum osteocalcin, a decrease in bone mass, loss of cancellous bone and increased osteoblast activity and recruitment (Movsowitz et al, 1988; Schlosberg et al, 1989; Epstein et al, 2001; Buchinsky et al, 1996).

CsA has been associated with a reduced bone mass, decreased trabecular number and increased trabecular separation. With regard to kinetic histomorphometric indices, CsA treated rats showed an increased percent mineralizing surface, mineral apposition rate, bone formation rate/tissue volume, and bone formation rate/bone volume (Epstein et al, 2001).

Fu et al (1999) found that CsA decreased bone formation and induced a massive resorption of the alveolar bone. Bone histomorphometry has shown a decreased percent of trabecular bone volume, increased multinucleated osteoclast number (increased resorption) and increased parameters on bone formation. Osteopenia was characterized by scattered and thin trabecular bone (Fu et al, 2001; Abdelhadi et al, 2002).

Besides the clinical evidences of bone loss after treatment with CsA in human and animals, *in vitro* studies have shown that CsA inhibits bone resorption. *In vitro* studies have demonstrated an inhibitory effect of CsA on bone resorption stimulated by PTH, IL-1, PGE_2 and 1,25 dihydroxyvitamin D_2 (Stewart et al, 1986; Klaushofer et al, 1987; Sasagawa et al, 1989; Chowdhury et al, 1991; Mccauley et al, 1991). In human osteoblast-like cells, CsA has a selective inhibitory effect on IL-1 (Skjodt et al, 1984). However, isolated culture systems may not represent the *in vivo* environment, as it is known that, *in vivo*, T lymphocytes are critical for CsA-induced bone resorption (Epstein, 1996) as well as in some *in vitro* systems (Buchinsky et al, 1996; Zahner et al, 1997).

Mechanism of Action of the CsA-Induced Bone Loss

The pathogenesis of post-transplantation bone disease is only partially understood. Increased serum PTH and decreased serum calcitriol ($25(OH)D_3$) levels are among previously reported causes (Compston et al, 1996). The influence of CsA on several parameters may be related with the pathogenesis of CsA-induced post-transplantation bone loss. Mechanistically, it is likely that these effects are largely mediated by the alteration of the balance between RANK-L and OPG, with CsA increasing RANK-L and decreasing OPG (Hofbauer et al, 2001; Cunninghan, 2005) (Fig. 2). Several factors can be involved and will be discussed in this review.

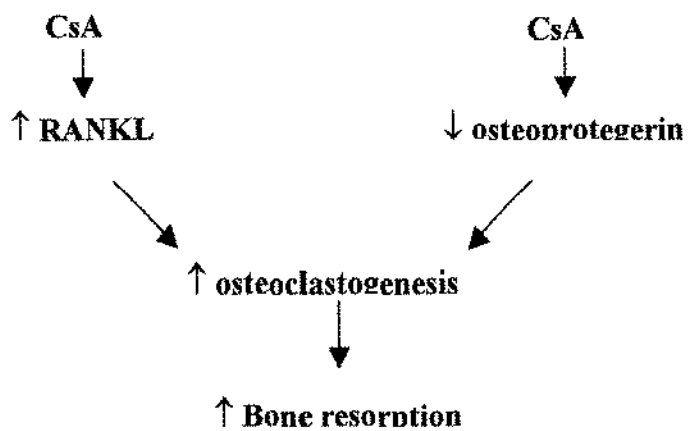

Figure 2. Proposed role of RANKL (receptor activator of NFKbeta ligand) and osteoprotegerin in the mediation of CsA toxicity. Figure based on Cunningham J. Posttransplantation Bone Disease *Transplantation* 2005; 79:629-634. All rights reserved to the author.

CsA and T Lymphocyte

It is thought that the CsA-induced osteoporosis may be mediated primarily by the presence of T lymphocytes, since CsA did not produce osteopenia in the T-cell-depleted nude rat (Buchinsky, 1996). In addition, the critical role of T lymphocytes on human osteoclastogenesis has been confirmed *in vitro* by Zahner et al (1997). T cells are known to be capable of producing a wide variety of citokines, including TGF-β, IFNγ, IL-4, interleukin 10 (IL-10), interleukin 13 (IL-13) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which inhibit osteoclastogenesis, and IL-6, interleukin 7 (IL-7) and TNF-α, which stimulate this process (Shinoda et al, 2003). Shinoda et al (2003) demonstrated that resting T cells negatively regulated the osteoclast generation via

production GM-CSF and IFNγ by CD4 T cells, and that CsA-induced bone loss may be due to the inhibition of these T-cell cytokines. In addition, previous reports have suggested that activated T cells stimulate osteoclast generation by expressing the key osteoclastogenic molecule, receptor activator of NF-κB ligand (RANKL) (Shinoda et al, 2003). Recent studies have reported that RANK-RANKL signaling induces NF-AT nuclear translocation from cytoplasm and CsA inhibits RANKL-induced NF-AT activation and osteoclast formation (Ishida et al, 2002; Takayanagi et al, 2002). These data suggest the T cells involvement in CsA-induced bone loss.

CsA-Induced Bone Loss and Parathyroid Hormone (PTH)

PTH increase has been proposed to be very important in post-transplantation bone disease in humans. Huang et al (1995) demonstrated that trabecular bone loss in human post-transplantation was associated with a significant increase in serum PTH. However, human studies are difficult to interpret since transplanted patients usually receive a cocktail of immunosuppressants and may have underlying disease. (Epstein et al, 2001)

The influence of CsA on PTH can be modulated by systemic factors since chronic renal failure is associated with a reduced glomerular filtration rate caused by CsA. However, secondary hyperparathyreoidism develops in response to impaired renal failure and intestinal calcium absorption and increased calciuria (Cunningham, 2005).

There is an interaction between CsA and circulating PTH levels resulting in a modified histomorphometric picture in parathyroidectomized (PTX) rats (Epstein et al, 2001). CsA and PTX have generally opposite effects on bone mineral metabolism, producing respectively, high turnover osteopenia or increased bone density. The individual effect of CsA negates the effect of PTX resulting in an indistinguishable histomorphometric picture with the BMD of normal rats (Epstein et al, 2001). Epstein et al, 2001 proposed that CsA and PTX could exert their effect via separate mechanisms, negating each other, resulting in a net effect similar to control. Another possible explanation is that to demonstrate the profound accelerated bone loss (cancellous bone) produced by CsA in normal rats, normal circulating levels of PTH must be present. That situation was demonstrated in Epstein's experiment, where there was no increase in circulating PTH with

CsA administration. In this experiment, the authors suggest that PTH, as part of a secondary hyperparathyroidism, may be involved in the bone disease caused by the cocktail of immunosuppressants used to prevent organ rejection, and they suggest that controlling post-transplantation levels of PTH may decrease the high turn over state and lessen risks and effects of transplantation bone disease.

Serum Osteocalcin and CsA

Serum osteocalcin (BGP) is a vitamin K-dependent non-collagenous protein product of mature osteoblasts. Increases in serum BGP may be an useful marker of osteoblast function. Serum BGP have raised in rats treated with CsA (Goodman et al, 2001; Movsowitz et al, 1988; Epstein et al, 2001) reflecting increased histomorphometric parameters of bone formation. Serum BGP was decreased when the combination of CsA and TGF-β was administered to the rats. (Goodman et al, 2001)

Serum 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D$) and CsA

Serum1,25-dihydroxyvitamin D3, a potent resorption inducer can be secreted by both monocytes and macrophages. Serum $1,25\text{-}(OH)_2D$ level has raised with CsA therapy in rats during 14 and 28 days.(Goodman et al, 2001; Epstein et al, 2001). $1,25\text{-}(OH)_2D$ is known to be a stimulator of BGP gene expression (Kerner et al, 1989; Lian et al, 1989). CsA increases 1α-hydroxylase activity and therefore produces significant elevations in serum $1,25\text{-}(OH)_2D$ levels in both rats and mice with a dose-dependent effect (Stein et al, 1991).

CsA and cytokines

The mechanism by which CsA affects the bone may involve the immune system. The pathogenetic role of cytokines in bone in post-transplanted patients is uncertain

In rat bone, CsA has been shown to increase mRNA expression of IL-1, IL-6 and TNF-α, cytokines known to be involved in bone resorption (Marshall et al. 1995). *In vitro* studies have shown that, in human osteoblast-like cells, CsA has a selective inhibitory effect on IL-1 (Skjodt et al, 1984). Several investigators have shown that IL-6 is modulated

by PTH (Littlewood et al, 1991; Papanicolaou et al, 1998), thus providing a possible link among PTH, IL-6, CsA and osteoporosis.

CsA and TGF-β

Transforming growth factor-beta (TGF-β), a member of a closely related multifunctional growth regulatory peptide family, is reported to be one of the key factors involved in the coupling of bone formation to previous bone resorption (Mundy, 1991-B). This potent osteotropic polypeptide is abundant in the bone matrix, and is produced in response to factors that stimulate osteoclastic bone resorption (Centrella et al, 1991; Mundy, 1991-B, Mundy & Bonewald, 1992). It is a potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation, and differentiation in osteoblasts. TGF-β has some complex effects on bone resorption, either stimulating or inhibiting bone resorption. *In vitro* studies have shown that TGF-β exerts its effects on both the proliferation as well as the differentiation and fusion of progenitors into mature multinucleated osteoclasts (Hattersley & Chambers, 1991; Mundy & Bonewald, 1992), suggesting that TGF-β may play a role in the stimulation of the osteoclasts (Hattersley & Chambers, 1991). However, it is thought that TGF-β does not directly stimulate osteoclast activity, but rather stimulates resorption through the coupling of osteoclast stimulation to previous osteoblast stimulation (Erlebacher & Derynck, 1996; Filvaroff et al, 1999; Hattersley & Chambers, 1991). In fact, it has been found that TGF-β1 inhibits osteoclast generation from murine bone marrow cells (Hattersley & Chambers, 1991). In addition, TGF-β can also down regulate osteoclast activity (Hock et al, 1990).

Recent work has shown that CsA stimulates the expression of TGF-β1 in both *in vitro* and *in vivo* models, (Khanna et al, 1997) It has been shown that CsA stimulates *in vivo* secretion of TGF-β-1 in humans, which, while improving immunosuppression, may also contribute to renal fibrosis (renal toxicity) (Pankewycz et al, 1996; Shin et al, 1997; Suthanthiran & Strom, 1994).

Goodman et al (2001) have shown that TGF-β administration alone did not alter bone mineral metabolism in rats. The combination of TGF-β and CsA administration

resulted in increased of bone formation parameters (increased percent of mineralizing surface and percent of osteoid perimeter) that may indicate an increase in osteoblast activity and recruitment and/or decrease in osteoclast function. Besides, TGF-β counteracted the increase in BGP induced by CsA. Thus, it appears as if TGF-β administration may have a potential in modulating the deleterious bone effects of CsA. However, the use of TGF-β in conjunction with CsA must be studied carefully with the monitoring of renal function.

CsA and Nitric Oxide (NO)

Several studies showed that CsA has an important inhibitory effect on production of nitric oxide synthase (NOS) enzyme and, consequently, nitric oxide (NO). NO is a mediator produced by various cells, which participates in inflammatory and autoimmune-mediated tissue destruction (Nathan, 1997).

NO is generated by the NOS enzymes from molecular oxygen (Feelish & Stamler, 1996). Three isoforms of NOS have been identified: a neuronal form (nNOS), an endothelial form (eNOS) and an inducible form (iNOS). Both nNOS and eNOS are constitutively expressed. The names used reflect the tissues of origin for the original protein and cDNA isolates, however it is known that all three isoforms are much more widely distributed (Looms et al, 2002). Recent data from several groups have shown that eNOS is widely expressed on a constitutive basis in bone marrow stromal cells, osteoblasts, osteocytes and osteoclasts (Brandi et al, 1995; Helfrich et al, 1997; Macpherson et al, 1999)

Expression of iNOS has been observed in fetal bone (Hukkanen et al, 1999) suggesting a role in skeletal development, but iNOS does not appear to be expressed constitutively in normal adult bone (Helfrich et al, 1997). Regulation of iNOS takes place at the level of gene transcription. Transcription of the iNOS gene is activated by pro-inflammatory cytokines such as IL-1, IL-6, TNFα, γIFN and endotoxin, whereas glucocorticoids and the anti-inflammatory cytokines IL-4, IL-10 and TGF-β are inhibitory (Damoulis & Hauschka, 1997; Vant Hof & Ralston, 2001).

The effects of the free radical NO on bone has received a great attention because of its important function on bone cells. Several studies suggests that NO has biphasic effects on osteoclastic bone resorption. (Ralston et al, 1995; Vant Hof & Ralston, 2001)

Low concentrations of NO have been shown to potentiate IL-1-induced bone resorption, based on the observation that NOS inhibitors inhibit IL-1 induced bone resorption *in vitro* (Ralston et al, 1995). Constitutive production of NO within osteoclasts has been suggested to be essential for normal osteoclast function, based on the observation that NOS inhibitors reduce activity and motility of isolated osteoclasts (Brandi et al, 1995). Nonetheless, examination of bones from animals with iNOS or eNOS deficiency has shown no major defect in bone resorption under physiological conditions, which suggests that these isoforms are not essential for osteoclast formation or activity (Vant Hof & Ralston, 2001). However, evidences suggest that the iNOS pathway plays an important role in cytokine and inflammation induced osteoporosis (Armour et al, 1999). Recent studies have shown that activation of the iNOS is essential for IL-1-stimulated bone resorption, both *in vivo* and *in vitro*. These studies suggest that IL-1 primarily acts on osteoblasts increasing NO synthesis by activation of the iNOS pathway and that this, in turn, promotes nuclear translocation of the transcription factor NF-κB in osteoclast progenitors. (Vant Hof et al, 2000).

When there is a high-level production, NO inhibits osteoclast formation and activity. Pro-inflammatory cytokines such as IFNγ in combination with IL-1 and/or TNF-α cause activation of the iNOS pathway in bone cells and the high NO levels derived from this pathway inhibit bone resorption (Brandi et al, 1995; Ralston et al, 1995; Vant Hof & Ralston, 2001). It has been shown that high NO concentrations are responsible for the inhibitory effect of IFNγ on IL-1 and TNF-α stimulated resorption (Ralston et al, 1995). The inhibitory effect of high NO levels appears to be due to the inhibition of both osteoclast formation and activity and NO-induced apoptosis of osteoclast progenitors (Vant Hof & Ralston, 1997). NO seems also to have biphasic effects on osteoblast activity. *In vitro* studies have indicated that the small amounts of NO, which are produced constitutively by osteoblasts, may act as autocrine stimulators of osteoblast growth and cytokine production (Riancho et al, 1995). Some investigators have shown that slow release NO donors stimulate osteoblast growth and differentiation *in vitro* (Mancini et al, 2000; Buttery et al, 1999; Koyama et al, 2000). High concentrations of NO have potent inhibitory effects on osteoblast growth and differentiation (Mancini et al, 2000). Recent evidence suggests that

this may be partly due to pro-apoptotic effect of NO on osteoblasts, (Damoulis & Hauschka, 1997; Mogi et al, 1999) and that these effects are mediated in part by cGMP (Mancini et al, 2000).

Based on the fact that NO is closely related in bone remodeling, the effects of CsA on NO concentrations may contribute to the bone loss induced by CsA. CsA inhibited NO production in human T84 epithelial cells and in murine J774 macrophages, measured as nitrite accumulation into the culture medium, in a dose-dependent manner in both cell lines (Hämäläinen et al, 2002).

Strestikova et al. (2001) have shown that CsA exhibits dose-dependent inhibitory effects on NO production in rat peritoneal macrophage culture experiments. The measurements of nitrite accumulation were made in supernatants of LPS-stimulated macrophages. The authors suggests that iNOS expression down-regulation by CsA occurs post-transcriptionally. In previous study, CsA inhibited cumulative nitrite accumulation and iNOS mRNA at level of iNOS transcription (Attur et al. 2000). CsA has shown to suppress iNOS expression via nuclear factor κB (NF-κB) transcriptional pathway in kidney (Kunz et al, 1995), whereas CsA was shown to inhibit iNOS expression by NF-κB-independent manner in human endothelial cell and J774 macrophage (Raffie et al, 2002; Hämäläinen et al, 2002). Kim et al (2004) has found that iNOS expression and NO production are inhibited by CsA in LPS stimulated macrophages. They showed that iNOS promoter activity and NF-κB activity are inhibited by CsA.

In vitro studies with two co-cultures of osteoclast-rich neonatal rat long-bone marrow cells and dentin sections with neonatal-rat calvarial osteoblasts incubated with CsA showed 90% of bone resorption inhibition. When L-arginine (NO substrate) were added it reversed this inhibition by 90%. The application NOS inhibitor L-NAME inhibited bone resorption by 87%. It demonstrated that NO-cGMP pathway is involved in CsA induced bone loss (Wimalawansa, 2000).

This inhibition of NO production by CsA may have an important function on the pathogeny of osteoporosis induced by CsA, since low concentrations of NO lead to bone resorption associated with IL-1, as described in this review.

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CAPÍTULO II

Artigo enviado para publicação no periódico *Calcified Tissue International*

Effect of Cyclosporin A therapy on femur bone mineral density (BMD) in mice: production of Nitric Oxide and Tumoral Necrosis Factor alpha.

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Running title: Effect of Cyclosporin A therapy on femur bone mineral density (BMD) in mice; production of Nitric Oxide and Tumor Necrosis Factor

Key words: **Cyclosporin A, nitric oxide, NF-AT receptor, bone loss**

ABSTRACT

Several reports suggest that Cyclosporin A (CsA) causes increased bone turnover and remodeling with resorption exceeding formation, resulting in bone loss. The mechanism of CsA-induced bone loss is complex and can involve cellular bone markers and osteoblasts and osteoclasts products. CsA have effects on nitric oxide (NO) production, which has a biphasic effect on osteoclastic bone resorption. The aim of the present study was to evaluate the effects of the CsA on bone by Bone Mineral Density (BMD), biochemical analysis (Total Alkaline Phosphatase – ALP and serum calcium) and to relate them with the expression of NO and Tumor necrosis factor alpha (TNF-α) by peritoneal macrophage of mice.

Sixty male mice were distributed into 4 groups and CsA (Sandimmun Novartis) was injected subcutaneously in a daily dose of 50mg/kg body weight during the periods of 7, 14, 28 and 60 days (n=10), whereas control mice received saline solution (n=5). Blood samples were obtained by direct cardiac puncture. Serum calcium and total alkaline phosphatase levels were obtained. Macrophages were obtained from the peritoneal cavities of treated and control mice 3 days after intraperitoneal administration of sodium thyoglycollate. Cells were incubated with lipopolysaccharide (LPS). After incubation, the culture supernatants were used for NO and TNF-α production assays. Digital radiographs of femurs were obtained with the use of a computerized imaging system, CDR^{R}. Bone mineral density (BMD) measurements were made with the help of the software Photoshop 7,0 (Microsoft, 2003).

The concentration of serum bone markers varied with the period of CsA administration. There was a significant decrease in the calcium levels after 28 days and ALP after 7 days. The BMD decreased significantly on distal epiphysis of femurs after 14 days and on proximal epiphysis after 60 days, in the mice submitted to CsA therapy. NO and TNF-α production had a significant increase in mice treated with CsA (7 and 14 days). Based on the results of the present study we confirmed that CsA induces bone loss *in vivo*. There was a synergism between the bone loss and the decrease of alkaline phosphatase, and calcium, and the increase of NO and TNF-α.

INTRODUCTION

CsA has improved graft survival after transplantation and some autoimmune diseases (Starzl et al, 1980; Borel, 1988). A frequent complication reported in patients following kidney, liver and heart transplantation is the development of bone loss and pathologic fractures (Shane et al, 1996; Ramsey-Goldman et al, 1999; Casez et al, 2002; Cohen et al, 2003; Marcen et al, 2005). The effect of the CsA on bone metabolism has been extensively investigated, but the positive and negative effects on bone mineral metabolism are still obscure. However, strong observations in human and animal studies have shown that CsA therapy induces bone loss (Movsowitz et al, 1988; Schlosberg et al, 1989; Derfus et al, 1991; Goodman et al., 2001; Fu et al, 1999; Epstein et al, 2001; Fu et al, 2001). It is suggested that CsA causes increased bone turnover and remodeling with resorption exceeding formation, resulting in bone loss. Osteopenia is characterized by scattered and thin trabecular bone (Fu et al, 2001; Goodman et al., 2001).

The probable mechanism of CsA-induced bone loss seems to be complex and can involve bone markers, as alkaline phosphatase (ALP) and/or serum osteocalcin (BGP) (Movsowitz et al, 1988; Withold, 1994; Bonnin, 1997; Goodman et al, 2001), besides immune system and consequently the function of osteoblasts and osteoclasts (Chowdhury et al, 1991; Mccauley et al, 1991). Components of the immune system have been shown to constitute and to partially integrate fracture healing, bone graft incorporation, and bone mineral metabolism. Cytokines secreted by inflammatory T-cells and activated macrophages have a high potential to induce bone resorption. For example, in rats, CsA has been shown to increase mRNA expression of interleukin 1 (IL-1) and interleukin 6 (IL-6), which are cytokines known to be involved in bone resorption (Marshall et al. 1995). CsA inhibits gene transcription and synthesis including interleukin-2 (IL-2), interleukin 3 (IL-3) and interleukin 4 (IL-4) (Kahan, 1989; Suthanthiran & Strom, 1994).

On the other hand, CsA seems to have an important inhibitory effect on nitric oxide synthase enzyme (NOS) production and, consequently, on nitric oxide (NO) in *in vitro* studies (Strestikova et al, 2001). NO is generated by the nitric oxide synthase enzymes (NOS) from molecular oxygen (Feelish et al, 1996). The transcription of the iNOS gene is

activated by pro-inflammatory cytokines such as IL-1, tumor necrosis factor alpha (TNF-α), gamma interferon (IFNγ) and endotoxin, whereas glucocorticoids and the anti-inflammatory cytokines IL-4, interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) are inhibitory (Vant Hof & Ralston, 2001). Therefore, due the inhibitory effects of iNOS and NO, other chemical mediators can be eventually altered and, consequently, change bone metabolism.

NO has received a great attention because of its effects on bone cells. Several studies suggest that NO has biphasic effects on osteoclastic bone resorption. Low concentrations of NO have been shown to potentiate IL-1 induced bone resorption *in vitro* (Ralston et al, 1995). Constitutive production of NO within osteoclasts has been suggested to be essential for normal osteoclast function (Brandi et al, 1995) but not essential for osteoclast formation or activity (Vant Hof & Ralston, 2001). However, evidences suggest that the iNOS pathway plays an important role in cytokine and inflammation induced osteoporosis (Armour et al, 1999).

When there is a high-level production, NO inhibits osteoclast formation and activity. Pro inflammatory cytokines, such as IFNγ in combination with IL-1 and/or TNF-α, cause the activation of the iNOS pathway in bone cells and the high levels of NO derived from this pathway inhibit bone resorption (Brandi et al, 1995; Ralston et al, 1995; Vant Hof & Ralston, 2001). Based on the fact that NO is closely related in bone remodeling, the effects of CsA on NO concentrations could contribute to the bone loss induced by CsA.

The purpose of the present study was to evaluate the effects of the CsA on bone by Bone Mineral Density (BMD) and biochemical analysis (Total Alkaline Phosphatase – ALP and serum calcium (CAL-L) and to relate them with the production of NO and TNF-α by peritoneal macrophage of mice.

MATERIAL AND METHODS

Animals

Sixty male mice Swiss (*Mus musculus*), with 4 weeks old, were housed under similar conditions and maintained on diet and water *ad libitum*. The mice were distributed into eight groups: four groups of ten animals were treated with CsA and four groups of five animals each were used as control.

Cyclosporin A

CsA (Sandimmun Novartis) was injected subcutaneously in a daily dose of 50mg/kg body weight during the periods 7, 14, 28 and 60 days. Control mice were daily injected subcutaneously with saline solution. All mice were killed after the mentioned period.

Biochemical analysis

At the corresponding end-time points, the animals were anesthetized with 0,1 mg/100g body weight ketamine (Francotar®, Virbac do Brazil Ind. Com. LTDA, São Paulo, São Paulo, Brazil) anesthesia and 1ml of blood was obtained by direct cardiac puncture. The blood samples were centrifuged and the serum was assayed at Araraquara Pharmacy School – UNESP – SP – Brazil. Total Serum calcium and total alkaline phosphatase (ALP) levels were obtained for each animal.

Total ALP activity was measured colorimetrically (ALP kit- Sera Pak, Bayer AG, Elitech, France) using *paranitrophenyl* phosphatase as the substrate. ALP activity was measured by the absorbance at 405nm, using a Technicon SMA-24 spectrophotometer (Technicon, Domont, France). The units (U/dl) of enzyme activity in experimental sample were calculated from this standard of Bayer units.

Peritoneal Macrophage cultures

Macrophages were obtained from the peritoneal cavities of treated and control mice 3 days after intraperitoneal administration of 3,0 ml sodium thyoglycollate (Difco). The cells were plated at concentration of 5 x 10^6 cells per ml in RPMI-1640 (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml penicillin-streptomycin, 1mM L-glutamine and β-mercapte (GIBCO Laboratories); this was named complete RPMI 1640 (1640-C). After incubation for 1h at 37°C in 5% CO_2 (95% humidity), non-adhering cells were washed off and the plates with adhering cells incubated with RPMI 1640-C plus 10μg/ml lipopolysaccharide (LPS) (E. coli 0111, B, DIFCO). Control wells were incubated only with RPMI-1640. After incubation for 24h at 37°C in 5% CO_2, the culture supernatants were colleted and used for NO and TNF-α production assays.

MTT assay (Viabilitycell measurement)

100μl (5mg/ml) of [3-(4,5-dimethyltiazol-2-yl)-(2,5- diphenyltetrazolium bromide] (MTT) were added to the supernatants on the adhered cells on the wells and incubated for 2h in culture. After incubation, the absorbance was recorded at 570nm with the 620nm filter of reference using an ELISA reader. The colorimetric assay of MTT have been used to detect living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method is used to measure cytotoxicity, proliferation or activation of cells. (Mossmann, 1983)

Nitric Oxide (NO) Production

The nitric oxide production was determined by measuring nitrite, a stable degradation product of nitric oxide, according to the method of Green et al. (1982). Briefly, 50 μl of cell supernatant was removed from each well and incubated with Griess reagent (1% sulfanil-amide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4) at room temperature, for 10 minutes. The absorbance was determined with an ELISA reader (multiskan Ascent, Labsystem Research Tech. Helsinki, Finland) at 540nm. The nitrite concentration was read from a standard curve generated with sodium nitrite. The value obtained was expressed in μM of NO/5x10^5 cells.

Determination of TNF-α (Tumoral Necrosis Factor-α) Production

The levels of TNF-α were measured in macrophage culture supernatants by enzyme linked assay (ELISA), employing a commercially available Kit (Mouse TNF mono/poly ELISA set kit - BD Biosciences Pharmingen, CA-USA), following the manufacturer's instructions.

Densitometric analysis

Femurs were carefully removed and soaked in 10% formalin. Standardized digital radiographs were obtained with the use of a computerized imaging system, CDR^{R} (Holzhausen et al, 2002; Nassar et al, 2004). Electronic sensors were exposed at 60KV and 10Ma for 10 seconds. The source-to-film distance was always set at 30 cm.

Bone mineral density measurements were made with the help of the software Photoshop 7,0 (Microsoft, 2003). Eleven points in radiographic images were made from femoral bone (three points on proximal epiphysis, three point on distal epiphysis and five points on diaphysis) (Fig.1). BMD was measured by the tool Histogram of the Photoshop software.

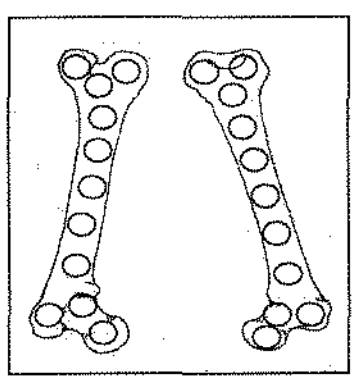

Figure.1 - Schematic points of measurement of femoral bone mineral density in mice.

Statistical analysis

Summary statistics included mean ± SEM. Comparisons between groups were determined by Kruskal Wallis and Wilcoxon tests. Significance level was always set at 95%.

RESULTS

Biochemical analysis

In the control groups, the serum calcium levels ranged from 8,08 ± 0,4 mg/dL to 12,00 ± 0,3 mg/dL. The CsA treated groups showed a significant decrease in the calcium levels in the periods of 28 and 60 days, respectively (12,00 ±0,33 mg/dL to 10,54 ± 0,17 mg/dL and 10.60 ± 0.17 mg/dL to 8,92 ± 0,33 mg/dL; $p<0,05$) (Fig.2A). The mice treated with CsA, a significant decrease ($p<0,05$) in serum ALP was observed on the 7th and 14th days (260,1 ± 27,1 U/L and 318,19 ± 19,99 U/L, respectively) compared to the control group (397,2 ± 42,1 U/L and 404,6 ± 16,26 U/L, respectively). After 28 and 60 days, serum ALP values returned to the normal values (Fig.2B).

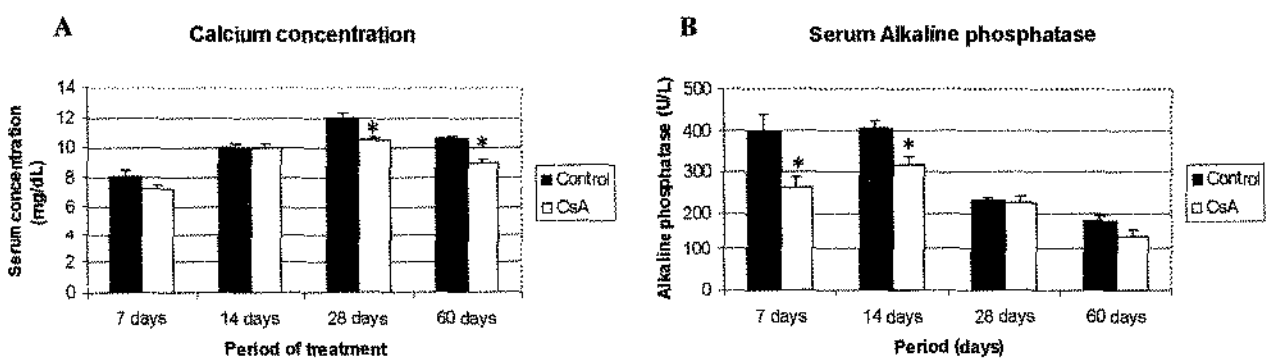

Fig. 2 – Serum calcium (A) and serum ALP (B) from mice control and treated with CsA, in the different periods of the experiment.

Bone Mineral Density

In the control group, the BMD at the distal epiphysis region were 64.23 ± 0.96, 65.1 ± 1.17, 72.4 ± 1.15 and 72.83 ± 1.77, in the periods of 7, 14, 28 and 60 days respectively. The distal epiphysis BMD decreased significantly in the mice submitted to CsA therapy (62.02 ± 0.8, 62.15 ± 0.9, 66.15 ± 0.9 and 66.52 ± 0.9, $p<0,05$, respectively) when compared to control (Fig.3-A). The values for distal epiphysis were statistically lower in the periods of 14, 28 and 60 days. At the proximal epiphysis, the BMD values of femurs in the control group were respectively, 51.4 ± 0.99, 53.47 ± 0.96, 59 ± 1.24 and 63.87 ± 1.11 for the periods of 7, 14, 28 and 60 days. The values for the CsA-treated mice were lower than control groups (50.26 ± 0.8, 51.41 ± 0.83, 57.26 ± 0.93 and 59.06 ± 0.81) but there was a statistically significant decrease of BMD only in the period of 60 days (63,87 ± 1,11 to 59,06 ± 0,81, $p<0,05$) (Fig.3-B). In the diaphysis, there was a small decrease between control groups and the CsA-treated groups, but it was not statistically significant ($p>0,05$).

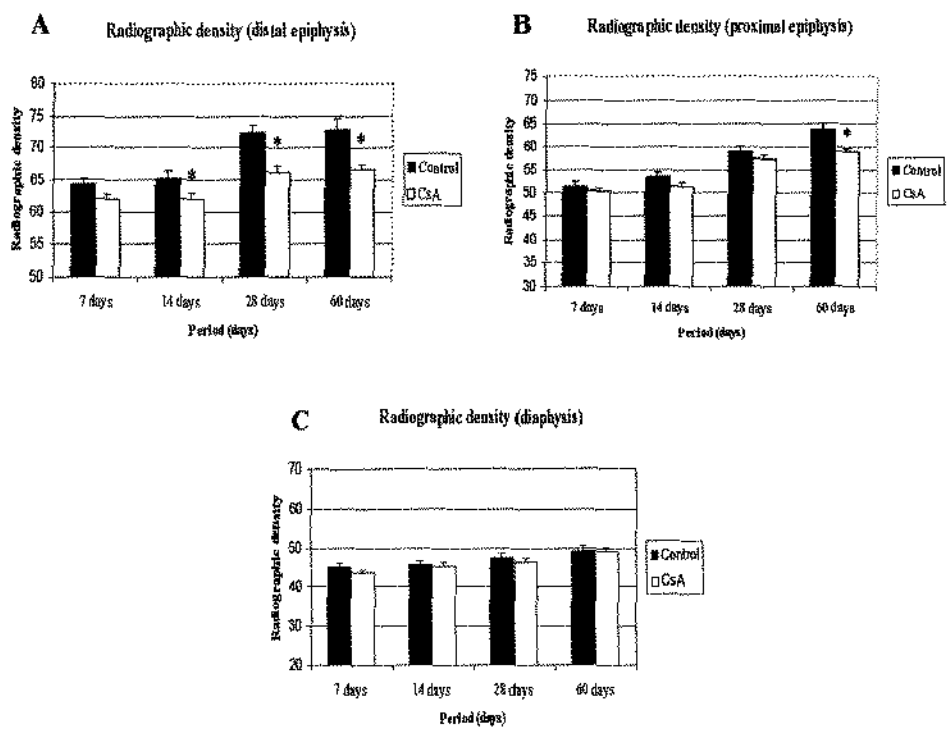

Figure 3 – Bone mineral density of different regions of femur from mice control and treated with CsA in the different periods. Distal epiphysis (panel A), Proximal epiphysis (panel B) and Diaphysis (panel C).

MTT, Nitric Oxide and TNF-α Measurements

MTT assay performed to verify the cell viability, by death cell measurement, revealed a cell viability above 90%.

The nitric oxide production in control groups were 46.19 ± 7.42, 33.67 ± 2.23, 51.09 ± 4.87 and 62.18 ± 5.71 μM of NO/5x10^5 cells, respectively for the periods of 7, 14, 28 and 60 days. In the CsA-treated mice, there was a significant increase in NO production was observed on the 7th (70.46 ± 8.52 μM of NO/5x10^5 cells) and 14th days (65.62 ± 4.97 μM of NO/5x10^5 cells).

The values for TNF-α production in control groups were 11.22 ± 1.91, 12.99 ± 0.92, 9.5 ± 2.15 and 9.15 ± 3.24 pg/ml, respectively for the periods of 7, 14, 28 and 60 days. There was also an increase in the CsA-treated groups on the 7th (17.25 ± 2.07 pg/ml) and 14th days (18.27 ± 1.89 pg/ml). The results obtained in the period of 28 days reveled that both, NO and TNF-α, increased but these values was not significant (p>0,05). The lowest values were observed after 60 days of the treatment, whose values returned to normal.

A

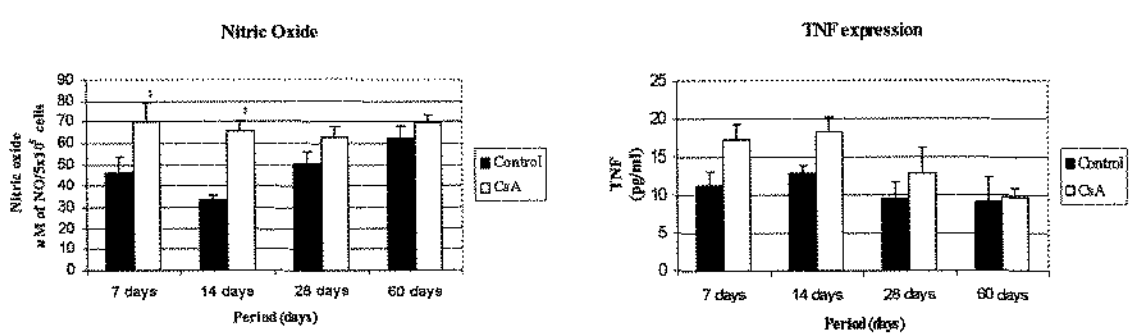

Figure 4. Nitric oxide (A) and TNF-α (B) production in mice control and treated with CsA in the different periods.

DISCUSSION

In the present study, we investigated the effects of the CsA on bone mass by biochemical and densitometry analysis in a mouse model and in different experimental periods. Animal model is an excellent protocol to study various side effects induced for drugs, because many variable are better controlled, such as genetic predisposition, gender, age, dose and duration of treatment (Kataoka et al, 2000). In accordance to Jee & Yao (2001), it was verified that the mouse is a good model for the study of osteoporosis.

The routes and dosage of CsA used in the present work was based on other works developed in our laboratory (Spolidorio et al, 2001; Spolidorio et al, 2004; Nassar et al, 2004) as well as described by others authors (Wassef et al, 1985; Morisaki et al, 1993)

The experimental treatment period with CsA was based on previous studies (Goodman et al, 2001; Fu et al, 1999, Fu et al, 2001), particularly on the study of Scholesberg et al (1989), who showed that the effect of CsA on bone and mineral metabolism is dependent of the duration of the treatment. They suggested an increased bone resorption, represented by a significant increase in the number of osteoclast-like cells, accompanied by a 79% reduction in bone volume within 28 days of CsA treatment.

Our results suggested that there was bone loss after 14 days of treatment. At the same time, there was a stability of the bone loss in the periods between 28 and 60 days. Our findings are in line with the findings of other authors (Fu et al, 1999; Fu et al, 2001; Epstein et al, 2001; Erben et al, 2003; Spolidorio et al, 2004; Nassar et al, 2004), that bone loss is dependent on treatment duration (Movsowitz et al, 1988; Schlosberg et al, 1989; Fu et al, 1999; Erben et al, 2003)

In the present study, we verified that bone regions are important for the study of bone metabolism and that the epiphysis was the main site altered after CsA treatment. There was a decrease of BMD at the sites of distal epiphysis in the first periods (as of the 14 day-period, and sustained for the 28- and 60-days periods) and at proximal epiphysis within longer periods (60 days). According to the Food and Drug Administration (FDA) guidelines for pre-clinical osteoporosis research, biomechanical investigation of two sites are required: the vertebral bodies and the femoral cortical bone (FDA, 1994). However, the

femoral neck site is very important from a clinical point of view, and it has been suggested that this site be included as a general test site (Mosekilde, 1995). In addition, as the proximal tibial or the distal femoral metaphysis are sites of choice concerning the assessment of dynamic and static histomorphometric changes due to ovriectomy (OVX) and treatment regimens (Kalu, 1991), one of these sites is recommended to merit mechanical assessment and longitudinal measurements of bone changes by use of densitometric analysis (Wronski et al, 1993; Noland et al, 1997).

Giavaresi et al (2002) investigated the mechanical and structural characteristics of cancellous bone from the femoral distal epiphysis. The data from this study showed osteopenia occurring in the rat distal femur after OVX and provided methodology for further investigations on osteoporosis and osteopenic bone. It showed that densitometric measurements decreased amounting 13-20% in the BMD and histomorphometric analysis showed a significant cancellous bone loss and the trabecular bone volume (BV/TV).

The findings obtained in the present work are in line with the findings of Erben et al. (2003) that showed a reduction of tibiae BMD in rats treated with CsA during 4 months. Clinically, in organ transplanted recipients bone loss occurs within the first year after transplantation (Shane et al, 1993; Sambrook et al, 1994; Lee et al, 2004). Lee et al (2004) showed that BMD decreased 4.8% at the lumbar spine and 12.3% at the proximal femur in patients treated with CsA. They point out that BMD decreased significantly with the cumulative CsA dose.

Indeed, there are several parameters that must be considered regarding the pathogenesis of CsA-induced bone loss, but it is suggested by several authors that it can be controlled by enhancing antiresorption molecule production from osteogenic cells (Suda et al, 1995). It is suggested that CsA-induced bone loss is mediated by alteration of the balance between the receptor activator of NF-κ-(RANK-L) (receptor activator of nuclear factor kappa B ligand) and osteoprotegerin (OPG), with CsA increasing RANK-L and decreasing OPG (Hofbauer et al, 2001; Cunninghan, 2005). OPG and RANK-L produced by osteogenic stromal cells play important roles in regulating osteoclastogenesis. NF-κ-(RANK-L) is involved in the differentiation and fusion of osteoclastic precursor mononuclear cells (Roodman, 1999). These precursor cells fuse to form large

multinucleated osteoclasts, which are the cell ultimately responsible for bone destruction. (Wiebe et al, 1996; Teitelbaum, 2000; Ross, 2000). OPG acts as a decoy receptor by blocking the interaction of RANK-L with its functional receptor RANK, thereby inhibiting osteoclastogenesis (Hofbauer et al, 2000). Therefore, regulation of the RANK-L expression is the key to the pathogenesis of many osteopenic disorders (Wei et al, 2005). The ratio of RANK-L to its soluble, antiosteoclastogenic decoy receptor, OPG is an indicator of the magnitude of systemic bone loss in pathological conditions (Wei et al, 2005). As CsA increases RANK-L, it directly increases osteoclast differentiation. The concept of bone remodeling is based on the hypothesis that osteoclastic precursors become actived and differentiated into osteoclasts, and thus the process of bone resorption begins. This phase is followed by a bone formation phase. The termination of bone resorption and the initiation of bone formation occur through a coupling mechanism that involves various lymphokines. Osteoclastogenesis is an important factor of the coupling process that can be modulated by CsA action.

Biochemical factor can also be involved with CsA-induced bone loss. In our findings, the biochemical assessment of biomarkers of bone turnover is in line with the radiographic findings. Our present experiment showed a significant decrease in serum calcium level, which, according to Mason (1990) and Ryffel (1986) could be a non-specific effect of CsA due to an increased excretion by the kidney. The data of the present study showed that there was a decrease in ALP level. Similar results were observed by Nassar et al. (2004).

In human renal transplant patients, an insignificant decrease in ALP was noted 1 week after CsA administration; the enzyme returned to normal 1 month after CsA therapy and then increased significantly 3-6 months after the drug was given (Withold et al., 1994). Similarly, in the rat model, there was a tendency of ALP to decrease 1-2 weeks after CsA administration and then return to normal at 4 weeks (Klein et al., 1994).

Besides the biochemical parameters, the measurement of immune markers like NO and TNF-α expression showed to be a relevant parameter in the CsA-induced bone loss. NO is a free radical generated by the nitric oxide synthase enzymes (NOS) that has important functions on bone cells (Feelish et al, 1996).

The experimental model of peritoneal macrophages cultures and BMD measures employed in the present study are commonly used in various research protocols (Strestikova et al, 2001; Kim et al, 2004). In the present study, we verified that NO and TNF-α amounts increased in the first periods of treatment (7 and 14 days) and then they returned to amounts similar to the control animals. Several studies showed that a high-level production of NO inhibits osteoclast formation and activity (Vant Hof & Ralston, 2001) and low doses of NO induced bone resorption *in vitro* (Ralston et al, 1995).

There are a lot of controversial findings about the role of NO on bone metabolism. *In vitro* studies suggest that the inhibition of iNOS with L-NIL prevents TNF-α-induced osteoclast differentiation (Lee et al, 2004). Previous studies have demonstrated that NO can induce apoptosis of osteoclast progenitors in inflammatory cytokine-stimulated bone marrow cell cultures (Vant Hof & Ralston, 1997). However, Wang et al (2004) showed that NO donors (NOC-18) increased OPG levels, but not RANK-L mRNA expression in bone marrow stromal cells culture. The authors suggested that an optimal dose of NO donor is required to promote cell proliferation and OPG mRNA and protein levels since a large amount of NO from a higher dose of NOC-18 suppressed OPG synthesis and cell proliferation. The results of this study support those of previous studies showing that NO has a biphasic effect on the proliferation and apoptosis of rat osteoblasts (Mancini et al, 2000; Chen et al, 2002). Therefore, the authors speculated that inhibitory effects of NO on osteoclast formation may be dependent on the model system used, the NO concentration, and the developmental potential of osteoclastogenic cells (Wang et al, 2004), suggesting that there are multiple osteoclastogenesis inhibitory pathways probably responsible for NO-modulated osteoclast formation.

Recent studies have added support to the theory that iNOS can promote osteoclast function. There is evidence that low levels of constitutive NO production may be essential for normal osteoclast function and maturation (Brandi et al, 1995), whereas other studies have shown that moderate induction of NO potentiates bone resorption induced by IL-1 and TNF (Ralston et al, 1995; Vant Hof & Ralston, 1997). Evidences suggest that the iNOS pathway plays an important role in cytokine- and inflammation-induced osteoporosis (Armour et al, 1999). Armour and colegues (1999) used an *in vivo* animal model of

inflammation-induced osteoporosis (IMO) and found a decrease in the BMD of rats that had a high production of NO. They also found that the BMD was associated with activation of iNOS, reduced osteoblast and decreased osteoclasts numbers. These clinical evidences are in accordance with the current work, where we found an increase in NO levels associated with BMD decrease.

Moreover, recent studies have shown that activation of the iNOS is essential for IL-1-stimulated bone resorption, both *in vivo* and *in vitro*. These studies suggest that IL-1 primarily acts on osteoblasts increasing NO synthesis by activation of the iNOS pathway and that this in turn promotes nuclear translocation of the transcription factor NFκB in osteoclast progenitors. (VANT HOF & Ralston, 1997 (B); Vant Hof et al, 2000).

Gyurko et al (2005) suggests that iNOS-generated NO is required for normal skeletal and alveolar bone development and that NO promotes bone resorption locally and that iNOS is an important signal for normal osteoclast differentiation. The mechanism suggested by which iNOS perform these functions is by enhacing M-CSF and RANK-L-induced osteoclast differentiation. Thus, similar to the CsA mechanism, the balance of RANK-L and OPG is also involved in the mechanism of NO-mediated bone functions.

Regulation of iNOS transcription is activated by pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IFNγ and endotoxin (Damoulis & Hauschka, 1997; Vant Hof & Ralston, 2001), recognized in bone resorption. Recent studies showed that TNF-α - important in inflammatory osteolysis (Ritchilin et al, 2003) - requires constitutive levels of RANK-L. Wei at al (2005) showed that TNF-α induces RANK-L expression by marrow stromal cells. Like TNF, IL-1, another major osteoclastogenic cytokine, promotes RANK-L expression by marrow stromal cells and osteoblasts (Hofbauer et al, 1999).

In this study we found that TNF-α levels increased simultaneously with the increase of NO levels, suggesting an important factor in NO concentrations. Based on the fact that NO is closely related in bone remodeling,, the effects of CsA on NO concentrations may contribute to the CsA-induced bone loss.

However, *in vitro* studies suggest that CsA inhibits NO production in several models of culture cells (Attur et al. 2000; Kim et al, 2004). Cyclosporin A inhibited NO production in human T84 epihtelial cells and in murine J774 macrophages, measured as

nitrite accumulation into the culture medium, in a dose-dependent manner in both cell lines (Hämäläinen et al, 2002). Strestikova et al. (2001) have shown that CsA exhibit dose-dependent inhibitory effect on NO production in rat peritoneal macrophage culture experiments suggesting that iNOS expression down-regulation by CsA occurs post-transcriptionally. CsA has shown to suppress iNOS expression via NF-κB transcriptional pathway in kidney (Kunz et al, 1995), whereas CsA was shown to inhibit iNOS expression by NF-κB-independent manner in human endothelial cell and J774 macrophage (Raffie et al, 2002; Hamalainen et al, 2002). It is important to consider that not always do model cultures correspond to *in vivo* model experiments.

In addition, an *in vitro* study with two co-cultures of osteoclast-rich neonatal rat long-bone marrow cells and dentin sections with neonatal-rat calvarial osteoblasts incubated with CsA showed 90% of bone resorption inhibition. When L-arginine (NO substrate) was added, it reversed this inhibition by 90%. The application of NOS inhibitor L-NAME inhibited bone resorption by 87%. It demonstrated that NO-cGMP pathway is involved in CsA-induced bone loss (Wimalawansa et al, 2000). The exact mechanism that modulates CsA-induced bone loss and the association of NO modulation in BMD in *in vivo* studies are not very well stablished. We suggest that NO synthesis has an important function in the modulation of CsA-induced bone loss since both, NO and CsA mechanisms interact in the balance of RANK-L and OPG with CsA increasing RANK-L and decreasing OPG (Hofbauer et al, 2001; Cunninghan, 2005) and NO enhacing RANKL-induced osteoclast differentiation. We suggest that the increase of NO production associated with CsA and the decreased bone mass, in this *in vivo* model, is due to uncoupling of bone resorption from bone formation process, mediated by the balance of RANK-L and OPG. Based on the results of this study, we confirmed that CsA induced bone loss, in accordance with the period of administration. There was a synergism between bone loss and the decrease of ALP and calcium, in addition to the increase of NO and TNF-α.

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CONCLUSÕES GERAIS

A partir dos resultados do presente trabalho, podemos concluir que:

A CsA induziu perda óssea nos camundongos de acordo com o período de administração;

Houve um sinergismo entre a perda óssea e a diminuição dos níveis de fosfatase alcalina e cálcio, assim como com o aumento do óxido nítrico e TNFα.

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