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TRATAMENTO DE ARTRITE COM VITAMINA D_3 LIVRE OU NANOENCAPSULADA: EFEITO SOBRE ECTOENZIMAS DE LINFÓCITOS EM MODELO ANIMAL

DISSERTAÇÃO DE MESTRADO

Karine Lanes da Silveira

Santa Maria, RS, Brasil
2014

TRATAMENTO DE ARTRITE COM VITAMINA D_3 LIVRE OU NANOENCAPSULADA: EFEITO SOBRE ECTOENZIMAS DE LINFÓCITOS EM MODELO ANIMAL

Karine Lanes da Silveira

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Análises Clínicas e Toxicológicas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Ciências Farmacêuticas.**

Orientadora: Prof. Drª. Daniela Bitencourt Rosa Leal

Santa Maria, RS, Brasil
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TRATAMENTO DE ARTRITE COM VITAMINA D_3 LIVRE OU NANOENCAPSULADA: EFEITO SOBRE ECTOENZIMAS DE LINFÓCITOS EM MODELO ANIMAL

elaborada por
Karine Lanes da Silveira

como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

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"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes".

Marthin Luther King

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

TRATAMENTO DE ARTRITE COM VITAMINA D_3 LIVRE OU NANOENCAPSULADA: EFEITO SOBRE ECTOENZIMAS DE LINFÓCITOS EM MODELO ANIMAL

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Data e local de Defesa: Santa Maria, 22 de abril de 2014.

A artrite reumatoide (AR) é uma doença inflamatória crônica, multissistêmica, caracterizada por sinovite simétrica e erosiva. O sistema de sinalização purinérgica desempenha um papel importante na modulação das respostas inflamatórias e imunes, através de biomoléculas extracelulares, como os nucleotídeos de adenina e seu derivado nucleosídeo adenosina, cujas concentrações extracelulares são controladas por ação de ectoenzimas, como ecto-nucleosídeo trifosfato difosfoidrolase (E-NTPDase) e ecto-adenosina desaminase (E-ADA) presentes na superfície de diversas células. A deficiência da vitamina D, um hormônio conhecido por desempenhar importante papel na homeostase do cálcio, vem sendo relacionada a diversas doenças autoimunes, entre elas a AR. Este fato deve-se a seu papel imunomodulador, onde atua na inibição da proliferação de linfócitos T, especialmente linfócitos Th_1, bem como na produção e na ação de citocinas. Como altas doses podem levar à hipercalcemia e à intoxicação, foi testada a utilização de um sistema de nanoencapsulação capaz de liberar, de forma controlada e sustentada, uma dose reduzida no local de ação. Com base nestes princípios, foi avaliado o efeito da vitamina D_3 livre e nanoencapsulada no escore de artrite, na hiperalgesia termal e no edema de pata, bem como em análises histológicas e na atividade das enzimas E-NTPDases e E-ADA de linfócitos de animais com artrite induzida por adjuvante Completo de Freund (CFA). Parâmetros hematológicos e bioquímicos, entre eles a concentração sérica de vitamina D, foram determinados. Ratas adultas Wistar foram divididas em dez grupos: I: controle (CN); II: artrite (AR); III: controle veículo (CV); IV: AR+V; V: vitamina D_3 livre na dose de 120 UI/dia (VD_3); VI: AR+VD_3 (120 UI/dia); VII: controle da formulação branca (CF); VIII: AR+F; IX: vitamina D_3 nanoencapsulada na dose de 15,84 UI/dia (LNC-D_3) e X: AR+LNC-D_3 (15,84 UI/dia). O início do tratamento foi após 15 dias da indução da artrite, em um período de 15 dias. Os resultados do presente estudo demonstraram que VD_3 foi capaz de reduzir escore de artrite, hiperalgesia térmica e edema da pata em ratos com AR. Ao mesmo tempo, o tratamento com LNC-D_3 reduziu apenas a hiperalgesia térmica e o edema da pata. As análises histológicas mostraram que ambas as formulações foram capazes de reduzir as alterações inflamatórias induzidas por CFA. Os níveis de AST apresentaram um aumento no grupo AR em comparação com os demais. No grupo tratado com a VD_3 observou-se um aumento nos níveis de 25(OH)D. A atividade da E-NTPDase em linfócitos de ratos que desenvolveram AR foi maior em comparação ao grupo controle, ao passo que a atividade da E-ADA foi menor. Este efeito foi revertido após 15 dias de tratamento com VD_3 e LNC-D_3. Além disso, ambas as formulações de vitamina D_3 não alteraram a atividade das enzimas E-NTPDase e E-ADA em animais saudáveis. Os dados do presente estudo apontam que a vitamina D_3, tanto na forma livre quanto nanoencapsulada, parece contribuir amenizando o processo inflamatório induzido pelo CFA, possivelmente por modular as atividades das ectonucleotidases, podendo após estudos adicionais ser utilizada como um agente terapêutico complementar para o tratamento da artrite reumatoide.

Palavras-chave: Artrite. Vitamina D_3. Nanocápsulas. Linfócitos. E-NTPDase. E-ADA.

ABSTRACT

ARTHRITIS TREATMENT WITH VITAMIN D_3 FREE OR VITAMIN D_3 LIPID-CORE NANOCAPSULES: ECTOENZYMES EFFECT ON LYMPHOCYTES IN ANIMAL MODEL

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Place and Date: Santa Maria, 22th April, 2014.

Rheumatoid arthritis (RA) is a chronic, multisystem inflammatory disease characterized by symmetric and erosive synovitis. The purinergic signaling system plays an important role in the modulation of inflammatory and immune responses through extracellular biomolecules as nucleotides adenine and its adenosine nucleoside derivative, whose extracellular concentrations are controlled by ectoenzymes action as ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-adenosine deaminase (E-ADA) present on the surface of various cells. The deficiency of vitamin D, a hormone known to play an important role in calcium homeostasis, has been related the several autoimmune diseases, including RA. This fact is due to its immunomodulatory role, where it inhibits the proliferation of T lymphocytes, especially Th1 lymphocytes as well as in the production and action of cytokines. Due to high doses of vitamin D can lead to hypercalcemia and intoxication it was tested the use of a nanoencapsulation system able to release in a controlled and sustainable manner, a reduced dose on site of action. Based on these principles, the effect of vitamin D_3 free and nanoencapsulated was evaluated in the score for arthritis, thermal hyperalgesia and paw edema as well as histological analyzes and in the activity of the E-NTPDase and E-ADA enzyme lymphocytes from animals with arthritis induced by complete Freud's adjuvant (CFA). Hematological and biochemical parameters, including serum concentrations of vitamin D, were determined. Adult Wistar rats were divided into ten groups: I: control (CN); II: adjuvant-induced arthritis (AR); III: control vehicle (CV); IV: AR+V; V: vitamin D_3 free at a dose of 120 IU/day (VD_3); VI: AR+VD_3 (120 IU/day); VII: control of white formulation (CF); VIII: AR+F; IX: vitamin D_3 lipid-core nanocapsules formulation at a dose of 15.84 IU/day (LNC-D_3) and X: AR+LNC-D_3 (15.84 IU/day). The initiation of treatment was 15 days after the induction of arthritis in a period of 15 days. The results of this study demonstrated that VD_3 was able to reduce arthritis score, thermal hyperalgesia and paw edema in rats with AR. At the same time, treatment with LNC-D_3 only reduced thermal hyperalgesia and paw edema. The histological analyzes showed that both formulations were able to reduce inflammatory changes induced by CFA. The levels AST showed an increased in the CN group compared to the others. In the group treated with VD_3 it was observed an increase in the levels of 25 (OH)D. The activity of the E-NTPDase in lymphocytes from rats that developed AR was higher in comparison with the control group, whereas the activity of E-ADA was lower. This effect was reversed after 15 days of treatment with VD_3 and LNC-D_3. In addition, both vitamin D_3 formulations did not alter the activity of E-NTPDase and E-ADA enzymes in healthy animals. Data from this study indicate that vitamin D_3, either in free or nanoencapsulated forms, seems to contribute mitigating the inflammatory process induced by CFA, possibly by modulating the activities of ectonucleotidases, can be used, after further studies, as a complementary therapeutic agent for the treatment of rheumatoid arthritis.

Keywords: Arthritis. Vitamin D_3. Nanocapsules. Lymphocytes. E-NTPDase. E-ADA.

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LISTA DE ABREVIATURAS

ADA: Adenosina desaminase
ADP: Adenosina difosfato
AIA: Artrite induzida por adjuvante
AINHs: Anti-inflamatórios não hormonais
AMP: Adenosina monofosfato
AR: Artrite reumatoide
ATP: Adenosina trifosfato
CIA: Colágeno bovino tipo II
CFA: Adjuvante Completo de Freund
DMARD: Drogas modificadoras do curso da doença
DMID: Diabetes melitus insulino dependente
DII: Doença inflamatória intestinal
DPB: Proteína ligante de vitamina D
E-ADA: Ecto- adenosina deaminase
EM: Esclerose múltipla
E-NTPDase: Ecto-nucleosídeo trifosfato difosfoidrolase
E-NPP: Ectonucleotídeo pirofosfatase/fosfodiesterase
E-5-NT: Ecto-5'-nucleotidase
IFA: Adjuvante incompleto de Freund
IL-1: Interleucina-1
IL-1β: Interleucina-1 beta
IL-2: Interleucina-2
IL-4: Interleucina-4
IL-5: Interleucina-5
IL-6: Interleucina-6
IL-7: Interleucina-7
IL-10: Interleucina-10
IL-12: Interleucina-12
IL-23: Interleucina-23
INF-γ: Interferon gama
LES: Lupus eritematoso sistêmico
MMP: Metaloproteinases
MRBs: Modificadores de resposta biológicas
NK: Células natural killer
NTPDase: Nucleosídeo trifosfato difosfoidrolase
PTH: Paratormônio
TGF-β: Fator transformador do crescimento beta
Th1: Linfócito helper 1
Th2: Linfócito helper 2
Th17: Linfócito helper 17
TNF-α: Fator de necrose tumoral alfa
Treg: Células T regulatórias
UI: Unidades internacionais
UV: Raio ultravioleta
VDR: Receptor de vitamina D
1,25 $(OH)_2D_3$: 1α,25 dihidroxivitamina D ou calcitriol
25 (OH)D: 25-hidroxivitamina D

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APRESENTAÇÃO

Esta dissertação está descrita na seguinte forma:

- Primeiramente são apresentados a introdução e os objetivos.
- A seguir, os resultados e a discussão são apresentados na forma de um manuscrito, que foi escrito seguindo as normas do periódico ao qual o mesmo será submetido.
- O item conclusão disposto após o manuscrito contêm interpretações e comentários gerais referentes ao manuscrito.
- As referências bibliográficas apresentadas no final da dissertação referem-se às citações que foram mencionadas no item Introdução.

O Manuscrito será submetido para a revista: **Clinical Science**.

1 INTRODUÇÃO

A artrite reumatoide (AR) é uma doença autoimune inflamatória crônica e multisistêmica, caracterizada por poliartrite periférica, simétrica, a qual leva à deformidade e à destruição de articulações (BRENOL et al., 2007; FIRESTEIN, 2003; LAURINDO et al., 2004; McINNES; SHETT, 2007; MOTA et al., 2011). É uma doença multifatorial de etiologia desconhecida, que resulta da interação entre fatores genéticos e ambientais, destacando-se como os principais fatores de risco: tabagismo, genética, idade, sexo, agentes infecciosos, fatores hormonais e fatores mecânicos (ALAMANOS; DROSOS, 2005; KLARESKOG et al., 2007, McINNES; SCHETT, 2011). Possui caráter progressivo, acometendo difusamente a membrana sinovial, produzindo dor, edema, calor e rubor articular e acentuada limitação de movimentos (FELLET; SCOTTON, 2004). Sua prevalência é de aproximadamente 1% na população brasileira, o que é similar à mundial, com uma incidência anual de 0,02% a 0,05%. É predominante no sexo feminino, afetando-o de duas a três vezes mais que o masculino. A incidência aumenta com a idade, surgindo geralmente na faixa etária de 30-50 anos (ALAMANOS et al., 2006; MOTA et al., 2011; PINCCUS; CALLAHAN, 1993).

A patogênese da AR parece ser bastante complexa e está relacionada com a resposta imune inata e adaptativa, como a resposta específica a antígenos mediado pelas células T e B (BEREK; SCHRODER, 1997; BOISSER et al., 2008; FIRESTEIN, 2003; PANAYI et al., 2001). Além disso, as citocinas pró-inflamatórias, tais como interleucina 1β (IL-1β), interleucina 6 (IL-6), interleucina 7 (IL-7), interleucina 12 (IL-12), interleucina 23 (IL-23) e o fator de transformação do crescimento beta (TGF-β), ativam múltiplas vias inflamatórias e destrutivas, sendo implicadas como componentes críticos da inflamação sinovial, já que auxiliam na diferenciação e perpetuação de respostas pró-inflamatórias, ocorrendo, assim, um equilíbrio entre respostas T auxiliar 1 (Th1) e/ou Th17 (BEREK; SCHRODER, 1997; BOISSIER et al., 2008; PANAYI et al. 2001).

O padrão de resposta Th1 ou Th17 é caracterizado por apresentar linfócitos T autoreativos, os quais produzem mediadores inflamatórios que resultam na destruição da cartilagem e do osso (CHABAUD et al., 1998; PANAYI et al., 2001).

Após ocorrer a perpetuação da doença, a membrana sinovial a qual é hipocelular, gera um ambiente hiperplástico, com a construção de uma camada de revestimento de células sinoviais e macrófagos (JIMENEZ-BOJ et al., 2005). Esse tecido neoformado é conhecido como sinovite crônica ou *pannus* e cresce sobre a articulação invadindo e destruindo a cartilagem (REDLICH et al., 2002; TEITELBAUM, 2000). A destruição da cartilagem ocorre devido à atividade da matriz de metaloproteinases (MMP), enzimas produzidas por macrófagos ativados e fibroblastos em resposta a citocinas como IL-1 e fator de necrose tumoral α (TNF-α), como representado na figura 1 (VINCENTI et al., 1994).

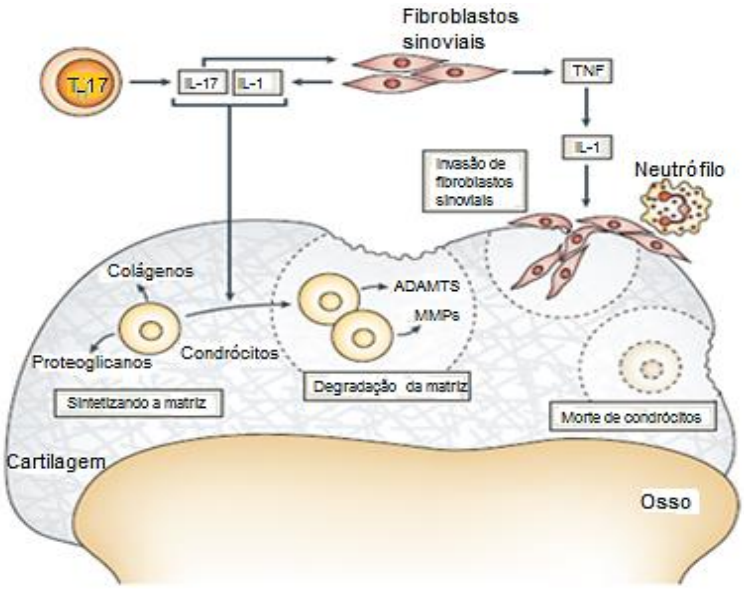

Figura 1 – Ativação de condrócitos e destruição da articulação (Adaptado de: McINNES; SCHEET, 2007).

Além do característico acometimento poliarticular, a AR pode envolver, direta ou indiretamente, diversos outros órgãos e tecidos, os quais representam uma sintomatologia extra-articular. Dentre os aspectos “não articulares” da AR temos o desenvolvimento de manifestações hematológicas, acometimento pulmonar e acometimento do fígado, envolvimento cardíaco, envolvimento renal e envolvimento ocular bem como vasculite reumatoide (FELLET; SCOTTON, 2004; MONTENEGRO; ROCHA, 2009).

O diagnóstico precoce e o início imediato do tratamento são fundamentais para o controle da doença, além de prevenir a incapacidade funcional e a lesão articular bem como diminuir a dor, a fim de melhorar a qualidade de vida dos pacientes, já que a remissão completa raramente é alcançada (CARRILHO, 2009; FELLET; SCOTTON, 2004; LAURINDO et al., 2004). O reconhecimento da AR depende da associação de uma série de sinais e sintomas clínicos, achados laboratoriais e radiográficos. Segundo Laurindo et al. (2004 apud ARNETT et al., 1988) e Mota et al. (2011) a orientação para o diagnóstico é baseada nos critérios de classificação do Colégio Americano de Reumatologia (Figura 2). Dos sete critérios analisados, quatro destes (de 1 a 4: rigidez matinal, artrite de três ou mais áreas articulares, artrite das articulações das mãos e artrite simétrica, respectivamente) são necessários para classificar um paciente como tendo AR e devem estar presentes por pelo menos seis semanas. Porém, aqueles pacientes que apresentarem dois ou três critérios não são excluídos da possibilidade do futuro desenvolvimento da doença (FELLET; SCOTTON, 2004).

Critérios do Colégio Americano de Reumatologia
1987 para classificação da artrite reumatoide

Critério	Definição
1) Rigidez matinal	Rigidez matinal com duração de pelo menos 1 hora até a melhora máxima
2) Artrite de três ou mais áreas articulares	Ao menos três áreas articulares simultaneamente afetadas, observadas pelo médico (interfalangeanas proximais, metacarpofalangeanas, punhos, cotovelos, joelhos, tornozelos e metatarsofalangeanas)
3) Artrite das articulações das mãos	Artrite em punhos ou metacarpofalangeanas ou interfalangeanas proximais
4) Artrite simétrica	Envolvimento simultâneo de áreas de ambos os lados do corpo
5) Nódulos reumatoides	Nódulos subcutâneos sobre proeminências ósseas, superfícies extensoras ou em regiões justa-articulares
6) Fator reumatoide sérico positivo	Presença de quantidades anormais de fator reumatoide
7) Alterações radiográficas	Radiografias posteroanteriores de mãos e punhos demonstrando rarefação óssea justa-articular ou erosões

Para a classificação como artrite reumatoide, o paciente deve satisfazer a pelo menos 4 dos 7 critérios. Os critérios 1 até o 4 devem estar presentes por, no mínimo, 6 semanas. Modificado a partir de Arnett *et al.*[73]

Figura 2 – Critérios de classificação do Colégio Americano de Reumatologia (MOTA et al., 2011).

Atualmente, a terapêutica do paciente vai variar de acordo com o estágio da doença, sua atividade e gravidade. Para o controle da dor e do processo inflamatório articular faz-se o uso de analgésicos e anti-inflamatórios não hormonais (AINHs), associados ou não a doses baixas de glicocorticoides (CARRILHO, 2009; MOTA et al., 2011). As drogas modificadoras do curso da doença (DMARDs), como hidroxicloroquina, metotrexato, sulfassalazina, leflunomida, dentre outras, sozinhas ou em combinação com drogas biológicas, são indicadas para qualquer paciente a partir da definição do diagnóstico de AR (BÉRTOLO et al., 2009; LAURINDO et al., 2004).

Aliado a estes tratamentos, avanços biotecnológicos também vem sendo estudados, como por exemplo, os chamados modificadores de respostas biológicas (MRBs), os quais têm como alvo substâncias químicas específicas do sistema imunológico envolvidas na AR, com papel central no início e na progressão da sinovite reumatoide e no consequente bloqueio da destruição óssea e cartilaginosa (CARRILHO, 2009; FELLET; SCOTTON, 2004).

A dificuldade da realização de estudos em pacientes por razões éticas ou técnicas leva à necessidade de modelos experimentais de artrite (ASQUITH et al., 2009). Para uma melhor compreensão da fisiopatologia da AR e na investigação de novas terapias para artropatias inflamatórias crônicas, diversos modelos são empregados pela comunidade científica, destacando-se os modelos por colágeno bovino tipo II (CIA) e por adjuvante completo de Freund (CFA) ou por adjuvante incompleto de Freund (IFA), ambos com *Mycobacterium tuberculosis* inativado (DONG et al., 2010; KLEINAU et al., 1991; OLIVEIRA et al., 2007).

O CFA é convencionalmente utilizado para induzir uma artrite crônica e progressiva em ratos conhecida como Artrite Induzida por Adjuvante (AIA). Neste modelo, a artrite é induzida com uma única injeção intraplantar de CFA, o qual atua como estímulo de respostas imunológicas a antígenos, desencadeando um edema na pata nas primeiras 24h perpetuando até o 11º dia, onde ocorre diminuição da inflamação aguda e subsequente desenvolvimento da inflamação crônica (OLIVEIRA et al., 2007). Após a indução, os animais desenvolvem uma inflamação poliarticular com uma consequente hiperplasia, desorganização da cartilagem e osso (BENDELE, 2001). Em decorrência disto, o processamento sensorial será anormal e, os estímulos ambientais que normalmente são inócuos, como um leve toque, produzem uma sensação de dor, ou seja, alodínia (LOESER; TREEDE, 2008).

Assim, após a inoculação do CFA, um processo inflamatório é desencadeado e, com isso, haverá a ativação das respostas imune e inflamatória. A inflamação é a resposta do organismo à lesão e é caracterizada por uma cascata de eventos celulares e moleculares que surgem independentemente do estímulo ou local (OLIVEIRA et al., 2007). Assim, sabendo-se que AR requer uma complexa resposta pró-inflamatória para que ocorra a sua manutenção, as moléculas sinalizadoras, como os nucleotídeos extracelulares, estão envolvidos nestes eventos auxiliando na perpetuação da AR. Entre os mediadores que sinalizam as respostas a diversas condições fisiológicas ou patológicas está o sistema purinérgico.

A sinalização purinérgica desempenha um importante papel na modulação da resposta imune através de biomoléculas extracelulares, como os nucleotídeos da adenina, adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP) e seu derivado nucleosídeo, a adenosina. Estas moléculas interagem com receptores purinérgicos presentes na superfície celular e desencadeiam cascatas enzimáticas que modulam diversos efeitos biológicos, incluindo inflamação, dor, proliferação e morte celular (Figura 3) (ATKINSON et al., 2006; RALEVIC; BURNSTOCK, 2003; YEGUTKIN, 2008).

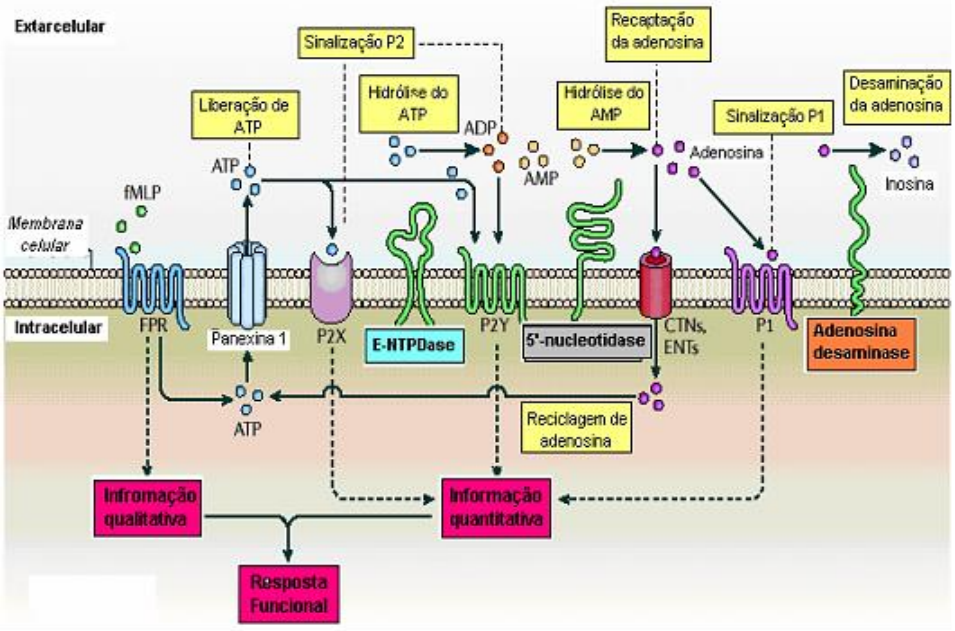

Figura 3 – Representação dos componentes do sistema purinérgico (Adaptado de: JUNGER, 2011).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO et al., 2001), sendo secretados por leucócitos, plaquetas e células endoteliais danificadas, (BOURS et al., 2006; VUADEN, 2006; ZIMMERMANN, 2000). Em altas concentrações essas biomoléculas podem ser citotóxicas e levarem à morte celular, pela formação de grandes poros na membrana plasmática (YOUNG et al., 1986). Já a adenosina pode ser liberada no meio extracelular como resultado da degradação do ATP e ADP por enzimas específicas (YEGUTKIN, 2008), ou através de transportadores na membrana das células que transportam a adenosina de dentro das células para o meio extracelular (BOROWIEC et al., 2006).

O ATP extracelular possui diversas funções fisiológicas sendo considerado uma molécula com atividade anti-inflamatória quando em baixas concentrações. Já em elevadas concentrações essa molécula desenvolve respostas pró-inflamatórias, atuando como citotóxica (FILIPPINI et al., 1990). O nucleotídeo ADP não possui um papel definido nos linfócitos (DI VIRGILIO et al., 2001). Porém, nas plaquetas, ele age como um importante mediador da agregação plaquetaria e da tromboregulação, podendo ser liberado na circulação sanguínea após danos teciduais (ZIMMERMANN, 1999). O AMP exerce a função de sinalizador em situações de desequilíbrio no metabolismo, além de servir como substrato para a formação da adenosina (LATINI; PEDATA, 2001). A adenosina desempenha funções importantes como efeitos neuromodulatorios, regulação de processos inflamatórios (agente anti-inflamatório endógeno), inibição da agregação plaquetaria e vasodilatadora (BOROWIEC et al., 2006; CRONSTEIN, 1994; ELLY; BERNE, 1992; SOSLAU; YOUNGPRAPAKORN, 1997). Também atua como imunossupressora (SPYCHALA et al., 1997) através da inibição da liberação de citocinas, da adesão de células imune e do funcionamento de linfócitos citotóxicos (CRONSTEIN et al., 1983).

Os nucleotídeos liberados no meio extracelular, antes de serem metabolizados pelas ectonucleotidases, interagem com receptores específicos da membrana plasmática, mediando os seus efeitos (BURNSTOCK, 2006; BURNSTOCK, 2007; DI VIRGÍLIO et al., 2001). Os receptores purinérgicos se dividem em duas famílias: P1 e P2 e são ativados pela adenosina e por ATP e ADP respectivamente. Os receptores P1 reconhecem a adenosina e são acoplados à proteína G (metabotrópicos) (BURNSTOCK, 2007). Os purinoreceptores P2 podem ser divididos em duas subclasses: metabotrópicos, chamados de P2Y e os ligados a

canais iônicos, designados P2X, que são específicos para o ATP (DI VIRGÍLIO et al., 2001). A sinalização purinérgica é concluída, então, pela ação de ectoenzimas que são as responsáveis por regular as concentrações de nucleotídeos extracelulares nos tecidos (ZIMMERMANN et al., 2007). Estas enzimas estão ancoradas à superfície celular e podem ser classificadas como membros das seguintes famílias: Ecto-difosfoidrolases (E-NTPDases); Ecto-nucleosídeo pirofosfato/fosfodiesterase (E-NPPs); Fosfatase Alcalina e Ecto-5'-nucleotidase (E-5'-NT) (Figura 4). Outra ectoenzima também importante no metabolismo purinérgico é a adenosina desaminase (E-ADA), a qual é responsável pela desaminação do nucleosídeo adenosina em inosina (ZIMMERMANN, 1996; ZIMMERMANN, 2001; ZIMMERMANN et al., 2012).

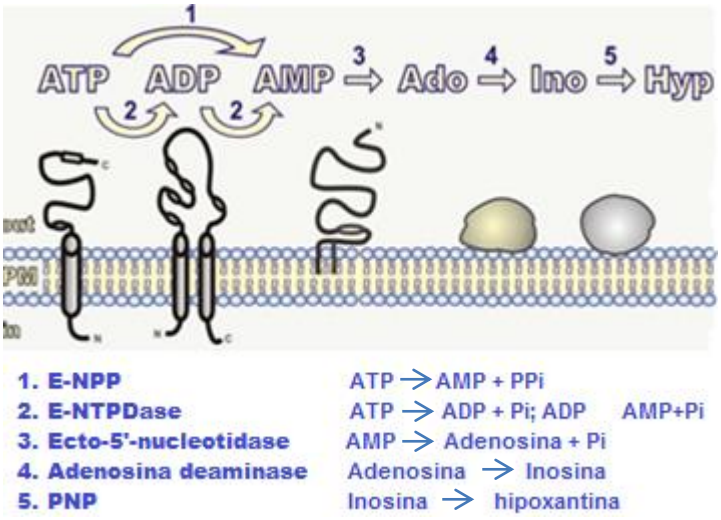

Figura 4 – Ectoenzimas envolvidas na degradação de nucleotídeos e nucleosídeo (Adaptado de: YEGUTKIN, 2008).

O conjunto de ações destas enzimas forma uma cadeia enzimática que tem início com a ação da E-NTPDase, a qual catalisa a hidrólise do ATP e do ADP, formando o AMP e da E-NPP, que hidrolisa ATP diretamente a formação do AMP (ZIMMERMANN et al., 2007). A seguir a enzima E-5'-NT hidrolisa a molécula do AMP formando adenosina (GODING, 2000; ZIMMERMANN, 2001). Ainda, seguindo a sequência de degradação dos nucleotídeos, a E-ADA atua regulando as

concentrações de adenosina, através da conversão e formação de inosina (BOURS et al., 2006; YEGUTKIN, 2008; ZIMMERMANN, 2000).

A E-NTPDase (CD39; E.C 3.6.1.5) tem sido relacionada a vários eventos celulares, tais como neurotransmissão, controle da agregação plaquetaria, sinalização purinérgica, tromboregulação (ZIMMERMAN, 2001) e participação na transdução de sinal através da adesão célula-célula (SARKIS et al., 1995; YAGI et al., 1992), além de importantes funções imunes e inflamatórias (DOMBROWSKI et al., 1995; LANGSTONE et al., 2003; ZIMMERMANN, 2001). Vários estudos têm mostrado uma atividade alterada da enzima E-NTPDase em pacientes com diferentes condições patológicas como o diabetes (LUNKES et al., 2003), a esclerose múltipla (SPANEVELLO et al., 2010), o infarto agudo do miocárdio (BAGATINI et al., 2008), na síndrome da imunodeficiência adquirida (AIDS) (LEAL et al., 2005). A atividade desta enzima também se encontra alterada em pacientes com hipercolesterolemia e processo inflamatório, onde a hidrólise do ATP e do ADP se encontra aumentada em plaquetas, assim como a expressão da CD39 na superfície da célula. Já em pacientes com AR, a atividade da E-NTPDase se encontra aumentada tanto em linfócitos como em plaquetas (BECKER et al., 2010; JAQUES et al., 2012).

A ecto-5´-nucleotidase (E-5´-NT, CD73, EC 3.1.3.5) é uma enzima que hidrolisa o AMP em adenosina, logo depois da hidrólise do ATP e ADP pela E-NTPDase (COLGAN et al., 2006; ZIMMERMANN, 2001), sendo por isto considerada a principal enzima responsável pela formação de adenosina. A adenosina desaminase (ADA, E.C. 3.5.4.4.) também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeos da adenina (YEGUTKIN, 2008). A ADA é uma enzima chave no metabolismo das purinas, já que é responsável por catalisar a deaminação irreversível da adenosina e 2'-deoxiadenosina em inosina e 2'-deoxinosina, respectivamente (RESTA et al., 1998; ROBSON et al., 2006). A E-ADA está presente no soro e em outros tecidos, particularmente nos tecidos linfoides e é essencial para a função e maturação dos linfócitos T, sendo também requerida para a maturação e proliferação dos monócitos e macrófagos no sistema imune (ADAMS; HARKNESS, 1976). Alterações em sua atividade têm sido consideradas indicadores de distúrbios imunológicos, pois vem sendo usada para monitorar várias patologias imunológicas (HITOGLU et al., 2001; POURSHARIFI et al., 2008). Esta enzima é encontrada em praticamente todos os

vertebrados. Em humanos existe na forma de duas isoenzimas classificadas como: ADA1 e ADA2, cada uma com suas particulares propriedades bioquímicas e distribuição tecidual (SHAROYAN et al., 2006). A principal localização da ADA1 é citosólica. Esta isoenzima é encontrada em todas as células e tecidos humanos, apresentando alta atividade em linfócitos e monócitos e é responsável por grande parte do desaparecimento da adenosina circulante nesse meio (TSUBOI et al., 1995). A ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos (ZUKKERMAN et al., 1980). Dados recentes têm sugerido que ADA2 no plasma humano pode ser secretada por monócitos ativados em processos inflamatórios, tendo a habilidade de regular a proliferação celular (IWAKI-EGAWA et al., 2006).

Além de possuir importante atividade na regulação dos níveis de nucleotídeos e nucleosídeo da adenina, as ectoenzimas exercem ações no sistema imunológico (BENREZZAK et al., 1999; SALAZAR-GONZALEZ et al., 1985). As enzimas do sistema purinérgico, como a E-NTPDase e a E-ADA, estão presentes na membrana dos linfócitos desempenhando um importante papel na resposta inflamatória. As respostas imunes pró-inflamatórias desencadeadas pela AR são moduladas por nucleotídeos e nucleosídeos, que se correlacionam diretamente com a atividade das ectonucleotidases.

Contudo, com o intuito de amenizar os diversos efeitos que as doenças autoimunes ocasionam, a utilização de novas terapias alternativas vem sendo pesquisadas e utilizadas.Estudos têm demonstrado que, através da suplementação dietética ou da administração oral de vitamina D_3, o aparecimento de artrite pode ser prevenido, além de que, se administrada em uma fase precoce, esta previne a progressão, diminui a atividade e melhora a evolução da artrite induzida (CANTORNA et al., 1998; CARRILHO, 2009). Estes efeitos terapêuticos estariam associados à capacidade de respostas imunomoduladoras da vitamina D sobre a diferenciação e produção de células, como as células dendríticas, T inflamatórias e pró-patogênicas; a inibição do desenvolvimento e função de células Th_1; a indução de células Th_2, entre outras (CUTOLO et al., 2007; DANTAS et al., 2009; PEIXOTO et al., 2012).

A vitamina D é uma molécula universalmente presente nos seres vivos e, apesar de chamada de vitamina, é na verdade um hormônio produzido fotoquimicamente na pele (UNGER, 2009). Isto justifica-se devido sua forma de

obtenção não ser exclusivamente através da alimentação como as outras vitaminas, mas por ser também produzida fotoquimicamente pela pele (PEREIRA; ALMEIDA, 2008; UNGER, 2009). Quimicamente, ela é um hormônio esteroide, com estrutura química de secoesteroide, ou seja, esteroide em que ocorre a quebra de um anel ciclopentanoperidrofenantreno com adição de dois átomos de hidrogênio em cada grupo terminal, como na estrutura molecular dos clássicos hormônios esteroides (estradiol, cortisol, aldosterona) (CARRILHO, 2009; NORMAN, 2008; PEREIRA; ALMEIDA, 2008). Segundo Luo et al. (2012), a vitamina D é classificada como sendo lipossolúvel e muito sensível a vários fatores ambientais, como luz, calor e oxigênio, que podem induzir a isomerização ou oxidação (McDOWELL, 2000, p.95), afetando negativamente sua estrutura química e benefícios fisiológicos.

Apesar de existirem várias formas nutricionais desta vitamina, duas delas são mais conhecidas: a vitamina D_2, calciferol ou ergocalciferol, de origem vegetal, e a vitamina D_3, colecalciferol ou calcitriol, sintetizada na pele em resposta à luz solar ou obtida através de alguns recursos alimentares (McDOWELL, 2000, p. 91; MISRA et al., 2008; DANTAS et al., 2009). A diferença química entre a vitamina D_2 e D_3 situa-se na cadeia lateral (Figura 5), o que faz que haja diferenças na sua ligação com a proteína de ligação da vitamina D (DPB) e no seu metabolismo (BIKLE, 2009). Mesmo com dados na literatura afirmando que ambas as formas possuem efeitos biológicos equivalentes em seres humanos, Norman (2008) relatou que a vitamina D_3 é substancialmente mais eficaz do que a vitamina D_2 em humanos, embora sejam metabolizadas de maneira equivalentes. Ambas sofrem o mesmo processo de metabolização para se tornarem ativas (BIKLE, 2009; CARRILHO, 2009). Por esta razão, a designação genérica “vitamina D” compreende tanto a vitamina D_2 como a D_3 (BIKLE, 2009; CARRILHO, 2009; McDOWELL, 2000, p. 94; PEREIRA; ALMEIDA, 2008; RAMALHO, 2010).

A vitamina D é considerada um nutriente essencial, onde a exposição solar é a maneira mais efetiva de sua síntese, a qual ocorre na epiderme após a exposição aos raios solares ultravioletas B (UV-B), tornando assim, a produção endógena nos tecidos cutâneos sua principal fonte. Devido a este fato, esta é conhecida vulgarmente como “vitamina do sol” (McDOWELL, 2000, p.91; PAIXÃO; STAMFORD, 2004; UNGER, 2009).

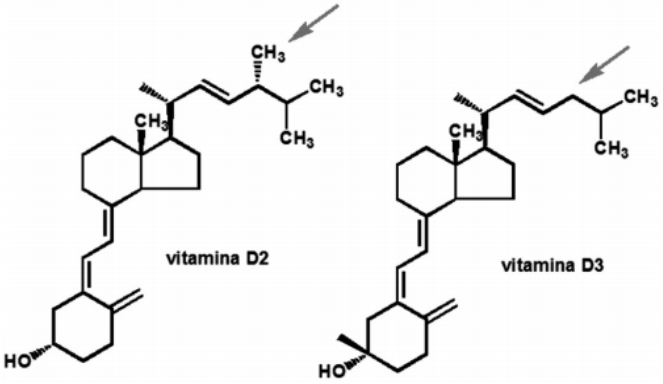

Figura 5 – Estrutura química do ergocalciferol (vitamina D_2) e do colecalciferol (vitamina D_3). As duas formas diferem pela presença de uma ligação dupla adicional e um grupo metil incorporados à cadeia lateral da forma biológica D_2 (seta) (PEIXOTO et al., 2012).

A produção exógena também é uma forma de obtenção de vitamina D, na qual as fontes alimentares contribuem na manutenção de seu status, além de assumirem grande importância nos indivíduos com baixa exposição solar (UNGER, 2009). Esses alimentos são uma fonte alternativa e menos eficaz, mas assumem um papel de maior importância em idosos, pessoas institucionalizadas e habitantes de climas temperados, onde a síntese cutânea é prejudicada (CARRILHO, 2009; DANTAS et al., 2009; PEREIRA; ALMEIDA, 2008; PREMAOR; FURLANETTO, 2006).

As recomendações de vitamina D são normalmente expressas por unidades internacionais (UI) ou microgramas (µg). Estudos de comparação entre a recomendação e a ingestão de vitamina D denotam, em diversas populações, ingestão inadequada do nutriente, sendo que muitos autores questionam estas recomendações, e indicam uma quantidade de 800 a 1000 UI/dia quando não há exposição solar adequada (HOLICK, 2007). Porém, parece que suplementação de 700 a 800 UI/dia de vitamina D, associada ao cálcio, é suficiente à manutenção da massa óssea e redução de fraturas (PREMAOR; FURLANETTO, 2006).

Para exercer sua função, a vitamina D necessita ser metabolizada até sua forma ativa, $1,25(OH)_2D_3$ (Figura 6). Quando exposto à radiação UV-B, o precursor cutâneo da vitamina D, o 7-dehidrocolesterol, sofre uma clivagem fotoquímica, originando a pré-vitamina D_3. Essa molécula termolábil, sofre um rearranjo molecular devido a temperatura corporal que a converte e resulta na formação da vitamina D_3 (colecalciferol) (DANTAS et al., 2009; McDOWELL, 2000, p. 97; NORMAN, 2008). A

pré-vitamina D_3 também pode sofrer um processo de isomerização, o qual origina produtos biologicamente inativos (BIKLE, 2009; HOLICK, 2005). Após a síntese cutânea, a vitamina D_3 entra na circulação e é transportada para o fígado, acoplada a DPB, embora uma pequena fração esteja ligada à albumina (McDOWELL, 2000, p. 101; PREMAOR; FURLANETTO, 2006). Pela absorção intestinal de alimentos ou suplementos, a vitamina D é incorporada em quilomicrons e transportada pelo sistema linfático até à circulação venosa e desta para o fígado (BIKLE, 2009; HOLICK, 2005).

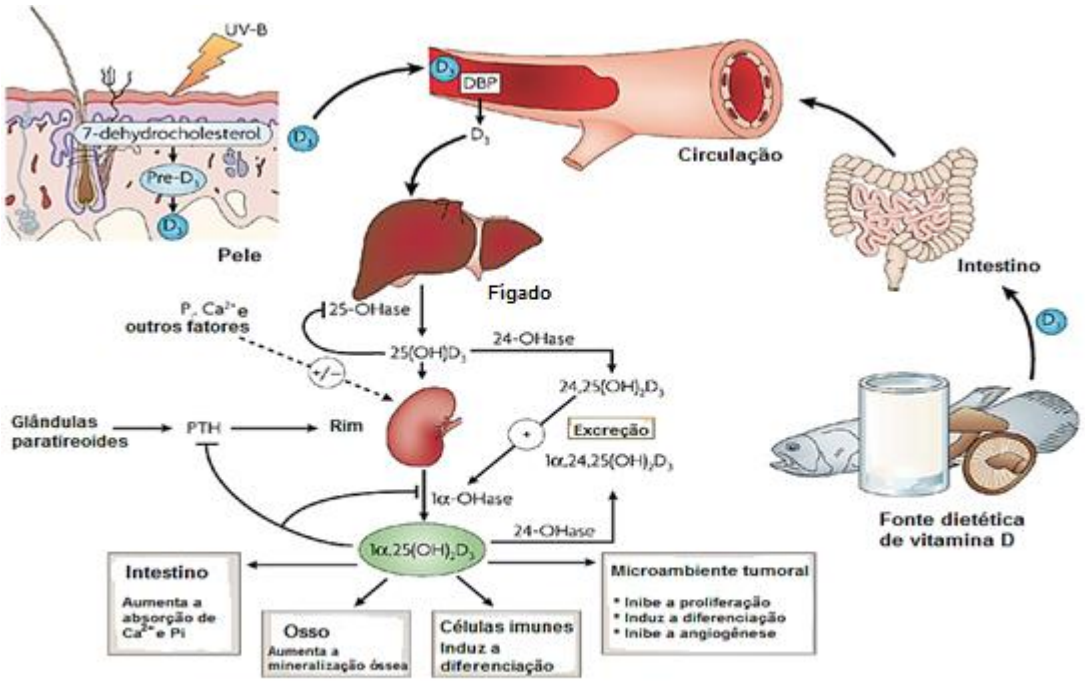

Figura 6 – Resumo das funções e metabolismo da vitamina D (Adaptado de: NATURE REVIEWS CANCER 7, 2007).

No fígado ocorre a hidroxilação por meio das 25-hidroxilases hepáticas, para a 25-hidroxivitamina D [25(OH)D] ou calcidiol, a qual será secretada no plasma. Esta forma representa a forma circulante em maior quantidade, porém biologicamente inerte (HOLICK, 2005; SCHUCH et al., 2009). Esta etapa de hidroxilação hepática é pobremente regulada, de forma que os níveis sanguíneos de 25(OH)D refletem a

quantidade de vitamina D que entra na circulação, sendo proporcionais à quantidade de vitamina D produzida na pele e ingerida (LEVENTIS; PATEL, 2008).

A etapa final da produção do hormônio é a hidroxilação adicional que acontece no rim, nas células do tubo contorcido proximal, através da enzima 25-hidroxivitamina D 1α-hidrolase (1α-OHase), originando a 1α,25 dihidroxivitamina D – $1,25(OH)_2D_3$ ou calcitriol, sua forma biologicamente ativa que exerce atividade biológica no organismo (McDOWELL, 2000, p.100 apud DeLUCA, 1990; PREMAOR; FURLANETTO, 2006). Este metabólito ativo é o que interage com receptores de vitamina D (VDR) no intestino delgado e nos osteoblastos, regulando o metabolismo de cálcio e fósforo, resultando na absorção de cálcio pelo intestino (CUTOLO et al., 2007; SCHUCH et al., 2009; ZITTERMANN, 2003).

Além disso, reconhece-se a existência de hidroxilação extrarrenal da vitamina D, originando a vitamina que agiria de maneira autócrina/ parácrina com funções de inibição da proliferação celular, promoção da diferenciação celular e regulação imune, já que a $1,25(OH)_2D_3$ neste caso é controlada por fatores locais como citocinas e fatores de crescimento e pelos níveis de 25(OH)D, tornando essa via mais sensível à deficiência de vitamina D (ANTICO, 2012; BIKLE, 2009; CARRILHO, 2009; LEVENTIS; PATEL, 2008). Após o catabolismo da $1,25(OH)_2D_3$ em compostos hidrossolúveis, a vitamina D é excretada principalmente através da bile e da urina. O mecanismo de conversão desta em compostos biologicamente inativos e excretáveis previne a intoxicação e auxilia na regulação de calcemia (HOLICK, 2007).

Para quantificar os níveis adequados da vitamina D no soro, esta deve ser associada à concentração de 25(OH)D, que representa o metabólito circulante em maior quantidade, sob condições normais, com meia-vida aproximadamente de duas a três semanas (HOLICK, 2007; McDOWELL, 2000, p. 99 apud LITTLEDIKE; HORST, 1982). Embora a forma ativa da vitamina D seja a $1,25(OH)_2D_3$, esta não deve ser utilizada para avaliar a concentração da vitamina, uma vez que sua meia-vida é de apenas 4 horas e sua concentração é muito menor do que a de 25(OH)D (HOLICK, 2007; UNGER, 2009). Não existe consenso sobre a concentração sérica ideal de vitamina D. Habitualmente, os valores normais são aqueles encontrados na média da população saudável, o que faz com que a maioria dos especialistas concordem que o nível de vitamina D deve ser mantido em uma faixa que não induza aumento dos níveis de paratormônio (PTH) (HOLICK, 2007; LEVENTIS; PATEL, 2008). Segundo Holick et al. (2011), valores de 25(OH)D de 30 a 100 ng/mL

são considerados suficientes por terem apresentado melhor correlação com a absorção de cálcio, densidade mineral e níveis de PTH.

Considerando este contexto, valores inferiores a 30 ng/mL podem ser indicadores de insuficiência ou deficiência, devendo ser correlacionados com a clínica e com os demais exames laboratoriais de avaliação do metabolismo do cálcio (HOLICK, 2007). Valores diminuídos estão associados com insuficiência dietética de vitamina D, doença hepática, má absorção, exposição ao sol inadequada e síndrome nefrótica, além de uma deficiente mineralização da matriz de colágeno nos ossos, gerando um retardo no crescimento e deformidades ósseas, conhecido como raquitismo (HOLICK, 2005). Porém, doses acima das usuais podem levar a casos de hipervitaminose, acarretando em efeitos como a hipercalcemia (MARQUES et al., 2010), bem como podem estar associados à intoxicação por vitamina D (UNGER, 2009; ZITTERMAN, 2003).

A vitamina D exerce suas ações através das vias não genômicas e genômicas. A via não genômica é responsável por gerar respostas rápidas, como a absorção intestinal de cálcio. Já a ação genômica depende da ligação desta com seu receptor nuclear VDR (do inglês *vitamin D receptor*, receptor específico da vitamina D). Estes receptores são semelhantes aos receptores para esteroides, hormônios tiroidianos e retinoides, os quais regulam a transcrição do DNA em RNA (BIKLE, 2009; NORMAN, 2008; SCHUCH et al., 2009; SZODORAY et al., 2008). Os VDR são expressos por vários tecidos e tipos celulares, incluindo intestino delgado, cérebro, próstata, mama, cólon, epitélio tubular renal, osteoblastos, osteoclastos, células hematopoiéticas, células epidérmicas, células pancreáticas, miócitos, neurônios, entre outros (DANTAS et al., 2009; NORMAN, 2008). Além desses, foram também identificadas em células do sistema imune, sendo que são amplamente expressos em monócitos, macrófagos, células dendríticas, células apresentadoras de antígenos, células NK e em linfócitos T e B ativados (ADORINI, 2002; CUTOLO et al., 2007; HOLICK, 2005). No entanto, sua maior concentração está nas células imunes imaturas no timo e nos linfócitos $CD8^+$ maduros, independente de seu estado de ativação. Além disso, linfócitos $CD4^+$ e macrófagos contêm quantidades menores, mas também significantes (DeLUCA; CANTORNA, 2001).

Embora a função clássica da vitamina D seja a mineralização óssea, também já foi demostrado uma multiplicidade de outras funções fisiológicas não relacionadas a isto, as denominadas funções “não clássicas” ou ações não calcêmicas, onde atua

de maneira endócrina, autócrina e parácrina (Figura 7) (HOLICK, 2005). Estas ações podem ser caracterizadas em três efeitos gerais: regulação da secreção do hormônio; regulação da função imunológica e regulação da proliferação e diferenciação celular (BIKLE, 2009; HOLICK, 2004).

Devido à presença do VDR em células do sistema imunológico, bem como a sua influência sobre genes responsáveis pela regulação de fatores neste sistema (CARRILHO, 2009), a vitamina D mostra ter um potente efeito de imunomodulação observado sobre linfócitos, macrófagos e células NK, além de interferir sobre a ação de citocinas (CARRILHO, 2009; DANTAS et al., 2009; PEREIRA; ALMEIDA, 2008).

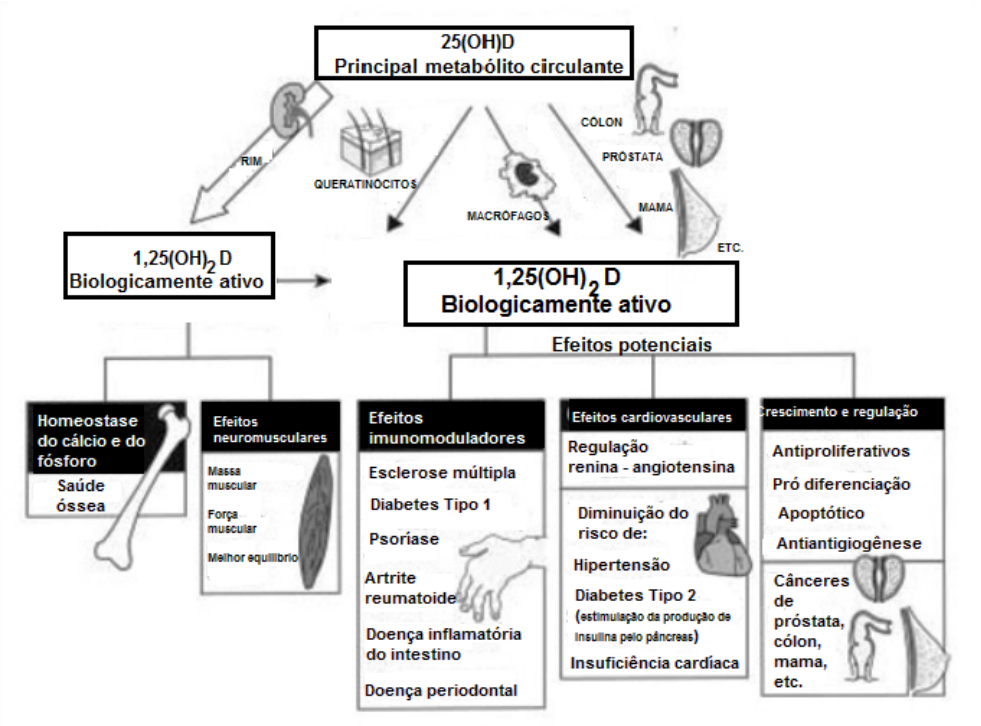

Figura 7 – Resumo das ações fisiológicas da vitamina D no que diz respeito à saúde cardiovascular, prevenção do câncer, regulação da função imunológica e diminuição do risco de doenças autoimunes (Adaptado de: HOLICK, 2004).

Os efeitos imunomoduladores da vitamina D foram demostrados através de vários mecanismos propostos para explicar sua participação no sistema imune, através de três importantes descobertas: a presença de VDR em células inflamatórias humanas ativadas (PROVVEDINI et al., 1983); a capacidade de $1,25(OH)_2D$ para inibir a proliferação de células T (RIGBY et al., 1984) e a

capacidade de macrófagos ativados em doença produzir 1,25$(OH)_2$D (ADAMS et al., 1983). A vitamina D exerce ação sobre o sistema imune, promovendo a imunidade inata e suprimindo a imunidade adaptativa por meio de uma ação inibitória (BIKLE, 2009). De maneira geral, a 1,25$(OH)_2$D suprime a proliferação e produção de imunoglobulinas e retarda a diferenciação dos precursores de linfócitos B em plasmócitos (CHEN et al., 2007; DANTAS et al., 2009; SZODORAY et al., 2008). Além disso, também inibe a proliferação de células T (RIGBY et al., 1984), em particular as células Th1, capazes de produzir IFN-γ e IL-2 e TNF (HOLICK, 2007; LEMIRE et al., 1995; WILLHEIM et al., 1999). Em contraste, a produção de IL-4, IL-5 e IL-10 podem ser aumentadas (ANTICO, 2012; BIKLE, 2009), deslocando a resposta para um fenótipo de células Th2. As células reguladoras (Treg) são também aumentadas por 1,25$(OH)_2D_3$ (PENNA; ADORINI, 2000).

A capacidade de 1,25 $(OH)_2$D em suprimir o sistema imune adaptativo parece ser benéfica para um número de condições em que o sistema imune é dirigida a si mesmo, isto é, a autoimunidade. Vários estudos têm relatado que em baixas concentrações ou na falta de vitamina D o sistema imune parece favorecer o desenvolvimento de células T autorreativas direcionadas contra tecidos do próprio organismo e a síntese de interleucinas pró-inflamatórias (IL-12, INF-γ), através de respostas de diferenciação, antiproliferação, indução de efeitos de apoptose ou imunomodulação (DANTAS et al., 2009; CASTRO, 2011). Essas respostas levariam a uma maior predisposição do indivíduo em desenvolver certas doenças autoimunes, incluindo diabetes melitus insulino-dependente (DMID), esclerose múltipla (EM), doença inflamatória intestinal (DII), lúpus eritematoso sistêmico (LES), bem como a AR (ADORINI, 2005; BIKLE, 2009; DeLUCA; CANTORNA, 2001).

Nos últimos anos, a participação da vitamina D na patogênese, atividade e tratamento da AR tem sido debatida com base nos resultados e observações de diversos estudos clínicos e experimentais, onde destaca-se: AR é uma doença imunomediada, cuja fisiopatologia envolve participação importante de células Th1; no líquido sinovial na AR, tem sido detectada 1,25$(OH)_2D_3$ e o VDR tem sido demonstrado em macrófagos, condrócitos e sinoviócitos; pacientes com AR e fator reumatoide positivo demonstraram linfócitos apresentando receptores para a vitamina D (CANTORNA et al., 1998; FRITSCHE et al., 2003; HOLICK, 2005; CUTOLO et al., 2007; SZODORAY et al., 2008). Ainda há estudos que demonstram uma deficiência de vitamina D em pacientes com AR (DANTAS et al., 2009). Em um

estudo realizado com mulheres, Merlino et al. (2004) mostrou que a ingestão regular de vitamina D foi inversamente associada ao risco de desenvolver AR, o que também foi mencionado por Dantas et al. ((2009) apud Andjelkovic et al. (1999)), onde se verificou que a suplementação com altas doses de vitamina D oral em pacientes com a referida patologia em tratamento com DMARDs convencionais, mostrou a redução da gravidade dos sintomas de AR em 89 % dos pacientes.

Em modelos animais experimentais, a adição de vitamina D na dieta tem se mostrado terapeuticamente efetiva, onde parece impedir o desenvolvimento da autoimunidade, como por exemplo, na artrite induzida por colágeno. Nesse caso, quando administrada numa fase precoce, ela previne a progressão da AR (ADORINI, 2002; ANTICO, 2012; CANTORNA et al., 1998; DANTAS et al., 2009).

Apesar de seus vários efeitos benéficos, foi demonstrado que a administração de doses orais elevadas de vitamina D são potencialmente tóxicas, podendo levar a mineralização generalizada de tecidos, ao desenvolvimento de hipercalcemia e hipercalciuria, o que torna a utilização sistêmica para o tratamento de desordens imunológicas bem limitada (DITTMER; THOMPSON, 2011; LARSSON et al., 1998). Com isso, uma forma alternativa de administração, como a nanoencapsulação, poderia ser um meio capaz de aumentar a bioatividade no sistema imune, através de uma entrega adequada, em um esquema terapêutico de doses bem menores que as usuais, a fim de tentar diminuir os efeitos secundários de hipervitaminose causados por uma dose excessiva (LUO et al., 2012).

A nanotecnologia farmacêutica é a área das ciências farmacêuticas envolvida no desenvolvimento, caracterização e aplicação de sistemas terapêuticos em escala nanométrica ou micrométrica. A maior parte dos estudos tem sido focada no desenvolvimento de formulações tecnológicas destinadas ao tratamento contra o câncer, doenças inflamatórias, cardiovasculares, neurológicas, entre outras (DIMER et al., 2013; PIMENTEL et al., 2007).

Entre as vantagens que os nanossistemas podem oferecer, destacam-se: a proteção do fármaco no sistema terapêutico contra possíveis instabilidades no organismo, promovendo manutenção de níveis plasmáticos em concentração constante; o aumento da eficácia terapêutica (bioatividade); a liberação progressiva e controlada do fármaco pelo condicionamento a estímulos do meio em que se encontra (variações de pH e/ou temperatura); a diminuição expressiva da toxicidade pela redução de picos plasmáticos de concentração máxima, devido à utilização de

dose reduzida; a diminuição da instabilidade e decomposição de fármacos sensíveis; a possibilidade de direcionamento a alvos específicos; a possibilidade de incorporação tanto de substâncias hidrofílicas como lipofílicas nos dispositivos; a diminuição da dose terapêutica e do número de administrações e o aumento da aceitação da terapia pelo paciente. Embora estas vantagens sejam significativas, alguns inconvenientes plausíveis não podem ser ignorados, como uma possível toxicidade, ausência de biocompatibilidade dos materiais utilizados e o elevado custo de obtenção dos nanossistemas, quando comparados com as formulações farmacêuticas convencionais (MORA-HUERTAS et al., 2010; PATEL; MARTINS-GONZALEZ, 2012; PIMENTEL et al., 2007).

Em uma situação ideal as nanopartículas, atuando como nanocarreadores, são capazes de transportar o fármaco para um local de destino específico (receptor, sítio alvo) para exercer a sua atividade terapêutica com o máximo de segurança (DIMER et al., 2013).

As nanopartículas poliméricas representam uma alternativa tecnológica, com excelente biocompatibilidade, não imunogênica, não tóxica e biodegradável. A mais importante classificação dessas nanopartículas consiste em separá-las em dois grandes grupos: os sistemas vesiculares (nanocápsulas) e os matricias (nanoesferas) (DIMER et al., 2013; MORA-HUERTAS et al., 2010; RAO, GECKELER, 2011).

Embora o encapsulamento de vitamina D venha sendo relatada desde 1998, muitas desvantagens ainda existem, o que inibe sua aplicação e industrialização. Por tratar-se de uma substância lipofílica, instável, sensível a vários fatores ambientais que podem afetar sua estrutura e levar a uma diminuição de seus efeitos fisiológicos, a aplicação de nanoencapsulação deve ser capaz de constituir um meio adequado para aumentar a estabilidade e preservar as propriedades durante o seu processamento e armazenamento (LUO et al., 2012). Além disso, pela utilização de doses reduzidas, poderá diminuir o aparecimento de síndrome da hipervitaminose e os efeitos secundários causados por uma excessiva ingestão (PATEL; MARTINS-GONZALEZ, 2012).

Recentemente, um estudo realizado por Wang et al. (2014) destacou o potencial das nanopartículas para entregar drogas de uma forma altamente precisa para as células imunes ativadas, podendo, assim, essa tecnologia, ser aplicada para o tratamento de uma ampla gama de doenças inflamatórias.

Sendo assim, torna-se relevante e de interesse científico e clínico a avaliação da atividade das ectonucleotidases participantes da degradação de nucleotídeos e nucleosídeo da adenina em linfócitos de ratos com artrite induzida por adjuvante e tratadas com vitamina $D_{3,}$ na sua forma livre ou nanoencapsulada. Além disso, ainda não existem estudos demostrando a relação da vitamina D_3 nanoestruturada no tratamento da AR, bem como sua interação com o sistema purinérgico. Desta forma, espera-se contribuir para a busca de uma nova terapia complementar que possa beneficiar pacientes com AR.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar e comparar o efeito da suplementação com vitamina D_3 livre ou nanoencapsulada em padrões hematológicos, bioquímicos, histológicos e sobre a atividade de ectoenzimas em linfócitos de modelo de artrite por adjuvante.

2.2 Objetivos específicos

Em ratos com artrite induzida por adjuvante e tratados com vitamina D_3 livre ou nanoencapsulada:

- Avaliar o escore de artrite, edema de pata e hiperalgesia termal;
- Avaliar o perfil histológico nas patas;
- Avaliar padrões hematológicos antes e após o tratamento;
- Avaliar a atividade das enzimas marcadoras de dano hepático, alanina aminotransferase (ALT), aspartato aminotransferase (AST), antes e após tratamento;
- Avaliar a atividade das enzimas marcadoras de danos renais, creatinina e ureia;
- Avaliar os níveis de cálcio, albumina e vitamina D circulantes, antes e após o tratamento;
- Avaliar a atividade das enzimas E-NTPDase e E-ADA em linfócitos;
- Comparar efeitos entre a vitamina D_3 livre e nanoencapsulada em parâmetros bioquímicos, hematológicos e histológicos, bem como nas atividades enzimáticas citadas.

3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito. Os itens Materiais e métodos, Resultados e Discussão e Referências bibliográficas encontram-se compondo o próprio manuscrito e representam a íntegra deste estudo.

O manuscrito será submetido à revista **Clinical Science**.

VITAMIN D_3 FREE AND LIPID-CORE NANOCAPSULES: EFFECT IN THE PURINERGIC SYGNALING ON ANIMAL MODEL WITH INDUCED ARTHRITIS

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ABSTRACT

The effect of vitamin D_3 free (VD_3) and nanoencapsulated (LNC-D_3) was analyzed in animals with arthritis induced by complete Freud's adjuvant (CFA). It was evaluated in the score for arthritis, thermal hyperalgesia and paw edema as well as histological analyzes and the activity of the E-NTPDase and E-ADA enzymes in lymphocytes of rats. Hematological and biochemical parameters, including serum concentrations of vitamin D were determined. The dosages administered were to 120UI/day of the VD_3 and 15.84UI/day of the LNC-D_3. The groups were treated for 15 days with vitamin D_3 initiating the treatment 15 days after the induction of AR. The results of this study demonstrated that VD_3 was able to reduce arthritis score, thermal hyperalgesia and paw edema in rat with AR. However, treatment with LNC-D_3 only reduced thermal hyperalgesia and paw edema. The histological analyzes showed that both formulations were able to reduce inflammatory changes induced by CFA. There was a significant increase in the levels of 25(OH)D in the group treated with VD_3. The activity of the E-NTPDase in lymphocytes from rats that developed AR was higher in comparison with the control group, whereas the activity of E-ADA was lower. This effect was reversed after 15 days of treatment with VD_3 and LNC-D_3. Data from this study indicate that vitamin D_3, either in free or nanoencapsulated forms, seems to contribute the inflammatory process induced by CFA, possibly altering the activities of ectonucleotidases, can be used as a complementary therapeutic agent for the treatment of rheumatoid arthritis.

INTRODUCTION

The role of vitamin D_3 in several vital cellular processes such as cell differentiation and proliferation, hormone secretion and some chronic no communicable diseases, drew attention to its part in the immune system [1-4]. The interaction of vitamin D_3 with immune system is by their participation in the regulation and differentiation of cells such as lymphocytes, macrophages and natural killer (NK) cells, interference with cytokine production, which leads to antiproliferative, immunosuppressive and immunomodulatory effects [1,3]. Clinical and experimental studies have provided evidence that in cases of low concentrations of vitamin D, immune system favors the development of auto-reactive T cells directed against the body's own tissues and the synthesis of pro-inflammatory interleukins (IL-12, INF-γ), predisposing them to an increased risk of developing autoimmune diseases such as rheumatoid arthritis (RA) [1, 5].

Despite its many benefits, administration of large oral doses of vitamin D are potentially toxic and may lead to the development of hypercalcemia and hypercalciuria, which makes the systemic use for the treatment of immunological disorders quite limited [6,7]. Thus, an alternative manner of administration, such as nanoencapsulation, may be a means capable of increasing the bioactivity immune system through an appropriate delivery scheme in a therapeutic much smaller than the usual doses in order to try to reduce the effects side of hypervitaminosis caused by an excessive dose [8].

Inflammation is a key player in the pathophisology of arthritis and the purinergic signaling system. Extracellular adenine nucleotides are signaling molecules that play an important role in the immune response regulation [9]. Among the mediators that modulate the actions of lymphocytes highlight the adenine nucleotides and nucleoside [10], during the inflammatory process that represent an important class of extracellular molecules which interact with the purine cell surface receptors, signaling pathways of great importance that intercede various biological effects [11].

Adenosine nucleotides (ATP, ADP and AMP) and their derived adenosine, are the important molecules in the mediation of many biological and pathological events [11] and are dynamically controlled during inflammation by a group of membrane-bound enzymes expressed in immune cells [12], such as ecto-nucleoside

triphosphate diphosphohydrolase (E-NTPDase; CD39; EC 3.6.1.5) and ecto-adenosine deaminase (E-ADA; EC 3.5.4.4) enzymes.

Knowing that rheumatic arthritis (RA) is characterized by autoimmune response and pro-inflammatory disease and the involvement of purinergic system in the modulation of these events, the purpose of this study was to investigate the activity of E-NTPDase and E-ADA in lymphocytes from CFA-induced arthritis before and after treatment with vitamin D_3 free (VD_3) and vitamin D_3 lipid-core nanocapsules (LNC-D_3) in order to achieve better comprehension of their immune status. It was also investigated the possible relationship of the presentation of the drug in both formulations with nucleoside and nucleotide adenine hydrolysis.

METHODS

Chemicals

The substrates adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate sodium salt (ADP), adenosine, bovine serum albumin, Trizma base and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ficoll-Hypaque (Lymphoprep) was obtained from Nycomed Pharma (Oslo, Norway). K_2HPO_4 was purchased from Reagen and Tetrabutylammonium chloride from Merck (Darmstadt, Germany). Vitamin D_3 used during the experiment was commercially obtained from pharmacies in Santa Maria, RS, Brazil. The formulation used was Addera D_3 (cholecalciferol – vitamin D_3 - 3300 IU/ mL) drops, oral solution, 10mL, FARMASA®. All the other chemicals used in this experiment were of analytical grade and of the highest purity.

Animals

Female Wistar adult rats (200-300g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. Animals were kept on a 12h light/12h dark cycle, at a temperature of 22±2^{o}C, with free access to water and standard chow *ad libitum*. The animals were used according to the guidelines of the Committee on Brazilian Society of Animal Science Lab [13] in accordance with international guidelines and were approved by the Committee on the

Use and Care of Laboratory Animals from the UFSM (protocol under number: 019/2013).

Experimental procedure

The treatment of animals with both vitamins D_3, free and nanocapsulated, began 15 days after induction of arthritis by CFA (described later). The animals were randomly divided into ten groups (n=5 rats in each group): I: CN (Control); II: AR (adjuvant-induced arthritis); III: CV (control vehicle/ corn oil); IV: AR-V (adjuvant-induced arthritis/ corn oil as vehicle); V: VD_3 (vitamin D_3 free); VI: AR+VD_3 (adjuvant-induced arthritis/ vitamin D_3 free); VII: CF (control white formulation); VIII: AR-F (adjuvant-induced arthritis/ white formulation); IX: LNC-D_3 (vitamin D_3 lipid-core nanocapsules formulation) and X: AR+LNC-D_3 (adjuvant-induced arthritis/ vitamin D_3 lipid-core nanocapsules formulation).

To test the confirmation of the induction of arthritis by development of the inflammatory process was realized in the both CN and AR groups after 15 days of induction with CFA, as described later. The groups VD_3 and AR+VD_3 received the formulation of 12 µg/kg/day [14], equivalent to 120 UI/day of the VD_3, dissolved in 0,3 mL of corn oil, administered by oral gavage during 15 days. The LNC-D_3 and AR+LNC-D_3 groups received a previously prepared suspension (52.8 IU/mL VD_3, which is equivalent to a dose of the 15.84 UI/day of the vitamin D_3) and were administrated by similar via and time of the treatment. The both corn oil (CV and AR-V groups) and white formulation (CF and AR-F groups) vehicle used in the preparation of free and nanocapsules VD_3 were administrated in equivalent doses as described early.

After the treatment period, the animals were anesthetized with inhalatory isoflurane and submitted to euthanasia. The blood was collected by cardiac puncture to separate the lymphocytes and serum. The ankle joint tissues were separated for histopathological analysis.

Adjuvant-induced chronic inflammation in rats

To induce arthritis complete Freud's adjuvant (CFA-0.6% suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) was used. Animals were slightly

anesthetized by inhalatory isoflurane and 50 µL of CFA or saline (used as control) was injected in to the right hind paw [18]. To confirm the inflammatory process development, fifteen days after the induction of arthritis were made score measurements, paw immersion latency (thermal hyperalgesia) and paw thickness. For investigate the effect of VD_3 (120 UI/day) and LNC-D_3 (15.84 UI/day) in chronic inflammation, new measurements these same tests were made after 15 days treatment.

Briefly, the measurements were made and the basal values of arthritis score, paw immersion latency (thermal hyperalgesia) and paw thickness (paw edema) (described after) were evaluated to confirm the development of inflammatory process.

Chronic inflammation evaluation

To confirm the development of chronic inflammation and the anti-inflammatory effect of VD_3 and LNC-D_3, we evaluate the development of the following alterations:

Arthritis score

To evaluate the progression of the arthritic response elicited by intraplantar CFA injection, animals were observed daily, before administration of both formulations vitamin D_3. The following signs of inflammation were observed and classified according to the scale: edema formation (0 – normal; 1 – slight swelling at the injection site; 2 – swelling at the injection site and toes or ankle; 3 – swelling at the injection site, toes and ankle), redness (0 – normal; 1 – slightly red/purple; 2 – red/purple) and claw position (0 – normal; 1 – slightly curved; 2 – almost closed). Individual scores were added to give the total arthritis score [19, 20].

Thermal hyperalgesia

To evaluate the hypersensitivity to heat stimulation, we used the paw immersion test [21]. Briefly, animals were held and the right hind paw was immersed in a water bath at 48°C. The time elapsed between onset of the stimulus and manifestation of the paw withdrawal response was measured automatically and was

taken as an index of the thermal nociceptive threshold. Significant decreases of paw withdrawal latency were interpreted as indicative of heat hyperalgesia.

Paw edema

To observe the development of edema, animals were held while right hind paw thickness was measured using a digital caliper [22]. Fifteen days later after the induction of inflammation and before each dose of vitamin D_3, new measurements were taken and compared to basal values. An increase in the difference between the measures was considered as representing edema values are expressed as the change relative to basal values.

Preparation of LNC-D_3

Vitamin D_3 lipid-core nanocapsules (LNC-D_3) was obtained by interfacial deposition of preformed polymer technique [15, 16]. Firstly, an organic solution were prepared by dissolution of poly(ε-caprolactone) (0.1 g), sorbitan monoestearate (0.038 g) and a commercial vitamin D_3 formulation (Addera D_3) (160 µL), which consists in a vitamin D_3 dissolved in an caprylic and capric acids mixture as an oily solution (3300 UI/mL) in acetone (27 mL) at 40°C. Following the organic solution was injected under magnetic stirring into an aqueous solution consisting of polysorbate 80 (0.077 g) in water (54 mL). Lastly the acetone and a portion of water were evaporated by evaporation under reduced pressure at a final volume of 10 mL. The LNC-D_3 formulations were prepared in triplicate and kept protected from light at room temperature. The theoretical concentration of LNC-D_3 formulation was 52.8 UI/mL. To perform the density gradient study, a vitamin D_3 nanoemulsion (NE-D_3) was prepared at the same conditions describe above, however the presence of poly(ε-caprolactone) into the organic solution was omitted.

Characterization of LNC-D_3

Particle size distribution and polydispersity index

Particle size distribution and polydispersity index (PDI) were determined by dynamic light scattering (Zetasizer Nano ZS®, Malvern Instruments, Malvern, UK) at 25°C after previous dilution of LNC-D_3 formulation with ultrapure water (500 x). To ensure the nanotechnological properties of LNC-D_3 formulations, particle size distribution of nanocapsules were also evaluated by laser diffraction (Mastersizer® 2000, Malvern Instruments, Malvern, UK), in order to check if just submicrometric particles composed the LNC-D_3 formulation. Additionally particle size distribution analyses were performed until the fifteenth day of storage aiming to ensure maintenance of the nanotechnological characteristics of LNC-D_3 formulation during this period.

Zeta potential

Zeta potential was determinate by electrophoretic mobility (Zetasizer Nano ZS®, Malvern Instruments, UK). Samples of LNC-D_3 formulations were diluted into a 10 mM NaCl aqueous solution (500 x) before analysis. Measurements were taken at 25°C.

Density gradient study

In order to evidence the lipid-core nanocapsules formation and to exclude the coexistence of the nanoemulsion droplets into the proposed lipid-core nanocapsules formulation, a density gradient study was carried out [16]. The lipid-core nanocapsules or nanoemulsion droplets density was determined by differential centrifugation in a colloidal silica gradient (Percoll® 54%, v/v, in NaCl 0.15 M). Percoll® (19.6 mL) was added to 0.4 mL of the formulation (lipid-core nanocapsules or nanoemulsion) without previous treatment. The samples were submitted to ultracentrifugation at 20°C and 45 0000 x g for 60 minutes. The bands height was measured from the dispersion meniscus to the central point of the band. The particles density were estimated from the polynomial curve ($y = 0.00005x^2 + 0.0004x + 0.9925$; $R^2 = 0.984$) obtained by plotting the distance against density of marker beads [16, 17].

Histopathological observation

Samples of ankle joints right were collected and fixed in 10% formalin solution and then dehydrated and embedded in paraffin, followed by sectioning and histological staining with hematoxylin and eosin (H&E). The slides were observed in optical microscope (400x) to evaluate a possible damage.

Hematological parameters

A complete hemogram was performed in the blood samples collected in the tubes containing 7.2 mg dipotassium EDTA as an anticoagulant and the quantitative determination the haematological parameters was performed by automated haematology analyzer (SYSMEX XT-1800i, Roche Diagnostic, USA).

Biochemical parameters and vitamin D levels

Biochemical analysis of serum samples was performed using a semi-automatic chemistry analyzer (Bioplus, BIO-2000), using commercial kits (Labtest, Minas Gerais, Brazil), according to the recommendations of the manufacturer. Biochemical parameters measured were urea, creatinine, alanine aminotransferase, aspartate aminotransferase, albumin and calcium.

Levels of vitamin D in serum were measured using the test LIASON® 25 OH Vitamin D TOTAL (DiaSorin) assay for the quantitative determination of 25-hydroxyvitamin D and other hydroxylated metabolites in serum.

Isolation of lymphocytes from blood

Lymphocyte-rich mononuclear cells were isolated from blood collected with EDTA as anticoagulant and separated on Ficoll–Histopaque density gradients [23] as previously described. The percentage of lymphocytes was higher than 93% as previously described [24]. Right after lymphocytes separation the cell viability was determined by measuring the activity of lactate dehydrogenase (LDH) present in the sample, using the kinetic method of the Labquest apparatus (Diagnostics Gold

Analyzer). The procedure was repeated before and after the incubation period and samples with more than 10% of disrupted cells were excluded.

Protein determination

Protein was measured by Coomassie Blue method using serum albumin as standard [25].

E-NTPDase activity determination

After lymphocytes isolation, E-NTPDase activity was determined as previously described [26]. This method is based on the measurement of inorganic phosphate (Pi) released by colorimetric assay. The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer at pH 8.0, with a final volume of 200 µL. Twenty microliters of intact lymphocytes suspended in saline solution were added to the reaction medium (2-4 µg protein) and pre-incubated for 10 min a 37 °C, and incubation proceeded for 70 min. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 µL 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was assayed by a method previously described [27] using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run triplicate and specific activity reported as nmol of Pi released/mim/mg of protein.

E-ADA activity determination

E-ADA activity was measured in lymphocytes by the method previously described [28] which is based on the direct measurement of the formation of ammonia produced, when E-ADA acts in excess of adenosine. Briefly, 25 µL of lymphocytes reacted with 21 mM of the adenosine pH 6.5 and was incubated at 37 °C for 60 min. The reaction was stopped by adding 106.2 mM phenol and 167.8 nM sodium nitroprissiate and hypochlorite solution. Ammonium sulfate (75 µM) was used as ammonium standard. All the experiments were performed in triplicate and the

values were expressed in units per milligram of protein (U/L) for E-ADA activity. One unit (1U) of E-ADA is defined as the amount of enzymes required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Statistical analysis

Data from enzyme assays, hematological and biochemical parameters as well as levels of vitamin D, after 15 days treatment, were submitted to analysis of variance one-way (ANOVA) followed by the Tukey's test *post hoc*. The results of evidences of arthritis induction were evaluated by analysis of variance two-way (ANOVA) followed by the Bonferroni's test. $P<0.05$ was considered to represent a significant difference among the all groups. All data were expressed as mean ± standard error of the mean (SEM).

RESULTS

Evidences of arthritis induction and anti-inflammatory effect of VD_3 and LNC-D_3

To first investigate the possible anti-inflammatory effect of both formulations of vitamins D_3 in chronic inflammation, were evaluated its effects on the chronic inflammation induced by intraplantar CFA. Were observed that 15 days after CFA injection there was an increase in the arthritis score (Figure 1A), a decrease in paw thermal latency (indicating thermal hyperalgesia development) (Figure 1B) and paw thickness increase (paw edema) (Figure 1C), in all the groups that received CFA, which was consistent until 30 days after CFA injection, in groups untreated.

Once insured the chronic inflammation, were started the treatment with VD_3 (120 UI/day) and LNC-D_3 (15.84 UI/day) for 15 days. The treatment with VD_3 was able to reduce arthritis score (Figure 1A), thermal hyperalgesia (Figure 1B) and paw edema (Figure 1C) in 22.2 ± 6.6, 58.5 ± 6.6 and 42.0 ± 6.1%, respectively. At the same time, the treatment with LNC-D_3 reduced only the thermal hyperalgesia (Figure 1B) and paw edema (Figure 1C) in 32.5 ± 10.6 and 42.9 ± 10.5%, respectively. The treatment with vehicle alone or vitamin D_3 (free and nanoestruturated), without injection of CFA, did not induce behavioral alterations.

Preparation and characterization of LNC-D_3

Vitamin D_3 lipid-core nanocapsules (LNC-D_3) formulations presented white bluish opalescent aspect after the preparation, a common feature of this kind of dispersed system. Regarding the physicochemical characteristics of LNC-D_3 the nanocapsules showed particle size of 189 ± 2 nm, polydispersity index (PDI) below 0.09 and negative zeta potential (-13 ± 1 mV). The particle size and zeta potential values are in agreement with other lipid-core nanocapsules formulations described previously [15-16, 29]. Analysis by laser diffraction showed the presence of particles only at the nanometric range, ensuring the nanotechnological properties of LNC-D_3 formulations (data not showed). Furthermore the LNC-D_3 particle size distribution profile remained unchanged until the fifteenth day of storage showing that nanotechnological characteristics of LNC-D_3 formulation were preserved during this period.

Concerning the density gradient studies, a single band was observed for vitamin D_3 lipid-core nanocapsules and for vitamin D_3 nanoemulsion formulations samples. The particles density estimated from the polynomial curve obtained by plotting the distance against density of marker beads were approximately 1.030 g/mL and 1.003 g/mL for lipid-core nanocapsules and nanoemulsion formulations respectively.

Hystological analysis

After 15 days of arthritis induction, ankle joint section of the control group (CN) showed organized collagen, normal connective tissue and no inflammatory infiltrate (Figure 2A). The other control groups (CV: vehicle/corn oil and CF: white formulation) also showed normal structures (data not showed). Adjuvant-induced arthritis groups (AR) showed periarticular connective tissue infiltrated by inflammatory lymphocytic cells, distended interjoint space, dilated vessels and destroyed bony structures by infiltration (0.5 mm) (Figure 2B). The AR groups without treatment (AR-V: vehicle/corn oil and AR-F: white formulation) also showed changes as observed in the AR group 15 days after induction arthritis (data not showed).

VD_3 groups (Figure 2C) showed that the collagen fibers in the interjoint space were separated by edema but without inflammatory infiltrate. Besides these aspects,

LNC-D_3 group (Figure 2D) showed breakdown of collagenous fibers. In the AR+VD_3 group treated for 15 days (Figure 2E) was observed the presence of an inflammatory infiltrate in the connective collagenic tissue with reduced edema when compared with the control groups (0.4 mm). Obliterating granulomatous reaction, an increase in interbone spaces and in the vessel associate with the reversion of the inflammatory process was observed in AR+LNC-D_3 group treated also for 15 days (Figure 2F).

Hematological parameters

The hematological parameters as RBC, Hb, Ht, MCV, MCHC, TPP, WBC, neutrophils, lymphocytes, monocytes and eosinophils no showed difference among the groups. However, it was observed a difference in platelet counts between (CN and AR) groups 15 days after induction and treated for 15 days with both forms vitamin D_3 forms, however, it is compatible with reference values previously described in the literature (638 – 1177 (10^3/μL) [30] (Table 1).

Biochemical parameters and levels of vitamin D

The biochemical parameters tested in the serum (albumin, ALT, calcium, creatinine and urea) did not differ among the different groups. However, AST levels showed a significant increase (P<0.05) when compared with others groups in the AR group when compared with others groups. It was observed a significant increase (P<0.05) in the serum levels of vitamin D in both VD_3 and AR+VD_3 groups, while LNC-D_3 and AR+LNC-D_3 groups did not represent alteration when compared with others groups (Table 2).

Cellular integrity

LDH activity measurement showed that approximately 5% of the lymphocytes of both groups was disrupted, indicating that the preparation was predominantly intact after the isolation procedure (data not showed).

E-NTPDase and E-ADA activity in lymphocytes 15 days after arthritis induction and 15 days after of treatment with VD_3 and LNC-D_3

The CV, AR-V, CF and AR-F groups showed very similar results to the groups CN and AR after 15 days induction, which showed no significant difference among them (data were not showed).

After confirmed the experimental model analysis of CN and AR groups 15 days after induction (described above), were evaluated the hydrolysis of adenine nucleotides (ATP and ADP) by the E-NTPDase activity and deamination of adenosine by E-ADA activity. The results obtained for activities these ectoenzymes in lymphocytes of adjuvant-induced arthritis rats treated with VD_3 dose 120UI/day and with LNC-D_3 dose 15.84 UI/day, both over 15 days, are shown in Figure 3.

The results obtained for ATP hydrolysis in lymphocytes (Figure 3A) showed a significant increase in the AR group (117.2 nmol Pi/ min/mg protein; SEM=15.17, n=5, P<0.05) compared to the CN group (70.94 nmol of Pi/ min/mg protein, SEM=4.94, n=5, P<0.05), VD_3 group (72.45 nmol Pi/min/mg protein, SEM=2.86, n=5, P<0.05), LNC-D_3 group (55.34 nmol Pi/min/mg protein; SEM=4.69, n=5, P<0.05) and AR+VD_3 group (46.03 nmol Pi/min/mg protein; SEM=4.89, n=5, P<0.05). The CN, VD_3, LNC-D_3 and AR+VD_3 groups represented a similar ATP hydrolysis. However, LNC-AR+D_3 group showed a decreased ATP hydrolysis when compared with all groups (30.50 nmol Pi/min/mg protein; SEM=4.64, n=5, P<0.05).

The results showed enhanced ADP hydrolysis (Figure 3B) in the AR group (105.0 nmol of Pi/min/mg of protein; SEM=9.88, n=5; P<0.05) when compared to CN group (47.54 nmol of Pi/min/mg of protein; SEM=9.17, n=5; P<0.05), VD_3 group (70.25 nmol of Pi/min/mg of protein; SEM=6.13, n=5; P<0.05), LNC-D_3 (45.87 nmol of Pi/min/mg of protein; SEM=3.09, n=5; P<0.05), AR+VD_3 (44.48 nmol of Pi/min/mg of protein; SEM=2.81, n=5; P<0.05); and AR+LNC-D_3 (46.88 nmol of Pi/min/mg of protein; SEM=3.51, n=5; P<0.05). The CN, VD_3, LNC-D_3, AR+VD_3 and AR+LNC-D_3 groups represented a similar ADP hydrolysis.

In addition, as can be seen in Figure 3C, E-ADA activity was decreased in AR group (3.43 U/L of ADA/mg of protein; SEM=0.66; n=5; P<0.05) when compared to CN group (8.48 U/L of ADA/mg of protein; SEM=1.43; n=5; P <0.05), VD_3 group (10.03 U/L of ADA/mg of protein; SEM=1.03, n=5; P<0.05), LNC-D_3 group (7.94 U/L of ADA/mg of protein; SEM=0.81, n=5; P<0.05), AR+VD_3 (9.59 U/L of ADA/mg of

protein; SEM=1.04, n=5; *P*<0.05) and AR+LNC-D_3 (8.10 U/L of ADA/mg of protein; SEM=0.55, n=5; *P*<0.05). The CN, VD_3, LNC-D_3, AR+VD_3 and AR+LNC-D_3 groups represented a similar adenosine deamination.

DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory and systemic autoimmune disease that mainly targets the synovial membrane, cartilage and bone [31-32]. It is well know that pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), the interleukin-1β (IL-1β), interleukin-6 (IL-6) and auto-reactive T cells are believe to contribute to joint destruction and other tissue dysfunction in RA [33]. Recent advances fibroblast proliferation and infiltration of inflammatory cells including T cells, B cells and macrophages lead to pannus development, which invades joint tissue [34].

In this study it was assessed the ability of vitamin D_3 to reverse process inflammatory in the rats with arthritis induced by CFA. Moreover, it was investigated the effects of vitamin D_3 and LNC-D_3 in the E-NTPDase and E-ADA activities in lymphocytes in this experimental model, in order to verify possible changes in the inflammatory reaction developed besides the possible efficacy of the formulations tested.

The results of this study showed that either VD_3 or LNC-D_3 improved the signal and the symptoms of the arthritis induced by CFA. The VD_3 was able to reduce arthritis score, thermal hyperalgesia and paw edema. However, the treatment with LNC-D_3 reduced only the thermal hyperalgesia and paw edema. Studies have shown that the dietary supplementation with vitamin D_3 or its metabolite is able to prevent the onset of arthritis, and when administered at an early stage, it can to prevent the progression, to decrease the activity and to halt the development of induced arthritis [35]. Additionally, it is know that the adjuvant-induced arthritis model has been showed to size areas of necrosis with the accumulation of inflammatory exudate on cartilage surfaces [36]. Then, the histological parameters realized could demonstrate that VD_3 treatment was able to mild reduce the edema, while LCN-D_3 treatment was able to reverse inflammatory processes induced by arthritis. However, there was the appearance of a granulomatous reaction in the AR+LNC-D_3 group. It is suggests that

this reaction probably would have been originated by the nanocapsules, either by is the formulation or by time of treatment.

During the evaluation of biochemical parameters, it can only be observed an increase in the AST activity in the serum of AR group. It is suggested that this is due to the injuries that happen to inflammatory process triggered by this pathology, since shows small increases serum AST occur in gangrene, muscle crush, hemolytic disease, progressive muscular dystrophy, dermatomyositis, cholangitis (inflammation of the bile ducts) and infection by parasites [37]. However, after the treatment, both forms of vitamin D_3 were able to reverse the serum AST levels in the groups with arthritis.

In relation to serum vitamin D levels, the results demonstrated that the groups treated with vitamin D_3 free (VD_3 and AR+VD_3) exhibit a high level of 25(OH)D circulating than the groups treated with LNC-D_3. This can be explained, since nanoparticles have the capability to carry and deliver therapeutics to target cells through the coating of ligands and antibodies on their surface. The potential of nanoparticles to deliver drugs in a highly precise manner to activated immune cells. This technology could potentially be applied to the treatment of a broad range of inflammatory diseases [38]. As asserted by different authors, nanoparticulated systems show promise as active vectors due to their capacity to release drugs, their subcellular size allows relatively higher intracellular uptake than other particulate systems, they can improve the stability of active substances and can be biocompatible with tissue and cells when synthesized from materials that are either biocompatible or biodegradable. Other advantages of nanoencapsulated systems as active substance carriers include high drug encapsulation efficiency due to optimized drug solubility in the core, low polymer content compared to other nanoparticulated systems such [8, 39].

These therapeutic effects observed, can be associated with the ability of immunomodulatory responses of the vitamin D by the production and differentiation of cells, such as dendritic cells, T and pro-inflammatory pathogens; inhibition of the development and function of T helper 1 (Th1) cells and induction of T helper 2 (Th2) cells [1-3, 6, 35, 40].

The ATP released into the extracellular environment at high concentrations activates the purinergic receptors P2X (ligand-gated ion channel) pro-inflammatory and contributes to tissue injury and inflammation [41]. Various studies have shown

that T lymphocytes are capable to release ATP in response to various extracellular stimuli as infections. Thus, purinergic signalling with ATP may be involved in migration and activation of T lymphocytes [42].

The results of this study showed an increase in the E-NTPDase activity (ATP and ADP as substrate) in lymphocytes after 15 days of adjuvant-induced arthritis. Nevertheless, after 15 days of treatment with both formulations of vitamin D_3 (free and nanoencapsulated) was observed the return close to baseline levels. The possible association between E-NTPDase activity and immune diseases has been evaluated considering that this enzymatic activity could be used as an activation marker of lymphocytes during the immune response [26, 43].

This increase of the E-NTPDase activity suggests that there is an increase in the hydrolysis of extracellular ATP and ADP with the purpose of reducing their levels during development of RA, as a dynamic response of probably lymphocytes in an attempt to the maintenance their appropriate levels [43]. Moreover, with the increase of E-NTPDase activity suggests a decrease of levels of ATP and ADP, generating a large amount of adenosine, which possesses anti-inflammatory and analgesic properties [44].

The results showed that both formulations of vitamin D_3 were able to reverse the E-NTPDase activity after treatment for arthritis. It is believed that the inflammatory process during arthritis may have been decreased due to predominance of a Th2 immune response with vitamin D_3 treatment, in relation to Th1 response developed by arthritis. The discovery of the vitamin D receptor (VDR) in the cells of the immune system, as mononuclear cells, antigen-presenting cells, activated T-B lymphocytes, and the fact that activated dendritic cells produce vitamin D hormone suggest that vitamin D could have immunoregulatory properties [1, 4, 40]. The effects of vitamin D_3 in immune response are characterized by inhibition of lymphocytes T proliferation, particularly of the Th1 response [1-3]. In addition, the vitamin D_3 leads to decreased secretion of IL-2 and INF-γ by CD4 T cells and promotes IL-5 and IL-10 production, which further shift the T cells response towards Th2 dominance response [4, 35, 40].

In addition, it was reported in the literature [43] a decrease in the E-ADA activity in patients with RA. In according to other study previously published by our research group, the level of adenosine in serum of RA patients was decrease [45]. Recently, it has been reported an relationship between disease activity and serum E-

ADA levels which in turn may predict disease activity actual as well as the response of treatment in RA [32]. It is important to note that adenosine, ADA substrate, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting the proliferation of T cells, the secretion of cytokines and the migration of leukocytes across endothelial barriers [46].

Corroborating with the discoveries in the literature, this study demonstrated that the AR group showed decreased E-ADA activity after 15 days of CFA-induced arthritis. Meantime, the treatment with both formulations of vitamin D_3 (free and nanoencapsulated) was able to reverse the activity of this ecto-enzyme, therefore, vitamin D_3 was able to reverse the E-ADA activity after treatment for arthritis.

At low levels, ATP can associate with P2Y receptors in surface the lymphocytes thus decreasing the pro-inflammatory cytokines. Furthermore, enzymatic cascade was able to form extracellular adenosine and this possible adenosine exerts its immunosuppressive effects, once E-ADA activity is elevated in humans and contributes to the pathogenesis of RA. The E-ADA activity represent the real status of RA since adenosine low levels are due to increase of its activity being related to a decreased IL-10 and TGF-β levels, both necessary to sustain the expansion of Treg cells, thus contributing to the pathogenesis of RA [31]. It is known that low ATP and high adenosine levels may counterbalance inflammatory stimuli by inhibiting the production of pro-inflammatory mediators, stimuli by inhibiting the production of Th1 response, such as TNF-α, INF-γ, IL-1, IL-12, impairing migration and skewing the immune response to a Th2 response [12, 33].

Many studies reported that the E-NTPDase and E-ADA have important implications roles in immune response once alteration in their activities have been observed in some in some autoimmune diseases and immunodeficiency such as cancer lung [47], HIV [26], lupus [48] and multiple sclerosis [49]. It is well established that these enzymes act in an enzymatic cascade and the data of this study suggest that increased E-NTPDase and decreased E-ADA activities in CFA-induced arthritis rats would be a way to compensate the co-stimulatory signalling in the immune synapse, which in increased proliferation of type Th1 cytokines [50] during the arthritis.

The purinergic signaling system is a key component of the immunoregulatory environment [10]. Adenosine and ATP have been implicated in the regulation of inflammatory response. Our data showed that there is an increase of the E-NTPDase

activity and a decrease of the E-ADA activity, resulting in increased level of adenosine in the extracellular milieu, suggest plays an important role in the inflammatory response. In additional, our data demonstrate that after 15 days of treatment with both formulations of vitamin D_3 (free and nanoencapsulated) was observed the return close to baseline levels the ATP, ADP and adenonise, which showing the formulations were able to modulation in the E-NTPDase and E-ADA activities. Moreover, both vitamin D_3 forms seems to contribute softening the inflammatory process induced by CFA.

CLINICAL PERSPECTIVES

Data from this study indicate that vitamin D_3, either free or nanoencapsulated forms, seems to contribute the inflammatory process induced by CFA, possibly altering the activities of ectonucleotidases. However, it is known that vitamin D has a beneficial effect on the modulation of immune system components responsible for the inflammatory process. Moreover, has not data in the literature showing the relationship between the effects of treatment with both vitamin D_3 forms (free and nanoencapsulated) of arthritis and the purinergic system, the results found in this study become great relevance. The establishment of responses to treatment front with both forms vitamin D_3 (free and nanoencapsulated) as well as knowledge about the biology of the immune system, may assist in understanding of the immunopathology and possible improvement of signs and symptoms that hinder the quality of life of patients with rheumatoid arthritis.

AUTHOR CONTRIBUTION

Karine Lanes da Silveira – animal care, arthritis induction, collection of biological material, enzymatic activity, writing of the manuscript.

Maria Luiza Prates Throstenberg – animal care, arthritis induction, administration the vitamin D_3 by gavagem, collection of biological material, enzymatic activity.

Livia Gelain Castilhos – animal care, arthritis induction, collection of biological material, enzymatic activity.

Fernanda Licker Cabral – animal care, arthritis induction, collection of biological material, enzymatic activity.

Leonardo Lanes da Silveira – animal care, arthritis induction, administration the vitamin D_3 by gavagem, collection of biological material.

João Felipe Peres Rezer – animal care, arthritis induction, collection of biological material, enzymatic activity.

Viviane do Carmo Gonçalves Souza – analysis of results, writing of the manuscrit

Tatiana Montagner Dalcin Bertoldo – collection of biological material, analysis hematological.

Cláudia de Mello Bertoncheli – animal care, collection of biological material, enzymatic activity, preparation of material for histological analysis, analysis of results.

Diego Fontoura de Andrade – Preparation and characterization of vitamin D_3 lipid-core nanocapsules (LNC-D_3) formulations.

Ruy Carlos Ruver Beck – Preparation and characterization of vitamin D_3 lipid-core nanocapsules (LNC-D_3) formulations.

Mateus Fortes Rossato – arthritis induction, analysis of results.

Heloísa Helfot – analysis biochemical and hematological.

Chintia Melazzo Mazzanti – analysis biochemical and hematological.

Renata Pereira – analysis vitamin D levels.

Nara Maria Martins – pathology analysis.

Daniela Bitencourt Rosa Leal – Orienting, analysis of results, writing of the manuscript.

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FIGURES AND TABLES

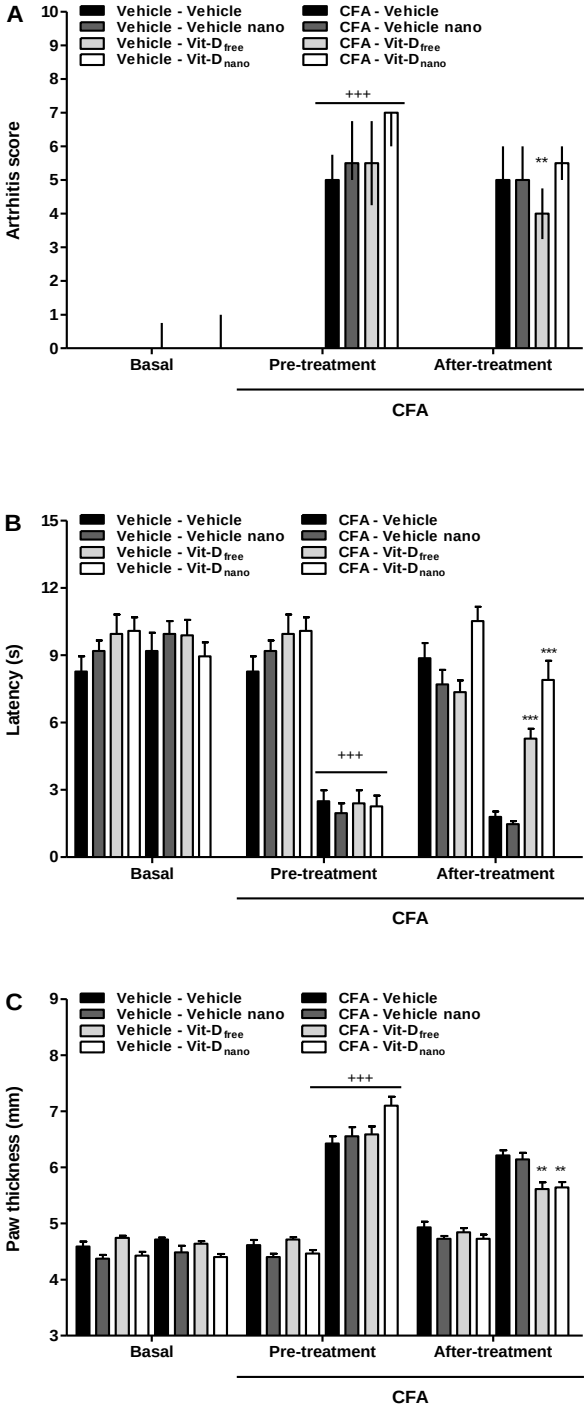

Figure 1 **–** Effect of both Vitamin D forms (free and nanoencapsulated) on some inflammatory changes induced by intraplantar CFA. Evaluation of the VD_3 (120 UI/day) and LNC-D_3 (15.84 UI/day) on the arthritis score increase (1A), thermal hyperalgesia (1B) and edema formation (1C) induced by intraplantar CFA. $^{+++}P<0.001$ in comparison to basal values, $^{**}P<0.005$ and $^{***}P<0.001$ in comparison to pre-treatment values according to two analysis of variance (ANOVA) followed by Bonferroni post-test (n = 4 – 5).

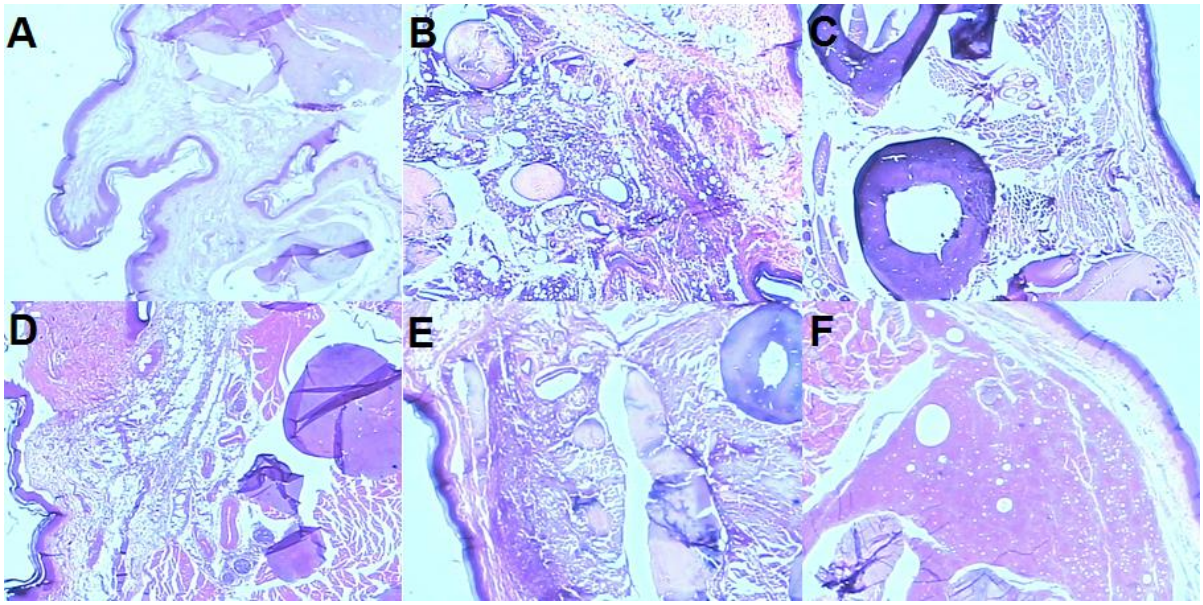

Figure 2 – Histological image of joint tissues from treated or non-treated rats. Groups: CN (control; 2A), AR (adjuvant-induced arthritis; 2B), VD_3 (vitamin D_3 free, 120 UI/day, for 15 days; 2C), LNC-D_3 (vitamin D_3 lipid-core nanocapsules, 15.84 UI/day, for 15 days; 2D), AR+VD_3 (adjuvant-induced arthritis treated with vit. D_3 free; 2E) and AR+LNC-D_3 (adjuvant-induced arthritis treated with vit. D_3 lipid-core nanocapsules; 2F).

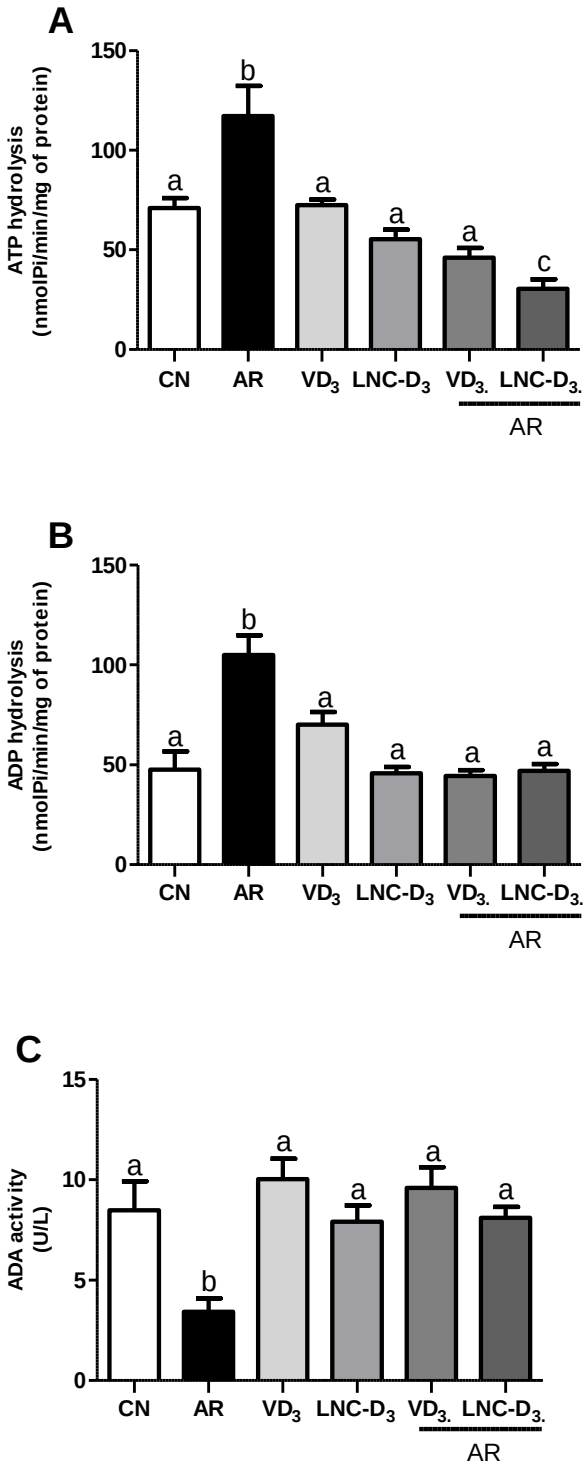

Figure 3 – E-NTPDase activity (ATP and ADP hydrolysis; 3A and 3B, respectively) and E-ADA activity (adenosine deamination; 3C) in lymphocytes obtained from the following groups: CN (control); AR (adjuvant-induced arthritis); VD_3 (vitamin D_3 free, 120 UI/day, for 15 days); LNC-D_3 (vitamin D_3 lipid-core nanocapsules, 15.84 UI/day, for 15 days); AR+VD_3 (adjuvant-induced arthritis treated with vit. D_3 free) and AR+LNC-D_3 (adjuvant-induced arthritis treated with vit. D_3 lipid-core nanocapsules). Data represent the means ± standard error. Groups with different letters are statistically differences (P<0.05; n= 5 for group). Same letter do not differ statistically. One-way ANOVA – Tukey's Test.

TABLE 1 – Hematological parameters of rats with adjuvant- induced arthritis and after the treatment with both vitamin D_3 (free and nanoencapsulated)

Tests	Groups	15 days after the induction		15 days after the treatment with both vitamin D_3			
		CN	AR	VD_3	LNC-D_3	AR+VD_3	AR+LNC-D_3
RBC (x10^6/µL)		7.3±0.24	7.2±0.27	7.4±0.11	7.5±0.10	7.3±0.24	7.4±0.13
Hemoglobin (g/dL)		13.41±0.44	13.28±0.52	14.14±0.30	14.53±0.17	13.69±0.49	14.43±0.20
Ht (%)		45.29±1.69	41.57±2.67	42.71±0.84	44.00±0.42	41.13±1.46	43.88±0.92
MCV (fL)		61.31±0.46	60.4±0.81	57.95±0.73	58.90±0.40	57.41±0.98	59.16±1.18
MCHC (%)		30.83±0.71	30.54±0.78	32.14±0.76	32.81±0.18	32.18±0.82	32.57±0.42
TPP (g/dL)		8.51±0.10	8.26±0.14	8.23±0.16	8.25±0.10	8.32±0.17	8.15±0.10
WBC (x10^3/µL)		7.66±0.64	5.64±0.60	5.63±0.66	7.42±0.60	5.17±0.60	6.44±0.53
Neutrophilis (x10^3/µL)		1.61±0.23	1.34±0.42	1.03±0.11	1.30±0.30	1.13±0.22	1.32±0.12
Lymphocytes (x10^3/µL)		5.82±0.55	4.11±0.38	3.93±0.56	5.93±0.47	3.94±0.49	4.93±0.46
Monocytes (x10^3/µL)		0.14±0.02	0.14±0.04	0.08±0.03	0.09±0.03	0.08±0.02	0.08±0.02
Eosinophilis (x10^3/µL)		0.06±0.01	0.06±0.01	0.04±0.01	0.08±0.03	0.02±0.01	0.09±0.03
Platelets (x10^5/µL)		7.02±5.42[a]	7.06±4.32[a]	9.41±4.38[b]	9.35±4.96[b]	9.15±4.86[b]	8.84±5.53[a,b]

Groups: CN (control); AR (adjuvant-induced arthritis); VD_3 (vitamin D_3 free, 120UI/day, for 15 days); LNC-D_3 (vitamin D_3 lipid-core nanocapsules, 15.84UI/day, for 15 days); AR+VD_3 (adjuvant-induced arthritis treated with vit. D_3 free) and AR+LNC-D_3 (adjuvant-induced arthritis treated with vit. D_3 lipid-core nanocapsules). One-way ANOVA – Tukey's multiple comparison test. Data are means ± SEM for n = 4 - 5 animals for group. Groups with different letters are statistically different ($P<0.05$). RBC: Erythrocytes; HCT: Hematocrit; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; TPP: Total plasma proteins; WBC: Leukocytes.

TABLE 2 – Biochemical parameters and vitamin D serum levels of rats with adjuvant- induced arthritis and after the treatment with both vitamin D_3 (free and nanoencapsulated)

Tests	Groups	15 days after the induction		15 days after the treatment with both vitamin D_3			
		CN	AR	VD_3	LNC-D_3	AR+VD_3	AR+LNC-D_3
Albumin (g/dL)		1.18±0.05	1.25±0.03	1.32±0.09	1.24±0.06	1.19±0.07	1.32±0.06
ALT (UI/L)		61.86±3.80	65.43±3.73	67.00±3.21	57.25±2.25	68.50±6.65	55.83±3.39
AST (UI/L)		192.70±23.80^a	267.10±14.30^b	148.20±17.20^a	135.30 ±8.10^a	129.30±15.90^a	130.70±8.70^a
Calcium (mg/dL)		11.31±0.42	10.93±0.30	10.60±0.35	11.06±0.33	10.86±0.61	11.42±0.50
Creatinine (mg/dL)		0.60±0.09	0.67±0.04	0.71±0.04	0.74±0.03	0.69±0.03	0.69±0.02
Urea (mg/dL)		45.83±3.23	47.36±2.55	53.52±2.05	44.06±2.25	49.54±2.55	43.70±1.70
Vit. D (ng/mL)		43.27±2.88^a	45.24±3.04^a	77.41±3.34^b	44.67±3.56^a	71.99±3.27^b	47.54±1.41^a

Groups: CN (control); AR (adjuvant-induced arthritis); VD_3 (vitamin D_3 free, 120UI/day, for 15 days); LNC-D_3 (vitamin D_3 lipid-core nanocapsules, 15.84UI/day, for 15 days); AR+VD_3 (adjuvant-induced arthritis treated with vit. D_3 free) and AR+LNC-D_3 (adjuvant-induced arthritis treated with vit. D_3 lipid-core nanocapsules). One-way ANOVA – Tukey's multiple comparison test. Data are means ± SEM for n = 4 - 5 animals for group. Groups with different letters are statistically different ($P<0.05$). Vit. D: 25-OH Vitamin D; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

SUMMARY STATEMENT

The effects promoted by both formulations of vitamin D_3 strongly suggest that the use of lipid-core nanocapsules may be an alternative to possibly inhibit pro-inflammatory response, might be an effective and promising agent to supplement the treatment of chronic arthritis.

4 CONCLUSÕES

- Evidências de indução de artrite foram bem estabelecidas, uma vez que escore de artrite, hiperalgesia termal e edema de pata foram alterados em ratos com artrite induzida por adjuvante em relação a ratos controles. Ambas as formas de vitamina D_3 (livre e nanoencapsulada) alteraram hiperalgesia térmica e edema de pata em ratos com artrite induzida em relação ao grupo controle. Porém, a vitamina D_3 livre mostrou reduzir o escore de artrite, o que não foi observado no tratamento com a vitamina D_3 nanoencapsulada.

- Apesar do aparecimento de uma reação granulomatosa, a vitamina D_3 nanoencapsulada foi capaz de reverter o processo inflamatório que o modelo de artrite por adjuvante ocasionou, sendo que a formulação de vitamina D_3 livre foi apenas capaz de amenizar as alterações.

- Também foi demonstrado que o uso de vitamina D_3 (livre ou nanoencapsulada) mostrou-se possivelmente seguro nas respectivas doses e tempo de tratamento, uma vez que não foram observadas alterações em índices hematológicos bem como em dosagens bioquímicas.

- Como ambos os tratamentos com a vitamina D_3 não mostraram ter influência sobre a atividade das enzimas E-NTPDase e E-ADA em linfócitos de ratos controle, os resultados obtidos foram em implicação da artrite induzida e da associação da artrite com o tratamento. Além disso, a menor atividade da E-NTPDase em linfócitos de ratos com artrite induzida e tratados com ambas as formulações de vitamina D_3, sugere que as formulações estão alterando o processo pró-inflamatório gerado por este modelo.

- A atividade da E-ADA em linfócitos também foi alterada em ratos com artrite induzida e tratados com vitamina D_3, demostrando influência deste agente no sistema purinérgico. A menor atividade da E-ADA poderia elevar as concentrações de adenosina, a qual exerce efeitos imunossupressores, protegendo o organismo de possíveis danos causados pela artrite induzida.

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Anexos

Anexo A – Carta de aprovação pelo Comitê de Ética Animal

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Atividade de ectonucleotidases em linfócitos e plaquetas em modelo de artrite reumatóide tratado com vitamina D_3 livre e nanoestruturada".

Número do Parecer: 019/2013

Pesquisador Responsável: Prof. Dr. Daniela Bitencourt Rosa Leal

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, com a ressalva da sugestão, pela CEUA, de uso de analgésico anterior à anestesia para punção cardíaca ao final do experimento. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 19/09/2013.

Santa Maria, 19 de setembro de 2013.

Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2° andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

Anexo B – Normas da revista Clinical Science

Clinical Science

At the interface of clinical research and discovery science

Guidance for Authors

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1 POLICY OF THE JOURNAL

1.1 Scope

Clinical Science is an international Journal publishing research in the field of translational science and medicine, defined as the whole range of biochemical, physiological, immunological and other approaches that may have relevance to disease in man, particularly that which explores integrative biology of systems and pathways and the translation of molecular mechanisms to clinical applications.
Its international Editorial Board is charged with selecting original papers of high scientific merit covering the broad spectrum of biomedical specialities including, although not exclusively, the cardiovascular system, cerebrovascular system, gastrointestinal tract and liver, genetics and functional genomics, infection and immunity, inflammation and oncology, metabolism, endocrinology and nutrition, nephrology and circulation, respiratory system and vascular biology.

The journal publishes six types of manuscript, namely invited Reviews, Hypotheses, Full Papers, Accelerated Publications (concise high-quality papers), Correspondence and invited Commentaries.

1.2 *Clinical Science* Online (**http://www.clinsci.org**)

Access to the previous 12 months' content in Clinical Science is restricted to current subscribers. To ensure that you have access, ask your subscribing librarian to contact sales@portland-services.com.
An electronic back archive from 1999 is freely available to all users. The archival content from 1970-1998 is available via a subscription top up fee or by outright purchase.

(a) Immediate Publications

PDFs of manuscripts are mounted on the Journal's website as Clinical Science Immediate Publications as soon as they are accepted, unless on submission the author has requested that this not be done. Clinical Science Immediate Publications are listed in and accessible through Medline.

(b) Medline links and inter-journal linking

Clinical Science Online provides links to Medline citations, to related papers in Medline, to Medline citations for downloading to citation management software, and from references to relevant abstracts in other online journals.

(c) Multimedia adjuncts

Clinical Science Online offers authors the opportunity to enhance their papers with multimedia adjuncts (e.g. time-lapse movies, three-dimensional structures). These will be submitted to peer review alongside the manuscript. To submit a paper with a multimedia adjunct, attach the file when you submit your manuscript online. Preferred formats are QuickTime for time-lapse movies and PDB for structures. There is no extra charge associated with the publication of a multimedia adjunct online.

1.3 The editorial process

A submitted paper is considered by an appropriate Editor together with (usually) one Referee from the Editorial Advisory Panel. The Editor returns it with a recommendation to the Associate Editor, who then writes formally to the authors. The ultimate responsibility of acceptance for publication lies with the Editor-in-Chief.

Authors may suggest potential reviewers for their paper in the letter of submission, but the journal will usually regard such suggestions as a guide only and is under no obligation to follow them. Authors may also specify the names of those they wish to be excluded from the review process for a particular paper; in such cases their wishes are usually respected, unless, of course, in the opinion of the journal such a request unreasonably excludes all of the expertise available to it in that scientific area.
It is accepted that the reviewers may from time to time come to decisions that are not easily accepted by authors. This may be because of a conflict of opinion or, for example, and as frequently happens, because the authors' point is felt by the reviewers to be obscured by the presentation. The journal is always willing to hear from authors and to consider their views sympathetically. Appeals against decisions will always be considered by the Editorial Board. In rare cases, and if the reviewers and the Editor-in-Chief agree, the usual anonymity of the reviewers may be set aside to allow discussion

between all parties concerned. In all cases the decision of the Editor-in-Chief will be final.

1.4 Ethics

(a) Human experimentation

Authors must state in the text of their paper that the research has been carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association, and has been approved by the Ethics Committee of the institution in which the work was performed. Consent must be obtained from each patient or subject after full explanation of the purpose, nature and risk of all procedures used, and the fact that such consent has been given should be recorded in the paper.

(b) Animal experimentation

Care must always be taken to ensure that experimental animals do not suffer unnecessarily. Authors must state in the text the anaesthetic procedures used in full, and all precautions they took to ensure that the animals did not suffer unduly during and after the experimental procedure. Authors must confirm that the work was undertaken as required by the appropriate institutional and national animal care committees, or, in the absence of such legislation, that the experimental procedures were carried out in accordance with the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication no. (NIH) 85–23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.].

(c) Scientific publication

Clinical Science is a member of COPE (Committee on Publication Ethics) and endorses its guidelines, which are available at http://www.publicationethics.org/. Complaints against the Journal must be submitted in writing to the Editor-in-Chief; if a complaint is not resolved to the satisfaction of the complainant they have the option of referring the matter to COPE.

Notwithstanding, the Editorial Board will not accept papers where the ethical aspects are, in the Board's opinion, open to doubt.

Clinical Science will not tolerate plagiarism in submitted manuscripts. Passages quoted or closely paraphrased from other authors (or from the submitting author's own published work) must be identified as quotations or paraphrases, and the sources of the quoted or paraphrased material must be acknowledged. Use of unacknowledged sources will be construed as plagiarism. If any manuscript is found to contain plagiarized material the review process will be halted immediately
Clinical Science will always investigate fully any matters of apparent misconduct that it becomes aware of.

Clinical Science follows the guidelines published by COPE in respect of the retraction of articles.

1.5 Originality of papers

Submission of a paper to *Clinical Science* implies that it has been approved by all the named authors, that all persons entitled to authorship have been so named, that it reports unpublished work that is not under consideration for publication elsewhere in any language, that proper reference is made to the preceding literature, and that if the paper is accepted for publication the authors will grant the Biochemical Society an exclusive licence to publish the paper (see 2.13 **Copyright policy**). The restriction on previous publication does not usually apply to previous publication of oral communications in brief abstract form or as preprints. In such cases authors should a copy of the abstracts of previous publications or provide the URL of the preprint server. However, the restriction does apply to complete papers published on the Web.

Clinical Science endorses the Vancouver Guidelines on authorship as defined in the International Committee of Medical Journal Editors' (ICMJE) Uniform Requirements for Manuscripts Submitted to Biomedical Journals http://www.icmje.org/#author, namely that entitlement to authorship should be based on all of the following criteria: (1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it for important intellectual content;(3) final approval of the version to be published. Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship. All contributors who do not meet the criteria for authorship should be listed in the Acknowledgments.

1.6 Declaration of financial interests

Authors are strongly encouraged to disclose all relevant competing interests and sources of research funding that could be perceived to compromise the integrity of their article published in Clinical Science.

2 ONLINE SUBMISSION OF PAPERS: GENERAL INFORMATION AND FORMAT

2.1 Online submission of papers

Papers should be submitted online at http://sirius.portlandpress.com/submit/cs/, where full instructions are available.

Submission checklist:

- Covering letter including the names, addresses and email addresses of four possible referees (two of whom should be from the editorial board; see the list on the Journal web page: http://www.clinsci.org/).
- Summary statement for publication: this should highlight the key finding of your study. The statement should not exceed 40 words and should be written for a broad audience without the use of abbreviations and acronyms that are not widely known.
- Master electronic copy of typescript, as a one-line-spaced PDF (or separate files of text and illustrations):
  - complete text in appropriate style, pages numbered
  - full names and addresses of authors
  - full name, address, telephone and fax numbers and email address of corresponding author (all correspondence and proofs will be sent to this author)
  - figures
- Related papers in press or under editorial consideration
- Evidence of approval of personal communications
- Evidence of submission of nucleic acid or protein sequences to an appropriate data bank.

2.2 Addresses and general information

(a) Correspondence about papers from the USA, Canada, Mexico and Central and South America should be sent to:

Dr Sharon Schendel, Administrative Editor, Clinical Science US Office, The Sanford-Burnham Institute for Medical Research, 10901 N Torrey Pines Road, La Jolla, CA 92037, U.S.A., telephone: +1 (858) 795 5283; fax: +1 (858) 795 5284; email: editorial@clinsciusa.org.

(b) Correspondence about papers from all other countries, proofs, offprints and requests for permission to reproduce material should be addressed to:

Dr James Mockridge, Executive Editor, Clinical Science, Third floor, Charles Darwin House, 12 Roger Street, London WC1N 2JU, U.K., telephone: (U.K.) 020 7685 2410; (from overseas) +44 20 7685 2410; fax: (U.K.) 020 7685 2469; (from overseas) +44 20 7685 2469; email: editorial@portlandpress.com.

Papers should be presented so that they are intelligible to the non-specialist reader of the journal. This is particularly important in highly specialized fields, and a very brief résumé of the current state of knowledge is usually helpful. Certain types of material, e.g. mathematical formulations requiring more than trivial derivations, should be given in a separate Appendix.

The Editorial Board reserves the right to reject papers that cannot adequately be assessed because of a poor standard of English.
Where the reader is referred to previous works by the same author(s) for important details relevant to the present work, three copies or reprints of the publication (including papers on the WWW) should be submitted alongside the manuscript. This is of particular importance in relation to methodology.

The dates of receipt and acceptance of the paper will be published. If the paper has to be returned to the authors for revision and is not resubmitted within 3 months, the date of receipt will be revised accordingly and the revised paper may be treated as a new submission. It is emphasized that badly presented or unduly long papers will be returned for revision and delays in publication will be inevitable. Similar delays will be incurred if the typescript is not prepared strictly in accordance with the instructions detailed below.

2.3 Accepted papers

On acceptance, authors will be requested to supply a Word file of the final version of their paper to the Editorial Office. Authors must ensure that the file has been updated to incorporate all revisions, and hence that the file matches the final version of the manuscript seen by the reviewers. Our preferred word-processing format is Microsoft Word for Windows version 6.0.

(a) Tables

Tables should be prepared using the Microsoft Word table editor.

b) Figures

No artwork should be incorporated into the text files. Figures should be supplied as electronic files. Full instructions will be provided on acceptance and guidance notes for the preparation of figures are available here.

c) Mathematics

In-line equations should be typed as text. Displayed equations (unless prepared by the 'MathType Equation Editor') are re-keyed by our typesetter.

2.4 Full Papers

These may be of any length that is justified by their content. Authors should, however, note that, because of pressure for space in the journal, no paper, whatever its scientific merits, will be accepted if it exceeds the minimum length required for precision in describing the experiments and clarity in interpreting them. As a guide, most papers published in the journal are of between six and eight printed pages. A concise well-written paper tends to be published more rapidly. Extensive sets of data can be made available online (see 2.9).

The authors should refer to a current issue of Clinical Science to make themselves familiar with the general layout. Manuscripts should, in general, be arranged as follows:

(a) Title page

Title: this should be short, snappy and as informative as possible, since titles of papers are used in indexing and coding for information storage and retrieval. The title should indicate the species in which the observations reported have been made. It should not contain any abbreviations. The numbering of parts in a series of papers is not permitted.

List of authors' names (degrees and appointments are not required).

Laboratory or Institute of origin, with full postal address.

Key words: for indexing the subject of the paper. Supply up to six key words of which at least three do not appear in the title of the paper; they should, if possible, be selected from the current issues of 'Medical Subject Headings' (MeSH) produced by the Index Medicus.

Short title: for use as a running heading in the printed text; it should not exceed 45 characters and spaces and should not contain any abbreviations.

Author for correspondence: the name, address and email address of the author to whom queries and requests for offprints should be sent.

(b) Abstract

This should be a brief statement of what was done, what was found and what was concluded, and should rarely exceed 250 words. Abbreviations should be avoided as far as possible and must be defined.

(c) Introduction

This should be comprehensible to the general reader and should contain a clear statement of the reason for doing the work, but should not include either the findings or the conclusions.

(d) Methods

The aim should be to give sufficient information in the text or by reference to permit the work to be repeated without the need to communicate with the author.

(e) Results

This section should not include material appropriate to the Discussion section.

(f) Discussion

This should not contain results and should be pertinent to the data presented.

(g) Clinical Perspectives

A short outline highlighting the clinical significance of the paper within approximately 100-150 words (about three sentences) should be provided. This section should (i) provide some background as to why the study was undertaken, (ii) provide a brief summary of the results and then (iii) indicate the potential significance of the results to human health and disease.

(h) Author contribution

The contribution of each author to the study should be provided.

(i) Acknowledgments

These should be as brief as possible and include any declaration of interest.

(j) Funding

This information should be provided in the form of a sentence with the funding agency written out in full followed by the grant number in square brackets.

(k) References

See 3.18 for the correct format.

(l) Figures and Tables

See 3.10.

(m) Summary statement

This should highlight the key finding of your study. The statement should not exceed 40 words and should be written for a broad audience without the use of abbreviations and acronyms that are not widely known.

2.5 Accelerated Publications

The passage of these papers through the editorial process will be expedited and contributors are encouraged to take advantage of this facility when data are novel and exciting, when rapid publication is of importance and when material can be presented concisely. Authors must include in their letter of submission a brief statement explaining the novelty of their work. Accelerated Publications should describe completed work and should not be merely a preliminary communication.

Accelerated Publications should be similar in format to full papers, except that they must occupy not normally more than four printed pages. This is about 3000 words, with appropriate deductions (at the rate of 1000 words/page) for Figures and Tables.

To achieve rapid publication, authors of accepted Accelerated Publications will not be sent proofs. Rejection of a paper submitted as an Accelerated Publication does not preclude its re-submission as a

full paper for publication in Clinical Science, in which event the paper would be reviewed and reports provided with the editorial decision in the normal way.

One colour Figure will be published free of charge in Accelerated Publications (a saving of £550) provided that it is deemed by the Editor to be necessary to illustrate a scientific point; subsequent Figures in the same paper will cost £300 each.

2.6 Correspondence

Letters containing critical assessments of material published in Clinical Science, including Reviews and Hypotheses, will be considered for the Correspondence section of the journal. All Letters received are subjected to the journal's peer-review procedure. Letters should be no longer than 750 words, with one Figure or Table and up to six references, or 1000 words maximum without a Figure or Table. Letters relating to material previously published in Clinical Science should be submitted within 6 months of the appearance of the article concerned. They will be sent to the authors for comment and both the letter and any reply by the author will be published together. Further correspondence arising therefrom will also be considered for publication. Consideration will also be given to publication of letters on ethical matters.

2.7 Reviews

These are normally commissioned. However, unsolicited reviews will be considered. Prospective authors should first submit a synopsis of their proposed review rather than the full typescript.

2.8 Hypotheses

Hypothesis articles are short manuscripts that present an original hypothesis. The article should set out a clearly identified hypothesis and be supported by appropriate references. The article should propose what experimental work might test the hypothesis, but should not contain new data. A conclusion should be provided focusing on translational medicine through to clinical practice.

The length should be up to a maximum of 2500 words and contain no more than 50 references. A short abstract of up to 250 words should be included that provides an overview of the hypothesis to be presented and its implications. Authors are strongly encouraged to include a number of figures or diagrams to improve the visual impact.

Hypothesis articles are normally commissioned. However, unsolicited reviews will be considered in which case prospective authors should first submit a synopsis of their proposed hypothesis to the Editorial Office rather than the full typescript.

2.9 Commentaries

These are normally commissioned by the Editorial Board.

2.10 Online data sets

It is impractical to publish very large sets of individual values or very large numbers of diagrams, and under these circumstances a summary of the information only should be included in the paper. The information from which the summary was derived should be submitted with the typescript and, if the latter is accepted, the Editor may ask for a copy of the full information and diagrams to be made available as an online data set.

2.11 Proof corrections

These are expensive and corrections of other than printers' errors may have to be charged to the author.

2.12 Offprints

Offprints may be obtained at terms, based upon the cost of production, that will be given with the proofs. All offprints should be ordered when the proofs are returned (except for Accelerated Publications, where they should be ordered when the subedited typescript is returned).

2.13 Availability on Medline and from Adonis

Summaries of papers in Clinical Science are available on the Medline system run by the National Library of Medicine, National Institutes of Health, Bethesda, MD, U.S.A.

Full text with illustrations of individual papers can be obtained from Adonis B.V., PO Box 993, 1000AZ Amsterdam, The Netherlands, telephone: +31 20 485 3870, fax: +31 20 485 3871, email: info@adonis.nl.

2.14 Copyright policy

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2.16 Portland Press books

Authors, Editors and all contributors to Portland Press journals may order books published by Portland Press, for their personal use, at 25% discount. A complete list of books can be found at http://www.portlandpress.com.

3 MISCELLANEOUS NOTES

3.1 Abbreviations

Abbreviations should be kept to a minimum compatible with clarity and conciseness; if used they must be defined at the first mention. Numbers, not initials, should be used for patients and subjects.

3.2 Anatomical nomenclature

This should follow the recommendations of the International Anatomical Nomenclature Committee [Nomina Anatomica (1966) 3rd edn, Excerpta Medica Foundation].

3.3 Animals, plants and micro-organisms

The full binomial specific names should be given at first mention for all experimental animals other than common laboratory animals. The strain and, if possible, the source of laboratory animals should be stated. Thereafter in the text, single letter abbreviations may be given for the genus; if two genera with the same initial letter are studied, abbreviations such as Staph. and Strep. should be used.

3.4 Array data

Clinical Science recommends that authors adhere to the MIAME guidelines for the submission of gene chip array data (see http://www.mged.org/Workgroups/MIAME/miame.html).

3.5 Biochemical nomenclature

As far as possible authors should follow the recommendations of the Nomenclature Committee of IUBMB and IUPAC–IUBMB Joint Commission on Biochemical Nomenclature [see Biochemical Nomenclature and Related Documents (1992) 2nd edn., Portland Press, London]; for corrections see Eur. J. Biochem. (1993) 213, 1–3. Further details are given at http://www.BiochemJ.org

3.6 Buffers and salts

The acidic and basic components should be given, together with the pH. Alternatively, a reference to the composition of the buffer should be given. Further details are provided at http://www.BiochemJ.org

When describing solutions containing organic anions and their parent acids, the salt designator (e.g. lactate, urate, oxalate) should be used in preference to the name of the acid (lactic, uric, oxalic) unless it is certain that virtually all of the acid is in the undissociated form.

The composition of incubation media should be described, or a reference to the composition should be given.

3.7 Computer modeling

Papers concerned primarily with computer modelling techniques are acceptable provided that use of such techniques leads to a clear choice between two or more alternative hypotheses, or to the formulation of a new hypothesis amenable to experimental challenge or verification, or provides some new insight into the behaviour of a particular physiological system. Extensive technical details of hardware and software should not be given.

3.8 Doses

Doses of drugs should be expressed in mass terms, e.g. milligrams (mg) or grams (g), and also (in parentheses) in molar terms, e.g. mmol, mol, where this appears to be relevant. Molecular masses of many drugs may be found in The Merck Index (1996) 12th edn., Merck & Co. Inc., Whitehouse Station, NJ.

3.9 Enzymes

Nomenclature should follow that given in Enzyme Nomenclature (1992, Academic Press, San Diego); for corrections and additions see Eur. J. Biochem. (1994) **223,** 1–5 and Eur. J. Biochem. (1995) **232**, 1–6. The Enzyme Commission (EC) number should be quoted at the first mention. Where an enzyme has a commonly used informal name, this may be employed after the first formal identification. A unit of enzyme activity can be expressed as that amount of material which will catalyse transformation of 1µmol of the substrate/s under defined conditions, including temperature and pH. This gives the unit of the amount of enzyme named the katal (symbol kat). Alternatively, or when the natural substrate has not been fully defined, activity should be expressed in terms of units of activity relative to that of a recognized reference preparation, assayed under identical conditions. Activities of enzymes should normally be expressed as units/ml or units/mg of protein.

3.10 Evaluation of measurement procedures

When a new measuring procedure has been used, or when an established procedure has been applied in a novel fashion, an estimate of the precision of the procedure should be given. This should, as far as possible, indicate what sources of variation have been included in this estimate, e.g. variation of immediate replication, variation within different times of day, or from day to day, etc.

If the precision of measurement varies in proportion to the magnitude of the values obtained, it can best be expressed as the coefficient of variation; otherwise it should be expressed by an estimate of the (constant) standard error of a single observation, or by estimates of several points within the range of observed values.

When recovery experiments are described, the approximate ratio of the amount added to the amount already present and the stage of the procedure at which the addition was made should be stated.

For methods or assays crucial to the understanding of the paper, information should normally be provided on the validity, accuracy and precision of those methods.

3.11 Figures and Tables

Their number should be kept to a minimum. References to Figures and Tables should be in arabic numerals, e.g. Figure 3, and they should be numbered in order of appearance. In general, the same data should not be presented in both a Figure and a Table.

Figures

Figures are not routinely relettered. Authors should ensure that nomenclature, abbreviations, etc. used in lettering of Figures correspond to those used in the text. Separate panels within Figures should be clearly marked (a), (b), (c), etc. so that they can be referred to easily in the legend and text. Acceptable symbols for experimental points are ● , ▲ , ■ , ○ , △ , □ . The symbols × or + should be avoided. Symbols should not be generated by using tints or a graphics program. The same symbols must not be used for two curves where the points might be confused. For scatter diagrams, solid symbols are preferred. When a particular variable appears in more than one Figure, the same symbol should be used for it throughout, if possible.

Curves should not be drawn beyond the experimental points, nor should axes extend appreciably beyond the data. Only essential information that cannot readily be included in the legend should be written within the Figure.

The use of tints should be avoided; however, if tints are necessary, please ensure that a dot fill of 100 lines per inch or lower is used. Columns in histograms should be differentiated by the use of simple hatching etc.

For half-tone figures where the magnification is to be indicated (e.g. on electron micrographs), this should be done by adding a bar representing a stated length.

Colour figures are accepted when, in the opinion of the Editorial Board, they are essential to illustrate a particular scientific point. Authors will normally be required to pay the full cost of colour separation and printing (at 2012 prices, approximately £550 for the first Figure and £300 for each subsequent Figure in the same paper). VAT is payable by authors in the European Union.

Captions for the Figures should make them **readily understandable** without reference to the text. Adequate statistical information, including that on regression lines, should be included in Figure legends where appropriate.

Care is needed when using powers in Figure to avoid numbers with too many digits (see 3.17).

Tables

Tables should be typed separately from the text. They should have a title followed by any legend. Parameters being measured, with units if appropriate, should be clearly indicated in the column headings.

Titles and legends for the Tables, should make them **readily understandable** without reference to the text.

Care is needed when using powers in Table headings to avoid numbers with too many digits (see 3.17).

Image acquisition and manipulation

Images will be checked for manipulation when a paper is accepted. The Editorial Board may request that authors supply the original data for comparison against the prepared figures. If authors are unable to comply with such a request, the acceptance of the paper may be withdrawn.

Clinical Science endorses the guidelines given in the Instructions for Authors of the Journal of Cell Biology, from where the following is adapted by kind permission of Rockefeller University Press:

The following information must be provided about the acquisition and processing of images:

1. Make and model of microscope
2. Type, magnification, and numerical aperture of the objective lenses
3. Temperature
4. Imaging medium
5. Fluorochromes
6. Camera make and model
7. Acquisition software
8. Any subsequent software used for image processing, with details about types of operations involved (e.g. type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

No specific feature within an image may be enhanced, obscured, moved, removed or introduced. The grouping of images from different parts of the same gel, or from different gels, fields or exposures must be made explicit by the arrangement of the figure (i.e. using dividing lines) and in the text of the figure legend. Adjustments of brightness, contrast or colour balance are acceptable if they are applied to the whole image and as long as they do not obscure, eliminate or misrepresent any information present in the original, including backgrounds.The background of figures should be clearly distinct from the surrounding page. Non-linear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Authors are encouraged to read the papers by M. Rossner and K. M. Yamada (2004) J. Cell Biol. 166, 11-15 http://www.jcb.org/cgi/content/full/166/1/11 and A. J. North (2006) J.Cell Biol. 172, 9-18 http://www.jcb.org/cgi/content/full/172/1/9

3.12 Footnotes

These should be avoided as far as possible, but where they are used in Tables they should be identified by the symbols * † ‡ § || ¶, in that order.

3.13 Gene nomenclature

Clinical Science encourages authors to use the symbols and nomenclature recommended by the HUGO Gene Nomenclature Committee which is responsible for approving and implementing unique human gene symbols and names, and works closely with the Mouse Genome Database and other organism databases. Considerable efforts are made to approve symbols acceptable to workers in the field, but sometimes it is not possible to use exactly what has previously appeared in the literature. In such cases the previously used symbols are listed as aliases for the approved nomenclature in the **Human Genome Nomenclature Database** and **Entrez Gene** to allow retrieval of all the information available for each gene.

3.14 'Homology'

The term 'homologous' has a precise meaning in biology of 'having a common evolutionary origin', but it has often been used in work on protein and nucleic acid sequences to mean simply 'similar'. A group of experts has urged that the interests of clarity are best served by restricting use to the more precise definition [Reeck, G.R. et al. (1987) Cell 50, 667; Lewin, R. (1987) Science **237**, 1570]. Clinical Science agrees with these arguments and seeks to preserve the distinction between 'homologous' and 'similar' in its pages.

3.15 Isotope measurements

Where possible, radioactivity should be expressed in absolute terms; the SI unit for radioactivity is the becquerel (Bq), defined as 1 disintegration/s, but the Curie (Ci; 1 Ci=3.7×1010 Bq) may also be used. Alternatively, radioactivity may be expressed as disintegrations (or counts) per unit of time, e.g. disintegrations/s (d.p.s.) or counts/min (c.p.m.).

3.16 Radionuclide applications in humans

If new or modified radionuclide applications in humans are described, an estimate of the maximal possible radiation dose to the body and critical organs should be given.

For the time being this can continue to be expressed in rems, but with the corresponding value in sieverts (Sv) given in parentheses after it.

3.17 Methods

In describing certain techniques, namely centrifugation (when the conditions are critical), chromatography and electrophoresis, authors should follow the recommendations published by the Biochemical Society (see http://www.BiochemJ.org).

3.18 Nomenclature of disease

This should follow the International Classification of Disease (1997, 9th revision. World Health Organization, Geneva) as far as possible. The correct abbreviation for insulin-dependent diabetes is Type I diabetes (notIDDM), and for non-insulin-dependent diabetes is Type II diabetes (not NIDDM).

3.19 Powers in Tables and Figures

Care is needed where powers are used in Table headings and in Figures to avoid numbers with an inconvenient number of digits. For example: (i) an entry '2' under the heading 10^3 *k* means that the value of *k* is 0.002; an entry '2' under the heading 10^{-3} *k* means that the value of *k* is 2000. (ii) A concentration 0.00015 mol/l may be expressed as 0.15 under the heading 'concn. (mmol/l)' or as 150 under heading 'concn. (μmol/l)' or as 15 under the heading '10^5 × concn. (mol/l)', but not as 15 under the heading 'concn. (mol/l × 10^{-5})'.

3.20 References

The number of references should not normally exceed 50, and 20–30 references are frequently adequate.

The Numbering System must be used. References should be cited in the text by sequential numbers in square brackets, e.g. '[1]', '[2–6]', '[4,5,7–10]'', etc. At the end of the paper references should be listed in numerical order, in the style shown in the following examples, preceded by the number. Thus:

1 Perlstein, T.S., Henry, R.R, Mather, K.J., Rickels, M.R., Abate, N.I., Grundy, S.M. , Mai, Y., Albu, J.B., Marks, J.B., Pool, J.L. and Creager, M.A. (2012) Effect of angiotensin receptor blockade on insulin sensitivity and endothelial function in abdominally obese hypertensive patients with impaired fasting glucose. Clin. Sci. **122**, 193-202

names and initials of all authors, the paper or chapter title, and first and last page numbers, should be provided for all references. Titles of journals should be abbreviated in accordance with the Chemical Abstracts Service Source Index (2004) and subsequent Quarterly Supplements (American Chemical Society; http://www.cas.org/).

When the quotation is from a book, the following format should be used, giving the relevant pages or chapter number:

2 Cornish-Bowden, A. (2004) Fundamentals of Enzyme Kinetics, 3rd edn, Portland Press Ltd, London

3 Warnholtz, A., Wendt, M., August, M. and Münzel, T. (2004) Clinical aspects of reactive oxygen and nitrogen species. In Free Radicals: Enzymology, Signalling and Disease (Cooper, C., Wilson, M. and Darley-Usmar, V., eds), pp. 121–133, Portland Press Ltd, London

References to a paper 'in the press' are permissible provided that it has been accepted for publication (the name of the journal and documentary evidence of acceptance must be provided):

5 Smith, A. (2008) Another paper. Clin. Sci., in the press

Alternatively, for papers 'in the press', if the doi (digital object identifier) is known, then this should be cited instead:

6 Bomfim, G., Dos Santos, R., Aparecida Oliveira, M., Giachini, F.R., Akamine, E., Tostes, R.C., Fortes, Z.B., Webb, R.C.and Carvalho, M.H.C. (2012) Toll like receptor 4 contributes to blood pressure regulation and vascular contraction in spontaneously hypertensive rat. Clin. Sci., doi:10.1042/CS20110523

References to 'personal communication' and 'unpublished work' should appear in the text only and not in the list of references. The name and initials of the source of information should be given. In the case of quotations from personal communications the authors must provide documentary evidence that permission for quotation has been obtained. When the reference is to material that has been accepted for publication but has not yet been published, this should be indicated in the list of references by "in the press" together with the name of the relevant journal and, if possible, the expected date of publication. If such a citation is of major relevance to the manuscript submitted for publication, authors are advised that the editorial process might be expedited by the inclusion of a copy of such work.

References are often the cause of many proof corrections, and inaccuracies hamper inter-journal linking and Medline links in Clinical Science Online. Please check the list carefully before submission.

3.21 Solutions

Concentrations of solutions should be described where possible in molar terms (mol/l and subunits thereof), stating the molecular particle weight if necessary. Values should not be expressed in terms of normality or equivalents. Mass concentration should be expressed as g/l or subunits thereof, for

example mg/l or µg/l. For solutions of salts, molar concentration is always preferred to avoid ambiguity as to whether anhydrous or hydrated compounds are used. Concentrations of aqueous solutions should be given as mol/l or mol/kg (g/l or g/kg if not expressed in molar terms) rather than % (w/v) or % (w/w). It should always be made clear whether concentrations of compounds in a reaction mixture are final concentrations or the concentrations in solutions added.

3.22 Spectrophotometric data

The general name for the quantity log (I_o/I) is attenuance, and it reduces to absorbance when there is negligible scattering or reflection. The more general term 'attenuance' should be used when scattering is considerable, e.g. when the quantity is measured to estimate the cell density of a culture. Otherwise the term absorbance should be used; neither should be called extinction or optical density. Symbols used are: *A*, absorbance; *D*, attenuance; *a*, specific absorption coefficient (litre·g^{-1}·cm^{-1}) (alternatively use $A\,{}^{1\%}_{1\ cm}$); ε, molar absorption coefficient (the absorbance of a molar solution in a 1 cm light-path) (litre·mol^{-1}·cm^{-1}, not cm^2·mol^{-1}).

3.23 Spelling

Clinical Science uses as standards for spelling the Concise or Shorter Oxford Dictionary of Current English (Clarendon Press, Oxford) and Butterworth's Medical Dictionary (Butterworths, London).

3.24 Statistics

Papers are frequently returned for revision (and their publication consequently delayed) because the authors use inappropriate statistical methods. Two common errors are the use of means, standard deviations and standard errors in the description and interpretation of grossly non-normally distributed data and the application of t-tests for the significance of difference between means in similar circumstances, or when the variances of the two groups are non-homogeneous. In some circumstances it may be more appropriate to provide a 'scattergram' than a statistical summary. Authors are recommended to consult the statistical guidelines presented by Altman et al. in 'Statistical guidelines for contributors to medical journals' (1983) Br. Med. J. **286**, 1489–1493.

The type of statistical test used should be stated in the Methods section. A reference should be given for the less commonly encountered statistical tests. Degrees of freedom should be indicated where appropriate. Levels of significance are expressed in the form $P<0.01$.

3.25 Trade names and drug names

The name and address of the supplier of special apparatus and of biochemicals should be given. Registered trademarks should be identified by the symbol ® where they appear in the text. In the case of drugs, approved names should always be given, with trade names and manufacturers in parentheses. Scientifically precise and unambiguous terms should be used to describe groups of drugs such as general anaesthetics and opioids.

4 UNITS: THE SI SYSTEM

The recommended Système International (SI) units [see Quantities, Units and Symbols in Physical Chemistry (1998) Blackwell Scientific Publications Ltd., Oxford] are used by Clinical Science. **All papers submitted should use these units** except for blood pressure values, which should be expressed in mmHg, and gas partial pressures, where values at the author's discretion may be given in mmHg (with kPa in parentheses) or as kPa (with mmHg in parentheses). Airways pressure should be expressed in kPa. Where molecular mass is known, the amount of a chemical or drug should be expressed in mol or in an appropriate subunit, e.g. mmol. Energy should be expressed in joules (J).

The basic SI units and their symbols are as follows:

Physical quantity	name	Symbol
length	metre	m
mass	kilogram	kg

time	second	s
electric current	ampere	A
thermodynamic temperature	kelvin	K
luminous intensity	candela	cd
amounts of substance	mole	mol

The following are examples of derived SI units:

Physical quantity	name	Symbol	Definition
energy	joule	J	$kg \cdot m^2 \cdot s^{-2}$
force	newton	N	$kg \cdot m \cdot s^{-2} = J \cdot m^{-1}$
power	watt	W	$kg \cdot m^2 \cdot s^{-3} = J \cdot s^{-1}$
pressure	pascal	Pa	$kg \cdot m^{-1} \cdot s^{-2} = N \cdot m^{-2}$
electric charge	coulomb	C	$A \cdot s$
electric potential difference	volt	V	$kg \cdot m^2 \cdot s^{-3} \cdot A^{-1} = J \cdot A^{-1} \cdot s^{-1}$
electric resistance	ohm	Ω	$kg \cdot m^2 \cdot s^{-3} \cdot A^{-2} = V \cdot A^{-1}$
electric conductance	siemens	S	$kg^{-1} \cdot m^{-2} \cdot s^3 \cdot A^2 = \Omega^{-1}$
electric capacitance	farad	F	$A^2 \cdot s^4 \cdot kg^{-1} \cdot m^{-2} = A \cdot s \cdot V^{-1}$
frequency	hertz	Hz	s^{-1}
volume	litre	l	$10^{-3}\ m^3$

The word 'litre' has been accepted as a special name for cubic decimetre (1 litre = 1 dm^3).

Both the basic and derived SI units, including the symbols of derived units that have special names, may be preceded by prefixes to indicate multiples and sub-multiples. The prefixes should be as follows:

Multiple	Prefix	Symbol	Multiple	Prefix	Symbol
10^6	mega	M	10^{-3}	milli	m
10^3	kilo	k	10^{-6}	micro	μ
10^2	hecto	h*	10^{-9}	nano	n
10	deka	da	10^{-12}	pico	p
10^{-1}	deci	d*	10^{-15}	femto	f
10^{-2}	centi	c*			

**To be avoided where possible (except for cm).*

Compound prefixes should not be used, e.g. 10^{-9} m should be represented by 1 nm, not 1 mμm.

Notes

(i) Full stops are not used after symbols.
(ii) Minutes (min), hours (h), days and years will continue to be used in addition to the SI unit of time [the second (s)].
(iii) The solidus may be used in a unit as long as it does not have to be employed more than once, e.g. mmol/l is acceptable, but ml/min/kg is not, and should be replaced by $ml \cdot min^{-1} \cdot kg^{-1}$.