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Doutorado

ANÁLISE DA AÇÃO DO RANELATO DE ESTRÔNCIO EM MODELO EXPERIMENTAL DE OSTEOARTRITE EM RATO

THIAGO ALVES RODRIGUES

SÃO LUÍS

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão, como requisito parcial para a obtenção do título de Doutor em Ciências da Saúde.

Orientador: Prof. Dr. João Batista Santos Garcia

Co-orientadora: Profª. Drª. Maria do Socorro de Sousa Cartágenes

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Aprovada em / /

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"A alegria que se tem em pensar e aprender
faz-nos pensar e aprender ainda mais."

Aristóteles

A Deus, que planeja cada segundo da minha vida.

A minha amada Camila.

A Larissa, Davi Antonio e Daniel, bênçãos divinas

personificadas em forma de filhos

A meus queridos e estimados pais

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

ACLT	*anterior cruciate ligament transection*
ADAM	*a disintegrin and metalloproteinase domain*
ADAMTS5	*a disintegrin and metalloproteinase with thrombospondin motifs*
AUSCAN	*Australian Canadian Questionnaire*
CINC-1	*cytokine-induced neutrophil chemoattractant*
CPK	*creatine phosphokinase*
CTX-I	*telopeptide of type I procollagen*
CTX-II	*C telopeptide of type II procollagen*
DeCS	*Health Descriptors*
DMOAD	*disease-modifying OA drugs*
EDTA	ácido etilenodiamino tetra-acético
ELISA	*enzyme linked immunosorbent assay*
EMA	*European Medicines Agency*
FDA	*Food and Drug Administration*
FIHOA	*Functional Index for Hand Osteoarthritis*
GRADE	*Grading of Recommendations Assessment, Development, and Evaluation*
IL1β	interleucina 1 β
LEED	Laboratório Experimental para Estudo da Dor
LNRP	limiar nociceptivo de retirada da pata
LNRPA	limiar nociceptivo de retirada da pata afetada
LNRPC	limiar nociceptivo de retirada da pata contralateral
MCII	*minimal clinical important improvement*

MeSH	*Medical Subject Headings*
MIA	monoiodoacetato de sódio
micro-CT	micro-computed tomography
MMP	metaloproteinases de matriz
MMT	*medial meniscal tears*
MPCI	*minimal perceptible clinical improvement*
mRNA	RNA mensageiro
MT1-MMP	*membrane type-1 matrix metaloproteinase*
MV	*Mechanical vibration*
NMR	*nuclear magnetic resonance*
NNT	número necessário para tratar
OA	osteoartrite
OARSI	*Osteoarthritis Research Society International*
OMERACT	*Outcome Measures in Rheumatology*
OPG	osteoprotegerina
OVX	ooforectomia
PCR	*polymerase chain reaction*
PET	*paw elevation time*
PICOS	*Patients/Intervention/Comparison/Outcomes/Study design*
PO	*per os*
PPA	peso da pata afetada
PPC	peso da pata contralateral
PRISMA	*Preferred Reporting Items for Systematic Reviews and Meta-analyzes*
RANKL	*receptor activator of nuclear factor kappa-B ligand*
SC	subcutâneo

SciELO	*Scientific Electronic Library Online*
SEKOIA	*Strontium ranelate Efficacy in Knee OsteoarthrItis triAl*
SF-36	*Medical Outcomes Study-36 questionnaire*
SOTI	*Spinal Osteoporosis Therapeutic Intervention trial*
SOX9	*sex-determining region Y – box 9*
SrRan	ranelato de estrôncio
TMJ	*Temporomandibular joint*
TNF- α	*tumor necrosis factor α*
TROPOS	*Treatment Of Peripheral Osteoporosis trial*
TUNEL	*transferase-mediated dUTP-TMR nick end labeling assay*
WOMAC	*Western Ontario and McMaster Universities Osteoarthritis Index*

LISTA DE TABELAS

Lista de Tabelas: Capítulo I

Lista de Tabelas: Capítulo III

LISTA DE FIGURAS

Lista de Figuras - Referencial Teórico

Lista de Figuras – Capítulo I

Lista de Figuras – Capítulo II

Lista de Figuras – Capítulo III

RESUMO

A osteoartrite (OA) é uma doença de etiologia multifatorial que envolve diartroses, caracterizada por estresse celular e degradação da matriz extracelular, associados a micro e macrolesões, que ativam respostas mal-adaptativas de reparação. O ranelato de estrôncio (SrRan) possui potencial de interferir na progressão da OA. O objetivo deste estudo foi testar os efeitos profilático e terapêutico do uso de SrRan na OA de joelho. No primeiro momento da pesquisa, realizou-se indução de OA de joelho, pela injeção intra-articular de monoiodoacetato de sódio. Trinta ratos Wistar foram divididos em cinco grupos com seis animais: grupo controle, sem intervenções; grupo que recebeu profilaticamente SrRan, via oral diária, de 25 mg/kg por 28 dias antes da indução de OA; grupos tratados com 25 ou 50 mg/kg/dia por 28 dias após a indução; e grupo que recebeu solução salina após a indução. Foram avaliados parâmetros clínicos de dor (incapacidade articular, hiperalgesia mecânica e atividade motora), nos dias zero, sete, 14, 21 e 28 após a indução. No segundo momento da pesquisa, 30 ratos Wistar foram distribuídos em grupos de forma semelhante à do primeiro momento, agora recebendo doses maiores de SrRan: o grupo profilático recebeu dose diária de 250 mg/kg por 28 dias antes da indução; e os grupos tratados receberam doses de 250 e 500 mg/kg/dia por 28 dias após a indução de OA; além do grupo controle e do grupo que recebeu salina. Foram realizados testes comportamentais, avaliação histológica e dosagem de citocinas inflamatórias no líquido sinovial (IL-6, IL-10, TNF-α e INF-γ). O uso de doses menores de SrRan não promoveu analgesia no modelo estudado. Com o aumento das doses, foi observada melhora no desconforto articular, tanto com utilização profilática, quanto terapêutica. O uso profilático e de doses de 500 mg/kg/dia também revelaram melhora da hiperalgesia mecânica. As dosagens de IL-6, IL-10, TNF-α e IFN-γ e a avaliação histopatológica dos grupos que receberam SrRan e do grupo com animais sadios demonstraram valores médios semelhantes. Este trabalho descreveu um possível efeito protetor do SrRan, tanto profilático quanto terapêutico, sobre a cartilagem articular, associado, provavelmente, a uma ação anti-inflamatória do fármaco, com efeito benéfico em parâmetros clínicos e morfológicos em modelo experimental de OA em ratos.

Palavras-chave: ranelato de estrôncio; osteoartrite; dor; inflamação.

ABSTRACT

Osteoarthritis (OA) is a multifactorial disease involving diarthrosis, characterized by cellular stress and degradation of the extracellular matrix, associated with micro and macrolesions, that activate maladaptive repair responses. Strontium ranelate (SrRan) has the potential to interfere with the progression of OA. The aim of this experiment was to test the prophylactic and therapeutic effects of the use of SrRan on knee OA. At the first moment of the research, an experimental protocol with induction of knee OA was performed by intra-articular injection of sodium monoiodoacetate. Thirty Wistar rats were divided into five groups with six animals: control group, without interventions; group which received prophylactically SrRan at a daily oral dose of 25 mg/kg for 28 days before induction of OA; groups treated with 25 or 50 mg/kg/day for 28 days after induction; and a group receiving saline after induction. Clinical parameters of pain, (joint incapacity, mechanical hyperalgesia and motor activity) were assessed at days zero, seven, 14, 21 and 28 after induction. In the second phase of the research, 30 Wistar rats were distributed in groups similar to the first one, now receiving higher doses of SrRan: the prophylactic group received daily dose of 250 mg/kg for 28 days before induction; and the treated groups received doses of 250 or 500 mg/kg/day for 28 days after induction of OA; besides the control group and the saline group. Behavioral tests, histological evaluation and dosage of synovial inflammatory cytokines (IL-6, IL-10, TNF-α and INF-γ) were performed. The use of smaller doses of SrRan did not promote analgesia in the model studied. As doses increased, an improvement in joint discomfort was observed, both with prophylactic and therapeutic use. The prophylactic use and doses of 500 mg/kg/day also showed improvement of mechanical hyperalgesia. IL-6, IL-10, TNF-α and IFN-γ dosages and histopathological evaluation for the groups receiving SrRan and the control group showed similar means. This work described a possible protective effect of SrRan, both prophylactic and therapeutic, on the articular cartilage, probably associated with an anti-inflammatory action of the drug, with beneficial effect in clinical and morphological parameters in experimental model of OA in rats.

Keywords: strontium ranelate; osteoarthritis; pain; inflammation.

1. INTRODUÇÃO

A maior compreensão acerca da fisiopatologia da osteoartrite (OA) tem feito com que o seu clássico conceito de doença degenerativa articular venha sendo gradativamente modificado, enfatizando fenômenos mecânicos e inflamatórios em sua gênese e manutenção (FELSON, 2013; BERENBAUM, 2013). Trata-se, portanto, de doença multifatorial, onde genética, padrão hormonal, fatores metabólicos e mecânicos, além do envelhecimento, interagem através de mecanismos moleculares complexos, regulando a biologia dos tecidos articulares (HERRERO-BEAUMONT et al., 2017).

A OA é caracterizada como condição que envolve diartroses, associada a estresse celular e degradação da matriz extracelular, provocados por micro e macro lesões que ativam respostas mal-adaptativas de reparação (KRAUS et al., 2015).

Dada a identificação da OA como uma relevante questão de saúde pública por sua prevalência crescente, várias estratégias farmacológicas ou não-farmacológicas têm sido estudadas e utilizadas para o manejo dessa entidade nosológica (NEOGI, 2013; REZENDE et al., 2013; LEMS; GEUSENS, 2014).

O ranelato de estrôncio (SrRan) tem figurado entre os fármacos com provável impacto sobre a progressão da OA (SMELTER; HOCHBERG, 2013; HAN et al., 2017). Seu mecanismo de ação possivelmente interfere no metabolismo do osso, corrigindo o desbalanço entre reabsorção e formação ósseas, pela interferência na atividade tanto doa osteoclastos, quando dos osteoblastos (ALMEIDA et al., 2016). Esses efeitos antirreabsortivo e anabólico ósseos podem promover melhora de sintomas álgicos relacionados à OA (FONSECA; BRANDI, 2010; NEOGI, 2013; LIU et al., 2013).

O estrôncio é um elemento fundamental semelhante ao cálcio e recebeu esse nome após sua descoberta na cidade de Strontian, na Escócia (FONSECA; BRANDI, 2010). As propriedades protetoras do estrôncio no osso foram sugeridas pela primeira vez em 1959, quando o lactato de estrôncio foi relatado como capaz de diminuir a dor e aumentar a densidade do osso avaliada radiograficamente, em pacientes com osteoporose (MCCASLIN; JANES, 1959). Dessa forma, sua utilização para tratamento

da osteoporose já ocorre há algumas décadas (MAEDA; LAZARETTI-CASTRO, 2014; LEMS; GEUSENS, 2014; O'DONNELL et al., 2016). Além de sua indicação terapêutica em osteoporose grave, especialmente em mulheres no climatério, tem sido descrita importante ação deste fármaco na prevenção de fraturas patológicas nessa população (REGINSTER et al., 2005; GIANNOTTI et al., 2013; CIANFEROTTI et al., 2013).

Por ser o ranelato de estrôncio uma medicação ainda com modesto número de estudos em OA, porém com possível impacto sobre a progressão desta doença, com indícios da sua ação tanto sobre a cartilagem articular, quanto sobre o osso subcondral, este estudo avaliou seus efeitos em modelo experimental de osteoartrite em ratos induzida pela injeção intra-articular de monoiodoacetato de sódio, a partir de avaliação clínica, radiológica e e dosagem de citocinas inflamatórias. O referido modelo pré-clínico comparou, ainda, um provável efeito de proteção à cartilagem articular pelo uso profilático, com a eventual ação terapêutica do referido fármaco sobre a OA.

2. REFERENCIAL TEÓRICO

2.1 Farmacologia do ranelato de estrôncio

2.1.1 Farmacocinética

O SrRan contém dois átomos de estrôncio, que é um cátion bivalente semelhante ao cálcio, além de uma metade orgânica chamada ácido ranélico, sendo este último uma molécula altamente polarizada e sem atividade farmacológica (MARIE, 2006; PILMANE et al., 2017) (Figura 1). O átomo em si tem uma forte afinidade pelo osso e, em certas condições, um metabolismo similar ao do cálcio. Porém, apesar da atração ao tecido ósseo, a integração do átomo ainda é baixa e teoricamente apenas um em cada dez átomos de cálcio podem ser substituídos pelo estrôncio (YU et al., 2013).

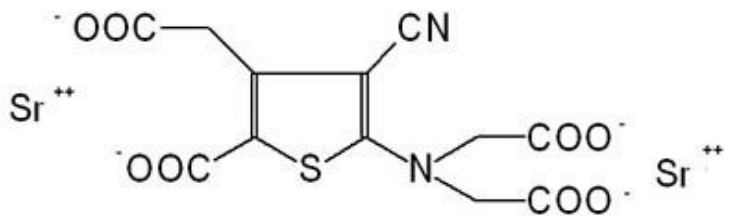

Figura 1: Representação da molécula de ranelato de estrôncio.

O SrRan é utilizado por via oral e absorvido no trato gastrointestinal, onde a molécula sofre dissociação entre sua parte orgânica e o cátion de estrôncio, que será posteriormente incorporado à superfície óssea. A integração dose-dependente do estrôncio ao osso aumenta a resistência deste tecido, alterando a estrutura e a composição dos cristais de apatita (MARIE et al., 2001; PILMANE et al., 2017).

A biodisponibilidade absoluta do estrôncio é de aproximadamente 25%, após ingestão de 2 g de SrRan, com concentrações plasmáticas máximas alcançadas em três a cinco horas depois da dose oral e o estado de equilíbrio ocorre em duas semanas de tratamento. O volume de distribuição é cerca de 1 L/kg e sua ligação às proteínas plasmáticas é considerada baixa (próxima de 25%). O SrRan não inibe as

enzimas do citocromo P450 e o cátion estrôncio não é metabolizado, sendo eliminado, independentemente da dose e do tempo de utilização, tanto pela via renal, quanto pelo trato gastrointestinal. Sua meia vida efetiva é de 60 horas (FONSECA; BRANDI, 2010).

2.1.2 Farmacodinâmica

Estudos com o SrRan sugerem que o fármaco pode promover tanto a formação óssea quanto a inibição da reabsorção. O exato mecanismo de ação da substância não está totalmente esclarecido, porém a regulação da diferenciação da célula óssea, a estimulação da proliferação dos osteoblastos e inibição da formação dos osteoclastos, com provável apoptose das células “maduras”, além da ativação de receptores sensíveis ao cálcio são colocados como possíveis mecanismos (BRENNAN et al., 2009; FONSECA; BRANDI, 2010). Demonstrou-se, ainda, que o SrRan tem potente efeito anabólico ósseo, por estimular, *in vitro,* a diferenciação de células-tronco mesenquimais e por ativar, *in vivo*, a via sinalizadora Wnt/β-catenina, contribuindo para o acúmulo da matriz extracelular (YANG et al., 2011). Desta forma, esta medicação pode ser classificada como um agente formador de osso.

O osteoblasto, como uma das mais importantes células do tecido ósseo, tem papel fundamental na formação do osso e, indiretamente, na modulação da diferenciação dos osteoclastos, pela expressão do ligante do receptor ativador do fator nuclear kappa-β (RANKL) e da osteoprotegerina (OPG), quando, juntamente com o receptor RANK, regulam a formação e a atividade osteoclástica. RANKL, uma proteína transmembrana altamente expressa em pré-osteoblastos, osteoblastos, células periosteais e osteócitos, é capaz de se ligar e ativar o receptor RANK, largamente presente na membrana dos osteoclastos e seus precursores. Após essa ligação, o RANKL estimula a formação, atividade e sobrevivência dos osteoclastos, resultando na reabsorção óssea. A OPG, por sua vez, tem alta afinidade pelo RANKL e compete pelo receptor RANK nos osteoclastos, impedindo a ligação e inibindo, por conseguinte, a osteoclastogênese. Mais ainda, esse sistema RANK/RANKL/OPG é regulado por várias citocinas pró-inflamatórias (interleucinas 1, 6, 11, 17 e TNF-α), hormônios (glicocorticoides, vitamina D e estrógeno) e outros, determinando a atividade dos osteoclastos (CAUDRILLIER et al., 2010; NARDONE et al., 2014)

(Figura 2). Outra via importante no processo de controle da osteogênese é a Wnt/β-catenina, que regula o gene da transcrição de proteínas importantes na função do osteoblasto, promovendo, em especial, a osteoblastogênese (BRENNAN et al., 2009; YANG et al., 2011; NARDONE et al., 2014).

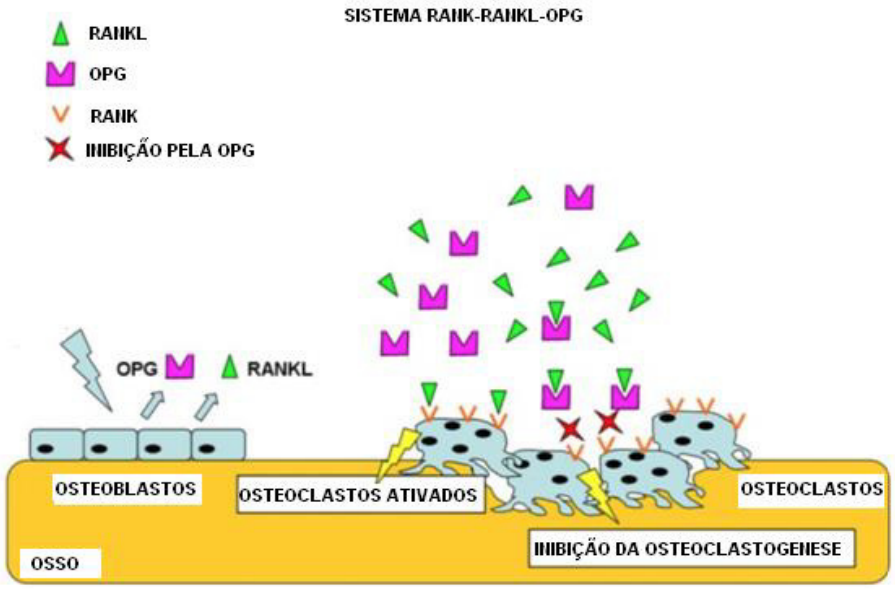

Figura 2. Representação esquemática do sistema RANK/RANKL/OPG no microambiente do osso. Adaptado de NARDONE et al., 2014.

Diretamente implicado nesse mecanismo, o SrRan está envolvido no aumento da produção de OPG e na redução da expressão de RANKL nos osteoblastos, dessa forma, intervindo na regulação negativa dos osteoclastos (BRENNAN et al., 2009; CAUDRILLIER et al., 2010). O SrRan possui, portanto, um efeito dual no metabolismo ósseo: promove regulação negativa no RNAm e nos níveis de proteínas indutoras de sinalização para a osteoclastogênese, a partir dos receptores sensíveis ao cálcio; por outro lado, tem efeito positivo na replicação, diferenciação e vida útil do osteoblasto (BRENNAN et al., 2009).

Os efeitos adversos mais comuns no uso do SrRan são náuseas e diarreia, que geralmente surgem no começo do tratamento e desaparecem após um período aproximado de três meses de uso. O fármaco também pode estar raramente relacionado a determinadas alterações fisiológicas graves e potencialmente letais,

como reações cutâneas, a exemplo da síndrome de Stevens-Johnson e necrose epidérmica tóxica (BERNABEI et al., 2014). Foi observado que os riscos do uso de SrRan parecem ser similares aos benefícios, e encontraram-se dados de efeitos adversos relevantes, como aumento do risco de tromboembolismo venoso, embolia pulmonar, infarto do miocárdio (BOLLAND; GREY, 2014). Assim, recomenda-se cautela na prescrição do fármaco a pacientes com hipertensão não controlada, histórico de doença cardíaca isquêmica, doença arterial periférica e doença cerebrovascular, indicando, nessas situações, o uso mais adequado dos bisfosfonatos, como alendronato, risendronato e zolendronato (REGINSTER, 2014; VESTERGAARD, 2014).

O estrôncio, como o cálcio, tem papel fundamental na eletrofisiologia do músculo cardíaco e anormalidades eletrocardiográficas são conhecidas consequências das variações plasmáticas deste elemento, da mesma forma o estrôncio tem um potencial efeito arritmogênico (TAUBEL et al., 2012). Entretanto, doses de 4g/dia têm se mostrado seguras, não apresentando repercussão eletrocardiográfica após uso pelo período de 15 dias; igualmente não foi descrita alteração no intervalo QT para a população em uso de dose 2g/dia (TAUBEL et al., 2012). Um estudo feito no Reino Unido não encontrou evidências para o risco aumentado de infarto do miocárdio com o uso de SrRan em mulheres em tratamento para osteoporose, quando comparadas à não utilização desse medicamento (COOPER et al., 2013). Em um estudo de coorte, o SrRan também não apresentou associação com o aumento do risco de síndrome coronariana aguda ou qualquer outra causa de mortalidade (SVANSTRÖM et al., 2014).

O SrRan não é aprovado pelo *Food and Drug Administration* (FDA) para uso nos EUA, porém a Agência Europeia de Medicamentos (EMA) referendou, por muito tempo, a utilização deste fármaco, tendo recentemente sua comercialização sido descontinuada pelo fabricante na Europa, considerando os efeitos adversos já descritos (REGINSTER, 2014; EMA, 2017).

A Agência Nacional de Vigilância Sanitária (ANVISA), por sua vez, mantém seu registro para tratamento de osteoporose grave em homens e mulheres, especialmente em casos em que outras medicações anti-osteoporose são inapropriadas (ANVISA, 2018). Publicações recentes, ainda buscando o melhor entendimento sobre os

mecanismos envolvidos na ação do SrRan, seguem agregando importantes informações sobre seus efeitos clínicos (CHU el al., 2017; RODRIGUES et al., 2017; ALVES et al., 2017; HAN et al., 2017; MIERZWA et al., 2017).

2.2 Uso do ranelato de estrôncio em osteoartrite

A osteoartrite é caracterizada pela degeneração da cartilagem e outras estruturas da articulação, incluindo a sinóvia e o osso subcondral. É a doença articular mais prevalente, estimando-se uma prevalência em torno de 40% em pessoas com mais de 65 anos. Ocorre mais frequentemente em mulheres, sendo diagnosticada basicamente com avalição clínica e radiográfica, tendo a dor como queixa mais frequente durante a consulta médica (REZENDE et al., 2013; REGINSTER et al., 2013). O padrão álgico varia entre os pacientes, de intermitente a constante, podendo ter caráter nociceptivo ou neuropático (NEOGI, 2013).

Dentre as situações fisiopatológicas que ocorrem na OA, a modificação tanto na estrutura quanto na função do osso subcondral inicia-se precocemente, implicando indiretamente em prejuízos para a cartilagem adjacente. Outro fator importante é que os condrócitos, assim como os osteoblastos e os osteoclastos, têm receptores sensíveis ao cálcio e participam de mecanismos físico-químicos semelhantes (TAT et al., 2010; TAT et al., 2011; YU et al., 2013).

A estrutura e a fisiologia da cartilagem articular do joelho, bem como os aspectos inflamatórios do processo degenerativo tem sido alvo de estudos (REZENDE et al, 2013). Entre os mediadores pró-inflamatórios destacam-se o fator de necrose tumoral alfa (TNF-α), a interleucina-1 (IL-1) e a interleucina-6 (IL-6), que possuem um papel essencial no desenvolvimento da dor na OA, assim como em outros eventos inflamatórios (REZENDE et al, 2013). Uma das primeiras citocinas liberadas após uma lesão tecidual é o TNF-α que desencadeia a liberação de IL-1β e IL-6, citocinas responsáveis pela estimulação da síntese de prostaglandinas e liberação de aminas simpáticas. Estas citocinas têm efeito catabólico e levam à destruição da cartilagem articular, pela indução da liberação de enzimas líticas, zinco-dependentes, conhecidas como metaloproteinases (colagenase, gelatinase, estromelisina), além da diminuição de produção de agentes inibitórios teciduais das metaloproteinases e dos inibidores

do plasminogênio (FITZGERALD et al, 2006). A IL-1β e o TNF-α inibem a síntese de componentes da matriz extracelular, sendo que a IL-1β suprime a síntese dos colágenos II e IX, que são constituintes próprios da cartilagem, além de aumentar a produção dos colágenos I e III, o que resulta em reparação tecidual deficiente (BERENBAUM, 2013).

O SrRan figura como um potente candidato para o uso na OA por atuar no osso subcondral, principalmente na via RANK/RANKL/OPG e pela modulação de metaloproteinases. Adicionalmente, estimula a produção do fator de crescimento semelhante a insulina (IGF-1) e potencializa o efeito deste sobre síntese de proteoglicanas nos condrócitos (TAT et al., 2011; YU et al., 2013; REGINSTER et al., 2013).

Avanços no entendimento da fisiopatologia da OA, relacionando-a a um estresse bioquímico ou biomecânico intra-articular anormal, e o esclarecimento das vias inflamatórias envolvidas permitiram que houvesse aumento considerável nos alvos terapêuticos. Assim, algumas medicações têm sido associadas à diminuição da progressão das lesões cartilaginosas, com redução da remodelação óssea subcondral (REZENDE; GOBBI, 2009; TAT et al., 2010; LANE et al., 2011; BIJLSMA et al., 2011; VARADY; GRODZINSKY, 2016). Tais medicações, denominadas como drogas modificadoras da doença osteoartrite (DMDOA), apresentam propriedade de reverter, estabilizar ou pelo menos retardar o curso da doença OA. Dentre as DMDOA de uso oral, merecem destaque a condroitina (HOCHBERG et al., 2008), a diacreína (FIDELIX et al., 2014), a glicosamina (KUCHARZ et al., 2016), a associação de glicosamina com condroitina (CLEGG et al., 2006) e a cloroquina (VUOLTEENAHO et al., 2005). Destacam-se, ainda, as de uso intra-articular, pela viscossuplementação com ácido hialurônico (REZENDE; CAMPOS, 2012).

Há uma constante busca por medicações com eficácia na redução da progressão da OA. Em tal cenário, o ranelato de estrôncio, já bastante difundido para uso na prevenção de fraturas e tratamento de osteoporose em mulheres pós-menopausa (MEUNIER et al., 2004; REGINSTER et al., 2005; MEUNIER et al., 2009), tem sido objeto de pesquisa para uso em OA (TAT et al., 2010; FONSECA; BRANDI, 2010; PELLETIER et al., 2012).

As primeiras observações do efeito clínico do uso do SrRan em OA são advindas de análises *post hoc* de ensaios randomizados com pacientes com diagnóstico primário de osteoporose. Estudos como o TROPOS (*Treatment of peripheral osteoporosis trial*) e o SOTI (*Spinal Osteoporosis Therapeutic Intervention trial*) demonstraram redução da progressão radiográfica de OA espinhal em mulheres com osteoporose e tratadas com SrRan, as quais apresentaram menores escores de dor após três anos de acompanhamento, apontando para um possível efeito modificador do SrRan sobre a doença. Ressalta-se, entretanto, que essas análises não mostraram diferença na qualidade de vida entre pacientes que usaram SrRan e aquelas que receberam placebo (MEUNIER et al., 2004; REGINSTER et al., 2005; BRUYÈRE et al, 2007; BRUYÈRE et al., 2014).

A avaliação do efeito do SrRan sobre a remodelação óssea subcondral também foi realizada em análise *post hoc* de ensaio clínico, incluindo mulheres com osteoporose, com ou sem diagnóstico concomitante de OA. Os níveis de CTX-II (*C telopeptide of type II procollagen*), um marcador urinário de degradação cartilaginosa, e de CTX-I (*C telopeptide of type I procollagen*), marcador sérico de reabsorção óssea, foram menores em usuárias de SrRan, assinalando uma ação protetora do fármaco sobre a matriz cartilaginosa articular (ALEXANDERSEN et al, 2010).

A maior pesquisa clínica desenvolvida, até então, especificamente em pacientes com OA foi o SEKOIA (*Strontium ranelate efficacy in knee osteoarthritis trial*), um estudo multicêntrico randomizado, duplo-cego, placebo-controlado com pacientes com OA de joelho, os quais foram tratados com SrRan (REGINSTER et al., 2013). Por três anos, 1683 pacientes, de ambos os gêneros, foram acompanhados e distribuídos em grupos que receberiam placebo, 1 g ou 2 g por dia de SrRan. O desfecho primário foi a avaliação de alterações radiográficas em relação ao basal. Os desfechos secundários foram a investigação de progressão radioclínica, análise de escores funcionais, de dor e avaliação das concentrações urinárias de CTX-II em intervalos semestrais. Os escores funcionais foram medidos pelo questionário WOMAC (*Western Ontario and McMaster Universities Osteoarthritis*), instrumento que mensura diferentes dimensões do estado de saúde de pacientes portadores de OA (com sub-escalas para dor, rigidez e função física), especialmente em joelho e quadril, e seus menores índices associam-se a uma melhor função articular e a reduzidos

níveis álgicos (BELLAMY et al., 1988). Os registros de dor foram feitos pela escala analógica visual. Foi observada menor progressão radioclínica em usuários de SrRan, especialmente em doses de 2 g/dia. Os escores de WOMAC e de dor só foram menores em doses diárias de 2 g de SrRan. Os usuários de SrRan também apresentaram menores níveis urinários de CTX-II, confirmando achados benéficos anteriormente descritos sobre o *turnover* de cartilagem articular (REGINSTER et al., 2013).

Os estudos pré-clínicos mostraram resultados variados em relação aos efeitos do uso do SrRan em OA. Em modelo de OA com zymosan, um potente indutor de expressão de COX-2, foi evidenciada redução da hipernocicepção, com doses de SrRan de 0.5, 5 e 50 mg/kg/dia, além de diminuição da expressão de TNF-α, sem alteração de contagem de leucócitos e das concentrações de IL1β, sugerindo, portanto, uma ação antinociceptiva por redução daquele mediador inflamatório (ALVES et al., 2017).

Foi relatada redução da progressão da OA com uso diário de 300 mg/kg de SrRan em ratas ooforectomizadas, associada à melhora da qualidade da matriz cartilaginosa por um estímulo direto sobre a síntese de proteoglicanos. Adicionalmente, registraram-se menores escores de OARSI (*Osteoarthritis Research Society International*), além de redução da expressão de caspase-3, enzima cuja expressão tem relação com apoptose celular. Esse efeito foi perdido com doses diárias de 625 mg/kg. A expressão de metaloproteinase 9 (MMP-9) não foi alterada com uso de SrRan e não se observou redução na produção de TNF- α (MIERZWA et al., 2017).

Por outro lado, a administração profilática e terapêutica de SrRan em doses diárias de 25 mg/kg e 50 mg/kg/dia não promoveu melhora da incapacidade articular em ratos com OA de joelho induzida com injeção intra-articular de monoiodoacetato de sódio (RODRIGUES et al, 2017).

O SrRan causou redução da liberação de TNF-α e IL-1β, promovendo analgesia em modelo de OA em ratos submetidos a transecção de ligamento cruzado anterior em OA. Além disso, foi observada reversão da analgesia promovida pelo SrRan nesses animais, com administração de naloxona, sugerindo um efeito opioide associado ao mecanismo de ação do fármaco (NUNES et al., 2015).

Uma redução na progressão de mudanças estruturais articulares também foi demonstrada com uso de SrRan em modelo experimental com cães submetidos a transecção de ligamento cruzado anterior e que receberam doses de 25, 50 ou 75 mg/kg/dia do fármaco. A expressão de genes de proteases de degradação osteocondral (como metaloproteinases e catepsina K) e IL-1β foi reduzida, especialmente com doses maiores do fármaco e por maiores períodos de tempo (PELLETIER et al., 2012).

Doses maiores de SrRan (de 625 e 1800 mg/kg/dia) foram testadas em ratos com OA induzida por lesão meniscal, evidenciando uma atenuação na degeneração articular, diminuição da apoptose de condrócitos, melhora importante da relação mineral óssea-colágeno e das propriedades mecânicas do osso subcondral. Assim, o tratamento com altas doses de SrRan apresentou resultados positivos sobre o controle da deterioração da cartilagem articular e do remodelamento ósseo subcondral (YU et al., 2013).

Resultados de pesquisa *in vitro* com culturas de osteoblastos subcondrais revelaram redução da expressão das metaloproteinases e aumento da síntese de OPG com administração de SrRan em concentração de 1 e 2 mM, além de aumento da expressão de RANKL e de suas isoformas. Enzimas associadas à clivagem do RANKL de membrana, como MT1-MMP (*membrane type-1 matrix metaloproteinase*), ADAM17 e ADAM19 (*a disintegrin and metaloproteinase domain 17 and 19*) não tiveram suas expressões alteradas (TAT et al., 2011).

Fundamentando-se nos dados científicos acima expostos, referentes à ação do ranelato de estrôncio, tanto na cartilagem articular, como na região subcondral, o presente estudo avaliou o impacto de sua utilização no perfil clínico em modelo experimental de OA em ratos e seus efeitos sobre o padrão histológico articular, visando ampliar o conhecimento sobre os mecanismos de ação deste fármaco, considerando os indícios de ação sobre o perfil inflamatório articular proporcionado por este fármaco.

3. OBJETIVOS

3.1 Geral

- Avaliar os efeitos do ranelato de estrôncio em modelo experimental de osteoartrite em joelhos de ratos.

3.2 Específicos

- Avaliar os efeitos profilático e terapêutico do ranelato de estrôncio sobre o grau de incapacitação articular, por meio de parâmetros clínicos;
- Verificar os efeitos profilático e terapêutico do ranelato de estrôncio sobre a lesão da cartilagem articular, por meio de análise histológica;
- Analisar os efeitos profilático e terapêutico na resposta inflamatória através da dosagem de citocinas (IL-6, IL-10, TNF-α e IFN-γ) no líquido sinovial.

4. RESULTADOS

4.1 CAPÍTULO I – ARTIGO I

TITLE: STRONTIUM RANELATE AS A POSSIBLE DISEASE-MODIFYING OSTEOARTHRITIS DRUG: A SYSTEMATIC REVIEW

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TITLE: STRONTIUM RANELATE AS A POSSIBLE DISEASE-MODIFYING OSTEOARTHRITIS DRUG: A SYSTEMATIC REVIEW

ABSTRACT

Considering that the osteoarthritis (OA) is the most prevalent joint disease worldwide, multiple pharmacological treatment have been proposed to alterate the articular structure with potential benefit in the progression of the disease. Those medicines are denominated as disease-modifying OA drugs (DMOAD) and are frequently investigated. However, they have not presented very conclusive findings. Strontium ranelate (SrRan) is a drug usually prescribed to treat osteoporosis, with proven effects of decreasing the risk of fractures and an indication of reducing the progression of osteoarthritis (OA). The objective of this review was to demonstrate the current panorama of knowledge on that subject, related to the use of SrRan in clinical and experimental models, aiming at the possibility of including SrRan in the group of DMOAD. The systematic review was based on the PRISMA statement and performed by selecting articles that are indexed in scientific databases. Fifteen studies were included: seven pre-clinical and eight clinical studies. Despite the limited number of studies, the results described suggest a positive effect of SrRan in patients with OA, through changes in functional capacity and reduction of progression of morphological parameters and joint degradation, with moderate quality of evidence for those clinical outcomes. More evidences are required, especially since most of them relate to one or a few randomized clinical trials. Novel studies are necessary to elucidate the molecular targets of SrRan, which will possibly lead to its inclusion in the group of OA-modifying medications, providing users with better quality of life with therapy applied.

Key words: Osteoarthritis; Strontium ranelate; Pain; Treatment; Prophylaxis

INTRODUCTION

Osteoarthritis (OA) is the most prevalent joint disease worldwide, which directly affects the performance of daily activities. Consequently, it increases the vulnerability and functional limitations of patients, contributing to the reduction of their well-being and quality of life (1). Thus, OA is a relevant public health problem, requiring special attention (1,2).

Of the several pathophysiological phenomena that occur in OA, modification in both the structure and function of the subchondral bone begins early, implying indirect damage to the adjacent cartilage. Another important factor is that chondrocytes, osteoblasts, and osteoclasts have calcium-sensitive receptors and participate in similar physicochemical mechanisms (3).

The structure and physiology of articular cartilage as well as the inflammatory aspects of the degenerative process have been well studied (3,4). Among the pro-inflammatory mediators, tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) all play essential roles in the development of pain in OA, and in other inflammatory events (4). Such mediators are responsible for stimulation of prostaglandin synthesis and release of sympathomimetic amines. These cytokines have a catabolic effect, leading to the destruction of articular cartilage by inducing the release of lytic, zinc-dependent enzymes, known as metalloproteinases (collagenase, gelatinase, stromelysin). Additionally, they decrease the production of tissue inhibitory agents of metalloproteinases and plasminogen inhibitors (5). IL-1β and TNF-α inhibit the synthesis of extracellular matrix components, with IL-1β inhibiting the synthesis of aggrecan and suppressing the synthesis of collagens II and IX (constituents of cartilage), besides increasing the production of collagen I and III, resulting in poor tissue repair (6). Regulatory factors of osteoclastic activity play a significant role in the natural history of OA, especially the osteoprotegerin-RANKL (receptor activator of nuclear factor kappa-B ligand) pathway (7,8).

In this context of complex mechanisms associated with the pathophysiology of OA, the search for optimal treatment for each stage of the disease has been challenging, with the objective of such studies being evaluations of non-pharmacological strategies (aimed at improving the functional state of the joint, postponing or avoiding surgical interventions) (9, 10), drug therapies (such as opioid

and non-opioid analgesics, anti-inflammatory drugs, chondroitin associated or not with glucosamine, diacerein, chloroquine, intra-articular hyaluronic acid, among others) (10-15), and surgical approaches for cases of clinical management failure (16).

Drugs with a probable effect on the alteration of the articular structure with potential benefit in the progression of the disease have been denominated as disease-modifying OA drugs (DMOAD) and are frequently investigated. However, they have not presented very conclusive findings (17). In such a scenario, strontium ranelate, already widespread for use in fracture prevention and treatment of osteoporosis in postmenopausal women (18,19), has shown favorable results that point to a probable use in the treatment of OA (3,20).

Considering the existing evidence of strontium ranelate action on both articular cartilage and subchondral bone, and the modest number of studies involving the action of this drug in OA, the objective of this present review was to demonstrate the current panorama of knowledge on the subject, related to the use of SrRan in clinical and experimental models, aiming at the possibility of, depending on the results, including strontium ranelate in the group of OA-modifying drugs.

MATERIAL AND METHODS

The present systematic review was conducted in accordance with the guidelines of PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyzes) for checklist and construction of the flowchart in four stages (identification, selection, eligibility, and inclusion) (21). A search was carried out for articles published in national and international journals indexed in the United States National Library of Medicine (PubMed), Scientific Electronic Library Online (SciELO), Science Direct and Biblioteca Virtual em Saúde/Centro Latino-Americano e do Caribe de Informação em Ciências da Saúde (VHL / BIREME), in September 2017.

The elaborate questioning used in the research was based on the acronym PICOS (*Patients/Intervention/Comparison/Outcomes/Study design*) (22). All *in vivo* and *in vitro* models of osteoarthritis as well as participants of all ages included in clinical trials were considered eligible. In this review, we listed all available dosages of SrRan administered orally for therapeutic and prophylactic purposes in

comparison with usual treatment for osteoarthritis or placebo. Regarding the outcome, we considered studies that evaluated the treatment by analysis of joint radiological alterations, besides those with histopathological analyses and inflammatory biomarkers. As for the design of the studies, original articles, both *in vivo* and *in vitro*, were considered as well as post hoc analyses of prospective studies. During the bibliographic research, the combination of descriptors and qualifiers, indexed in the Medical Subject Headings (MeSH) and Health Descriptors (DeCS), and certain free terms were used to construct the search strategy. The descriptors used were "arthritis" OR "osteoarthritis" AND "strontium ranelate" AND "treatment".

The inclusion criteria that were decisive for selecting the articles were as follows: presence of the descriptors chosen in the title of the study or inserted in the abstract; full-text articles available on the internet; publications in Portuguese or English; and studies published between January 2000 and August 2017. We excluded descriptive studies that did not provide accurate information about the method used and/or results obtained, as well as incomplete articles, reviews, editorials, comments, and studies that did not have the descriptors used in the search as the main object of the research as well as articles in a language besides Portuguese or English.After refinement of the research, duplicate studies were identified and excluded. All abstracts of the remaining articles were read. In cases where reading the abstract was not enough to establish whether the article should be included considering the defined inclusion criteria, the article was read in its entirety to determine its eligibility for subsequent inclusion in the study.

The included studies were submitted to a critical analysis by the authors of the review through reading, focusing on the method used and the instruments for evaluating the clinical manifestations of OA, as well as on the detail of the exposure of the results obtained with the interventions. The evaluated articles were presented in the form of tables, separated between experimental studies and clinical trials.

Considered as a positive event related to treatment, the reduced progression of the joint lesions radiologically evidenced in clinical trials, after the initial analysis of the studies, an evaluation of the patients was conducted if the studies generated these outcomes. To describe the quality of the evidence for this outcome, a GRADE (Grading of Recommendations Assessment, Development, and Evaluation) method

was adopted (23). By this method, through the investigation of factors such as study limitations, inconsistency of results, inaccuracies, and biases of publication, the quality of the evidence was classified into four levels: high, moderate, low, or very low.

RESULTS

The search in the databases resulted in 78 articles related to the descriptors. Of these, duplicate studies were excluded, leaving behind 43 studies. These studies had their abstracts read and, after a joint critical analysis of the authors, those that did not present as outcomes of the OA evaluation and did not test the effects of the SrRan, were removed, leaving behind 15 articles. These studies were read in full and divided between clinical trials and experimental studies (Figure 1). The results of the studies were summarized in tables (Tables 1 and 2). Evaluation of the quality of evidence of change in radiological progression with treatment with SrRan or placebo by the GRADE method is detailed in Table 3.

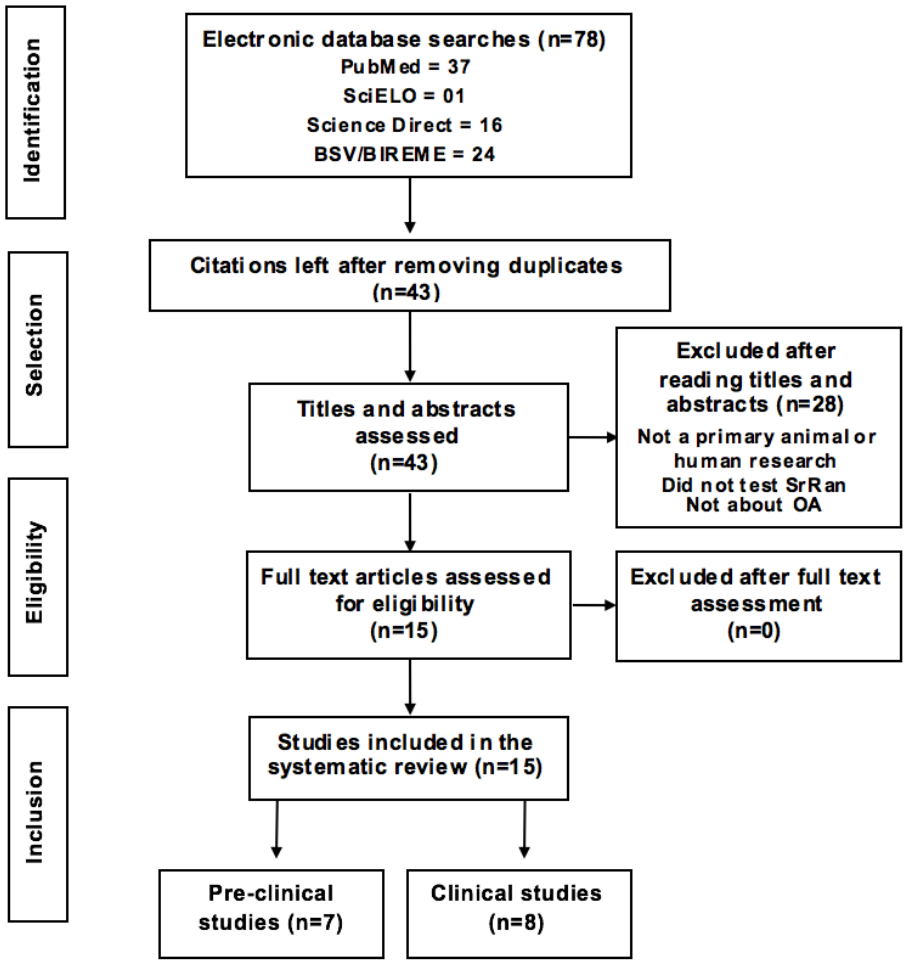

Figure 1. Systematic review flow diagram.

Table 1. Experimental studies of the use of strontium ranelate (SrRan) in osteoarthritis.

STUDY	OA MODEL	GROUP DESCRIPTION	TESTS USED	RESULTS
Rodrigues et al, 2017 (28)	Sodium MIA: 2 mg in the knee of Wistar rats	Five groups with 6 animals each: - SrRan 25 mg/kg/d prophylactically PO (28 days before induction); - SrRan 25 mg/kg/d for treatment (28 days after induction); - SrRan 50 mg/kg/d for treatment (28 days after induction); - 0.9 % saline; - Control: no intervention	Joint incapacitation (Weight bearing test); mechanical hyperalgesia (Randall Selitto test) and motor activity (Rotarod test) on days 0, 7, 14, 21 and 28.	SrRan did not promote analgesia in the prophylactic and treated groups at the doses tested.
Alves et al, 2017 (29)	Zymosan: 2 mg in TMJ of Wistar rats 1 h after treatment with SrRan	Seven groups with 6 animals each: - Sham: no induction and receiving 0.9 % saline PO; - Control: indomethacin 5 mg/kg SC; - Pretreated with zinc protoporphyrin IX 3 mg/kg, followed by SrRan 0.5 mg/kg/d after 30 min PO - 3 groups: SrRan 0.5, 5 and 50 mg/kg/d each - 1 group without treatment Period: 3 months	Von Frey test at the 4th hour after induction; Myeloperoxidase activity; Evans Blue Extravasation Assay; histopathological analysis; cytological evaluation for polymorphonuclear joint wash; immunohistochemical analysis and ELISA for TNF-α and IL-1β	Reduction of mechanical hypernociception with the use of SrRan, possibly by reduction of TNF-α expression in the periarticular tissue and trigeminal ganglion, without impact on the other inflammatory parameters evaluated.
Mierzwa et al, 2017 (30)	OVX in Wistar rats at 6 months of age.	Five groups with 10 animals each: - Control: OVX + saline; - SrRan300: OVX + SrRan 300 mg/kg/d PO; - SrRan625: OVX + SrRan 625 mg/kg/d PO; - MV: OVX + MV; -SrRan625 + MV: SrRan 625 mg/kg/d with MV Period: 3 months	Histological analysis of femoral articular cartilage using the OARSI score; quantitative evaluation of glycosaminoglycan sulfate and hyaluronic acid; immunohistochemical analysis for caspase 3, collagen type II, TNF-α and MMP-9	Increased expression of caspase-3 in MV group with SrRan 625 mg/kg/d. SrRan alone at a dose of 300 mg/kg/d reduced caspase-3 expression and OARSI score. No reduction of TNF-α expression.
Nunes et al, 2015 (31)	ACLT in Wistar rats and group with injection of zymosan 1 mg in knee.	Nine groups with 6 rats each: - Zymosan-induced group: 30 mg/kg 30 min before induction; - Zymosan-induced group: 300 mg/kg 30 min before induction; - Control: intra-articular saline and PO; - Naïve: only received saline PO; - ACLT + SrRan (A1): 300 mg/kg of SrRan between the 1st and 4th days; - ACLT + Saline (A2): saline between the 1st and 4th days; - ACLT + SrRan (B1) 300 mg/kg/day on the 14th and 16th days; - ACLT + Saline (B2) saline on the 14th and 16th days; - Sham: no transection + Saline. In addition, groups received naloxone 2 mg/kg SC 30 minutes prior to administration of SrRan.	PET in the forced ambulation test and Von Frey test at the 4th hour after induction with zymosan; PET at the 4th hour after administration on day 4 in groups A1 and A2; Von Frey test in the 0, 2nd, 4th and 6th hours of days 14 and 16 and only once a day on the other days in groups B1 and B2; Cytologic analysis and ELISA for TNF-α, IL-1β and CINC-1 of synovial fluid.	Reduction of PET with SrRan in zymosan-induced groups. Dose of 300 mg/kg/d decreased joint pain by the Von Frey test, with no change in influx of inflammatory cells to the joint compared to saline. SrRan increased paw withdrawal threshold in operated groups. Reversal of effects of SrRan with naloxone. Decreased TNF-α and IL-1β expression after zymosan-induced OA with SrRan.

Pelletier et al, 2012 (32)	ACLT in female dogs aged 1-3 years.	4 groups with 10 animals each: - Group 1: placebo; - Group 2: SrRan 25 mg/kg/d; - Group 3: SrRan 50 mg/kg/d; - Group 4: SrRan 75 mg/kg/d. The treatment was performed between the 4th and 12th week after induction.	Plasma and synovial fluid levels of SrRan. Macroscopic and microscopic classification (OARSI) of the lesions. Histomorphometric analysis of subchondral bone. PCR for quantification of MMP-1, MMP-13, ADAMTS5 and cathepsin K in cartilage and MMP-3 and IL-1β in the synovial membrane. ELISA for plasma CTX-II.	Reduction in the depth and size of the lesions of the groups treated with SrRan, as well as the disorganization of the articular cartilage collagen networks. Reduction of subchondral bone plaque thickness in the group treated with SrRan 50 mg/kg/d. Decreased mRNA levels for MMP-1, MMP-13 and cathepsin K in the articular cartilage with SrRan 75 mg/kg/d. Decreased expression of IL-1β in the synovial membrane at doses of 50 and 75 mg/kg/d and MMP-3 at all doses. Decreased plasma CTX-II levels with SrRan 50 (8^{th} week) and 75 mg/kg/d (16^{th} week).
Yu et al, 2013 (33)	Sprague Dawley rats submitted to MMT in knee	4 groups with 32 animals each: - Low dose: 625 mg/kg/d SrRan; - High dose: 1800 mg/kg/d SrRan; - MMT + saline group; - Sham + saline group; Half of the animals in each group were sacrificed in the 3^{rd} week and the other half in the 6^{th} week.	Histological analysis (OARSI), immunohistochemistry (TUNEL and for SOX9), micro-CT, microspectroscopy and nanoindentation.	SrRan at a dose of 1800 mg/kg attenuated joint degeneration at week 6, improved apoptotic chondrocyte indexes, increased SOX9 expression, improved microstructural abnormality indexes and increased joint elasticity compared to MMT + saline group.
Tat et al, 2011 (34)	Culture of osteoblasts from subchondral bone samples	Culture media containing 0.1, 1 and 2 mM SrRan Control group with subchondral bone sample of patients without OA.	PCR for quantification of MMP-2, MMP-9, OPG, RANKL3 and RANKL1. Flow cytometry for RANKL membrane, ELISA for OPG and Western blot for MT1-MMP, ADAM17 and ADAM19.	Reduction of MMP-2 and MMP-9 expression and increased OPG synthesis and expression with concentrations of 1 and 2 mM SrRan. Elevation of RANKL levels and reduction of RANKL-3 isoform at 2 mM SrRan concentration No effect on MT1-MMP and ADAM17 and ADAM19.

ACLT: Anterior cruciate ligament transection; ADAM: a disintegrin and metalloproteinase domain; ADAMTS5: a disintegrin and metalloproteinase with thrombospondin motifs; TMJ: Temporomandibular joint; CINC-1: cytokine-induced neutrophil chemoattractant; CTX-II: C telopeptide of type II procollagen; ELISA: Enzyme linked immunosorbent assay; IL1β: interleukin 1 β; MIA: sodium monoiodoacetate; micro-CT: computed tomography; MMP: matrix metalloproteinases; MMT: medial meniscal tears; MT1-MMP: membrane type-1 matrix metalloproteinase; MV: Mechanical vibration; OARSI: Osteoarthritis Research Society International index; OPG: osteoprotegerin; OVX: oophorectomy; PCR: polymerase chain reaction; PET: paw elevation time; PO: *per os*; mRNA: Messenger RNA; RANKL: receptor activator of nuclear factor kappa-B ligand; SC: subcutaneous; SOX9: sex-determining region Y – box 9; TNF- α: tumor necrosis factor α; TUNEL: transferase-mediated dUTP-TMR nick end labeling assay.

Table 2. Clinical trials of use of strontium ranelate (SrRan) in osteoarthritis.

STUDY	STUDY DESIGN	DESCRIPTION	OUTCOMES	RESULTS
SEKOIA TRIAL				
Reginster et al, 2013 (38)	Multicenter placebo-controlled double-blind randomized clinical trial, initially with 1683 patients diagnosed with knee OA.	Three groups: -Placebo (n=559) -1g/d SrRan (n=558) -2g/d SrRan (n=559) Follow-up period: Three years	- Primary outcome: radiographic changes in relation to baseline. - Secondary outcomes: radioclinical progression, WOMAC score, visual analog scale, and urinary CTX-II levels.	- Less radiological and radioclinical progression in users of SrRan. - WOMAC and pain scores were only lower with doses of 2 g/d over placebo. -CTX-II: lower levels with SrRan users - CPK transient increase
SEKOIA post hoc analysis				
Pelletier et al, 2015 (40)	Of the 1683 patients with OA in the SEKOIA trial, a subgroup of 330 submitted to Knee MRI at 12, 24 and 36 months of follow-up	Three groups of patients who performed MRI: -Placebo (n=113) -1g/d SrRan (n=105) -2g/d SrRan (n=112) Follow-up period: Three years	- Primary outcome: changes in the global volume of cartilage in the knee and in the medial and lateral compartments in 36 months. - Secondary outcome: changes in bone marrow lesions associated with OA	- Use of 2 g/d SrRan reduced the overall loss of cartilage volume in the tibial plateau at 12 and 36 months. -Medial compartment, doses of 1 g/d showed increased loss of cartilage. -Lateral compartment: 2 g/d of SrRan reduced cartilage volume loss in the tibial plateau from the first year of treatment, while 1 g/d doses reduced the loss after the second year of treatment. -Reduction of lesions in bone marrow with use of 1 g and 2 g/d of SrRan
Cooper et al, 2013 (41)	Analysis of radiological response to the treatment of SEKOIA trial patients.	Three groups: -Placebo (n=472) -1 g/d SrRan (n=445) -2 g/d SrRan (n=454) Follow-up period: Three years	Identification of responders to the treatment, in the criterion of reduction of the radiological progression of OA. Responders were divided into three levels of joint narrowing cut (≤ -0.1; -0.2; or -0.3 mm).	-For the three cuts of joint narrowing, there was a higher proportion of patients responding to SrRan 1g/d or 2g/d compared to placebo -NNT=13 for 1g/d and NNT=9 for 2g/d (for joint space reductions ≤ -0.3 mm).
Maheu et al, 2013 (42)	Of the patients included in the SEKOIA trial, 999 performed hand radiography to assess OA	Three groups: -Placebo (n=472) -1 g/d SrRan (n=445) -2 g/d SrRan (n=454) Follow-up period: Three years.	Secondary outcome of SEKOIA trial: radiographic and clinical evaluation of hand OA. Evaluation with radiological scores for hand OA (Kellgren-Lawrence; Kallman and Verbruggen) - Clinical evaluation by FIHOA and AUSCAN criteria.	-Discrete radiological progression of hand OA with placebo. No statistical difference for use of 1g and 2g/d. -Tendency to improve pain scores with use of SrRan 2g/d, especially in patients with severe hand OA.

Bruyere et al, 2014 (43)	Analysis of treatment response of the 1683 patients with OA in the SEKOIA trial	Three groups: -Placebo (n=472) -1 g/d SrRan (n=445) -2 g/d SrRan (n=454) Follow-up period: Three years.	Identification of responders to treatment, based on WOMAC scores, OMERACT-OARSI index and MPCI and MCII criteria.	-There was no effect on symptoms at doses of 1 g/d over placebo - At doses of 2 g/d there was an improvement in the response to the WOMAC pain score. Response above the MPCI threshold in WOMAC for pain, stiffness, and physical function and above the MCII threshold for physical function.
Roubille et al, 2015 (48)	Of the 1683 patients with OA in the SEKOIA trial, a subgroup of 330 submitted to knee MRI at 12, 24 and 36 months of follow-up	Three groups of patients who performed MRI: -Placebo (n=113) -1 g/d SrRan (n=105) -2 g/d SrRan (n=112) Follow-up period: Three years	Identification of meniscal extrusion or bone marrow lesion in the medial compartment of the knee.	Placebo group with more meniscal extrusion and/or bone marrow lesions, associated with greater reduction of joint space. Patients with 2 g/day had less loss of cartilage in cases of meniscal extrusion and/or bone marrow lesions.
OTHERS				
Bruyere et al, 2007 (36)	Post hoc analysis of patients in the TROPOS and SOTI trials who presented spine OA, in addition to osteoporosis.	1105 women with osteoporosis and spine OA (SOTI = 399 and TROPOS = 706) on 2 g/d of SrRan versus placebo for three years	Radiographic evaluation of narrowing of intervertebral lumbar spaces; presence of osteophytes and sclerosis. Low back pain (Likert scale in SOTI trial). Quality of life (SF-36).	Reduction of spinal OA progression with SrRan. Improvement of pain. No change in quality of life.
Alexandersen et al, 2010 (37)	Post hoc analysis of a randomized placebo-controlled study of 2617 postmenopausal women with osteoporosis who also had OA.	Use of SrRan 2 g/d or placebo for three years.	Evaluation of bone resorption (serum CTX-I) and cartilage degradation (urinary CTX-II).	SrRan reduced levels of CTX-I and CTX-II in the three years.

AUSCAN: Australian Canadian Questionnaire; CPK: creatine phosphokinase; CTX-I: C telopeptide of type I procollagen; CTX-II: C telopeptide of type II procollagen; FIHOA: Functional Index for Hand Osteoarthritis; MCII: minimal clinical important improvement; MPCI: minimal perceptible clinical improvement; NNT: number needed to treat; OA: osteoarthritis; OMERACT-OARSI: Outcome Measures in Rheumatology – Osteoarthritis Research Society International index; NMR: nuclear magnetic resonance; SEKOIA: Strontium ranelate Efficacy in Knee OsteoarthrItis triAl; SF-36: Medical Outcomes Study-36 questionnaire; SOTI: Spinal Osteoporosis Therapeutic Intervention trial; TROPOS: Treatment Of Peripheral Osteoporosis trial; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index.

Table 3. Radiological progression of osteoarthritis with strontium ranelate treatment at different doses *versus* placebo (GRADE method).

Certainty assessment						# of patients		Effect		Certainty
# of the studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirect evidence**	**Inaccuracy**	**Strontium ranelate**	**Placebo**	**Relative (95 % CI)**	**Absolute (95 % CI)**	
Treatment with 1 g of SrRan per day (follow-up: mean 36 months; evaluated with: radiological progression)										
1 Reginster et al, 2013 (38)	rando-mized clinical trials	serious [a]	not serious	not serious	not serious	899/1371 (65.6 %)	472/1371 (34.4 %)	RR 2.90 (-16.54 to -5.06)	**654 more per 1000**	⊕⊕⊕◯ MODERATE
Treatment with 2 g of SrRan per day (follow-up: median 36 months; evaluated with: radiological progression)										
2 Reginster et al, 2013 (38) **Bruyere et al, 2007 (36)**	rando-mized clinical trials	serious [b]	not serious	not serious	not serious	1020/2031 (50.2 %)	1011/2031 (49.8 %)	RR 3.00 (-13.34 to -1.66)	**996 more per 1000**	⊕⊕⊕◯ MODERATE

CI: Confidence interval; **RR:** Risk ratio.

a. The possibility of different phenotypes in osteoarthritis, suggesting that strontium ranelate might be more effective in certain subgroups of osteoarthritis patients, e.g, those with dominant subchondral bone changes

b. Many patients had prior osteoporosis and spinal osteoarthritis, with no data on peripheral OA being available

DISCUSSION

The present systematic review sought to analyze articles related to the use of SrRan in the management of OA, aiming to obtain state-of-the-art information for its use as a disease-modifying drug.

The protective properties of strontium in bone were first described in 1959 when strontium lactate was reported as capable of decreasing pain and increasing bone density assessed radiographically in a small study of patients with osteoporosis (24). Thus, its use for the treatment of osteoporosis, for example, has been occurring for some decades (25). SrRan contains two strontium atoms, which is a bivalent calcium-like cation, in addition to an organic moiety called ranelic acid, the latter being a highly polarized molecule with no pharmacological activity (26). The atom itself has affinity for the bone and, under certain conditions, a metabolism similar to that of Ca^{+2}. However, despite the attraction to the bone tissue, the atomic integration is still low and theoretically only one in ten Ca^{+2} atoms can be replaced by strontium (26).

The majority of studies on SrRan published so far involved patients with osteoporosis and promoted the developing knowledge on its probable clinical effects, which raised the hypothesis of its use in OA (18,19,27). Although the mechanism of action of SrRan has not yet been fully elucidated, it is possibly associated with an effect on bone metabolism, correcting the imbalance between bone resorption and bone formation observed in these osteoarticular conditions (7,8).

The preclinical studies reported in the present review have shown mixed results regarding the benefit of using SrRan in OA, especially regarding the variety of doses used and the multiple induction models employed. It is also worth noting that many positive results were obtained with administration of increased doses of SrRan (as 625 to 1800 mg/kg/day), unlikely to be transposed into clinical trials.

In a recent survey of rats that had knee OA induced by intra-articular injection of MIA (sodium monoiodoacetate), prophylactic administration of SrRan at daily doses of 25 mg/kg and post-induction use of this drug at doses of 25 and 50 mg/kg/day did not promote improvement in mechanical hyperalgesia (assessed by

the Randall Selitto test), joint incapacitation (assessed by Weight-bearing test) and motor activity (assessed by the Rotarod test) (28).

Additionally, OA models with zymosan, a potent inducer of COX-2 expression, were used in an experimental study in the temporomandibular joint. Clinical evaluation by Von Frey's test showed a reduction in hypernociception with SrRan doses of 0.5, 5, and 50 mg/kg/day. Furthermore, there was a decrease in TNF-α expression, with no change in leukocyte counts and IL-1β levels, suggesting an antinociceptive action by reducing that inflammatory mediator (29).

Oophorectomy has also been used for the induction of osteoporosis and osteoarthritis in rats by establishing early menopause. Either 300 or 625 mg/kg/day of SrRan associated with vibratory stimuli, or not, were used for histological investigation of articular cartilage quality, as well as immunohistochemical analysis for caspase-3, collagen type II, TNF-α, and MMP- 9 (matrix metalloproteinase-9) (30). It is worth mentioning that the expression of caspase-3 is related to cellular apoptosis. Metalloproteinases, in turn, are lytic enzymes responsible for extracellular matrix degradation; hence, also called matrixins. In this study, SrRan at a dose of 300 mg/kg/day was efficient in attenuating the progression of osteoarthritis, improving the quality of the cartilaginous matrix by a direct stimulus on the synthesis of proteoglycans, preserving the cellular viability in oophorectomized rats, with reduced expression of caspase-3 and lower OARSI (Osteoarthritis Research Society International) scores. This effect was lost with daily doses of 625 mg/kg administered along with mechanical vibration. The expression of MMP-9 was not altered with the use of SrRan. Contrary to what was found in a previous study, no reduction in TNF-α expression was observed in this study (30).

Another method used in preclinical studies for induction of OA is anterior cruciate ligament (ACLT) transection. One study analyzed paw elevation time (PET) and Von Frey test in groups of rats submitted to ACLT or zymosan induction, with subsequent use of SrRan at doses of 30 and 300 mg/kg/day. Additionally, cytological analysis and ELISA for TNF-α, IL-1β, and CINC-1 (cytokine-induced neutrophil chemoattractant) using the synovial fluid were performed. Reduction in PET was observed in zymosan-induced models receiving SrRan, whereas in rats subjected to ACLT, there was an increase in the paw withdrawal threshold at the

administered doses. It was suggested that SrRan promoted analgesia in the two OA models evaluated, associated with reduced release of cytokines TNF-α and IL-1β, but not CINC-1, at doses of 300 mg/kg/day. In the same study, reversal of analgesia promoted by SrRan with naloxone administration was observed, suggesting an opioid effect associated with the mechanism of drug action (31).

A reduction in the progression of joint structural changes was also demonstrated using SrRan in an experimental model with dogs submitted to anterior cruciate ligament transection and receiving doses of 25, 50 or 75 mg/kg/day of the drug. Effects such as decreased depth and size of joint lesions, in addition to greater preservation of the articular collagen network were observed by histomorphometric analysis. Expression of osteochondral degradation protease genes (such as metalloproteinases and cathepsin K) and IL-1β was reduced, especially with higher doses of the drug and for longer periods of time (32).

Higher doses of SrRan (625 and 1800 mg/kg/day) were tested in mice with OA induced by meniscal injury, demonstrating an attenuation in joint degeneration. The reduction of apoptotic chondrocyte indices was proven by the TUNEL method (transferase-mediated dUTP-TMR nick end labeling assay). Using computed micro-tomography to evaluate bone mineral density, there was an improvement in the abnormality indexes in the microarchitectures of the knees investigated. Microspectroscopy determined an increase in the mineral:collagen ratio with the use of SrRan. Additionally, it verified an increase in joint elasticity, through nanoindentation techniques, a dynamic test to determine the hardness of the materials. Increased expression of SOX-9 (sex-determining region Y – box 9), a transcription factor of fundamental importance in chondrogenesis, was also observed. Thus, treatment with high doses of SrRan presented positive results on the control of articular cartilage deterioration and subchondral bone remodeling (33).

Moreover, subchondral osteoblast cultures were used to investigate the action of SrRan on the bone resorption process by quantifying the expression of MMP-2, MMP-9, OPG (osteoprotegerin) and total RANKL and isoforms (34). Osteoblasts play a key role in promoting bone formation and, indirectly, modulating osteoclast differentiation through the expression of RANKL and OPG when, together with the RANK receptor, they regulate osteoclast formation and activity. RANKL is

a transmembrane protein highly expressed in pre-osteoblasts, osteoblasts, periosteal cells, and osteocytes, capable of binding and activating the RANK receptor, the latter widely present in the osteoclast membrane and its precursors. After this binding, RANKL stimulates the formation, activity, and survival of osteoclasts, resulting in bone resorption. OPG, on the other hand, has high affinity for RANKL and competes for the RANK receptor on osteoclasts, preventing binding, and therefore inhibiting osteoclastogenesis (7,8). The findings of such research revealed reduced metalloproteinase expression and increased OPG synthesis in osteoblast cultures of bones with OA concentration of 1 and 2 mM SrRan, in addition to increased expression of total RANKL and isoforms. Enzymes associated with membrane RANKL cleavage, such as MT1-MMP (membrane type-1 matrix metalloproteinase), ADAM17 and ADAM19 (a disintegrin and metalloproteinase domain 17 and 19), did not have their expression altered in the cultures with SrRan (34).

Although indications point to the potential benefits of SrRan in OA, its prescription for this purpose has not yet been approved by international control organizations such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), and the latter only allows it for the treatment of severe osteoporosis (35).

The first observations of the clinical effect of the use of SrRan in OA are derived from post hoc analyses of randomized trials with patients with a primary diagnosis of osteoporosis. Studies such as TROPOS (Treatment of peripheral osteoporosis trial) and SOTI (Spinal Osteoporosis Therapeutic Intervention trial) demonstrated a reduction in the radiographic progression of spinal OA in women with osteoporosis, with lower pain scores after three years of follow-up, pointing to a possible modifying effect of the SrRan on the disease. It should be noted, however, that such analyses did not demonstrate a difference in quality of life between patients who used SrRan and those who received placebo (36).

The evaluation of the effect of SrRan on subchondral bone remodeling was also performed in post hoc clinical trial analysis including women with osteoporosis, with or without concomitant diagnosis of OA. The levels of CTX-II (C telopeptide of type II procollagen), a urinary marker of cartilaginous degradation, and CTX-I (C

telopeptide of type II procollagen), serum marker of bone resorption, were lower in SrRan users, indicating a protective action on the articular cartilaginous matrix (37).

The largest clinical research ever developed specifically in patients with OA was SEKOIA (Strontium ranelate efficacy in knee osteoarthritis trial), a multicenter randomized, double-blind, placebo-controlled study with patients with knee OA who were treated with SrRan (38). For three years, 1683 patients of both sexes were followed-up and divided into groups that received placebo, 1 g or 2 g per day of SrRan. The primary outcome was the evaluation of radiographic changes from baseline. Secondary outcomes were the investigation of radioclinical progression, analysis of functional, pain, and urinary CTX-II scores at half-yearly intervals. Functional scores were measured by the WOMAC (Western Ontario and McMaster Universities Osteoarthritis) questionnaire, an instrument that measures different dimensions of the health status of patients with OA (with subscales for pain, stiffness, and physical function), especially in the knee and hip, and their lower indexes are associated with better algofunctional profiles (39). The pain records were made by visual analog scale. Lower radioclinical progression was observed in SrRan users, especially at doses of 2 g/day. The WOMAC and pain scores were only lower in users of 2 g/day doses of SrRan. Users of SrRan also had lower urinary CTX-II levels, confirming beneficial findings previously reported on articular cartilage turnover (38).

Several analyses were performed on subgroups of SEKOIA trial patients, giving greater weight to the evidence of the effect of SrRan on OA. An evaluation in SEKOIA patients was conducted in a subgroup that performed annual nuclear magnetic resonance, aiming to verify alterations in the global volume of cartilage of the knee and in its lateral and medial compartments (femoral and tibial components), in addition to bone marrow lesions associated to OA, demonstrating varied patterns in relation to the different regions of said joint. The daily use of 2 g of SrRan was related to a lower overall loss in articular cartilage volume, which was not observed in smaller doses in the medial component of the knee. In the lateral compartment, the loss of cartilage was reduced in the first and second years of patients receiving 2 g/day and from the second year in patients with doses of 1

g/day. Both doses were shown to be effective in decreasing bone marrow lesions related to OA (40).

Additional interpretations with radiography were also performed aiming at the identification of responders to SrRan treatment from the SEKOIA trial, based on the reduction of joint narrowing progression, with three cut levels (joint reduction ≤ - 0.1, - 0.2 or - 0.3 mm). Preservation of articular cartilage was observed in comparison with placebo, with NNT=13 (number needed to treat) with use of 1 g/day and NNT=9 with 2 g/day to promote joint space reductions ≤ - 0.3 mm (41).

Another subgroup of SEKOIA trial patients, submitted to hand radiography to assess OA in this joint component, showed a slight radiological progression in the placebo, with no statistical difference in the use of 1 g or 2 g/day. There was a trend toward lower pain scores with 2 g/day, especially in more severe cases of hand OA, determined through FIHOA (Functional Index for Hand Osteoarthritis) and AUSCAN (Australian-Canadian questionnaire) clinical classification, with the latter evaluating pain patterns, joint stiffness, and physical function (42).

In a study of response analysis, for the demonstration of magnitude of the clinical effect of SrRan in SEKOIA trial patients, WOMAC, OMERACT-OARSI (Outcome Measurements in Rheumatology – Osteoarthritis Research Society International) scales and MPCI (minimal perceptible clinical improvement) and MCII (minimal clinical important improvement) criteria were used for response analysis (43). OMERACT-OARSI criteria are internationally validated for response analysis of clinical trials in OA, evidencing effects on symptoms through dichotomous responses to specific questions (44,45). Other indexes used in analyzes of response to the WOMAC score are MPCI and MCII, which determine, respectively, the lowest values at which the patient begins to perceive clinical improvement and from which the patient classifies this improvement as important. These values have their thresholds previously determined (46,47). In this study, no effect on symptoms was observed for daily doses of 1 g of SrRan over placebo. Doses of 2 g/day led to better WOMAC scores for pain, in addition to a response above the MPCI threshold in the overall WOMAC score (for pain, stiffness, and physical function) and above the MCII threshold in the WOMAC score for physical function (43).

In SEKOIA patients, in whom meniscal extrusion and/or bone marrow lesion were identified in the medial knee compartment, there was a greater reduction of joint space and loss of cartilage when using placebo, in contrast to the use of 2 g/day of SrRan, which reduced the progression of OA, with less loss of cartilage in the medial plateaus. Such findings are relevant because they reinforce SrRan's protective effect of articular cartilage, even in cases of greater severity, with meniscal lesions and already established subchondral bone remodeling (48).

Despite the limited number of studies available, the results described in this review suggest a positive effect of the use of SrRan in patients with OA, through changes in functional capacity and reduction of progression of morphological parameters and joint degradation. Moderate quality of evidence for this outcome was observed, possibly due to diversity of OA phenotypes, in addition to the differences among the patients included in the analysis of this endpoint. This property attributed to SrRan is compatible with its pharmacological effect obtained through experimental studies, by improving the quality of the cartilaginous matrix and viability of the chondrocytes, as well as endpoints involving hypernociception and joint discomfort. However, more evidences are required, especially since most of them relate to one or a few randomized clinical trials. It is necessary to reinforce the signs of articular action of SrRan through novel studies to elucidate the molecular targets of this drug, which will possibly lead to its inclusion in the group of OA-modifying medications, providing users with better quality of life with therapy applied.

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4.2 CAPÍTULO II – ARTIGO II

TITLE: EFFECT OF STRONTIUM RANELATE ON PAIN BEHAVIOR IN AN EXPERIMENTAL MODEL OF OSTEOARTHRITIS

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ABSTRACT

Strontium ranelate (SrRan) is a drug usually prescribed to treat osteoporosis, with proven effects of decreasing the risk of fractures and an indication of reducing the progression of osteoarthritis (OA). This study aimed to investigate the effects of SrRan as either a prophylactic or a treatment drug, using an OA rat model to assess pain behavior. A monoiodoacetate (MIA)-induced knee joint OA model in Wistar rats was used. Thirty Wistar rats (both sexes, 60 days old) were distributed in five groups of 6 rats each: the control group, that received no intervention; a prophylactic group, that received oral administration of 25 $mg \cdot kg^{-1} \cdot day^{-1}$ of SrRan for 28 days before induction of OA; a group treated with 25 $mg \cdot kg^{-1} \cdot day^{-1}$ of SrRan for 28 days after OA induction; a group treated with 50 $mg \cdot kg^{-1} \cdot day^{-1}$ during 28 days after OA induction; and a group that received oral saline for 28 days after induction. The assessment of pain behavior was performed considering articular incapacitation (weight-bearing test), mechanical hyperalgesia (Randall Selitto test) and motor activity (rotarod test), on days 0, 7, 14, 21, and 28. This experiment did not yield a significant difference when comparing the group that received SrRan prophylactically with the groups treated with 25 or 50 $mg \cdot kg^{-1} \cdot day^{-1}$ and the group that received oral saline. Thus, SrRan did not provide analgesia in either treated rats or as a prophylactic drug with the tested doses. Higher doses should be tested further to achieve possible significant results.

Key words: Osteoarthritis; Strontium ranelate; Pain; Treatment; Prophylaxis.

INTRODUCTION

Advances in the understanding of the pathophysiology of osteoarthritis (OA), such as the influence of biochemical stress or abnormal intra-articular biomechanics, and the inflammatory pathways involved, have allowed for a considerable increase in therapeutic targets for the disease. Some medications have been associated with reduction of cartilaginous lesions and decreased subchondral bone remodeling, changing the progression of OA (1-5). These drugs, known as disease-modifying osteoarthritis drugs (DMOADs), present the properties of reversing, stabilizing, or at least delaying the course of OA. Oral DMOADs of note include chondroitin (6), diacerein (7), glucosamine (8), glucosamine combined with chondroitin (9), and chloroquine (10). Among the intra-articular treatments, viscosupplementation with hyaluronic acid (11) is also worth highlighting.

Studies have been conducted to increase the number of medications that effectively reduce the progression of OA. The drug strontium ranelate has shown promising results in the prevention of fractures and treatment of osteoporosis in postmenopausal women (12-14), indicating its probable utility in the treatment in OA (2 3 4,15-17).

Strontium (Sr, group II of the periodic table with atomic number = 38) is a fundamental element. Its nucleus is similar in size to calcium, making it easily absorbed, carried, and incorporated into bones, as is calcium (15). Its ability to decrease pain and increase bone density was first assessed radiographically in 1959, in a small study including patients with osteoporosis treated with strontium lactate (18). Thus, it has already been used in the treatment of osteoporosis for decades (19).

Currently, strontium ranelate (SrRan - C12H6N2O8SSr2) is indicated for the treatment of severe OA and osteoporosis, especially in postmenopausal women with a high risk of fractures (16,19–21). It has also been postulated that SrRan inhibits the resorptive activity of osteoblasts, thus reducing the synthesis of metalloproteinase. In addition, it modulates the osteoprotegerin-RANKL (receptor activator of nuclear factor kappa-B ligand) signaling pathway, and inhibits osteoclastic differentiation (15). SrRan has also been associated with the formation

of cartilaginous matrix. Recent data show that it can reduce the progression of radiological findings in spinal OA, along with leading to improvement of lower back pain in women with osteoporosis (15).

Although SrRan shows the ability to reduce the progression of OA, few relevant studies have been published to date. Therefore, the present study aimed to test the effects of SrRan in an experimental animal model of OA induced by intra-articular injection of sodium monoiodoacetate (MIA). Clinical assessment of motor activity, articular incapacitation, and mechanical hyperalgesia was conducted.

MATERIAL AND METHODS

This study was conducted in the Experimental Laboratory for Pain Study (LEED) following approval from the Animal Ethics Committee of the Universidade Federal do Maranhão (CEUA-UFMA No. 23115.012456/2016-4).

Animals

Thirty Wistar rats, *Rattus norvegicus* species (albino variety), were used in the study. The animals were male and female adults, approximately 60 days old. This study did not aim to evaluate differences between genders. The rats were obtained from the Central Animal Facility of the Universidade Federal do Maranhão. The animals remained in cages and were housed at the LEED lab, where they were fed standard chow and water *ad libitum* and maintained under controlled conditions of light and temperature.

Experimental design

The animals were divided into five groups (PROF25, SR25, SR50, SAL, and Control), with 6 rats each. Group PROF25 (prophylactic group) began treatment with SrRan 4 weeks prior to the induction of OA with sodium MIA. SrRan was administered in a dose of 25 mg/kg by gavage, once daily in the morning, two hours before the subsequent feeding. Groups SR25 and SR50 (treatment groups) received 25 mg/kg and 50 mg/kg of SrRan, respectively, by gavage, once daily in

the morning, 2 h before subsequent feeding, for a period of 4 weeks. Group SAL received 0.9% saline solution by gavage after OA induction. The day of OA induction in groups PROF25, SR25, SR50 and SAL was considered day zero (D-0). The Control group did not undergo OA induction nor received intervention. The administered doses in that model were chosen based on previously described animal models using SrRan (15). Throughout the experiment, all groups were periodically evaluated for articular incapacitation, motor activity, and mechanical hyperalgesia on days 0, 7, 14, 21, and 28, as described below.

Model of osteoarthritis induced by sodium monoiodoacetate

To induce OA, the animals were anesthetized using an intraperitoneal injection of 40 mg of sodium thiopental. The joint injury was induced with a single intra-articular injection of 2 mg of sodium MIA into the right knee, diluted in a maximum volume of 25 µL solution (22,23).

Evaluation of motor activity - forced ambulation (rotarod test)

The animals were placed on a rotarod (IITC Life Science, USA) at a speed of 16 rpm for a period of 300 s. The use of the affected limb was evaluated through forced ambulation. The use of the affected paw was graded by a subjective measure, on a numerical scale ranging from 5 to 1, in which: 5=normal use of the paw; 4=mild limping; 3=severe limping; 2=intermittent disuse of the affected paw; 1=complete disuse of the affected paw (24).

Incapacitation test - distribution of weight on the hindpaws (weight bearing test)

The animals were placed in a glass chamber, angled and positioned so that each hindpaw rested on a different platform. The weight exerted on each hindpaw (measured in grams) was evaluated over a period of 5 s. The final weight distribution was calculated using the average of three measurements. The variations in distribution of the weight on the hindpaws were calculated using the following formula:

$$\text{Weight distribution } (\%) = \frac{APW}{APW + CPW} \; X \; 100$$

where APW was affected paw weight and CPW was contralateral paw weight.

Mechanical hyperalgesia (Randall Selitto test)

The Randall Selitto test is a useful test to evaluate the hypernociception, based on the induction of hyperalgesia by the increasing paw pressure using a special device. Mechanical hyperalgesia was assessed using the nociceptive paw-withdrawal threshold (NPWT) to mechanical pressure using an analgesy-meter (IITC Life Science) (25,26) in both paws, and then calculating the mean of three measures. The paw withdrawal reflex is considered representative of the hypernociceptive threshold. The NPWT was recorded in grams for both paws, and then the percentage was calculated using the results of affected and contralateral paws, with the following formula:

$$\text{NPWT } (\%) = \frac{NAPWT}{NAPTW + NCPWT} \; X \; 100$$

where NAPWT is nociceptive affected paw-withdrawal threshold and NCPWT is nociceptive contralateral paw-withdrawal threshold.

Statistical analysis

Comparison of the means of different experimental groups was performed using the Student's *t*-test or univariate analysis of variance (one-way ANOVA), followed by the Bonferroni's test. A value of $P<0.05$ was considered indicative of significance and the data obtained were analyzed using the GraphPad Prism® software, version 6.00 for Windows (USA).

RESULTS

Evaluation of articular incapacitation

The analysis of data regarding articular incapacitation, assessed using the weight bearing test, demonstrated that the OA induction was effective, as we observed a statistically significant difference between the OA group that received saline (group SAL) and Control group (healthy animals that received saline). A statistically significant difference was observed between the PROF25 (prophylactic) group and the Control group ($P<0.05$). However, no difference was observed between groups PROF25 and SAL. These results showed that the animals that received SrRan prior to OA induction did not approach the healthy standard of the Control group, nor were they significantly different from those that received saline (Figure 1).

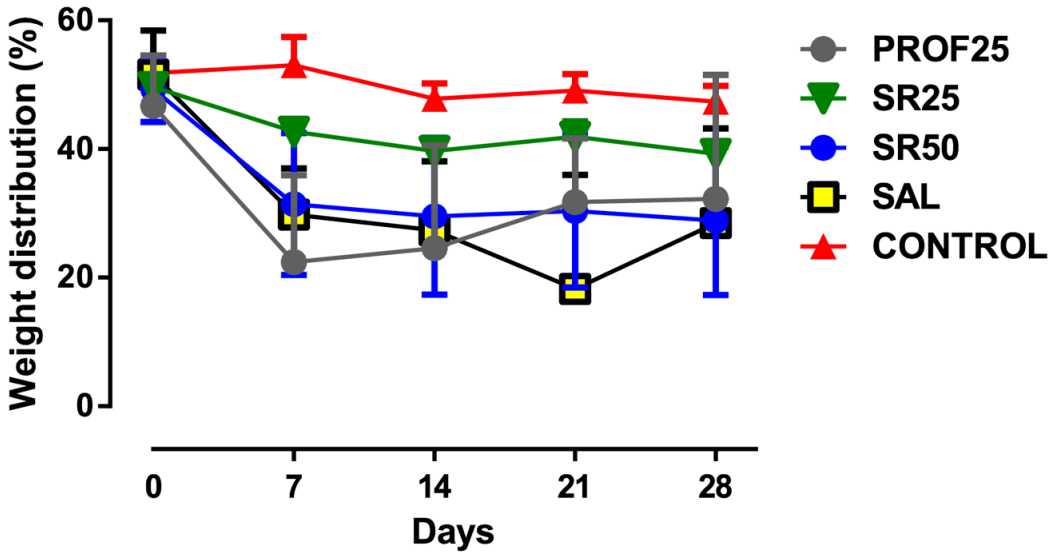

Figure 1. Evaluation of articular incapacitation using the weight bearing test. Group PROF25: administration of prophylactic 25 mg/kg of strontium ranelate (SrRan) 4 weeks prior to the induction of monoiodoacetate (MIA)-induced osteoarthritis (OA); Groups SR25 and SR50 (treatment groups) received 25 mg/kg and 50 mg/kg of SrRan, respectively, after OA induction for a period of 4 weeks; SAL: MIA-induced OA receiving only saline; Control group: no treatment and no OA induction. Results are reported as means±SD. $P<0.05$ between groups PROF25, SR25 and SR50, and Control; $P>0.05$ between those groups and SAL (one-way ANOVA, followed by the Bonferroni's test).

We observed a difference between the groups that received 25 and 50 mg/kg SrRan (SR25 and SR50), and the group of healthy animals (Control). These groups did not significantly differ from group SAL (Figure 1).

Evaluation of motor activity/forced ambulation

The rotarod test showed that the OA induction was effective, resulting in a statistically significant difference between groups SAL and Control. We also observed a statistically significant difference between the PROF25 and the Control groups. The same difference was found between the groups that received treatment with SrRan (SR25 and SR50) and the Control group. A difference was not observed between groups PROF25 and SAL, SR25 and SAL, or SR50 and SAL. These findings indicated that there was no change between the animals receiving SrRan and those receiving only saline (Figure 2).

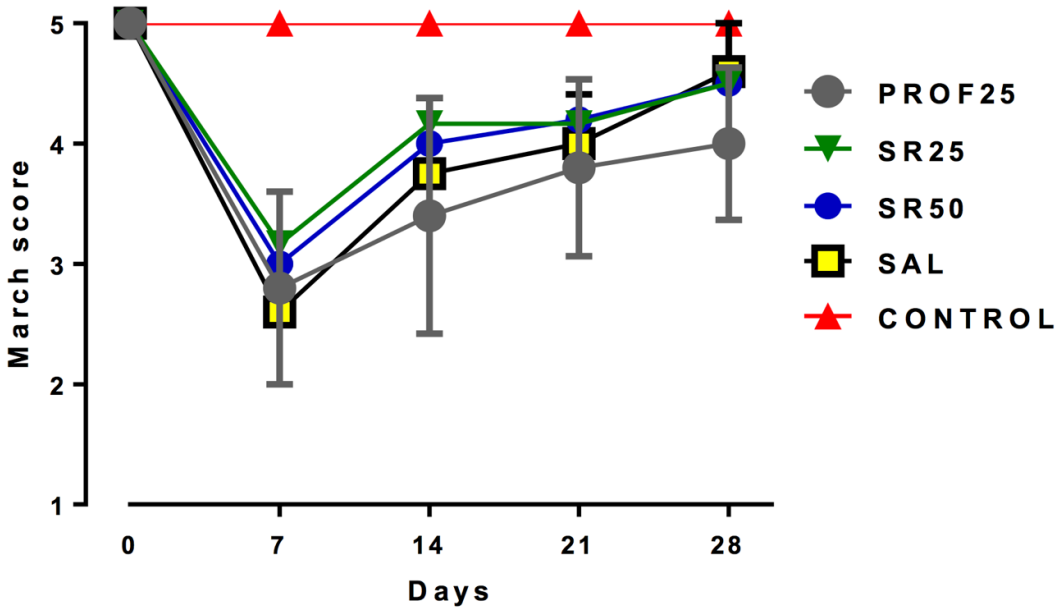

Figure 2. Evaluation of motor activity/forced ambulation using the rotarod test. Group PROF25: administration of prophylactic 25 mg/kg of strontium ranelate (SrRan) 4 weeks prior to the induction of monoiodoacetate (MIA)-induced osteoarthritis (OA); Groups SR25 and SR50 (treatment groups) received 25 mg/kg and 50 mg/kg of SrRan, respectively, after OA induction for a period of 4 weeks; SAL: MIA-induced OA receiving only saline; Control group: no treatment and no OA induction. Results are reported as means±SD. $P<0.05$ between groups PROF25, SR25 and SR50, and Control; $P>0.05$ between those groups and SAL (one-way ANOVA, followed by the Bonferroni's test).

Evaluation of mechanical hyperalgesia

Evaluation of hyperalgesia by means of the Randall Selitto test also showed that the OA induction was effective, with a statistically significant difference observed between groups SAL and Control. A statistically significant difference was also found with this test between the prophylactic group and the healthy group. In addition, a difference was observed when comparing the groups treated with SrRan (SR25 and SR50) and the group consisting of healthy animals (Control). We did not observe any difference between the prophylactic group, groups receiving treatment, and group SAL (Figure 3).

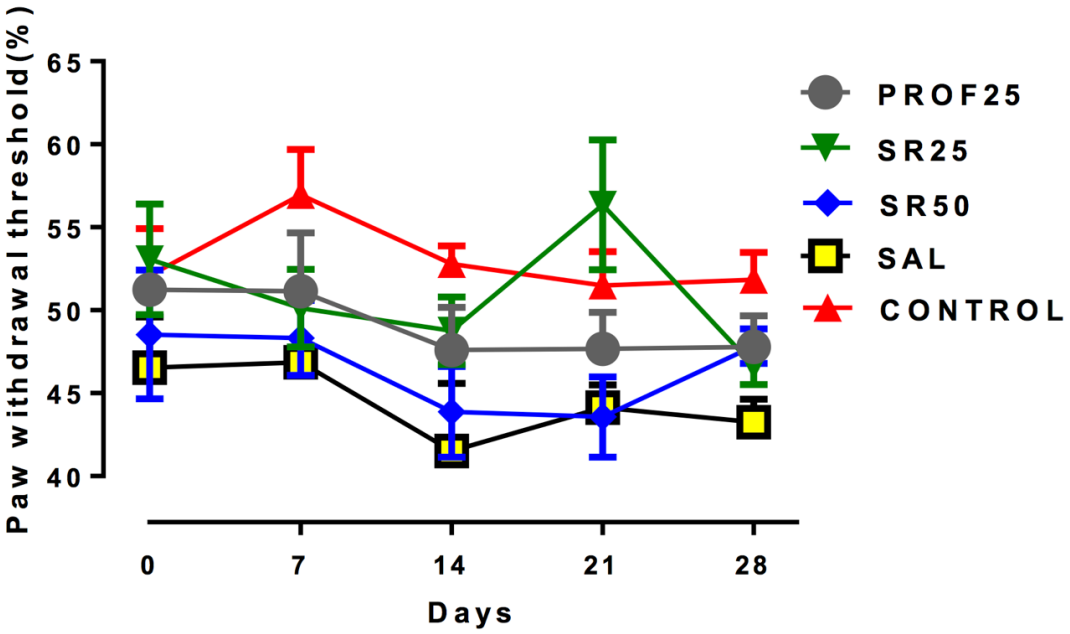

Figure 3. Evaluation of mechanical hyperalgesia using the Randall Selitto test. Group PROF25: administration of prophylactic 25 mg/kg of strontium ranelate (SrRan) 4 weeks prior to the induction of monoiodoacetate (MIA)-induced osteoarthritis (OA); Groups SR25 and SR50 (treatment groups) received 25 mg/kg and 50 mg/kg of SrRan, respectively, after OA induction for a period of 4 weeks; SAL: MIA-induced OA receiving only saline; Control group: no treatment and no OA induction. Results are reported as means±SD. $P<0.05$ between groups PROF25, SR25 and SR50, and Control; $P>0.05$ between those groups and SAL (one-way ANOVA, followed by the Bonferroni's test).

DISCUSSION

The present study assessed pain behavior in an experimental model of OA, using a drug that has been studied as a potential pharmaceutical to be included in the class of DMOADs.

At the administered doses and with the tests employed, there was no observed improvement with the use of SrRan in cases of established OA. This outcome differs from the findings of another study in which OA was induced in rats by zymosan. In that case, the animals were treated with higher doses of SrRan than in our study, ranging from 30 to 300 mg/kgday for a shorter period of time (27). The difference could be probably attributed to the different model applied, with higher doses used in that study.

The choice of the SrRan doses administered in our study was based on a study with dogs that underwent OA induced by the section of the anterior cruciate ligament. The doses used were 25, 50, or 75 mg/kg for a longer period (12 weeks, beginning 4 weeks after the surgery) than in our study (15). That study was the first to demonstrate *in vivo* the effect of SrRan in reducing the OA progression. The main differences between our study and the above one were the OA induction method and the duration of the treatment. Such differences could have influenced the varying results between the studies.

The prophylactic effect of SrRan was not shown in this experimental study using a model of MIA-induced OA. We did not observe a reduction in articular incapacitation after OA induction, nor was there an improvement in the motor response in the group that received 25 mg/kg SrRan for 1 month prior to OA induction. Otherwise, the prolonged prophylactic use of SrRan has already been shown to be associated with a reduced fracture risk in some clinical trials, especially in postmenopausal women with osteoporosis (13,21). It has been suggested that this prophylactic effect is due to an anti-resorptive and pro-formation action in bone metabolism processes (14,21).

The exact mechanism of action of the substance is not entirely clear (17). However, the regulation of bone cell differentiation, the stimulation of osteoblast proliferation, and the inhibition of osteoclast formation with probable apoptosis of

"mature" cells have been suggested as possible mechanisms, in addition to the activation of receptors sensitive to calcium – the latter being the most probable (17,19,28 29–30). It has also been shown *in vitro* that SrRan increases the synthesis of collagen and non-collagenous proteins, improves the proliferation of pre-osteoblast cells, and that it should, therefore, be classified as a bone-forming agent (20).

At the doses used in the present study, SrRan did not promote analgesia in the treatment and prophylactic groups. There was no improvement in pain behavior in the animals studied, with no impact on articular mobility, motor activity, or mechanical hyperalgesia in comparison to the control group. This finding could be related to the doses used in this experimental model, which were smaller than those used in other studies on this drug, varying from 300 to 625 mg/kg (27,31). That could probably be a limitation of our study, including the time period of medication usage. Experimental improvements were obtained with higher doses (27,31) and for longer periods (15,31). This experimental model determined that additional studies examining the use of SrRan in the treatment of OA are required, particularly investigations using higher doses of this drug.

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4.3 CAPÍTULO III – ARTIGO III

TITLE: PROPHYLACTIC AND THERAPEUTIC USE OF STRONTIUM RANELATE REDUCES THE PROGRESSION OF EXPERIMENTAL OSTEOARTHRITIS.

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TITLE: PROPHYLACTIC AND THERAPEUTIC USE OF STRONTIUM RANELATE REDUCES THE PROGRESSION OF EXPERIMENTAL OSTEOARTHRITIS

Abstract

Strontium ranelate (SrRan) has the potential to interfere in the progression of osteoarthritis (OA), multifactorial disease associated with mechanical problems and articular inflammatory changes. This study aimed to test the effects of prophylactic and therapeutic use of SrRan on clinical parameters of pain, the inflammatory process, and degradation of the articular cartilage. This was an experimental study, using a model of knee OA induced by intra-articular injection of monoiodoacetate. Thirty Wistar rats were divided into five groups and treated as indicated: control, without intervention; prophylactic, received SrRan at a daily oral dose of 250 mg/kg for 28 days before OA induction; SrRan treatments, administered 250 or 500 mg/kg/day for 28 days after the induction; and model control, received saline solution after the induction. Behavioral tests (joint incapacity, mechanical hyperalgesia, tactile sensitivity, and forced ambulation), histological evaluation of articular cartilage, and determination of inflammatory cytokines in the synovial fluid (interleukin [IL]-6, IL-10, tumor necrosis factor [TNF]-α, and interferon [INF]-γ) were performed. Both prophylactic and therapeutic treatments improved the articular discomfort. A prophylactic dose of 500 mg/kg/day also improved mechanical hyperalgesia and the same dose was beneficial on tactile sensitivity. SrRan did not improve ambulation. Levels of IL-6, IL-10, TNF-α, and IFN-γ in SrRan-treated groups with OA were similar when compared with those in the normal control animals. The histopathological evaluation showed less articular damage in the SrRan-treated and control groups compared to the saline-treated group. The prophylactic and therapeutic administration of SrRan was associated with improved behavioral patterns of pain, especially joint discomfort. SrRan administration mitigated histological changes in the articular cartilage and reduced the inflammatory process, which beneficially reduced the progression of OA in the experimental model studied.

Keywords: strontium ranelate; osteoarthritis; pain; treatment; inflammation.

1. Introduction

A greater understanding of the pathophysiology of osteoarthritis (OA) has gradually transformed the classic concept of articular degenerative disease, emphasizing mechanical and inflammatory phenomena in its pathogenesis and progression (Berenbaum, 2013; Felson, 2013). OA is characterized by cellular stress and degradation of the extracellular matrix, caused by macro- and micro-injuries that activate maladaptive repair responses (Kraus et al., 2015).

The emergence of OA can be related to a response to mechanical insults, leading to an abnormal increase of forces in certain areas of the joints. Congenital or acquired anatomical alterations and joint overload are commonly associated with both increased static and dynamic local stress, generating bad alignment and related injuries. Consequently, the articular tissues lose their ability to adequately support the loads imposed on them (Felson, 2013).

Besides cartilage, other joint components including synovial fluid, ligaments, and adjacent bone tissue play relevant roles in the pathophysiology of OA. The importance of the subchondral bone in the pathogenesis and development of OA has been recently highlighted, suggesting that it interferes with chondrocyte metabolism (Funck-Brentano and Cohen-Solal, 2015).

The most accepted proposed mechanism implicates vascular changes in the subchondral bone in changes in the cartilaginous matrix, through the reduction of intra-osseous perfusion and consequent hypoxia (Aaron et al., 2017). Furthermore, the consequent physical-chemical disturbances are capable of generating responses by osteoblasts with the production of a modified profile of cytokines involved in the degeneration of the articular cartilage and catabolic activity of the chondrocytes (Aaron et al., 2017). The chondrocytes and synovial cells produce increased levels of inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which decrease the synthesis of collagen and increase catabolic mediators, such as metalloproteinases (MMPs), and other inflammatory mediators, such as IL-8, IL-6, prostaglandin E2 (PGE2), and nitric oxide (NO) (Berenbaum, 2013).

There has been a constant search for substances that can be combined with conventional therapy for OA. Currently, conventional therapy consists of a combination of non-pharmacological measures such as aerobic exercises, weight loss, and joint

protection techniques, as well as symptomatic pharmacological treatments including anti-inflammatory non-steroidal analgesics and corticosteroids or local intra-articular lubricants until, eventually, surgical intervention is required (Gelber, 2015; Lafeber and Van Laar, 2013).

Strontium ranelate (SrRan), an antiresorptive agent and bone proforming agent already proven effective in patients with severe osteoporosis, has been the subject of clinical and experimental studies in OA because of a probable effect on both bone turnover and inflammation associated with this disease (Reginster et al., 2005; Reginster, 2014; Rodrigues et al., 2017; Tenti et al., 2014).

The exact mechanism of action of SrRan is not fully understood. However, regulation of bone cell differentiation, stimulation of osteoblast proliferation, and inhibition of osteoclast formation with probable apoptosis of "mature" cells, in addition to the activation of calcium-sensitive receptors have been considered as possible mediators of the pharmacological properties of this medication (Brennan et al., 2009; Fonseca and Brandi, 2010; Lems and Geusens, 2014). The inhibition of osteoclastic activity by SrRan has been demonstrated to be related to the reduction in MMP synthesis and modulation of the osteoprotegerin-receptor activator of nuclear factor kappa-B ligand (RANKL) pathway (Tat et al., 2011).

In line with the current trend of investigating drugs that can control the inflammatory component of OA, pre-clinical studies with SrRan have been performed to test such a hypothesis. However, results showing the probable antinociceptive effects of SrRan have conflicted with those of alterations in levels of inflammatory mediators such as TNF-α and IL1-β (Alves et al., 2017; Mierzwa et al., 2017; Nunes et al., 2015).

There is accumulating evidence of the action of SrRan on both articular cartilage and subchondral bone, but few studies have investigated the actions of this drug on OA. In addition, considering that inflammation is one of the main mediators of OA progression, this study aimed to examine the effects of SrRan in an experimental rat model of OA, using clinical and histological evaluations as well as the assessment of inflammatory cytokines. We also aimed to use this preclinical model to compare the potential protective effects of SrRan following prophylactic use with its eventual therapeutic action on articular cartilage in OA.

2. Material and Methods

The study was conducted at the Experimental Laboratory for Study of Pain (LEED), after approval by the Ethics Committee on Animal Use of the Federal University of Maranhão - Brazil (CEUA-UFMA number 23115.012456/2016-4).

2.1 Animals

Thirty male, approximately 60-day-old Wistar rats, *Rattus norvegicus* species (albinus variety) were used in the study. The animals were obtained from the Central Animal Facility of the Universidade Federal do Maranhão. They remained in the bioterium of the LEED and were fed a standard ration and water *ad libitum* and maintained under controlled conditions of light and temperature.

2.2 Experimental Protocol

The animals were divided into five groups (PROF250, SR250, SR500, SAL, and CONTROL) of six rats each. For the PROF250 ("prophylactic") group, from 4 weeks before the induction of OA with sodium monoiodoacetate (MIA), each rat received SrRan at a dose of 250 mg/kg by gavage once daily in the morning, 2 h before the subsequent feeding. The SR250 and SR500 ("treated") groups received SrRan at doses of 250 and 500 mg/kg, respectively, by gavage once daily in the morning, 2 h before the subsequent feeding for 28 days. The SAL group received saline solution (0.9% sodium chloride) by gavage for 21 days from day 7 after the induction of OA. The day of OA induction in groups PROF250, SR250, and SR500 and saline administration was considered D0. Further, OA was not induced in the CONTROL group, which was administered only saline solution by gavage. Throughout the experiment, all groups were evaluated periodically for joint incapacity, mechanical allodynia, mechanical hyperalgesia, and motor activity by forced ambulation on D0, D7, D14, D21, and D28 (Figure 1). On D28, after completing the clinical evaluations, synovial fluid was collected from the affected joint of each rat for the laboratory analysis of cytokines, the animals were euthanized with a lethal injection of sodium

thiopental), and then, the articular cartilage was harvested for histopathological analysis.

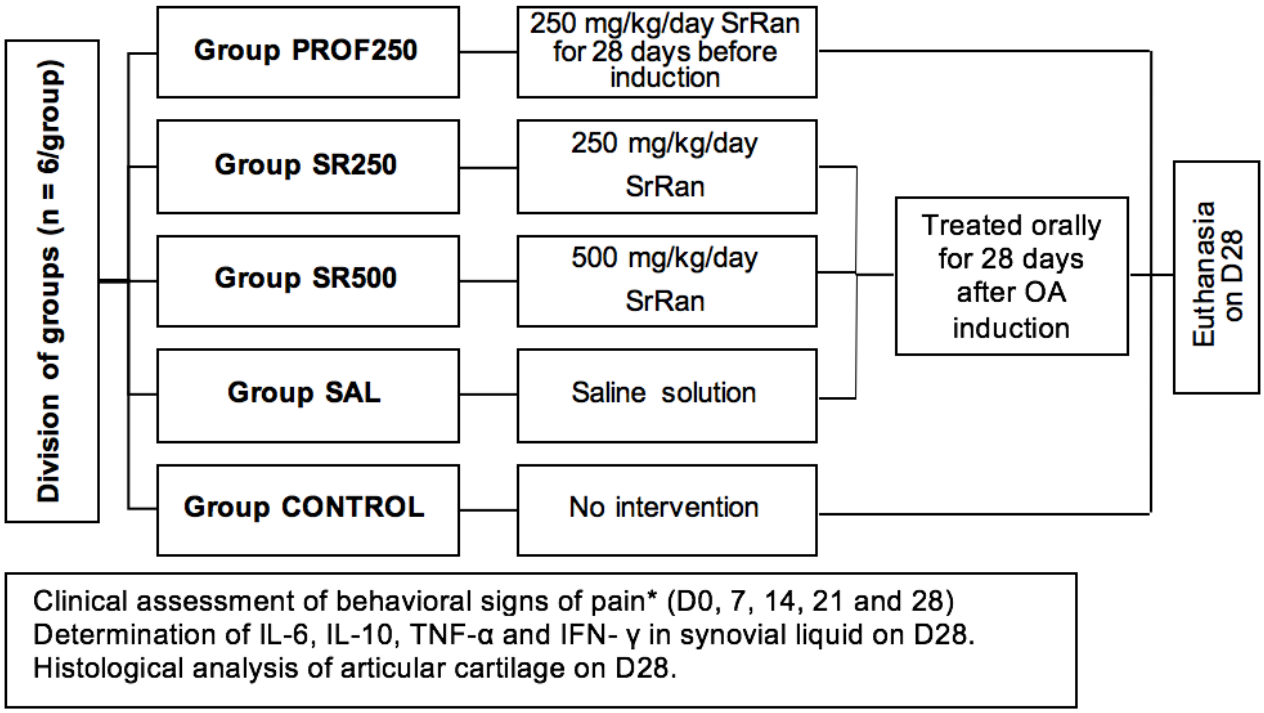

Figure 1. Experimental protocol.

2.3 Sodium MIA-induced OA model

For the induction of OA, the animals were anesthetized with intraperitoneal injections of 40 mg sodium thiopental. After certifying the anesthetic plane, a trichotomy was performed in the right knee and, subsequently, a topical solution of 10% iodopovidone was applied for local asepsis. An articular lesion was induced by a single intra-articular injection of 2 mg sodium MIA (diluted in a maximum volume of 25 µL) into the right knee through the patellar ligament (Fernihough et al., 2004; Silva et al., 2008).

2.4 Clinical Evaluations

2.4.1 Weight-bearing test /weight distribution test on hind legs

The animals were placed in a glass bowl angled and positioned so that each hind leg laid on different platforms. The weight exerted on each back paw (measured in grams) was evaluated for 5 s. The final measurement of weight distribution was the mean of three measurements. Changes in the weight distribution on the paws were calculated as follows:

$$\text{Weight distribution } (\%) = \frac{\text{APW}}{\text{APW} + \text{CPW}} \text{ X } 100 \quad ,$$

where APW was affected paw weight and CPW was contralateral paw weight. (Schött et al., 1994).

2.4.2 Quantification of mechanical allodynia (*Von Frey test*)

The evaluation of mechanical allodynia was performed using an electronic device (Model 1601C, Life Science, CA, USA), which consisted of a pressure transducer connected to a digital force counter expressed in grams (g) and calibrated to record a maximum force of 150 g. The animals were placed in individual transparent acrylic boxes on raised platforms to allow access to the lower part of their bodies. The holes in the platforms provided access to the transducer tip, allowing its contact with the animals' paws. The response of the paw withdrawal frequency to the filament stimulus was measured in five applications, lasting 1 s each, always performed by the same evaluator, and the final result was the mean of all measurements.

2.4.3 Mechanical hyperalgesia (*Randall Selitto test*)

Mechanical hyperalgesia was assessed by evaluating the nociceptive threshold paw withdrawal following the application of mechanical pressure using an analgesiometer (model IITC, Life Science). A wedge-shaped device (area, 1.75 mm²) was applied to the dorsal surface of the hind paws with increasing linear pressure until the animal responded by withdrawing the paw. Three measurements were performed

in the ipsilateral and contralateral paws. A cut-off threshold pressure of 250 g was programmed to prevent tissue damage.

The paw withdrawal reflex was considered to represent the hypernociceptive threshold. The nociceptive paw withdrawal threshold (NPWT) was recorded in grams and defined as the percentage pressure required to provoke a withdrawal of the ipsilateral affected paw, and was calculated as follows:

$$\text{NPWT}\ (\%) = \frac{\text{NAPWT}}{\text{NAPWT} + \text{NCPWT}}\ \text{X}\ 100 \quad ,$$

where NPWT was nociceptive paw withdrawal threshold, NAPWT was nociceptive affected paw-withdrawal threshold and NCPWT was nociceptive contralateral paw-withdrawal threshold (Nogueira et al., 2012; Randall and Selitto, 1957).

2.4.4 Evaluation of motor activity/forced ambulation (*rotarod test*)

The animals were placed on a swivel bar (model IITC, Life Science) at a speed of 16 rpm for a period of 300 s. The use of the affected limb was assessed by forced ambulation. The use of the paw was graded using a numerical scale ranging from 5 to 1, where: 5 = normal use of the paw, 4 = mild claudication, 3 = severe claudication, 2 = intermittent disuse of affected paw, and 1 = complete disuse of affected paw (Monville, 2006).

2.5 Laboratory Analysis of Cytokines

Laboratory analysis of the synovial fluid to quantify IL-6, IL-10, TNF-α, interferon (IFN)-γ was performed using an enzyme-linked immunosorbent assay (ELISA, R&D Systems®, MN, US).

The synovial fluid samples were obtained on D28 by washing out the affected knee joint twice with 200 µL phosphate-buffered solution (0.15 M, pH 7.4) containing 37.2 mg ethylenediaminetetraacetic acid (EDTA, 0.01 M).

2.6 Histopathological Analysis of Articular Cartilage

On D28, the articular cartilage and subchondral bone of the knee of each animal were removed after euthanasia. The excised components were embedded in paraffin blocks, cut into 5 µm sections, and the proteoglycans of the organic cartilage matrix were specifically stained using 0.5% safranin-O.

The histopathological evaluation was performed according to the guidelines of the Osteoarthritis Research Society International (OARSI). The slides were analyzed blindly by two pathologists, who graded them on a scale of 0 to 6, according to the severity of the articular cartilage lesion. The classification considered the most severe lesion observed on the slide regardless of the extent of the lesion. Grade 0 indicated morphologically intact cartilage, grade 1 indicated an intact surface with possible focal lesions or abrasion, grade 2 showed discontinuity in the articular surface, grade 3 showed vertical fissures, grade 4 presented erosion, grade 5 exhibited denudation with sclerotic bone or fibrocartilaginous tissue repair or both, and grade 6 showed remodeling and bone deformation with changes in the contour of the articular surface (Pritzker et al., 2016).

2.7 Statistical Analysis

The means of different experimental groups were compared using the Student's *t*-test or a univariate (one-way) analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons. A $p < 0.05$ was considered statistically significant, and the data obtained were analyzed using the GraphPad Prism 7.0® software for Windows® (CA, USA).

3. Results

3.1 Assessment of Joint Incapacity using *Weight Bearing Test*

A significant difference was observed in joint disability among the OA-induced groups treated with saline (SAL) and the CONTROL group (healthy animals that

received saline). Comparisons on D7, D14, D21, and D28 among the prophylactic group, the group pre-treated with SrRan before OA induction (PROF250), and the CONTROL), showed no statistically significant differences. A similar trend was observed when the SR250 and SR500 groups were compared with the CONTROL, and on D7, D14, D21, and D28, the treated animals approached the normal status of the CONTROL group without any significant differences between the groups. The results of the PROF250, SR250, and SR500 groups were statistically different from those of the SAL group (Figure 2).

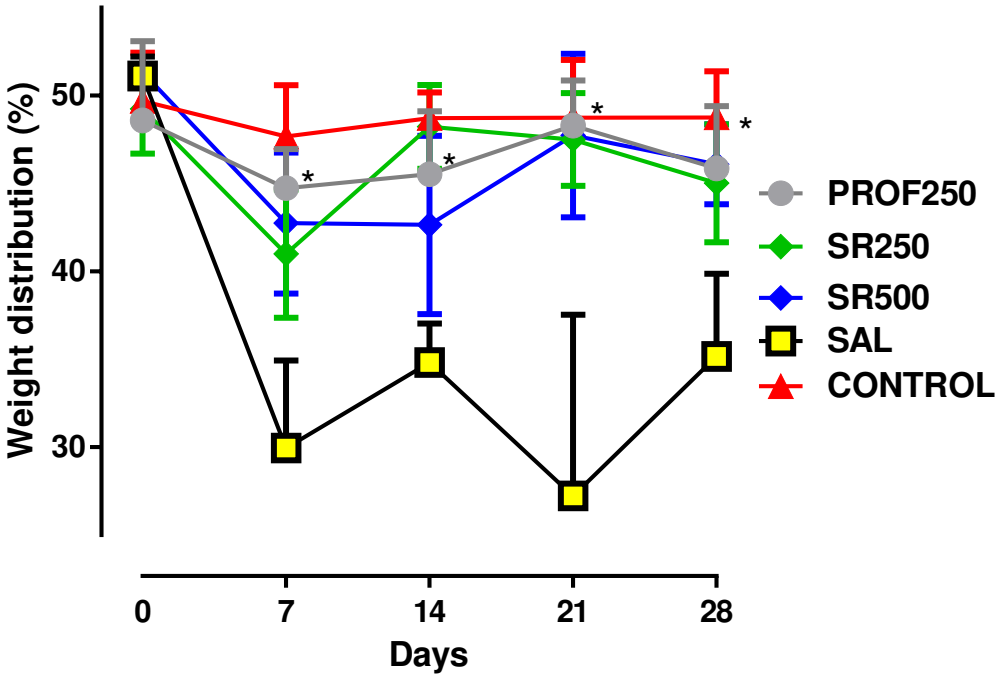

Figure 2. Evaluation of joint disability using weight bearing test. Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD); *$p > 0.05$ between PROF250, SR250, and SR500 and CONTROL groups on D7, D14, D21, and D28. A $p < 0.05$ comparing groups PROF250, SR250, and SR500 with SAL, using one-way analysis of variance (ANOVA) followed by the Bonferroni test. X-axis = Days; Y-axis = Weight distribution of paws (%).

3.2 Quantification of Mechanical Allodynia (*Von Frey Test*)

The Von Frey test demonstrated differences between the SAL and CONTROL groups. The PROF250 group did not show any improvement in spontaneous pain behaviors compared to the CONTROL group ($p < 0.05$). A similar trend was observed when the SR250 and CONTROL groups were compared, and no beneficial effect was observed in the analysis. A beneficial effect was observed in animals administered 500 mg/kg SrRan from D14 compared to the CONTROL group (Figure 3).

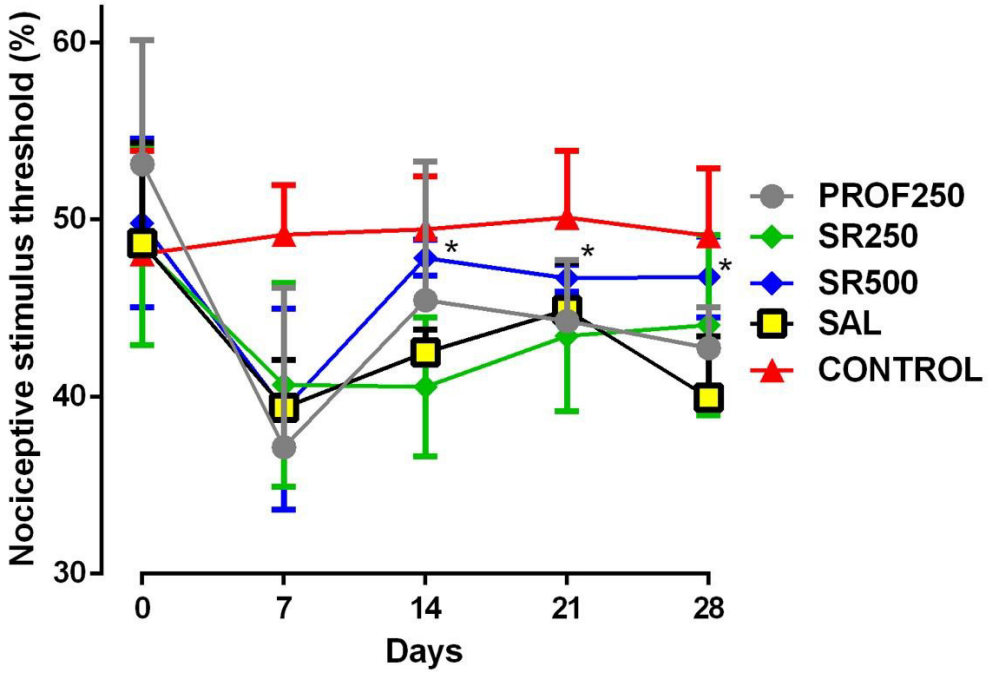

Figure 3. Evaluation of tactile sensitivity using *Von Frey test.* Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD); *$p > 0.05$ from D14 onward, comparing SR500 and CONTROL groups using one-way analysis of variance (ANOVA) followed by the Bonferroni test. X-axis = Days; Y-axis = nociceptive stimulus threshold (%).

3.3 Mechanical Hyperalgesia (*Randall Selitto Test*)

The Randall Selitto test revealed a statistically significant difference between the SAL and CONTROL groups. Prophylactic use of SrRan in the PROF250 group caused the animals to exhibit responses that were similar to those of the CONTROL group on D7, D14, and D21, but the effect did not persist up to D28. No beneficial effect was observed on mechanical hyperalgesia with 250 mg/kg SrRan compared to the untreated CONTROL group. At a dose of 500 mg/kg SrRan, isolated improvement in mechanical hyperalgesia was found only on D14 and D21 (Figure 4).

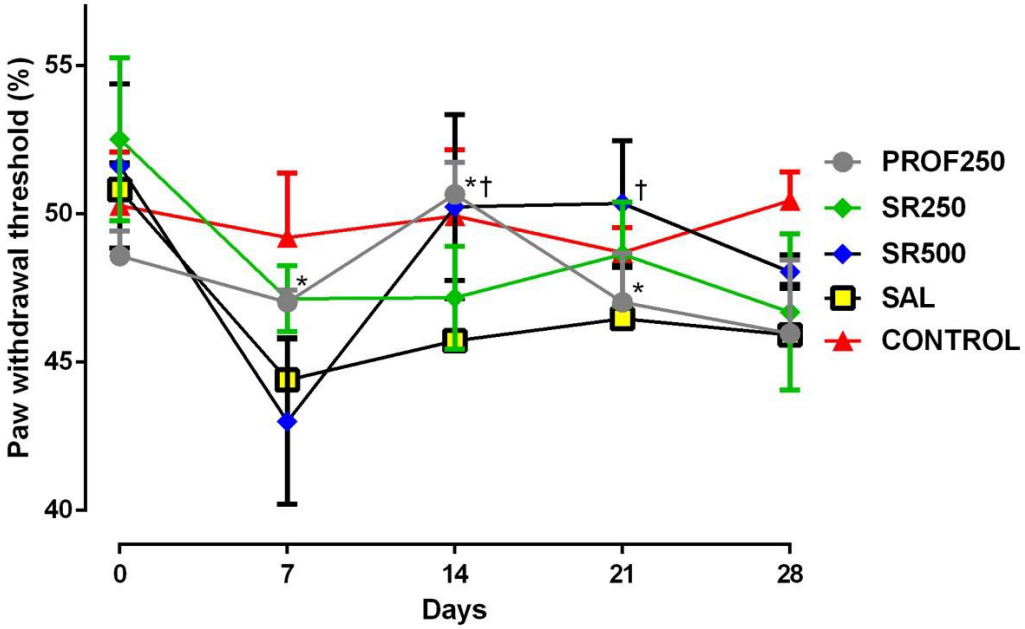

Figure 4. Evaluation of mechanical hyperalgesia using the Randall Selitto test. Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD); *p > 0.05 between groups PROF250 and CONTROL on D7, D14, and D21, using one-way ANOVA followed by the Bonferroni test. † p>0.05 at D14 and D21, in the comparison between SR500 and CONTROL groups, using *one-way* ANOVA followed by the Bonferroni test. X-axis = Days; Y-axis = Paw withdrawal threshold (%).

3.4 Assessment of Motor Activity/Forced Ambulation (*Rotarod Test*)

Statistically significant difference between the SAL and CONTROL groups were observed in the rotarod test. No improvement of motor function was observed with prophylactic use and treatment with 250 and 500 mg/kg SrRan compared with the CONTROL group, and statistically significant differences were observed at all points of comparison ($p < 0.05$, Figure 5).

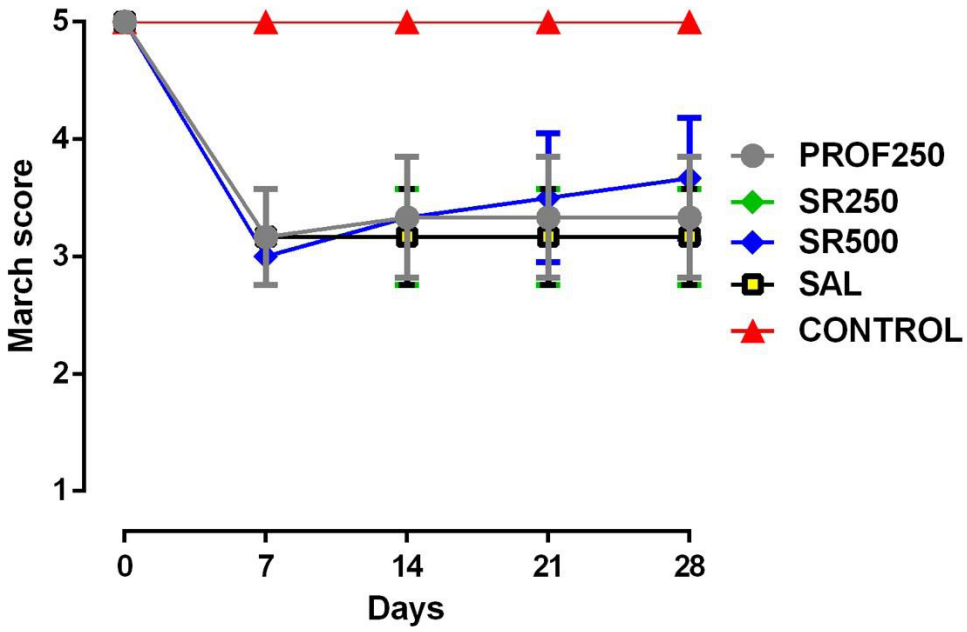

Figure 5. Evaluation of motor activity/forced ambulation using the rotarod test. Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD), $p < 0.05$, comparing all time points using one-way analysis of variance (ANOVA) followed by Bonferroni test. X-axis: Days; Y-axis: March score**.**

3.5 Laboratory Analysis of Cytokines

The determination of cytokines in the synovial fluid samples collected at the end of the procedure is shown in Table 1. The levels of IL-6, IL-10, TNF-α, and IFN-γ in the SrRan-treated PROF250, SR250, and SR500 groups were close to those of the normal

CONTROL group, and there were no statistically significant differences among them. However, a similar trend was not observed between the SAL and CONTROL groups, which showed significantly different values. Of note was the lack of significant differences between the prophylactic and 250 and 500 mg/kg/day SrRan-treated groups.

Table 1. Determination of cytokine levels in synovial fluid using enzyme-linked immunosorbent assay (ELISA)

GROUPS	TNF-α	IL-6	IFN-γ	IL-10
PROF250	378.2 ± 33.29 (p=0.78)	95.3 ± 13.8 (p=0.81)	1377 ± 110.2 (p=0.82)	638.8 ± 87.5 (p=0.9)
SR250	392.2 ± 43.89 (p=0.95)	133.7 ± 14.7 (p=0.54)	1558 ± 101.5 (p=0.11)	632.3 ± 87.5 (p=0.9)
SR500	411.5 ± 25.26 (p=0.99)	99.4 ± 12.8 (p=0.92)	1173 ± 137.7 (p=0.45)	476.2 ± 83.8 (p=0.35)
SAL	569.9 ± 41.1 (p=0.01)	173.4 ± 14.6 (p<0.01)	1891 ± 139.2 (p<0.01)	303.4 ± 92.8 (p=0.01)
CONTROL	416.1 ± 33.29	110.5 ±15.7	1311 ± 115.9	634.9 ± 112.0

Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD); p-values express comparison between OA-induced groups and CONTROL. TNF-α, tumor necrosis factor-α; IL, interleukin; interferon-γ, IFN-γ.

3.6 Histopathological Analysis of Articular Cartilage

The histopathologic evaluation using the OARSI scoring system revealed that the SAL group had a higher grade classification than the other groups (4.25 ± 0.47), indicating a greater involvement of articular cartilage in those animals. The SrRan-treated groups (PROF250, SR250, and SR500) showed average grade classifications of 0.5 ± 0.22, 1 ± 0.7, and 0.75 ± 0.25, respectively, which were statistically similar to

that of the CONTROL group (0.16 ± 0.16), with no significant difference between the two doses used (Figures 6 and 7).

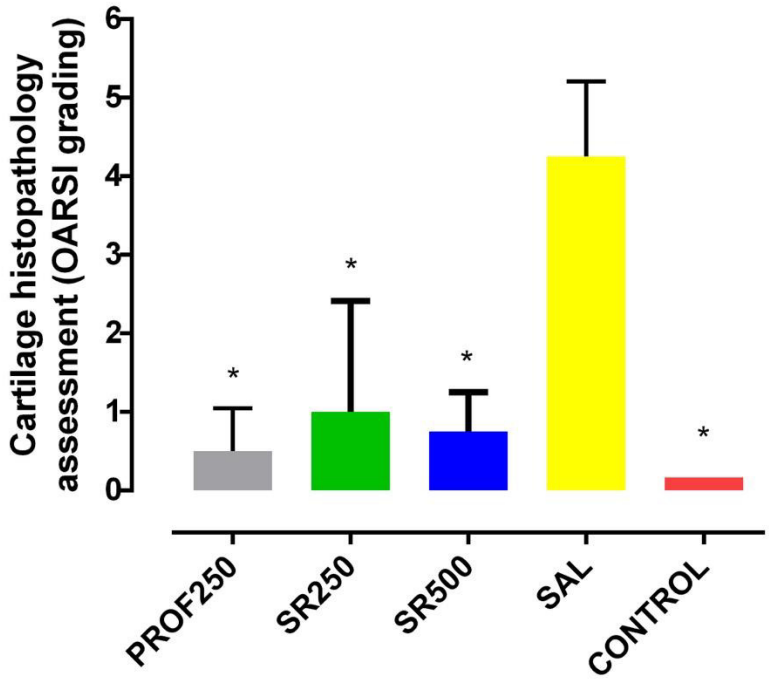

Figure 6. Histopathologic assessment of cartilage classified using the Osteoarthritis Research Society International (OARSI) scoring system. Groups: PROF250, prophylactically administered strontium ranelate (SrRan) 250 mg/kg 4 weeks before osteoarthritis (OA) induction; SR250 and SR500, OA-induced and treated with SrRan 250 and 500 mg/kg, respectively for 4 weeks after induction; SAL, OA-induced and receiving only saline; CONTROL, untreated and not OA-induced. Results are means ± standard deviation (SD); *p > 0.05, comparing PROF250, SR250, and SR500 and CONTROL groups, using one-way analysis of variance (ANOVA), followed by Bonferroni test. Y-axis: Cartilage histopathology assessment (OARSI grading).

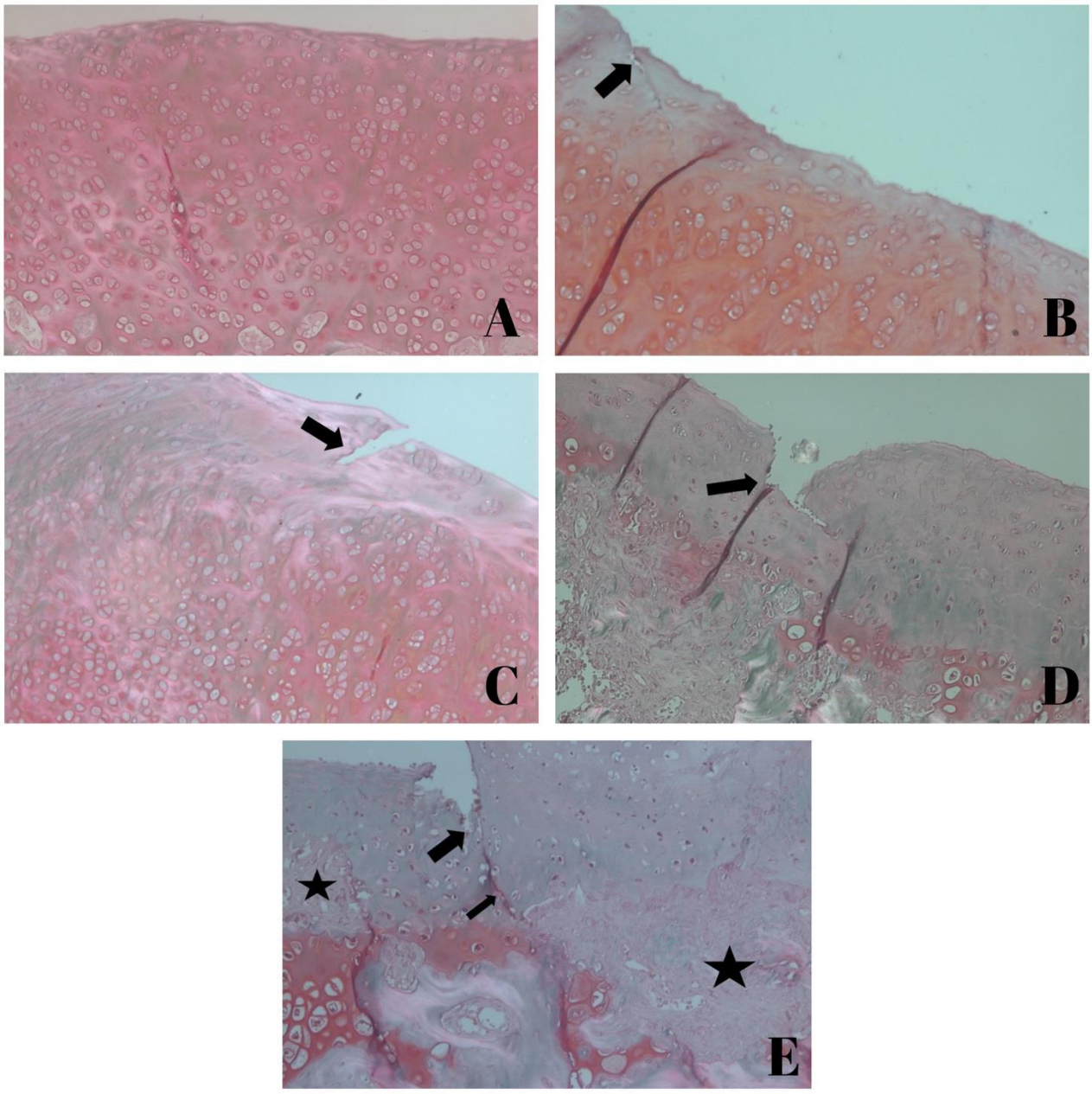

Figure 7. Sections of articular cartilage with different grades of degenerative changes in osteoarthritis (OA) classified using Osteoarthritis Research Society International (OARSI) scoring system. (A) Example of the CONTROL group - Grade 0 - normal cartilage. (B) Example of PROF250 group - grade 1, indicates intact surface with slight discontinuity in surface area (arrow). (C) Example of SR250 group - grade 3, demonstrates vertical fissure in superficial layer (arrow). (D) Example of SAL group - grade 4, with erosion reaching mid layer. (E) Example of SAL group - grade 5, with ulceration and fissure reaching the deep layer of articular cartilage (arrow), with areas of fibrocartilaginous repair (stars).

4. Discussion

The present study investigated the effects of SrRan, a medication that has been evaluated for possible inclusion in the class of disease-modifying OA drugs (DMOADs). To this end, we evaluated the effects of SrRan on pain behaviors in an experimental rat model of OA and described its effects on inflammation by determining cytokine levels in the synovial fluid and histopathological changes in affected joint sections.

The experiment was designed to mimic conditions with that would lead to the development of OA in the knee such as direct trauma, joint overload, improper alignment of the limb, quality of bone mass, and obesity (Allen and Golightly, 2015). Thus, the drug would have a preventive indication for administration before degenerative articular changes arise, which was assessed in the PROF250 group. In addition, we also assessed its effectiveness as a treatment in groups SR250 and SR500.

An improvement was observed in the joint incapacity following prophylactic use at the doses tested, and in the treatment of already established OA, although this effect lasted only up to D28. A study performed using a different rat model of OA induced with zymosan, a potent inducer of cyclooxygenase (COX)-2 expression, used doses ranging from 30 to 300 mg/kg/day for a shorter period (Nunes et al., 2015) and yielded similar results to those of this study. An experiment using much lower doses (25 and 50 mg/kg/day) and a method of OA induction similar to that used in the current study showed no reduction of joint incapacitation in animals treated prophylactically with SrRan or in those treated after the establishment of OA (Rodrigues et al., 2017). Therefore, the present study was the first to identify the beneficial effects of the preventive use of SrRan on joint activity.

SrRan (0.5, 5, and 50 mg/kg/day) also exhibited antinociceptive effects on the temporomandibular joint of rats in a zymosan-induced model of OA by reducing hypernociception, assessed using the Von Frey test. This phenomenon was verified, for the first time, in the present study at doses of 500 mg/kg/day, probably because the magnitude of OA induced in the knee was different from that induced in the temporomandibular joint (Alves et al., 2017). This was also verified at 300 mg/kg/day but not at 30 mg/kg/day in zymosan-induced knee OA (Nunes et al., 2015).

The benefit of the prophylactic use of SrRan was also verified by analyzing its effects on hyperalgesia induced by increasing the pressure on the rat's paw, based on the fact that inflammation lowers the threshold of pain reactions. SrRan showed similar effects in other studies; however, these studies treated already established OA models and did not investigate the preventive effects (Alves et al., 2017; Rodrigues et al., 2017).

The model of OA induced by intra-articular injection of MIA enables pain assessment at an early stage of OA (Fernihough, 2004). Although the rats already presented structural changes consistent with OA as early as day 7 after induction, it is likely that the reduction of hypernociception by SrRan did not correlate only with structural modification, but also with changes in subtle biochemical mechanisms that mediate pain. OA is an autoinflammatory disease caused by chondrocyte- and synoviocyte-mediated responses, and the serum and synovial levels of inflammatory cytokines are higher in patients with OA than in those without the condition (Berenbaum, 2013; Goldring and Otero, 2011). To clarify the possible mechanisms mediating in this phenomenon, we determined the inflammatory mediators involved.

The results of the determination of inflammatory mediators in the synovial fluid following treatment with SrRan at different doses demonstrated a significant reduction of IL-6, TNF-α, and IFN-γ. These cytokines are known to be involved in the inflammatory cascade in the progression of OA, whereas there was an increase in IL-10, an anti-inflammatory cytokine (Berenbaum, 2013; Goldring and Otero, 2011; Pelletier et al., 2001; Wojdasiewicz et al., 2014). On the contrary, there was no the difference between the treatment and prophylactic doses in the reduction of pro-inflammatory substances. This result corroborates available data showing a reduction in synovial fluid levels of TNF-α and IL-1β in zymosan-induced OA rats treated with SrRan at a dose of 300 mg/kg/day (Nunes et al., 2015). A decrease in the expression of TNF-α without changes in leukocyte count and IL-1β levels was also observed in another pre-clinical study of periarticular tissue samples, with lower doses of SrRan (up to 50 mg/kg/day) (Alves et al., 2017). These results were not confirmed in a study with rats subjected to oophorectomy and treated with SrRan at doses of 300 and 625

mg/kg/day, with no reduction of TNF-α and MMPs, which are metal-dependent endopeptidases that remodel the extracellular matrix (Mierzwa et al., 2017).

The histopathological analysis of articular cartilage using the OARSI classification in animals treated with SrRan showed encouraging results, suggesting the possible efficacy of SrRan in the prophylaxis and treatment of OA at the doses tested. The hyaline cartilage is the most relevant joint tissue in the pathogenesis of OA (Xia et al., 2014). Therefore, a drug with the potential to protect this tissue has excellent relevance. Thus, the protective effect of SrRan on cartilage demonstrated in the present study might justify its use for this purpose.

The scarcity of published experimental studies on SrRan use in OA, especially prophylaxis, makes a comparative evaluation of the results in this study a challenge. In rats subjected to oophorectomy, SrRan at doses of 300 mg/kg/day effectively attenuated the progression of OA, improving the quality of cartilaginous matrix by directly stimulating the synthesis of proteoglycans Furthermore, the cellular viability in those animals was preserved, with lower OARSI scores and reduced expression of caspase-3, an enzyme associated with apoptosis. This effect was abrogated with daily doses of 625 mg/kg associated with mechanical vibration (Mierzwa et al., 2017).

The SrRan-induced attenuation of degeneration of the articular architecture was also demonstrated in another rat model of OA induced by meniscal injury, with higher doses of the drug (625 and 1,800 mg/kg). The reduction of apoptotic indices of the chondrocytes was confirmed using terminal deoxynucleotidyl transferase-dUTP-nick end labeling (TUNEL) assay. Using micro-computed tomography to evaluate bone mineral density, an improvement in the rates of abnormality in the microarchitecture of the joints was observed. Microspectroscopy revealed an increase in the mineral-to-collagen ratio with SrRan treatment. Moreover, an increase in joint elasticity was observed using nanoindentation testing, a dynamic test for determining hardness. Additionally, an increase in the expression of sex determining region Y - box 9), a transcription factor with fundamental importance in chondrogenesis. Thus, treatment with high doses of SrRan showed positive results in controlling the deterioration of articular cartilage and subchondral bone remodeling (Yu et al., 2013).

A reduction in the progression of joint structural changes was also demonstrated by SrRan 25, 50, or 75 mg/kg/day in an experimental model of dogs subjected to anterior cruciate ligament transection. Effects such as reduction of the depth and size

of articular lesions, in addition to greater preservation of the articular collagen network, were observed at doses of 25, 50, or 75 mg/kg/day using histomorphometric analysis. The expression of osteochondral degradation proteases (such as MMPs and cathepsin K) and IL-1β was reduced, especially with larger doses of the drug and longer time periods (Pelletier et al., 2012).

In contrast, a recent study with pigs administered a therapeutic dose of 625 mg/kg SrRan did not reveal any protective effects against degeneration of the articular cartilage in OA, determined using the OARSI classification scoring (Chu et al., 2017). The treated group in this study showed results similar to the control, which only showed improvements in the profile of the subchondral bone (Chu et al., 2017).

The results of the effects of SrRan on pain behavior, the inflammatory process, and the histological progression of OA presented in the current study were impressive with both prophylactic and therapeutic administration. However, this study has some limitations that need to be highlighted. Many of the beneficial effects observed with SrRan treatment in this and other pre-clinical studies, were observed at high doses of the drug, in contrast to doses of up to 2 g/day typically used in humans (Reginster, 2014). It is also worth noting the difficulty in reproducing OA with similar changes and evolution observed in humans since the experimental models still fail in this correlation (Pelletier et al., 2010). The lack of standardized clinical and experimental research methods for determining the effects of drugs for potential DMOAD classification is also a challenge in this field (Reginster et al., 2013).

5. Conclusions

The administration of SrRan in an MIA-induced experimental model of knee OA indicated its beneficial effect on pain, particularly in the improvement of articular incapacitation. This medication also mitigated histological changes in the articular cartilage and reduced the inflammatory process with lower synovial levels of IL-6, TNF-α, and IFN-γ than those in the untreated groups. In the challenging search for drugs that modify the normal pathogenesis and progression history of OA, pre-clinical results including those of this present study, support the potential use of SrRan for this purpose, both prophylactically and as a therapeutic agent.

Conflicts of interest and fundind statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. They have no funding to report.

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5. CONSIDERAÇÕES FINAIS

Com mecanismos fisiopatológicos associados a fenômenos mecânicos e inflamatórios, os fatores envolvidos na progressão e no tratamento da OA ainda necessitam de maior esclarecimento, o que justifica constantes pesquisas para elucidação destas questões.

Neste contexto, a pesquisa experimental com uso do ranelato de estrôncio em OA traz informações relevantes, que evidenciam o efeito do fármaco sobre o metabolismo ósseo, gerando importantes benefícios morfofuncionais à articulação.

Os resultados apresentados neste estudo fornecem evidências de que altas doses do ranelato de estrôncio em modelo experimental de OA em ratos se associaram à melhora no comportamento doloroso, especialmente na incapacitação articular. Adicionalmente, foi verificado controle das lesões histológicas articulares relacionadas à OA.

A utilização do ranelato de estrôncio se relacionou com a diminuição da liberação local de citocinas pró-inflamatórias, sugerindo benefício da administração do referido fármaco no controle do processo inflamatório descrito na OA.

Vale ressaltar que, além de relatar efeitos positivos do uso terapêutico do SrRan em modelo experimental de OA, o presente estudo demonstrou, de forma inédita, um efeito profilático favorável deste fármaco, prevenindo ou minimizando o desenvolvimento desta doença.

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ANEXO A – COMPROVANTE DE SUBMISSÃO DE ARTIGO À REVISTA *BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH*

Brazilian Journal
of medical and biological research

STRONTIUM RANELATE AS A POSSIBLE DISEASE-MODIFYING OSTEOARTHRITIS DRUG: A SYSTEMATIC REVIEW

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Keywords:	osteoarthritis, strontium ranelate, treatment, pain, symptoms
Special Sections:	Clinical Investigation/Skeletal, muscle and nervous systems

ANEXO B – NORMAS PARA SUBMISSÃO À REVISTA *BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH*

Brazilian Journal
of medical and biological research

Scope and policy

The purpose of the *Brazilian Journal of Medical and Biological Research* is to publish the results of original experimental research that contribute significantly to knowledge in medical and biological sciences. Major criteria for acceptance are scientific quality, originality, and conciseness. Preference will be given to manuscripts that develop new concepts or experimental approaches and are not merely repositories of data. Papers that report negative results require special justification for publication. Methodological papers shall be considered for publication provided they describe new principles or a significant improvement of an existing method.

The following papers will not be accepted for publication

- Studies on people not approved by an accredited Ethics Committee or without written informed consent from the subject or legal guardian.
- Studies on animals not approved by an accredited Ethics and Animal Care Committee.
- Manuscripts that report preliminary results or only confirm previously reported results.
- Manuscripts that describe the pharmacokinetics, bioavailability and toxicity of drugs in people or animals.
- Manuscripts that deal with transcultural adaptation and validation of instruments of measurements.
- Manuscripts that translate a text published in another language and validate it on local patients.
- Manuscripts that use questionnaires translated from the language of another country and their validation in local patients.
- Manuscripts that present only *in silico* analysis.
- Manuscripts from the area of veterinary medicine.
- The Journal does not publish toxicological studies.

Publication charges

- The authors are responsible for "publication charges" of all accepted papers. Publication charges will be billed to the Corresponding Author when the paper is accepted.

- The charge is R$3.300,00/paper for Brazilian authors and US$1.600,00/paper for authors outside Brazil and is independent of the length of the paper.

Preparation of Research Manuscripts

The ***Brazilian Journal of Medical and Biological Research*** publishes original research articles of outstanding scientific significance. Manuscripts must be submitted in English. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a limited number of experiments. The key criteria are that the research clearly demonstrates its novelty, its importance to a particular field as well as its interest to those outside that discipline, and conclusions that are justified by the data.

Authorship requirements

Only those persons who contributed directly to the intellectual content of the paper should be listed as authors. Authors should meet all of the following criteria, thereby allowing persons named as authors to take public responsibility for the content of the paper.

- Conceived, planned and carried out the experiments that led to the paper or interpreted the data it presents, or both.
- Wrote the paper, or reviewed successive versions.
- Approved the final version.
  Holding positions of administrative leadership, contributing patients, and collecting and assembling data, however important to the research, are not by themselves criteria for authorship. Other persons who have made substantial, direct contributions to the work but cannot be considered authors should be cited in the Acknowledgment section, with their permission, and a description of their specific contributions to the research should be given.

Cover Letter

It is important that you include a cover letter with your manuscript. Take the time to consider why this manuscript is suitable for publication in the *Brazilian Journal of Medical and Biological Research*. Why will your paper inspire the other members of your field, and how will it drive research forward? Please explain this in your cover letter.

The cover letter should also contain the following information:

- Title of article.
- Name(s) of all author(s).
- Name, complete mailing address, including zip code, telephone number, fax number and e-mail of author to whom correspondence should be sent.
- If a version of the manuscript has been previously submitted for publication to another journal, include comments from the peer reviewers and indicate how the authors have responded to these comments.
- Papers in the area of Clinical Investigation should include a statement indicating that the protocol has been approved by the Hospital Ethics Committee (Hospital with which at least one of the authors is associated) and that written informed

consent was obtained from all participants. This information must also be cited in the Material and Methods section of the manuscript.

Text format

- The text of a manuscript can only be accepted as a Microsoft Word file created with MS Word as a "doc", "docx" or "rtf" document.
- Each page should contain the page number in the upper right-hand corner starting with the title page as page 1.
- Report all measurements in Système International, SI (http://physics.nist.gov/cuu/Units) and standard units where applicable (see below).
- Do not use abbreviations in the title and limit their use in the abstract and text.
- The length of the manuscript and the number of tables and figures must be kept to a minimum.
- Ensure that all references are cited in the text.
- Generic names must be used for all drugs. Instruments may be referred to by proprietary name; the name and country or electronic address of the manufacturer should be given in parentheses in the text.

Guidance on grammar, punctuation, and scientific writing can be found in the following sources:

- Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers. 8th edn. Rockefeller University Press, Reston, 2006. http://www.scientificstyleandformat.org/Home.html
- Medical Style and Format. Huth EJ (Editor). ISI Press, Philadelphia, 1987, Marketed by Williams & Wilkins, Baltimore, MD.
- Writing scientific articles like a native English speaker: top ten tips for Portuguese speakers. Clinics (Sao Paulo). Mar 2014; 69(3): 153-157. doi: 10.6061/clinics/2014(03)01. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3935133/

The *Brazilian Journal of Medical and Biological Research* follows the reference format of the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, which can be found on the website of the National Library of Medicine (http://www.icmje.org/).

The writing style should be concise and accessible. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

Although we encourage submissions from around the globe, we require that manuscripts be submitted in American English. As a step towards overcoming language barriers, we encourage authors to seek the assistance of professional services available on the homepage of the journal/Service and Information.

Footnotes

Text footnotes, if unavoidable, should be numbered consecutively in superscript in the manuscript and written on a separate page following the abstract.

Abbreviations

Abbreviations should be kept to a minimum. Define all abbreviations upon first use in the abstract and the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

- Explain all abbreviations in the abstract, text, figure and table legends **when they first appear**. Keep the number of abbreviations to a minimum.
- Do not explain abbreviations for units of measurement [3 mL, not 3 milliliters (mL)] or standard scientific symbols [Na, not sodium (Na)].
- Abbreviate long names of chemical substances and terms for therapeutic combinations. Abbreviate names of tests and procedures that are better known by their abbreviations than by the full name (VDRL test, SMA-12).
- Use abbreviations in figures and tables to save space, but they **must be defined in the legend**.

Nomenclature

vThe use of standardized nomenclature in all fields of science and medicine is an essential step toward the integration and linking of scientific information reported in published literature. We will enforce the use of correct and established nomenclature wherever possible: We strongly encourage the use of SI units.

- s for second
- min for minute
- h for hour
- L for liter
- m for meter
- kDa for mass in kilodaltons
- 5 mM rather than ^{5} x 10-3 M or 0.005 M

Species names (e.g., *Homo sapiens*), genes, mutations, genotypes, and alleles should be italicized. Use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text.

The Recommended International Non-Proprietary Name (rINN) of drugs should be provided.

Manuscript categories

Authors should state in the cover letter that the manuscript is intended to be a Full-length Paper, Short Communication, Review Article, Overview, Concepts and Comments, or Case Report.

Full-length paper

Each manuscript should clearly state its objective or hypothesis; the experimental design and methods used (including the study setting and time period, patients or participants with inclusion and exclusion criteria, or data sources and how these were selected for the study); the essential features of any interventions; the main outcome measures; the main results of the

study; and a discussion placing the results in the context of published literature.

The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text should be divided into separate sections (Introduction, Material and Methods, Results, Discussion), without a separate section for conclusions
- no more than 40 references (without exceptions)

Short communication

A short communication is **a report on a single subject**, which should be concise but definitive. The scope of this section is intended to be wide and to encompass methodology and experimental data on subjects of interest to the readers of the Journal.

The manuscript should contain:

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Review article

A review article should provide a synthetic and critical analysis of a relevant area and should not be merely a chronological description of the literature. A review article by investigators who have made substantial contributions to a specific area in medical and biological sciences will be published by invitation of the Editors. However, an outline of a review article may be submitted to the Editors without prior consultation. If it is judged appropriate for the Journal, the author(s) will be invited to prepare the article for peer review. A minireview is focused on a restricted part of a subject normally covered in a review article.

The manuscript should contain:

- abstract of no more than 250 words
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Overview

An overview does not contain unpublished data. It presents the point of view of the author(s) in a less rigorous form than in a regular review or minireview and is of interest to the general reader.

The manuscript should contain:

- abstract of no more than 250 words
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Concepts and Comments

The Concepts and Comments section provides a platform for readers to present ideas, theories and views.

The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
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- the text may be divided into sections with appropriate titles and subtitles
- no more than 40 references (without exceptions)

Case report

A case report should have at least one of the following characteristics to be published in the Journal:

- special interest to the clinical research community
- a rare case that is particularly useful to demonstrate a mechanism or a difficulty in diagnosis
- new diagnostic method
- new or modified treatment
- a text that demonstrates relevant findings and is well documented and without ambiguity

The manuscript should contain:

- abstract of no more than 250 words
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- a running title to be used as a page heading, which should not exceed 60 letters and spaces

- the text may be divided into sections with appropriate titles and subtitles
- no more than 20 references (without exceptions)
- no more than three illustrations (figures and/or tables)

Organization of the Manuscript

Most articles published in the *Brazilian Journal of Medical and Biological Research* will be organized into the following sections:
Title, Authors, Affiliations, Abstract, Key words, Running Title, Author for Correspondence and email address
Introduction
Material and Methods
Results
Discussion
Acknowledgments
References
Tables with a short descriptive title and footnote legends
Figures with a short descriptive title, descriptive legends and uniformity in format

Continuous page numbers are required for all pages including figures. There are no specific length restrictions for the overall manuscript or individual sections. However, we urge authors to present and discuss their findings concisely. We recognize that some articles will not be best presented in our research article format. If you have a manuscript that would benefit from a different format, please contact the editors to discuss this further.

Title Page

Title - The title should be as short and informative as possible, should not contain non-standard acronyms or abbreviations, and should not exceed two printed lines. Example: **Single-step purification of crotapotin and crotactine from *Crotalus durissus terrificus* venom using preparative isoelectric focusing**

Authors and Affiliations

Initials and last name(s) of author(s) (matched with superscript numbers identifying institutions). Institution(s) (Department, Faculty, University, City, State, Country) of each author (in Portuguese if authors are from Brazil). Example:
A.S. Aguiar[1], A.R. Melgarejo[1], C.R. Alves[2] and S. Giovanni-De-Simone[2,3]
[1]Divisão de Animais Peçonhentos, Instituto Vital Brazil, Niterói, RJ, Brasil
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[3]Departamento de Biologia Celular e Molecular, Universidade Federal Fluminense, Niterói, RJ, Brasil

Abstract
Since abstracts are published separately by Information Services, they should contain sufficient hard data, to be appreciated by the reader. The *Brazilian Journal* publishes **unstructured abstracts** in a single paragraph. The abstract should not exceed 250 words.

The abstract should briefly and clearly present the objective, experimental approach, new results as quantitative data if possible, and conclusions. It should mention the techniques used without going into methodological detail and mention the most important results.

Abbreviations should be kept to a minimum and should be defined in both the Abstract and text.
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Running title
This short title, to be used as a page heading, should not exceed 60 letters and spaces.

Corresponding author

One of the authors should be designated as the corresponding author. It is the corresponding authors responsibility to ensure that the author list is accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and affiliations should be listed in the Acknowledgments section. Provide the name and email address of the author to whom correspondence should be sent.

Introduction

The Introduction should put the focus of the manuscript into a broader context. As you compose the Introduction, think of readers who are not experts in this field. This should state the purpose of the investigation and justification for undertaking the research and relationship to other work in the field. An extensive listing or review of the literature should not be used. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The Introduction should

conclude with a brief statement of the overall aim of the experiments and a comment about what was achieved.

Material and Methods

Sufficient information should be provided in the text or by referring to papers in generally available journals to permit the work to be repeated. This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. We encourage authors to submit, as separate files, detailed protocols for newer or less well-established methods. These will be linked to the article and will be fully accessable.

Results

The results should be presented clearly and concisely. Tables and figures should be used only when necessary for effective comprehension of the data. The Results section should provide results of all of the experiments that are required to support the conclusions of the paper. There is no specific word limit for this section, but a description of experiments that are peripheral to the main message of the article and that detract from the focus of the article should not be included. The section may be divided into subsections, each with a concise subheading. Large datasets, including raw data, should be submitted as supplementary files; these are published online linked to the article. The Results section should be written in past tense. In some situations, it may be desirable to combine Results and Discussion into a single section.

Discussion

The purpose of the Discussion is to identify new and relevant results and relate them to existing knowledge. Information given elsewhere in the text, especially in Results, may be cited but all of the results should not be repeated in detail in the Discussion. The Discussion should spell out the major conclusions and interpretations of the work including some explanation of the significance of these conclusions. How do the conclusions affect the existing assumptions and models in the field? How can future research build on these observations? What are the key experiments that must be done? The Discussion should be concise and tightly argued. If warranted, the Results and Discussion may be combined into one section.

Acknowledgments

When appropriate, briefly acknowledge technical assistance, advice and contributions from colleagues. People who contributed to the work, but do not fit the criteria for authors should be listed in the Acknowledgments section, along with their contributions. Donations of animals, cells, or reagents should also be acknowledged You must also ensure that anyone named in the Acknowledgments agrees to being so named. Financial support for the research and fellowships should be acknowledged in this section (agency and grant number).

Figures

Figures must be submitted in high-resolution version (600 dpi). Please ensure that the files conform to our Guidelines for Figure Preparation when preparing your figures for production and/or "Image Quality Specifications". The link contains important information about image quality from PubMed Central where the Brazilian Journal of Medical and Biological Research is indexed. The Brazilian Journal of Medical and Biological Research requires the same quality as PubMed Central. Please follow these instructions when you submit figures to the Brazilian Journal.

Preparing figure files for submission

The *Brazilian Journal of Medical and Biological Research* encourages authors to use figures where this will increase the clarity of an article. The use of color figures in articles is free of charge. The following guidelines must be observed when preparing figures. Failure to do so is likely to delay acceptance and publication of the article.

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- The aim of the figure legend should be to describe the key messages of the figure, but the figure should also be discussed in the text.
- An enlarged version of the figure and its full legend will often be viewed in a separate window online, and it should be possible for a reader to understand the figure without moving back and forth between this window and the relevant parts of the text.
- Each legend should have a concise title of no more than 15 words. The legend itself should be succinct, while still explaining all symbols and abbreviations. Avoid lengthy descriptions of methods. Statistical information should be given as well as the statistical tests used.
- Arrows or letters should be used in the figure and explained in the legend to identify important structures.
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- Detailed information about the microscope used should be included in the Material and Methods section.
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- Tables must be numbered consecutively with Arabic numerals in the text.
- Tables must have a concise and descriptive title.
- All explanatory information should be given in a footnote below the table. Footnotes should be used to explain abbreviations and provide statistical information, including statistical tests used.
- All abbreviations must be defined in this footnote, even if they are explained in the text.
- Tables must be understandable without referring to the text.
- Tables occupying more than one printed page should be avoided, if possible.
- Vertical and diagonal lines should not be used in tables; instead, indentation and vertical or horizontal space should be used to group data.
- Tables in Excel must be cell-based; do not use picture elements, text boxes, tabs, or returns in tables.

References

Only published or accepted manuscripts should be included in the reference list. Meeting abstracts, conference talks, or papers that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only. All personal communications should be supported by a letter from the relevant authors. Authors are responsible for the accuracy and completeness of their references and for correct text citation. When possible, references which are easily available in English should be cited.

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without a space (1,5,7) . Where there are 3 or more sequential citations, they should be given as a range (4-9).

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Please use the following style for the reference list:

Published Papers. First 6 authors followed by et al., Title, Journal (abbreviation in italics), Year, Volume, Complete Pages. Lammers AE, Hislop AA, Flynn Y, Haworth SG. The 6-minute walk test: normal values for children of 4-11 years of age. *Arch Dis Child* 2008; 93: 464-468. Zhang Q, Malik P, Pandey D, Gupta S, Jagnandan D, Belin de CE, et al. Paradoxical activation of endothelial nitric oxide synthase by NADPH oxidase. *Arterioscler Thromb Vasc Biol* 2008; 28: 1627-1633.

Article accepted for publication but not yet published. First 6 authors followed by et al., Title, Journal (abbreviation), Year of expected publication, (in press) at the end of the citation.
Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CXC, et al. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. *J Biol Chem* 2005 (in press).

Internet Communication. Ensure that URLs are active and available. Provide DOI, if available.
Developmental toxicology. http://www.devtox.org/nomenclature/organ.php. Accessed April 10, 2015.
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Physician's Desk Reference (PDR). Release 2003.1AX. [CD-ROM]. Montvale: Thomson PDR; 2003.

Computer Program
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Larsen CE, Trip R, Johnson CR. Methods for procedures related to the electrophysiology of the heart. Patent No. 5.529.067. Novoste Corporation; 1995.

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American College of Sports Medicine. *Diretrizes do ACSM para os testes de esforço prescrição.* Rio de Janeiro: Guanabara Koogan; 2007.

Book, Chapter. Authors, Chapter Title, Editors, Book title, Edition, City, Publisher, Year, Pages of citation.
Kronfol A. Behavioral effects of cytokines: a psychiatrist's perspective. In: Plotnikoff NP, Faith RE, Murgo AJ, Good RA (Editors), *Cytokines, stress and immunity*. London: CRC Press; 2007. p 1-16.
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Report
WHO (World Health Organization), IPCS (International Program in Chemical Safety). *Environmental health criteria: 118 Inorganic mercury.* Geneva: World Health Organization; 1991.
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National Institute of Health. National Heart, Lung and Blood Institute publication No. 02-3659; 2006.

Thesis
Joselevitch C. Visão no ultravioleta em Carassius auratus (Ostariophysi, Cypriformes, Cyprinidae): estudo eletrofisiológico do sistema cone - células horizontais. [Master's thesis]. São Paulo: Instituto de Psicologia, USP; 1999.

Conference, Symposium Proceedings. Cite papers only from published proceedings.
Hejzlar RM, Diogo PA. The use of water quality modelling for optimising operation of a drinking water reservoir. *Proceedings of the International Conference Fluid Mechanics and Hydrology.* 1999 Jun 23-26; Prague. Prague: Institute of Hydrodynamics AS CR; 1999. p 475-482.

"Unpublished results", "Personal communication" and "Submitted papers". Reference should appear in the text with the individual name(s) and initials and not in the reference list. (Santos CS, da-Silva GB, Martins LT, unpublished results).
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- *PLoS Biology* Guidelines for Table and Figure Preparation (http://www.plosbiology.org/static/figureGuidelines)

ANEXO C: COMPROVANTE DE SUBMISSÃO DE ARTIGO À REVISTA *FRONTIERS IN PHARMACOLOGY*

Prophylactic and Therapeutic Use of Strontium Ranelate Reduces the Progression of Experimental Osteoarthritis

Thiago A. Rodrigues[1*], Abner d. Freire[1], Gyl Eanes B. Silva[1], José Wanderley Vasconcelos[1], Maria Do Socorro d. Cartagenes[1], João Batista S. Garcia[1]

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ANEXO D – NORMAS PARA SUBMISSÃO À REVISTA *FRONTIERS IN PHARMACOLOGY*

Author Guidelines

3.10. Support and Ethical concerns

1. Summary Table

Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal.

	Abstract (max. length)	Running title (5 words)	Figures and/or tables (combined)	Manuscript (max. length)	Author fees	Submitted to PubMed Central or other indexing databases
Book Review	✗	✗	1	1'000 words	✗	✓
Brief Research Report	250 words	✓	2	2'000 words	✓	✓
Classification	250 words	✓	10	2'000 words	✓	✓
Case Report	350 words	✓	4	3'000 words	✓	✓
Clinical Study Protocol	350 words	✓	15	12'000 words	✓	✓
Clinical Trial	350 words	✓	15	12'000 words	✓	✓
Code	250 words	✓	3	3'000 words	✓	✓
Community Case Study	350 words	✓	5	5'000 words	✓	✓
Conceptual Analysis	350 words	✓	10	8'000 words	✓	✓
CPC	250 words	✓	6	2'500 words	✓	✓
Curriculum, Instruction, and Pedagogy	350 words	✓	5	5'000 words	✓	✓
Data Report	✗	✓	2	3'000 words	✓	✓
Editorial	✗	✗	0	1'000 words*	✗	✓
Empirical Study	350 words	✓	10	8'000 words	✓	✓
Evaluation	350 words	✓	5	6'000 words	✓	✓
Field Grand Challenge	✗	✓	1	2'000 words	✗	✓
Focused Review (1)	350 words	✓	5	5'000 words	✗	✓
Frontiers Commentary (1)	✗	✗	1	1'000 words	✗	✓
General Commentary	✗	✗	1	1'000 words	✓	✓
Hypothesis and Theory	350 words	✓	15	12'000 words	✓	✓
Methods	350 words	✓	15	12'000 words	✓	✓
Mini Review	250 words	✓	2	3'000 words	✓	✓
Opinion	✗	✓	1	2'000 words	✓	✓
Original Research	350 words	✓	15	12'000 words	✓	✓
Policy & Practice Reviews	350 words	✓	15	12'000 words	✓	✓
Policy Briefs	125 words	✓	5	3'000 words	✓	✓
Protocols	350 words	✓	15	12'000 words	✓	✓
Perspective	250 words	✓	2	3'000 words	✓	✓
Review	350 words	✓	15	12'000 words	✓	✓
Specialty Grand Challenge	✗	✓	1	2'000 words	✗	✓
Systematic Reviews	350 words	✓	15	12'000 words	✓	✓
Technology Report	350 words	✓	15	12'000 words	✓	✓

(1) Tier 2 article - field level article reserved to authors of selected Tier 1 articles.
* Editorials for Research Topics with 5 to 10 published articles have a maximum of 1'000 words, for Research Topics with more than 10 published articles the following applies: 1'100 words for 11 articles, 1'200 for 12 articles, 1'300 for 13 articles etc. up to maximum 5'000 words, for 50 or more papers.
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CORRESPONDENCE:

Dr. Max Maximus
maximus@gmail.com

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2.3.1.17. Cover Letter

When you submit your manuscript, you will be required to add a cover letter directed to the Editor.

Please indicate, in the first paragraph, the title of the manuscript, the article type, the Journal and specialty to which the manuscript is being submitted, and whether it is part of a Research Topic. You must also state that the manuscript has not been submitted for publication elsewhere; any closely related works submitted for consideration in other publications should be noted and you may be asked to provide a copy.

It is essential as well that you provide a short description of the significance of the manuscript. While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your cover letter should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings - whether positive or negative - contribute to progress in your research discipline. This will assist the Chief Editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed cover letter will also facilitate the identification of the Editors and Reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

2.3.2. References

All citations in the text, figures or tables must be in the reference list and vice-versa. The references should only include articles that are published or accepted. Data sets that have been deposited to an online repository should be included in the reference list, include the version and unique identifier when available. For accepted but unpublished works use "in press" instead of page numbers. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for the article types that allow such inclusions. Personal communications should be documented by a letter of permission. Website urls should be included as footnotes. Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source.

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

- **SCIENCE, ENGINEERING, and HUMANITIES: For articles submitted in the domains of SCIENCE, ENGINEERING and HUMANITIES please apply Author-Year system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be called according to the surname of the first author, followed by the year. For works by 2 authors include both surnames, followed by the year. For works by more than 2 authors include only the surname of the first author, followed by et al., followed by the year. For Humanities and Social Sciences articles please include page numbers in the in-text citations.

ARTICLE IN A PRINT JOURNAL:

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

ARTICLE IN AN ONLINE JOURNAL:

Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

ARTICLE OR CHAPTER IN A BOOK:

Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes,

ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

BOOK:

Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

ABSTRACT:

Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

PATENT:

Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

DATA:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

THESES AND DISSERTATIONS:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style.

Frontiers Science Endnote Style

Frontiers Science, Engineering and Humanities Bibstyle

- **HEALTH, PHYSICS AND MATHEMATICS: For articles submitted in the domain of HEALTH or the journal Frontiers in Physics and Frontiers in Applied Mathematics and Statistics please apply the Vancouver system for in-text citations.**

Reference list: provide the names of the first six authors followed by et al. and doi when available.

In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis for Health articles, and in square brackets for Physics and Mathematics articles.

ARTICLE IN A PRINT JOURNAL:

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

ARTICLE IN AN ONLINE JOURNAL:

Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

ARTICLE OR CHAPTER IN A BOOK:

Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

BOOK:

Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

ABSTRACT:

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

PATENT:

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

DATA:

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of

Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

THESES AND DISSERTATIONS:

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

For examples of citing other documents and general questions regarding reference style, please refer to Citing Medicine.

Frontiers Health Endnote Style

Frontiers Health and Physics Bibstyle

2.3.3. Disclaimer

Any necessary disclaimers which must be included in the published article should be clearly indicated in the manuscript.

2.3.4. Supplementary Material

Frontiers journals do not support pushing important results and information into supplementary sections. However, data that are not of primary importance to the text, or which cannot be included in the article because it is too large or the current format does not permit it (such as movies, raw data traces, power point presentations, etc.) can be uploaded during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to FigShare for permanent storage, during the publication stage of the article, and receive a DOI.

The Supplementary Material can be uploaded as Data Sheet (word, excel, csv, cdx, fasta, pdf or zip files), Presentation (power point, pdf or zip files), Supplementary Image (cdx, eps, jpeg, pdf, png or tif), Supplementary Table (word, excel, csv or pdf), Audio (mp3, wav or wma) or Video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv).

Supplementary material is not typeset so please ensure that all information is clearly presented, the appropriate caption is included in the file and not in the manuscript, and that the style conforms to the rest of the article. For Supplementary Material templates (LaTex and Word) see Supplementary Material for Frontiers.

2.3.5. File Requirements

2.3.5.1. Word Files

If working with Word please use Frontiers Word.

2.3.5.2. LaTeX Files

If you wish to submit your article as LaTeX, we recommend our Frontiers LaTeX templates. These templates are meant as a guide, you are of course welcome to use any style or formatting and Frontiers journal style will be applied during typesetting.

When submitting your article please ensure to upload all relevant manuscript files including:

- tex file
- PDF
- .bib file (if the bibliography is not already included in the .tex file)

Figures should be included in the provided pdf. In case of acceptance, our Production Office might require high resolution files of the figures included in the manuscript in eps, jpg or tif format. In order to be able to upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file, and upload them as 'Supplementary Material Presentation'.

To facilitate the review process, please include a Word Count at the beginning of your manuscript, one option is teXcount which also has an online interface.

During the Interactive Review, authors are encouraged to upload versions using 'Track Changes'. Editors and Reviewers can only download the PDF file of the submitted manuscript .

2.3.6. Additional Requirements per article types

2.3.6.1. CrossMark Policy

CrossMark is a multi-publisher initiative to provide a standard way for readers to locate the current version of a piece of content. By applying the CrossMark logo Frontiers is committing

to maintaining the content it publishes and to alerting readers to changes if and when they occur. Clicking on the CrossMark logo will tell you the current status of a document and may also give you additional publication record information about the document.

2.3.6.2. Commentaries on Articles

For General Commentaries, the title of your manuscript must have the following format: "Commentary: Title of the original article". At the beginning of your Commentary, please provide the citation of the article commented on. Authors commenting on a Frontiers article must submit their commentary for consideration to the same Journal and Specialty as the original article.

Rebuttals may be submitted in response to Commentaries; our limit in place is one commentary and one response. Rebuttals should be submitted as General Commentary articles and the title should have the following format: "Response to: Commentary: Title of the original article".

2.3.6.3. Book Reviews

For book Reviews, you must provide the full book details at the beginning of the article in this format: "A book review on: Full book reference"

2.3.6.4. Focused Reviews

For Tier 2 invited **Focused Reviews**, to shape the paper on the importance of the research to the field, we recommend structuring the Review to discuss the paper's Introduction, Materials and Methods, Results and Discussion. In addition the authors must submit a short biography of the corresponding author(s). This short biography has a maximum of 600 characters, including spaces

A picture (5 x 5 cm, in *.tif or *.jpg, min 300 dpi) must be submitted along with the biography in the manuscript and separately during figure upload.

Focused Reviews highlight and explain key concepts of your work. Please highlight a minimum of four and a maximum of ten key concepts in bold in your manuscript and provide the definitions/explanations at the end of your manuscript under “Key Concepts”. Each definition has a maximum of 400 characters, including spaces.

2.3.6.5 Systematic Reviews

For Systematic Reviews, the following article structure applies.

- Title: include systematic review/meta-synthesis/meta-analysis as appropriate in the title

Each of the sections should include specific sub-sections as follows

- Abstract
  - Background
  - Methods
  - Results
  - Conclusions
- Introduction
  - Rationale
  - Objectives
  - Research question
- Methods
  - Study design
  - Participants, interventions, comparators
  - Systematic review protocol
  - Search strategy
  - Data sources, studies sections and data extraction
  - Data analysis
- Results
  - Provide a flow diagram of the studies retrieved for the review
  - Study selection and characteristics
  - Synthesized findings

- Risk of bias
- Discussion
  - Summary of main findings
  - Limitations
  - Conclusions

2.3.6.6. Data Reports

For Data Reports, please make sure to follow these additional specific guidelines.

1. The data sets (defined as a collection of data that contains individual data units organized in a standardized reusable format, including pre-processed or raw data) must be deposited in a public repository for long-term data preservation prior to submission of the Data Report. The data set(s) is to be fixed and made publicly available upon publication of the Data Report.

2. Our data sharing policy also requires that the dataset be made available to the Frontiers editors and reviewers during the review process of the manuscript. Prior to submission of your Data Report manuscript, please ensure that the repository you have selected supports confidential peer-review. If it does not, we recommend that the authors deposit the datasets to figshare or Dryad Digital Repository for the peer-review process. The data set(s) can then be transferred to another relevant repository before final publication, should the article be accepted for publication at Frontiers.

Note that it is the authors' responsibility to maintain the data sets after publication of the Data Report. Any published Frontiers Data Report article will be considered for retraction should the data be removed from the final selected repository after publication or the access become restricted.

3. The submitted manuscript must include the following details:

- Detailed cover letter (including a link to the data set)
- Name of the data set
- Name of the database/repository where the data set has been submitted
- Link to the data set for confidential peer-review (which can be updated after acceptance, prior to publication once the data is made public)
- Description of how the data was acquired, data collection period
- Filters applied to the data
- Overview of the data files and their formats
- Reference to and/or description of the protocols or methods used to collect the data
- Information on how readers may interpret the data set and reuse the data

All these elements will be peer-reviewed and are required for the publication of the Data Report.

Any future updates to the data set(s) should be deposited as independent versions in a repository and the relevant information may be published as General Commentaries linked on the Frontiers website to the initial Data Report.

Any detailed analyses or new scientific insights relating to the Data Report can be submitted as independent research articles which can also be linked on the Frontiers website to the Data Report article. The protocols and methodology used to collect the data can also be submitted as Methods articles.

2.3.6.7. Case Reports

For Case Reports the following sections are mandatory:

1 **Introduction** Include symptoms at presentation, physical exams and lab results.

2 **Background** This section may be divided by subheadings. Include history and review of similar cases.

3 **Discussion** This section may be divided by subheadings. Include diagnosis and treatment.

4 **Concluding Remarks**

2.3.6.8. Policy & Practice Reviews

For Policy and Practice Reviews, the following article structure applies:

- Abstract
- Introduction

- Sections on assessment of policy/guidelines options and implications
- Actionable Recommendations and Conclusions

2.3.6.9. Policy Briefs

For Policy Briefs, the following article structure applies:

- Abstract (bullet point format)
- Introduction
- Sections on Policy Options and Implications
- Section on Actionable Recommendations
- Conclusions

2.3.6.10. Protocols

For Protocols articles, please make sure to follow these additional specific guidelines.

1 The submitted manuscript must include the following sections:
   - An Abstract.
   - An Introduction outlining the protocol and summarizing its possible applications.
   - A Materials and Equipment section providing a list of reagents or other materials and/or equipment required to carry out the protocol. For basic-science protocols, the formulation of any solutions, e.g. buffers, should be clearly indicated in the Materials and Equipment section.
   - A Stepwise Procedures section listing, stepwise, the stages of the protocol. The timing of each step or related series of steps should be indicated, as should points at which it is possible to pause or halt the procedure without adversely influencing the outcome. For steps requiring repeated measurements, details of precision and accuracy should be presented. Limits of detection or quantification should also be stipulated where appropriate.
   - An Anticipated Results section describing, and illustrating with figures, where possible, the expected outcome of the protocol. Any analytical software or methods should be presented in detail in this section, as should possible pitfalls and artifacts of the procedure and any troubleshooting measures to counteract them. These last may also be described in an optional Notes section.
   - Code or training data sets referenced by the protocol and useful in its execution should be hosted in an online repository; their accession numbers or other stable identifiers should be referenced in the Anticipated Results.
2 The following additional information should be presented in the cover letter accompanying your manuscript:
   - Significance of the protocol and references to any relevant primary research manuscript(s) in which it has been previously employed.
   - Any advance represented by the method compared with other, similar methods.
   - Appropriateness of the manuscript to the Specialty Section to which it has been submitted.
   - Associate Editors with suitable expertise to handle the manuscript.

2.3.6.11. Code

The code should be novel and presented in human-readable format, adhere to the standard conventions of the language used (variable names, indentation, style and grammar), be well documented (comments in source), be provided with an example data set to show efficacy, be compilable or executable free of errors (stating configuration of system used).

The code should only call standard (freely accessible) libraries or include required libraries, and include a detailed description of the use-scenarios, expected outcomes from the code and known limitations of the code.

Please therefore make sure to provide access to the following upon submission:

1 Abstract explicitly including the language of code
2 Keywords including the language of the code in the following format:"code:language"" e.g.: "code:matlab"
3 Cover Letter including the utility of the code and its language

4 Main Text including:
- code description
- application and utility of the code
- link to an accessible online code repository where the most recent source code version is stored and curated (with an associated DOI for retrieval after review)
- access to test data and readme files
- methods used
- example of use
- known issues
- licensing information (Open Source licenses recommended)

5 Compressed Archive (.zip) of the reviewed version of the code as supplementary material (.zip archives are currently available under the “Presentation” dropdown menu).

2.4. Figure and Table Guidelines

2.4.1. General Style Guidelines for Figures

The maximum number of figures and tables for all article types are shown in the Summary Table. Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript, the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order.

For graphs, there must be a self-explanatory label (including units) along each axis. For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be added during typesetting according to Frontiers journal style. Please note that figures which are not according to the guidelines will cause substantial delay during the production process.

Permission must be obtained for use of copyrighted material from other sources (including re-published/adapted/modified/partial figures and images from the internet). It is the responsibility of the authors to acquire the licenses, to follow any citation instructions requested by third-party rights holders, and cover any supplementary charges.

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (e.g. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. Image grouping and splicing must be clearly stated in the manuscript and the figure text.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.2. General Style Guidelines for Tables

Tables should be inserted at the end of the manuscript. If you use a word processor, build your table in word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material on the online article abstract page at the time of acceptance. The author will notified during the typesetting of the final article if this is the case. A link in the final PDF will direct to the online material.

For additional information, please see our Editorial Policies: 3.5 Image Manipulation.

2.4.3. Figure and Table Requirements

Legends

Figure and table legends are required to have the same font as the main text (12 point normal Times New Roman, single spaced). Legends should be preceded by the appropriate label, for example "Figure 1" or "Table 4". Figure legends should be placed at the end of the manuscript (for supplementary images you must include the caption with the figure, uploaded as a

separate file). Table legends must be placed immediately before the table. Please use only a single paragraph for the legend. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image Size

Figure images should be prepared with the PDF layout in mind, individual figures should not be longer than one page and with a width that corresponds to 1 column or 2 columns.

- **All articles are prepared using the 2 column layout:** 2 column articles can contain images 85 mm or 180 mm wide.

2.4.4. Format

The following formats are accepted:

TIFF (.tif) TIFF files should be saved using LZW compression or any other non-lossy compression method. JPEG (.jpg)

EPS (.eps) EPS files can be uploaded upon acceptance

Color Image Mode

Images must be submitted in the color mode RGB.

Resolution Requirements

All images must be uploaded separately in the submission procedure and have a resolution of **300 dpi at final size**. Check the resolution of your figure by enlarging it to 150%. If the resolution is too low, the image will appear blurry, jagged or have a stair-stepped effect.

Please note saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software. EPS files can be uploaded upon acceptance.

Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program according to the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt. Scale 100%Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for incorporation into the manuscript.

Legibility

Figures must be legible. Check the following:

- The smallest visible text is no less than 8 points in height, when viewed at actual size.
- Solid lines are not broken up.
- Image areas are not pixilated or stair stepped.
- Text is legible and of high quality.
- Any lines in the graphic are no smaller than 2 points width.

2.5. Funding disclosure

Details of all funding sources must be provided in the funding section of the manuscript including grant numbers, if applicable. All Frontiers articles are published with open access under the CC-BY Creative Commons attribution license. Articles published with Frontiers automatically fulfil or exceed the requirements for open access mandated by many institutions and funding bodies, including the National Institutes of Health, the Medical Research Council, Research Councils UK, and the Wellcome Trust. Frontiers submits funding data to the Open Funder Registry which is a funder identification service from CrossRef resulting from collaboration between scholarly publishers and funding agencies.

2.6. Materials and Data Policies

Frontiers is committed to open science and open data, and we strongly encourage authors to maximize the availability of data included in their articles by making generated data publicly available where possible, and ensuring that published data sets are cited in accordance with our data citation guidelines. We aim to achieve the best community standards regarding data availability, ensuring increased levels of transparency and reproducibility in our published

articles.
Our policies on data availability are informed by community-driven standards, which Frontiers endorses, such as the Transparency and Openness (TOP) guidelines, and the joint declaration of data citation principles produced by FORCE 11.

2.6.1. Availability of Materials

Authors are strongly encouraged to make all materials used to conduct their research available to other researchers. Research materials necessary to enable the reproduction of an experiment should be clearly indicated in the Materials and Methods section. Relevant materials such as protocols, analytic methods, and study material should preferably be uploaded to an online repository providing a global persistent link/identifier. If this is not possible, authors are strongly encouraged to make this material available upon request to interested researchers, and this should be stated in the manuscript.

Resource Identification Initiative

Authors wishing to participate in the Resource Identification Initiative should cite antibodies, genetically modified organisms, software tools, data, databases, and services using the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here.

2.6.2. Availability of Data

Frontiers requires that authors make all data relevant to the conclusions of the manuscript available to editors and reviewers during peer-review to enable complete and objective evaluation of the work described.
We strongly encourage authors to make the raw data supporting the conclusions of the manuscript available in publicly accessible repositories. To comply with best practice in their field of research, authors are required to make certain types of data available to readers at time of publication in specific stable, community-supported repositories such as those listed below. Authors are encouraged to contact our data availability office at datapolicy@frontiersin.org prior to submission with any queries concerning data reporting.

2.6.3. Data Citation Guidelines

Authors are encouraged to cite all datasets generated or analyzed in the study. Where datasets are cited, they should be included in the references list to maximize future usability. The following format should be used:
[Dataset] Author names. (year) Data Title. Repository name. Version. Persistant identifier

2.6.4. Data Availability Statements

Frontiers requires that every published article contains a data availability statement detailing where the data supporting the conclusions of the manuscript can be found. This includes both data generated by the authors and data analyzed for the study. Submitted manuscripts that contain incomplete or incorrect statements will be prevented from entering the review process. Datasets referred to in the data availability statement should be cited in accordance with our data citation guidelines, and should also be included in the statement.

Examples of acceptable statements

1 **Datasets are in a publicly accessible repository:** The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]

2 **Datasets are available on request:** The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

3 **All relevant data is contained within the manuscript:** All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

4 **Restrictions apply to the datasets:** The datasets for this manuscript are not publicly available because: [VALID REASON]. Requests to access the datasets should be directed to [NAME, EMAIL].

5 **Data has been obtained from a third party:** The data analyzed in this study was obtained

from [SOURCE], the following licenses/restrictions apply [RESTRICTIONS]. Requests to access these datasets should be directed to [NAME, EMAIL].

6 **No datasets were generated for this study**

2.6.5. Recommended and Required Repositories

Authors are required to deposit the following data-types in public, community-supported repositories, such as those listed below, prior to publication of an associated Frontiers manuscript:

Data-type	Recommended Repositories	Metadata Standard
Genetic and genomic sequence (DNA/ RNA)^	GenBank DNA Data Bank of Japan (DDBJ) European Nucleotide Archive (ENA)	MiXS
Metagenomic sequence	EBI Metagenomics	MiXS
DNA and RNA trace or short-read sequencing data	NCBI Trace Archive NCBI Sequence Read Archive	MiXS
Genetic polymorphism data, including SNP and CNV data	dbSNP dbVar European Variation Archive DGVa	MiXS
Gene expression data; chromatin immunoprecipitation data (deep-sequencing or microarray)	ArrayExpress Gene Expression Omnibus (GEO)	MIAME / MINSEQE
Data linking genotype to phenotype	dbGaP	
Protein sequence data	UniProt	
Proteome profiling data	PRIDE PeptideAtlas ProteomeXchange	MIAPE
Small molecule, protein, protein complex data structural data	Crystallography Open Database Cambridge Structural Database wwPDB (Protein DataBank) Electron Microscopy Databank	CIF
Taxonomy data	Zoobank	

^ Genetic sequence variants should be annotated according to the guidelines established by the Human Variome Project.

Authors are encouraged to consider deposition in public, community-supported repositories of the data-types listed below:

Data-type	Recommended Repositories	Metadata Standard
Protein-protein interaction data	Database of Interacting Proteins (DIP)	MIMIx
Metabolite and metabolome profiling data	MetaboLights Human Metabolome Database	MSI

Small-molecule screening data, chemical compound data	PubChem	CIF
Flow cytometry data	Flow Repository	
Brain Imaging data / Neuroimaging data	OpenNeuro INDI NITRC NeuroVault [Statistical maps]	BIDS
Trait data	TRY database	
Phenology data	National Phenology Network	
Any data	FigShare Dryad Digital Repository	None

2.6.6. Inclusion of Zoological Nomenclature

The International Code of Zoological Nomenclature, in a recent 2012 amendment to the 1999 Zoological Code, allows all electronic-only papers, such as those published by the Frontiers journals, to have valid new taxon names and nomenclatural acts. However, these new names or nomenclatural acts must be registered in ZOOBANK and have associated Life Science Identifiers (LSIDs). Registration must be done by the authors before publication. Should your manuscript include any zoological new taxon names and/or nomenclatural acts, please ensure that they are registered prior to final publication.

2.6.7. Inclusion of RNAseq Data

Studies employing RNASeq for comparative transcriptomic analyses must contain at least 3 biological replicates (unless otherwise justified). Each biological replicate should be represented in an independent library, each with a unique barcode if libraries are multiplexed for sequencing. Validation on a number of key transcripts highlighted in the study is also highly recommended.

Full data accompanying these experiments must be made available to reviewers at the time of submission in a freely accessible resource e.g the sequence read archive (SRA) or European Nucleotide Archive (ENA). Depending on the question addressed in a manuscript, de novo assemblies of transcriptomes may also require multiple replicates and assembled sequences together with sequence annotation must be made freely available e.g figshare or dryad.

2.7. Statistics

Frontiers requires that all statements concerning quantitative differences should be based on quantitative data and statistical testing. For example, if a quantitative statement is made regarding the abundance of a certain protein based on a western blot, we request that the blot be scanned and the abundance assessed quantitatively using the correct analytic software (e.g. ImageJ) and statistics in order to support that statement.

Statistics should/must be applied for independent experiments. The number of independent samples and the deviation parameters (e.g. Standard Error of the Mean, Standard Deviation, Confidence Intervals) should be clearly stated in the Methods or the Figure legends. In general, technical replicates within a single experiment are not considered to be independent samples. Where multiple comparisons are employed (e.g. microarray data or Genome-wide association studies), any analysis should correct for false positive results. Descriptions of statistical procedures should include the software and analysis used, and must be sufficiently detailed to be reproduced.

3.1. Authorship and Author Responsibilities

Frontiers follows the International Committee of Medical Journal Editors guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Provide approval for publication of the content;
- Agree to be accountable for all aspects of the work in ensuring that questions related to the

accuracy or integrity of any part of the work are appropriately investigated and resolved. Contributors, who do not meet these criteria, but nonetheless provided important contributions to the final manuscript should be included in the acknowledgements section. It is the authors responsibility to get written approval by persons named in the acknowledgement section. In order to provide appropriate credit to all authors, as well as assigning responsibility and accountability for published work, individual contributions should be specified as an Author Contributions statement. This should be included at the end of the manuscript, before the References. The statement should specify the contributions of all authors. You may consult the Frontiers manuscript guidelines for formatting instructions. Please see an example here:
AB, CDE and FG contributed conception and design of the study; AB organized the database; CDE performed the statistical analysis; FG wrote the first draft of the manuscript; HIJ, KL, AB, CDE and FG wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.
The corresponding author takes primary responsibility for communication with the journal and editorial office during the submission process, throughout peer review and during publication. The corresponding author is also responsible for ensuring that the submission adheres to all journal requirements including, but not exclusive to, details of authorship, study ethics and ethics approval, clinical trial registration documents and conflict of interest declaration. The corresponding author should also be available post-publication to respond to any queries or critiques.

3.2. Research Integrity

Material submitted to Frontiers must comply with the following policies to ensure ethical publication of academic work:

i Original content and duplicate publication: Frontiers only publishes original content. Authors confirm the submission of original content in the Terms & Conditions upon submission. Manuscripts submitted to Frontiers must not have been previously published or be under consideration for publication elsewhere, either in whole or in part. If an article has been previously submitted for publication elsewhere, Frontiers will only consider publication if the article has been definitively rejected by the other publisher(s) at the point of submission to Frontiers.

ii Redundant publication: Frontiers considers the submission and publication of very similar articles based on the same experiment or study to be unethical.

iii Fabrication and falsification: Frontiers opposes both the fabrication of data or images (i.e. fake or made up data) and the falsification of data or images (i.e. the intentional misrepresentation or deceptive manipulation of data).

iv Plagiarism: Plagiarism occurs when an author attempts to present previously published work as original content. Every manuscript submitted to Frontiers is screened for textual overlap by the software CrossCheck, powered by iThenticate. Manuscripts found to contain textual overlap are not considered for publication by Frontiers. For more details on what constitutes plagiarism, please see here.

We reserve the right to contact the affiliated institutions of authors, who have not acted according to good research and publication practices.

3.3. Translations

Frontiers accepts manuscript submissions that are exact translations of previously published work. This should be clearly stated upon manuscript submission, in the cover letter and in the manuscript. Permission from the original publisher and authors needs to be sought and also stated, and the relevant documents should be provided as supplementary data for verification by the Editor and the editorial office. The original work from which the manuscript has been translated should be clearly referenced.

> "This is a (‘language’) language translation/reprint of (‘insert title here’) originally published in (‘insert name here’). (‘Insert name here’) prepared this translation with support from (insert name of funding source, if any). Permission was granted by (‘Insert name here’)."

Please note that Frontiers may request copies of related publications if there are any concerns about overlap or possible redundancy.

3.4. Plagiarism and Duplication

Frontiers checks all submitted manuscripts for plagiarism and duplication, and publishes only original content. Those manuscripts where plagiarism or duplication is shown to have occurred will not be considered for publication in a Frontiers journal. It is required that all submissions must consist as far as possible of content that has not been published previously. In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work.

For submissions adapted from theses, dissertations, conference abstracts or proceedings papers, please see the following sections for more information.

Theses and Dissertations

Frontiers allows the inclusion of content which first appeared in an author's thesis so long as this is the only form in which it has appeared, is in line with the author's university policy, and can be accessed online. If the thesis is not archived online, it is considered as original unpublished data and thus is subject to the unpublished data restrictions of some of our article types. This inclusion should be noted in the Acknowledgements section of the manuscript and the thesis should be cited and referenced accordingly in the Reference list. For some examples, please check our in Manuscript Requirements and Style Guide at 2.3.1

Conferences, Proceedings and Abstracts

Manuscripts that first appeared as conference papers must be expanded upon if they are to be considered as original work. At least 30% of content must be original. Authors submitting such work are required to:

- Cite the conference in the Acknowledgements section, or the references section if applicable
- Seek permission for reuse of the published conference paper if the author does not hold the copyright (proof of permission should be submitted as supplementary material, or sent to editorial.office@frontiersin.org with the manuscript ID upon submission)

Blogs

Although permissible, extended manuscript content which previously appeared online in non-academic media, e.g. blogs, should be declared at the time of submission in a cover letter or in communication with the relevant editorial office for consideration.

3.5. Image Manipulation

Frontiers takes concerns regarding image manipulation seriously. We request that no individual features within an image are modified (eg. enhanced, obscured, moved, recycled, removed or added). Image processing methods (e.g. changes to the brightness, contrast or color balance) must be applied to every pixel in the image and the changes should not alter the information illustrated in the figure. Where cropped images of blots are shown in figures, a full scan of the entire original gel(s) must be submitted as part of the supplementary material. Where control images are re-used for illustrative purposes, this must be clearly declared in the figure legend. If any form of image processing is legitimately required for the interpretation of the data, the software and the enhancement technique must be declared in the methods section of the manuscript. Image grouping and splicing must be clearly stated in the manuscript and the figure text. Any concerns raised over undeclared image modifications will be investigated and the authors will be asked to provide the original images.

3.6. Conflicts of Interest

A conflict of interest can be anything potentially interfering with, or that could reasonably be perceived as interfering with, full and objective peer review, decision-making or publication of articles submitted to Frontiers. Personal, financial and professional affiliations or relationships can be perceived as conflicts of interest.

All authors and members of Frontiers Editorial Boards are required to disclose any actual and potential conflicts of interest at submission or upon accepting an editorial or review assignment. The Frontiers review system is designed to guarantee the most transparent and objective

editorial and review process, and because handling editor and reviewers' names are made public upon the publication of articles, conflicts of interest will be widely apparent.
Failure to declare competing interests can result in the rejection of a manuscript. If an undisclosed competing interest comes to light after publication, Frontiers will take action in accordance with internal policies and Committee on Publication Ethics guidelines.

What Should I Disclose?

As an author, disclosure of any potential conflicts of interest should be done during the submission process. Consider the following questions and make sure you disclose any positive answers:

1 Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work?
2 Do you have financial relationships with entities that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work?
3 Do you have any patents and copyrights, whether pending, issued, licensed and/or receiving royalties related to the research?
4 Do you have other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

If you failed to disclose any of the potential conflicts of interest above during submission, or in case of doubt, please contact as soon as possible the Frontiers Editorial Office at editorial.office@frontiersin.org with the details of the potential conflicts.
The handling editors and reviewers will be asked to consider the following potential conflicts of interest before accepting any editing or review assignment:

FAMILY	1. Are any of the authors a spouse or significant other, a member of the same family or a very close personal friend? Review Editors should also not be a member of the same family as the handling editor.
COLLABORATIONS	2. Are you currently hosting or have hosted a Frontiers Research Topic with any of the authors within the past 2 years? Are you currently hosting a Frontiers Research Topic with the Editor? 3. Are you currently collaborating or have you collaborated on a research project or a publication with any of the authors within the past 2 years? 4. Are you currently collaborating or have you collaborated with any of the authors as an advisor or in any other direct supervisory capacity in the past five years? 5. Are you currently collaborating or have you collaborated with any of the authors as a student or in any other direct subordinate capacity in the past five years? Note: Review Editors should not accept assignments if they have a close professional relationship with the handling editor, which in their view could affect the objectivity of the review.
AFFILIATION	6. Are you affiliated with the same institution as the editor? Are you affiliated with the same institution as any of the authors? If so, has this resulted in interactions, collaborations, or mutual interests with the authors that would compromise your impartiality in conducting this review? 7. Are you a current member of a committee or department that coincides with an affiliation with the editor or any of the authors?

FINANCIAL	8. Do you have a business or professional partnership with any author? 9. Do you have financial interests or business relations with any organization involved in this research or in the preparation of the manuscript? 10. Do you have any financial interest or competing interests in the content of the manuscript that might affect your ability to perform an objective review?

3.7. Bioethics

All research submitted to Frontiers for consideration must have been conducted in accordance with Frontiers guidelines on study ethics. In accordance with COPE guidelines, Frontiers reserves the right to reject any manuscript that editors believe does not uphold high ethical standards, even if authors have obtained ethical approval or if ethical approval is not required.

3.7.1. Studies involving animal subjects

All research involving regulated animals (i.e. all live vertebrates and higher invertebrates) must be performed in accordance with relevant institutional and national guidelines and regulations. Prior approval of research involving regulated animals must be obtained from the relevant institutional review board or ethics committee prior to commencing the study. Confirmation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee].

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the cover letter and manuscript. Studies involving privately owned animals should demonstrate the best practice veterinary care and confirm that informed consent has been granted by the owner/s, or the legal representative of the owner/s. Frontiers supports and encourages authors to follow the ARRIVE guidelines for the design, analysis and reporting of scientific research. (http://www.veteditors.org/consensus-author-guidelines-on-animal-ethics-and-welfare-for-editors)

Humane Endpoints

All manuscripts describing studies where death is an endpoint will be subject to additional ethical considerations. Frontiers reserves the right to reject any manuscripts lacking in appropriate justification.

3.7.2. Studies involving human subjects

Research involving human subjects is expected to have been conducted in accordance with the World Medical Association's Declaration of Helsinki. Studies involving human participants must be performed in accordance with relevant institutional and national guidelines, with the appropriate institutional ethics committee's prior approval and informed written consent from all human subjects involved in the study including for publication of the results. Conformation of this approval is required upon submission of a manuscript to Frontiers; authors must provide a statement identifying the full name of the ethics committee that approved the work and confirm that study subjects (or when appropriate, parent or guardian) have given written informed consent. For most article types, this statement should appear in the Materials and Methods section. An example ethics statement:

This study was carried out in accordance with the recommendations of [name of guidelines], [name of committee]. The protocol was approved by the [name of committee]. All subjects gave **written informed consent** in accordance with the Declaration of Helsinki.

Should the study be exempt from ethics approval, authors need to clearly state the reasons in the cover letter and manuscript. In order to protect subject anonymity, identifying information should not be included in the manuscript unless such information is absolutely necessary for scientific purposes AND explicit approval has been granted by the subjects.

3.7.3. Inclusion of identifiable human data

Frontiers follows the ICMJE recommendations on the protection of research participants, which state that patients have a right to privacy that should not be violated without informed consent. We require non-essential identifiable details to be omitted from all manuscripts, and written informed consent will be required if there is any doubt that anonymity can be maintained.

It is the responsibility of the researchers and authors to ensure that these principles are complied with, including the obtaining of written, informed consent.

Written informed consent can be documented on a form provided by an institution or ethics committee, and it must clearly state how the identifiable data will be used. Frontiers also makes available its own form , which may be used for this purpose, but use of the Frontiers form is not required if a suitable alternative form of consent, meeting the ICMJE recommendations, is used. We consider it to be the author' duty to encourage participants or patients whose consent for publication is required to read and understand the ICMJE guidelines, for their information prior to completing the consent form. Participants should also be encouraged to ask any questions and to ensure they are comfortable before they sign the consent form.

The completed consent forms should be stored by authors or their respective institutions, in accordance with institutional policies. Frontiers does not need to view the completed form, and this should not be included with the submission. The completed form should be made available on request from the editor or editorial office, both during the review process and post-publication.

The determination of what constitutes identifiable data lies with our editors and editorial office staff, and manuscripts may be rejected if the required consent documents cannot be provided. Please note that written informed consent for publication is required for all case report articles where the patient or subject is identified or identifiable.

3.7.4. Clinical Trials

The World Health Organization defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes." In accordance with the Clinical Trial Registration Statement from the International Committee of Medical Journal Editors (ICMEJ), all clinical trials must be registered in a public trials registry at or before the onset of participant enrolment. This requirement applies to all clinical trials that begin enrolment after July 1, 2005. To meet the requirements of the ICMJE, and Frontiers', clinical trials can be registered with any Primary Registry in the WHO Registry Network or an ICMJE approved registry.

Clinical trial reports should be compliant with the Consolidated Standards of Reporting Trials (CONSORT) both in terms of including a flow diagram presenting the enrolment, intervention allocation, follow-up, and data analysis with number of subjects for each and taking into account the CONSORT Checklist of items to include when reporting a randomized clinical trial. The information on the clinical trial registration (Unique Identifier and URL) must be included in the abstract.

3.8. Corrections

Frontiers recognizes our responsibility to correct errors in previously published articles. If it is necessary to communicate important, scientifically relevant errors or missing information, and compelling evidence can be shown that a major claim of the original article was incorrect, a Correction should be submitted detailing the reason(s) for and location(s) of the change(s) needed in the cover letter. Corrections can be submitted if a small portion of an otherwise reliable publication proves to be misleading, e.g. an error in a figure that does not alter conclusions OR an error in statistical data not altering conclusions OR mislabeled figures OR wrong slide of microscopy provided, or if the author / contributor list is incorrect when a deserving author has been omitted or somebody who does not meet authorship criteria has been included.

The title of the submission should have the following format: "Corrigendum: Title of original article". It is advised to use the corrigendum Word and LaTeX templates.

If the error was introduced during the publishing process, the Frontiers Production Office

should be contacted.

3.9. Retractions

As a member of the Committee on Publication Ethics (COPE), Frontiers abides by their guidelines and recommendations in cases of potential retraction.

Frontiers also abides by two other key principles, as recommended by COPE:

- Retractions are not about punishing authors.
- Retraction statements should be public and linked to the original, retracted article.

While all potential retractions are subject to an internal investigation and will be judged on their own merits, Frontiers considers the following reasons as giving cause for concern and potential retraction:

- Clear evidence that findings are unreliable, either as a result of misconduct (e.g. data fabrication) or honest error (e.g. miscalculation or experimental error)
- Findings have previously been published elsewhere without proper attribution, permission or justification (i.e. cases of redundant publication)
- Major plagiarism
- The reporting of unethical research, the publication of an article that did not have the required ethics committee approval
- Legal issues pertaining to the content of the article e.g. libellous content
- Major authorship issues i.e. proven or strongly suspected cases of ghostwriting or sold (‘gift’) authorship
- Politically-motivated articles where objectivity is a serious concern
- The singling out of individuals or organizations for attack
- Faith issues (e.g. intelligent design)
- Papers that have made extraordinary claims without concomitant scientific or statistical evidence (e.g. pseudoscience)

Readers who would like to draw the editors' attention to published work that might require retraction should contact the authors of the article and write to the journal, making sure to include copies of all correspondence with authors.

Please find more details on our comments and complaints policy here

3.10. Support and Ethical concerns

In our commitment to continuously improve our website, we welcome your feedback, questions and suggestions. Please visit our Help Center to find guidance on our platform or contact us at support@frontiersin.org.

For any ethical concerns, please contact us at editorial.office@frontiersin.org.

ANEXO E – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE FEDERAL DO MARANHÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-CEUA
CIAEP: 01.0341.2014

CERTIFICADO

Certificamos que a proposta intitulada " **Analise do ranelato de estroncio em modelo experimental de osteoartrite em rato"** registrada com o nº **23115.012456/2016-04**, sob a responsabilidade de **João Batista Santos Garcia**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi considerado **Aprovada** pela Comissão de Ética no Uso de Animais (CEUA- UFMA) da Universidade Federal do Maranhão.

FINALIDADE	()ENSINO(**X**)PESQUISA ( ) EXTENSÃO
Vigência da autorização	01/ 09/2016 à 01/02/2017
Espécie/linhagem/raça	Roedores da linhagem Wistar (*Rattus norvegicus albinus*)
Nº de animais	30
Peso/Idade	60 dias
Sexo	Macho e Fêmeas
Origem	Biotério Central - UFMA

Profa. Dra. Lucilene Amorim Silva
Presidente da Comissão de Ética no uso de animais-CEUA
UFMA