



**UNESP – Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara**



Cassio Rocha Scardueli

**Utilização sistêmica de estrôncio não radioativo como potencializador da
formação óssea em ratos**

Araraquara

2018



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formação óssea em ratos**

Tese apresentada ao Programa de Pós-Graduação em
Odontologia – Área de Implantodontia, da Faculdade de
Odontologia de Araraquara, da Universidade Estadual
Paulista para Obtenção do título de Doutor em
Odontologia, na Área de Implantodontia

Orientador: Prof. Dr. Elcio Marcantonio Junior

ARARAQUARA

2018

Scardueli, Cassio Rocha

Utilização sistêmica de estrôncio não radioativo como potencializador da formação óssea em ratos / Cassio Rocha Scardueli. – Araraquara: [s.n.], 2018

128 f. ; 30 cm

Tese (Doutorado em Odontologia) – Universidade Estadual Paulista, Faculdade de Odontologia

Orientador: Prof. Dr. Elcio Marcantonio Junior

Co-orientadora: Profa. Dra. Rosemary Adriana Chiérici Marcantonio

1. Estrôncio 2. Osseointegração 3. Remodelação óssea
4. Estrôncio - Toxicidade I. Título

CASSIO ROCHA SCARDUELI

**UTILIZAÇÃO SISTÊMICA DE ESTRÔNCIO NÃO RADIOATIVO COMO
POTENCIALIZADOR DA FORMAÇÃO ÓSSEA EM RATOS**

Comissão julgadora

Tese para obtenção do grau de Doutor em Odontologia

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Araraquara, 22 de março de 2018.

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Dedico esse trabalho...

À **Deus**, que tornou possível a realização de cada etapa de minha jornada.

Aos meus pais, **Gilmar e Maíza**, exemplos de dedicação, esforço, humildade e amor.
Pais estes que abriram mão de suas vontades pela minha prosperidade e realização de meus sonhos.

Aos meus avós, **Átila Ribeiro Rocha** (in memoriam), **José Scardueli Netto**, e **Zenaide Barreto Scardueli**, exemplos de honestidade, força de vontade e amor.

Ao meu irmão **Érico** e minha cunhada **Tati** pelo companheirismo, confiança depositados, durante toda a trajetória desta etapa.

Aos meus **avós, tios, tias, e primos** que de certa forma dividem momentos passados por mim, e acreditam na minha competência e trabalho.

à toda a família Rocha Scardueli.

Agradeço especialmente

Ao **Prof. Dr. Elcio Marcantonio Junior** e à **Profa. Dra. Rosemary Adriana C. Marcantonio**, meus orientadores, que são ótimos e exemplares professores, sinônimos de dedicação, competência, transmissão de conhecimentos, além de serem pessoas amigas com um coração muito grande. Me acolheram em meus primeiros passos, como cirurgião dentista, permitindo que me tornasse também pesquisador, saciando minhas dúvidas, anseios, para que num futuro possa associar ambas características e tornar um profissional completo.

Ao **Prof. Dr. Rubens Spin Neto** e a **Carol Bizelli**, pela oportunidade que me deram e pela confiança em meu trabalho depositados. Ótimos amigos, companheiros. Tenho orgulho de tê-los próximos a mim, pessoas exemplares que levarei durante toda vida.

Ao meu tio **Rodney Garcia Rocha**, além de tio, um companheiro e amigo. Através de conselhos, idéias, transmissão de experiências e conversas, o que para mim é muito gratificante e um espelho para a vida.

À minha namorada **Isabella**, que além de toda sua ajuda nesta tese, me apoia e dá todo carinho e amor necessário. Exemplo de companheirismo, dedicação e união.

Agradeço...

À **Faculdade de Odontologia de Araraquara**, na pessoa de sua Diretora, Profa. Dra. Elaine Maria Sgavioli Massucato, e do Vice-Diretor, Prof. Dr. Edson Alves de Campos, responsável pela minha formação e pela estrutura oferecida para a realização dessa pesquisa.

Ao **Coordenador do Curso de Pós-Graduação em Odontologia**, Área de Periodontia, Prof. Dr. Joni Augusto Cirelli, e a todos os docentes do Curso de Pós-Graduação do Programa de Periodontia, sempre dispostos a ensinar, pela disponibilidade e dedicação ao programa e aos seus alunos.

Aos **diretores** da Faculdade de Odontologia de Araraquara-UNESP, onde tive a oportunidade de estar presente em diversas gestões.

À **todos os professores** da Faculdade de Odontologia de Araraquara-UNESP, que de certa forma sempre me puderam ajudar por meio da amizade, conversas, e ensinamentos passados durante a graduação podendo-me fazer crescer profissionalmente durante a graduação podendo-me fazer crescer profissionalmente.

Aos **funcionários da Disciplina de Periodontia e do Departamento de Diagnóstico e Cirurgia** pela paciência, ajuda e confiança depositados.

Aos **funcionários do setor de Pós-graduação**, Cristiano e José Alexandre pela atenção dada aos momentos que precisei.

Aos **funcionários**, em geral, da Faculdade de Odontologia de Araraquara- (FOAr-UNESP).

Aos **amigos especiais, aos colegas da faculdade, da pós-graduação e colegas** fora do ambiente institucional.

“Não basta ensinar ao homem uma especialidade, porque se tornará assim uma máquina utilizável e não uma personalidade. É necessário que adquira um sentimento, senso prático daquilo que vale a pena ser empreendido, daquilo que é belo, do que é moralmente correto”.

Albert Einstein

Scardueli CR. Utilização sistêmica de estrôncio não radioativo como potencializador da formação óssea em ratos [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

RESUMO

O uso de medicamentos a base de estrôncio tem se tornado notável na prevenção e tratamento de enfermidades relacionadas ao tecido ósseo. Essa notabilidade se dá devido a sua ação conjunta em células distintas e envolvidas na neoformação óssea, osteoblastos e osteoclastos. Devido a seus resultados positivos o estrôncio passou a ser utilizado também como coadjuvante de processos de neoformação óssea, principalmente procedimentos cirúrgicos maxilo-faciais. Sendo assim, este estudo teve como objetivos a avaliação de diferentes compostos, e dosagens a base de estrôncio em procedimentos de osseointegração, enxertia óssea. Para tal, os resultados foram distribuídos em 4 artigos para melhor compreensão. No estudo 1, revisão de literatura, foram analisados estudos que utilizaram estrôncio sistêmico como terapia para processos de osseointegração e enxertia óssea em animais. No estudo 2, osseointegração, foram analisadas diferentes dosagens de ranelato de estrôncio (50 e 625mg), e carbonato/cloreto de estrôncio (30 e 365 mg), administrados sistemicamente, durante o processo de osseointegração. Semelhantemente, o estudo 3 analisou a influência dos mesmos compostos a base de estrôncio em defeitos críticos ósseos em calvarias. E por ultimo, no estudo 4, uma análise relacionada a toxicidade do suplemento foi realizada nas dosagens que obtiveram melhores resultados. Como resultados, os estudos 2 e 3 apresentaram resultados semelhantes e positivos para os suplementos a base de estrôncio nas maiores concentrações (ranelato de estrôncio 625 mg e carbonato/cloreto estrôncio 365mg). Diante dessas posologias, o estudo 4 mostrou a ausência de efeitos danosos para os órgãos envolvidos no metabolismo ósseo. Devido a esses resultados, podemos crer que o estrôncio exerce atividades positivas sobre o tecido ósseo, e novos estudos associando a uma terapia local poderiam indicá-lo como suplementos clínicos.

Palavras-Chave: Estrôncio. Osseointegração. Remodelação óssea. Estrôncio-toxicidade.

Scardueli CR. Use of systemic strontium non-radioactive as potentiator of bone formation in rats. [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

ABSTRACT

The use of strontium medications has become notable in prevention and treatment of bone tissue related diseases. This notability is related to double action in distinct cells involved in bone neoformation, osteoblasts and osteoclasts. Due to its positive results, strontium has also been used as a coadjuvant of bone neoformation processes, mainly maxillofacial surgical procedures. Thus, this study had as objectives the evaluation of different compounds, and dosages based on strontium in procedures of osseointegration and bone grafting. For this, the results were distributed in 4 studies for better understanding. In study 1, literature review, we analyzed studies that used systemic strontium as a therapy for osseointegration processes and bone grafting in animals. In study 2, osseointegration, different dosages of strontium ranelate (50 and 625mg), and strontium carbonate/strontium chloride (30 and 365mg), systemically administered during the osseointegration process, were analyzed. Similarly, study 3 analyzed the influence of the same strontium-based compounds on critical bone defects in calvarias. Finally, in study 4, an analysis related to toxicity of the supplement was performed at the dosages that obtained the best results.

As results, studies 2 and 3 presented similar and positive results for the strontium-based supplements at the highest concentrations (strontium ranelate 625 mg and strontium carbonate / 365 mg strontium). Considering these dosages, study 4 showed the absence of harmful effects for the organs involved in bone metabolism. Because of these results, we may believe that strontium exerts positive activities on bone tissue, and further studies associating it with local therapy could indicate it as clinical supplements.

Keywords: Strontium. Osseointegration. Bone remodeling. Toxicity.

SUMÁRIO

1 INTRODUÇÃO.....	11
1.1 Estrôncio Não Radiativo (Sr²⁺).....	11
1.2 Mecanismos de Ação.....	12
1.3 Tratamentos de Patologias Sistêmicas a Base de Estrôncio.....	14
1.4 Cloreto de Estrôncio e Carbonato Estrôncio (Novas Formulações).....	15
1.5 Suplementos a Base de Estrôncio para Formação Óssea.....	16
2 PROPOSIÇÃO.....	17
2.1 Proposição Geral.....	17
2.2 Objetivos Específicos.....	17
3 PUBLICAÇÕES.....	18
3.1 Publicação 1.....	18
3.2 Publicação 2.....	38
3.3 Publicação 3.....	58
3.4 Publicação 4.....	75
4 DISCUSSÃO.....	94
5 CONCLUSÃO.....	98
REFERÊNCIAS.....	99
APÊNDICE A – METODOLOGIA DETALHADA DA PUBLICAÇÃO 2.....	103
APÊNDICE B – METODOLOGIA DETALHADA DA PUBLICAÇÃO 3.....	111
APÊNDICE C – METODOLOGIA DETALHADA DA PUBLICAÇÃO 4.....	118
ANEXO A - COMITÊ DE ÉTICA NO USO DE ANIMAL.....	125
ANEXO B - COMITÊ DE ÉTICA NO USO DE ANIMAL.....	126
ANEXO C - SUBMISSÃO DA PUBLICAÇÃO 1.....	127
ANEXO D - SUBMISSÃO DA PUBLICAÇÃO 4.....	128

1 INTRODUÇÃO

Diferentes estratégias têm sido rotineiramente utilizadas com o intuito de acelerar ou melhorar a formação óssea. Sendo assim, inúmeros tratamentos de ordem sistêmica^{1,2} ou local^{3,4} têm sido pesquisados e/ou propostos para favorecer a neoformação óssea, como a administração ou incorporação de substâncias e/ou medicamentos.

Notavelmente, a quantidade dessas substâncias empregadas é bem expressiva. Com atuações diretas sobre células formadoras de osso, osteoblastos, ou sobre células reabsortivas, osteoclastos. As substâncias que agem em osteoblastos atuam de modo a estimular sua replicação ou aumentar sua atividade, estimulando uma maior produção óssea¹. Distintivamente à essas substâncias estariam as atuantes em osteoclastos, que agem negativamente, inibindo ou induzindo a morte celular. Com a inativação ou morte dessas células haveria uma sobreposição da formação óssea frente a reabsorção, agindo também de modo a favor da formação óssea¹ (indiretamente).

Em sua grande maioria tais substâncias são indicadas para tratamento sistêmico de patologias ou enfermidades relacionadas à manutenção ou formação de tecido ósseo. No entanto, nem todas substâncias quando administradas sistemicamente ou localmente apresentam bons resultados no âmbito odontológico, seja por uma baixa atividade^{5,6} ou efeitos adversos^{7,8} que vêm a impossibilitar o uso coadjuvante a favor de uma maior ou mais rápida formação óssea, como ocorre nos procedimentos de enxertos ósseos e instalação de implantes osseointegrados.

Em virtude dessas dificuldades e contraindicações, a busca por novas substâncias representa uma alternativa para uma melhor condição ósseo tecidual, tal qual o uso de suplementos a base de estrôncio.

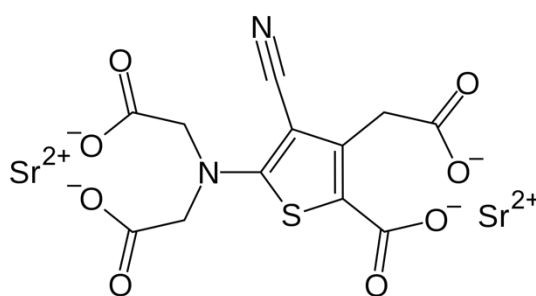
1.1 Estrôncio Não Radiativo (Sr^{2+})

O estrôncio é um elemento químico não radiativo pertencente ao grupo dos metais alcalinos-terrosos, localizado abaixo do cálcio na tabela periódica, e possui muitas semelhanças com o mesmo, como absorção, eliminação, e afinidade pelos tecidos duros. Devido ao seu mimetismo estrutural ao cálcio, o organismo assimila e incorpora o estrôncio para os tecidos ósseos e dentes⁹. Sua absorção máxima se dá em torno de 3-5 horas após sua ingestão, podendo ser reduzido pela influência da

ingestão de alimentos (cálcio, magnésio e alumínio), atingindo seu estado de equilíbrio 2 semanas após o início do tratamento. A permanência do estrôncio no organismo (meia-vida) é em torno de 60 horas e sua excreção é feita pela via renal e trato gastrointestinal. A curto prazo o estrôncio se deposita sobre cristais de hidroxiapatita e a longo prazo permutam com o cálcio, mantendo-se ligados com o tecido ósseo.

Dentre os compostos que contém estrôncio está o ranelato de estrôncio (Figura 1), que diferentemente das substâncias inicialmente citadas, possui o potencial de agir de forma conjunta nos mecanismos de formação quanto de reabsorção óssea, tendo um duplo efeito no metabolismo ósseo, resultando em um desequilíbrio ósseo a favor da neoformação óssea¹⁰.

Figura 1 - Molécula Ranelato de Estrôncio



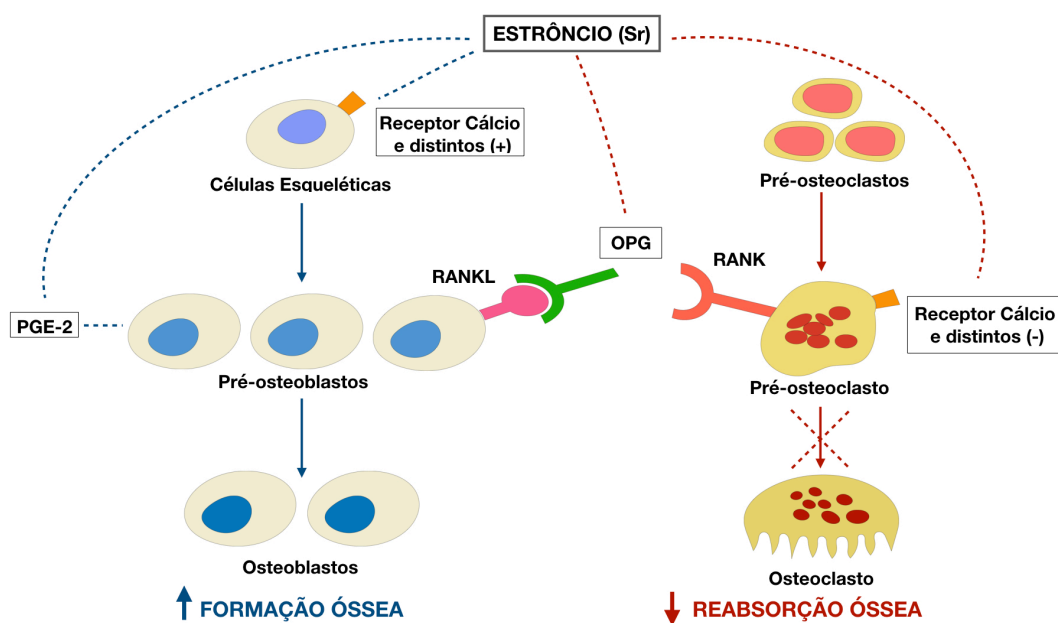
Fonte: Elaboração própria.

1.2 Mecanismos de Ação

Apesar dos mecanismos ainda não estarem totalmente elucidados, estudos recentes mostraram avanço na descoberta das ações do estrôncio. O estrôncio tanto quanto o cálcio pode ativar o receptor sensível ao cálcio (CaR), presente em células não esqueléticas e esqueléticas, induzindo a apoptose de osteoclastos ou aumentar a replicação de osteoblastos¹¹. Em outros receptores distintos ao cálcio o estrôncio também pode agir, resultando em moléculas sinalizadoras que poderiam mediar o aumento no recrutamento, diferenciação e sobrevivência de pré-osteoblastos¹². Outros mecanismos também podem estar envolvidos no efeito do estrôncio na formação óssea, como mostrado por Marie¹³, onde o ranelato de estrôncio aumentou a produção de prostaglandinas E2 (PGE2) e consequentemente a diferenciação de células da medula (pré-osteoblastos) em osteoblastos¹³.

Já outros estudos mostraram que o estrôncio pode agir sobre o metabolismo ósseo através da regulação do fator do núcleo Kappa B (RANK) expresso em pré-osteoclastos e osteoclastos através dos ligantes RANKL (membro do fator de necrose tumoral TNF) ou da osteoprotegerina (OPG), proteína solúvel produzida por osteoblastos e seus precursores. Quando da presença da ligação RANK/RANKL ocorre a ativação de vias de sinalização que promovem a formação, diferenciação e aumento da sobrevivência dos osteoclastos¹⁴. No entanto a ligação RANK/OPG atua como um receptor antagonista ao RANKL, impedindo a osteoclastogênese. Assim, o estrôncio teria capacidade de modular o eixo RANKL/OPG, regulando negativamente RANKL e estimulando OPG em células osteoblásticas¹³ (Figura 2).

Figura 2 - Principais mecanismos de ação do estrôncio



Fonte: Elaboração própria.

1.3 Tratamentos de Patologias Sistêmicas a Base de Estrôncio

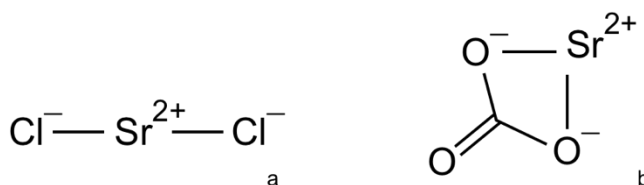
Diferentes estudos puderam comprovar a atuação do medicamento ranelato de estrôncio em tratamentos sistêmicos de patologias por meio de diversos programas de acompanhamento.

Dois programas para tratamento de osteoporose em diferentes regiões do esqueleto foram realizados, sendo denominados SOTI¹⁵ (Intervenção Terapêutica de Osteoporose Espinhal) e TROPOS¹⁶ (Tratamento de Osteoporose Periférica). Esses estudos foram randomizados, duplo-cegos, com grupo controle e associado a um público alvo que eram mulheres. Em ambos os programas houve uma redução significativa das fraturas associadas a osteoporose. Em consequência da aplicabilidade destes estudos somente em mulheres, um novo estudo também foi realizado para avaliar a influência do estrôncio frente a homens osteoporóticos (MALEO)¹⁷. Os resultados se apresentaram similares aos encontrados em mulheres com osteoporose (SOTI e TROPOS), suportando a idéia da sua ação ser independente do sexo. Sendo assim, o estrôncio também passou a ser uma alternativa para tratamento de outras patologias ósseo-reabsortivas como a osteoartrite (doença degenerativa crônica dos ossos), denominado programa de acompanhamento SEKOIA¹⁸. Mais uma vez houve um aumento significativo da estrutura e menor degradação óssea, quando o estrôncio foi administrado via oral. A partir desses estudos, o estrôncio pode ser comprovado e sugerido, trazendo benefícios a diferentes classes de pacientes. Sendo assim o uso do ranelato de estrôncio é um medicamento comprovado e muito usado atualmente para tratamento de diversas enfermidades, fornecendo benefícios clínicos em várias populações de pacientes especiais¹⁵. Portanto, devido a sua efetividade, surgem novos suplementos a base de estrôncio para atuar na prevenção, tratamento de patologias ósseas e em benefício de procedimentos cirúrgicos.

1.4 Cloreto De Estrôncio e Carbonato Estrôncio (Novas Formulações)

Na apresentação de uma nova substância, o cloreto de estrôncio (Cl_2Sr) seria composto por cloro e estrôncio (figura 3a), e poderia vir a substituir o ranelato de estrôncio, atuando como um suplemento alimentar. Porém, sua utilização se torna mais dificultada por se tratar de um sal ácido solúvel (sal resultante da neutralização parcial de um ácido) podendo ser compensado pela adição de uma outra molécula, carbonato de estrôncio (SrCO_3 -figura 3b), um sal básico pouco solúvel, mantendo o pH equilibrado e consequentemente favorecendo a absorção do estrôncio.

Figura 3 - Molécula Cloreto Estrôncio (a) e Carbonato de Estrôncio (b)



Fonte: Elaboração própria.

Em virtude da ação do estrôncio, não só o tratamento de patologias pode ser beneficiado, como também outras terapias que dependam da neoformação óssea em qualidade e quantidade, em detrimento de eventos de reabsorção óssea. No caso dos procedimentos de instalação de implantes de titânio e fechamento de defeitos críticos, aonde se busca um processo de neoformação óssea mais rápida e eficiente^{1,19}, sendo um fator importante para a atividade clínica, permitindo reabilitações em menor tempo²⁰. Dessa forma, qualquer fármaco que possa agir a favor da formação óssea em detrimento da reabsorção pode auxiliar no alcance de maiores taxas de sucesso em Implantodontia.

1.5 Suplementos a Base de Estrôncio para Formação Óssea

Levando em consideração as desvantagens das substâncias ou medicamentos usados atualmente, que não trazem vantagens com seu uso adjunto a tratamentos reabilitadores, o potencial dos medicamentos a base de estrôncio vem demonstrando ser efetivo na prevenção e tratamento de patologias, e seu uso adjunto a procedimentos cirúrgicos maxilo-faciais e a também pela pobre literatura a respeito da remodelação óssea em enxertos, achamos pertinente avaliar a utilização do estrôncio associado a implantes e defeitos ósseos.

2 PROPOSIÇÃO

2.1 Proposição Geral

Os objetivos deste projeto de pesquisa foram avaliar, em ratos, as respostas teciduais da utilização sistêmica do estrôncio não radioativo em implantes osseointegráveis e defeitos ósseos críticos. Para responder estes objetivos, este trabalho foi dividido em 4 publicações.

2.2 Objetivos Específicos

Publicação 1: Systemic strontium to enhance the osseointegration of implants: systematic review of animal studies.

Publicação 2: Use of non-radioactive strontium supplementation to enhance the osseointegration of titanium implants.

Publicação 3: Use of non-radioactive strontium supplementation systemic to enhance the bone formation in bone calvaria defects.

Publicação 4: Toxicity assessment of non-radioactive strontium systemic supplementation.

3 PUBLICAÇÕES

3.1 Publicação 1

Systemic strontium to enhance the osseointegration of implants: systematic review of animal studies*

Running Head: Systemic strontium in implantology.

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* Artigo formatado de acordo com as normas da revista International Journal Implant Dentistry.

Abstract

Background: The literature states that Strontium (Sr) is able to simultaneously stimulate bone formation and suppress bone resorption. Recent animal studies suggest that the systemic administration of Sr would enhance the osseointegration of implants.

Purpose: The purpose of the present study was to undertake a systematic review on animal studies evaluating the systemic administration of Sr to enhance the osseointegration of titanium implants and the remodeling of bone grafts. The MEDLINE (PubMed) and Scopus bibliographic databases were searched from 1950 to October 2017 for reports on the use of systemic and non-radioactive Sr to enhance the osseointegration of titanium implants and the remodeling of bone grafts in animals. The search strategy was restricted to English language publications using the combined terms: “strontium” and “implant or graft or biomaterial or bone substitute”.

Results: Five studies were included, all related to the systemic administration of Sr and its effects on osseointegration of titanium implants. No studies on the use of Sr-based therapy to enhance the remodeling of bone grafts was found. The studies differed notably with respect to the study population (healthy female rats, healthy male rats, and female rats with induced osteoporosis) and Sr dose (in the form of Sr Ranelate - SRAN) (ranging from 500 to 1000 mg/Kg/day). Results were diverse, but a tendency suggesting positive influence of systemic Sr administration on the osseointegration of titanium implants was observed. No major side-effects due to strontium administration were reported.

Conclusion: Systemic Sr administration, in the form of SRAN, seems to enhance peri-implant bone quality and implant osseointegration in animals, however at a moderate extent. Further studies, evaluating both the effects of this drug on implant osseointegration and the risk/benefit of its use, are needed to provide a rationale of this therapeutic approach.

Keywords: *Strontium, systemic use, osseointegration, bone remodeling.*

Introduction

Following the trauma induced to the bone tissue during dental implant installation, wound healing involves the fine-tuned coupling of bone resorption and formation [1, 2], which finally leads to the direct bone-to-implant contact, i.e. implant osseointegration [3]. The same biological mechanisms are involved in the wound healing (i.e. remodeling) of a bone defect filled with bone graft and/or bone substitute material [4]. In order to enhance wound healing and thereby achieve an optimized osseointegration and/or bone defect closure, systemic- and local administration of drugs, including growth- and differentiation factors, and/or implant and graft drug-based surface modifications have been employed with variable success [5, 6]. In the past years, the literature regarding the local interventions (mostly based on the modification of implant surfaces to improve the osseointegration process) became highly developed [6]. On the other hand, the additional effect of systemic therapies supplementing such local modification factors, although acknowledged, was not developed in a similar manner [5].

Examples of systemic administration of anabolic and anti-catabolic substances include estrogen [7], parathyroid hormone [8], and bisphosphonates [9, 10]. In particular, systemic administration of bisphosphonates, which are widely used for cancer and osteoporosis treatment, is based on the rationale that suppression of bone resorption – achieved by this type of drugs – results in denser peri-implant bone and thereby in larger amounts of bone-to-implant contact [11]. Indeed, a recent review of animal studies indicated that systemic administration of bisphosphonates enhances implant osseointegration, especially in animals with induced osteoporotic conditions [12]. Nevertheless, the increasing number of reports in recent years of bisphosphonate-related osteonecrosis of the jaws, has raised alarm regarding the side-effects of bisphosphonate treatment [13] and has led to the search of alternatives to this group of drugs.

Another type of drug recently developed for osteoporosis treatment is strontium (Sr) ranelate (SRAN) [14-16]. This drug is a salt consisting of two atoms of stable strontium (Sr^{2+}) and an organic acid (Ranelic acid), and it is usually administrated orally [17, 18]. Sr ions possess a high affinity to hydroxyapatite (HA) [19], and in contrast to bisphosphonates that decrease bone resorption, Sr exerts a dual action, i.e. it is able to simultaneously stimulate bone formation and suppress bone resorption [17, 20, 21]. This has been demonstrated in both animal- (by means of bone mineral content

analysis [22], dual-energy X-ray absorptiometry [23], and histomorphometric assessment [24]), and clinical studies (by means of microtomography and histomorphometric assessment) [25]. Further, relatively recent reports from animal studies suggest that the systemic administration of Sr would enhance the osseointegration of implants.

Thus, the aim of this review was to undertake a systematic review of the literature on the available evidence – deriving from animal studies – on the systemic administration of non-radioactive Sr to enhance the osseointegration of titanium implants and/or the bone regeneration (i.e. remodeling) in association with bone grafting techniques.

Material and methods

This review was executed in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [26], and the Cochrane Handbook for Systematic Reviews of Interventions [27].

Criteria for considering studies for this review

Animal studies in which the methodology/results included parameters regarding the use of systemic administration of non-radioactive Sr to enhance the osseointegration of implants and/or the remodeling of bone (and bone substitute materials) grafts qualified for inclusion. Retrieval of information focused on (1) the used posology (dose and timing), (2) the type of treatment, (3) the assessed outcome, and (4) the side effects of the treatment. Studies which failed reporting one or more of these four topics still qualified for inclusion, but the missing information was acknowledged as non-declared.

Search strategy for identification of studies

Electronic search

The MEDLINE (Medical Literature Analysis and Retrieval System Online, via PubMed) and Scopus databases were searched until October 2017 for studies evaluating the use of systemic non-radioactive Sr to enhance to enhance the osseointegration of titanium implants and the remodeling of bone grafts. The search strategy was restricted to English language publications using the combined terms: (strontium) and (implant

or graft or biomaterial or bone substitute). Systematic reviews, reviews, and case reports were immediately excluded.

Hand-searching

Unpublished data and hand-searching

Unpublished data were sought by searching a database listing unpublished studies (OpenGray - www.opengrey.eu). A manual search was additionally conducted based on the reference lists of the selected papers. Further, electronic databases of the following journals, which were considered important to this review, were separately manually searched: *Clinical Oral Implants Research*, *Bone*, *International Journal of Implant Dentistry*, *Osteoporosis International*, *Journal of Periodontology*, *Journal of Clinical Periodontology*, *Journal of Dentistry*, *Dentomaxillofacial Radiology*, and *Oral Surgery*, *Oral Medicine*, *Oral Pathology and Oral Radiology*. Further, the bibliographic references of the included studies were also sought for possible relevant studies.

Titles, abstracts, and full texts of the search results were independently screened in duplicate by three reviewers (CRS, CBS, and RSN). When there was a disagreement, the reviewers discussed the study and reached consensus.

Study selection and data extraction

Three independent researchers (CRS, CBS, and RSN) conducted data extraction and validity assessment of the studies that met the inclusion criteria. Data was extracted focusing on the animal model, study groups, treatment start, duration of the treatment, period of examination, implant specification, evaluation methods, and results (outcomes), according to what was reported in each study (i.e. histomorphometry, biomechanical, microtomography, and serum analysis).

Study outcomes

Any qualitative and/or quantitative bone-related parameters which could explain the effects of the systemic administration of non-radioactive Sr on bone tissue of the animals, determining if and how this substance interacts with the bone tissue and bone-tissue regeneration, were included as relevant outcomes.

Quality assessment and risk of bias of included studies

Quality assessment of the studies was made according to SYRCLE's risk of bias tool for animal studies [28]. All ten domains (sequence generation, baseline characteristics, allocation concealment, random housing, performance blinding, random outcome assessment, detection blinding, incomplete outcome data, selective outcome reporting, and other sources of bias) of SYRCLE'S tool were individually evaluated in terms of the risk of bias (no summary scores for the studies were included). Three reviewers (CRS, CBS, and RSN) independently assessed the studies for their quality. When there was a disagreement regarding the assessed data, a consensus meeting was carried out.

Results

Search results

The initial search for publications yielded 578 titles in MEDLINE (PubMed) database, and 152 in Scopus database. After duplicates were removed, there were a total of 553 titles to be screened. After initial screening, using the abstracts and key words, 37 publications remained (31 from PubMed and 6 from Scopus), that potentially met the inclusion criteria. Hand-searching did not reveal any additional publications. After full-text reading, publications which did not fulfill the inclusion criteria were excluded. From the selected full-texts, the main reason for exclusion was the methodology based on the local delivery of Sr, instead of its systemic use.

Finally, five studies reporting on the impact of systemic administration of Sr on the osseointegration of titanium implants were identified as eligible to be included in this systematic review. No additional publications were found from the bibliographic references of the included studies. No information on the use of systemic administration of strontium to enhance the remodeling of bone or bone substitute materials following grafting procedures were found. The study selection procedure is presented in the PRISMA flowchart (Figure 1).

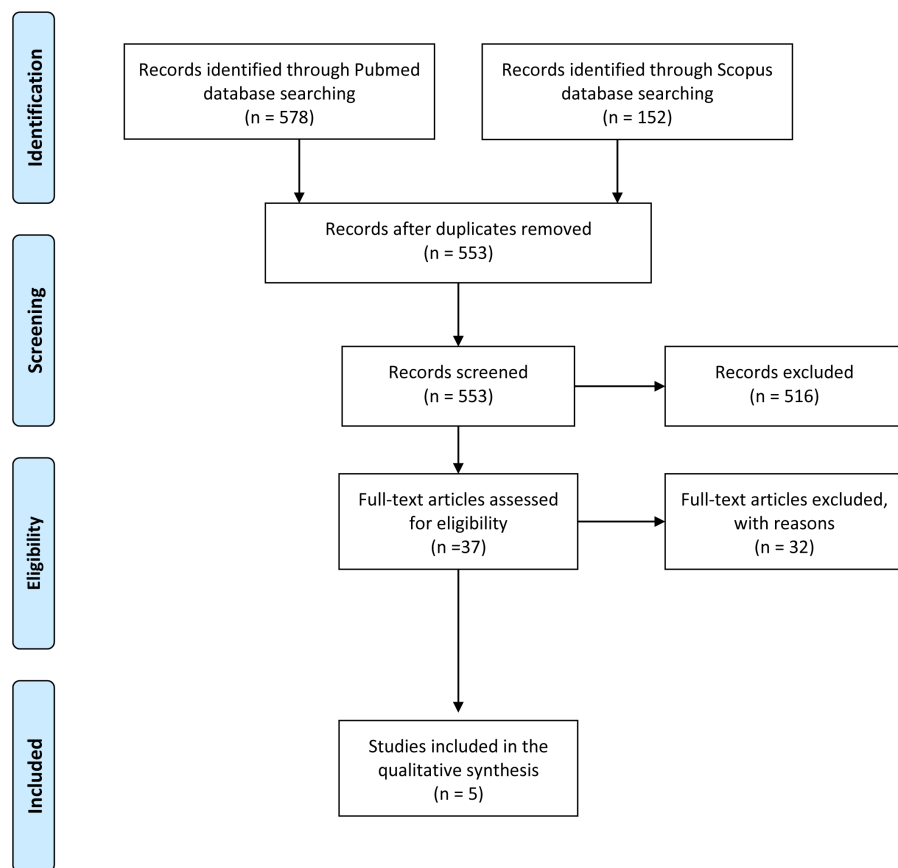


Figure 1. PRISMA flow diagram.

Study outcomes

Tables 1 and 2 show the information retrieved from the included studies. The studies differed notably with respect to the study population, Sr dose, time-point of medication start, duration of the treatment and period of evaluation, implant specification, and the methods.

Study	Sample characteristics					
	Animal model	Study groups	Treatment start	Duration of the treatment and period of examination	Implant specification	Evaluation methods
Maimoun et al., 2010 [29]	female rats	C: 0.5% carboxymethylcellulose aqueous solution (gavage, 5 days a week, n=15) SR: 625 mg/Kg of strontium ranelate, for 8 weeks following implantation (gavage, 5 days a week, n=15)	nd	8 weeks	1.0 x 4.1 mm pure titanium, with sand-blasted, acid-etched surface, inserted in the tibial metaphysis, bilaterally	microtomography, pull-out biomechanical test, and nanoindentation tests
Li et al., 2010 [32]	female rats	Cs: sham operation as a control group, and did not receive any drug treatments (n=10) OVX: bilateral ovariectomy 12 weeks before implantation (n=10) OVX + SR _L : bilateral ovariectomy 12 weeks before implantation, plus special diet providing 500 mg/Kg/day of strontium ranelate after implantation (n=10) OVX + SR _H : bilateral ovariectomy 12 weeks before implantation, plus special diet providing 1000 mg/Kg/day of	started on the day of implantation	12 weeks	1.8 x 3.5 mm, sand-blasted and with sprayed HA-coating, inserted in the tibiae, unilaterally	microtomography, push-out biomechanical test
Li et al., 2012 [33]	female rats	OVX: bilateral ovariectomy, 12 weeks before implantation (n=10) OVX + SR: bilateral ovariectomy 12 weeks before implantation, plus special diet providing 625 mg/Kg/day of strontium ranelate after implantation (n=10)	started on the day of implantation	12 weeks	1.2 x 10.0 mm commercially pure titanium, machined and grit-blasted with aluminum oxide, inserted in the distal femur, bilaterally	microtomography, histomorphometric (BA and BIC), serum analysis (OCN and TRAP), and push-out biomechanical test
Linderbäck et al., 2012 [30]	male rats	C: normal diet (n=20) BP _A : 20 µg/Kg/day subcutaneous alendronate, three times a week (n=20) SR: special diet containing 800 mg/Kg/day strontium ranelate (n=20)	BP _A group started within 24 hours after implantation SR group started on the day of implantation	4 and 8 weeks	1.6 x 2.5 mm stainless steel and polymethyl methacrylate (PMMA) screws, inserted in the tibial metaphysis, bilaterally	microtomography, pull-out biomechanical test, histomorphometric analysis (BA), and bone mineral analyses (ash weight)
Chen et al., 2013 [31]	female rats	Cs: sham operation as a control group, and did not receive any drug treatments (n=12) OVX: bilateral ovariectomy 4 weeks before implantation, and did not receive any drug treatments (n=11) OVX+ BP _A : bilateral ovariectomy 4 weeks before implantation, alendronate 7 mg/kg/week orally (n=10) OVX+ SR: bilateral ovariectomy 4 weeks before implantation, strontium ranelate 500 mg/Kg/day orally (n=12) OVX+ BP _Z : bilateral ovariectomy 4 weeks before implantation, single dose of zoledronic acid 0.1 mg/kg intravenous (n=11)	one week after implantation	11 and 12 weeks	1.0 x 10.0 mm titanium plasma-sprayed with hydroxyapatite, inserted in the tibial metaphysis, bilaterally	bone mineral density, histological analysis, and push-out biomechanical test

C_s= sham operation as a control positive group; **C**= control; **SR**= strontium; **OVX**= ovariectomy; **SR_L**= strontium low dose; **SR_H**= strontium high dose; **BP**= bisphosphonate; **BP_A**= alendronate; **BP_Z**= zoledronate, **nd**= non declared

Table 1. Included studies and sample characteristics. Animal model, study groups, treatment start point and its duration, period of evaluation, implants specification, and evaluation methods.

Study	Tests and overall results					
	Bone mineral density	Histomorphometric	Biomechanical	MicroCT	Nano indentation	Serum analysis
Maimoun et al., 2010 [29]	–	–	SR > C	SR > C (trabecular bone microarchitecture and BIC)	SR > C (elastic modulus, tissue hardness, and working energy)	–
Li et al., 2010 [32]	–	–	OVX+SR _H =C _S >OVX+SR _L >OVX	OVX+SR _H >C _S >OVX+SR _L >OVX	–	–
Li et al., 2012 [33]	–	OVX+SR > OVX (BIC and BA)	OVX+SR > OVX	OVX+SR > OVX	–	OVX+SR > OVX (OCN) OVX+SR < OVX (TRAP)
Linderbäck et al., 2012 [30]	BP _A >SR=C (4 and 8 weeks)	BP _A >SR=C (4 and 8 weeks)	BP _A >SR=C (4 and 8 weeks)	BP _A >SR=C (around implant) BP _A >SR>C (growth plate)	–	–
Chen et al., 2013 [31]	BP _Z =C _S > BP _A =SR>OVX	BP _Z =C _S > BP _A =SR>OVX	BP _Z =C _S > BP _A =SR>OVX	–	–	–

C_S= sham operation as a control positive group; **C**= control; **SR**= strontium; **OVX**= ovariectomy; **SR_L**= strontium low dose; **SR_H**= strontium high dose; **BP**= bisphosphonate; **BP_A**= alendronate; **BP_Z**= zoledronate; **BIC**= bone contact implant; **BA**= bone area; **OCN**= osteocalcin; **TRAP**= tartrate-resistant acid phosphatase.

Table 2. Included studies and the evaluation methods employed, together with major outcomes for each method.

In one of the studies included in the present review, including healthy female rats, SRAN at a dose of 625 mg/Kg/day induced 33.9% higher pull-out strength values, improved trabecular bone microarchitecture (bone volume/total volume, trabecular thickness, structure model index, and connectivity), bone biomechanical characteristics, and bone-to-implant contact, compared with a control (C) group [29].

In another study, including healthy male rats, SRAN at a dose of 800 mg/Kg/day did not lead to higher implant pullout values, but showed increased bone volume fraction, trabecular number, and decreased trabecular separation compared with the control group, based on microtomographic and histomorphometric findings. Further, SRAN was not able to significantly enhance implant pullout values when compared to the treatment with bisphosphonates (alendronate) of four and eight weeks [30].

In three studies from the same research group, female rats with ovariectomy-induced (OVX) osteoporosis were included [31-33]. Rats receiving a high dose of SRAN (SRAN_H - 1000 mg/Kg/day) showed an increased ratio between bone and total voxels in direct contact to the implant (1.9 fold) compared with a non-supplemented

OVX group. Similar trends were observed regarding trabecular thickness (by 1.2 fold), bone volume / tissue volume (by 1.1 fold), trabecular number (by 90%), and connectivity density (by 85.1%), evaluated by microtomography. In fact, the OVX+SRAN_H group showed similar or even statistically significantly better values regarding the above-mentioned parameters when compared with a non-osteoporotic group, while the improvements induced with a smaller dose of SRAN (SRAN_L - 500 mg/Kg/day) did not reach statistical significance [32] compared with the control group. However, SRAN_L animals showed improved implant osseointegration (as assessed by microtomography and histomorphometric evaluation) compared to a non-supplemented OVX group [33], but did not enhance bone-quality-related parameters when compared with a group receiving bisphosphonates (zoledronate and alendronate) [31]. Animals receiving bisphosphonates, however, showed significantly increased bone mineral density, and bone-to-implant contact and implant push-out values, when compared with a non-supplemented OVX group [31].

Quality assessment and risk of bias

According to SYRCLE's risk of bias tool for animal studies [28], the studies were classified in relation to the risk of bias regarding their selection, performance, detection, attrition, and reporting characteristics. All studies successfully met the criterion of not reporting their outcomes in a selective manner, and all proposed aims and elaborated hypotheses were adequately addressed in the results and discussion sessions of the papers. On the other hand, most studies were unclear regarding relevant steps, e.g. the housing of the animals, allocation concealment of the animals, whether outcome assessment was blinded, and whether incomplete outcome data existed and were not reported. Another possible source of bias may be the lack of consistent reporting regarding selection and justification of the posology of Sr supplementation. All these parameters represent relevant biases regarding the findings of the selected studies. The overall scoring for the included studies is presented in Table 3.

Risk of bias	Study				
	Maimoun et al., 2010 [29]	Li et al., 2010 [32]	Li et al., 2012 [33]	Linderbäck et al., 2012 [30]	Chen et al., 2013 [31]
<i>Selection</i>					
Sequence generation	L	L	U	L	L
Baseline characteristics	L	L	L	L	L
Allocation concealment	U	L	U	U	U
<i>Performance</i>					
Random housing	U	U	U	U	U
Blinding	U	L	U	L	U
<i>Detection</i>					
Random outcome assessment	U	U	U	U	U
Blinding	U	U	L	U	U
<i>Attrition</i>					
Incomplete outcome data	U	U	U	U	U
<i>Reporting</i>					
Selective outcome reporting	L	L	L	L	L
Other sources of bias	H	H	H	H	H

L=low risk of bias; **H**=high risk of bias; **U**=unclear

Table 3. Assessment of risk of bias using SYRCLE's tool

Discussion

Mechanism of action

Sr is a metabolic trace element closely related to calcium. Sr^{2+} ions are incorporated into bone by two main mechanisms: a) a rapid uptake mechanism, dependent on osteoblast activity, whereby Sr^{2+} becomes absorbed via ion exchange processes with Ca^{2+} or binding to osteoid proteins, and b) Sr^{2+} ions incorporate into the crystal lattice of the bone mineral phase.[34] When Sr^{2+} is present in higher levels than those required for normal cell physiology, it induces pharmacological effects on bone, through the activation of diverse cellular pathways [34].

Systemic use of non-radioactive Sr has showed promising results regarding the treatment of bone pathologies, such as osteoporosis and osteoarthritis [35-37], as it directly and positively interferes with bone mass, quality, and strength [38]. Further, *in vitro* studies have shown that Sr has the ability to increase bone formation [39, 40] while inhibiting osteoclast differentiation [41]. This dual action on bone remodeling, distinguishes Sr from the other traditional anti-osteoporotic agents, which either increase bone formation (intermittent parathyroid hormones) or inhibit bone resorption (bisphosphonates) [42]. Several mechanisms have been discussed for how systemic Sr-based therapy enhances bone formation [43-46]. Sr might activate the calcium-sensing (or another functionally different cation-sensing) receptor on osteoblasts,

thereby leading to enhanced bone matrix production [46]. Another plausible mechanism is that Sr induces prostaglandin production and cyclooxygenase expression, thereby increasing osteoblastic differentiation [44]. Further, Sr might interact with fibroblast growth factor receptors, thereby increasing osteoblast synthetic activity [43]. At last, Sr also interacts with the mitogen-activated protein kinase (MAPK) signaling pathway, enhancing the differentiation of mesenchymal stem cells in osteogenic cells [45]. In regard with interfering with bone resorption, Sr enhances osteoprotegerin (OPG) expression in osteoblasts [40, 47, 48] thereby reducing osteoclastogenesis [42].

Based on this dual effect, studies have suggested the use of Sr as an adjunct therapy in situations where enhanced bone tissue formation is needed [24, 46, 49]. One could hypothesize that any event dependent on bone formation, such as the osseointegration of implants, could benefit of strontium supplementation, i.e., osseointegration is enhanced due to improved bone formation rate and bone quality surrounding the implants [1, 2].

Sr and implant osseointegration

All studies included in this review tested the hypothesis that non-radioactive Sr supplementation would enhance implant osseointegration. Overall, the results of the included studies suggest that the systemic Sr administration enhances peri-implant bone quality and implant osseointegration, however to a moderate extent. Positive results regarding implant osseointegration and the quality of peri-implant bone tissue, as evaluated by various methods (microtomography, histomorphometry, biomechanical, nanoindentation, and serum analysis) were observed in studies with both osteoporotic and healthy animals [29, 30, 32, 33]. However, the effect of Sr was not so pronounced as that achieved with systemic administration of bisphosphonates [30, 31]. As mentioned, systemic administration of bisphosphonates is known to lead to denser peri-implant bone, larger amounts of bone-to-implant contact, and overall enhancement of osseointegration-related parameters [11, 12, 50]. However, the reports on bisphosphonate-related osteonecrosis affecting the jaws [13] have practically removed these drugs from the group of possible alternatives for enhancing osseointegration [51].

In the present review, Sr was always used in the form of SRAN. In contrast to what is reported for bisphosphonates, no reports of major side-effects due to the SRAN treatment were found in the literature. However, it is relevant to mention that the literature is limited regarding the risks and pitfalls associated to systemic SRAN therapy. In fact, one population study conducted in France showed that osteoporotic patients with a history of venous thromboembolism presented cardiovascular side effects related to SR (104 cases in 39 months) [52]. In the same study, another important side effect, cutaneous toxicity, was mentioned connected to the first few weeks of drug administration. In this context, Sr dosage may be an important issue. SRAN dose in the studies included in the present review varied immensely (from 500 to 1000 mg/Kg/day). It is known fact that the anabolic effects of SR on bone remodeling are dose-dependent [23, 24], and indeed the positive effects of SR herein were observed with the higher doses [32, 33]. Clinical studies, show efficacy regarding reduction of fracture risk in postmenopausal women with osteoporosis with a Sr dose of 2 g/day [53]. Thus, a corresponding effective dose in a human adult with 60 Kg of body weight would be 30 – 60 g/day. Despite the fact that high serum levels of strontium are required in rats in order to generate significant anabolic bone response [54], one could consider that if a dose 15 and 30 times higher than the clinical dose would be needed to have an effect on osseointegration, this could lead to toxicity in humans [55-57]. Clearly, this issue deserves further investigation.

The variability in the results of the studies included in this review, may not only relate to SRAN dose, but may somehow relate to differences in the time-period after ovariectomy, before the animals were included in the study, which differed greatly among studies (from 4 to 12 weeks). Although it is already defined in the literature that initial osteoporosis features appear already at 4 weeks after ovariectomy [58], in not a single study included in this review, were the osteoporotic conditions after ovariectomy confirmed by a specific test [31-33]. Thus, comparison of animals 4 and 12 weeks after ovariectomy in terms of bone architecture characteristics may not be considered optimal, since they represent diverse stages of osteoporotic state [58]. In the same context, in some studies SRAN treatment started the same day of implant installation [30-33], while in other studies treatment started 7 days after implantation [31]; SRAN treatment duration also varied much from among studies (from 4 to 12 weeks). Considering the fact that implant osseointegration in rats is completed within maximum 8 weeks after installation [59], and the fact that it is not yet known for how long should

SRAN be used before it exerts a measureable effect on bone architecture, differences in the time-point of SRAN treatment start and its duration may have contributed to the variability in the results. Finally, differences in the evaluation methods used in the various studies – ranging from the gold-standard for osseointegration assessment, i.e., histomorphometry, to diverse biomechanical tests, and microtomography that has the inherent drawback of metal artifacts hampering osseointegration evaluation [60], may have also contributed to the observed variability in the results

Quality assessment and risk of bias

According to SYRCLE's tool for assessing risk of bias [28], most studies were unclear regarding relevant steps in the selection, performance, detection, and attrition characteristics. On the other hand, there was a low risk for bias related to outcome reporting, so that the conclusions were often and straight-forward related towards the listed aims. Finally, a high risk for other biases, mostly related to inconsistency when defining and reporting the SRAN posology was seen in all included studies, something highly relevant, since it could directly reflect on the results and interfere in the reproducibility of the studies.

Conclusion

Based on the few studies included in this systematic review, it is possible to state that the systemic administration of Sr, in the form of SRAN, seems to enhance peri-implant bone quality and implant osseointegration, however to a moderate extent. Further studies should focus on standardization of the study designs to properly assess the effects of Sr, including parameters such Sr dose, administration start point and duration of administration, further allowing the assessment of potential risks/benefits of Sr use.

Abbreviations

Sr: strontium; SRAN: strontium ranelate; Medline: Medical Literature Analysis and Retrieval System Online; SYRCLE: Systematic Review Centre for Laboratory animal Experimentation; mg: milligrams; g: grams; kg: kilograms; OVX: ovariectomy.

References

1. Davies JE. Mechanisms of endosseous integration. *The International journal of prosthodontics*. 1998;11:391-401.
2. Davies JE. Understanding peri-implant endosseous healing. *Journal of dental education*. 2003;67:932-49.
3. Sakka S, Baroudi K, Nassani MZ. Factors associated with early and late failure of dental implants. *Journal of investigative and clinical dentistry*. 2012;3:258-61.
4. Bastos AS, Spin-Neto R, Conte-Neto N, Galina K, Boeck-Neto RJ, Marcantonio C, et al. Calvarial autogenous bone graft for maxillary ridge and sinus reconstruction for rehabilitation with dental implants. *J Oral Implantol*. 2014;40:469-78.
5. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. *BMC Med*. 2011;9:66.
6. Apostu D, Lucaciu O, Lucaciu GD, Crisan B, Crisan L, Baciut M, et al. Systemic drugs that influence titanium implant osseointegration. *Drug Metab Rev*. 2017;49:92-104.
7. Qi MC, Zhou XQ, Hu J, Du ZJ, Yang JH, Liu M, et al. Oestrogen replacement therapy promotes bone healing around dental implants in osteoporotic rats. *Int J Oral Maxillofac Surg*. 2004;33:279-85.
8. Gabet Y, Muller R, Levy J, Dimarchi R, Chorev M, Bab I, et al. Parathyroid hormone 1-34 enhances titanium implant anchorage in low-density trabecular bone: a correlative micro-computed tomographic and biomechanical analysis. *Bone*. 2006;39:276-82.
9. Li Y, Gao Y, Song G, Liu X, Hu J. Additive effects of estrogen replacement therapy and bisphosphonates on osseointegration of hydroxyapatite-coated titanium screws in ovariectomized rats. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2010;109:700-5.
10. Viera-Negron YE, Ruan WH, Winger JN, Hou X, Sharawy MM, Borke JL. Effect of ovariectomy and alendronate on implant osseointegration in rat maxillary bone. *J Oral Implantol*. 2008;34:76-82.

11. Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, et al. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer*. 2000;88:2961-78.
12. Vohra F, Al-Rifaiy MQ, Almas K, Javed F. Efficacy of systemic bisphosphonate delivery on osseointegration of implants under osteoporotic conditions: lessons from animal studies. *Arch Oral Biol*. 2014;59:912-20.
13. Polymeri AA, Kodovazenitis GJ, Polymeris AD, Komboli M. Bisphosphonates: Clinical Applications and Adverse Events in Dentistry. *Oral Health Prev Dent*. 2015;13:289-99.
14. Pemmer B, Hofstaetter JG, Meirer F, Smolek S, Wobrauschek P, Simon R, et al. Increased strontium uptake in trabecular bone of ovariectomized calcium-deficient rats treated with strontium ranelate or strontium chloride. *J Synchrotron Radiat*. 2011;18:835-41.
15. Peng S, Liu XS, Zhou G, Li Z, Luk KD, Guo XE, et al. Osteoprotegerin deficiency attenuates strontium-mediated inhibition of osteoclastogenesis and bone resorption. *J Bone Miner Res*. 2011;26:1272-82.
16. Seeman E, Vellas B, Benhamou C, Aquino JP, Semler J, Kaufman JM, et al. Strontium ranelate reduces the risk of vertebral and nonvertebral fractures in women eighty years of age and older. *J Bone Miner Res*. 2006;21:1113-20.
17. Tournis S. Improvement in bone strength parameters. The role of strontium ranelate. *J Musculoskelet Neuronal Interact*. 2007;7:266-7.
18. Tournis S, Economopoulos D, Lyritis GP. Strontium ranelate: a novel treatment in postmenopausal osteoporosis. *Ann N Y Acad Sci*. 2006;1092:403-7.
19. Likins RC, Posner AS, Kunde ML, Craven DL. Comparative metabolism of calcium and strontium in the rat. *Arch Biochem Biophys*. 1959;83:472-81.
20. Burlet N, Reginster JY. Strontium ranelate: the first dual acting treatment for postmenopausal osteoporosis. *Clin Orthop Relat Res*. 2006;443:55-60.
21. Reginster JY, Sarlet N, Lejeune E, Leonori L. Strontium ranelate: a new treatment for postmenopausal osteoporosis with a dual mode of action. *Curr Osteoporos Rep*. 2005;3:30-4.

22. Marie PJ, Hott M, Modrowski D, De Pollak C, Guillemain J, Deloffre P, et al. An uncoupling agent containing strontium prevents bone loss by depressing bone resorption and maintaining bone formation in estrogen-deficient rats. *J Bone Miner Res.* 1993;8:607-15.
23. Ammann P, Shen V, Robin B, Mauras Y, Bonjour JP, Rizzoli R. Strontium ranelate improves bone resistance by increasing bone mass and improving architecture in intact female rats. *J Bone Miner Res.* 2004;19:2012-20.
24. Buehler J, Chappuis P, Saffar JL, Tsouderos Y, Vignery A. Strontium ranelate inhibits bone resorption while maintaining bone formation in alveolar bone in monkeys (*Macaca fascicularis*). *Bone.* 2001;29:176-9.
25. Arlot ME, Jiang Y, Genant HK, Zhao J, Burt-Pichat B, Roux JP, et al. Histomorphometric and microCT analysis of bone biopsies from postmenopausal osteoporotic women treated with strontium ranelate. *J Bone Miner Res.* 2008;23:215-22.
26. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Bmj.* 2009;339:b2535.
27. JP H, S G. *Cochrane Handbook for Systematic Reviews of Interventions*, version 5.1.0. : The Cochrane Collaboration; 2011.
28. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW. SYRCLE's risk of bias tool for animal studies. *BMC Med Res Methodol.* 2014;14:43.
29. Maimoun L, Brennan TC, Badoud I, Dubois-Ferriere V, Rizzoli R, Ammann P. Strontium ranelate improves implant osseointegration. *Bone.* 2010;46:1436-41.
30. Linderback P, Agholme F, Wermelin K, Narhi T, Tengvall P, Aspenberg P. Weak effect of strontium on early implant fixation in rat tibia. *Bone.* 2012;50:350-6.
31. Chen B, Li Y, Yang X, Xu H, Xie D. Zoledronic acid enhances bone-implant osseointegration more than alendronate and strontium ranelate in ovariectomized rats. *Osteoporos Int.* 2013;24:2115-21.

32. Li Y, Feng G, Gao Y, Luo E, Liu X, Hu J. Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats. *J Orthop Res.* 2010;28:578-82.
33. Li Y, Li X, Song G, Chen K, Yin G, Hu J. Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats. *Clin Oral Implants Res.* 2012;23:1038-44.
34. Dahl SG, Allain P, Marie PJ, Mauras Y, Boivin G, Ammann P, et al. Incorporation and distribution of strontium in bone. *Bone.* 2001;28:446-53.
35. Deeks ED, Dhillon S. Spotlight on strontium ranelate: in postmenopausal osteoporosis. *Drugs Aging.* 2010;27:771-3.
36. Kaufman JM, Audran M, Bianchi G, Braga V, Diaz-Curiel M, Francis RM, et al. Efficacy and safety of strontium ranelate in the treatment of osteoporosis in men. *J Clin Endocrinol Metab.* 2013;98:592-601.
37. Reginster JY, Felsenberg D, Boonen S, Diez-Perez A, Rizzoli R, Brandi ML, et al. Effects of long-term strontium ranelate treatment on the risk of nonvertebral and vertebral fractures in postmenopausal osteoporosis: Results of a five-year, randomized, placebo-controlled trial. *Arthritis Rheum.* 2008;58:1687-95.
38. Marie PJ, Felsenberg D, Brandi ML. How strontium ranelate, via opposite effects on bone resorption and formation, prevents osteoporosis. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA.* 2011;22:1659-67.
39. Canalis E, Hott M, Deloffre P, Tsouderos Y, Marie PJ. The divalent strontium salt S12911 enhances bone cell replication and bone formation in vitro. *Bone.* 1996;18:517-23.
40. Peng S, Liu XS, Wang T, Li Z, Zhou G, Luk KD, et al. In vivo anabolic effect of strontium on trabecular bone was associated with increased osteoblastogenesis of bone marrow stromal cells. *J Orthop Res.* 2010;28:1208-14.
41. Bonnelye E, Chabadel A, Saltel F, Jurdic P. Dual effect of strontium ranelate: stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption in vitro. *Bone.* 2008;42:129-38.

42. Peng S, Pan H, Lu WW. Osteoprotegerin plays a key role in the dual action of strontium on bone remodeling: Evidences from bench to clinic. *Bone*. 2012.
43. Caverzasio J, Thouverey C. Activation of FGF receptors is a new mechanism by which strontium ranelate induces osteoblastic cell growth. *Cell Physiol Biochem*. 2011;27:243-50.
44. Choudhary S, Halbout P, Alander C, Raisz L, Pilbeam C. Strontium ranelate promotes osteoblastic differentiation and mineralization of murine bone marrow stromal cells: involvement of prostaglandins. *J Bone Miner Res*. 2007;22:1002-10.
45. Peng S, Zhou G, Luk KD, Cheung KM, Li Z, Lam WM, et al. Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway. *Cell Physiol Biochem*. 2009;23:165-74.
46. Pors Nielsen S. The biological role of strontium. *Bone*. 2004;35:583-8.
47. Atkins GJ, Welldon KJ, Halbout P, Findlay DM. Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*. 2009;20:653-64.
48. Brennan TC, Rybchyn MS, Green W, Atwa S, Conigrave AD, Mason RS. Osteoblasts play key roles in the mechanisms of action of strontium ranelate. *Br J Pharmacol*. 2009;157:1291-300.
49. Verberckmoes SC, De Broe ME, D'Haese PC. Dose-dependent effects of strontium on osteoblast function and mineralization. *Kidney Int*. 2003;64:534-43.
50. Berardi D, Carlesi T, Rossi F, Calderini M, Volpi R, Perfetti G. Potential applications of biphosphonates in dental surgical implants. *Int J Immunopathol Pharmacol*. 2007;20:455-65.
51. Javed F, Almas K. Osseointegration of dental implants in patients undergoing bisphosphonate treatment: a literature review. *J Periodontol*. 2010;81:479-84.
52. Jonville-Bera A, Autret-Leca E. Ranélate de strontium (Protelos) : effets indésirables rapportés en France. *Presse Med*. 2011;40:9.

53. O'Donnell S, Cranney A, Wells GA, Adachi JD, Reginster JY. Strontium ranelate for preventing and treating postmenopausal osteoporosis. *Cochrane Database Syst Rev.* 2006;CD005326.
54. Fuchs RK, Allen MR, Condon KW, Reinwald S, Miller LM, McClenathan D, et al. Strontium ranelate does not stimulate bone formation in ovariectomized rats. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA.* 2008;19:1331-41.
55. Morohashi T, Sano T, Yamada S. Effects of strontium on calcium metabolism in rats. I. A distinction between the pharmacological and toxic doses. *Jpn J Pharmacol.* 1994;64:155-62.
56. Sobel AE, Cohen J, Kramer B. The nature of the injury to the calcifying mechanism in rickets due to strontium. *Biochem J.* 1935;29:2640-5.
57. Reinholt FP, Hjerpe A, Jansson K, Engfeldt B. Stereological studies on the epiphyseal growth plate in strontium-induced rickets. With special reference to the distribution of matrix vesicles. *J Bone Joint Surg Am.* 1984;66:1274-80.
58. Lelovas PP, Xanthos TT, Thoma SE, Lyritis GP, Dontas IA. The laboratory rat as an animal model for osteoporosis research. *Comp Med.* 2008;58:424-30.
59. Glosel B, Kuchler U, Watzek G, Gruber R. Review of dental implant rat research models simulating osteoporosis or diabetes. *The International journal of oral & maxillofacial implants.* 2010;25:516-24.
60. Ejima K, Omasa S, Motoyoshi M, Arai Y, Kai Y, Amemiya T, et al. Influence of metal artifacts on in vivo micro-CT for orthodontic mini-implants. *J Oral Sci.* 2012;54:55-9.

3.2 Publicação 2

Use of non-radioactive strontium supplementation to enhance the osseointegration of titanium implants.*

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Running title: Systemic strontium to enhance osseointegration

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* Artigo formatado de acordo com as normas da revista Clinical Oral Implant Research.

Abstract

Purpose: To present diverse formulas for non-radioactive strontium (Sr) systemic supplementation and assess if they enhance the osseointegration of titanium implants inserted in rats' tibiae by means of biomechanical, histomorphometric, immunohistochemical, and microtomographic evaluation.

Methods: Seventy male rats (*Rattus Norvegicus*) were randomly divided into 5 groups, according to the systemic supplementation they received: Control - saline solution; SRAN₅₀ - strontium ranelate (SRAN) 50mg/kg/day; SRAN₆₂₅ - SRAN 625mg/kg/day; SCAR/SCHL₃₀ - strontium carbonate and strontium chloride (SCAR/SCHL) 30mg/kg/day; SCAR/SCHL₃₆₅ - SCAR/SCHL 365mg/kg/day. The drugs were administered via gavage, once a day, starting 15 days before surgery (1 titanium implant in each tibia), and persisted during 15 or 60 days. The right tibiae were used for biomechanical (removal torque) and immunohistochemical (Osteocalcin – OCN, and bone morphogenetic protein - BMP-2) evaluation. The left tibiae were used for microtomographic, and histomorphometric evaluation.

Results: Biomechanical evaluation showed increased removal torque for SRAN₆₂₅ and SCAR/SCHL₃₆₅ when compared with the Control, in 15 days. However, no differences were found in the 60-days period among the groups. Microtomographic evaluation showed larger bone volume at 60 days, compared to 15 days, for all groups but SCAR/SCHL₃₀. When all groups were compared, no differences were seen in the 15-days period, while in the 60-days SRAN₆₂₅ and SCAR/SCHL₃₆₅ were statistically higher than the Control. In the immunohistochemical analysis, higher doses of Sr (SRAN₆₂₅ and SCAR/SCHL₃₆₅) lead to an increase of BMP-2 in 15 days. Histomorphometric analysis revealed no differences among the groups regarding bone-to-implant-contact and bone area around the implant threads.

Conclusions: This study suggests that higher concentrations of systemic Sr lead to variably improved osseointegration-related parameters regarding the biomechanical and microtomographic evaluation. No differences were found on the other assessment levels.

Key-words: *Strontium; systemic use; osseointegration; bone remodeling; implants.*

1. Introduction

Strontium (Sr) is a chemical element that belongs to the group of alkaline earth metals, which presents similarities to Calcium, such as incorporation with the bone tissue and high affinity for hydroxyapatite(Blake and Fogelman 2006). Sr is easily absorbed in bone and teeth(Blake and Fogelman 2006) and can exert a “dual action” by stimulating the production of osteoblasts, and inducing the apoptosis of osteoclasts, simultaneously(Marie 2006, Reginster, Deroisy et al. 2009). However, the mechanisms for this dual action are not totally understood. A theory suggests that these mechanisms are associated with the interaction of Sr and calcium-sensing receptors (CaR)(Mentaverri, Wattel et al. 2005, Chattopadhyay, Quinn et al. 2007). Other theory is that Sr downregulates the nuclear factor-kappa B (RANK), preventing the ligation of ligand (RANKL), and therefore leading to an enhanced production of osteoprotegerin (OPG)(Brennan, Rybchyn et al. 2009). Both mechanisms suggest that Sr could interfere favorably towards bone formation(Marie 2006, Reginster, Deroisy et al. 2009).

Emerging Sr-based medications for the treatment / prevention of bone-related pathologies, such as osteoporosis and osteoarthritis, showed good initial outcomes in human trials(Reginster, Seeman et al. 2005, Deeks and Dhillon 2010, Kaufman, Audran et al. 2013, Reginster, Beaudart et al. 2013). Currently, the most known representative of this class of medicaments is Sr ranelate (SRAN). This drug is usually administered in humans daily, in a concentration of 2 g/day(Meunier, Roux et al. 2009). When this drug was tested in animals, such as rats, the tested concentration was 625 mg/kg/day(Ammann 2006), which would, based on the diminished intestinal absorption of the medication by the rats', leading to plasmatic levels which would be similar to that tested in humans(Ammann 2006). Studies testing such concentrations showed promising results regarding in healthy and osteoporosis animals(Ammann, Shen et al. 2004, Li, Luo et al. 2010).

In face of these findings, Sr-based therapies could also benefit in others research areas which also depend on new bone formation, with increased quality and quantity of newly formed bone, such as implant-dentistry(Maimoun, Brennan et al. 2010, Li, Li et al. 2012). As an example, one could speculate on possible implant-osseointegration enhancement effects, which would allow faster and more efficient bone formation surrounding the implants, allowing the rehabilitation of the patients to be concluded in in less time (Li, Feng et al. 2010, Maimoun, Brennan et al. 2010).

Not only SRAN, but also diverse forms of strontium supplementation could be tested for such application. One example would be a new formula based on Sr carbonate and Sr chloride. The protocol for using this new formula was based on the molecular weight of strontium ranelate, being the quantity of the Sr equivalent for the new supplement. For this creation two molecules were mixed in distilled water, being one substance with acid pH (strontium chloride) and one substance with basic pH (strontium carbonate), providing a neutral salt, which would, in theory, keep the pH equilibrium, leading to a better strontium intestinal absorption.

The aim of study was to develop a new Sr-based supplement which could substitute strontium ranelate as an adjunct therapy following the placement of implants, and assess if it enhances the osseointegration of titanium implants inserted in rats' tibiae by means of biomechanical, histomorphometric, immunohistochemical, and microtomographic evaluation.

2. Materials and methods

2.1 Animals and diet

This experimental protocol was approved by the Animal Ethics Committee of the University of São Paulo – UNESP - Araraquara-SP (04/2012). Seventy male rats (3 month-old *Rattus Norvegicus*, *albinus* variation) were maintained housed in a room with controlled temperature, and access to water and food *ad libitum*. The animals were randomly divided into five groups (n=14): Control, treated with saline solution; SRAN₅₀, treated with strontium ranelate in the concentration of 50mg/kg/day (Protos; Laboratory Servier, São Paulo, Brasil); SRAN₆₂₅, treated with strontium ranelate in the concentration of 625mg/kg/day; SCAR/SCHL₃₀, treated with strontium carbonate and strontium chloride (50/50% proportion) in the concentration of 30mg/kg/day (Cromoline Química Fina, Diadema, São Paulo, Brasil); SCAR/SCHL₃₆₅, treated with strontium carbonate and strontium chloride (50/50% proportion) in the concentration of 365mg/kg/day. All animals received the supplement via gavage, starting 15 days before the installation of implants on their tibiae. The period of 15 days was selected to allow the supplement reaching adequate plasmatic levels before the surgery took place (Dahl, Allain et al. 2001). Supplementation was always provided in the morning, fitting the rats' circadian cycle (Almon, Yang et al. 2008).

During treatment, the animals were weighted weekly, and their respective supplement dose was adjusted. The adjustment of the dose for the SRAN groups was

based on a high dose recommended for rats (625mg/kg/day SRAN) (Ammann 2006) and on a low dose-equivalent used in humans (50mg/kg/day SRAN). After the two doses were defined for the ranelate group, the new supplement (strontium carbonate and strontium chloride) dose was defined based on the number of molecules of SRAN, 365mg/kg/day and 30mg/kg/day.

2.2 Implantation technique

In this study, specially produced titanium implants (2.2 mm Ø and 4 mm long, Neodent, Curitiba, Brasil), with an acid-etched surface were used.

The animals were anesthetized by an intramuscular injection of ketamine (hydrochloride of ketamine 10%) (Francotar; Virbac, São Paulo, Brasil) and xylazine (hydrochloride of xylazine 2%) (Virbaxil; Virbac, São Paulo, Brasil). The bilateral tibiae were cleaned using a solution containing 0.2% chlorhexidine. An incision of 1.5 centimeters was made exposing the bone tissue. Using a hand-piece (Mont Blanc Anthogyr, São Paulo, Brasil), attached to an electrical motor BLM 600 (Driller, Carapicuíba, Brasil). A progressive sequence of drills (1.4 mm, KG Sorensen Implant; 2 mm Neodent, Curitiba, Brasil) was used to prepared the bone tissue, always accompanied by saline irrigation, and not exceeding the speed of 900 rpm. The implants were inserted into the cavity (figure 1) using a specific key-set (Neodent, Curitiba, Brasil), connected to the same hand-piece used to prepare the bone cavity at 15 rpm. After of the insertion of the implants, the tissues were sutured in planes (Vycril; Ethicon, Spreintenbach, Switzerland; and Seda; Ethicon, Spreintenbach, Switzerland). After surgery, the animals were medicated by a subcutaneous injection of penicillin associated to streptomycin 0.1mg/kg (Pentabiotic; FortDodge, Campinas, Brasil), and an intramuscular injection of sodic dipirone 5mg/kg (Febrax; Lema Injex Biologic, São Paulo, Brasil) twice a day for 2 days.



Figure 1. Microtomographic representation of the implant positioned at the proximal epiphysis of the tibiae.

After 15 or 60 days the animals were euthanized by means of prolonged general anesthesia, and the tibiae were retrieved and stored in 70% alcohol. Biomechanical evaluation was performed for the implants placed in the right tibiae, assessing their removal torque. The bone piece from which the implant was removed was also used for immunohistochemical evaluation. The implants in the left tibiae were used for microtomographic, histological, and histomorphometric evaluation.

2.3 Biomechanical analysis

The tibiae were mounted on a stabilization table and a hexagonal key (Neodent, Curitiba, Brasil) was connected to the implant and to a torque wrench (Tohnichi ATG24CN-S, Tokyo, Japan), graduated on a scale of 0.05 N/cm. The maximum value needed to remove the implant was assessed.

2.4 Immunohistochemical analysis

The same specimens which were utilized for the biomechanical evaluation (without the implant) were then utilized for the immunohistochemical evaluation. The specimens were decalcified in 7% buffered EDTA, dehydrated and then embedded in paraffin. Histological serial sagittal sections, with a thickness of 4 μ m were mounted on silanized slides (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK).

The tissues were treated with an avidin-biotin-peroxidase (ABC) complex using an ABC staining system kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the detection of two antibodies associated to bone formation, Osteocalcin (OCN) and Bone Morphogenetic Protein (BMP-2). The analysis was made in bone region around the first 2-3 threads and with the use of the optical microscope (DIASTAR Microscope, Leica Reichert & Jung products, Wetzlar, Hessen, Germany) for make the counting. The intensity of the proteins markers was evaluated by one blinded examiner for the experimental groups according a four-grade scale: negative (–), positive (+), superpositive (++), and hyperpositive (+++) (de Oliveira, de Paula et al. 2014).

2.5. Microtomography analysis

The tibiae area containing the implant were imaged using a micro CT scanner (SkyScan 1176 Bruker, Aatselaar, Belgium), in sections of 9 µm, rotation step 0.3 mm, and aluminum and copper filters. The obtained images were reconstructed using dedicated software (NRecon, SkyScan, Aatselaar, Belgium), properly positioned using a dedicated data viewer (SkyScan, Aatselaar, Belgium), and evaluated for the bone volume around the implant (region of interest - ROI) also using dedicated software (CTAnalyser, SkyScan, Aatselaar, Belgium). The ROI was defined as a rectangular (4.5 x 3.2 mm) region extending 0.5 millimeters around the implant (figure 2A). Using a fixed, manually determined threshold (25-90), the area inside the ROI was transformed to a binary image, in which the bone volume around the implants was measured. As sometimes there was bone formation inside the in the region of the implant used to fit the placement key, this areas were later subtracted from the final results (figure 2B).

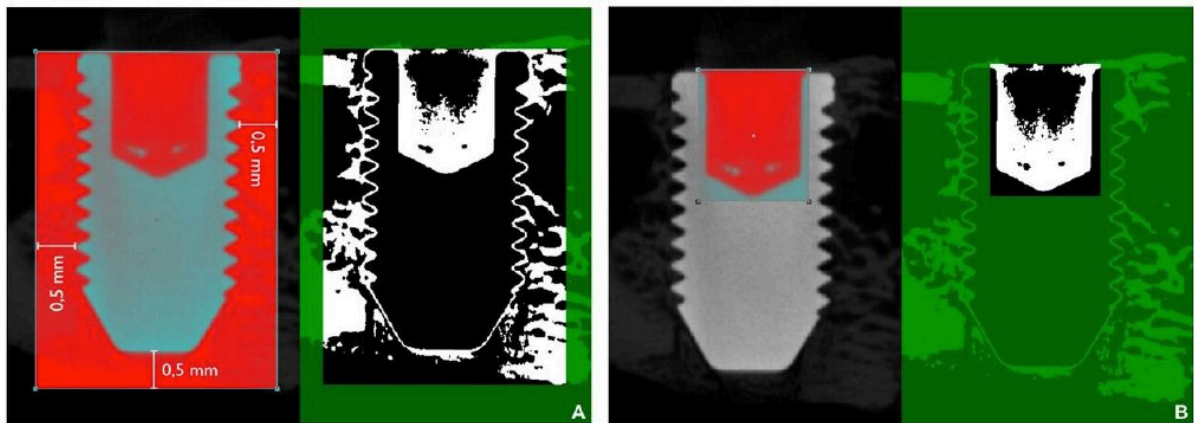


Figure 2. Representation of the evaluated ROI for the microtomographic assessment.
(A) *total ROI*, and B *partial ROI*.

2.6 Histological and histomorphometric evaluation

The same samples that were scanned for the micro CT evaluation were later prepared for non-decalcified histological evaluation, according to the protocol previously described by Donath & Breuner (Donath and Breuner 1982). The sections were stained using Stevenel's Blue, and photographed using light microscopy (Diastar microscope, Leica Reichert & Jung products, Germany), connected to a camera (Leica Microsystems DFC-300-FX, Leica Reichert & Jung Products, Germany). Obtained images were used for descriptive and quantitative evaluation of the tissues formed around the implants.

For the quantitative evaluation, osseointegration was assessed by means of the percentage of bone-to-implant-contact (BIC) and the bone area (BA) around the implants threads. The three first threads of the implants were analyzed, showing representative amounts of both cortical and cancellous bone. Dedicated software (Image J 1.42q, Bethesda, Maryland, USA) was used to make these assessments in the histological images.

2.7 Statistical methods

All assessments were conducted by the same trained observer, blinded to the groups being evaluated. Results were always expressed as means and standard deviation. As the sample is rather small, data was treated as non-parametrical and the Kruskal-Wallis followed by Dunn's post-test, for the evaluation among the groups in the same

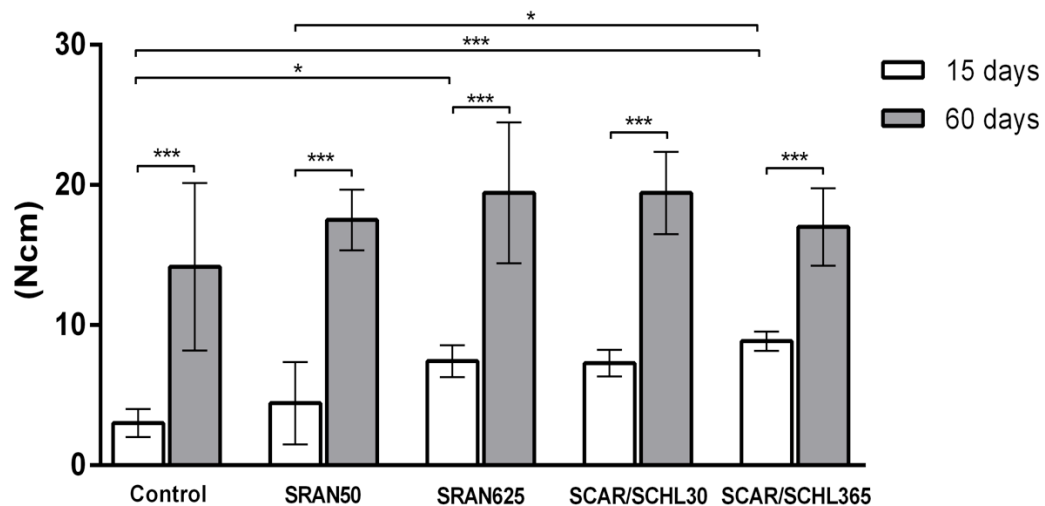
period. For the comparison between the periods of evaluation, the Mann-Whitney test was applied.

3. Results

3.1 Biomechanical evaluation

In the short period (15 days), the animals of SRAN₆₂₅ and SCAR/SCHL₃₆₅ groups showed higher torque values when compared to Control. However in the longer period of evaluation (60 days) all groups were statistically equal.

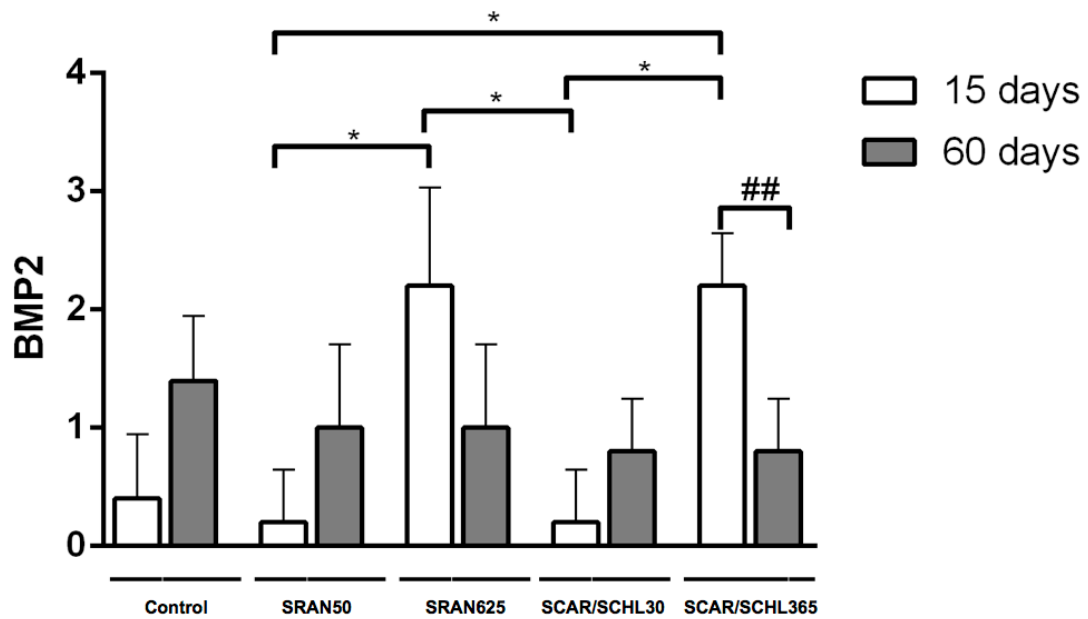
When the two evaluation periods were compared, there was a significant increase of removal torque from 15 days to 60 days for all evaluated. Graphic 1 shows these results.



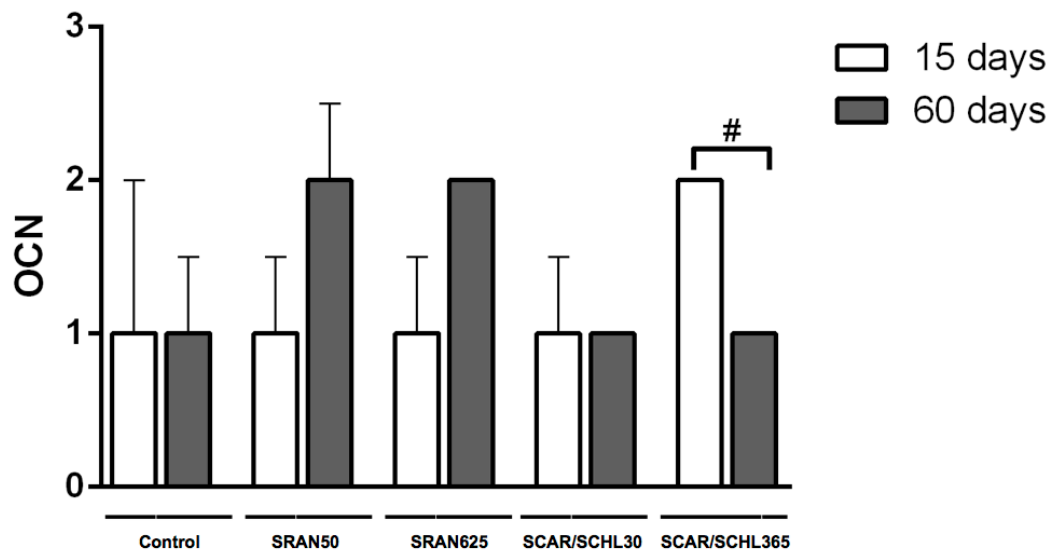
Graphic 1. Mean and standard deviation of the values assessed during the biomechanical analysis, for all studied groups and periods (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.2 Immunohistochemical evaluation

The groups SRAN₆₂₅ and SCAR/SCHL₃₆₅ presented higher expression of BMP-2 at 15 days than the groups SRAN₅₀ and SCAR/SCHL₃₀, however these statistical differences were not detected at 60 days. There were no statistical differences in the expression of the OCN between the groups in the both periods of evaluation. The expression of the BMP-2 and OCN were reduced at 60 days in the SCAR/SCHL₃₆₅.



Graphic 2. Mean and standard deviation of the values assessed during the BMP-2 immunohistochemical analysis, for all studied groups and periods (* $p < 0.05$; ## $p = 0.0079$).

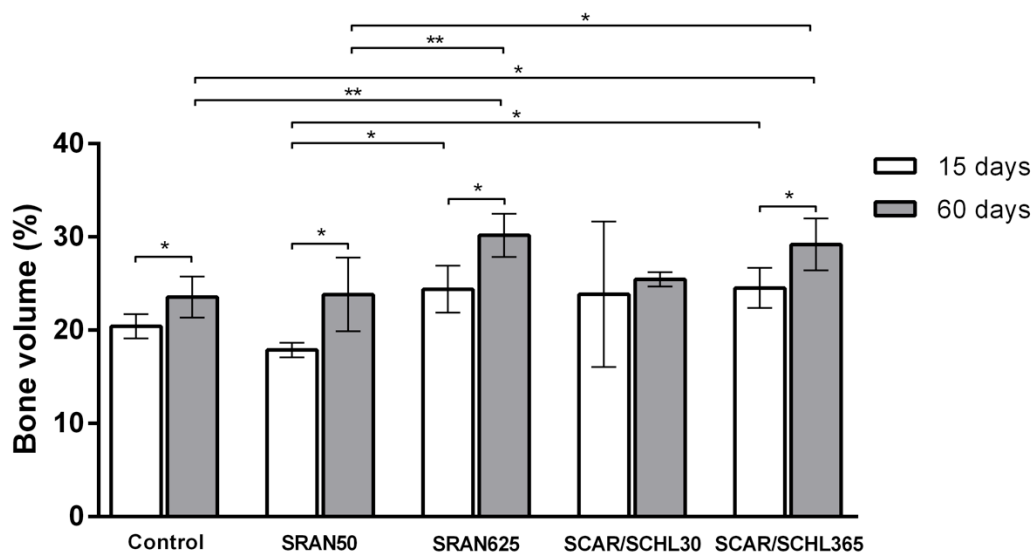


Graphic 3. Mean and standard deviation of the values assessed during the OCN immunohistochemical analysis, for all studied groups and periods (# $p = 0.04$).

3.3 Microtomography evaluation

In the period of 15 days, there was a larger bone formation around the implants in the groups SRAN₆₂₅ and SCAR/SCHL₃₆₅ compared to SRAN₅₀. In the period of 60 days, Control and SRAN₅₀ groups showed the lowest values for bone volume percentage compared to SRAN₆₂₅ and SCAR/SCHL₃₆₅ groups.

When the two evaluation periods were compared, an increase in the percentage of bone around the implants from 15 to 60 days was verified for all groups except SCAR/SCHL₃₀. Graphic 4 shows these results.

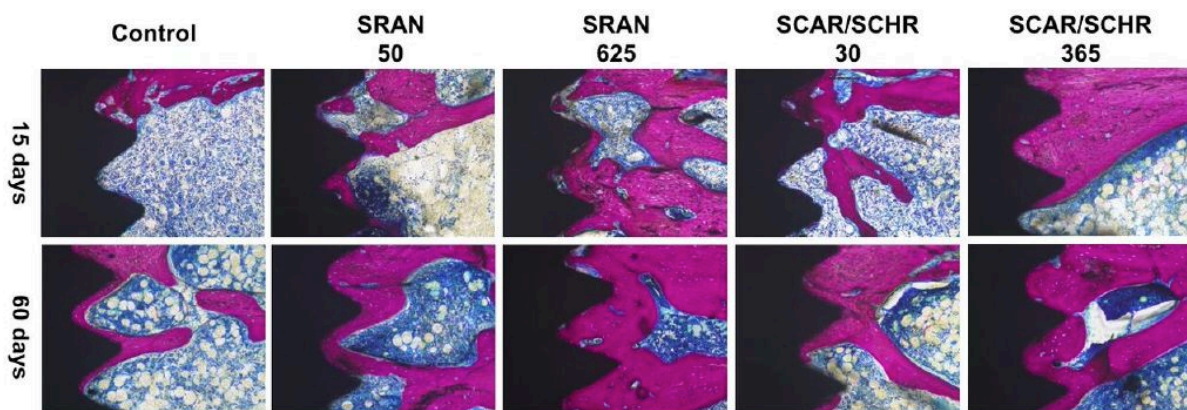


Graphic 4. Mean and standard deviation of the values assessed in the microtomography analysis, for all evaluated groups and periods (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.4 Histologic description

For all groups and periods, elements of compact and medullary bone could be seen. Compact bone was composed by circumferential lamellae involving vascular figures, interstitial lamellae, and osteocytes. Further, it was in intimate contact with the titanium (implants), in some regions. The regions presenting medullary bone were morphologically consistent with the anatomical features of the rat's tibiae (Conte Neto, de Andrade et al. 2014). Representative images of each group are shown in figure 3.

Figure 3. Microphotographies representing each evaluated group and period. These sections were posteriorly evaluated in for histomorphometry.



3.5. Histomorphometric evaluation

There were no statistical differences among the groups regarding assessed BIC and BA. When the two evaluation periods were compared, only the Control and SCAR/SCHL₃₀ groups had a progressive increase from 15 to 60 days in BIC values. Regarding BA, Control, SCAR/SCHL₃₀, and SRAN₆₂₅ showed significant increase from 15 to 60 days. These values are presented in table 1.

Table 1. Mean and standard deviation of BIC (%) and BA (%), as assessed in the histomorphometric evaluation, for all evaluated groups and periods.

Groups/ Analysis		Control	SRAN ₅₀	SRAN ₆₂₅	SCAR/SCHL ₃₀	SCAR/SCHL ₃₆₅
BIC	15	32.75	40.22	42.48	34.36	49.14
	days	±8.06	±12.0	±15.60	±13.66	±13.23
	60	48.72	47.52	57.84	67.09	58.71
	days	±10.64	±12.92	±15.09	±9.76	±13.87
BA	15	32.75	36.59	35.65	31.24	44.04
	days	±8.06	±10.32	± 9.63	±11.40	±12.12
	60	54.06	50.32	60.59	60.43	61.20
	days	±12.76	±19.86	±15.97	±12.03	±16.91

4. Discussion

The use of the Sr in the medical field brought favorable results in the prevention and treatment of the bone metabolism diseases such as osteoporosis(Blake and Fogelman 2006, Deeks and Dhillon 2010). Following this idea, one could speculate that not only bone-related diseases, but also bone-related surgical procedures could benefit from Sr supplementation(Maimoun, Brennan et al. 2010, Li, Li et al. 2012). This involves any procedures which depend on new bone formation, like the osseointegration of titanium implants used for oral rehabilitation (Branemark, Adell et al. 1983). However, to the present, only one medication has been tested for such task, which is Sr ranelate (Scardueli CR 2016).

The literature has shown promising, but at same time controversial results from the systemic use of Sr, in the form of SRAN to enhance the osseointegration of titanium implants (Scardueli CR 2016). Studies have shown that the systemic administration of SRAN could improve the bone formation around the implants, both in healthy (Maimoun, Brennan et al. 2010) and in osteoporotic rats(Li, Feng et al. 2010, Li, Li et al. 2012). However, other studies showed that SRAN had a weak effect over healthy rats (Linderback, Agholme et al. 2012). Independent of the controversial results, no

variation for SR supplementation have been tested aiming to potentialize Sr absorption and action. A relatively simple way to vary Sr administration and potentialize its action would be to find forms to enhance its absorption. In this study, we suggest a formula containing Sr in the form of carbonate and chloride, which would in theory lead to a better Sr absorption, and which could act as a supplement for bone neoformation, acting for a short period.

Dose choice was defined based on the molecular weight of SRAN ($C_{12}H_6N_2O_8SSr_2$ - 513.49 mg, being 175.24 mg of Sr), which has been the standard drug tested for the same aim to the present. Therefore, the association between Sr carbonate ($SrCO_3$ - 147.63 mg) and Sr chloride ($SrCl_2$ - 158.52 mg) adds the same 175.24 mg of Sr. The proportion, in weight, between SRAN and the new test supplement is approximately 1.7, in such a way that 625 mg of SRAN leads to the same amount of Sr as in 365 mg of the test formula, and 50 mg of SRAN compares to 30 mg of the test supplement formula. Therefore, this association between the two molecules was due to prove a neutral salt, which would keep the pH equilibrium, leading to a better Sr intestinal absorption and consequently greater use of the same, once time that generally the Sr is poorly absorbed in the intestinal tract. (Pors Nielsen 2004)

The biomechanical evaluation (removal torque), although seen as an indirect form to measure (assess) osseointegration, is a valid evaluation method (Margonar, Sakakura et al. 2003, Sakakura, Margonar et al. 2003). The present results suggest that only the formulas providing higher Sr doses were able to induce positive effects towards bone formation, based on the results seen in the 15-days period of evaluation. The lack of differences among the groups in the 60-days period is possibly related to the fact that this relates to a period in which the osseointegration of implants is already consolidated in healthy animals (de Molon, Morais-Camilo et al. 2013, Conte Neto, de Andrade et al. 2014, de Oliveira, de Paula et al. 2014).

In the immunohistochemical analysis, higher doses of Sr (SRAN₆₂₅ and SCAR/SCHL₃₆₅) lead to an increase of BMP-2 in 15 days, suggesting the Sr action in initial bone formation. These results are according with Lv H et al. (Lv, Huang et al. 2013) and Li Z et al. (Li, Wang et al. 2011), which showed similar results in vitro, for diverse concentrations of SRAN. However, although a tendency of increased OCN for the Sr groups in 15 (SCAR/SCHL₃₆₅) and 60 days (SRAN₅₀ and SRAN₆₂₅) was seen, the results showed no difference among groups. Differently, the study of Li Y et al. (Li, Li

et al. 2012) and Ibrahim MR et al.(Ibrahim, Singh et al. 2016) showed increased values for OCN, but also considering a different dosage of SRAN.

The microtomographic analysis allows quantitative evaluation, assessing the bone quantity and quality around the implants, as means of the bone volume in the region around the implant. Assessing BIC based on microtomographies is not suggested, due to the artefacts related to metal which could lead to false results regarding the bone surrounding the implants (Ejima, Omasa et al. 2012). In an attempt to lead to a better standardization of the results, in the present study we used, for all animals, the same (fixed) threshold for bone detection, making the results comparable within the study.

The results of the microtomographic analysis were in agreement with those of the biomechanical analysis in the period of the 15 days. The groups with high Sr concentration (SRAN₆₂₅ and SCAR/SCHL₃₆₅) showed a superior bone formation in the early periods of evaluation, in comparison to SRAN₅₀. In the period of 60 days, the same groups showed larger values for bone volume in comparison to Control and SRAN₅₀. These larger values found both in 15 and 60 days might suggest that the Sr acts both in early and late stages of bone healing/formation as suggested by Dahl SG et al., 2001(Dahl, Allain et al. 2001). In addition, there was a significant increase in the assessed bone volume from 15 to 60 days in all groups, except for SCAR/SCHL₃₀, suggesting that the effects of Sr might exist all through the dynamic process of bone formation. These findings are compatible with those showed by Li et al., 2012 (Li, Li et al. 2012), who, however, found larger values than these reported in the present study. This difference can be explained by the fact that the used thresholds were not the same, added to the fact that Sr administration occurred for a longer period, and that the sample consisted of ovariectomized rats, simulating the osteoporotic state, that could accentuate the effect of the tested supplement.

Thus, Sr can act both in the early and late stages of bone maturation through two diverse mechanisms of action, according to the cellular intake dynamics. First, a mechanism of fast intake, which is directly associated to the osteoblastic activity, in which Sr is absorbed by ionic exchanges with Calcium (Ca) or by binding to osteoid proteins and/or both. The slower mechanism occurs through of the incorporation of Sr ions into the bone mineral crystals, which takes place gradually, due to exchanges in the structure of the mature bone. In the end, these processes are related both to bone

neof ormation and bone maturation bone, being able to exert its effect in both osseointegration stages (Dahl, Allain et al. 2001).

In the histological evaluation, bone tissue showed normal morphology, with characteristics of healthy tissue. This suggests that the process of bone formation around the implants was not only a response to their primary stability (Branemark, Ohnrell et al. 1998), but a true osseointegration process, in which secondary (away from the implant) bone formation takes place. For the quantitative evaluation of osseointegration, the bone tissue was measured by means of BIC and BA assessment. The results did not show any statistical differences among the groups, and only a suggestive tendency indicating larger values for the groups with higher Sr concentration (625mg and 365mg). These controversial results, when compared with the other assessment methods used in the present study can be explain by the small number of sections (only the most central one) used for the histomorphometrical evaluation. This happened due to methodological limitations which cannot be overcome when such small implants are used (Donath and Breuner 1982). In this way, we believe that this method does not fully represent the real condition of the bone around the implants, for the present study.

High Sr concentrations can lead to many benefits when incorporated into the bone tissue. However, Sr favorable results depends on the use of ideal concentrations, the correct period of administration even before the surgical procedure (allowing proper plasmatic levels of the drug to be reached), and also which are not so high to induce toxicity (Dahl, Allain et al. 2001). The low concentrations tested in the present study did not lead to any side effects, but higher concentrations could lead to adverse effects, not only locally, to the bone. Other studies showed that high concentrations can cause, at the more severe cases, cardiac diseases (Reginster 2014). However, other studies showed controversial results, not being able to stabilish the link between Sr administration and systemic alterations (Cooper, Fox et al. 2014). In any case, finding the parameters for Sr administration which could lead to bone tissue enhanced formation, with no (or reduced) side effects, is the next challenge. This would be the only way in which the suggested new formulas could be used as a supplement, in substitution of SRAN, in those cases in which bone formation needs to be enhanced.

CONCLUSION

This study shows that higher concentrations of systemic Sr lead to variably improved osseointegration-related parameters regarding the biomechanical and microtomographic evaluation. No differences were found on the other assessment levels.

REFERENCES

- Almon, R. R., E. Yang, W. Lai, I. P. Androulakis, D. C. DuBois and W. J. Jusko (2008). "Circadian variations in rat liver gene expression: relationships to drug actions." *J Pharmacol Exp Ther* 326(3): 700-716.
- Ammann, P. (2006). "Strontium ranelate: a physiological approach for an improved bone quality." *Bone* 38(2 Suppl 1): 15-18.
- Ammann, P., V. Shen, B. Robin, Y. Mauras, J. P. Bonjour and R. Rizzoli (2004). "Strontium ranelate improves bone resistance by increasing bone mass and improving architecture in intact female rats." *J Bone Miner Res* 19(12): 2012-2020.
- Blake, G. M. and I. Fogelman (2006). "Strontium ranelate: a novel treatment for postmenopausal osteoporosis: a review of safety and efficacy." *Clin Interv Aging* 1(4): 367-375.
- Branemark, P. I., R. Adell, T. Albrektsson, U. Lekholm, S. Lundkvist and B. Rockler (1983). "Osseointegrated titanium fixtures in the treatment of edentulousness." *Biomaterials* 4(1): 25-28.
- Branemark, R., L. O. Ohnells, R. Skalak, L. Carlsson and P. I. Branemark (1998). "Biomechanical characterization of osseointegration: an experimental in vivo investigation in the beagle dog." *J Orthop Res* 16(1): 61-69.
- Brennan, T. C., M. S. Rybchyn, W. Green, S. Atwa, A. D. Conigrave and R. S. Mason (2009). "Osteoblasts play key roles in the mechanisms of action of strontium ranelate." *Br J Pharmacol* 157(7): 1291-1300.
- Chattopadhyay, N., S. J. Quinn, O. Kifor, C. Ye and E. M. Brown (2007). "The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation." *Biochem Pharmacol* 74(3): 438-447.

- Conte Neto, N., C. R. de Andrade, L. C. Spolidorio, S. Planeta Cda, F. C. Cruz, A. de Souza Bastos and E. Marcantonio, Jr. (2014). "Effects of chronic stress and alendronate therapy on the osseointegration of titanium implants." *Clin Implant Dent Relat Res* 16(5): 762-771.
- Cooper, C., K. M. Fox and J. S. Borer (2014). "Ischaemic cardiac events and use of strontium ranelate in postmenopausal osteoporosis: a nested case-control study in the CPRD." *Osteoporos Int* 25(2): 737-745.
- Dahl, S. G., P. Allain, P. J. Marie, Y. Mauras, G. Boivin, P. Ammann, Y. Tsouderos, P. D. Delmas and C. Christiansen (2001). "Incorporation and distribution of strontium in bone." *Bone* 28(4): 446-453.
- de Molon, R. S., J. A. Morais-Camilo, M. H. Verzola, R. S. Faeda, M. T. Pepato and E. Marcantonio, Jr. (2013). "Impact of diabetes mellitus and metabolic control on bone healing around osseointegrated implants: removal torque and histomorphometric analysis in rats." *Clin Oral Implants Res* 24(7): 831-837.
- de Oliveira, G. J., L. G. de Paula, R. Spin-Neto, A. Stavropoulos, L. C. Spolidorio, E. Marcantonio, Jr. and R. A. Marcantonio (2014). "Effect of avocado/soybean unsaponifiables on osseointegration: a proof-of-principle preclinical in vivo study." *Int J Oral Maxillofac Implants* 29(4): 949-957.
- Deeks, E. D. and S. Dhillon (2010). "Spotlight on strontium ranelate: in postmenopausal osteoporosis." *Drugs Aging* 27(9): 771-773.
- Donath, K. and G. Breuner (1982). "A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique." *J Oral Pathol* 11(4): 318-326.
- Ejima, K., S. Omasa, M. Motoyoshi, Y. Arai, Y. Kai, T. Amemiya, H. Yamada, K. Honda and N. Shimizu (2012). "Influence of metal artifacts on in vivo micro-CT for orthodontic mini-implants." *J Oral Sci* 54(1): 55-59.
- Ibrahim, M. R., S. Singh, A. M. Merican, H. R. Raghavendran, M. R. Murali, S. V. Naveen and T. Kamarul (2016). "The effect of strontium ranelate on the healing of a fractured ulna with bone gap in rabbit." *BMC Vet Res* 12(1): 112.
- Kaufman, J. M., M. Audran, G. Bianchi, V. Braga, M. Diaz-Curiel, R. M. Francis, S. Goemaere, R. Josse, S. Palacios, J. D. Ringe, D. Felsenberg and S. Boonen (2013).

"Efficacy and safety of strontium ranelate in the treatment of osteoporosis in men." *J Clin Endocrinol Metab* 98(2): 592-601.

Li, Y., G. Feng, Y. Gao, E. Luo, X. Liu and J. Hu (2010). "Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats." *J Orthop Res* 28(5): 578-582.

Li, Y., X. Li, G. Song, K. Chen, G. Yin and J. Hu (2012). "Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats." *Clin Oral Implants Res* 23(9): 1038-1044.

Li, Y. F., E. Luo, G. Feng, S. S. Zhu, J. H. Li and J. Hu (2010). "Systemic treatment with strontium ranelate promotes tibial fracture healing in ovariectomized rats." *Osteoporos Int* 21(11): 1889-1897.

Li, Z., Y. Wang, X. N. Wang, A. P. Lan and W. Wu (2011). "[Strontium ranelate promotes osteogenic differentiation of rat bone marrow mesenchymal stem cells by increasing bone morphogenetic protein-7 expression]." *Nan Fang Yi Ke Da Xue Xue Bao* 31(11): 1949-1953.

Linderback, P., F. Agholme, K. Wermelin, T. Narhi, P. Tengvall and P. Aspenberg (2012). "Weak effect of strontium on early implant fixation in rat tibia." *Bone* 50(1): 350-356.

Lv, H., X. Huang, S. Jin, R. Guo and W. Wu (2013). "[Strontium ranelate promotes osteogenic differentiation of rat bone mesenchymal stem cells through bone morphogenetic protein-2/Smad signaling pathway]." *Nan Fang Yi Ke Da Xue Xue Bao* 33(3): 376-381.

Maimoun, L., T. C. Brennan, I. Badoud, V. Dubois-Ferriere, R. Rizzoli and P. Ammann (2010). "Strontium ranelate improves implant osseointegration." *Bone* 46(5): 1436-1441.

Margonar, R., C. E. Sakakura, M. Holzhausen, M. T. Pepato, R. Alba j and E. Marcantonio j (2003). "The influence of diabetes mellitus and insulin therapy on biomechanical retention around dental implants: a study in rabbits." *Implant Dent* 12(4): 333-339.

Marie, P. J. (2006). "Strontium ranelate: a physiological approach for optimizing bone formation and resorption." *Bone* 38(2 Suppl 1): S10-14.

- Mentaverri, R., A. Wattel, A. S. Lemaire-Hurtel, S. Kamel, A. Blesius and M. Brazier (2005). "[Partnership between academic research and industry to study a new anti-osteoporotic drug]." *Med Sci (Paris)* 21(6-7): 663-668.
- Meunier, P. J., C. Roux, S. Ortolani, M. Diaz-Curiel, J. Compston, P. Marquis, C. Cormier, G. Isaia, J. Badurski, J. D. Wark, J. Collette and J. Y. Reginster (2009). "Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis." *Osteoporos Int* 20(10): 1663-1673.
- Pors Nielsen, S. (2004). "The biological role of strontium." *Bone* 35(3): 583-588.
- Reginster, J. Y. (2014). "Cardiac concerns associated with strontium ranelate." *Expert Opin Drug Saf* 13(9): 1209-1213.
- Reginster, J. Y., C. Beaudart, A. Neuprez and O. Bruyere (2013). "Strontium ranelate in the treatment of knee osteoarthritis: new insights and emerging clinical evidence." *Ther Adv Musculoskelet Dis* 5(5): 268-276.
- Reginster, J. Y., R. Deroisy, A. Neuprez, M. Hiligsmann, B. Zegels and O. Bruyere (2009). "Strontium ranelate: new data on fracture prevention and mechanisms of action." *Curr Osteoporos Rep* 7(3): 96-102.
- Reginster, J. Y., E. Seeman, M. C. De Vernejoul, S. Adami, J. Compston, C. Phenekos, J. P. Devogelaer, M. D. Curiel, A. Sawicki, S. Goemaere, O. H. Sorensen, D. Felsenberg and P. J. Meunier (2005). "Strontium ranelate reduces the risk of nonvertebral fractures in postmenopausal women with osteoporosis: Treatment of Peripheral Osteoporosis (TROPOS) study." *J Clin Endocrinol Metab* 90(5): 2816-2822.
- Sakakura, C. E., R. Margonar, M. Holzhausen, F. H. Nociti, Jr., R. C. Alba, Jr. and E. Marcantonio, Jr. (2003). "Influence of cyclosporin A therapy on bone healing around titanium implants: a histometric and biomechanic study in rabbits." *J Periodontol* 74(7): 976-981.
- Scardueli CR, Bizelli-Silveira C, Marcantonio RAC, Marcantonio-Jr E, Stavropoulos A, Spin-Neto R. (2016). "Systemic strontium to the osseointegration of titanium implants in animals: systematic review of the literature." *Clin Implant Dent Relat Res* Submitted.

3.3 Publicação 3

Use of non-radioactive systemic strontium supplementation to enhance bone formation in bone calvarial defects.*

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Running title: Systemic strontium to enhance bone formation in bone defects

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* Artigo formatado de acordo com as normas da revista Bone.

Abstract

Purpose: The aim of this study was to evaluate the effect of diverse formulas of non-radioactive strontium (Sr) systemic supplementation in the healing of critical rats' calvaria defects by means of microtomographic, histomorphometric, and immunohistochemical evaluation.

Methods: Eighty male rats (*Rattus Norvegicus*) were randomly divided into 5 groups, according to the systemic supplementation they received: Control, treated with saline solution; SRAN₅₀, treated with strontium ranelate in concentration of 50mg/kg/day; RAN₆₂₅, treated with strontium ranelate in concentration of 625mg/kg/day; SCAR/SCHL₃₀, treated with strontium carbonate and strontium chloride in concentration of 30mg/kg/day; SCAR/SCHL₃₆₅, treated with strontium carbonate and strontium chloride in concentration of 365mg/kg/day. The drugs were administered via gavage, once a day, starting 15 days before surgery (defects created in the calvaria), and persisted during 15 or 60 days. Microtomography, histomorphometric, and immunohistochemical evaluation was performed to assess bone formation within the created defects.

Results: In 15 and 60 days, the histomorphometric evaluation showed larger values of bone formation in groups SRAN₆₂₅ and SCAR/SCHL₃₆₅ when compared to the Control group. In the immunohistochemical analysis the SCAR/SCHL₃₆₅ group showed increased BMP-2 in 15 days when compare to Control group. Differently, in 60 days, the SRAN₆₂₅ showed larger values of BMP-2 and OCN when compared to the Control group. Microtomographic evaluation showed larger bone volume at 60 days, compared to 15 days (SCAR/SCHL₃₀, SCAR/SCHL₃₆₅, and SRAN₆₂₅), and higher Sr concentrations showed an increased bone formation in 15 days (SCAR/SCHL₃₆₅) and 60 days (SRAN₆₂₅ and SCAR/SCHL₃₆₅), compared to the other groups.

Conclusions: High concentrations of systemic Sr (SRAN₆₂₅; SCAR/SCHL₃₆₅) lead to improved bone formation parameters in rats' calvarial defects.

Key-words: *Strontium; systemic use; osteogenesis; bone remodeling.*

1. Introduction

The use of Strontium(Sr)-based medications has been broadly indicated for the prevention and/or treatment of systemic bone pathologies, mainly osteoporosis(1-3) and osteoarthritis(4), once that Sr might decrease bone degradation. Several studies suggested a positive action of Sr in bone metabolism both in animals(5, 6) and in humans(2, 7). However, the process of Sr action is complex and still not totally discovered(8-10). It is known that the Sr can act in two axes of bone repair, both in osteoclasts and/or osteoblasts, increasing bone formation and/or inhibiting bone resorption(10). The two most accepted theories in relation to Sr, suggest that it can be associated to calcium-sensing receptors and/ or to axis RANK/RANKL/OPG(8). Therefore, both theories could interfere favorably in bone formation through osteoblasts cells, increasing cell activity and number of cells, and/or through osteoclasts cells, decreasing cell activity and number of cells(10).

Due to these good results with Sr-based medications and Sr's potential to positively interfere with bone formation, new studies have emerged in other areas also dependent of bone formation (11). It is the case of dentistry procedures, which depend on better or faster bone formation. In this area, countless studies have been made with the intent of improve of quality or quantity bone. (11) Therefore, the use of Sr can be applied in basically two opportunities: for the prevention(3) or treatment of bone pathologies (4, 7, 12), the Sr (medication) is used on long-term, and for procedures that need an increase of bone formation and/or a shorter time for maturation of bone tissue, the Sr (supplement) should be ingested during a short-term period, corresponding to the bone healing period (11), without leading to any side effects (13).

The use of Sr in humans is well defined, through strontium ranelate (SRAN) salt and a fixed posology (2g/day)(12). However, new Sr-based salts have been suggested in studies(6, 10, 14), which could come to substitute the strontium ranelate, and different concentrations of Sr have been tested in animals, showing controversial results (15). This divergent concentration used in studies for animals is based in the diverse levels of Sr intestinal absorption, according with the animal species. However, it is known that in rats, the dose must be adjusted according to the animal's weight, and these values are higher than in humans, once the intestinal absorption is smaller in rats than in humans. Many studies test Sr supplementation during the process of osseointegration (11, 15), alveolar extraction(16), expansion of mid-palatal suture(17,

18), and orthodontic treatment(19). In these studies, the majority showed positive of Sr, however, to the present there are no studies to teste the Sr efficacy enhancing bone formation in bone defects.

The aim of study was to test a new Sr-based supplement compared with SRAN (formula already defined) in diverse concentrations, as an adjunct therapy for the healing of critical size bone defects and assess if it enhances the bone formation by means of microtomography, histomorphometric, and immunohistochemical evaluation.

2. Materials and methods

2.1 Animals and diet

This experimental protocol was approved by the Animal Ethics Committee of the University of São Paulo – UNESP - Araraquara-SP (04/2012). Sixty male rats (3 month-old *Rattus Norvegicus*, *albinus* variation) were maintained housed in a room with controlled temperature, and access to water and food *ad libitum*. The animals were randomly divided in five groups: Control, treated with saline solution (n=14); SRAN₅₀, treated with strontium ranelate in the concentration of 50mg/kg/day (Protos; Laboratory Servier, São Paulo, Brasil) (n=14); SRAN₆₂₅, treated with strontium ranelate in the concentration of 625mg/kg/day (Protos; Laboratory Servier, São Paulo, Brasil) (n=14); SCAR/SCHL₃₀, treated with strontium carbonate and strontium chloride (50/50% proportion) in the concentration of 30mg/kg/day (Cromoline Química Fina, Diadema, São Paulo, Brasil) (n=14); SCAR/SCHL₃₆₅, treated with strontium carbonate and strontium chloride (50/50% proportion) in the concentration of 365mg/kg/day (Cromoline Química Fina, Diadema, São Paulo, Brasil) (n=14). All animals received the supplement via gavage, starting 15 days before of defects surgical. The period of 15 days was selected to allow the supplement reaching adequate plasmatic levels before the surgery took place(20). Supplementation was always provided in the morning, fitting the rats' circadian cycle(21).

2.2 Group preparation

The adjustment of the dose for the strontium ranelate groups was based on a high dose recommended for rats (625mg/kg/day strontium ranelate)(22) and on a low dose-equivalent used in humans (50mg/kg/day strontium ranelate). After the two doses were defined for the ranelate group, the new supplement (strontium carbonate and strontium chloride) dose was defined based on the number of molecules of strontium ranelate,

365mg/kg/day and 30mg/kg/day, respectively. During the treatment, the animals were weighted weekly, and their respective supplement dose was adjusted.

2.3 Surgical Procedure

In this study, the animals were anesthetized by an intramuscular injection of ketamine (hydrochloride of ketamine 10%) (Francotar; Virbac, São Paulo, Brasil) and xylazine (hydrochloride of xylazine 2%) (Virbaxil; Virbac, São Paulo, Brasil). The superior regions were cleaned using a solution containing 0.2% chlorhexidine and shaved. An incision of 2 centimeters, was made and subcutaneous tissue was dissected, exposing the bone tissue. Using a hand-piece (Mont Blanc Anthogyr, São Paulo, Brasil), attached to an electrical motor BLM 600 (Driller, Carapicuíba, Brasil), a dental trephine burr (5 mm-diameter, Neodent, Curitiba, Brasil) was used to prepare two defects in the bone tissue, always accompanied by saline irrigation, and not exceeding the speed of 900 rpm.

The defects were positioned laterally to avoid interference of the cranial suture in the center of the calvaria, and were filled naturally only with blood clot, without collagen membranes (Figure 1). After the defects were created, soft tissues were repositioned and sutured in planes (Vycril; Ethicon, Spreintebach, Switzerland and Seda; Ethicon, Spreintebach, Switzerland).

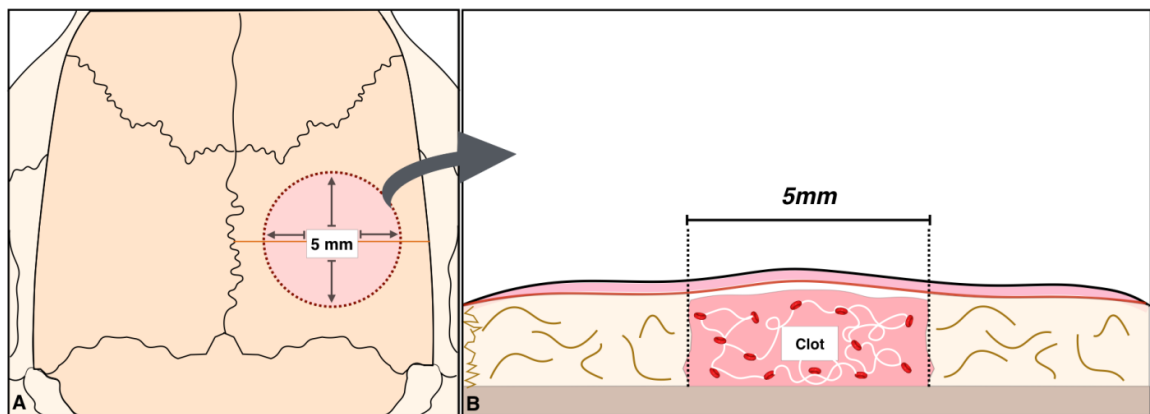


Figure 1. Representation of the calvarial defects. **A-** The defects were positioned laterally to the cranial suture; **B-** Coronal vision of the calvarial defects.

After surgery, the animals were medicated by a subcutaneous injection of penicillin associated to streptomycin 0.1mg/kg (Pentabiotic; FortDodge, Campinas,

Brasil), and an intramuscular injection of sodic dipirone 5mg/kg (Febrax; Lema Injex Biologic, São Paulo, Brasil) twice a day for 2 days.

In periods of 15 or 60 days, the animals were euthanized by means of prolonged general anesthesia, and the calvaria were retrieved and stored in alcohol 70%. The specimens were used for microtomographic, histological, histomorphometric, and immunohistochemical evaluation.

2.4 Microtomography analysis

The calvaria area containing the defect were scanned using a micro CT scanner (SkyScan 1176 Bruker, Aatselaar, Belgium), in sections of 18 μ m, rotation step 0.4 mm, aluminum and copper filters, voltage of 80 kV, and 300 μ A of intensity. The obtained images were reconstructed using dedicated software (NRecon, SkyScan, Aatselaar, Belgium), with -1000 to 2300 Hounsfield values, being this value was determined for provided analysis of density in same software. For the reconstruction, no additional settings were applied, such as smoothing, misalignment compensation, object larger than field of view, or ring artifacts reduction. Before the reconstruction, the images were properly positioned using dedicated data viewer software (SkyScan, Aatselaar, Belgium). At last, with the aid of software CTAn (CTAnalyser, SkyScan, Aatselaar, Belgium) the more central image central of the defect (by subtraction of the starting and ending image) was chosen, and a rectangular ROI (5,0 x 0,5mm) was defined in the center of defect.

According with previous density measurements, and visual observation, the threshold to define the bone tissues was determined initially and manually as 45-165. However, to reach a more adequate threshold, which could lead to a values with would be more closely related with the histomorphometric results, four more thresholds (35-155; 40-160; 50-170; 55-175) were also used, At last, one new threshold interval was also applied, with the minimum and maximum values used in the other tested thresholds (35-175).

2.5 Histomorphometric analysis

The specimens were decalcified in 7% buffered EDTA, dehydrated and then embedded in paraffin, the according with method described by Donath & Breuner(23). Histological serial sagittal sections, with a thickness of 4 μ m, were removed and mounted on slides (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK). The

sections were stained using hematoxylin-eosin (H&E), and photographed using light microscopy (Diastar microscope, Leica Reichert & Jung products, Germany), connected to a camera (Leica Microsystems DFC-300-FX, Leica Reichert & Jung Products, Germany). One section, which represented the region more central of the defect chosen to the histomorphometric evaluation.

For the quantitative evaluation, the software Image J (Image J 1.42q, Bethesda, Maryland, USA) was used to make the counting of the tissues present tissues. With the aid of a digital grid measuring 75 x 75 pixels (5625 square pixels) positioned on top of the image, each intersection between the grid lines was counted according of the present tissue (1- empty space; 2- soft tissue; 3-bone tissue). At last, the values were showed as a percentage of the measured area.

2.6 Immunohistochemical analysis

For the immunohistochemical evaluation, the specimens passed through the same processing methods as described for the histomorphometric analysis. The tissues were treated with an avidin-biotin-peroxidase (ABC) complex using an ABC staining system kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the detection of three antibodies associated to bone formation, Osteocalcin (OCN), Bone Morphogenetic Protein (BMP-2), and Osterix (OTX). The analysis was made in the bone region around of defects and with the use of the optical microscope (DIASTAR Microscope, Leica Reichert & Jung products, Wetzlar, Hessen, Germany) for counting marked cells. Then, descriptions of the positively marked cell types were performed.

2.7 Statistical methods

All assessments were conducted by the same trained observer, blinded to the groups being evaluated. The results were evaluated by a normality test. Histomorphometric and microtomographic data was treated by unpaired t-student test to compare the groups in the same period. For the comparison between the periods of evaluation, the One-way ANOVA test was applied, following the post-test of Tukey. The immunohistochemical data was not distributed according the Gaussian curve, so the non-parametric test of Kruskal-Wallis complemented by Dunn test was used for inferential statistical analysis. All the tests were applied at a significance level of 5%.

3. Results

Histomorphometric evaluation

In 15 days, the group SCAR/SCHL₃₆₅ presented an increase of bone formation when compared to SCAR/SCHL₃₀, SRAN₅₀, and Control. The SRAN₆₂₅ group also showed larger percentage of bone tissue, however, only when compared to Control. In the period of 60 days, the SCAR/SCHL₃₆₅ and SRAN₆₂₅ showed larger percentage of bone tissue compared to SCAR/SCHL₃₀, and Control.

When the two evaluation periods were compared, an increase in the percentage of bone tissue from 15 to 60 days was verified for SRAN₅₀, SRAN₆₂₅, and Control. Figure 2 expresses these values in a graphic, and figures 3 (15 days) and 4 (60 days) show representative images of defects and respective groups.

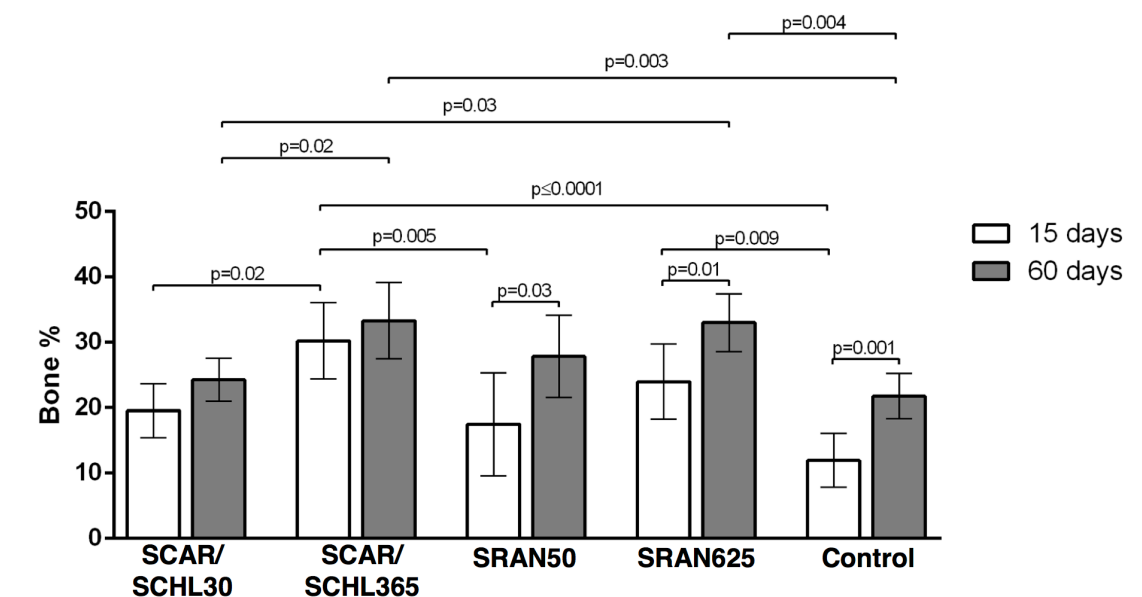


Figure 2. Mean and standard deviation of the values assessed in the histomorphometric analysis, for all evaluated groups and periods.

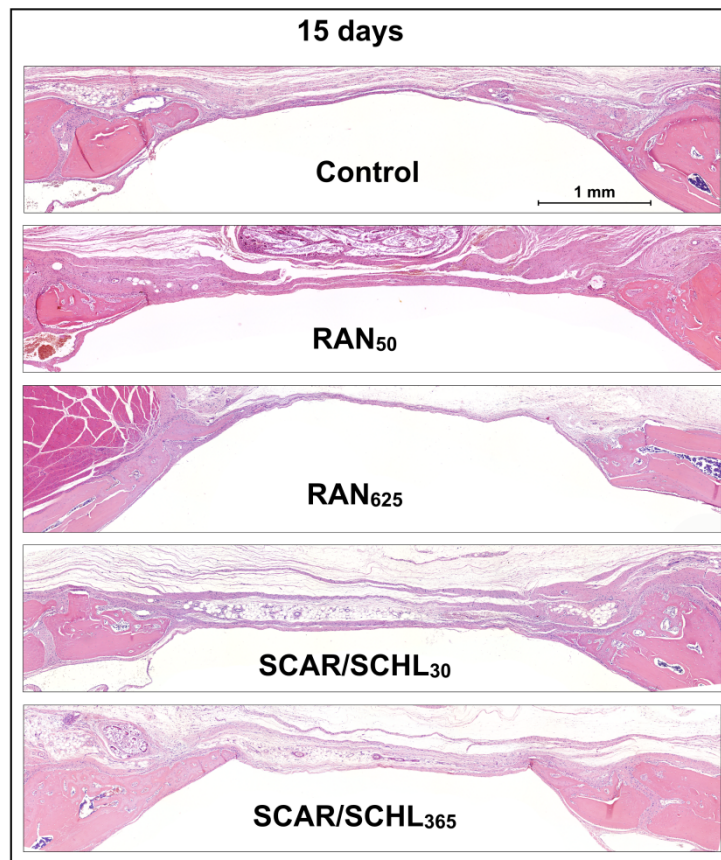


Figure 3. Representative images of defects in respective groups with 15 days.

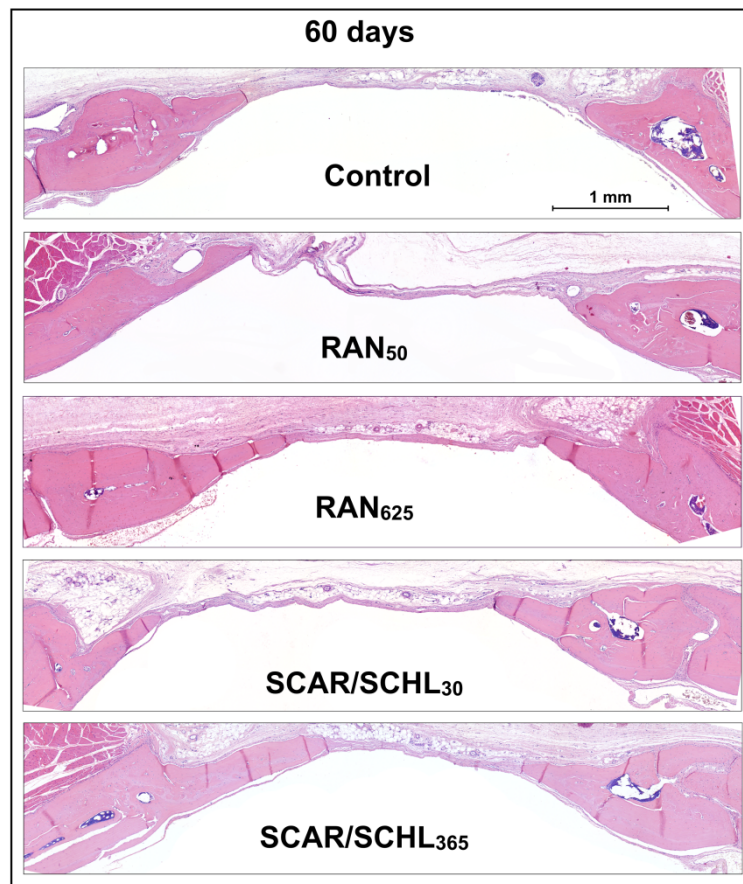


Figure 4. Representative images of defects in respective groups with 60 days.

Immunohistochemical evaluation

In the period of 15 days, the groups SRAN₆₂₅ and SCAR/SCHL₃₆₅ showed higher expression of BMP-2 than the groups SRAN₅₀ and SCAR/SCHL₃₀, however, only the SCAR/SCHL₃₆₅ group was statistically different from the Control group. There were no statistical differences in the expression of the OCN between the groups in both periods of evaluation. At last, the OTX expression was higher only in SCAR/SCHL₃₆₅ group with relation to SRAN₅₀ and SCAR/SCHL₃₀.

In the period of 60 days, the SRAN₆₂₅ group showed higher expression of BMP-2 than the SRAN₅₀ and Control groups. Similarly, when the OCN was evaluated, the SRAN₆₂₅ group showed higher expression than the Control group. At last, OTX

expression was higher in SRAN₆₂₅ and SCAR/SCHL₃₆₅, being statistically different in relation to SRAN₅₀ and SCAR/SCHL₃₀. Figure 5 expresses these values in graphics.

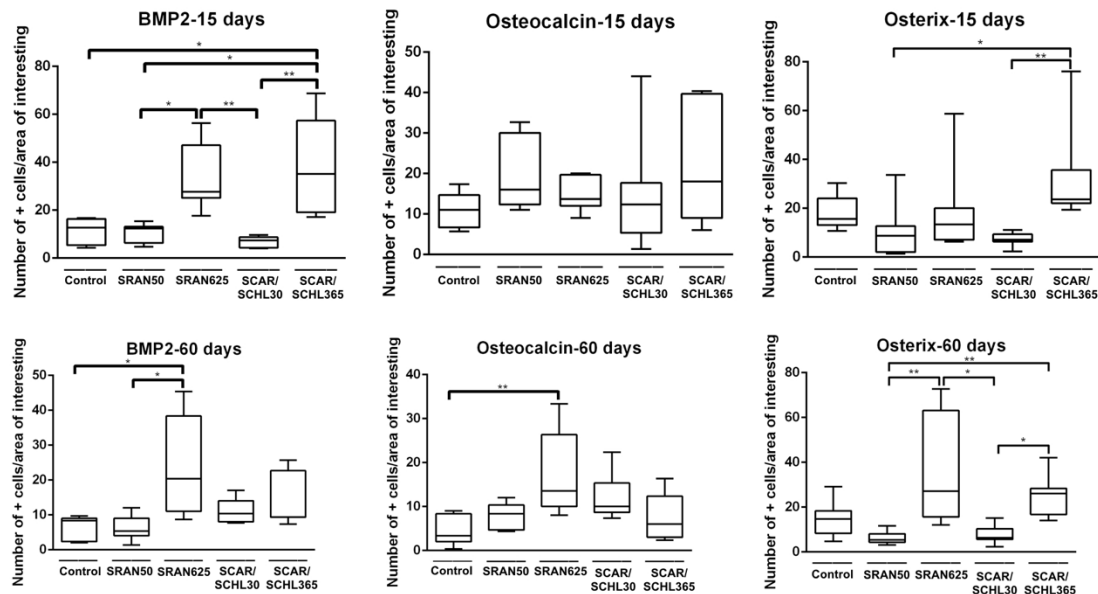


Figure 5. Mean and statistical difference among groups in the immunohistochemical analysis, for all evaluated groups and periods (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Microtomography evaluation

In the initial period of 15 days, the SCAR/SCHL₃₆₅ group presented a larger bone formation when compared to Control. The other groups showed no significant statistical difference. In the period of 60 days, the SCAR/SCHL₃₆₅ group showed significantly larger amount of bone tissue compared to the SRAN₅₀, SCAR/SCHL₃₀, and Control groups. The SRAN₆₂₅ group also showed larger bone formation when compared to the Control and SCAR/SCHL₃₀ groups.

When the two evaluation periods were compared, an increase for bone formation from 15 to 60 days was verified for SCAR/SCHL₃₀, SCAR/SCHL₃₆₅, and SRAN₆₂₅. Figure 6 has expresses these values in a graphic.

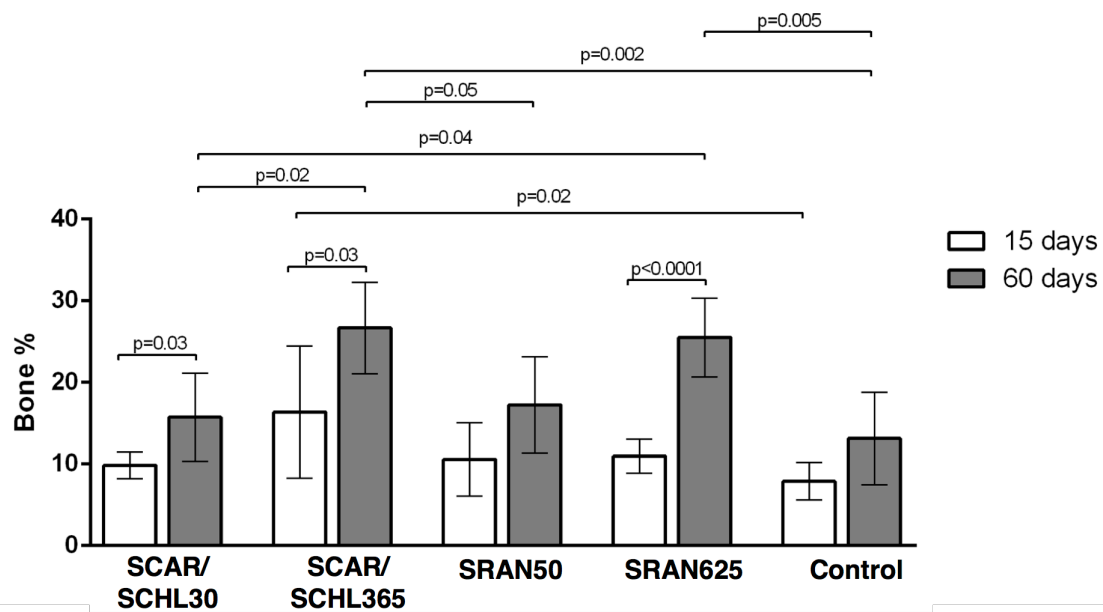


Figure 6. Mean and standard deviation of the values assessed in the microtomography analysis, for all evaluated groups and periods.

4. Discussion

The use of systemic Sr has presented good results in diverse medical areas, acting for prevention(3), and treatment of pathologies(1, 2) or boosting tissue healing in those procedures that depend on bone formation(15), such implants' osseointegration and healing of bone defects(11). Sr supplementation has been used in many animal studies to accelerate and/or increase the bone formation around the implants and the results suggest a positive influence of systemic strontium administration on osseointegration (24-27). Other studies also suggest that the use of systemic Sr supplementation during short periods do not cause side-effects in the metabolism-related organs(13). However, the absence of critical defects, and the lack of dose standardization in those studies testing systemic Sr supplementation, although suggesting positive effects of Sr, could not elucidate or ratify its use(11). Therefore, the aim this study was to evaluate diverse formulas and salts of Sr in the healing of bone defects.

In the histomorphometric analysis at 15 days, the higher Sr doses (SRAN₆₂₅ and SCAR/SCHL₃₆₅) showed an increase of bone formation when compared to Control. These results suggest a positive acting of Sr in initial periods of bone repair with high doses (considered ideal for rats), such as those showed by Maïmoun et al. 2010(27) and Li et al. 2012(26). The Control group presented a no statistically significant

difference to the groups with low doses (SRAN₅₀ and SCAR/SCHL₃₀), suggesting a weak or absent effect in 15 days. Similarly, in 60 days, period corresponding to final stages of bone maturation, the groups SRAN₆₂₅ and SCAR/SCHL₃₆₅ showed higher bone formation values than SCAR/SCHL₃₀ and Control. According with Dahl SG et al. 2001(20), the Sr could act both in early and late stages through fast or slow Sr absorption mechanisms. The slower mechanism occurs through the incorporation of Sr ions into the bone mineral crystals, which takes place gradually, due to exchanges in the structure of the mature bone, and the faster intake is directly associated to the osteoblastic activity in which Sr is absorbed by ionic exchanges with Calcium (Ca) or by binding to osteoid proteins and/or both(20). In the end, these processes are related both to bone neoformation and bone maturation, and Sr is able to exert its effect in both bone formation stages(20). In an evaluation from 15 to 60 days within the same group, the SRAN₆₂₅, SRAN₅₀, and Control showed significant statistical differences between the periods.

In the immunohistochemical analysis, proteins relationship to bone formation or osteoblast differentiation were labeled and showed significant difference in relation to Control group. In the initial period of evaluation the SCAR/SCHL₃₆₅ group showed an increase of BMP-2, however, OCN and OTX did not show statistical difference among groups. Therefore, one can speculate that a greater activity of bone formation can be to see at SCAR/SCHL₃₆₅ group. One other study that evaluated the bone formation around implants with the same groups and periods showed similar results (15). Differently, in the period of evaluation that corresponds to bone maturation (60 days), the SRAN₆₂₅ group showed a higher expression of BMP-2 and OCN in relation to Control group, and OTX did not show statistical difference among groups. In other study with same Sr dosage, Li Y et al., 2012 (26) showed a greater OCN expression in Sr group with ovariectomized rats during the 12 weeks period. These results suggest that Sr in high doses can act in the initial and/or the final periods of bone formation, according to the present histomorphometric and immunohistochemical results.

Microtomographic analysis showed that the SCAR/SCHL₃₆₅ was the single group with statistical differences to Control, in 15 days. These differences between the microtomography and the histomorphometric and microtomography evaluation can be associated to the difficulty of assessing immature bone (i.e. poorly mineralized tissue) using microtomographic analysis. Microtomography quantifies the amount of bone tissue through the gray-levels distribution in a sample, however, the threshold which

determine the range of values used to define what is / is not to be considered as mineralized tissue hardly contains immature bone, which could be considered as noise(28). This is not the case of the other analysis performed in the present study. At the final evaluation period, the SCAR/SCHL₃₆₅ group showed superiority in relation to the lower Sr doses (SRAN₅₀, SCAR/SCHL₃₀), and Control group. The SRAN₆₂₅ group showed to differences when compared to SCAR/SCHL₃₀ and Control. In this way, the microtomographic evaluation was compatible with the histomorphometric for the longest period of evaluation, mostly associated of the presence of more mature bone. According with Scardueli et al., 2017 (29), the use of the same threshold for different periods and substances/drugs that can change the bone density could derail the analysis, and consequently impair the analysis.

The use of Sr supplement showed as a potential alterer of bone activity through systemic application, however, the use of local Sr incorporate in biomaterials could be interesting in clinical studies.

5. Conclusion

High concentrations of systemic Sr (SRAN₆₂₅; SCAR/SCHL₃₆₅) lead to improved bone formation parameters in rats' calvarial defects regarding the histomorphometric, immunohistochemical, and microtomographic evaluation.

6. References

1. Kaufman JM, Lapauw B, Goemaere S. Current and future treatments of osteoporosis in men. *Best Pract Res Clin Endocrinol Metab.* 2014;28(6):871-84.
2. Meunier PJ, Roux C, Ortolani S, Diaz-Curiel M, Compston J, Marquis P, et al. Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis. *Osteoporos Int.* 2009;20(10):1663-73.
3. Reginster JY, Deroisy R, Dougados M, Jupsin I, Colette J, Roux C. Prevention of early postmenopausal bone loss by strontium ranelate: the randomized, two-year, double-masked, dose-ranging, placebo-controlled PREVOS trial. *Osteoporos Int.* 2002;13(12):925-31.

4. Reginster JY. Efficacy and safety of strontium ranelate in the treatment of knee osteoarthritis: results of a double-blind randomised, placebo-controlled trial. *Ann Rheum Dis*. 2014;73(2):e8.
5. Pemmer B, Hofstaetter JG, Meirer F, Smolek S, Wobrauschek P, Simon R, et al. Increased strontium uptake in trabecular bone of ovariectomized calcium-deficient rats treated with strontium ranelate or strontium chloride. *J Synchrotron Radiat*. 2011;18(Pt 6):835-41.
6. Peng S, Liu XS, Huang S, Pan H, Zhen W, Zhou G, et al. Intervention timing of strontium treatment on estrogen depletion-induced osteoporosis in rats: bone microstructure and mechanics. *J Orthop Res*. 2014;32(3):477-84.
7. Reginster JY, Seeman E, De Vernejoul MC, Adami S, Compston J, Phenekos C, et al. Strontium ranelate reduces the risk of nonvertebral fractures in postmenopausal women with osteoporosis: Treatment of Peripheral Osteoporosis (TROPOS) study. *J Clin Endocrinol Metab*. 2005;90(5):2816-22.
8. Fonseca JE. Rebalancing bone turnover in favour of formation with strontium ranelate: implications for bone strength. *Rheumatology (Oxford)*. 2008;47 Suppl 4:iv17-9.
9. Stepan JJ. Strontium ranelate: in search for the mechanism of action. *J Bone Miner Metab*. 2013;31(6):606-12.
10. Yamaguchi M, Weitzmann MN. The intact strontium ranelate complex stimulates osteoblastogenesis and suppresses osteoclastogenesis by antagonizing NF-kappaB activation. *Mol Cell Biochem*. 2012;359(1-2):399-407.
11. Scardueli CR, Bizzeli-Silveira C, Marcantonio RAC, Marcantonio E-Jr, Stavropoulos A, Spin-Neto R. Systemic strontium to enhance osseointegration of implants: systematic review of animal studies. *Journal of Oral Implantology*. 2017;sent.
12. Deeks ED, Dhillon S. Spotlight on strontium ranelate: in postmenopausal osteoporosis. *Drugs Aging*. 2010;27(9):771-3.
13. Scardueli CR, Spin-Neto R, Arcaro CA, Brunetti IL, Baviera AM, Bizzelli-Silveira C, Marcantonio RAC, Marcantonio E-Jr. Toxicity assessment of non-radioactive strontium systemic supplementation. *Journal of Oral Medicine and Toxicology*. 2017;sent.

14. Ma B, Zhang Q, Wu D, Wang YL, Hu YY, Cheng YP, et al. Strontium fructose 1,6-diphosphate prevents bone loss in a rat model of postmenopausal osteoporosis via the OPG/RANKL/RANK pathway. *Acta Pharmacol Sin.* 2012;33(4):479-89.
15. Scardueli CR S-NR, Oliveira GJLP, Stavropoulos A, Marcantonio RAC, Marcantonio E Jr. Use of non-radioactive strontium supplementation to enhance the osseointegration of titanium implants. *Bone* 2017;sent.
16. Buehler J, Chappuis P, Saffar JL, Tsouderos Y, Vignery A. Strontium ranelate inhibits bone resorption while maintaining bone formation in alveolar bone in monkeys (*Macaca fascicularis*). *Bone.* 2001;29(2):176-9.
17. Chao K, Xuxia W, Qianqian W, Yuanyuan H, Shuya Z, Jun Z. [Effects of strontium ranelate on the rats' palatal suture after rapid maxillary expansion]. *Hua Xi Kou Qiang Yi Xue Za Zhi.* 2016;34(4):336-40.
18. Zhao S, Wang X, Li N, Chen Y, Su Y, Zhang J. Effects of strontium ranelate on bone formation in the mid-palatal suture after rapid maxillary expansion. *Drug Des Devel Ther.* 2015;9:2725-34.
19. Kirschneck C, Wolf M, Reicheneder C, Wahlmann U, Proff P, Roemer P. Strontium ranelate improved tooth anchorage and reduced root resorption in orthodontic treatment of rats. *Eur J Pharmacol.* 2014;744:67-75.
20. Dahl SG, Allain P, Marie PJ, Mauras Y, Boivin G, Ammann P, et al. Incorporation and distribution of strontium in bone. *Bone.* 2001;28(4):446-53.
21. Almon RR, Yang E, Lai W, Androulakis IP, DuBois DC, Jusko WJ. Circadian variations in rat liver gene expression: relationships to drug actions. *J Pharmacol Exp Ther.* 2008;326(3):700-16.
22. Ammann P. Strontium ranelate: a physiological approach for an improved bone quality. *Bone.* 2006;38(2 Suppl 1):15-8.
23. Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique. *J Oral Pathol.* 1982;11(4):318-26.
24. Chen B, Li Y, Yang X, Xu H, Xie D. Zoledronic acid enhances bone-implant osseointegration more than alendronate and strontium ranelate in ovariectomized rats. *Osteoporos Int.* 2013;24(7):2115-21.

25. Li Y, Feng G, Gao Y, Luo E, Liu X, Hu J. Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats. *J Orthop Res.* 2010;28(5):578-82.
26. Li Y, Li X, Song G, Chen K, Yin G, Hu J. Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats. *Clin Oral Implants Res.* 2012;23(9):1038-44.
27. Maimoun L, Brennan TC, Badoud I, Dubois-Ferriere V, Rizzoli R, Ammann P. Strontium ranelate improves implant osseointegration. *Bone.* 2010;46(5):1436-41.
28. Song JW, Cha JY, Bechtold TE, Park YC. Influence of peri-implant artifacts on bone morphometric analysis with micro-computed tomography. *Int J Oral Maxillofac Implants.* 2013;28(2):519-25.
29. Scardueli CR, Marcantonio RAC, Marcantonio E-Jr, Wenzel A, Spin-Neto R. Micro CT to evaluate bone tissue formation in rat calvarial defect models: a systematic review *Journal of Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology.* 2017;sent.

3.4 Publicação 4

Toxicity assessment of non-radioactive strontium systemic supplementation.*

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Running title: Strontium systemic toxicity during short term.

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* Artigo formatado de acordo com as normas da revista Basic & Clinical Pharmacology & Toxicology.

Introduction

Some drugs can be used to prevent and/or treat the loss of bone mass caused by systemic diseases, such as osteoporosis, and their use have been widely studied in diverse areas [1, 2]. The drugs for treatment of bone loss could act decreasing the number of osteoclast cells, acting in an antiresorptive way, and/or increasing the number of osteoblast cells, enhancing bone formation, directly or indirectly [3]. Bisphosphonates (BPs) have been largely used by its direct action in osteoclasts [4], showing successful results [5, 6]. However, this medication is not indicated as supplement for procedures which depend on bone formation, due to its side-effects (e.g. osteonecrosis of the jaws [7], bone fractures [8], and renal, gastrointestinal lesions as severe as cancers lesions [9]). Consequently, any suggested medication which would interfere in the bone tissue metabolism, must be evaluated regarding both its real purpose and possible systemic side-effects [10]. Therefore, the search for new drugs with this purpose and an alternative medication for bisphosphonates have been studied, such as Strontium (Sr) based medications [11, 12].

Non-radioactive Sr is a chemical element which belongs to the group of alkaline earth metals, presenting similarities to Calcium, such as incorporation to the bone tissue and high hydroxyapatite affinity [13]. Sr is easily incorporated to bone and teeth, and can exert a “dual action” by simultaneously stimulating the production of osteoblasts, and inducing the apoptosis or less activation of osteoclasts [14]. Many studies refer to the beneficial action of Sr in the treatment of osteoporotic animals [15, 16] and humans (SOTI [17], TROPOS [18], MALEO [19]). Sr has been also used to treat others pathologies related to bone metabolism, such as osteoarthritis (SEKOIA [20]), for the enhancement of process that demand bone formation as maxillofacial procedures [21, 22], or improving the characteristics of bone tissue [23]. Nonetheless, it has also been found that the beneficial effects of Sr supplementation appear in a dose-dependent fashion [24, 25].

The Sr dose (Sr ranelate-SRAN) for humans is well defined as of 1mg/day as prevention [26] or 2mg/day for treatment [17, 18] of bone pathologies. However, for studies in animals and like a supplement for surgical procedures, the dose is a controversial topic [27]. It is known that higher doses could develop toxic effects [25, 28], and lower doses will have lower expression of Sr beneficial effects, being a relevant factor regarding its bone-tissue action [23, 25]. However, mammalian animals show different levels of Sr absorption [29] and consequently the posology (dose and

timing for supplementation) may be distinct. Other important factor of Sr use is related to its administration time, being considered long, when used for prevention or treatment, or short, when used as supplement for surgical bone therapies. However, when used on the long term can bring side-effects, in a sparse and non-specific manner [30-33].

Based in other studies, two diverse Sr-salts were proposed to be tested in this study. One salt is the one normally suggested for systemic supplementation in humans (SRAN), and the other is an alternative salt containing Sr (Sr carbonate and Sr chloride). The selected dose reflects the amount of Sr needed to achieve the same plasmatic levels of Sr in a human being treated with 2 g of SRAN per day. In this way, the aim of the present study was to evaluate the Sr-supplementation toxicity, in the main organs related to metabolism, used in periods of 30 and 75 days, in rats.

Methods

Animals

The experimental protocol was approved by the Animal Ethics Committee in Animal Experimentation from the School of Pharmaceutical Sciences, (64/2016), and School of Dentistry (04/2012) in São Paulo State University (UNESP), Araraquara, SP. Thirty-three male rats (one month-old *Rattus norvegicus*, *albinus* variation), weighing 160-180g were maintained housed under environmentally controlled conditions of temperature ($23\pm1^{\circ}\text{C}$) and humidity ($55\pm5\%$) and with 12 hours light/dark cycle. The animals had free access to water and food *ad libitum*.

Experimental design and systemic supplementation

The animals were randomly divided in three groups: Control, treated with saline solution (n=11); SRAN₆₂₅, treated with strontium ranelate in the concentration of 625 mg/kg/day (Protos; Laboratory Servier, São Paulo, Brasil) (n=11); SCAR/SCHL₃₆₅, treated with strontium carbonate and strontium chloride (50/50% proportion) in the concentration of 365 mg/kg/day (Cromoline Química Fina, Diadema, São Paulo, Brasil) (n=11). The adjustment of the dose for the strontium ranelate groups was based on an ideal dose recommended for rats (625 mg/kg/day strontium ranelate) [29] and the new supplement (strontium carbonate and strontium chloride) dose was defined based on the number of molecules of strontium ranelate, 365 mg/kg/day, being equivalent to

each other. During the treatment, the animals were weekly weighted, and their respective supplement doses were adjusted.

All animals received the supplement via gavage. Twenty-four animals were used for the biochemical analysis, which was carried out in the periods of 0, 15, and 30 days. Nine animals were used for the histological evaluation, carried out after 75 days of supplementation. To all animals, the supplementation was provided in the morning, fitting the rats' circadian cycle [34].

Samples collection

In the periods of 0 and 15 days, blood samples were collected from the rat's tail in heparinized tubes (5,000 UI/mL) for the analysis of urea, creatinine, sodium, potassium, uric acid, total protein, albumin, protein fractions, aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). At the end of the treatment, in the period of 30 days, blood samples were collected in the same way as for the other periods, and the same biochemical tests as previously described were carried out. The rats were then euthanized by decapitation and the blood collected and used for analysis of malondialdehyde (MDA), biomarker of lipid peroxidation. The liver and kidneys of the animals were immediately removed and stored at -80°C until the analysis of oxidative stress parameters and activities of antioxidant enzymes.

Organ collection for histological evaluation

For the histological evaluation, the nine rats treated for 75 days had their kidneys and liver removed and preserved in 10% neutral buffered formalin solution for 24 h, and then washed with running water. Tissues were then placed in small metal caskets, stirred by a magnetic stirrer, dehydrated using alcohol series from 70% to 100% alcohol and embedded in paraffin using an embedding machine.

Biochemical analysis

The biochemical analysis of urea, creatinine, sodium, potassium, uric acid, total protein, albumin, protein fractions, AST, ALT, and ALP were quantified in the plasma with the Ortho-Clinical Diagnostics®kits (Johnson & Jonhson Company).

The MDA concentration was analyzed in the liver and blood plasma. Liver samples (0.25 g) were homogenized in 1 mL of 1.15% potassium chloride at 4°C. The

homogenates were centrifuged at 10,000 g for 10 min at 4°C and the supernatants were used for the analysis. Plasma and liver supernatants were previously deproteinized according to Pilz [35]. The MDA levels were measured through thiobarbituric acid reaction [36]. The MDA levels in liver were measured spectrophotometrically at 535 nm and the MDA levels in plasma were measured fluorometrically with excitation and emission wavelengths of 510 and 553 nm, respectively; 1,1,3,3-tetramethoxypropane was used as a standard. Results were expressed as mmol/L (plasma) or mmol/L/g tissue (liver).

Histological analysis

Histological serial sagittal sections of the animal's liver and kidney, with 4 µm in thickness, were stained with hematoxylin-eosin (H&E). Using a light microscope (Diastar microscope, Leica Reichert & Jung products, Germany), connected to a camera (Leica Microsystems DFC-300-FX, Leica Reichert & Jung Products, Germany) images were obtained, which were used for descriptive evaluation of the tissues.

Statistical analysis

All assessments were conducted by the same trained observer, blinded to the groups being evaluated. Results were always expressed as means and standard deviation. The data were treated as non-parametrical and the Kruskal-Wallis followed by Dunn's post-test, for the evaluation among the groups in the same period. For the comparison between the periods of evaluation, the t-Student test was applied.

Results

Biochemical evaluation

For a better understanding, the results were divided according to the function of the studied biochemical markers in the respective tissues – kidneys, kidneys and liver, or liver and other tissues.

Kidneys

For all kidney-related biomarkers evaluated in the plasma, the groups treated with the Sr-based supplements (SRAN₆₂₅;SCAR/SCHL₃₆₅) showed no significant differences

when compared with the Control group in the periods of 0,15, and 30 days. These results are shown in Table 1.

	Control			SRAN ₆₂₅			SCAR/SCHL ₃₆₅		
	0	15	30	0	15	30	0	15	30
Creatinine (mg/dL)	0.35 ±0.02	0.30 ±0.01	0.34 ±0.01	0.33 ±0.02	0.30 ±0.01	0.31 ±0.01	0.31 ±0.02	0.31 ±0.01	0.33 ±0.01
Urea (mg/dL)	47.20 ±3.42	42.80 ±1.76	45.51 ±1.69	51.38 ±3.55	44.01 ±0.69	45.33 ±1.33	46.93 ±1.29	44.46 ±0.99	47.48 ±1.08
Potassium (mmol/L)	6.40 ±0.32	5.48 ±0.35	5.23 ±0.11	6.14 ±0.22	5.28 ±0.10	5.03 ±0.22	6.12 ±0.21	5.12 ±0.09	5.35 ±0.19
Sodium (mmol/L)	140.18 ±0.67	145.50 ±0.70	153.75 ±1.48	144.15 ±1.27	145.55 ±0.71	155.25 ±2.24	146.33 ±0.68	147.50 ±0.56	155.32 ±1.34

Table 1. Mean and standard deviation of creatinine, urea, potassium, and sodium plasma levels for kidneys evaluation. The data showed no significant differences compared with the Control group in the periods of 0, 15, and 30.

Kidneys and liver

To represent possible tissue alterations in the kidneys and liver, the plasma levels of uric acid, total protein, albumin, and protein fractions were evaluated. There were no changes in the plasma levels of these biomarkers during the treatment with the Sr-based supplement (SRAN₆₂₅; SCAR/SCHL₃₆₅), for the periods of 0, 15, and 30 days, when compared with the Control group. Table 2 shows the results expressed as means.

	Control			SRAN ₆₂₅			SCAR/SCHL ₃₆₅		
	0	15	30	0	15	30	0	15	30
Uric acid (mg/dL)	0.67 ±0.05	0.57 ±0.01	0.72 ±0.07	0.60 ±0.02	0.59 ±0.02	0.57 ±0.03	0.57 ±0.03	0.51 ±0.02	0.59 ±0.05
Albumin (g/dL)	2.73 ±0.04	2.82 ±0.05	3.02 ±0.08	2.94 ±0.06	2.79 ±0.07	2.95 ±0.06	2.9 ±0.06	2.97 ±0.02	3.03 ±0.04
Total protein (g/L)	5.40 ±0.10	5.57 ±0.05	5.64 ±0.06	5.57 ±0.10	5.49 ±0.09	5.53 ±0.12	5.51 ±0.10	5.66 ±0.04	5.56 ±0.07
Protein fractions (g/L)	2.67 ±0.08	2.74 ±0.06	2.62 ±0.06	2.62 ±0.05	2.7 ±0.07	2.57 ±0.09	2.61 ±0.05	2.68 ±0.04	2.52 ±0.06

Table 2. Mean and standard deviation of uric acid, albumin, total protein, and protein fractions in plasma for kidneys evaluation. The data showed no significant differences compared with the Control group in the periods of 0, 15, and 30.

Liver and others tissues

The enzymes and biomarkers used for the detection of liver and others tissues disorders were AST, ALT, ALP, and MDA. The findings for AST and ALT did not show significant differences, in all periods of evaluation, when comparing Sr-treated with the Control group (Table 3). Blood plasma and liver MDA levels, at the period of 30 days, also did not have differences among the studied groups, as shown in Table 3.

	Control			SRAN ₆₂₅			SCAR/SCHL ₃₆₅		
	0	15	30	0	15	30	0	15	30
ALT (U/L)	63.5 ±1.5	75.25 ±2.67	65.25 ±2.80	58.62 ±3.13	72 ±3.29	60,5 ±2.74	63.5 ±1.56	79.37 ±4.27	62 ±1.82
AST (U/L)	99 ±3.09	100 ±2.17	96.25 ±2.83	101.25 ±3.2	107.16 ±2.1	92.16 ±2.54	100.12 ±2.05	104.25 ±5.28	91.37 ±2.88
MDA plasma (mM)	-	-	5.11 ±0.21	-	-	5.33 ±0.21	-	-	5.82 ±0.22
MDA liver (mM/g tissue)	-	-	150.26 ±7.82	-	-	145.79 ±11.29	-	-	142.85 ±6.02

Table 3. Mean and standard deviation of ALT, AST, and MDA plasma levels, and MDA liver levels for liver and other tissues evaluation. The data showed no significant differences compared with the Control group in the periods of 0, 15, and 30.

ALP analysis showed difference between the Sr-treated groups (SRAN₆₂₅; SCAR/SCHL₃₆₅) and the Control group. At baseline, all the groups showed resembling results, showing no significant statistical differences. In the periods of 15 and 30 days, the values for the Sr-treated groups were significantly higher than those of the control group ($p \leq 0.05$). However, in the period of 15 days, the Sr-treated groups did not show significant statistical difference when compared to each other. In the period of 30 days, the ALP levels in the SRAN₆₂₅ showed superior values when compared with the SCAR/SCHL₃₆₅ ($p \leq 0.05$).

In the temporal evaluation, in the Control group there was a decrease of values with significant statistical difference, from 0 to 15 and 30 days ($p < 0.05$). In the SCAR/SCHL₃₆₅ group, there was a tendency of increase from 0 to 30 days, however, without significant statistical difference when the periods were compared. The SRAN₆₂₅ group showed a progressive increase between the periods, with significant statistical difference ($p \leq 0.05$) whenever one period of evaluation was compared to any of the other two. ALP values are shown in Figure 1.

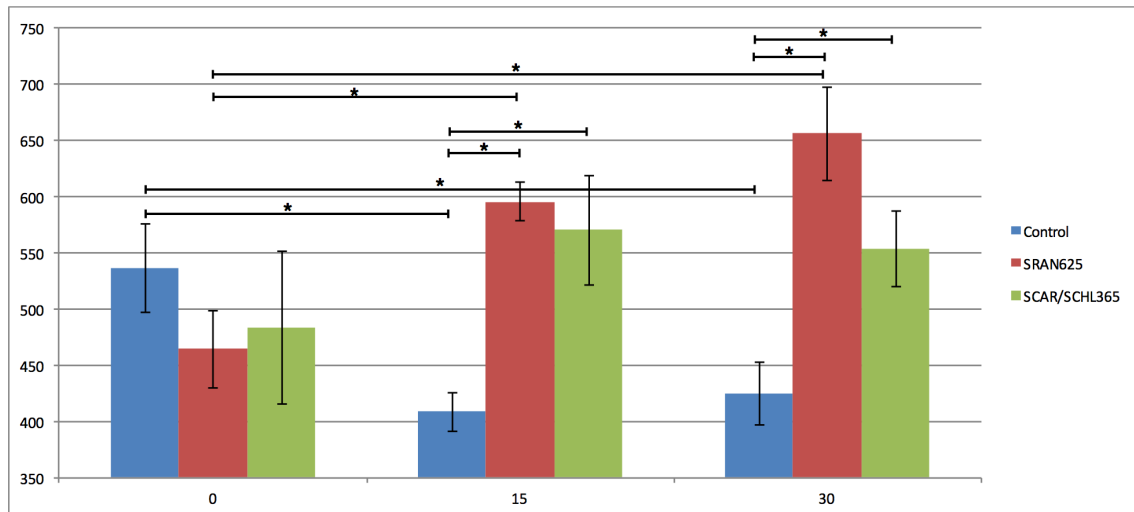


Figure 1. The graphic represents the ALP plasma levels during the evaluation periods. An increase of ALP levels in Sr-treated groups can be seen at 15 and 30 days, when compared with the C group (* $p < 0.05$).

Histological evaluation

Liver

In all the evaluated groups, the sections revealed hepatocytes with no visible alterations, as described in the literature [37]. The hepatocytes revealed distinctly stained nuclei, of varied shapes (most common aspect were cells with one, or sometimes two oval nuclei), arranged in plates or cords around the central vein and extending to the portal triads, being separated by sinusoidal capillaries and small amount of connective tissue [38]. There were no signs of necrosis or edema that could indicate changes in the liver tissue. Images, showing the region of the central vein and the portal triad of the liver, for each of the studied groups, are shown in Figure 2.

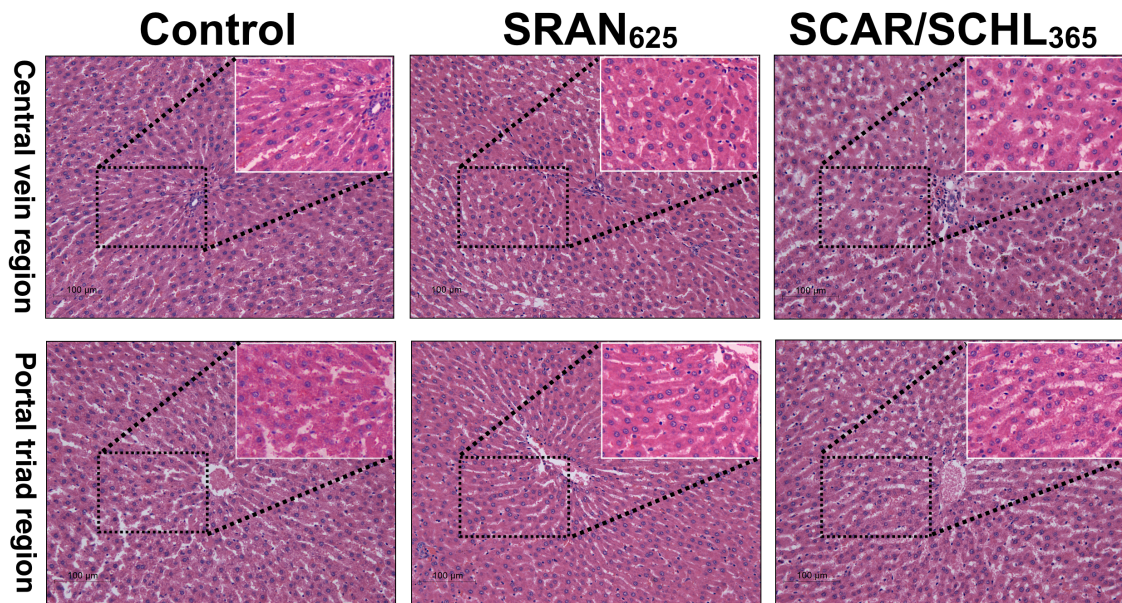


Figure 2. The images represent histological samples (stained with HE and photographed in 100x magnification) of all groups in the period of 75 days and different regions of the liver: central vein and portal triad. The photomicrographs show characteristics of the normal tissue, and the boxes represent a greater magnification of region.

Kidneys

In all the evaluated groups, sections revealed normal cell patterns in the two kidneys regions, and did not observe tissue alterations, such as edema, swell, disruption, congestion, and inflammation. In cortical or cortex region can observe the presence of renal corpuscles, which consists of a glomerulus surrounded by Bowman's capsule, and few tubules around these capsules. In contrast with the cortical region, the medular region there is not presence of corpuscles, and there is a lot of tubules that tend to be parallel (Figure 3).

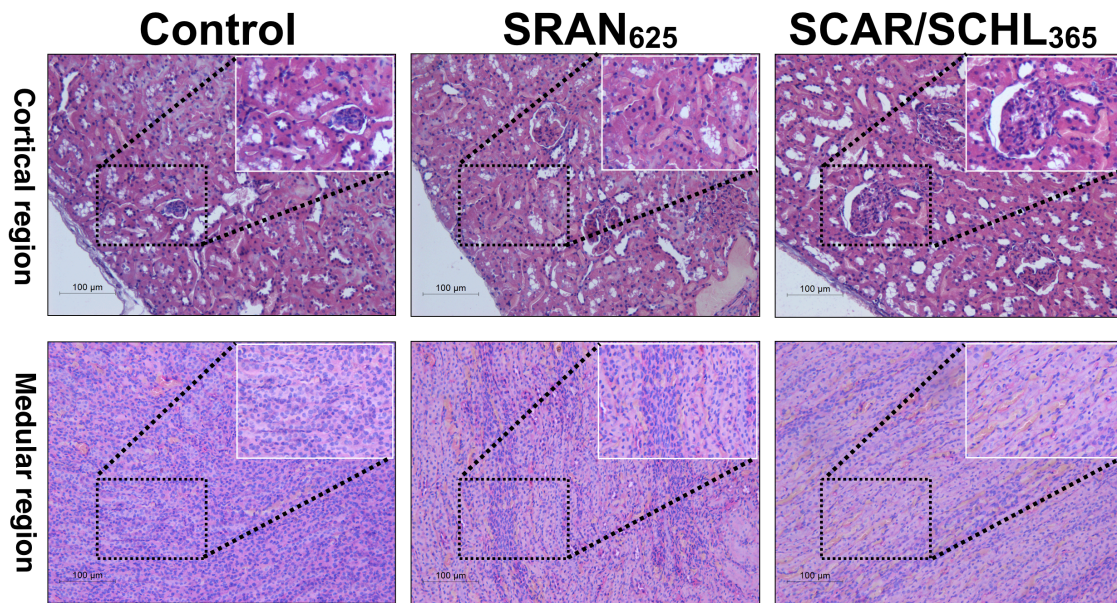


Figure 3. The images represent histological samples (stained with HE and photographed in 100x magnification) of all groups in the period of 75 days and in different regions of kidneys: cortical and medullary region. The photomicrographs show characteristics of the normal tissue and the boxes represent a greater magnification of region.

Discussion

The use of Sr as a medication was initially proposed to the treatment of pathologies related with bone loss (e.g. osteoporosis) [30, 31]. Due to the good results for this tested treatments [17-20], Sr was suggested to be used for other procedures which also depend on new bone formation, with increased quality and quantity of newly formed bone, such as implant-dentistry [27].

Recently, the use of Sr, when used to the treatment of bone pathologies, was associated to many reports for cardiac diseases, skin reactions, hepatitis, and kidney problems [31, 32, 39, 40]. However, these side effects occur in the long term of use, once the treatment is continuous [41]. Differently of the treatment for bone pathologies, the suggestion for using Sr as supplement should occur during the period of bone repair, in other words, in the short-term [27]. Like this, due to good results of Sr like a supplement in maxillofacial procedures [12, 42-44], and a lack of toxicity studies with different Sr formulas in short term, this study evaluated the potential toxicological risk of Sr in the main organs involved in metabolism and excretion [45]. In this way, liver

and kidneys toxicity was evaluated in two steps, chemical analysis and histologic evaluation.

For the chemical analysis, the choice was to assess specific enzymes and biomarkers that suggest alteration in the liver and kidneys, during the period of 30 days [46, 47]. In others studies, the administration of Sr occurred for periods longer than 30 days [27], however, this short period is enough to detect the presence or not of tissue damages related to toxicity [48].

For the evaluation of kidney function, the urea, creatinine, sodium, and potassium markers were analyzed in the blood, once that impaired amounts of these markers in blood can suggest a malfunction of kidneys [47]. However, the lack of statistical difference among the studied groups and the control (not treated group) suggests that there is no toxic effect of the tested Sr-based supplementation on the kidneys. Other markers were also used to detect both alterations in kidneys and livers, like uric acid, total protein, albumin, protein fractions [47], and also showed no difference among the groups. In the vent of difference among the groups, the results could generate alerts regarding possible toxic effects.

Lastly, were analyzed many enzymes and biomarkers to the detection of liver and other tissues disorders. The increased circulating levels of AST are associated with hepatitis and problems of skeletal and heart muscles [38], nevertheless, this enzyme did not change. Similarly, increased plasma levels of ALT are associated with liver disorders, and increased levels of MDA are associated to oxidative damage, mainly in lipids [46]; these biomarkers also did not show differences among the studied groups. However, the ALP enzyme, which is associated with disorders in the liver and bone tissues [38], showed expressive changes in Sr groups (SRAN₆₂₅; SCAR/SCHL₃₆₅), during the periods of 15 and 30 days. The increase of ALP, by character exclusive, could be associated with alterations in bone tissue [49], since the other hepatic enzymes and biomarkers did not showed significant differences among the groups. In this way, the SRAN₆₂₅ group showed regularly high ALP values, which could be associated with important alterations in bone tissue metabolism, when compared with SCAR/SCHL₃₆₅ and Control groups. However, the animals diet could change the values for Sr absorption, and consequently the bone activity [40, 45]. These results are in agreement with Meunier [17] and Bain [50].

Adding corroborative and visual features, the histological evaluation was made in the period of 75 days, checking on the effects of the continuous use of Sr [12, 42-

44]. The results showed similarity with the chemical analysis and reinforce the idea that there is no toxicity associated to the tested Sr-based therapies.

We believe that the present results must be born in mind comparatively to other therapies also constantly used to act in favor of bone formation or an enhancer of bone metabolism, such as bisphosphonates [51] and bone morphogenetic proteins (BMP-2) [52]. Bisphosphonates are indicated to decrease bone resorption processes [3], however, as when used as a supplement, it could bring many unwanted side effects (e.g. osteonecrosis of the jaws [7], bone fractures [8], and renal, gastrointestinal lesions as severe as cancers lesions [9]), associated to a long half-life (years). One of the most assessed toxic-related aspects of the bisphosphonates is related to its association with gastrointestinal intolerance. The use of Alendronate, one of the most known and used drug in the bisphosphonates family, has been associated with upper gastrointestinal intolerance and injury in postmarketing experience [53]. Unfortunately, clinical trials often underestimate the rate of adverse events in clinical practice, specially for bisphosphonates, in which most studies focus on the osteonecrosis issue. Allied to that, ethics prohibits direct evaluation of toxicity in high-risk patients. Although restricted to local application, the increasing clinical use of BMP-2 must also be considered. From BMP-2-based therapies, side effects also have emerged, such as inflammatory complications, ectopic bone formation (bone cysts), activation of osteoclasts, urogenital events, and risk of tumor formation [54]. To the present, no toxicity reports were published regarding possible systemic toxic effects of BMP-2 therapy.

Nonetheless, with the results showed, the use of Sr for short periods, as a supplement could bring to advantages for the procedures which depending of bone remodeling process. Therefore, surgical procedures, as osseointegration, could be speed up and/or achieve a better quality of bone formation, as showed in others studies [27]. Then, Sr could be to use not only for prevention or treatment of pathologies, but also as supplement for bone formation [55].

In conclusion, the systemic Sr supplementation did not lead to plasma, hepatic, or nephrological signs of toxicity in rats, during the proposed periods. However high concentrations of ALP can be associated to higher bone activity when the Sr supplementation was administrated.

Acknowledgments

The authors would like to thank Leandro Alves dos Santos, laboratory technician.

Funding

This study was financed by the Brazilian agencies CAPES (003628/2015-09) and CNPq (scholarship)

Conflict of interest

There is no conflict of interest in the present study.

References

1. Reginster JY, Neuprez A, Dardenne N, et al. Efficacy and safety of currently marketed anti-osteoporosis medications. *Best Pract Res Clin Endocrinol Metab.* 2014; 28(6): 809-834.
2. Harvey N, Dennison E, and Cooper C. Osteoporosis: impact on health and economics. *Nat Rev Rheumatol.* 2010; 6(2): 99-105.
3. Rachner TD, Khosla S, and Hofbauer LC. Osteoporosis: now and the future. *Lancet.* 2011; 377(9773): 1276-1287.
4. Rodan GA and Reszka AA. Bisphosphonate mechanism of action. *Curr Mol Med.* 2002; 2(6): 571-577.
5. Stoch SA, Saag KG, Greenwald M et al. Once-weekly oral alendronate 70 mg in patients with glucocorticoid-induced bone loss: a 12-month randomized, placebo-controlled clinical trial. *J Rheumatol.* 2009; 36(8): 1705-1714.
6. Chen L, Wang G, Zheng F et al. Efficacy of bisphosphonates against osteoporosis in adult men: a meta-analysis of randomized controlled trials. *Osteoporos Int.* 2015; 26(9): 2355-2363.
7. Lazarovici TS, Yahalom R, Taicher S et al. Bisphosphonate-related osteonecrosis of the jaw associated with dental implants. *J Oral Maxillofac Surg.* 2010; 68(4): 790-796.

8. Blum L, Cummings K, Goulet JA et al. Atypical femur fractures in patients receiving bisphosphonate therapy: etiology and management. *Eur J Orthop Surg Traumatol*. 2016; 26(4):371-7.
9. Vestergaard P. Occurrence of gastrointestinal cancer in users of bisphosphonates and other antiresorptive drugs against osteoporosis. *Calcif Tissue Int*. 2011; 89(6): 434-441.
10. Yip VL, Hawcutt DB, and Pirmohamed M. Pharmacogenetic Markers of Drug Efficacy and Toxicity. *Clin Pharmacol Ther*. 2015; 98(1): 61-70.
11. Wu Y, Adeeb SM, Duke MJ et al. Compositional and material properties of rat bone after bisphosphonate and/or Strontium ranelate drug treatment. *J Pharm Pharm Sci*. 2013; 16(1): 52-64.
12. Chen B, Li Y, Yang X et al. Zoledronic acid enhances bone-implant osseointegration more than alendronate and strontium ranelate in ovariectomized rats. *Osteoporos Int*. 2013; 24(7): 2115-2121.
13. Blake GM and Fogelman I. Strontium ranelate: a novel treatment for postmenopausal osteoporosis: a review of safety and efficacy. *Clin Interv Aging*. 2006; 1(4): 367-375.
14. Marie PJ. Strontium ranelate: a dual mode of action rebalancing bone turnover in favour of bone formation. *Curr Opin Rheumatol*. 2006; 18 Suppl 1: S11-5.
15. Ma B, Zhang Q, Wu D et al., Strontium fructose 1,6-diphosphate prevents bone loss in a rat model of postmenopausal osteoporosis via the OPG/RANKL/RANK pathway. *Acta Pharmacol Sin*. 2012; 33(4): 479-489.
16. Peng S, Liu XS, Huang S et al. Intervention timing of strontium treatment on estrogen depletion-induced osteoporosis in rats: bone microstructure and mechanics. *J Orthop Res*. 2014; 32(3): 477-484.
17. Meunier PJ, Roux C, Ortolani S et al. Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis. *Osteoporos Int*. 2009; 20(10): 1663-1673.
18. Reginster JY, Seeman E, De Vernejoul MC et al. Strontium ranelate reduces the risk of nonvertebral fractures in postmenopausal women with osteoporosis:

- Treatment of Peripheral Osteoporosis (TROPOS) study. *J Clin Endocrinol Metab.* 2005; 90(5): 2816-2822.
19. Kaufman JM, Audran M, Bianchi G et al. Efficacy and safety of strontium ranelate in the treatment of osteoporosis in men. *J Clin Endocrinol Metab.* 2013; 98(2): 592-601.
 20. Reginster JY. Efficacy and safety of strontium ranelate in the treatment of knee osteoarthritis: results of a double-blind randomised, placebo-controlled trial. *Ann Rheum Dis.* 2014; 73(2): e8.
 21. Zacchetti G, Dayer R, Rizzoli R et al. Systemic treatment with strontium ranelate accelerates the filling of a bone defect and improves the material level properties of the healing bone. *Biomed Res Int.* 2014; 2014: 549785.
 22. Buehler J, Chappuis P, Saffar JL et al., Strontium ranelate inhibits bone resorption while maintaining bone formation in alveolar bone in monkeys (*Macaca fascicularis*). *Bone.* 2001; 29(2): 176-179.
 23. Ammann P, Shen V, Robin B et al. Strontium ranelate improves bone resistance by increasing bone mass and improving architecture in intact female rats. *J Bone Miner Res.* 2004; 19(12): 2012-2020.
 24. Meunier PJ, Slosman DO, Delmas PD et al. Strontium ranelate: dose-dependent effects in established postmenopausal vertebral osteoporosis--a 2-year randomized placebo controlled trial. *J Clin Endocrinol Metab.* 2002; 87(5): 2060-2066.
 25. Morohashi T, Sano T, and Yamada S. Effects of strontium on calcium metabolism in rats. I. A distinction between the pharmacological and toxic doses. *Jpn J Pharmacol.* 1994; 64(3): 155-162.
 26. Reginster JY, Deroisy R, Dougados M et al. Prevention of early postmenopausal bone loss by strontium ranelate: the randomized, two-year, double-masked, dose-ranging, placebo-controlled PREVOS trial. *Osteoporos Int.* 2002; 13(12): 925-931.
 27. Scardueli CR, Bizelli-Silveira C, Marcantonio RAC et al. Systemic strontium to the osseointegration of titanium implants in animals: systematic review of the literature. *J Oral Implantol.* 2017. sent

28. Reinholt FP, Hjerpe A, Jansson K et al. Stereological studies on the epiphyseal growth plate in strontium-induced rickets. With special reference to the distribution of matrix vesicles. *J Bone Joint Surg Am.* 1984; 66(8): 1274-1280.
29. Ammann P. Strontium ranelate: a physiological approach for an improved bone quality. *Bone.* 2006; 38(2 Suppl 1): 15-18.
30. Lewiecki EM. Current and emerging pharmacologic therapies for the management of postmenopausal osteoporosis. *J Womens Health (Larchmt).* 2009; 18(10): 1615-1626.
31. Reid IR. Efficacy, effectiveness and side effects of medications used to prevent fractures. *J Intern Med.* 2015; 277(6): 690-706.
32. (MHRA)., M.a.H.P.R.A. Strontium ranelate (Protelos): risk of serious cardiac disorders-restricted indications, new contraindications, and warnings. *Drug Saf Update* 2013. 6(S1).
33. Fischer DC, Jensen C, Rahn A et al. Moderate strontium loading induces rickets in rats with mild chronic renal failure. *Kidney Blood Press Res.* 2011; 34(6): 375-381.
34. Almon RR, Yang E, Lai W et al. Circadian variations in rat liver gene expression: relationships to drug actions. *J Pharmacol Exp Ther.* 2008; 326(3): 700-716.
35. Pilz J, Meineke I, and Gleiter CH. Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *J Chromatogr B Biomed Sci Appl.* 2000; 742(2): 315-325.
36. Kohn HI and Liversedge M. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. *Pharmacol Exp Ther.* 1944; 82(3): 292-300.
37. Baratta JL, Ngo A, Lopez B et al. Cellular organization of normal mouse liver: a histological, quantitative immunocytochemical, and fine structural analysis. *Histochem Cell Biol.* 2009; 131(6): 713-726.
38. Mori DM, Baviera AM, de Oliveira Ramalho LT et al. Temporal response pattern of biochemical analytes in experimental diabetes. *Biotechnol Appl Biochem.* 2003; 38(Pt 2): 183-191.

39. Jonville-Bera AP and Autret-Leca E. [Adverse drug reactions of strontium ranelate(Protelos((R)) in France]. *Presse Med.* 2011; 40(10): e453-462.
40. Rizzoli R, Dawson-Hughes B, Kaufman JM et al. Correction of vitamin D insufficiency with combined strontium ranelate and vitamin D3 in osteoporotic patients. *Eur J Endocrinol.* 2014; 170(3): 441-450.
41. Cianferotti L, D'Asta F and Brandi ML. A review on strontium ranelate long-term antifracture efficacy in the treatment of postmenopausal osteoporosis. *Ther Adv Musculoskelet Dis.* 2013; 5(3): 127-139.
42. Li Y, Feng G, Gao Y et al. Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats. *J Orthop Res.* 2010; 28(5): 578-582.
43. Li Y, Li X, Song G et al. Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats. *Clin Oral Implants Res.* 2012; 23(9): 1038-1044.
44. Maimoun L, Brennan TC, Badoud I et al. Strontium ranelate improves implant osseointegration. *Bone*; 2010; 46(5): 1436-1441.
45. Deeks ED and Dhillon S. Strontium ranelate: a review of its use in the treatment of postmenopausal osteoporosis. *Drugs.* 2010; 70(6): 733-759.
46. Arcaro CA, Gutierrez VO, Assis RP et al. Piperine, a natural bioenhancer, nullifies the antidiabetic and antioxidant activities of curcumin in streptozotocin-diabetic rats. *PLoS One.* 2014; 9(12): e113993.
47. Gutierrez VO, Pinheiro CM, Assis RP et al. Curcumin-supplemented yoghurt improves physiological and biochemical markers of experimental diabetes. *Br J Nutr.* 2012; 108(3): 440-448.
48. Afshar S, Farshid AA, Heidari R et al. Histopathological changes in the liver and kidney tissues of Wistar albino rat exposed to fenitrothion. *Toxicol Ind Health.* 2008; 24(9): 581-586.
49. Gade TP, Motley MW, Beattie BJ et al. Imaging of alkaline phosphatase activity in bone tissue. *PLoS One.* 2011; 6(7): e22608.

50. Bain SD, Jerome C, Shen V et al. Strontium ranelate improves bone strength in ovariectomized rat by positively influencing bone resistance determinants. *Osteoporos Int.* 2009; 20(8): 1417-1428.
51. Vohra F, Al-Rifaiy MQ, Almas K et al. Efficacy of systemic bisphosphonate delivery on osseointegration of implants under osteoporotic conditions: lessons from animal studies. *Arch Oral Biol.* 2014; 59(9): 912-920.
52. Teng FY, Chen WC, Wang YI et al. Effects of Osseointegration by Bone Morphogenetic Protein-2 on Titanium Implants In Vitro and In Vivo. *Bioinorg Chem Appl.* 2016; 2016:3837679.
53. Marshall JK. The gastrointestinal tolerability and safety of oral bisphosphonates. *Expert Opin Drug Saf.* 2002; 1(1): 71-78.
54. James AW, LaChaud G, Shen J et al. A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Eng Part B Rev.* 2016; 22(4):284-297.
55. Zhao S, Wang X, Li N et al. Effects of strontium ranelate on bone formation in the mid-palatal suture after rapid maxillary expansion. *Drug Des Devel Ther.* 2015; 9: 2725-2734.

4 DISCUSSÃO

Diante dos objetivos deste projeto de pesquisa que foi avaliar as respostas teciduais da utilização sistêmica do estrôncio não radioativo em implantes osseointegráveis e defeitos ósseos críticos, os resultados se mostraram interessantes e puderam se complementar. Esses resultados demonstraram favoráveis a neoformação óssea, confirmando uma ação positiva dos suplementos a base de estrôncio. Suplementos esses que podem agir de forma conjunta nas células osteoblásticas e osteoclásticas, levando a um balanço a favor da formação óssea. Sendo assim, procedimentos que demandam de nova formação óssea podem ser beneficiados, acelerando o processo de reparo ou aumentando a quantidade de tecido ósseo.

Devido esse tema associado a procedimentos maxilo-faciais ser pouco abordado na literatura, uma revisão científica foi realizada de modo a comprovar e explorar as metodologias associadas ao uso do estrôncio frente a procedimento para instalação de implantes e enxertos ósseos. Na revisão de literatura, publicação 1, os poucos estudos encontrados mostraram-se pouco conclusivos, uma vez que diferentes metodologias associadas a diferentes públicos alvo, concentrações e posologias do estrôncio foram usadas, dificultando a comparação entre os mesmos. No entanto, mesmo com diferenças metodológicas existentes entre os estudos podemos notar uma ação positiva e moderada do estrôncio. Em virtude dessa pequena quantidade de trabalhos e a necessidade de uma conclusão mais fiel sobre o assunto, novos trabalhos foram desenvolvidos seguindo uma sequência. Para tal, estudos relacionados a osseointegração, defeitos ósseos críticos e toxicologia associado ao estrôncio foram realizados.

No trabalho que avaliou a osseointegração, os resultados se mostraram similares a alguns estudos encontrados na revisão de literatura. Nos dois estudos^{19,20} que utilizaram a mesma concentração do estrôncio para avaliar a osseointegração (625mg/kg/dia) os resultados foram bem próximos aos encontrados em nosso estudo, nos testes biomecânicos e microtomográficos. No entanto, no estudo de Li Y et al.²¹, o público-alvo foram ratas ovariectomizadas. Já quando diferentes dosagens foram utilizadas, como no estudo de Li et al 2010¹⁹ (500mg/kg/dia e 1000mg/kg/dia) e Chen et al.²² (500mg/kg/dia) os resultados também se mostraram positivos a favor da utilização do estrôncio. Diferentemente desses resultados, o estudo de Linderback et al.²³(800mg/kg/dia) e a utilização de um público-alvo de ratos machos mostrou efeitos

fracos ou inexistentes quando comparado ao grupo controle. Diante desses resultados fracos ou inexistentes poderíamos considerar a diferença entre públicos-alvo, porém um estudo em humanos no ano de 2013 pode comprovar que a atuação do estrôncio em pessoas do sexo masculino¹⁸, comprovando sua atuação ser independente do sexo. Acredita-se também que a dosagem excessiva pode influenciar negativamente os resultados, como mostrado por Morohashi et al.²⁴ e Storey E²⁵, e sendo assim o estudo de Li et al 2010¹⁹ que utilizou a dosagem de 1000mg/kg/dia não estaria de acordo com esta afirmação.

Diante dos resultados positivos encontrados em 4 dos 5 artigos da revisão frente a utilização do estrôncio, acreditamos que nosso trabalho associado a osseointegração com dosagens de 625mg/kg/dia está de acordo com a literatura até o momento, e que a dosagem aplicada deverá ser uma dosagem ideal e equivalente a 2g/day de ranelato de estrôncio em humanos. Alguns estudos também puderam comprovar que doses baixas envolvendo o estrôncio não apresentaram efeitos positivos²⁶⁻²⁹, como pudemos comprovar nas dosagens de 50mg/kg/dia com ranelato de estrôncio, e 30mg/kg/dia com carbonato e cloreto de estrôncio.

De acordo com esses resultados e a ausência de estudos com estrôncio associado a defeitos críticos, um novo estudo com metodologias similares foi realizado. Na análise histomorfométrica, imunohistoquímica e microtomográfica os resultados de um modo geral apresentaram-se favoráveis. Na avaliação histomorfométrica (publicação 3) maiores taxas de osso neoformado foram observadas nos grupos de altas dosagens de estrôncio (ranelato de estrôncio 625mg/kg/dia e carbonato e cloreto de estrôncio 365mg/kg/dia). Esses resultados estão de acordo com o estudo de Li et al.²¹ que avaliou a formação óssea ao redor de implantes e um recente estudo associado ao preenchimento de defeitos ósseos³⁰. Quando comparamos os resultados histomorfométricos da publicação 2 com a 3, os mesmos se apresentam como divergentes, uma vez que cortes histológicos únicos no centro da região implantar podem não representar a realidade em torno do implante. Na avaliação microtomográfica novamente os resultados com maiores dosagens se mostraram compatíveis aos estudos com implantes^{20,21}. Por fim, quando comparamos a estudos semelhantes para fechamento de defeitos ósseos, os dados estão de acordo com os poucos estudos existentes^{31,32}. Já na análise imunohistoquímica os resultados se mostraram favoráveis somente quando BMP-2 foi analisada. Porém nenhum estudo comparativo foi encontrado com BMP-2. Quando a osteocalcina foi

avaliada não foram encontradas diferenças significantes entre os grupos, semelhantemente a publicação 2. Diferentemente da publicação 2 e 3 no estudo de Li et al. 2012²¹ diferenças positivas foram encontradas no grupo ranelato de estrôncio 625mg/kg/dia quando comparado ao grupo controle.

Diante da possibilidade da indicação do estrôncio para novos estudos e procedimentos de cicatrização óssea seja sistemicamente ou localmente, e em virtude dos mais expressivos resultados estarem associados a maiores dosagens (ranelato de estrôncio 625mg/kg/dia e carbonato e cloreto de estrôncio 365mg/kg/dia), um novo estudo foi realizado afim de validar a ação positiva do estrôncio frente a toxicidade gerada nos animais.

Na avaliação de toxicidade, frente a órgãos envolvidos no processo de metabolização e excreção do estrôncio os resultados se mostraram complementares. Em uma análise dos rins, fígados e outros tecidos associados os resultados mostraram uma ausência de diferenças entre os grupos controle e estrôncio (ranelato de estrôncio 625mg/kg/dia ; carbonato e cloreto de estrôncio 365mg/kg/dia), com exceção para a avaliação da enzima fosfatase alcalina (ALP), que está associada a desordens no fígado e tecido ósseo³³. Diante das outras análises realizadas para a avaliação de toxicidade no fígado e ausência de indícios toxicológicos, levamos a crer que o aumento de ALP está associado a um aumento da atividade óssea³⁴. Estes resultados além de mostrarem a ausência de danos teciduais frente a administração de estrôncio durante curtos períodos também pode vir a mostrar sua atividade no tecido ósseo, como mostrado nas publicações 2 e 3. Esses resultados estão de acordo Meunier et al.³⁵, que utilizou estrôncio para avaliar o risco de fraturas associadas a menopausa em humanos, e Bain et al.³⁶ em ratos.

Em virtude dos resultados apresentados acima, e a ausência maiores estudos, acreditamos que não somente a administração sistêmica poderia vir a beneficiar a cicatrização óssea, e sim uma aplicabilidade local também poderia melhorar a cicatrização óssea. Para isso a incorporação de estrôncio a veículos poderia vir a trazer inúmeros benefícios em procedimentos médicos e odontológicos para cicatrização óssea. Essa idéia não seria novidade uma vez que diversos estudos com estrôncio e biomateriais^{37,38} ou sobre superfícies de implantes^{39,40} tem sido realizados, no entanto, esse trabalho comprova que a utilização em dosagens ideais traz grandes benefícios sem danos toxicológicos, sendo um estudo base para as diferentes indicações e aplicabilidades. Além da atuação em tecidos ósseos, novos estudos

também têm mostrado que o estrôncio pode atuar na proliferação do ligamento periodontal^{41,42}, melhorando o reparo, e sendo um importante elemento para tratamento de doenças periodontais.

5 CONCLUSÃO

Estes estudos mostraram que doses ideais de estrôncio podem vir a melhorar o reparo tecidual ósseo ao redor de implantes osseointegráveis e defeitos críticos ósseos, sem danos teciduais em órgãos envolvidos na metabolização e excreção. Novos estudos podem comprovar a indicação do estrôncio em diferentes formas e outras finalidades.

REFERÊNCIAS*

1. Apostu D, Lucaciu O, Lucaciu GDO, Crisan B, Crisan L, Baciut M, et al. Systemic drugs that influence titanium implant osseointegration. *Drug Metab Rev.* 2017;49(1):92–104.
2. Dikicier E, Karaçaylı Ü, Dikicier S, Günaydin Y. Effect of systemic administered zoledronic acid on osseointegration of a titanium implant in ovariectomized rats. *J Cranio-Maxillofacial Surg.* 2014;42(7):1106–11.
3. Kellesarian S V., Abduljabbar T, Vohra F, Malignaggi VR, Malmstrom H, Romanos GE, et al. Role of local alendronate delivery on the osseointegration of implants: a systematic review and meta-analysis. *Int J Oral Maxillofac Surg.* 2017;46(7):912–21.
4. Song W, Seta J, Chen L, Bergum C, Zhou Z, Kanneganti P, et al. Doxycycline-loaded coaxial nanofiber coating of titanium implants enhances osseointegration and inhibits *Staphylococcus aureus* infection. *Biomed Mater.* 2017;12(4):045008. doi: 10.1088/1748-605X/aa6a26.
5. Altundag O, Altundag K, Selim Silay Y, Gunduz M, Demircan K, Gullu I. Calcium and vitamin D supplementation during bisphosphonate administration may increase osteoclastic activity in patients with bone metastasis. *Med Hypotheses.* 2004;63(6):1010–3.
6. Kim H-C, Song J-M, Kim C-J, Yoon S-Y, Kim I-R, Park B-S, et al. Combined effect of bisphosphonate and recombinant human bone morphogenetic protein 2 on bone healing of rat calvarial defects. *Maxillofac Plast Reconstr Surg.* 2015;37(1):16. doi: 10.1186/s40902-015-0015-3.
7. Yarom N, Goss A, Lazarovici TS, Elad S. Bisphosphonate-Related Osteonecrosis of the Jaw. *J Am Dent Assoc.* 2016;147(10):776–7.
8. Vestergaard P. Occurrence of Gastrointestinal Cancer in Users of Bisphosphonates and Other Antiresorptive Drugs Against Osteoporosis. *Calcif Tissue Int.* 2011;89(6):434–41.
9. Blake GM, Fogelman I. Strontium ranelate: a novel treatment for postmenopausal osteoporosis: a review of safety and efficacy. *Clin Interv Aging.* 2006;1(4):367–75.
10. Meunier PJ, Roux C, Ortolani S, Diaz-Curiel M, Compston J, Marquis P, et al. Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis. *Osteoporos Int.* 2009;20(10):1663–73.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

11. Chattopadhyay N, Quinn SJ, Kifor O, Ye C, Brown EM. The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation. *Biochem Pharmacol.* 2007;74(3):438–47.
12. Fromigué O, Haÿ E, Barbara A, Petrel C, Traiffort E, Ruat M, et al. Calcium sensing receptor-dependent and receptor-independent activation of osteoblast replication and survival by strontium ranelate. *J Cell Mol Med.* 2009;13(8 B):2189–99.
13. Marie PJ. Strontium ranelate: New insights into its dual mode of action. *Bone.* 2007;40(5):S5–8.
14. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev.* 1999;20(3):345–57.
15. Deeks ED, Dhillon S. Spotlight on strontium ranelate in postmenopausal osteoporosis. *Drugs and Aging.* 2010;27(9):771–3.
16. Reginster J-Y, Deroisy R, Neuprez A, Hiligsmann M, Zegels B, Bruyere O. Strontium ranelate: new data on fracture prevention and mechanisms of action. *Curr Osteoporos Rep.* 2009 Sep;7(3):96–102.
17. Kaufman J-M, Audran M, Bianchi G, Braga V, Diaz-Curiel M, Francis RM, et al. Efficacy and safety of strontium ranelate in the treatment of osteoporosis in men. *J Clin Endocrinol Metab.* 2013;98(2):592–601.
18. Reginster J-Y, Beaudart C, Neuprez A, Bruyère O. Strontium ranelate in the treatment of knee osteoarthritis: new insights and emerging clinical evidence. *Ther Adv Musculoskelet Dis.* 2013;5(5):268–76.
19. Li Y, Feng G, Gao Y, Luo E, Liu X, Hu J. Strontium ranelate treatment enhances hydroxyapatite-coated titanium screws fixation in osteoporotic rats. *J Orthop Res.* 2010;28(5):578–82.
20. Maïmoun L, Brennan TC, Badoud I, Dubois-Ferriere V, Rizzoli R, Ammann P. Strontium ranelate improves implant osseointegration. *Bone.* 2010;46(5):1436–41.
21. Li Y, Li X, Song G, Chen K, Yin G, Hu J. Effects of strontium ranelate on osseointegration of titanium implant in osteoporotic rats. *Clin Oral Implants Res.* 2012;23(9):1038–44.
22. Chen B, Li Y, Yang X, Xu H, Xie D. Zoledronic acid enhances bone-implant osseointegration more than alendronate and strontium ranelate in ovariectomized rats. *Osteoporos Int.* 2013;24(7):2115–21.
23. Linderbäck P, Agholme F, Wermelin K, Närhi T, Tengvall P, Aspenberg P. Weak effect of strontium on early implant fixation in rat tibia. *Bone.* 2012;50(1):350–6.

24. Morohashi T, Sano T, Yamada S. Effects of Strontium on Calcium Metabolism in Rats. I. A Distinction between the Pharmacological and Toxic Doses. *Jpn J Pharmacol.* 1994;64(3):155–62.
25. Storey E. Intermittent bone changes and multiple cartilage defects in chronic strontium rickets in rats. *J Bone Joint Surg Br.* 1962;44B:194–208.
26. Grynpas MD, Hamilton E, Cheung R, Tsouderos Y, Deloffre P, Hott M, et al. Strontium increases vertebral bone volume in rats at a low dose that does not induce detectable mineralization defect. *Bone.* 1996;18(3):253–9.
27. Grynpas MD, Marie PJ. Effects of low doses of strontium on bone quality and quantity in rats. *Bone.* 1990;11(5):313–9.
28. Buehler J, Chappuis P, Saffar JL, Tsouderos Y, Vignery A. Strontium ranelate inhibits bone resorption while maintaining bone formation in alveolar bone in monkeys (*Macaca fascicularis*). *Bone.* 2001;29(2):176–9.
29. Marie PJ, Garba MT, Hott M, Miravet L. Effect of low doses of stable strontium on bone metabolism in rats. *Min Electrolyte Metab.* 1985;11(1):5–13.
30. Lavet C, Mabilieu G, Chappard D, Rizzoli R, Ammann P. Strontium ranelate stimulates trabecular bone formation in a rat tibial bone defect healing process. *Osteoporos Int.* 2017;28(12):3475–87.
31. Zacchetti G, Dayer R, Rizzoli R, Ammann P. Systemic Treatment with Strontium Ranelate Accelerates the Filling of a Bone Defect and Improves the Material Level Properties of the Healing Bone. *Biomed Res Int.* 2014;2014:549785. doi: 10.1155/2014/549785.
32. Komrakova M, Weidemann A, Dullin C, Ebert J, Tezval M, Stuermer KM, et al. The Impact of Strontium Ranelate on Metaphyseal Bone Healing in Ovariectomized Rats. *Calcif Tissue Int.* 2015;97(4):391–401.
33. Mori DM, Baviera AM, de Oliveira Ramalho LT, Vendramini RC, Brunetti IL, Pepato MT. Temporal response pattern of biochemical analytes in experimental diabetes. *Biotechnol Appl Biochem.* 2003;38(2):183–91.
34. Gade TP, Motley MW, Beattie BJ, Bhakta R, Boskey AL, Koutcher JA, et al. Imaging of alkaline phosphatase activity in bone tissue. *PLoS One.* 2011;6(7):e22608. doi: 10.1371/journal.pone.0022608.
35. Meunier PJ, Roux C, Ortolani S, Diaz-Curiel M, Compston J, Marquis P, et al. Effects of long-term strontium ranelate treatment on vertebral fracture risk in postmenopausal women with osteoporosis. *Osteoporos Int.* 2009;20(10):1663–73.
36. Bain SD, Jerome C, Shen V, Dupin-Roger I, Ammann P. Strontium ranelate improves bone strength in ovariectomized rat by positively influencing bone resistance determinants. *Osteoporos Int.* 2009;20(8):1417–28.

37. Silva GAB, Bertassoli BM, Sousa CA, Albergaria JD, de Paula RS, Jorge EC. Effects of strontium ranelate treatment on osteoblasts cultivated onto scaffolds of trabeculae bovine bone. *J Bone Miner Metab*. 2018;36(1):73–86.
38. Wei L, Ke J, Prasad I, Miron RJ, Lin S, Xiao Y, et al. A comparative study of Sr-incorporated mesoporous bioactive glass scaffolds for regeneration of osteopenic bone defects. *Osteoporos Int*. 2014;25(8):2089–96.
39. Querido W, Farina M, Anselme K. Strontium ranelate improves the interaction of osteoblastic cells with titanium substrates: Increase in cell proliferation, differentiation and matrix mineralization. *Biomater*. 2015;5:e1027847. doi: 10.1080/21592535.2015.1027847.
40. Tian A, Zhai J, Peng Y, Zhang L, Teng M, Liao J, et al. Osteoblast Response to Titanium Surfaces Coated with Strontium Ranelate–Loaded Chitosan Film. *Int J Oral Maxillofac Implants*. 2014;29(6):1446–53.
41. Wu C, Zhou Y, Lin C, Chang J, Xiao Y. Strontium-containing mesoporous bioactive glass scaffolds with improved. *Acta Biomater*. 2012;8(10):3805–15.
42. Römer P, Desaga B, Proff P, Faltermeier A, Reicheneder C. Strontium promotes cell proliferation and suppresses IL-6 expression in human PDL cells. *Ann Anat*. 2012;194(2):208–11.

APÊNDICE A – METODOLOGIA DETALHADA DA PUBLICAÇÃO 2

Amostra

Neste estudo foram utilizados 70 ratos *Rattus Norvegicus*, variação *albinus*, *Holtzman*, machos, adultos, de aproximadamente 3 meses de idade, com massa corporal entre 300 e 350 gramas, mantidos no Biotério da Faculdade de Odontologia da UNESP de Araraquara (FOAr-UNESP). Os animais foram mantidos em ambiente com luz e temperatura controladas e alimentados com ração sólida e com acesso a água ad libitum antes e durante todo o período experimental.

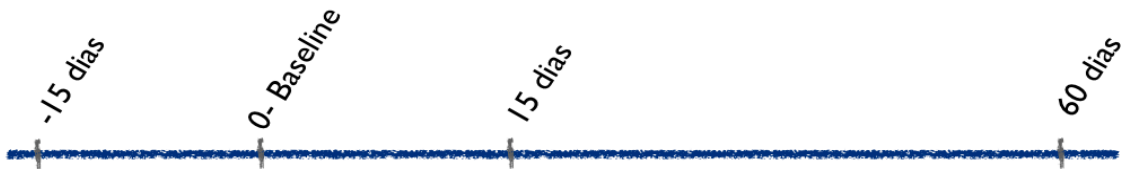
Grupos Experimentais

Os animais foram divididos aleatoriamente em 5 grupos com 7 animais em cada grupo de acordo com o medicamento e dosagem:

- **Grupo C:** administração diária de 1 ml de soro fisiológico
- **Grupo SRAN50:** administração diária de 50 mg/kg/dia de Ranelato de Estrôncio (Protos®, Laboratórios Servier do Brasil)
- **Grupo SRAN625:** administração diária de 625 mg/kg/dia de Ranelato de Estrôncio (Protos®, Laboratórios Servier do Brasil).
- **Grupo SCAR/SCHL30:** administração diária de 30 mg/kg/dia da combinação entre 50% de Carbonato de Estrôncio e 50% de Cloreto de Estrôncio (manipulado por Cromoline Química Fina, Diadema- São Paulo).
- **Grupo SCAR/SCHL365:** administração diária de 365 mg/kg/dia da combinação de 50% de Carbonato de Estrôncio e 50% Cloreto de Estrôncio (manipulado por Cromoline Química Fina, Diadema- São Paulo).

Os medicamentos foram administrados 15 dias antes do procedimento cirúrgico persistindo até o final do experimento (15 ou 60 dias).

Figura 1 – Linha do tempo



Fonte: Elaboração própria.

-15 dias: Início da administração dos medicamentos.

0- Baseline: Procedimento cirúrgico de instalação dos implantes osseointegráveis

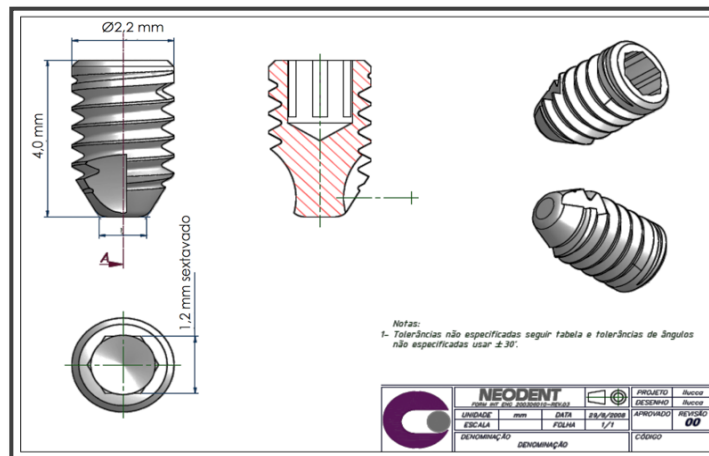
15 dias: Eutanásia dos animais

60 dias: Eutanásia dos animais

Especificação dos implantes

Foram utilizados implantes especialmente desenvolvidos para o projeto, confeccionados pela empresa *Neodent*[®] (Curitiba-PR- Brasil), com superfície tratada por ataque ácido e diâmetros de 4 mm de comprimento por 2.2 mm de espessura e formato especialmente desenhados para a instalação na tíbia de ratos (figura 1).

Figura 2 - Desenho esquemático do tamanho e configuração tridimensional do implante



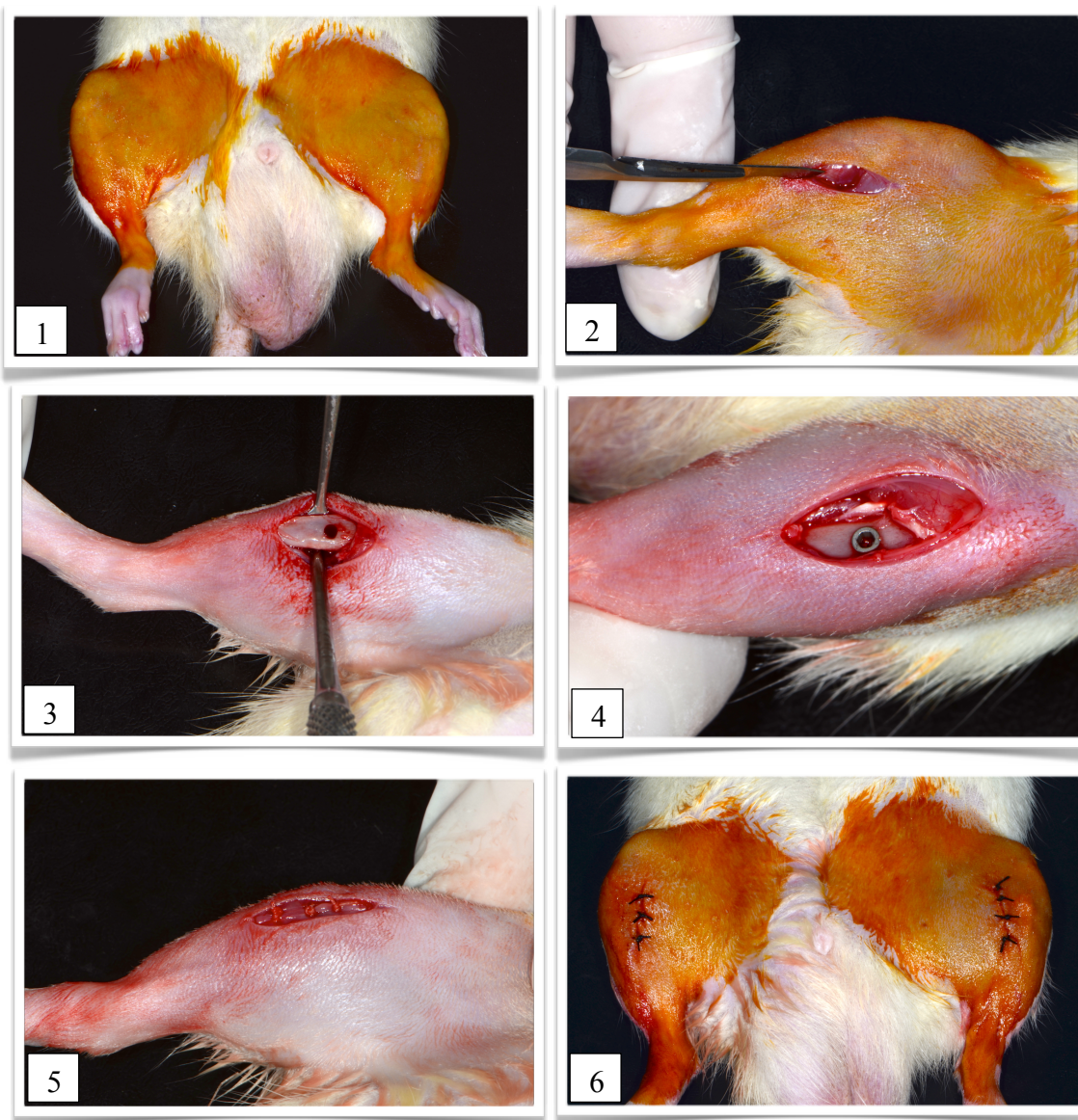
Fonte: Neodent[®].

Cirurgia para instalação dos implantes

Os animais foram anestesiados por uma combinação de Quetamina com Xilazina, na proporção de 0,08 ml/100g de massa corporal (Cloridrato de Quetamina 10% – Francotar – Virbac do Brasil Ind. Com. Ltda.) e 0,04 ml/100g massa corporal (Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda.), respectivamente.

Posteriormente foram submetidos à tricotomia da região interna das patas direita e esquerda e realizada a antisepsia com gaze estéril embebida em solução de povidine 10%(Figura 2(1)). Uma incisão de aproximadamente 10 mm foi realizada em planos sobre a metáfise tibial (Figura 2(2)). Após uma dissecação delicada o tecido ósseo foi submetido à osteotomia, realizada por meio de uma sequência progressiva de fresas (fresa lança KG Implant 1,4 – KG Sorensen – Medical Burs Ind. e Com. de Pontas e Brocas Cirúrgicas Ltda; fresa espiral de 2.0 mm – *Neodent*®- Curitiba- PR- Brasil) para instalação do implante de titânio (Figura 2 (3)). Todas as perfurações foram realizadas com motor elétrico (BLM 600 – Driller), ajustado a 1200 rpm, sob abundante irrigação com solução salina estéril. O implante foi instalado (Figura 2(4)) com a ajuda de uma chave hexagonal de 1,2 mm (*Neodent*®- Curitiba- PR- Brasil) acoplada ao contra ângulo (Mont Blanc Anthogyr, São Paulo- Brasil). O ferimento foi suturado em planos, internamente com fio reabsorvível Vicryl Ethicon 4.0 (Ethicon, Johnson & Johnson, Brasil) (Figura 2(5)) e externamente com fio de seda Ethicon 4.0 (Ethicon, Johnson & Johnson, Brasil) (Figura 2(6)). Os animais receberam, em dose única, penicilina associada à estreptomicina (Pentabiótico pequeno porte, FortDodge, Brasil) na dosagem 0,1 ml/kg de peso e 5mg/kg de dipirona sódica intramuscular (Febrax, Lema Injex Biologic, Brasil).

Figura 3 - Protocolo cirúrgico para instalação dos implantes tibiais



1) Tricotomia e antissepsia da face interna da tíbia; 2) Incisão e descolamento para exposição do tecido ósseo receptor do implante; 3) Cavidade óssea preparada; 4) Posição final do implante na tíbia 5) Tecido muscular suturado internamente com fio 4.0 Vicryl; 6) Tecido epitelial suturado com fio de seda 4.0. Ao total foram instalados 140 implantes, sendo 1 em cada metáfise tibial.

Fonte: arquivo pessoal do autor.

Obtenção das peças cirúrgicas

Após os períodos experimentais propostos (15 e 60 dias) os animais foram eutanaziados com sobredose anestésica e as tíbias removidas. As tíbias direitas (70 implantes) foram utilizadas para a avaliação biomecânica (torque de remoção) e imunohistoquímica, e as tíbias esquerdas (70 implantes) foram fixadas em paraformaldeído a 4% por 48 horas, lavadas em água corrente por 24 horas e armazenadas em álcool 70%. Estas peças foram avaliadas através de análise radiográfica tridimensional (Micro-CT) e análise histomorfométrica.

Avaliação biomecânica (torque de remoção)

As tíbias removidas foram estabilizadas em uma pequena morsa. Uma chave hexagonal foi conectada no implante e no torquímetro (Tohnichi ATG24CN-S, Japan), com escala graduada de 0.05 N/cm, com possibilidade de aferir a força no intervalo de 1 a 30 N/cm. Foi realizado um movimento anti-horário com o objetivo de desrosquear o implante. O valor máximo necessário para movimentar o implante foi anotado como o valor do torque de remoção. As avaliações foram realizadas por um examinador treinado e cego para os grupos experimentais.

Avaliação radiográfica tridimensional (Micro-Ct)

As amostras armazenadas em álcool 70%, foram primeiramente submetidas a análise por varredura de feixe de raios-X em um sistema de microtomografia digital computadorizada. As peças foram escaneadas pelo microtomógrafo SkyScan (SkyScan 1176 Bruker MicroCT, Aatselaar, Bélgica, 2003) utilizando cortes de 9 µm de espessura (50Kv e 500µ), com filtro de cobre e alumínio e passo de rotação de 0.3 mm. As imagens obtidas pela projeção dos raios-X nas amostras foram armazenadas e reconstituídas determinando a área de interesse pelo software NRecon (SkyScan, 2011; Versão 1.6.6.0). No software Data Viewer (SkyScan, Versão 1.4.4 64-bit) (figura 14-B) as imagens foram reconstruídas para adequação do posicionamento padrão para todas as amostras, podendo ser observada em três planos (transversal, longitudinal e sagital). Em seguida, utilizando o software CTAnalyser – CTAn (2003-11SkyScan, 2012 Bruker MicroCT Versão 1.12.4.0) foi definida uma área ao redor do implante (ROI) delimitada por 0,5 mm em torno de todo o implante. Esta área ficou definida como Área Total (0,5mm de margem ao redor dos implantes- ROI 4,5mm x 3,2mm) (figura 3A e 3B). O software CTAn analisa e mede a imagem de acordo com

a escalas de cinza (*threshold*). O *threshold* utilizado na análise foi de 25-90 tons de cinza, que possibilitou obter o volume de osso formado ao redor dos implantes. Como os implantes osseointegrados colocados não receberam o *Cover Screw* (vedante) em alguns casos houve a formação óssea dentro da plataforma protética. Para que esta formação óssea não interferisse com a análise da osseointegração foi definido um segundo ROI que compreendeu a região da plataforma protética (Área da Plataforma) (figura 4A e 4B). Com os resultados obtidos nos dois ROIs, foi possível definir a Área de formação óssea utilizando a fórmula:

$$\text{Área Total} - \text{Área Plataforma} = \text{Área de formação óssea}$$

Figura 4 - A) Imagem ilustrando a definição do ROI 4,5mm x 3,2mm; B) Área Total delimitada pelo ROI (regiões brancas correspondentes ao tecido ósseo neoformado)



Figura 4A

Fonte: Elaboração própria.

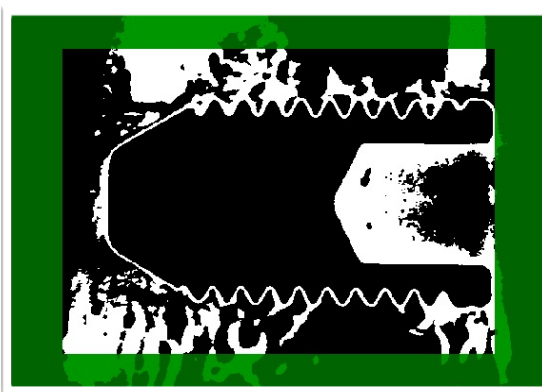


Figura 4B

Figura 5 - A) Área Plataforma (ROI); B) Área Plataforma delimitada pelo ROI (regiões brancas correspondentes ao tecido ósseo neoformado na região da plataforma)

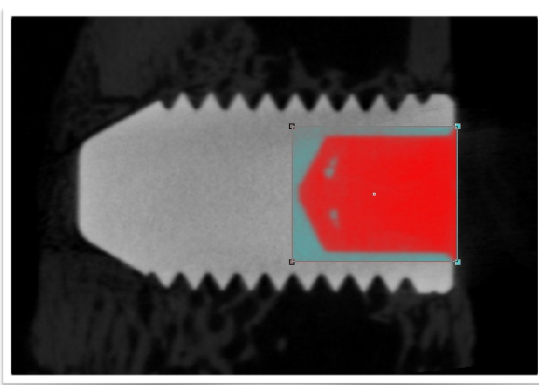


Figura 5A

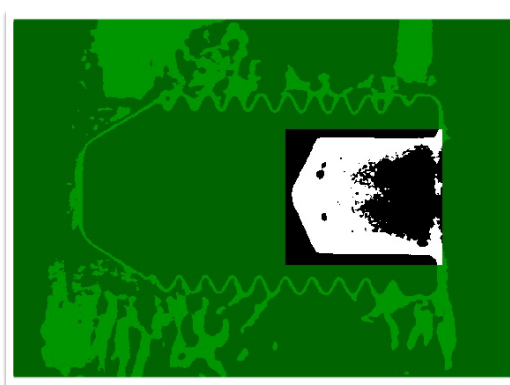


Figura 5B

Fonte: Elaboração própria.

O software CTAn, a partir da delimitação da região de interesse em 2D, promove a análise em 3D da mesma pela soma coletiva de todos os “ROI”s de um conjunto contíguo de fatias de imagens transversais. Deste modo foi obtido o percentual de volume do tecido ósseo presente nas áreas de interesse previamente delimitadas (ROIs). Os dados obtidos foram anotados em % de volume ao redor dos implantes.

Avaliação histomorfométrica

Após o processo de fixação, posterior lavagem com água, armazenamento em álcool 70% e escaneamento, o espécime foi desidratado progressivamente em soluções de álcool etílico (80%, 90% e absoluto). A infiltração plástica foi realizada com misturas de glicolmetacrilato (Technovit 7200 VLC) e álcool etílico, seguindo variações gradativas, finalizando com duas infiltrações de glicolmetacrilato puro, sob agitação constante. Após a infiltração plástica, os espécimes foram incluídos em resina e polimerizados. Os blocos foram montados em lâmina acrílica com o auxílio da resina Tecnovit 4000 (Kulzer, Wehrheim, Alemanha). Utilizando um sistema de corte e microdesgaste (Exact-Cutting, System, Apparatebau GmbH, Hamburgo, Alemanha) as lâminas foram processadas até a espessura aproximada de 70 μm . As lâminas foram coradas com Stevenel’s Blue. Para a avaliação do padrão de osseointegração foi observado o percentual de osseointegração-BIC (contato osso/implante); e de formação óssea sobre a superfície- BA (área óssea ao redor da superfície da rosca). As análises foram feitas nas 3 primeiras roscas de cada implante. As mensurações foram realizadas utilizando-se um microscópio de luz convencional DIASTAR (Leica Reichert & Jung products, Alemanha), com objetiva para aumento de 4.0/10 vezes. As imagens foram captadas e enviadas para um microcomputador, com o auxílio de uma câmera Leica Microsystems DFC-300-FX (Leica Microsystems, Alemanha), com resolução de 1.3 megapíxels, e salvas sob a extensão TIFF. A determinação dos valores foi feita utilizando um software analisador de imagens (ImageJ 1.45s, NIH, EUA). Os valores para a extensão linear de contato osso/implante e para a área óssea foram obtidos em μm e μm^2 , respectivamente. As avaliações foram realizadas por um examinador treinado, calibrado e cego para os grupos experimentais.

Avaliação Imunohistoquímica

As amostras que receberam o teste biomecânico também foram destinadas a análise imunohistoquímica. Foi empregado o método de imunohistoquímica para detecção e localização da expressão das proteínas ligadas à remodelação óssea: BMP-2 (proteína morfogenética óssea) e OCN (osteocalcina). Cortes histológicos com 4 a 5 µm de espessura foram montados em lâminas silanizadas (Fisher Superfrost plus), seguido de tramitação laboratorial de rotina para desparafinização e reidratação. Em seguida, os cortes foram tratados pelo método do complexo avidina-biotina-peroxidase (ABC) com a utilização do kit ABC Staining System (sc-2023, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) segundo as instruções do fabricante. Quando necessário, a recuperação do antígeno foi realizada pela fervura dos cortes em solução tampão apropriada (Dako; Glostrup, Denmark), em uma câmara de pressão (Biocare Medical; Concord, CA) por 20 min e resfriadas à temperatura ambiente.

Anticorpos primários de rato para as proteínas avaliadas tiveram sua diluição otimizada individualmente. Como controle negativo, cortes histológicos foram tratados por IgG normal (R&D Systems), em substituição aos anticorpos primários, nas mesmas diluições destes. Os cortes serão corados com solução de hematoxilina para visualização dos núcleos. A intensidade e localização da coloração foi realizada ao redor das 2-3 primeiras rocas com o uso de microscópio óptico (DIASTAR, Leica). A intensidade de proteínas marcadas foi avaliada por um examinador cego para os grupos experimentais de acordo com a escala: negativo(-), positivo(+), superpositivo(++), e hiperpositivo(+++).

APÊNDICE B – METODOLOGIA DETALHADA DA PUBLICAÇÃO 3

Amostras

Neste estudo foram utilizados 70 ratos *Rattus Norvegicus*, variação *albinus*, *Holtzman*, machos, adultos, de aproximadamente 3 meses de idade, com massa corporal entre 300 e 350 gramas, mantidos no Biotério da Faculdade de Odontologia da UNESP de Araraquara (FOAr-UNESP). Os animais foram mantidos em ambiente com luz e temperatura controladas e alimentados com ração sólida e com acesso a água ad libitum antes e durante todo o período experimental.

Grupos Experimentais

Os animais foram selecionados aleatoriamente e divididos em 5 grupos e que foram avaliados em dois períodos experimentais (15 e 60 dias), com 7 animais em cada grupo. Os grupos foram divididos de acordo com o uso ou não do medicamento que foi fornecido aos animais, e ainda de acordo com sua dosagem, calculada de tal modo a mimetizar a quantidade de Estrôncio utilizada em terapias atuais em humanos, e também para seguir as dosagens utilizadas em pesquisas com animais, geralmente mais altas:

- Grupo C: administração diária de 1 ml de soro fisiológico
- Grupo SRAN50: administração diária de 50 mg/kg/dia de Ranelato de Estrôncio (Protos®, Laboratórios Servier do Brasil)
- Grupo SRAN625: administração diária de 625 mg/kg/dia de Ranelato de Estrôncio (Protos®, Laboratórios Servier do Brasil).
- Grupo SCAR/SCHL30: administração diária de 30 mg/kg/dia da combinação entre 50% de Carbonato de Estrôncio e 50% de Cloreto de Estrôncio (manipulado por Cromoline Química Fina, Diadema- São Paulo).
- Grupo SCAR/SCHL365: administração diária de 365 mg/kg/dia da combinação de 50% de Carbonato de Estrôncio e 50% Cloreto de Estrôncio (manipulado por Cromoline Química Fina, Diadema- São Paulo).

No total, foram confeccionados 70 defeitos ósseos, de acordo com a tabela a seguir:

Tabela 1 - Número de animais/defeitos segundo período (dias), de acordo com grupos

	15	60	Total
Grupo C	7	7	14
Grupo SRAN50	7	7	14
Grupo SRAN625	7	7	14
Grupo SCAR/SCHL30	7	7	14
Grupo SCAR/SCHL365	7	7	14
Total	35	35	70

Fonte: Elaboração própria.

Similarmente a metodologia anterior, os medicamentos foram administrados 15 dias antes do procedimento cirúrgico persistindo até o final do experimento (15 ou 60 dias).

Procedimento Cirúrgico

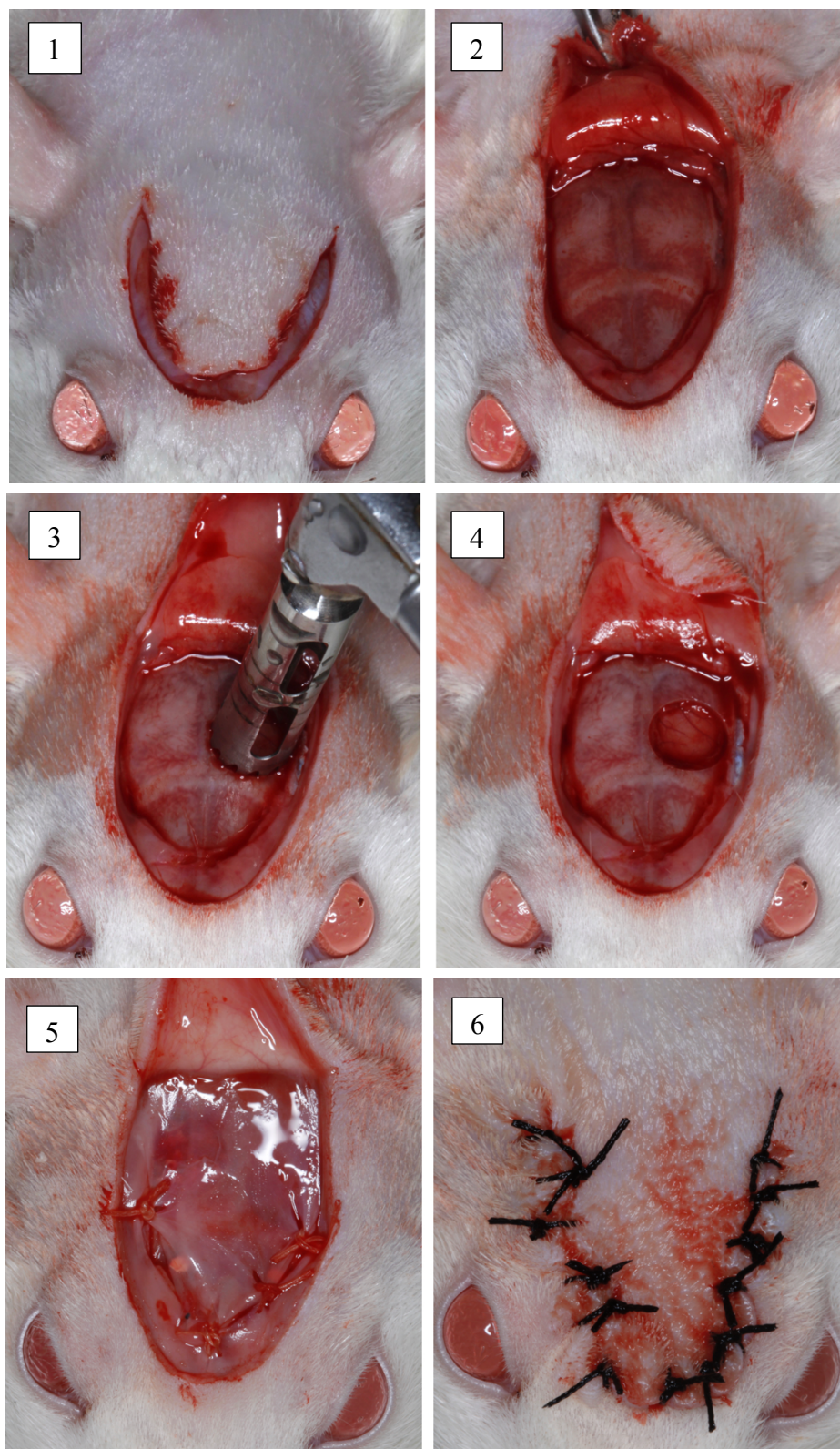
Os animais foram anestesiados por uma combinação de Quetamina com Xilazina, na proporção de 0,08 ml/100g de massa corporal (Cloridrato de Quetamina – Francotar – Virbac do Brasil Ind. Com. Ltda.) e 0,04 ml/100g massa corporal (Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda.), respectivamente. Posteriormente, os animais foram submetidos à tricotomia na região da calvária e será realizada antisepsia do campo cirúrgico com gaze estéril, embebida em solução de povidine, com o animal sendo então posicionado em decúbito ventral sobre a mesa cirúrgica.

Foi então utilizada a técnica cirúrgica descrita por Chesmel e colaboradores, na qual o acesso cirúrgico à porção posterior da calvária foi obtido com uma incisão bicoronal cutânea e muscular, com aproximadamente 3 cm de extensão (Figura 1(1)), permitindo a exposição do tecido ósseo, utilizando-se cabo para bisturi com lâmina intercambiável nº 15.

Em seguida, os tecidos foram divulsionados com o auxílio de uma tesoura pequena com a extremidade romba e uma pinça de dissecação, até a exposição do periósteo, sendo este incisado e removido com o mesmo tipo de lâmina até a exposição do tecido ósseo (Figura 1(2)). Foram confeccionados defeitos ósseos circulares, na porção lateral da calvária, desviando da sutura craniana do animal, com 5 mm de diâmetro e aproximadamente 1,5 mm de espessura, dada pela remoção total

do tecido ósseo, utilizando uma fresa trefina (Dentoflex, São Paulo, Brasil) com 5 mm de diâmetro externo(Figura 1(3)), montada em contra-ângulo (Anthogyr – Injecta – Diadema, Brasil) com redução de 16:1, acoplado em motor para implante (BML 600 Plus Driller - CK Driller – Brasil), com 1500 rpm, sob irrigação constante com solução salina. Todos os cuidados foram mantidos para evitar qualquer lesão à dura-máter. Após a confecção do defeito (Figura 1(4)) e preenchimento com coágulo, os tecidos serão suturados em planos, internamente com fio reabsorvível Vicryl Ethicon 4.0 (Ethicon, Johnson & Johnson, Brasil) (Figura 1(5)) e externamente com fio de seda Ethicon 4.0 (Ethicon, Johnson & Johnson, Brasil). Os animais receberam, em dose única, penicilina associada à estreptomicina na dosagem 0,1 ml/kg e paracetamol 50 ml/100g de peso.

Figura 1 - Protocolo cirúrgico para criação de defeitos em calvárias



1) Tricotomia e incisão; 2) Incisão e descolamento para exposição do tecido ósseo; 3) Trefina 5mm posicionada sobre a região e acoplada no contra-ângulo; 4) Defeito ósseo; 5) Perióstio suturado com fio reabsorvível 4.0 Vicryl; 6) Tecido epitelial suturado com fio de seda 4.0.
Fonte: Arquivo pessoal do autor.

Obtenção das peças cirúrgicas

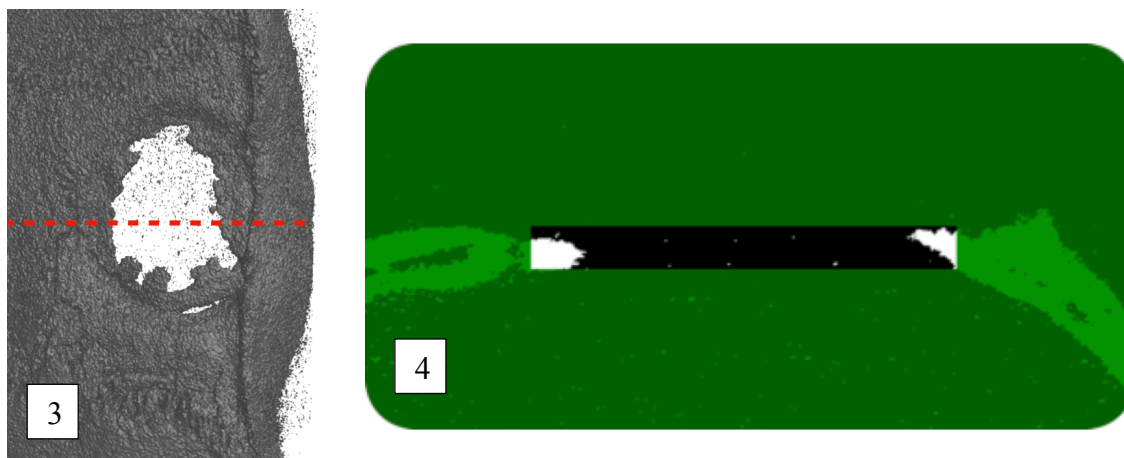
Foi realizada uma incisão bicoronal no animal, removendo toda a porção superior da calvária (em bloco), incluindo tecidos moles e duros, utilizando tesouras e alicate de corte. As biópsias obtidas foram identificadas e fixadas em paraformaldeído a 4%, aproximadamente 48 horas, para manutenção das estruturas que se deseja avaliar. As peças foram então mantidas em álcool até o escaneamento tomográfico, e após descalcificadas em solução de EDTA a 20%, trocada a cada 48 horas, durante 60 dias. Constatada a correta descalcificação das peças, as mesmas foram divididas com base no centro do defeito, sendo que a partir deste momento uma das metades foi utilizada para avaliação histomorfométrica, e a outra seguiu para a avaliação imunohistoquímica.

Avaliação radiográfica tridimensional (Micro-Ct)

Os animais de cada grupo foram avaliados quanto a formação óssea através do micro-CT no dia da cirurgia, 15 e 60 dias após cirurgia. Estes foram posicionados em posição de decúbito dorsal e tiveram suas calvárias escaneadas através do aparelho Skyscan (SkyScan 1176, Kontich, Bélgica), em cortes de 18 μ m, passo de rotação 0.4mm, uso de filtro de cobre-alumínio, voltagem de 80 kV e 300 μ A de intensidade. As imagens foram reconstruídas usando software específico (NRecon, SkyScan, Aatselaar, Belgium), com -1000 a 2300 valores de Hounsfield, sendo esse valor determinado por análises prévias no mesmo software, e nenhum ajuste adicional foi realizado. Após a reconstrução, as imagens foram posicionadas usando software data viewer software (SkyScan, Aatselaar, Belgium). Por último, com o uso do software CTAn (CTAnalyser, SkyScan, Aatselaar, Belgium), uma imagem mais central do defeito foi escolhida (final-inicial) e um ROI retangular foi aplicado (5,0 x 0,5mm).

De acordo com mensurações prévias, e uma visualização observacional, o threshold foi escolhido inicialmente como 45-165. No entanto, para alcançar um adequado threshold, mais 4 thresholds foram usados (35-155; 40-160; 50-170; 55-175). E por último um novo threshold foi aplicado, com o valor mínimo e máximo dos valores dos thresholds usados (35-175).

Figura 2 - Imagem 3D e 2D esquemático do corte central analisado na avaliação microtomográfica



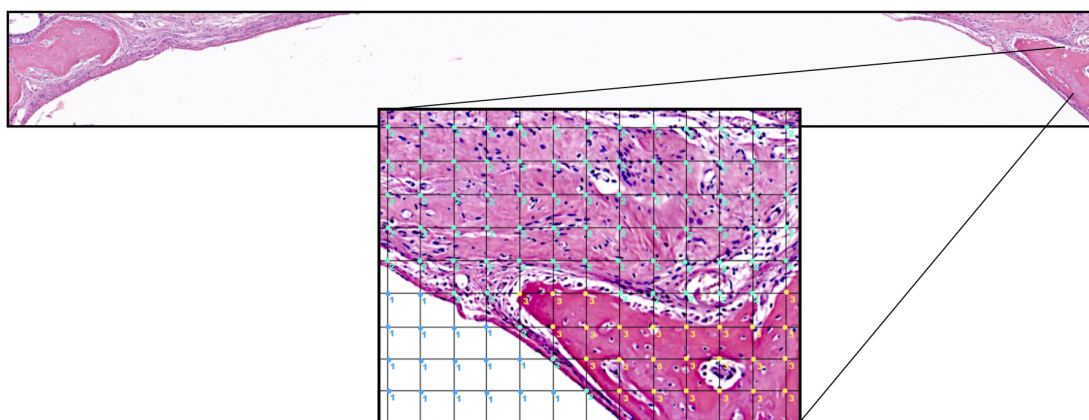
Fonte: Arquivo pessoal do autor.

Avaliação histomorfométrica

Foram obtidos cortes com 6 μm de espessura de cada peça, divididos em lâminas com 5 cortes cada, corados pela técnica da Hematoxilina-Eosina, permitindo a correta avaliação histológica das estruturas teciduais na região dos defeitos criados.

A avaliação histológica foi feita utilizando um microscópio de luz DIASTAR (Leica Reichert & Jung products, Germany), com objetivas para aumento de 5 até 100 vezes, e oculares com aumento de 10 vezes, através do qual as imagens foram observadas e avaliadas. As imagens representativas à avaliação foram enviadas para um microcomputador, através de uma câmera fotográfica digital Leica Microsystems DFC-300-FX (Leica Microsystems, Alemanha), com resolução de 1.3 megapíxels, acoplada ao microscópio de luz comum, para sua posterior utilização na descrição dos resultados obtidos. Para uma análise quantitativa, o software Image J (Image J 1.42q, Bethesda, Maryland, USA) foi usado para realizar a contagem de tecidos presentes. Com o auxílio de uma grade digital medindo 75x 75 pixels (5625 pixels) posicionado no centro do defeito, sendo cada intersecção entre linhas contada de acordo com o tecido presente (1- espaço vazio, 2-tecido mole, 3- tecido ósseo) (Figura 3). Por último os valores foram apresentados como percentagens da área mensurada.

Figura 3 - Corte histológico central e grade posicionada sobre a região para contagem das intersecções



Fonte: Elaboração própria.

Avaliação Imunohistoquímica

Foi empregado o método de imunohistoquímica para detecção e localização da expressão das proteínas ligadas à remodelação óssea: BMP-2 (proteína morfogenética óssea), OCN (osteocalcina), e OTX (Osterix). Cortes histológicos com 4 a 5 µm de espessura foram montados em lâminas silanizadas (Fisher Superfrost plus), seguido de tramitação laboratorial de rotina para desparafinização e reidratação. Em seguida, os cortes foram tratados pelo método do complexo avidina-biotina-peroxidase (ABC) com a utilização do kit ABC Staining System (sc-2023, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) segundo as instruções do fabricante. Quando necessário, a recuperação do antígeno foi realizada pela fervura dos cortes em solução tampão apropriada (Dako; Glostrup, Denmark), em uma câmara de pressão (Biocare Medical; Concord, CA) por 20 min e resfriadas à temperatura ambiente.

Anticorpos primários de rato para as proteínas avaliadas tiveram sua diluição otimizada individualmente. Como controle negativo, cortes histológicos foram tratados por IgG normal (R&D Systems), em substituição aos anticorpos primários, nas mesmas diluições destes. Os cortes foram corados com solução de hematoxilina para visualização dos núcleos. A intensidade e localização da coloração foi realizada ao redor do defeito com o uso de microscópio óptico (DIASTAR, Leica). A intensidade de proteínas marcadas foi avaliada por um examinador cego para os grupos experimentais de acordo com a escala: negativo(-), positivo(+), superpositivo(++), e hiperpositivo(+++).

APÊNDICE C – METODOLOGIA DETALHADA DA PUBLICAÇÃO 4

Amostras

Neste estudo foram utilizados 24 ratos *Rattus Norvegicus*, variação *albinus*, *Holtzman*, machos, adultos, de aproximadamente 3 meses de idade, com massa corporal entre 250 e 300 gramas, mantidos no Biotério da Faculdade de Odontologia da UNESP de Araraquara (FOAr-UNESP). Os animais foram mantidos em ambiente com luz e temperatura controladas e alimentados com ração sólida e com acesso a água *ad libitum* antes e durante todo o período experimental.

Grupos Experimentais

Os animais foram selecionados aleatoriamente e divididos em 3 grupos e foram avaliados em três períodos experimentais (0, 15 e 30 dias), com 6 animais em cada grupo. Os grupos foram divididos de acordo com o uso ou não do medicamento (dosagens mais altas que apresentaram bons resultados nos estudos anteriores).

-Grupo C: administração diária de 1 ml de soro fisiológico

-Grupo SRAN625: administração diária de 625 mg/kg/dia de Ranelato de Estrôncio (Protos®, Laboratórios Servier do Brasil).

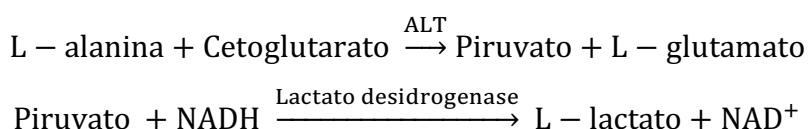
-Grupo SCAR/SCHL365: administração diária de 365 mg/kg/dia da combinação de 50% de Carbonato de Estrôncio e 50% Cloreto de Estrôncio (manipulado por Cromoline Química Fina, Diadema- São Paulo).

Obtenção das peças cirúrgicas

Nos períodos 0, 15 e 30 dias foram colhidos sangue do animal para análise de toxicidade sanguínea. Para a determinação de efeitos nocivos ao animal foram analisadas as dosagens bioquímicas de Alanina aminotransferase (ALT), Aspartato aminotransferase (AST), Fosfatase alcalina (ALP), ureia, creatinina, ácido úrico, albumina, proteína total, sódio e potássio foram quantificados no plasma sanguíneo utilizando *kits* comerciais da ©Ortho-Clinical Diagnostics (Johnson&Johnson Company) no autoanalisador Vitrus® 250. A concentração de Malondialdeído foi realizada após a eutanásia dos animais e analisada no plasma sanguíneo e no fígado. Abaixo os processos reacionais de cada análise.

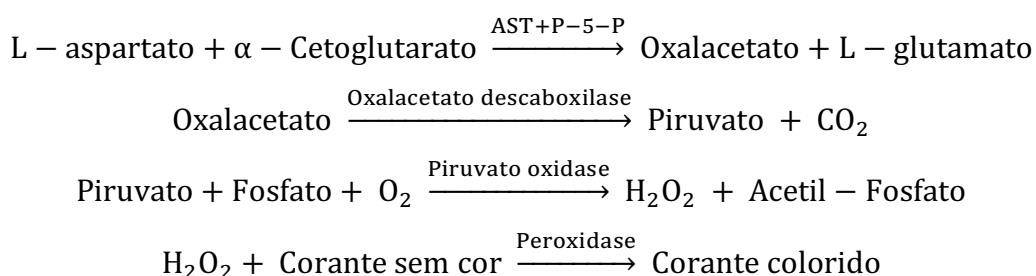
Alanina aminotransferase (ALT)

ALT catalisa a transferência do grupo amino da alanina para o alfa-cetoglutarato com a formação de glutamato e piruvato. O piruvato é reduzido à lactato sob ação catalisadora da lactato desidrogenase (LDH), enquanto que a coenzima NADH é oxidada a NAD⁺. A velocidade de oxidação do NADH é monitorizada por espectrofotometria de reflectância. A alteração da densidade de reflexão é proporcional à atividade da enzima.



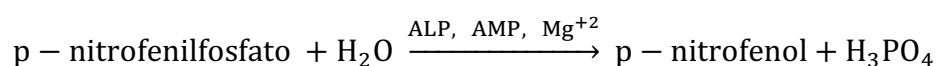
Aspartato aminotransferase (AST)

O grupo amino de L-aspartato é transferido para α-cetoglutarato na presença de piridoxal-5-fosfato (P-5-P) para produzir glutamato e oxaloacetato. O oxaloacetato formado na desaminação do L-aspartato é convertido em dióxido de carbono e piruvato pela oxaloacetato descarboxilase. O piruvato é oxidado em acetil-fosfato e peróxido de hidrogénio pela piruvato oxidase. O passo final da reação envolve a oxidação catalisada por peroxidase de um corante sem cor para produzir um corante colorido. A taxa de oxidação do corante sem cor é monitorizada por espectrofotometria de reflectância. A variação da densidade de reflectância é proporcional à atividade da enzima na amostra



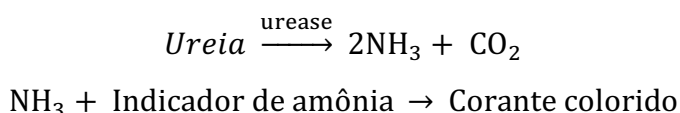
Fosfatase alcalina (ALP)

A fosfatase alcalina do soro, em meio alcalino, hidrolisa o p-nitrofenilfosfato, liberando p-nitrofenol e fosfato inorgânico. A quantidade de p-nitrofenol produzida é monitorada em 405 nm e é diretamente proporcional à atividade da fosfatase alcalina na amostra. A alteração da densidade de reflexão é convertida para atividade enzimática.



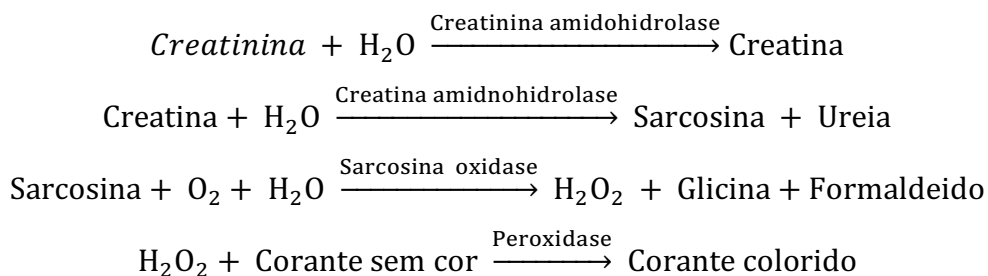
Ureia plasmática

A ureia é transformada em amônia pela ação catalítica da urease. A membrana semipermeável permite apenas amoníaco passar para a camada de formação de cor, onde reage com o indicador para formar cor. A densidade de reflexão do corante é medida e é proporcional à concentração da ureia na amostra.



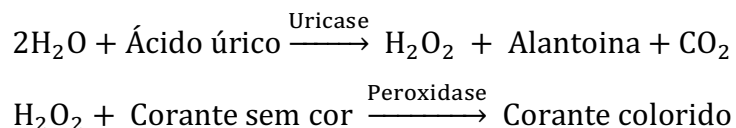
Creatinina

Creatinina é hidrolisada a creatina, através da creatinina amidohidrolase. A creatina é convertida em sarcosina e uréia pela creatina amidinohidrolase. A sarcosina é oxidada na presença de sarcosina oxidase, e gera glicina, peróxido de hidrogênio e formaldeído. A reação final envolve a oxidação catalisada por peroxidase de um corante sem cor com a produção de um produto colorido. As mudanças na densidade de reflexão é medida em dois tempos. A diferença na densidade de reflexão nestes tempos é proporcional à concentração de creatinina presente na amostra.



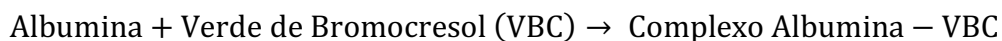
Ácido Úrico

Ácido úrico é oxidado na presença de uricase para formar alantoína e peróxido de hidrogénio. O peróxido de hidrogénio oxida um corante sem cor na presença de peroxidase para produzir um corante colorido. A densidade de reflexão do corante é medida por espectrofotometria de reflectância.



Albumina

A albumina liga-se com o verde de Bromocresol (VBC). Esta ligação resulta em uma mudança no comprimento de onda da reflectância máxima do corante livre, medida por espectrofotometria de reflectância. A quantidade de corante ligado à albumina é proporcional à concentração de albumina na amostra.



Proteína Total

As proteínas se complexam com o tartarato de cobre em meio alcalino resultando em um complexo colorido medido por espectrofotometria de reflectância.



Sódio

A determinação consiste de dois elétrodos seletivos que medem o potencial eletroquímico produzido entre os eletrodos, sendo a diferença de potencial proporcional à quantidade de sódio na amostra.

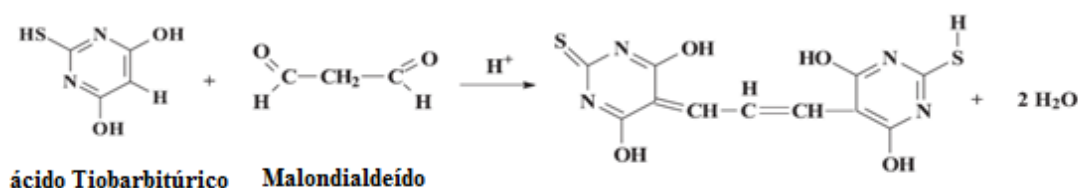
Potássio

A determinação consiste de dois elétrodos seletivos que medem o potencial eletroquímico produzido entre os eletrodos, sendo a diferença de potencial proporcional à quantidade de potássio na amostra.

Malondialdeído (MDA) no plasma e fígado

Durante o período de 30 dias, o plasma e o sobrenadante do homogenato de fígado foram submetidos a um processo de desproteínização descrito segundo Pilz et al.¹. A concentração de MDA foi determinada (Figura 1) segundo a metodologia descrita por Kohn e Liversedge². O MDA no fígado foi determinada espectrofotometricamente em 535 nm e a concentração de MDA no plasma foi determinada fluorimetricamente em comprimentos de onda de excitação e emissão de 510 e 553 nm, respectivamente. Foi realizada uma curva analítica com o padrão 1,1'3,3'-tetrametoxipropano para a quantificação de MDA nas amostras. Os resultados foram expressos em mol de MDA/L (plasma) ou μmol de MDA/g de tecido (fígado).

Figura 1- Reação entre o ácido 2-tiobarbitúrico e o malondialdeído, formando o composto colorido, medindo espectrofotometricamente a 535 nm



Fonte: OSAWA et al.³.

Após o período necessário para uma detecção de toxicidade (30 dias) os animais foram eutanaziados por decapitação e os órgãos, rins e fígados, removidos e armazenados em formol e/ou congelados a -80°C . A decapitação ocorreu para obtenção de maior volume sanguíneo para análise de Malondialdeído, uma vez que há a necessidade de separação do plasma.

Avaliação toxicidade nos órgãos

Os órgãos foram removidos dos animais nos estudos anteriores (2 e 3) nos períodos de 60 dias, mantidos em formol, incluídos em parafina, submetidos a lavagem com água corrente por 4 horas, armazenados em álcool 70° GI e então processados da seguinte maneira:

I – 1 hora em álcool 90° GI;

II – 5 trocas sucessivas em frascos com álcool absoluto, com intervalos de 30 minutos entre cada uma;

III – 30 minutos em solução (1:1) de álcool absoluto e solvente xilol;

IV – 30 minutos em solução de xilol puro;

V – 1 hora em outra solução de xilol puro;

VI – 1 hora em parafina líquida em estufa a 61 °C;

VII – 2 horas em nova parafina líquida em estufa a 61°C;

VIII – Confeção dos blocos em inclusora padrão, com uso de parafina líquida, formas metálicas e placa fria.

Após a confecção dos blocos de parafina foram feitos cortes histológicos em micrótomo semi-automático, com espessura de 4 µM. Estes cortes foram submetidos à solução de etanol 10º GL e posteriormente à água aquecida à 43º C, a fim de serem esticados mantendo-se a qualidade necessária. Posteriormente, foram dispostos em lâminas de vidro lapidadas, sendo então encaminhadas a estufas a 61°C para que ocorra a adesão dos cortes e dissolução do excesso de parafina. Após a adesão, foi realizada a coloração histológica, seguindo o protocolo da coloração da Hematoxilina de Harris com adição de Eosina amarelada.

No fígado, foram analisados os seguintes parâmetros:

- Presença de áreas de necrose
- Edema ou inchaço celular
- Desestruturação tecidual
- Congestão sanguínea
- Inflamação
- Gordura

Nos rins, foi verificado:

- Edema ou inchaço celular
- Desestruturação
- Preservação do espaço na cápsula de Bowman
- Congestão
- Inflamação

Referências

1. Pilz J, Meineke I, Gleiter CH. Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. J Chromatogr B Biomed Sci Appl. 2000 Jun 9;742(2):315–25.

2. Kohn HI, Liversedge M. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. *J Pharmacol Exp Ther*. 1944;82(3):292–300.
3. Osawa CC, Felício PE de, Gonçalves LAG. Teste de TBA aplicado a carnes e derivados: métodos tradicionais, modificados e alternativos. *Quim Nova*. 2005 Aug;28(4):655–63.

ANEXO A - COMITÊ DE ÉTICA NO USO DE ANIMAL



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara



FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 04/2012

Araraquara, 09 de Maio de 2012

Senhores Pesquisadores:

A Comissão de Ética no Uso de Animal - CEUA desta Faculdade reunida em 09/05/2012, após a avaliação do projeto de sua responsabilidade intitulado "Utilização do estrôncio não radioativo como potencializador da remodelação óssea em áreas enxertadas e da ósseointegração de implantes. Avaliação histológica, radiológica, microtomográfica e imunoinflamatória em ratos" (Proc. CEUA nº 04/2012) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para ABRIL/2015.

Atenciosamente.

Profª Drª DÉBORA SIMÕES DE ALMEIDA COLOMBARI
Vice-Coordenadora no exercício da Coordenadoria da CEUA

À

Profª Drª ROSEMARY ADRIANA CHIÉRICI MARCANTONIO
DD. Pesquisadora Responsável
a/c Cássio Rocha Scardueli
Departamento de Diagnóstico e Cirurgia

ANEXO B - COMITÊ DE ÉTICA NO USO DE ANIMAL**C E R T I F I C A D O**

Certificamos que a proposta intitulada "Avaliação sistêmica do uso de medicamentos derivados de Estrônico", registrada com o Protocolo CEUA/FCF/CAR: 64/2015, sob a responsabilidade de Cássio Rocha Scardueli e Profa. Dra. Rosemary Adriana C. Marcantonio - 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 aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS da Faculdade de Ciências Farmacêuticas do Campus de Araraquara da UNESP em reunião de 20 de maio de 2016.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	Março de 2017
Espécie/linhagem/raça	Rato (Rattus Norvegicus). Variação Albinus, Holtzman
Nº de animais	24
Peso/Idade	150 g 6 semanas
Sexo	Macho
Origem	Biotério Campus de Araraquara

Araraquara, 09 de Junho de 2016.



Prof. Dr. CARLOS CESAR CRESTANI
Coordenador da CEUA



ANEXO C – SUBMISSÃO DA PUBLICAÇÃO 1

International Journal of Implant Dentistry

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ANEXO D – SUBMISSÃO DA PUBLICAÇÃO 4

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Araraquara, 22 de março de 2018

Cassio Rocha Scardueli