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NANOPARTÍCULA DE ÓXIDO DE GADOLÍNIO AMINO-MODIFICADA UTILIZADA COMO AGENTE DE CONTRASTE PARA IMAGENS EM RESSONÂNCIA MAGNÉTICA

JOÃO ELITON BONIN

UBERLÂNDIA, MG
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina da Universidade Federal de Uberlândia, como requisito parcial para a obtenção do título de Mestre em Ciências da Saúde.

Área de concentração: Ciências da Saúde.

Orientador: Prof. Dr. Luiz Ricardo Goulart Filho

Co-orientador: Prof. Dr. Túlio Augusto Alves Macedo

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Dedico este trabalho:

A Deus por não fazer de mim um comum em ideias e práticas.

A minha mãe pelos costumeiros atos de apoio/incentivo e a meu pai que “foi embora antes do combinado” - saudades!

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“Cabeças e relógios querem-se conforme o clima e a moral de cada terra.”
João Paulo de Emílio Cristovão dos Santos Coelho Barreto (João do Rio)

RESUMO

Introdução: A Ressonância Magnética (RM) é uma modalidade propedêutica que utiliza radiação não-ionizante para aquisição de imagens médicas de maneira não-invasiva, úteis no diagnóstico de diversas patologias. Para melhorar a sensibilidade da RM utilizam-se meios de contraste (MCs) paramagnéticos, principalmente à base de gadolínio (Gd). Apesar de amplamente utilizados, falta especificidade aos MCs para o diagnóstico de diferentes tipos de neoplasias, por exemplo. **Objetivo:** analisar o comportamento cinético *in vitro* e *in vivo* de uma nova nanopartícula de óxido de gadolínio funcionalizada com um grupo amino (NP-Gd_2O_3:NH_2) como agente de contraste para RM. **Materiais e Métodos:** estudos *in vitro* com *Peripheral Blood Mononuclear Cells* (PBMC) e células endoteliais foram realizados estimulando-as com NP-Gd_2O_3:NH_2 e Gd-DOTA (ácido gadolínico-tetraazaciclododecanotetra-acético), contraste convencional usado como controle. Citocinas pró-inflamatórias e apoptose foram quantificadas por testes ELISA e citometria de fluxo, respectivamente. Estudos pré-clínicos com ratos Wistar (***Rattus norvegicus***) também foram realizados para comparar os dois contrastes, utilizando duas doses de NP-Gd_2O_3:NH_2 (1 mg/mL and 2 mg/mL) e Gd-DOTA (10 mg/mL). **Resultados:** análises *in vitro* com ambas doses de NP-Gd_2O_3:NH_2 e Gd-DOTA indicaram ausência de toxicidade nas linhagens celulares e baixa toxicidade em PBMC. Ao analisar as medidas de intensidade de pixel (relacionada neste estudo à intensidade de sinal (IS)) em cada animal e órgão (cérebro, fígado, rim e baço), observaram-se hiporrealce da NP-Gd_2O_3:NH_2 a 1 mg/mL em relação ao Gd-DOTA (controle), e hiperrealce da NP-Gd_2O_3:NH_2 a 2 mg/mL em relação ao controle. Notaram-se, ainda, menor efeito da NP no rim, e contraste de longo prazo por mais de 24 horas. **Conclusão:** em resumo, a nova NP-Gd_2O_3:NH_2, além de mostrar baixa toxicidade e boa biocompatibilidade, também foi capaz de aumentar a IS em vários órgãos e com lavagem lenta. O grupo amino ligado à NP pode permitir a conjugação de sondas específicas para o monitoramento de doenças e seus tratamentos, conferindo-lhe potencial para substituir os contrastes convencionais.

PALAVRAS-CHAVE: óxido de gadolínio amino-modificado, nanopartículas, meios de contraste, ressonância magnética, toxicidade.

ABSTRACT

Introduction: Magnetic Resonance Imaging (MRI) is a propaedeutic modality that uses non-ionizing radiation for the acquisition of non-invasive medical images, useful in the diagnosis of various pathologies. To improve the sensitivity of MR, paramagnetic contrast media (MCs) are used, mainly gadolinium (Gd). Although widely used, MCs lack specificity for the diagnosis of different types neoplastic, for example. **Objective**: to analyze the in vitro and in vivo kinetic behavior of a novel nanoparticle of gadolinium oxide functionalized with an amino group (NP-Gd_2O_3:NH_2) as a contrast agent for MRI. **Materials and Methods**: in vitro studies with Peripheral Blood Mononuclear Cells (PBMC) and endothelial cells were performed by stimulating them with NP-Gd_2O_3:NH_2 and Gd-DOTA (gadolinic acid-tetraazacyclododecanotetra-acetic acid). Proinflammatory cytokines and apoptosis were quantified by ELISA and flow cytometry tests, respectively. Pre-clinical studies with Wistar rats (***Rattus norvegicus***) were also performed to compare the two contrasts using two doses of NP-Gd_2O_3:NH_2 (1 mg / mL and 2 mg / mL) and Gd-DOTA (10 mg / mL). **Results**: análises *in vitro* com ambas doses de NP-Gd_2O_3:NH_2 e Gd-DOTA indicaram ausência de toxicidade nas linhagens celulares e baixa toxicidade em PBMC. Ao analisar as medidas de intensidade de pixel (relacionada neste estudo à intensidade de sinal (IS)) em cada animal e órgão (cérebro, fígado, rim e baço), observaram-se hiporrealce da NP-Gd_2O_3:NH_2 a 1 mg/mL em relação ao Gd-DOTA (controle), e hiperrealce da NP-Gd_2O_3:NH_2 a 2 mg/mL em relação ao controle. Notaram-se, ainda, menor efeito da NP no rim, e contraste de longo prazo por mais de 24 horas. **Conclusion**: in summary, the new NP-Gd_2O_3:NH_2, besides showing low toxicity and good biocompatibility, was also able to increase IS in several organs and with slow washing. The amino group bound to NP may allow the conjugation of specific probes for the monitoring of diseases and their treatments, giving it the potential to replace the conventional contrasts.

Keywords: amino-modified gadolinium oxide, nanoparticles, contrast media, magnetic resonance imaging, toxicity.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

AXI	Axial
Ca_2^+	Íon Cálcio
FDA	*Food and Drug Administration*
Fig.	Figura
FNS	Fibrose Nefrogênica Sistêmica
FOV	*Field-Of-View*
FS	*Fat Saturation*
Gd	Gadolínio
Gd_3^+	Íon Gadolínio
Gd_2O_3	Óxido de Gadolínio
GE	General Electric
HU - UFU	Hospital Universitário da Universidade Federal de Uberlândia
MC	Meio (s) de Contraste
NP	Nanopartícula
NP-$Gd_2O_{3:}NH_2$	Nova nanopartícula de óxido de gadolínio amino-modificada
PBMC	*Peripheral Blood Mononuclear Cells*
RM	Ressonância Magnética
ROI	*Region Of Interest*
SE	SPIN ECHO
TE	Tempo de Eco
TR	Tempo de Repetição
UFU	Universidade Federal de Uberlândia

SUMÁRIO

1. INTRODUÇÃO

1.1. Caracterização do problema

Diversas formas de meios de contraste (MCs) foram utilizadas com o objetivo de melhorar o diagnóstico por imagem de Ressonância Magnética (RM), mas estes agentes não são completamente isentos de risco.

O gadolínio (Gd) é um elemento químico não radioativo, paramagnético, e o principal componente ativo de vários MCs, sendo possível identificar processos neoplásicos, inflamatórios e infecciosos com melhor precisão. Contudo, apesar de amplamente utilizados nos exames de diagnóstico por imagem, falta especificidade nesses MCs para o diagnóstico de tumores como mamário, ovariano, intracraniano e intraperitoneal.

Nesse contexto, várias tentativas (1,2,6,7,8,11) têm sido realizadas com novos agentes de contraste, e particularmente uma nova classe com base em nanopartículas (NP) de Óxido de Gadolínio (Gd_2O_3) tem surgido, apresentando grande estabilidade, mas seus efeitos tóxicos tem sido analisados apenas *in vitro*.

1.2. Justificativa do trabalho

Embora existam NP de Gd_2O_3 ultra pequenas em sistemas coloidais, pouco se sabe sobre o comportamento das mesmas ao longo do tempo e sua toxicidade *in vivo*.

Com o advento desses novos MCs à base de NP de Gd_2O_3 perceberam-se a possibilidade de aumentar a expressão à RM dos agentes de contraste, reduzir seu volume/dose a ser injetado no sistema venoso, além de diminuir sua citotoxicidade *in vitro* e *in vivo*.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Ressonância magnética

A RM é uma modalidade propedêutica que utiliza radiação não-ionizante para a aquisição de imagens médicas, de maneira não invasiva, úteis no diagnóstico de diversas doenças (1,2). O contraste da imagem em RM depende, principalmente, das diferenças nos tempos de relaxamento e na densidade de prótons fornecida pela água entre os tecidos vizinhos (3,4).

Um equipamento de RM consiste em um grande e potente ímã onde o paciente se posiciona. Uma antena de ondas de rádio é utilizada para enviar sinais para o corpo e, em seguida, receber sinais de volta. Estes sinais de retorno são convertidos em imagens por um computador. Estas imagens podem ser obtidas em qualquer plano-axial, sagital e coronal (3).

Para melhorar a sensibilidade da RM na detecção de algumas doenças utilizam-se MCs paramagnéticos. O MC paramagnético mais consumido é o denominado quelato de gadolínio (Gd), que pode ser aplicado clinicamente para o estudo de vários órgãos e se caracteriza por permitir o relaxamento longitudinal dos prótons nas moléculas de água e aumentar do sinal nas sequências ponderadas em T1.

2.2. Meios de contraste

Diversas formas de MCs são utilizados com o objetivo de melhorar o diagnóstico por imagem. O uso disseminado em todo o mundo atesta o reconhecimento destas substâncias.

Dos agentes de contraste para RM desenvolvidos até o momento citam-se dois: um deles é o dextrano, composto superparamagnético revestido com nanopartículas de óxido de ferro, que possui grande relaxamento transversal dos prótons nas moléculas de água; e o outro é o quelato de Gd, composto paramagnético, que possui relaxamento longitudinal dos prótons nas moléculas de água (2,5). Hoje em dia, o quelato de Gd é mais amplamente utilizado na prática do diagnóstico por imagem, por poder ser empregado para a avaliação de todos os órgãos, enquanto que o dextrano possui maior especificidade para o fígado (6).

O Gd é um elemento químico não radioativo, paramagnético, da série dos lantanídeos. É o principal componente ativo de vários agentes de contraste sendo possível identificar processos neoplásicos, inflamatórios e infecciosos com melhor precisão, além de serem utilizados amplamente nos serviços de diagnóstico por imagem.

Os MCs à base de Gd foram aprovados pelo *Food and Drug Administration* (FDA) para uso parenteral no final dos anos 1980. Estes compostos podem ser diferenciados com base na química do quelato, estabilidade, viscosidade, osmolalidade e, em alguns casos, na eficácia para aplicações específicas. MCs à base de Gd são extremamente bem tolerados pela maioria dos pacientes nos quais são injetados (7).

No entanto, igualmente aos demais produtos farmacológicos, estes agentes não são completamente isentos de risco. As taxas de efeitos adversos para MCs à base de Gd injetados em doses clínicas (0,1 a 0,2 mmol/kg) variam de 0,07% a 2,4%. A maioria dessas reações adversas é leve e fisiológica. Reações alérgicas são incomuns e são encontradas com uma frequência inferior à observada após a administração dos MCs iodados, por exemplo (8). Reações anafiláticas graves com risco de morte ocorrem (9,10,11), mas são extremamente raras (12,13). Reações fatais ao quelato de Gd ocorrem, mas são extremamente raras (14).

A injeção de MCs à base de Gd em pacientes com lesão renal aguda ou doença renal crônica grave pode resultar na síndrome da Fibrose Nefrogênica Sistêmica (15,16).

O íon Gd_3^+ tem tamanho aproximado ao do íon Ca_2^+, deste modo, pode comportar-se como um eficaz e tóxico íon bloqueador de canal de cálcio, por isso o Gd_3^+ necessita estar ligado a um quelato. A ligação de íons Gd_3^+ reduz a sua interação com os tecidos e facilita a sua excreção, que ocorre pelo rim, predominantemente. Os quelatos podem ser iônicos ou não iônicos e suas cadeias podem ser lineares ou cíclicas. Geralmente, quelatos iônicos e cíclicos se ligam mais fortemente ao gadolínio do que os não iônicos e lineares (17,18).

2.2.1. Complexos gadolínio

Todos os agentes de contraste extracelulares, atualmente disponíveis, são produzidos à base de Gd. Este íon metálico exibe um forte efeito no tempo de relaxamento longitudinal T1.

2.2.1.1. Complexos com quelatos acíclicos (Gd-DTPA)

Gd-DTPA (gadopentetato, Magnevist) é um íon metálico coberto por um ligante polidenteado como uma garra. O íon metálico central tem nove locais de coordenação. Ele está ligado a três átomos de nitrogênio e cinco porções de carboxilato. Uma única molécula de água é capaz de coordenar o nono local vago resultando em um forte aumento da taxa de relaxamento dos prótons nas moléculas de água. A toxicidade do Gd-DTPA é mais de dez vezes menor que a toxicidade do íon de gadolínio e do ligante. O seu perfil de segurança é muito bem conhecido

e de uma notavelmente baixa incidência de eventos adversos. Relatos indicam sua utilidade no diagnóstico por RM de lesões no encéfalo, rins, neoplasias, isquemia miocárdica e lesões inflamatórias/infecciosas, entre outras (6,7,18,19).

2.2.1.2. Complexos com quelatos macrocíclicos (Gd-DOTA)

Gd-DOTA (gadoterate, Dotarem) é uma segunda classe de contraste para RM (sendo o primeiro complexo macrocíclico de gadolínio a entrar no mercado). A ciclização é realizada com um rendimento elevado uma vez que os segmentos de hidrocarbonetos entre os heteroátomos são curtos, e segmentos relativamente iguais do macrociclo alvo são condensados. A estrutura macrocíclica dentro do sítio de ligação do ligante metálico é mais encapsulada e a entropia é reduzida mediante a incorporação de metal. Como resultado, a estabilidade da maioria dos quelatos macrocíclicos é maior do que a dos complexos acíclicos. Geralmente os complexos macrocíclicos exibem uma maior estabilidade cinética (18).

2.3. Complexos e conjugados de Gd-DOTA e seus efeitos

Estes complexos e conjugados propiciaram o desenvolvimento do MC mais utilizado atualmente nos exames de RM e aprovado para avaliação em imagens de alterações cerebrais e na medula espinhal com quebra da barreira hematoencefálica ou vascularização anômala, bem como para imagens corporais (19).

2.3.1. Dendrímeros e dendrímeros nanoagrupados

A preparação de quelatos DOTA bifuncionais aumentou ainda mais a relaxividade de dendrímeros Gd-rotulados e reduziu a possibilidade do íons Gd ficarem livres no interior do núcleo dendrímero durante o processo de quelação. Estes agentes apresentam meia-vida no soro de 1,6 hora e baixa retenção no tecido (24 horas). Portanto, esses agentes apresentam uma direção interessante para futuras aplicações de imagem molecular (20).

2.3.2. Lipossomas e “polymersomes”

Lipossomas foram transformados em agentes de contraste paramagnéticos, quer por encapsulação de quelato de gadolínio dentro do lúmen aquoso ou por imobilização do quelato Gd na superfície da membrana. Embora, os lipossomas com quelatos de Gd encapsulados tenham sido previamente utilizados em RM, o fluxo lento da água através da camada dupla de membrana prejudica a taxa de trocas de água com o gadolínio encapsulado e, portanto, conduz a uma redução significativa da relaxividade. Estudos de biodistribuição e RM indicaram gradual desestabilização em circulação e excretada por filtração renal (20).

2.3.3. Micelas e nanoemulsões

Agentes de contraste macromoleculares de alta relaxividade envolvem o desenvolvimento de compostos de gadolínio autoagregados em nanopartículas micelares. As micelas formadas exibem uma relaxividade alta. Este aumento deve-se à incorporação de colesterol para o interior hidrofóbico, além do aumento da rigidez e flexibilidade rotacional reduzida do quelato de gadolínio.

Para reduzir qualquer potencial de toxicidade, estas nanopartículas com base em gadolínio-metoxi-DOTA-PE também foram desenvolvidas. A alta relaxividade permitiu que esses meios de contraste sejam utilizados em uma variedade de aplicações moleculares, incluindo o diagnóstico por imagem de tumores, placas ateroscleróticas e estenoses vasculares (20).

2.3.4. Nanopartículas de óxido de gadolínio e nanotubos de gadolínio

Recentemente tem havido um interesse crescente pelo uso de partículas inorgânicas, por exemplo, Gd_2O_3 e nanotubos de gadolínio, como agentes de contraste para RM. Nanopartículas pequenas e ultrapequenas de Gd_2O_3, com diâmetros entre 3 e 40 nm, foram desenvolvidas e possuem relaxividades comparáveis ao quelato de gadolíno por DOTA. No entanto, as preocupações com a liberação de íons livres Gd_3^+ podem inibir a sua utilidade clínica, embora não existam relatos na literatura sobre os efeitos tóxicos dessas nanopartículas, mas mesmo assim esforços recentes incidiram sobre o revestimento de Gd_2O_3 com diversos materiais, de modo a reduzir a toxicidade potencial (20).

2.3.5. Nanopartículas biológicas naturais

Partículas semelhantes a vírus são uma classe especial de proteínas multiméricas que formam invólucros de proteína com um espaço interior vazio. Estes blocos de proteínas altamente ordenadas em uma nanoescala formam uma plataforma interessante para o desenvolvimento de agentes multifuncionais, devido a sua estrutura de núcleo-invólucro altamente uniforme e à elevada densidade de grupos quimicamente reativos na superfície exterior. Esta nanoplataforma biocompatível pode expandir a eventual aplicação de nanopartículas de lipoproteínas em diagnóstico por imagem (20).

3. OBJETIVOS

3.1. Objetivo primário

Desenvolver uma nanopartícula de óxido de gadolínio modificada com um grupo amino em sua superfície (NP-Gd_2O_3:NH_2) que apresente intensidade de pixel (representada por intensidade de sinal (IS)) similar ou superior ao do Gd-DOTA (contraste convencional) na avaliação de órgãos e sistemas e que seja estável e não tóxica *in vivo*.

3.2. Objetivos secundários

Demonstrar os comportamentos *in vitro* e *in vivo* desta nanopartícula amino-modificada como MC específico para órgãos ou sistemas do corpo, e mostrar sua segurança em modelos pré-clínicos.

4. CÓPIA DO ARTIGO SUBMETIDO

4.1. ARTIGO

AMINO-MODIFIED GADOLINIUM OXIDE NANOPARTICLE AS A CONTRAST AGENT FOR MAGNETIC RESONANCE IMAGING

João Eliton Bonin[1]†, Túlio Augusto Alves Macedo[1]†, Letícia de Souza Castro Filice[2], Aline Teodoro de Paula[3], Larissa Prado Maia[3], Robinson Sabino-Silva[4], Léia Cardoso de Sousa[4], Anielle Christine Almeida[5,6], Noelio Oliveira Dantas[5,6], Luiz Ricardo Goulart[3*]

[1]Department of Radiology and Diagnostic Imaging of the Clinics Hospital of Uberlandia Federal University, Federal University of Uberlândia, Uberlândia, MG, Brazil
[2]School Medicine, Federal University of Uberlândia, Uberlândia, MG, Brazil.
[3]Institute of Biotechnology, Laboratory of Nanobiotechnology, Federal University of Uberlândia, Uberlândia, MG, Brazil
[4]Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil
[5] Laboratory of New Insulating and Semiconductors Materials, Institute of Physics, Federal University of Uberlândia, Uberlândia, MG, Brazil.
[6] Laboratory of New Nanostructured and Functional Materials, Institute of Physics, Federal University of Alagoas, Maceio, AL, Brazil.

† The authors had equal contribution and are considered co-first authors.

***Corresponding author:**

Luiz R. Goulart. Institute of Biotechnology, Laboratory of Nanobiotechnology, Federal University of Uberlândia, Campus Umuarama, Bl. 2E, Sl. 248, CEP 38400-902, Uberlândia, MG, Brazil. Phone: (55+34) 3225-8440. E-mail: lrgoulart@ufu.br

ABSTRACT

Magnetic resonance imaging (MRI) is a modality that uses non-ionizing radiation for acquisition of non-invasive medical images for pathological diagnosis. Although paramagnetic contrast media based on gadolinium (Gd) has been widely used, there is a lack of specificity for different malignancies. Our aim was to analyze *in vitro* and *in vivo* kinetics of a novel amino-functionalized nanoparticle (NP-Gd_2O_3:NH_2) as a contrast agent for MRI. *In vitro* cellular analyses were performed with peripheral blood mononuclear cells (PBMC) and HUVEC (human umbilical vein endothelial cell) line, which were stimulated with NP-Gd_2O_3:NH_2 and Gd-DOTA (gadolinium-tetraazacyclododecanetetraacetic acid), a conventional contrast used as control. Pro-inflammatory cytokines and apoptosis were quantified by ELISA and flow cytometry tests, respectively. Preclinical study with Wistar rats (*Rattus norvegicus*) was performed to compare the two contrasts using two doses of NP-Gd_2O_3:NH_2 (1 mg/mL and 2 mg/mL) and Gd-DOTA (10 mg/mL). Intensity signals (IS) were obtained from brain, liver, kidneys and spleen for each animal. *In vitro* analyses with both NP-Gd_2O_3:NH_2 doses and Gd-DOTA indicated absence of toxicity in cell lines and low toxicity in PBMCs. We have observed a weak enhancement of NP-Gd_2O_3:NH_2 at 1 mg/mL in relation to Gd-DOTA (control), but higher enhancement of NP-Gd_2O_3:NH_2 at 2 mg/mL. Interestingly, a low enhancement of NPs in kidneys was also observed after 24 hours. Briefly, the new NP-Gd_2O_3:NH_2, in addition to showing low toxicity and good biocompatibility, it was also capable of enhancing the IS in several organs with slow washing out. The Amino-group linked to the NP may allow its conjugation with specific probes for monitoring diseases and their treatments, conferring to it a potential to replace conventional contrasts.

Keywords: amino-modified gadolinium oxide, nanoparticles, contrast media, magnetic resonance imaging, toxicity.

Magnetic Resonance Imaging (MRI) is a propaedeutic modality that uses non-ionizing radiation for the acquisition of non-invasive medical images useful in the diagnosis of various diseases[1,2]. The MRI contrast depends mainly on differences of the relaxation times and density of protons supplied by water between neighboring tissues[3,4]. Contrast media (CM) are commonly used to improve MRI sensitivity. Among MRI contrast agents developed so far, one is a dextran, superparamagnetic compound coated with iron oxide nanoparticles, which has great transverse relaxation of protons, in water molecules, and the other is a gadolinium paramagnetic chelate compound, which has longitudinal relaxation of protons, in water molecules[2,5]. Nowadays, the later is the most widely used in clinical practice, due to its ability to evaluate all organs, but with high specificity for the liver[6].

Gadolinium-based CMs have been approved by the Food and Drug Administration (FDA) for parenteral use in late 1980s. These compounds can be differentiated based on chelating chemistry, stability, viscosity, osmolality and, in some cases, the efficacy for specific applications[7]. Similar to other pharmacological products, these CMs are not completely risk-free and may potentially present adverse effects, athough they may rarely cause serious illness, such as systemic nephrogenic fibrosis, or even more rarely death[8, 9,10,11,12]. The Gd_3^+ ion is approximately the size of the Ca_2^+ ion, so it can behave as an effective and toxic calcium channel blocker ion, which explains the need to bind Gd_3^+ to a chelate. The binding of Gd_3^+ ions reduces their interaction with tissues and facilitates their excretion, which occurs predominantly in the kidney. The chelates may be ionic or non-ionic and their chains may be linear or cyclic. Generally, ionic and cyclic chelates bind more strongly to gadolinium than non-ionic and linear chelates[13,14].

Gd-DOTA (gadoterate, Dotarem®) is a second class of MRI contrast (the first macrocyclic gadolinium complex to enter the market). The macrocyclic structure within the binding site of the metal linker is more encapsulated and the entropy is reduced by the incorporation of metal. As a result, the stability of most macrocyclic chelates is greater than that of acyclic complexes. In addition, generally the macrocyclic complexes exhibit a higher kinetic stability[14]. These complexes led to the development of the CM that is currently used in MRI examinations, and approved for evaluation in brain and spinal cord images with rupture of the blood-brain barrier or anomalous vascularization, as well as for body images[15]. It is noteworthy that, although widely used in imaging tests, there is a lack of specificity in these CMs for the evaluation of tumors such as mammary, ovarian, intracranial and intraperitoneal. In this context, several attempts have been made with new contrasts, and particularly a new

class based on Gd_2O_3 nanoparticles has emerged, presenting great stability, but its toxic effects have been analyzed only *in vitro*[16,17,18,19]. These Gd-based nanoparticles have shown good biocompatibility and enhanced MRI, because they present greater longitudinal relaxivity of water protons than those of Gd(III)-chelates due to a high density of Gd(III) per nanoparticle. In general, the higher the concentration of the contrast agent, the bigger the change in tissue relaxation will be[19]. Interestingly, such nanoparticles may also present a potential role as therapeutic agents. Biocompatible ligand-coated gadolinium oxide nanoparticles may also be used as drug carriers, including molecules attached to its surface for theranostic applications[20].

In this sense, our study sought to develop a modified nanoparticle with an amino group in its surface (Gd_2O_3:NH_2), which presented signal intensity similar or superior to that of Gd-DOTA (conventional contrast) in the evaluation of organs and systems, and it was also shown increased stability and absence of toxicity *in vivo*. Our aim in this investigation was to demonstrate the *in vitro* and *in vivo* behaviors of this specific amino-modified nanoparticle as an imaging agent for organs or body systems, and to show it safety in pre-clinical models.

This study was carried out as a cooperation among the Laboratory of Nanobiotechnology (NANOS) of the Institute of Biotechnology of the Federal University of Uberlândia, Laboratory of New Materials and Semiconductors (LMNIS) of the Institute of Physics of the Federal University of Uberlandia, and the Department of Radiology and Imaging Diagnostics of the Clinics' Hospital of the Federal University of Uberlândia (HC-UFU). The Gd-DOTA contrast agent (Dotarem®) used in this study for MRI was obtained from the Radiology and Imaging Diagnostic Service of the HC-UFU. The NP-Gd_2O_3:NH_2 nanoparticle manipulated in this study were synthesized and characterized at LMNIS. *In vitro* and *in vivo* tests were performed at the Nanobiotechnology Laboratory of Federal University of Uberlândia, and all the MRI images were obtained in the HC-UFU.

The CMs used in this experiment were:

1) Gd-DOTA - gadolinium (III) - {l,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate} - (Dotarem®, Guerbet, Rio de Janeir, RJ, Brazil), used as control, approved by FDA and ANVISA - National Health Surveillance Agency - (Brazilian Regulatory Agency for Food and Drug) in 2013 as the first macrocyclic CM for use in humans[21]. Dotarem® is in the concentration 279.32 mg/mL and has the following physical characteristics: osmolarity: 851 mOsm/L; osmolarity: 1350 mOsm/kg^{-1}; viscosity at 20°C: 3.2 mPa.s; viscosity at 37ºC: 2.0 mPa.s; pH: 6.5-8.0[22].

2) Gd_2O_3:NH_2 nanocrystals were synthesized by coprecipitation[23] with minor modifications, with thermal annealing at 800ºC/4h. High-resolution transmission electron microscopy (HRTEM) images and X-ray dispersive energy (EDS) spectrometry results were obtained by high-resolution transmission electron microscopy (JEOL SIOD) of acceleration voltage of 200 kV[24]. The *Fourier-transform infrared spectroscopy* (FTIR) spectra of the samples were recorded using a Shimadzu Fourier Transform IR (FT-IR) (Vertex 70, Bruker Optik) spectrophotometer in the spectral range 500 to 4000 cm^{-1} via a coupled total attenuated reflectance (ATR) element with resolution of 2 cm^{-1}. All characterizations were performed at room temperature.

For the imaging acquisition, we have used the GE-brand magnetic resonance device, model Optima MR360 Advance 1.5T, with OpTix technology and Express coil coil assembly and flexible for studies in animal models, 8 channels. Gradients 33/120, with automation system through "Slide Bar" and "Ready Brain". It has applications for sensitization to contrast media, such as Inhance 2.0 Suite - Visualization of arterial and venous flow in any part of the body, 3D ASL - Quantitative evaluation of cerebral perfusion, Heart - Generation of coronary artery image without apnea, DWI - Generation of multi-b-weighted diffusion image and multidirectional acceleration tools[25]. In addition, this equipment has phantons capable of producing images *in vitro* and *in vivo*. For this experiment the following parameters were used: spine coil; sequence T1 FAT SAT; TR 600; TE 9.8; Flip Angle 90º; coronal plane; cut thickness 4.0 mm; FOV 22 x 22 cm; approximate duration of the acquisition two minutes and eighteen seconds.

In vitro studies were characterized as follows:

1) Test 1: after synthesis of Gd_2O_3:NH_2 nanoparticles, successive dilutions were carried out for *in vitro* tests. The solutions were placed in eppendorf tubes, always in the proportion of 1 mL of solution with nanoparticles in the following concentrations: 0.125, 0.25, 0.5 and 1 mg/mL Gd_2O_3:NH_2; 0.125 and 0.5 mg/mL of Gd-DOTA as control in this experiment, both mixed in 4 mL of agarose gel (1%).

2) Test 2: additional experiments were carried out with Gd_2O_3:NH_2 and Gd-DOTA in the proportions 1, 2.5 and 4 mL of Gd_2O_3:NH_2 in the concentration of 1 mg/mL and 4, 2.5 in 1 mL of gel, respectively. In addition to 1 mL of the Gd-DOTA control at concentrations 1 and 0.5 mg/mL in 4 mL of H_2O.

3) Cell culture: the human bronchial epithelial cell line (BEAS-2B) and the monkey kidney epithelial cell line (Vero) were provided by the Federal University of Triângulo Mineiro (UFTM, Uberaba, Brazil) and by the Cell Bank of Rio de Janeiro (BCRJ), respectively. The

BEAS-2B strain was cultured in Dulbecco's modified Eagle's medium-F12 (DMEM-F12/Cultilab) medium, and the Vero strain was grown in Dulbecco's modified Eagle's medium (DMEM/Cultilab) medium. Both media were supplemented with 10% fetal bovine serum and 1% gentamicin. In parallel, peripheral blood mononuclear cells (PBMC) from three healthy volunteers were isolated from whole blood using Ficoll density gradient Histopaque-1077 (Sigma), following the manufacturer's recommendations. PMBC cells were cultured in RPMI medium. All cell lines were maintained under standard culture conditions (37ºC, 95% humidified air and 5% CO_2).

The viability of Gd_2O_3:NH_2 and Gd-DOTA drugs in different cells was determined using diphenyltetrazolium 3-(4,5-dimethylthiazolyl-2)-2,5-bromide (MTT). BEAS-2B and Vero cell lines were plated in 96-well plates (1×10^4 cells/well) in their culture media and incubated overnight. PBMC cells were plated and incubated under the same conditions, differentiating the incubation period of 1 h. All cells were stimulated with Gd_2O_3:NH_2 (autoclaved) and sterile Gd-DOTA (commercial) in different concentrations (100; 50; 25; 12.5; 6.25; 3.12 and 1.56 µg / mL) 24 hours and subsequently incubated with 10 µL of MTT solution (5 mg/mL) for 4 h. Then, 50 µl of a solution containing 20% sodium dodecyl sulfate (SDS) and 50% N, N-dimethylformamide (pH 4.7) was added and incubated for 2 h. Viable cells were determined by the absorbance (570 nm) of formazan crystals solubilized in a Thermo Plate reader (TP-Reader). All incubations were performed following the standard cell culture condition (37ºC, 95% humidified air and 5% CO_2). Cell assays were performed in triplicates and at different times.

For the *in vivo* studies, Wistar rat model were infused with 1X Gd_2O_3:NH_2, 2X Gd_2O_3:NH_2 and Gd-DOTA (Dotarem®). The venous contrast material (abdominal vein) was infused to obtain MRI images in brain, liver, kidneys, and spleen. For MRI analyses, animals were anesthetized with ketamine hydrochloride and xilasin hydrochloride at doses of 90mg/kg and 10mg/kg, respectively. Afterwards, animals remained confined for 36 hours in metabolic cages, with MRI data collection in 2', 5', 20' and 24 hours post nanoparticle injection, and vital data collection and volumes of urinary excretions were performed. For the MRI analysis, after the MC infusion, the impregnation of the different tissues and organs in the rat model was evaluated. T1 and T2-weighted sequences were programmed for MRI imaging. RadiAnt DICOM Viewer version 4.2.1 (64-bit) (Medixant, Poznan, Poland) was used to evaluate the images. For the measurement of the minimum, medium and maximum pixel intensities (relative to signal intensities), the Region of Interest (ROI) was used with a mean area of 0.1 cm^2 in the desired organs (brain, liver, kidney and spleen), as exemplified in Fig 1 (pre-contrast images).

It should be noted that these same measurements were also performed on images of "tests 1 and 2" *in vitro*.

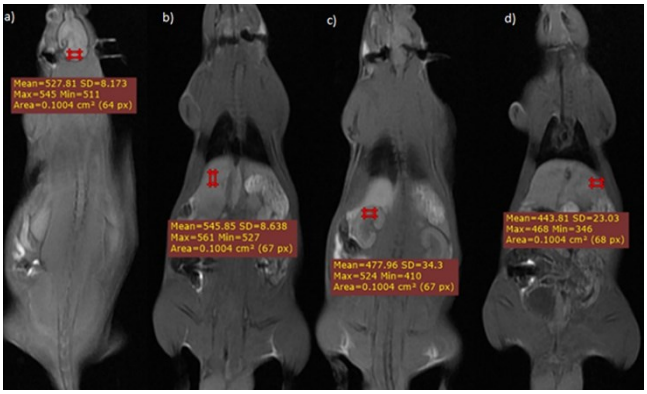

Fig. 1: MRI images of T1-weighted images (TR 600, TE 9,8) obtained pre-contrast in Wistar rats (*Rattus norvegicus*) demonstrating how measurements of pixel intensities (related to signal intensity) were performed on the four objectives organs of this study: a) brain, mean of 527 ; b) liver, mean of 545; c) kidney, mean of 477 and d) spleen, mean of 443.

To analyze vital data and renal excretion of animals, three groups of animals were established: 1) Sham (saline-control); 2) Gd-DOTA (Dotarem®) and 3) Gd_2O_3:NH_2. These groups were evaluated twenty-four hours before and twenty-four hours after CM injection, for the following parameters: weight, tidal volume before and after, respiratory rate before and then ingestion of water before and after and urinary volume before and after. Eleven (11) Wistar rats (*Rattus norvegicus*) were used for *in vivo* tests, being that, three (3) rats received saline solution (negative control) and eight (8) rats received CM administration according to the following distribution (three (3) rats received 1X Gd_2O_3:NH_2; two (2) rats mice received - 2X Gd_2O_3:NH_2 - and another three (3) rats mice received - Gd-DOTA (positive control)}. As inclusion criteria, we have used mice 60 (sixty) days old. The exclusion criteria were following: death of the animal after anesthetic induction and/or failure of catheterization.

For statistical analysis the experiments were performed in triplicates and results presented by means ± standard deviation (S.D.). The Gd_2O_3:NH_2 values were compared to the Gd-DOTA using two-way ANOVA test, followed by the Bonferroni post-test using the Prism GraphPad 7 software (GraphPad Software, Inc.). The value of $p < 0.05$ was considered significant. In addition, one-way ANOVA, Kruskal-Wallis and Duns post-test were used in the analysis of the signal intensities of MR images.

High-resolution transmission electron microscopy (HRTEM), Energy Dispersive X-ray Spectrometry (EDS) and X-Rays Diffraction (XRD) were employed to investigate the size,

composition, morphologies and crystalline structure of the samples as illustrated in Figure 2. Figure 2 (A) shows HRTEM image of which it is observed some agglomerates due to the high concentration used in measurements (1 mg/mL). The sample comprised of 3 nm nanocrystals (Figure 2B, inset) in its large majority followed by 15 nm nanocrystals in small quantity. The inset shows an HRTEM image of inidividual nanocrystals formed with size extremality small size. The EDS result (Figure 2 (B) also confirmed that the nanocrystals were constituted by Gd_2O_3. In Figure 2 C, the crystalline structure was confirmed by XRD patterns of cubic Gd_2O_3 nanocrystals (JCPDS: 12-0797). Thus, based on these results, we can confirm the formation of ultrasmall Gd_2O_3 nanocrystals.

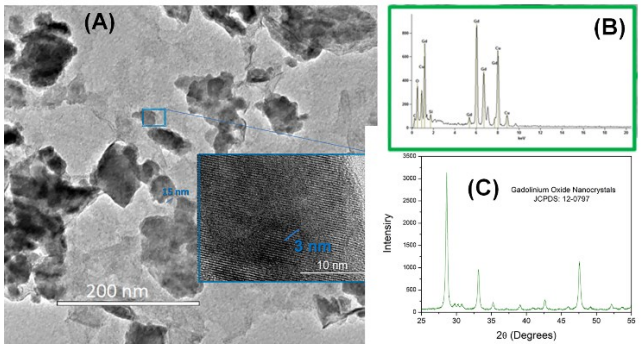

Fig. 2: (A) HRMET image with inset, (B) EDS and (C) XRD patterns of the Gd_2O_3 nanocrystals.

The Fourier Transform Infrared Spectroscopy (IR-FT) was performed to investigate the vibrational modes of the samples, as well as to confirm the functionalization and coating of nanocrystals. In the Figure 3 shows infrared spectra of the bare Gd_2O_3 nanocrystal and the NH_2-functionalized Gd_2O_3. In the spectrum (a) the band at 550 cm^{-1} is characteristics the Gd-O vibrations in the cubic Gd_2O_3 phase[26]. The additional bands observed are characteristic to C=O and $(CO_3)^{2-}$ of the to the CO_2 and H_2O groups present on the surface of nanocrystals due heat treatment in the air[26,27]. In the spectrum (b) the bands at 1490 cm^{-1} e 1567 cm^{-1} are assigned to the vibrational mode of symmetric $–NH_3^+$ deformation mode and to scissor vibration of the terminal $–NH_2$ group of the APTMS molecule, respectively[24]. The presence of the $-NH_2$ group confirms that APTMS successfully functionalized the Gd_2O_3 nanocrystals.

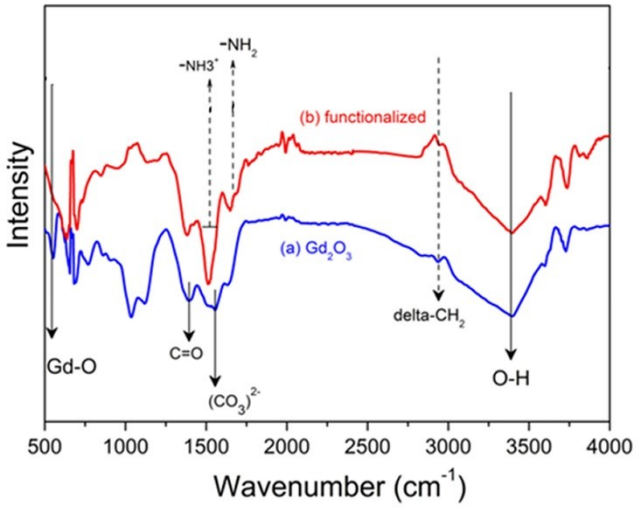

Fig. 3: FTIR spectra of Gd_2O_3 (a) and NH_2-functionalized Gd_2O_3 (b).

To evaluate whether the signal intensity of the Gd_2O_3:NH_2 nanoparticles was superior to Gd-DOTA, we have mixed different concentrations of the NPs into an agarose gel for proper distribution of the agents for imaging analyses. It was observed that the Gd_2O_3:NH_2 (Tube 2) had a mean signal intensity similar to that of the Gd-DOTA control (Tube 6), measuring 1677 and 1659, respectively (Fig. 4).

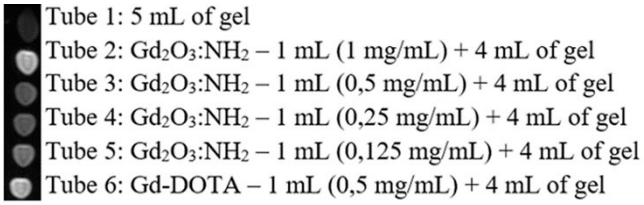

Fig. 4: Image obtained in SPIN-ECHO T1 sequence of eppendorf tubes with varied contents and concentrations, using TR 700, TE 10, flip angle 90° and 4.0 mm thickness. It is observed that there is similarity between the pixel intensities (related to the signal intensity) of tubes 2 and 6 (Gd_2O_3:NH_2 and Gd-DOTA, respectively).

Subsequently, new dilutions and new tests were performed to confirm the finding. The MRI images were acquired at different times of Eco (TE) and Time of Repetition (TR), according to AXI TR 100/TE 15, TR 200/TE 15, TR 400/TE 15, TR 200/TE 15, TR 400/TE 15 and TR 600/TE 15[17] (Fig. 5). The sample "3" (Gd_2O_3:NH_2) of this experiment showed expression similar to Gd-DOTA-control samples "6" and "7", with mean signal intensities in "Tubes 3, 6 and 7", measuring 1316, 1269 and 1358, respectively.

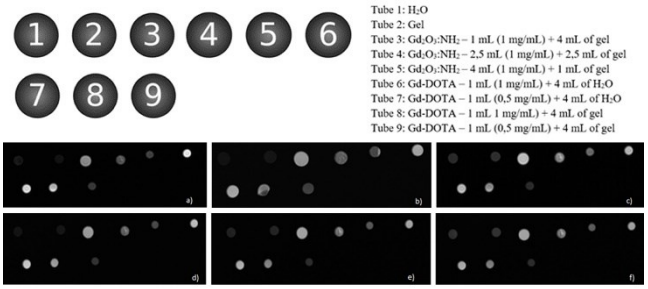

Fig. 5: Images obtained in SPIN-ECHO T1 sequence of eppendorf tubes with varied contents, sequences and concentrations. a) TR 100/TE 15; b) TR 200/TE 15; c) TR 400/TE 15; d) TR 200/TE 15; e) TR 400/TE 15; f) TR 600/TE 15. All sequences were acquired with 90 ° flip angle and 4.0 mm thickness. Similar pixel intensity (relacionadas at signal intensity) is observed in tubes 3 (Gd_2O_3:NH_2) and 6 and 7 (Gd-DOTA).

The viability of the BEAS-2B, Vero and PBMC cell lines (Fig. 6) were evaluated by the MTT assay. In the present study, the Vero line presented no significant difference when compared to both treatments. In contrast, BEAS-2B presented a statistical difference for concentration of 100 µg/mL when compared to Gd_2O_3:NH_2 and Gd-DOTA. All concentrations tested in the PBMC assay showed significant differences ($p < 0.05$) when compared to both treatments. The maximum toxicity of Gd_2O_3:NH_2 was 3.12 µg/mL when compared to Gd-DOTA at the lowest concentrations tested in peripheral blood mononuclear cells.

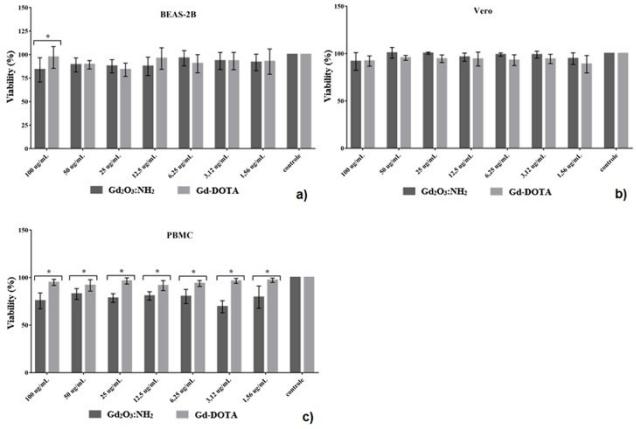

Fig. 6: Viability analysis of Gd_2O_3:NH_2 and Gd-DOTA in BEAS-2B, Vero and PBMC cells by means of the MTT assay. The cultures of BEAS-2B (A), Vero (B) and PBMC (C) were treated with different concentrations (100; 50; 25; 12,5; 6,25; 3,125 and 1,56 µg/mL) of Gd_2O_3:NH_2 and Gd-DOTA for 24 h. These results are representative with 3 independent experiments. Data show the mean ± standard deviation (S.D.). * Statistically significant difference ($p < 0.05$) of Gd_2O_3:NH_2 compared to Gd-DOTA.

Signal intensity measurements were performed in each animal according to acquisition times (PRE-contrast and after injection of the MC in the 2', 5' and 24h times) in organs (brain,

liver, kidney and spleen) (Fig. 7). It was observed that in animal 2 (Gd_2O_3:NH_2) there was a low post contrast enhancement in relation to animal 1 (control - Gd-DOTA) in all organs propose (brain, liver, kidney and spleen).

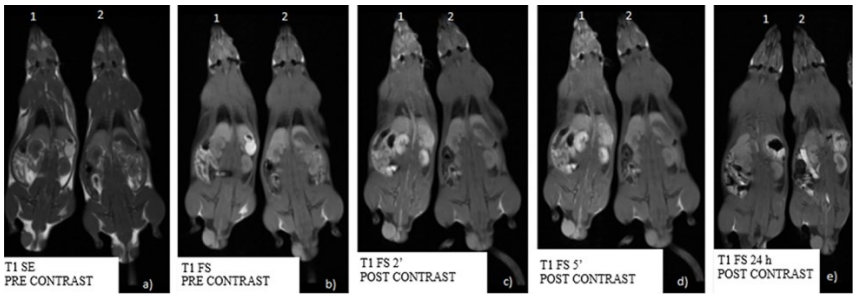

Fig. 7: MRI images obtained pre and post-contrast in Wistar rats (*Rattus norvegicus*): a) CORONAL T1 SE (TR 383.3/TE 9.7); b) CORONAL T1 FS PRE (TR 650/TE 9.7); c) CORONAL T1 FS POST 2 '(TR 650/TE 9.7); d) CORONAL T1 FS POST 5 '(TR 650/TE 9.7); e) CORONAL T1 FS POST 24 h (TR 650/TE 9,7). All sequences were acquired with 90° flip angle and 4.0 mm thickness. Animal 1) Control (Gd-DOTA- Dotarem®) - Concentration: 279.32 mg/mL; Dose: 0.02 mL. Animal 2) Test (Gd_2O_3:NH_2) - Concentration: 1 mg/mL; Dose: 0.02 mL. In animal 2 (Gd_2O_3:NH_2) there was a low post contrast enhancement in relation to animal 1 (control - Gd-DOTA) in all organs propose.

The tests in different periods of MRI detection was performed with Gd_2O_3:NH_2 in two doses (1 mg/mL and 2 mg/mL) and Dotarem® as control (10 mg/mL) to determine variations in intensity signals for 24 hours (Fig. 8). The signal intensity for 1X Gd_2O_3:NH_2 (animal 2) in each organ (brain, liver, kidney and spleen) was observed, which showed low enhancement in relation to the control (animal 1; Dotarem®). However, a significant signal enhancement was observed in the animal 3 (2X Gd_2O_3:NH_2), in relation to animal 1 (Dotarem®), which was more evident in kidney and liver (Fig. 9).

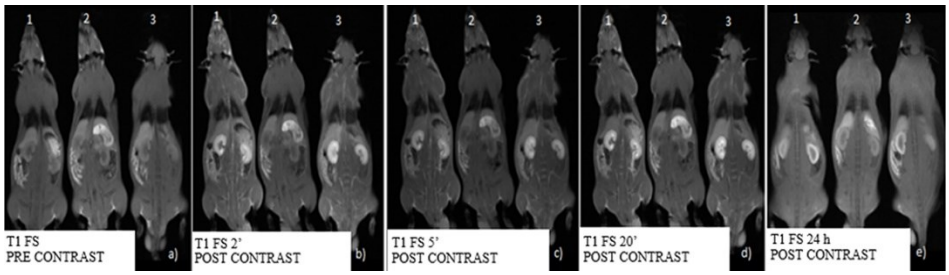

Fig. 8: MRI images obtained pre and post-contrast in Wistar rats (*Rattus norvegicus*): a) CORONAL T1 FS PRÉ (TR 600/TE 9.8); b) CORONAL T1 FS POS 2 '(TR 600/TE 9.8); c) CORONAL T1 FS POS 5 '(TR 600/TE 9.8); d) CORONAL T1 FS POS 20 '(TR 600/TE 9.8); e) CORONAL T1 FS POS 24 h (TR 600/TE 9,8). All sequences were acquired with 90° flip angle and 4.0 mm thickness. Animal 1) Control (Gd-DOTA - Dotarem®) - Concentration: 279.32 mg / mL; Dose: 0.02 mL. Animal 2) Test (1X Gd_2O_3:NH_2) - Concentration: 1 mg / mL; Dose: 0.02 mL. Animal 3) Test (2X Gd_2O_3:NH_2) DOUBLE DOSE - Concentration: 2 mg / mL; Dose: 0.02 mL. 1X Gd_2O_3:NH_2 (animal 2) in each organ was observed, which showed low enhancement in relation to the control (animal 1; Dotarem®). Significant signal enhancement was observed in the animal 3 (2X Gd_2O_3:NH_2), in relation to animal 1 (Dotarem®), which was more evident in kidney and liver.

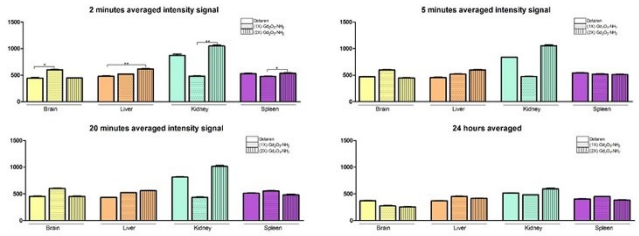

Fig. 9: Mean signal intensities in brain, liver, kidney and spleen, in the following times of image acquisition after MC injection (2 ', 5', 20 'and 24h).

Regarding the analysis of vital data and renal excretion of animals (Fig. 11), considering that animals presented similar weights (~310 g), none has shown significant changes in tidal volume and water intake before and after the injection of CM, but showed significant alterations ($P<0.05$) regarding the increase in respiratory rate and urinary volume after injection of MC in group C (Gd_2O_3:NH_2) e group B (Control - Dotarem®), respectively.

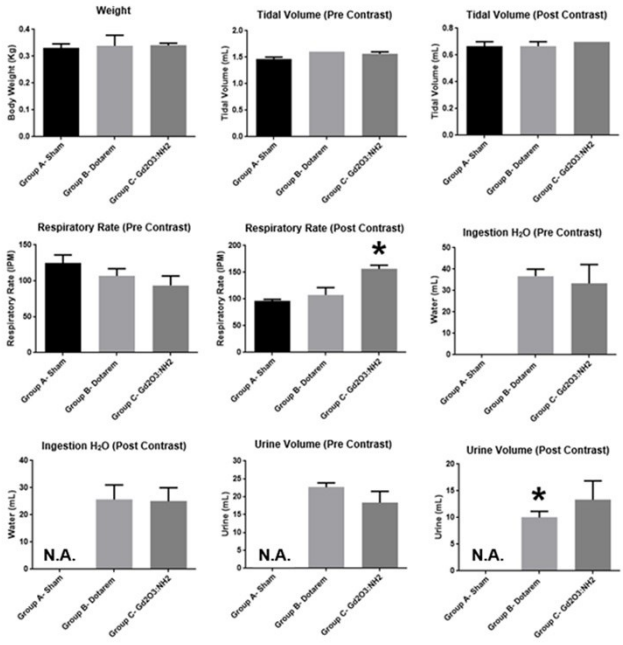

Fig. 10: Histogram data referring to animals 24 h before and 24 h after injection of contrast agents divided into three groups: A) Sham (saline - control); B) Dotarem® (Gd-DOTA) and C) Gd_2O_3:NH_2 nanoparticles, which presented significant increased respiratory rate and urinary volume. NA = Not Analysed.

It should be emphasized that intravenous CMs are a tool that has gained great importance in recent years for directing therapy, and in many cases for dispensing additional propaedeutic in the diagnosis of various diseases. However, there is an urgent need to advance MRI possibilities due to its lack of specificity with current contrast agents. In this investigation, we have introduced a novel Gd_2O_3:NH_2 nanoparticle aiming its future use for specific imaging

and/or directed therapy. This new nanoparticle showed stimulants results and good biocompatibility with a long exposure time without damaging the organs or animal physiology in any of the evaluations performed. We also explore the kinetics of this new nanomaterial, which is further discussed herein.

High resolution transmission electron microscopy (HRTEM) has shown that the new NP-Gd_2O_3:NH_2 has an average diameter of 3 nm in its large majority, whereas other studies with Gd_2O_3 nanoparticles had an average diameter of 5 to 14 nm[16,17]. It is noteworthy to observe that these studies did not have an amino group coupled to the Gd_2O_3 nanoparticle, justifying the larger size of the nanoparticle in this investigation.

The Gd_2O_3:NH_2 nanoparticle has also shown good biocompatibility in vivo, evidenced by the absence of toxicity in cell lines (BEAS-2B and Vero) and low toxicity in the PBMC, with viability greater than 80% even at higher concentration 100 µg/mL. Similar viability results have also been found in other studies[16,20], suggesting that the Gd_2O_3:NH_2 did not cause any significant cytotoxic effect.

Regarding the MR imaging, some researchers have used intermediate / high repetition times for MR imaging, which could lead to confusion due to the transition between longitudinal relaxation T1 and T2 relaxation[16,17,18,19,28]. In this study, we obtained better signal intensity in the images acquired in longitudinal T1 relaxation, with low repetition time. For in vitro images, we have also found good similarity between this study and others[16,17,18,19,20,28], achieving signal intensities like that found for GD-DOTA (control). Analysis of the graphs that deal with signal intensities of MR images *in vivo*, animals that received 2X NP-Gd_2O_3:NH_2 presented a significant higher signal than animals that received Gd-DOTA. Other relevant information was the signal intensity conferred by the 2X NP-Gd_2O_3:NH_2, which started in the second minute after infusion and continued for the next 24 h. Most of the references regarding the first moment of capturing signal intensities post-injections of Gd_2O_3 nanoparticles, as contrasts in MRI, varied between 15 and 90 minutes, in various organs and structures evaluated[16,17,18,19,20,28]. It should be noted that in this analysis, the concentration of Gd-DOTA was 10 mg/mL, whereas 2X Gd_2O_3:NH_2 was 2 mg/mL, that is, five times lower. Inferring that, even in significantly lower concentrations, NP- Gd_2O_3:NH_2 presented better results in MRI.

The differential of this study in relation to the current scientific literature is the behavior of vital data and renal excretion of animals before and after the injection of CMs. In our study, we have observed a significant increase in the respiratory rate of the animal that received NP-Gd_2O_3:NH_2-based CM injection, with a significant reduction in urinary volume in the animal that received the control CM infusion (Gd-DOTA). To investigate whether this was a specific

effect on the animal lungs or kidneys, we have also tested the *in vitro* NPs toxicity in the human bronchial epithelial cell line (BEAS-2B) and the monkey kidney epithelial cell line (Vero), which demonstrated to be non-toxic. So the physiological effect may be due to unknown physical factors.

That way, the synthesis of new complexes that are more efficient for certain pathological phenomena is highly desired, thus increasing the sensitivity and specificity of this important tool in the diagnosis and treatment of neoplastic, inflammatory and infectious diseases, besides avoiding invasive and mutilating approaches in many cases.

Although one of the limitations of this study may be considered the small sample size for *in vivo* assays, the pre-clinical study was highly significant when considered that *in vitro* assays had replicated most of the findings reported in other articles[16,17,18,19] Similar to another study with Gd_2O_3[16], the *in vitro* and *in vivo* analyses of the Gd_2O_3:NH_2 nanoparticle showed that even at concentrations significantly lower than the Gd-DOTA (Dotarem®) used as control, our NP-Gd_2O_3:NH_2 showed significant signal intensity in the MRI and low toxicity in PBMC. It remains to be demonstrated if the long period of detection is due to phagocytosis by tissue and blood macrophages with slow and long-term excretion.

Finally, our results indicated a significant enhancement of the MRI, good biocompatibility, and long-exposure time for 24 hours of the Gd_2O_3:NH_2 nanoparticle, which allows clinical monitoring of a disease treatment for longer time without major toxicity detected in the evaluations carried out in this study. Most important, this is one of the earliest studies that monitors the kinetics of a novel Gd-contrast agent based on a nanoparticle for 24 hours in comparison to the current contrast used in clinical settings.

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Authors' Contributions

LRG, TAAM, NOD, RSS, LSCF and ACAS conceived and designed the experiments; JEB, TAAM processed and analyzed all the MRI images; LPM, LSCF and ATP performed the *in vitro* assays and analyzed the data; LSCF, LCS and RSS performed the *in vivo* experiments;

JEB, TAAM and LRG interpreted the data and wrote the manuscript. All authors approved the final version of the manuscript.

Competing Interests

The authors declare there is no competing financial interest.

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ANEXO I

Universidade Federal de Uberlândia
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética na Utilização de Animais (CEUA)
Rua Ceará, S/N - Bloco 2T, sala 113 – CEP 38405-315
Campus Umuarama – Uberlândia/MG – Ramal (VoIP) 3423;
e-mail:ceua@propp.ufu.br; www.comissoes.propp.ufu.br

ANÁLISE FINAL Nº 146/15 DA COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS PARA O PROTOCOLO REGISTRO CEUA/UFU 063/15

Projeto Pesquisa: "Meios de contraste alvo para ressonância magnética sintetizados a partir de nanotecnologia".

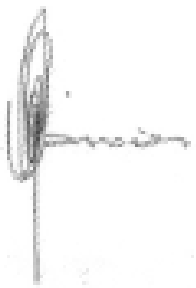
Pesquisador Responsável: Túlio Augusto Alves Macedo

O protocolo não apresenta problemas de ética nas condutas de pesquisa com animais nos limites da redação e da metodologia apresentadas. Ao final da pesquisa deverá encaminhar para a CEUA um relatório final.

SITUAÇÃO: PROTOCOLO DE PESQUISA APROVADO.

OBS: O CEUA/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEUA PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

Uberlândia, 31 de julho de 2015.



Prof. Dr. César Augusto Garcia
Coordenador da CEUA/UFU

ANEXO II

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

CERTIFICADO

Certificamos que o projeto intitulado "Meios de contraste alvo para ressonância magnética sintetizados a partir de nanotecnologia", protocolo nº 063/15, sob a responsabilidade de **Túlio Augusto Alves Macedo** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – 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 da Experimentação Animal (CONCEA), e foi **APROVADO** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião de **10 de julho de 2015**.

(We certify that the project entitled "Meios de contraste alvo para ressonância magnética sintetizados a partir de nanotecnologia", protocol 063/15, under the responsibility of Túlio Augusto Alves Macedo - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of July 10th, 2015.

Vigência do Projeto	Início: 01/08/2015 / Término: 31/07/2017
Espécie / Linhagem / Grupos Taxonômicos	*Rattus norvegicus* / Wistar
Número de animais	53
Peso / Idade	200-300g / 60-65 dias
Sexo	Machos e Fêmeas
Origem / Local	CBEA
Número da Autorização SISBIO	-
Atividade(s)	-

Uberlândia, 31 de julho de 2015.

Prof. Dr. César Augusto Garcia
Coordenador da CEUA/UFU