UNIVERSIDADE FEDERAL DO PAMPA

CAROLINE SILVEIRA MARTINEZ

EFEITOS DA EXPOSIÇÃO AO ALUMÍNIO SOBRE PARÂMETROS NEUROLÓGICOS, REPRODUTORES, CARDIOVASCULARES E BIOQUÍMICOS EM RATOS

Uruguaiana
2017

CAROLINE SILVEIRA MARTINEZ

EFEITOS DA EXPOSIÇÃO AO ALUMÍNIO SOBRE PARÂMETROS NEUROLÓGICOS, REPRODUTORES, CARDIOVASCULARES E BIOQUÍMICOS EM RATOS

Tese apresentada ao programa de Pós-graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Bioquímica.

Orientadora: Profª. Drª. Giulia A. Wiggers Peçanha

Co-orientadora: Drª. Marta Miguel Castro

Uruguaiana
2017

M298e Martinez, Caroline Silveira
EFEITOS DA EXPOSIÇÃO AO ALUMÍNIO SOBRE PARÂMETROS NEUROLÓGICOS, REPRODUTORES, CARDIOVASCULARES E BIOQUÍMICOS EM RATOS / Caroline Silveira Martinez.
111 p.

Tese(Doutorado)-- Universidade Federal do Pampa, DOUTORADO EM BIOQUÍMICA, 2017.
"Orientação: Giulia Alessandra Wiggers Peçanha".

1. Efeitos do alumínio sobre a memória e comportamento.. 2. Efeitos do alumínio sobre o sistema reprodutor masculino.. 3. Efeitos do alumínio sobre o sistema cardiovascular.. I. Título.

CAROLINE SILVEIRA MARTINEZ

EFEITOS DA EXPOSIÇÃO AO ALUMÍNIO SOBRE PARÂMETROS NEUROLÓGICOS, REPRODUTORES, CARDIOVASCULARES E BIOQUÍMICOS EM RATOS

Tese apresentada ao programa de Pós-graduação *Stricto Sensu* em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutora em Bioquímica.

Área de concentração: Bioprospecção Molecular.

Tese defendida e aprovada em 13 de dezembro de 2017.
Banca examinadora:

Profª. Drª. Giulia Alessandra Wiggers Peçanha
Presidente / Orientadora
(Universidade Federal do Pampa/ UNIPAMPA)

Prof. Dr. Dalton Valentim Vassallo
(Universidade Federal do Espírito Santo / UFES)

Prof. Dr. Paulo Bayard Dias Gonçalves
(Universidade Federal de Santa Maria / UFSM)

Prof. Dr. Robson Luiz Puntel
(Universidade Federal do Pampa / UNIPAMPA)

Prof. Dr. Fabiano Elias Xavier
(Universidade Federal do Pernambuco / UFPE)

Dedico aos meus pais Júlio e Glória,
por todo amor, compreensão, incentivo
e apoio incondicionais.

AGRADECIMENTOS

Eis que hoje estou encerrando um ciclo, como a lagarta que trabalhou duro para que finalmente pudesse ver suas asas de borboleta cumprirem suas funções. A parte mais fácil talvez agora seja a mais difícil e, esquecer de citar alguém nessa etapa infelizmente é possível uma vez que muitos anjos passaram pelo meu caminho ao longo desses quase 4 anos. Quando aceitei esse desafio a primeira coisa que fiz foi entregar nas mãos de Deus e pedir que Ele me orientasse, guiasse e capacitasse para que ao final dessa jornada Ele pudesse se orgulhar como um pai sente orgulho de uma filha. Hoje eu agradeço a Deus e, mesmo que clichê, agradeço por absolutamente tudo, sejam lutas ou vitórias (que não foram poucas – as duas!). Paizinho, eu não seria capaz de nada se não fosse a sua proteção, cuidado, direção e sabedoria.

Seguindo os agradecimentos, agradeço a minha família mãe, pai e queridos irmãos. A minha raiz nessa terra, o meu ir e o meu voltar, o significado do amor incondicional. A mãe, minha melhor amiga que resolve desde os problemas do dia a dia até como colocar Madrid dentro de uma (ou quatro!) malas. E ao pai, o mais cuidadoso e zeloso sempre com sua clássica pergunta "você já comeu?", independente da região do planeta e fuso horário que você se encontre e, o qual, com toda a sabedoria direciona e soluciona tuas "questões insolucionáveis" em 5 minutos.

Quero agradecer especialmente a minha orientadora Profª Drª Giulia Alessandra Wiggers Peçanha, sem dúvida muito mais que uma orientadora, foi e é uma grande amiga, irmã e em alguns momentos (ou muitos!) como uma mãe. Me orientando e oportunizando desde desafios acadêmicos e científicos até a tomada de decisões pessoais importantes. Foram muitos anos de aprendizagem, trabalho, admiração, amizade e amor. Como toda relação madura e firme, a nossa não termina aqui, mas permite o início de outro grande ciclo, o qual tenho certeza que será ainda mais bonito e iluminado. Muito obrigada por tudo.

Agradeço ao grande Mestre Franck (também conhecido por Prof Dr. Franck Maciel Peçanha), lembro como se fosse ontem o primeiro dia de aula na graduação com "o bicho" e não, não foi de Fisioterapia Cardiorrespiratória, o primeiro que aprendi com o mestre foi a aplicação de recursos terapêuticos manuais (sim ele já deu aula de RTM!). Desde então, foram incontáveis os ensinamentos, conselhos e ajudas recebidas deste professor ao qual alimento uma admiração "inenarrável". Hoje cumpro parte do "Meu Currículo dos Sonhos", planejado na sua disciplina no dia 18/08/2008 por uma aluna sonhadora que mal sabia a diferença entre

Stricto e *Lato Sensu*. Desejo que muitos outros "alunos sonhadores" tenham a mesma sorte de cruzar o seu caminho. Obrigada, muito obrigada.

Uma segunda família, definição dos irmãos que encontrei no laboratório quando voltei ao Brasil. Paola, Elane e José (grupo PB com intrusas do Hg e Al!) muito obrigada por todo apoio, incentivo, carinho, abraços e "chichos". Vocês fizeram esse ano mais leve, alegre e especial. Paola, minha amiga muito obrigada pelo seu poio, amizade e carinho, sempre me "colocando" para cima, você é uma joia rara. Elane, foi tão lindo compartilhar contigo esse ano e saber que a UNIPAMPA me trouxe uma amiga para a vida toda, nunca se esqueça: "É porque Deus quer!". José, a energia boa e luz em pessoa, obrigada por todo o carinho e sábias palavras de sempre. Eu quero estar bem pertinho (fisicamente ou no coração de vocês) para ver a alegria e as vitórias de cada um e, então brindaremos e "zumbaremos" juntos a felicidade e o sucesso de vocês. Agradeço a tia Estela, muito mais do que a responsável pela manutenção da limpeza laboratório mas sim uma pessoa usada por Deus para trazer luz e conforto a todos os seus "sobrinhos". Não posso deixar de agradecer todos os demais colegas do GPFisCar e os incansáveis IC´s que passaram pelo laboratório ao longo desses quatro anos, muitos dos quais hoje são colegas de pós-graduação: Patrícia, Alyne, Pedro, Janaína, Danize, Verônica, Marina, Geovana e Natália.

Não poderia faltar um agradecimento especial ao "Chefe" ou Prof. Dr. Dalton Valentim Vassallo, nosso padrinho mesmo antes da formação do GPFisCar o qual eu arriscaria chamar de "o culpado de tudo". Chefe, obrigada por estar presente em todos os nossos principais momentos e, por compartilhar conosco suas ideias, conselhos e experiências (haa e por aquele tinto de verano em Puerta del Sol!). É uma honra poder dividir esse momento com o senhor, muito obrigada!

Gostaria de lembrar aqui minhas queridas amigas e agradecer pelo constante incentivo, amizade e carinho que sempre recebi de todas, me sinto extremamente abençoada em manter amizades verdadeiras há pelo menos 17 anos, obrigada Vivi, Tanize, Marília, Daniele, Kelli, Carol e meu presente da Fisioterapia Juliana Bordin.

Creo que si pudiera dividir mi vida en dos, sería antes y después de Madrid. No solamente por los retos y logros profesionales sino que también por el cambio personal. Por lo tanto, la primera persona a quien quiero agradecer es mi tutora y directora Drª Marta Miguel Castro. La persona que me recibió no solamente en su laboratorio pero también en su hogar, que ha compartido conmigo su familia sobretodo sus padres Emilio y Juana y su hermana Natalia, a quienes les agradezco muchísimo todo el cariño y amor que recibí. Marta, te lo

agradezco por todo el aprendizaje, confianza y oportunidades que me has dado mientras estuve en Madrid. Me alegra muchísimo saber que hoy, en el día de la lectura, vas a poder estar aquí con nosotros, será un gran honor y alegría. Muchísimas gracias por todo.

A ver, empezamos con los agradecimientos por las Universidades o gran grupos que he tenido el placer y honor de trabajar en España, que son 3!! Madre mía, vaya agradecimientos! Lo bueno es que fueron 3 despedidas también ☺ (o malo porque me hicieron llorar muuucho jajajajaj).

Universidad Rey Juan Carlos, mi primera grande casa en Madrid, agradezco enormemente por todo lo que he aprendido y por todas las amistades que hice y que voy a llevar para siempre en mi corazón. Sobretodo para las familias de Farmacología, Histología y Bioquímica! Mi familia de Farma, ninguna palabra será lo suficiente para expresar el amor que tengo por vosotros, la cena de despedida que habéis organizado sin duda fue una de las sorpresas más bonitas que ya he tenido en mi vida! Agradezco a mis "mamás" Lup y Maica por todo el cariño, preocupación y cuidados (y por los mejores dasayunos sin gluten que he probado!). Profª Drª Gema Vera Pasamontes una de las primeras personas que conocí y seguramente de las más importantes que pasaron por mi vida en Madrid, Gema gracias por toda la enseñanza, amistad y por compartir tu familia conmigo en fechas tan importantes y claro por mis regalos de Reyes! Drª Nancy Paniagua, mi amiga y hermana para toda la vida, soy muy afortunada en haberte conocido y tenerte a ti y a tu familia como mi familia también, gracias por todo! Mi amigo Dr. Miguel Ángel Martínez García, gracias por tu amistad, consejos y cervecitas después del trabajo! Agradezco a mis amigas y compañeras de aventuras por Madrid Marta, Paula y Lorena. Marta Martín muchísimas gracias por la sorpresa y la carta, te quiero mucho mi amiga, Lore muchas gracias por grabar mi pre-lectura en Madrid y mi amiga Polaca Paula, thank you for everything and mainly for your advices in our shoppings days!! Muchísimas gracias Profª Drª Raquel Abalo, Drª Ana Bagues y a los jefes del departamento Dr. Carlos Goicoechea e Drª Maribel Martín Fontelles.

Agradezco a la familia del laboratorio de histología de la URJC, especialmente al Prof Dr. José Antonio Uranga-Ocio, el que fue un gran director y amigo para mí mientras estuve en la URJC. Muchísimas gracias a los compañeros y amigos Antonio, Julio y Raquel vosotros sois como uma gran familia y os quiero muchísimo (aún no he tenido oportunidad de utilizar a mi regalo, creo que la llevaré en mi defensa!!). Agradezco imensamente a mi amiga del corazón Davínia, amiga que también compartió conmigo su familia y hogar, te quiero mucho Dav ☺

Me gustaría agradecer a la familia del departamento de Bioquímica de la URJC, especialmente a la Professora Drª María Jesus Alonso, muchísimas gracias por todo apoyo, enseñanza y cariño, eres un gran ejemplo a seguir como persona y profesional. Agradezco muchísimo a la Profª Drª Àngela Martín Cortés, muuuuchímas gracias *Angelines* por todo desde las enseñazas hasta los consejos en los sitios más raros jajajajaj! Guapa, gracias de verdad por tu amistad y cariño! Agradezco mucho por todo el cariño y amistad que recebí de todos del departamento, especialmente a Patrícia Corrales, Profª Drª Adriana Izquierdo, Ismael Velasco González, Elia Escasany y Lucía Torres Carrillo. No puedo olvidarme de las señoras encargadas de la limpieza y de los de la seguridad, mis compañeros de todas las horas sobretodo en Navidad, siempre me saludaban con una sonrisa "Hola, cómo estas Señorita Caroline".

En 2013, mientras hacía mi master en Brasil, he tenido el placer de conocer a el grupo de la Profª Drª Mercedes Salaices en el Departamento de Farmacología de la Universidad Autónoma de Madrid, en la ocasión me acuerdo muy bien que la idea de volver para hacer el doctorado era como un sueño y hacía brillar a mis ojos. Pues hoy, agradezco muchísimo la oportunidad y puedo decir que convivir con vosotros eses meses fue algo inolvidable, no solamente por el aprendizaje (que fue mucho) pero especialmente por conocer a un grupo que trabaja con el amor y el corazón en las manos. Estoy segura que eso es un reflejo de la jefa que tenéis, una verdadera reina en el sentido más puro de la palabra, muchísimas gracias Prof. Drª Mercedes Salaices por enseñarnos que la ciencia puede y debe ser sinónimo de amor y amabilidad. Agradezco muchísimo a la Drª Ana Briones, mi gran amigo Dr. Roberto Palacios, Drª Ana Gracia Redondo, Drª Raquel Rodrígues y MsC Maria Gonzalez, por toda ayuda y amistad.

Agradezco a las amigas y compañeras del Instituto de Investigación en Ciencias de la Alimentación (CIAL/CSIC), especialmente a Silvia Moreno, Estefanía Jimenez, Sara Moreno, Francisca Fernandez y Drª Marta Garcés, gracias por todo chicas!

Agradezco también a mis amigos y compañeros de piso Raul y Andrea, mi pequeña gran family en Madrid. Andrea gracias por tu amistad, por nuestros viajes, por estar conmigo y hacer de mí cumple un día especial. Ami me alegro mucho en haberte pasado un poquito de mi amor por la ciencia y espero luego verte Doctora!

I would like to say thank you to Dr. Christopher Exley from Keele University for the excellent opportunity of work at your laboratory. Surely, it was a brilliant opportunity and made me grown up as a scientist, thank you for the constant incentive and important scientific support and advices. Thank you for my attendance and participation at 12th Keele, Vancouver, Canada,

It was a unique and very special event on Aluminum. I am also gratefull for your research group, especially Dr. Matthew Mold for his help and expertise on staining techniques especially with lumogallion, Dr. Emma Shardlow and my friends MsC. Isabel Rodríguez and sister MsC Jacqueline Cerdas. Dr. Chris, work at your laboratory besides of my scientific improve allowed me to make big friends. Thank you for everything!!

Finalmente um agradecimento especial aos professores componentes da banca examinadora deste trabalho Prof. Dr. Dalton V. Vassallo, Prof. Dr. Paulo Bayard Dias Gonçalves, Prof. Dr. Robson Puntel e Prof. Dr. Fabiano Elias Xavier, obrigada pelo aceite em avaliar esse trabalho, será uma grande honra tê-los com suas *expertises* colaborando e enriquecendo esse momento.

Gostaria de agradecer a Universidade Federal do Pampa, ao Programa de Pós-Graduação em Bioquímica, a coordenadora Profª Drª Daiana Silva de Ávila, a Profª Drª Cristiane Denardin e a todo excelente corpo docente deste programa de pós-graduação pela oportunidade de realizar e concluir mais esta etapa.

Agradeço as agências de fomento do governo brasileiro Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [406715/2013-0, 203503/2015-5], Programa Nacional de Cooperação Acadêmica, Pró-reitoria de Pesquisa − Universidade Federal do Pampa [No. 10.134.14] e ao governo da Espanha [MINECO −AGL2012-32387, CSIC − Intramural 201570I028 and SAF2015-69294- R] pelo auxílio financeiro e concessão de bolsas de estudo que garantiram o sustento e possibilitaram a realização desta tese de Doutorado. I would like to say thank you for Keele University and the Keele Meeting committee for the bursary for attendance at the 12th Keele Meeting in Vancouver, Canada.

Y para acabar con los agradecimientos (que ya van 5 páginas!), quiero decir que vivir fuera de mí país fue el experimento más importante que hice en mi tesis. Suelo siempre decir que España me ha dado muchas cosas y ahora quiero agradecer a la persona que conocí exactamente 30 días antes de volver a Brasil. Andrés Martínez Artal, muchas gracias por tu apoyo, cariño y amor, seguramente fue la fuerza motriz que necesitaba para llegar hasta aquí y cerrar esa etapa tan importante. Hoy entiendo el significado de la preeminencia “Y si tuviese profecía, y entendiese todos los misterios y toda ciencia, y si tuviese toda la fe, de tal manera que trasladase los montes, y no tengo amor, nada soy.” Corintios 13:2.

“Precisamos perseguir nossos
mais belos sonhos.”

Augusto Cury

“E agora, deixe-a voar.
Disse ao vê-la sair do casulo.”

Caroline Silveira Martinez

RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa

EFEITOS DA EXPOSIÇÃO AO ALUMÍNIO SOBRE PARÂMETROS NEUROLÓGICOS, REPRODUTORES, CARDIOVASCULARES E BIOQUÍMICOS EM RATOS

Autora: Caroline Silveira Martinez
Orientadora: Profª. Drª. Giulia Alessandra Wiggers Peçanha
Co-orientadora: Drª. Marta Miguel Castro
Local e Data da defesa: Uruguaiana, 13 de dezembro de 2017.

O Alumínio (Al) é o metal de maior exposição humana, no entanto os efeitos do metal em nível de exposição humana ainda são pouco conhecidos. Assim, o objetivo desse estudo foi investigar os efeitos da exposição ao Al por 60 dias em dose equivalente a exposição humana ao metal através da dieta sobre o Sistema Nervoso Central (SNC), Sistema Nervoso Periférico (SNP), sistema reprodutor masculino e sistema cardiovascular e, comparar com os efeitos de uma exposição alta ao metal com efeitos tóxicos conhecidos. Para isso, ratos *Wistar* com 3 meses de idade foram divididos em: 1) Grupo 1: baixas doses de Al, onde durante 60 dias os ratos receberam por água de beber: a) Controle – água ultrapura; b) Al na dose de 1,5 mg/kg de peso corporal e, c) Al na dose de 8,3 mg/kg de peso corporal e, 2) Grupo 2: alta dose de Al, onde durante 42 dias os ratos receberam por gavagem: a) Controle – água ultrapura; b) Al na dose de 100 mg/kg de peso corporal. O tratamento com Al mesmo em baixas doses prejudicou a memória de reconhecimento de objetos e promoveu o desenvolvimento de catalepsia nos ratos. Somado a isso, a exposição ao Al aumentou os níveis de espécies reativas de oxigênio (EROs) e de peroxidação lipídica, reduziu a capacidade antioxidante e inibiu a atividade da acetilcolinesterase no hipocampo dos animais. No SNP, o Al promoveu o desenvolvimento de alodínea mecânica, aumentou o estresse oxidativo sistêmico, induziu inflamação com recrutamento de macrófagos e, o metal foi capaz de depositar-se entre as fibras do nervo ciático. Já no sistema reprodutor masculino, a exposição ao Al reduziu a contagem espermática, a motilidade e a produção diária de espermatozides, aumentou a porcentagem de espermatozoides com anormalidades morfológicas, alterou a estrutura testicular, aumentou os níveis de estresse

oxidativo e a inflamação testicular, demonstrando que uma baixa concentração do metal nos testículos (3.35 μg/g) é o suficiente para comprometer a espermatogênese e a qualidade dos gametas masculinos. No sistema cardiovascular, o Al aumentou a pressão arterial sistólica, reduziu a resposta vasodilatadora a acetilcolina, aumentou a resposta vasoconstritora a fenilefrina, reduziu a modulação endotelial na resposta vasoconstritora, reduziu a biodisponibilidade de óxido nítrico, o envolvimento dos canais de potássio nas respostas vasculares e aumentou a produção de EROs principalmente via NAD(P)H oxidase e de prostanóides contráteis da via da COX-2. A exposição ao Al aumentou o estresse oxidativo em artérias aorta e mesentérica, reduziu a expressão de mRNA de eNOS e SOD1 e aumentou a expressão da isoforma da NAD(P)H oxidase 1, COX-2 e a expressão de TXA-2 R. Tomados em conjunto, nossos dados demonstram que a exposição subcrônica ao Al por 60 dias em baixa dose, que reflete a exposição humana ao metal através da dieta, alcança um limiar tóxico suficiente para promover efeitos adversos no SNC, SNP, sistema reprodutor masculino e sistema cardiovascular. Além disso, os efeitos de uma exposição em baixa dose são praticamente os mesmos de uma exposição alta ao metal.

Palavras-chave: metal; disfunção cognitiva; disfunção periférica; qualidade espermática; disfunção vascular; estresse oxidativo; inflamação.

ABSTRACT

Dissertation of Ph.D - Doctor of Philosophy
Graduate Program in Biochemistry
Federal University of Pampa

EFFECTS OF ALUMINUM EXPOSURE ON NEUROLOGICAL, REPRODUCTIVE, CARDIOVASCULAR AND BIOCHEMICAL PARAMETERS IN RATS

Author: Caroline Silveira Martinez
Advisor: Drª Giulia Alessandra Wiggers Peçanha
Co-advisor: Drª Marta Miguel Castro
Date and Place of Defense: Uruguaiana, December 13, 2017.

Aluminum (Al) is the most important environmental and human contaminant. While a good deal of research has been conducted on the acute toxic effects of Al, little is known about the effects of longer-term exposure at human dietary Al levels. Therefore, the purpose of this study was to investigate the effects of 60-day Al exposure at low doses on Central Nervous System (CNS), Peripheral Nervous System (PNS), male reproductive system and cardiovascular system for comparison with a model of exposure known to produce toxicity in rats. Three-month-old male *Wistar* rats were divided into two major groups: 1) Grou 1, low aluminum levels - rats were treated orally by drinking water for 60 days as follows: a) Control – received ultrapure drinking water; b) Aluminum at 1.5 mg/kg b.w. and c) Aluminum at 8.3 mg/kg b.w. and 2) Group 2, high aluminum level - rats were treated through oral gavages for 42 days as follows: a) Control – received ultrapure water; b) Aluminum at 100 mg/kg b.w. Al treatment even at low doses promoted recognition memory impairment seen in object recognition memory testing and catalepsy behavior in rats. Moreover, Al increased hippocampal reactive oxygen species (ROS) and lipid peroxidation levels, reduced antioxidant capacity and decreased acetylcholinesterase activity. On PNS, Al promoted the development of mechanical allodynia, increased inflammation in the sciatic nerve, systemic oxidative stress and, is able to be retained in the sciatic nerve. Regarding the male reproductive system, Al decreased sperm count, daily sperm production, sperm motility, normal morphological sperm, impaired testis histology; increased oxidative stress in reproductive organs and inflammation in testis, showing that low concentrations of Al in testes (3.35 μg/g) are sufficient to impair

spermatogenesis and sperm quality. On cardiovascular system, Al increased systolic blood pressure, decreased acetylcholine-induced relaxation, increased response to phenylephrine, decreased endothelial modulation of vasoconstrictor responses, the bioavailability of nitric oxide, the involvement of potassium channels on vascular responses, as well as increased ROS production from NAD(P)H oxidase and contractile prostanoids mainly from COX-2 in both aorta and mesenteric resistance arteries (MRA). Al exposure increased vascular ROS production and lipid peroxidation as well as altered the antioxidant status in aorta and MRA. Al decreased vascular eNOS and SOD1 mRNA levels and increased the NAD(P)H oxidase 1, COX-2 and TXA-2 R mRNA levels. Taken together, our data demonstrate that 60-day subchronic exposure to low doses of Al from feed and added to the water, which reflect human dietary Al intake, reaches a threshold sufficient to promote adverse effects on SNC, PNS, male reproductive system and cardiovascular system. Moreover, these effects were almost the same as Al exposure at much higher levels.

Keywords: metal; cognitive dysfunction; peripheral neuropathy; sperm quality; vascular dysfunction; oxidative stress; inflammation.

LISTA DE FIGURAS

PARTE I

PARTE II

PARTE III

PARTE IV

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ACh - Acetilcolina

AChE - Acetilcolinesterase

Al - Alumínio

$AlCl_3$ - Cloreto de alumínio

COX-2 - Ciclooxigenase 2

DA - Doença de Alzheimer

EFSA - European Food Safety Authority

eNOS - Óxido nítrico sintase endotelial

EROs - Espécies reativas de oxigênio

FAO/WHO - Food and Agriculture Organization of the United Nations / World Health Organization

FSH - Hormônio folículo estimulante

GSH - Glutationa

H_2O_2 - Peróxido de hidrogênio

HPV - Papiloma Vírus Humano

i.p. - Intraperitoneal

JECFA - Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives

LH - Hormônio luteinizante

MDA - Malondialdeído

mg/kg - Miligramas por quilograma

mL - Mililitros

Mrna - Messenger RNA

NO - Óxido nítrico

NOX - NADPHoxidase

OMS - Organização Mundial de Saúde

$ONOO^-$ - Radical peroxinitrito

PDE - Produção diária espermática

Phe - Fenilefrina

SNC - Sistema Nervoso Central

SNP - Sistema Nervoso Periférico

SOD - Superoxido dismutase

TGI - Trato gastro-intestinal

TXA2 - Tromboxano A2

TXA-2 R - Receptor de tromboxano A2

μg/g - Microgramas por grama

μg/kg - Microgramas por quilograma

μg/L - Microgramas por litro

μM - Micromolar

SUMÁRIO

PARTE I

PARTE II

PARTE I

INTRODUÇÃO

1 ALUMÍNIO

1.1 Propriedades Físico-químicas do Alumínio

O alumínio (Al) é o metal mais presente e o terceiro elemento mais abundate da crosta terreste, após oxigênio e silício (EXLEY, 2012). Apesar da sua abundância, não apresenta função essencial em nenhum sistema bioquímico de qualquer organismo existente (EXLEY, 2013). Pertencente ao terceiro período da família do Boro (família IIIA) da Tabela Periódica, possui número atômico 13 e, na sua forma elementar, o Al é um metal de cor branca prateada (AZEVEDO; CHASIN, 2003).

Inicialmente extraído da bauxita, principal minério rico em Al com 52% de óxido de alumínio, o metal tem uma valência fixa de III, de modo que os compostos de Al podem libertar o íon Al^{3+}em solução aquosa. No entanto, apenas pequenas quantidades de Al^{3+} existem em solução aquosa uma vez que em pH neutro o metal apresenta uma tendência a precipitar-se e/ou formar compostos polinucleares complexos / polihidróxidos moderadamente solúveis, o que não ocorre em pH ácido (HALLIWELL; GUTTERIDGE, 1999).

A alta valência do íon Al^{3+} (+3) juntamente com seu pequeno raio atômico conferem ao metal características físico-químicas altamente desejáveis como alta reatividade, condutividade para calor e eletricidade, maleabilidade, leveza, baixo ponto de fusão, resistência à corrosão e grande reciclabilidade (ALDRIDGE; DOWNS, 2011). Devido as suas características e grande versatilidade de uso, o Al tornou-se após a Segunda Guerra Mundial o metal não-ferroso mais utilizado no mundo (OECD, 2015). No Brasil, a demanda por Al na industria, transporte, transmissão elétrica e infraestruturas em geral, estimulou a produção do metal e, tornou o Brasil o 4º maior produtor mundial de bauxita e o 3º maior produtor mundial de alumina (BRASIL, 2012; CNI, 2012).

Além das importantes características físicas e baixo custo de produção o Al ainda possui um potencial de reciclagem praticamente ilimitado e igualmente pouco custoso (IAI, 2009). Assim, ainda que o Al possa ser substituído por outros materiais em algumas de suas aplicações como por magnésio, titânio, aço ou cobre, essa susbtituição não é economicamente vantajosa

para a indústria e, reduziria a qualidade, eficiência, leveza e, a praticamente infinita capacidade de reciclagem do Al.

1.2 Exposição Humana ao Alumínio

A habilidade de separar o Al de seus minérios em escala industrial transformou o metal que antes era em grande parte decorativo para o metal mais utilizado do século XXI (AZEVEDO; CHASIN, 2003). As sociedades industrializadas, a queima de combustíveis fósseis e a versatilidade dos compostos de Al contribuíram para o estabelecimento de um espaço temporal recentemente batizado de "A Era do Alumínio", período compreendido entre o século XX até os dias atuais (EXLEY, 2013).

Diariamente os seres humanos estão expostos ao Al onde, fontes dietéticas e não-dietéticas são responsáveis pela carga corporal humana do metal. A exposição humana ao Al pela dieta ainda é a principal forma pela qual os humanos estão expostos ao metal. Sais de Al são adicionados aos alimentos processados por inúmeras razões, como por exemplo: corantes, emulsificantes, conservantes, para ajuste de pH, como agentes neutralizantes e tamponamento. A água também é uma fonte de exposição humana ao Al uma vez que o sulfato de Al é utilizado para o tratamento da água potável, em um processo chamado de coagulação-floculação (WALTON, 2014).

A Organização Mundial de Saúde (OMS) aponta que em média a exposição derivada de alimentos para um adulto é de 5 mg/dia, chegando a 5g/dia para usuários regulares de antiácidos e analgésicos contendo Al (*Food and Agriculture Organization of the United Nations / World Health Organization - FAO/WHO, 2007*). A exposição por meio da água para um adulto é de aproximadamente 0,1 mg/L, representando 4% do total de exposição oral. No Brasil o Ministério da Saúde estipulou uma concentração máxima de Al na água para o consumo humano de 0,2 mg/L (Portaria 2914/2011, Ministério da Saúde, 2011). Estima-se que a ingesta de Al exclusivamente pela dieta corresponda a um montante de 1 a 95 mg/Al/dia, no entanto essa é uma estimativa altamente variável uma vez que toda e qualquer ingesta alimentícia representa uma carga de exposição humana ao Al, o que praticamente inviabiliza uma mensuração mais precisa (GREGER, 1993). Além disso, o uso de medicamentos contendo Al aumenta consideravelmente a exposição humana ao metal. Antiácidos e aspirinas tamponadas podem fornecer doses de 50-1000 mg / Al por dia (REINKE *et al*., 2003).

Recentemente, agências reguladoras internacionais ajustaram de 7 para 1 mg/Al/kg de peso corporal a exposição semanal máxima ao Al pela dieta (*Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives JECFA - WHO 2007 and, European Food Safety Authority - EFSA 2008*), no entanto esse valor é fácilmente excedido por uma grande parcela da população (FEKETE *et al*., 2013; GONZALEZ-WELLER *et al*., 2010; YANG *et al*., 2014). Além disso, levando em consideração as inúmeras fontes de exposição humana ao Al, não há uma normativa internacional que regule ou estabeleça os valores máximos seguros advindos das multiplas formas de exposição (EXLEY, 2013).

Dentre as formas de exposição humana não-dietética ao Al estão o uso do metal de forma intencional na formulação de produtos estéticos, cosméticos, desodorantes, protetores solares, cremes corporais e medicamentos e/ou através da contaminação com o ambiente. Antitranspirantes podem fornecer até 2g de Al diretamente na pele todos os dias e, o uso de protetores solares pode fornecer até 5g de Al em um único dia de aplicação (EXLEY, 1998; NICHOLSON; EXLEY, 2007; PINEAU *et al*., 2012). O uso do Al para fabricação de próteses cirúrgicas e odontológicas também estão entre as formas de exposição não-dietética (NICHOLSON; CZARNECKA, 2009; ZAFFE; BERTOLDI; CONSOLO, 2004). Incluído nessa categoria está o uso do Al como adjuvante em vacinas onde os sais hidróxido de alumínio, $[Al(OH)_3]$e o óxido hidróxido de alumínio, [AlO(OH)], são os adjuvantes mais utilizados e, encontrados em vacinas contra o tétano, hepatite A, hepatite B, *Haemophilus influenzae* B, pneumocócica, meningocócica e antraz. O uso dos compostos de Al para otimizar a resposta imunológica á vacinação não tem sido apontado como algo inócuo, ao contrário tem sido relacionado com o desencadeamento de diversas complicações neurológicas e imunológicas (GHERARDI *et al*., 2015). Recentemente, o uso nas vacinas contra o Papiloma Vírus Humano (HPV) de um novo adjuvante contendo Al vem sendo relacionado com o surgimento de efeitos adversos como danos neurológicos irreversíveis e morte de crianças e adolescentes em países onde foi estabelecida a vacinação preventiva contra o cancer do colo do útero. Segundo a OMS, foram registrados mais de 167.900 reações adversas relacionadas a vacinação e a notificação de 280 mortes (OMS, 2017).

1.3 Rotas Corporais de Entrada do Alumínio

A carga corporal humana de Al é uma relação dinâmica entre as rotas / vias de entrada do Al e características indivíduais de metabolismo e excreção (EXLEY, 2013). A figura 1 representa brevemente como o corpo humano é impactado pela exposição diária ao Al (Fig 1).

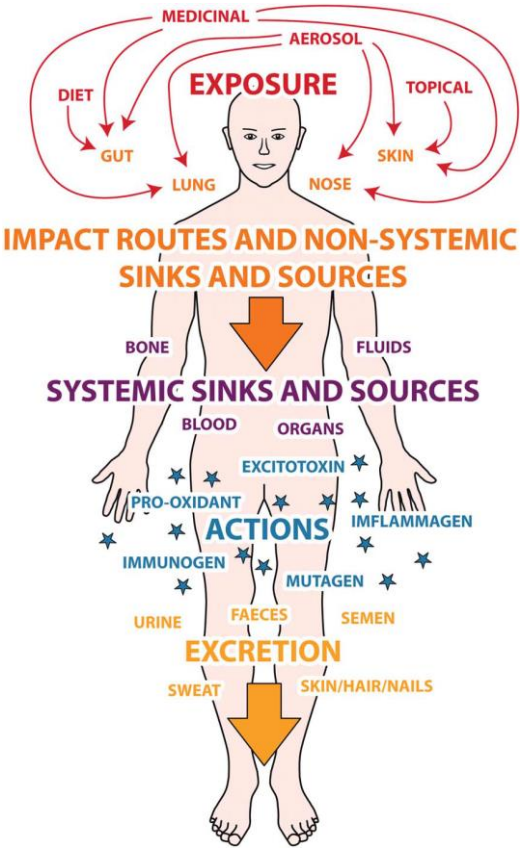

Fig 1. *Aluminium's exposome*, adaptado de EXLEY, 2013. Esquema representando a relação entre exposição ao Al, fontes de exposição, órgãos-alvo do metal, possíveis mecanismos de ação e excreção.

Primeiramente descritas apenas como importantes barreiras frente a exposição ao Al, as 4 principais superfícies corporais: pele, nariz, pulmão e trato gastro-intestinal (TGI) contribuem também para a carga corporal humana de Al além de serem alvos da toxicidade do metal (EXLEY, 2013).

A natureza dos compostos de Al que estão presentes em aplicações tópicas, a quantidade e a frequência de uso sugerem que a pele pode ser considerada uma rota de entrada de Al tanto local como sistêmica (YANAGISHITA *et al.*, 2012). A aplicação tópica de Al como por uso de antitranspirante ou protetor solar e, consequentemente transporte do metal pelo estrato córneo da epiderme envolveria a difusão passiva por rotas trans e paracelulares, entretanto, acredita-se que seja mínimo (FLAREND *et al.*, 2001) (Fig. 2). No entanto, a presença intacta de outras

estruturas da epiderme como glândulas sudoríparas e folículos pilosos facilitariam o transporte e acesso do Al a derme e hipoderme, sistema linfático e sanguíneo bem como possibilitaria a persistência do metal nessas estruturas (Fig. 3) (YANAGISHITA *et al*., 2012).

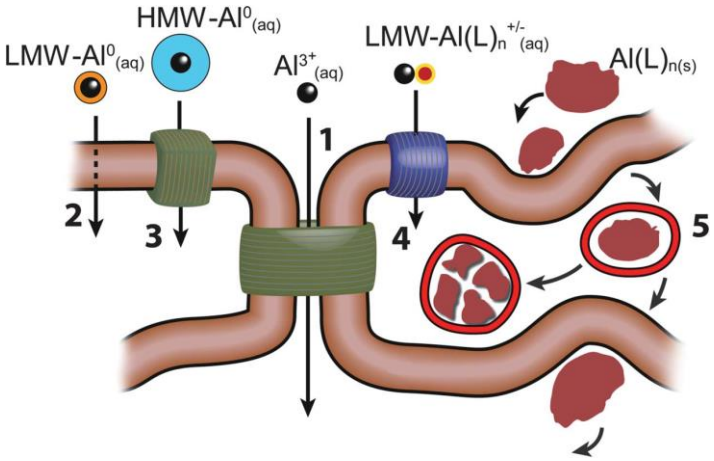

Fig 2. Rotas de transporte e distribuição do Al no corpo humano. Esquema representando as 5 possíveis rotas pelas quais o Al pode ser transportado entre membranas ou células epiteliais: (1) paracelular; (2) transcelular; (3) transporte ativo; (4) canais; (5) absortiva ou endocitose mediada por receptores (*LMW: low molecular weight, neutral, soluble complexes; HMW: high molecularweight, neutral, soluble complexes; LMW-Al(L)n: low molecular weight, charged, soluble complexes +/-; Al(L)n(s): nano and micro-particulates*), adaptado de EXLEY, 2013.

A cavidade nasal também representa uma importate rota de entrada do Al no corpo humano. Após exposição a partículas de Al, como no uso de antitranspirantes aerosóis ou a presença ambiente de Al, o metal entra na cavidade nasal e, após impactar-se com o epitélio pode permanecer entre as camadas mucosas ou ser transportado pela *clearance* mucociliar. O último será direcionado para o TGI enquanto que o primeiro permanecerá entre as camadas da mucosa respiratória, sendo ambos fontes locais ou sistêmicas de Al para o corpo (PERL; GOOD, 1987). Além disso, o epitélio olfativo apresenta continuidade com o nervo e bulbo olfatório, o que poderia representar uma rota de entrada de Al para o cérebro (DIVINE *et al*., 1999).

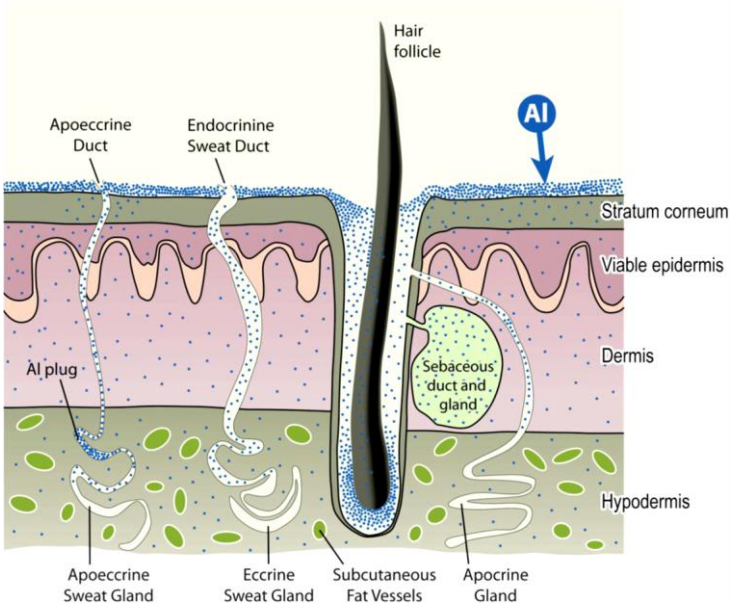

Fig 3. Exposição tópica ao Al. Figura representando a exposição tópica ao Al, onde a pele atuará como fonte de Al biologicamente ativo, tanto para as camadas mais internas da pele como para a circulação sistêmica, adapatado de EXLEY, 2013.

O sistema respiratório apresenta uma grande área de superfície para interações gás-partículas, presença de diferentes tipos celulares, proteínas transportadores e canais, podendo assim constituir uma importante rota de entrada, acúmulo e distribuição sistêmica de Al (RIIHIMAKI; AITIO, 2012).

Já o TGI é o acolhedor imediato de todo Al ingerido pela dieta e do Al depurado pelo processo de limpeza de vias aéras após exposição a suas partículas. Além das inúmeras funções inerentes ao TGI relacionadas a absorção de nutrientes e auxílio no controle de funções imunológicas, por meio das alterações de pH o TGI oferece oportunidades para absorção, retenção no tecido ou mucosa e eliminação do Al através das fezes, além de recentemente apontado como um órgão-alvo do Al (POWELL; JUGDAOHSINGH; THOMPSON, 1999).

1.4 Absorção, Distribuição e Excreção do Alumínio

A forma de exposição ao Al determinará a entrada, absorção e possível acúmulo do metal no corpo humano. Ainda que a entrada do Al possa ocorrer principalmente por 4 diferentes vias (TGI, nariz, vias aéreas / pulmões ou pele), somente a absorção via TGI tem recebido adequeda atenção no meio científico e, ainda assim o mecanimo de absorção ainda não está totalmente esclarecido (EXLEY *et al.*, 1996; ITTEL, 1993). Sugere-se que

características individuais como estado de saúde do indíviduo e do TGI possam influenciar a absorção do metal (EXLEY *et al*., 1996).

A absorção do Al pelo TGI é inferior a 1%, ainda que controversa essa afirmação pode sugerir uma importante participação das demais rotas de entrada contribuindo para a carga corporal de Al (XU *et al*., 1992). A mucosa do TGI parece ter um importante papel na absorção do Al por essa via, podendo dificultar ou facilitar a absorção do metal (COCHRAN *et al*., 1993). O Al, em determinadas condições, pode alterar características reológicas do muco, o que poderia comprometer barreiras protetoras epiteliais (EXLEY *et al*., 1996). Em condições ácidas como no ambiente estomacal o Al é solubilizado e dissociado o que favorece a sua absorção, no entanto sugere-se que ao entrar em contato com o intestino o metal volte a precipartar-se reduzindo assim a absorção do Al (POWELL *et al*.,1994). No entanto, dietas ricas em substâncias ácidas como o citrato poderiam facilitar a absorção do Al pelo TGI (YOKEL *et al*., 1996).

Esforços têm sido feitos para o entendimento do mecanismo pelo qual o Al torna-se sistêmico. Sugere-se o envolvimento de internalização celular (transporte transcelular) ou transporte paracelular de Al. A internalização celular de Al seria um processo passivo e incluiria endocitose, a simples difusão de complexos eletricamente neutros ou possivelmente lipofílicos e a difusão facilitada através de canais iônicos específicos para catións (EXLEY; BIRCHALL, 1992). Já o transporte paracelular seria uma consequência da toxicidade induzida pelo Al e não apenas devido a presença de Al no meio extracelular. Por exemplo, um desequilílbrio na homeostase do cálcio intracelular induzido por Al parece reduzir a eficácia de junções compactas intracelulares (*tight junctions*) em peixes, aumentando assim a permeabilidade epitelial. Somado a isso, a associação do Al a membranas celulares e consequente dano as barreiras protetoras parece estar associado a toxicidade induzida pelo metal (EXLEY; CHAPPELL; BIRCHALL, 1991; EXLEY, 1996). Similar mecanismo foi atribuído ao citrato,o qual parece aumentar a absorção de Al via TGI por alterar a permeabilidade de junções *tight* intracelulares (FROMENT *et al*., 1989). Assim, a taxa de absorção do Al via TGI vai depender desses dois mecanismos de absorção, no entanto, suspeita-se que o transporte paracelular ocorra com uma velocidade maior que a internalizaçõo celular de Al (EXLEY; CHAPPELL; BIRCHALL, 1991). Outro importante fator a influenciar as taxas de absorção de Al é a forma (química / complexado) na qual o metal é carregado para o interior celular ou, tratando-se de transporte paracelular, a forma com a qual o metal entra na corrente sanguínea e fluídos intersticiais. Por exemplo, a incorporação de um complexo Al-transferrina ou de uma molécula

lipofílica neutra poderão influenciar os efeitos diretos da internalização e absorção do metal (EXLEY; CHAPPELL; BIRCHALL, 1991; HARRIS *et al.*, 1996).

Distribuição é o termo utilizado para abordar o transporte do Al e o seu acúmulo em diferentes compartimentos corporais. A presença do metal já foi registrada em sangue total (GRANADILLO *et al.*, 1995), tecidos e órgãos (WALKER *et al.*, 1994) e, em diferentes compartimentos celulares como citosol, mitocôndria, lisossomos e núcleo (KING *et al.*, 1994). O acúmulo do metal em diferentes órgãos e tecidos tem recebido especial atenção. Os primeiros registros apontavam o tecido ósseo e cerebral como principais alvos parao depósito de Al (CRAPPER *et al.*, 1973; ROMANSKI *et al.*, 1993), seguido do tecido renal (SPENCER *et al.*, 1995), hepático (LOTE *et al.*, 1993), múscular, cardíaco e testículo (NOREMBERG *et al.*, 2016).

No entanto, o transporte e distribuição do Al no corpo estão sobre um controle termodinâmico e cinético. O primeiro refere-se a disponibilidade biológica do Al a partir de uma pespectiva de equilíbrio químico, enquanto que, o último refere-se a rota dinâmica que o Al percorre na busca de um equilíbrio. O último é o mais importante tratando-se da disponibilidade biológica do Al, já que a carga coporal do metal está em contínuo estado de fluxo entre os diferentes compartimentos corporais e sob influência das taxas de absorção e excreção (EXLEY *et al.*, 1996). Além disso, a busca de um equilíbrio ou a ocorrência de uma situação (por ex. um trauma) em que desencadeie uma redistribuição de Al entre os compartimentos corporais pode transformar uma carga corporal de Al em um determinado órgão antes benigna em níveis tóxicos do metal. Importante ponto na toxicidade sistêmica do Al sugere-se inclusive que a disponibilidade biológica no cérebro e consequente desencadeamento de doenças atribuídas ao metal, possa estar relacionado com sua redistribuição antes acúmulado em outros compartimentos corporais como o tecido ósseo (EXLEY *et al.*, 1996; KHAN *et al.*, 2013).

O sangue é provavelmente o principal distribuidor do Al sistêmico, uma vez que não existem dados concretos da presença de Al na linfa. No sangue o Al pode ser encontrado no soro ou associado a frações celulares. Estudos sugerem que o Al sérico esteja ligado a transferrina e que esta proteína seja a principal responsável pelo transporte e distribuiçao do Al sistêmico. No entanto, estudos cinéticos contestam (EXLEY; BEARDMORE; RUGG, 2007; BEARDMORE; RUGG; EXLEY, 2007) essa afirmativa e apontam bioligantes de baixo peso molecular para o Al^{3+}como o citrato, fosfato e hidróxido atuando na distribuição do metal entre o sangue e tecidos (BEARDMORE; EXLEY, 2009).

Assim como ocorre com a absorção e distribuição sistêmica do Al, o mecanismo de excreção do metal é pouco conhecido. O Al é excretado do corpo pelas fezes (GREGER; BAIER, 1983), urina, pele, cabelo, unhas (IYENGAR, 1998), suor (GENUIS *et al*., 2011), e também parece ser excretado pelo sêmen (HOVATTA *et al*., 1998). Devido a baixa solubilidade do Al (principalmente os sais), a grande maioria do metal ingerido e considerado não-sistêmico parece ser excretado pelas fezes, enquanto que a urina parece ser a principal rota de excreção do Al sistêmico (EXLEY, 2013).

1.5 Toxicidade do Alumínio

Respostas celulares e sistêmicas desencadeadas pela exposição ao Al e pelo alcance de uma carga corporal do metal podem ser chamadas de metabolismo do Al e, incluem os efeitos tóxicos do metal. Esses efeitos já foram observados em diferentes sistemas e órgãos e, ainda que pouco esclarecidos, tem se atribuído as características pró-oxidantes, citotóxicas, inflamatórias, imunogênicas ou mutagênicas do metal (EXLEY, 2013).

O Al é um potente pró-oxidante podendo agir por duas diferentes vias, pode ligar-se ao radical ânion superóxido ($O2^{\bullet-}$) formando o complexo Al-ânion superóxido, uma espécie potencialmente mais reativa que o $O2^{\bullet-}$ (EXLEY, 2004), assim como por sua ação sinérgica com o ferro promovendo a chamada *Aluminum Fenton Reaction Promotion Cycle*. Ao promover a reação de Fenton através do ciclo: I) O Al é capaz de estabilizar o $O_2^{\bullet-}$; II) O complexo resultante Al (III)-superóxido é capaz de reduzir o Fe (III) para o Fe (II); e então, III) O Fe (II) pode induzir a formação de radicais –OH pela reação de Fenton. Ao final desses passos, que podem ser visualizados na figura 4, serão gerados espécies reativas de oxigênio e a recuperação do Al na sua forma hidrolítica (Al^{3+}) o qual estará pronto para dar início a um novo ciclo (RUIPÉREZ *et al*., 2012).

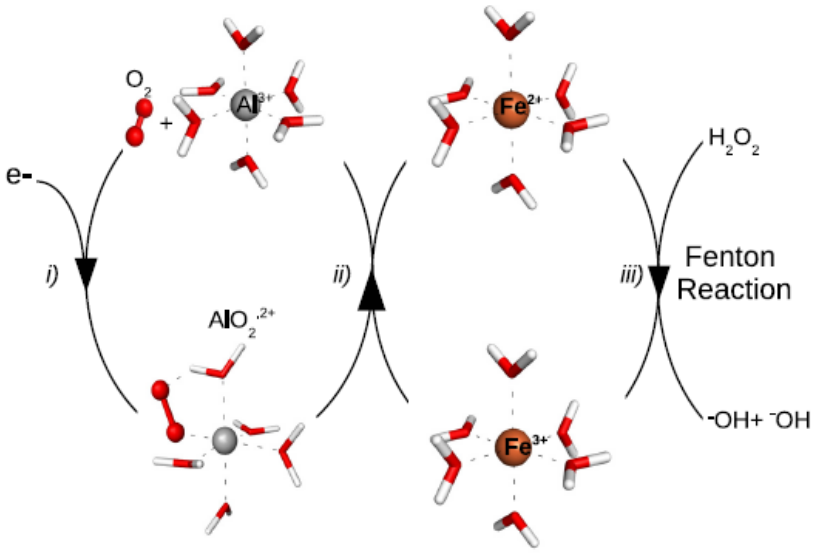

Fig 4. *Aluminum Fenton Reaction Promotion Cycle*, adaptada de RUIPÉREZ *et al*., 2012.

A citotoxicidade causada pelo Al tem sido evidenciada em estudos com animais experimentais e, parece estar implicada nas doenças neurodegenerativas. Uma característica comum entre as avaliações de citotoxicidade causada por Al é a elevada e sustentada presença de cálcio livre intracelular, como ocorre na Doença de Alzheimer (DA) (EXLEY, 1999; EXLEY, 2012; KHACHATURIAN, 1994; SASS *et al*., 1993).

Além disto, a exposição humana ao Al tem sido fortemente relacionada com o desencadeamento de cascatas inflamatórias com a ativação de mediadores e biomarcadores pro-inflamatórios, estes relacionados com a ação pró-oxidante do metal (JOHNSON; SHARMA, 2003; LUKIW; PERCY; KRUCK, 2005) em um amplo número de doenças (CAMPBELL; BONDY, 2000; GHERARDI *et al*., 2001; PERL *et al*., 2000).

A capacidade excitatória do Al sobre o sistema imunológico é conhecida há pelo menos 100 anos e, é a base para o uso dos sais de Al como adjuvantes em vacinas e terapias antialérgicas. No entanto, o seu mecanismo de ação assim como a causa da crescente toxicidade induzida por adjuvantes em indivíduos susceptíveis são, em sua maior parte, desconhecidos (KHAN *et al*., 2013; TOMLJENOVIC; SHAW, 2011).

A mutagenicidade induzida por Al tem sido sugerida há anos, no entanto estudos enfocando a imunogenicidade, cardiogenicidade e/ou teratogênicidade são escassos (BANASIK *et al*., 2005). Células epiteliais da mama na presença de Al em concentrações similares as encontradas em células cancerígenas acumulam mutações, possibilitando assim o desenvolvimento de tumores e metástases (SAPINO *et al*., 2012; MANDRIOTA *et al*., 2016).

No entanto, muito ainda precisa ser esclarecido no que tange a relação do Al e o câncer de mama (MANDRIOTA *et al*., 2017; EXLEY, 2013; LINHART *et al*., 2017).

1.6 Efeitos do Alumínio no Sistema Nervoso Central e Periférico

Após exposição ao Al, a taxa do metal que não é excretada pode acumular-se em diferentes órgãos do corpo humano (CRÉPEAUX *et al*., 2017; FLATEN, 2001; LI *et al*., 2011). A presença do metal em diferentes regiões cerebrais desencadeia respostas tóxicas já observadas *in vitro* ou *in vivo* (BONDY, 2015; WALTON, 2014). Fenotipicamente, o acúmulo do metal no cérebro parece desencadear alterações comportamentais, danos a memória e aprendizado e, redução da atividade locomotora em animais experimentais (LAKSHMI *et al*., 2015; KASBE *et al*., 2015).

Especial atenção tem sido empregada no estudo da relação entre a exposição ao Al e o desenvolvimento de doenças neurodegenerativas (FLATEN, 2001; WALTON, 2014; WANG *et al*., 2016). Por muitos anos o Al tem sido apontado como uma neurotoxina envolvida direta ou indiretamente no desencadeamento da DA. Estudos epidemiológicos mostram a associação positiva entre a exposição ao Al através do consumo de água ou exposição ocupacional com maiores casos da DA (FLATEN, 2001; WALTON, 2014; WANG *et al*., 2016). Pacientes com DA apresentam grandes concentrações do metal nas artérias que irrigam o hipocampo e, já foi demonstrado que o Al é capaz de desencadear respostas neuroquímicas e neuropatotógicas similares às encontradas em pacientes com a doença (BHATTACHARJEE *et al*., 2013, WALTON, 2014). Recentemente, concluiu-se que o acúmulo e retenção do Al no cérebro contribui tanto para o início quanto para a progressão agressiva de todas as formas da DA (EXLEY, 2017, MIRZA *et al*., 2017).

Efeitos do Al no Sistema Nervoso Periférico (SNP) são menos relatados, entretanto sugere-se que o metal possa estar envolvido no desenvolvimento de neuropatias periféricas. O consumo de água contaminada por Al (13.17 - 15.70 ppm) em Kirazli, Península Biga, NW Turquia, foi relacionado a maiores casos de neuropatia periférica na região (BAKAR *et al*., 2010). Além disso, a dor neuropática causada pela cisplatina em camundongos e o concomitante acúmulo de Al em neurônios de gânglio da raiz dorsal foram aliviados pela administração de glutationa e, os autores atribuem os efeitos a capacidade antioxidante e quelante da glutationa (LEE *et al*. 2017).

1.7 Efeitos do Alumínio no Sistema Reprodutor

O aumento dos casos de infertilidade masculina e a concomitante redução da qualidade espermática nas últimas décadas sugerem o envolvimento de fatores ambientais e/ ou ocupacionais (CARLSEN *et al.*, 1992; NELSON *et al.*, 1974; SHEINER *et al.*, 2003). Com o advento da globalização e sociedades modernas o Al tem se tornado cada vez mais presente no dia a dia da população, sendo assim, começaram a ser postulados possíveis efeitos dessa crescente exposição sobre a saúde reprodutiva (DAWSON *et al*., 1998; HOVATTA *et al*., 1998; JAMALAN *et al*., 2016). Recentemente, verificou-se que a presença de Al no sêmen humano está relacionado à redução da qualidade espermática, especificamente, pacientes com oligozoospermia apresentaram maior concentração de Al quando comparados aos pacientes sem disfunção reprodutiva (KLEIN *et al*., 2014).

Experimentalmente, animais expostos ao Al apresentaram alterações hormonais, redução de parâmetros de qualidade espermática, anormalidades histopatológicas em órgãos reprodutores e infertilidade (ABDEL-MONEIM, 2013; GUO *et al*., 2005; IGE; AKHIGBE, 2012; JAMALAN *et al*., 2016; MOHAMMAD; ARAFA; ATTEIA, 2015; SUN *et al*., 2011; ZHU *et al*., 2014). No entanto, estudos experimentais abordando os efeitos tóxicos do Al sobre o sistema reprodutor foram conduzidos com doses altas que não correspondem a níveis próximos de exposição humana ao Al (ODA, 2016; SUN *et al*., 2011; ZHU *et al*., 2014).

1.8 Efeitos do Alumínio no Sistema Cardiovascular

Os efeitos do Al no sistema cardiovascular são pouco conhecidos, no entanto estudos têm reconhecido um possível risco cardiovascular após exposição ao metal (KORCHAZHKINA *et al*., 1999; LIND *et al*., 2012; SUBRAHMANYAM *et al*., 2016). Nos últimos anos, dados epidemiológicos apontam um papel do Al no desencadeamento de doenças cardiovasculares, demonstrando que trabalhadores expostos a partículas aerodinâmicas de Al com diâmetro <2.5 μm apresentam maiores riscos para cardiopatia isquêmica (COSTELLO *et al*., 2014; NEOPHYTOU *et al*., 2016; SUBRAHMANYAM *et al*., 2016). Somado a isso, o Al parece apresentar grande afinidade por células endoteliais humanas, uma vez que demonstrou-se o acúmulo do metal em diferentes leitos vasculares e, a presença circulante do Al parece

contribuir para a rigidez arterial e formação de placas ateroscletóticas (BHATTACHARJEE *et al*., 2013; LIND *et al*., 2012; MINAMI *et al*., 2001; SUBRAHMANYAM *et al*., 2016).

Estudos experimentais sobre o efeito do Al no sistema cardiovascular dão suporte aos dados epidemiológicos. Animais expostos por 120 dias ao Al na dose de 256 mg Al/kg de peso corporal apresentaram um aumento na pressão arterial e danos a membrana eritrocitária (ZHANG *et al.*, 2016). Além disso, uma única dose intraperitoneal (i.p.) de Al a 0.5 mg/kg aumentou a expressão de renina em ratos (EZOMO *et al*., 2009). Igualmente, nosso grupo demonstrou que uma única exposição ao Al na dose de 100 mg/kg de peso corporal via i.p. é capaz de alterar a resposta vascular reduzindo a reatividade vascular (SCHMIDT *et al*., 2016). Entretanto, como demonstrado os estudos conduzidos até o momento empregaram doses de Al consideradas altas.

Ainda que evidências experimentais e epidemiológicas apontem o Al como um potente agente tóxico frente a diferentes sistemas e órgãos, muito ainda precisa ser estudado sobre os efeitos da presença corporal desse metal não essencial. Atualmente, não há uma normativa internacional que regule os níveis seguros de exposição ao Al tomando como base as inúmeras fontes de exposição humana ao metal e, os dados experimentais disponíveis advém de estudos realizados com altos níveis de exposição ao metal. Nesse sentido, são necessários estudos experimentais que simulem a exposição humana ao Al para que assim, os efeitos da presença do Al no corpo humano possam ser melhor esclarecidos.

2 JUSTIFICATIVA

O crescente avanço na expectativa de vida da população mundial vem acompanhado de um similar aumento no número de casos de doenças crônicas. Mesmo com o grande avanço médico e farmacológico, as doenças cardiovasculares ainda representam a maior causa de morbidade e mortalidade no mundo (GORADEL *et al*., 2017). Já as doenças neurodegenerativas representavam em 2010 um total de 35,6 milhões de diagnósticos e, a projeção é que em 2050 existam cerca de 1,25 bilhões de pessoas com demência no mundo, o que representaria 22% da população mundial (WIMO; PRINCE, 2010). E, em relação ao sistema reprodutor, recentes descobertas evidenciam um declínio da qualidade do esperma humano e o aumento da infertilidade masculina inclusive em países que possuíam os melhores índices de fertilidade, sugerindo assim o envolvimento de fatores externos nessa mudança de paradigma (LEVINE *et al*., 2017).

É crescente o número de evidências que apontam a presença de fatores ambientais interferindo negativamente na saúde da população e no desencadeamento de doenças crônicas (QIU *et al*., 2009). O Al é o metal de maior exposição humana e, a sua versatilidade e ampla utilização nos setores industriais e domésticos aumentaram consideravelmente a exposição humana ao metal que ocorre principalmente pela dieta, cosméticos e medicamentos (EXLEY, 2013; EXLEY, 2012). Sem apresentar função biológica, a presença do Al no corpo humano parece estar relacionada com o desenvolvimento de doenças crônicas como DA, aumento do risco cardiovascular e redução da fertilidade masculina (COSTELLO *et al*., 2014; KLEIN *et al*., 2014; NEOPHYTOU *et al*., 2016; WALTON, 2014; WANG *et al*., 2016). No entanto, o conhecimento acerca dos efeitos da exposição ao Al são em parte especulatórios e inconclusivos (CHIN-CHAN *et al*., 2015) e, até o momento advém de estudos experimentais conduzidos com doses de exposição consideradas irrealistas e altas.

Soma-se ao exposto o fato de que mesmo com o recente ajuste do nível de exposição semanal segura ao Al para 1 mg/kg de peso corporal (OMS, 2007), esse valor de exposição é constantemente excedido pelos humanos (FEKETE *et al*., 2013; GONZALEZ-WELLER *et al*., 2010; YANG *et al*., 2014). Nesse sentido, o estudo do impacto da carga corporal total de Al e consequentemente os efeitos sobre a saúde humana precisam ser investigados. Assim, torna-se necessário o desenvolvimento de trabalhos experimentais que melhor simulem a exposição humana ao Al, verificando os efeitos e mecanismos de ação do metal sobre possíveis órgãos-alvo. Ainda, o melhor esclarecimento acerca das ações do Al permitirá o desenvolvimento de

estratégias que reduzam o impacto da crescente e, por muitas vezes imensurável exposição humana ao Al.

3 OBJETIVOS

3.1 Objetivo Geral

Estabelecer um modelo animal de exposição ao Al que simule a exposição humana ao metal pela dieta e, verificar os efeitos dessa exposição sobre o Sistema Nervoso Central (SNC) e SNP, sistema reprodutor masculino e sistema cardiovascular. Ainda, comparar os efeitos da exposição em nível de contaminação humana com uma dose alta de exposição ao metal.

3.2 Objetivos Específicos

Mensurar a ingesta total de Al pelos animais e, investigar os efeitos da exposição ao Al sobre:

Sistema Nervoso Central e Periférico:

- As atividades locomotora e exploratória dos animais;
- Memória de longa duração;
- Características comportamentais (ansiedade e catalepsia);
- Funcionamento do sistema colinérgico em hipocampo e córtex pré-frontal;
- Sensibilidades táctil e dolorosa;
- Quanto a presença de inflamação periférica;
- Biomarcadores inflamatórios e de estresse oxidativo;
- Capacidade do metal em depositar-se no nervo ciático.

Sistema Reprodutor Masculino:

- Acúmulo do metal nos órgãos reprodutores;
- Peso dos órgãos reprodutores;
- Qualidade espermática (motilidade, contagem, produção de espermatozoides e morfologia);
- Análise histopatológica do testículo e epidídimo;

- Biomarcadores inflamatórios e de estresse oxidativo em testículo, epidídimo e próstata.

Sistema Cardiovascular:

- A distribuição do metal no sangue e fígado;
- Pressão arterial;
- Reatividade vascular em artérias de condutância e resistência bem como as possíveis vias envolvidas nesta resposta;
- Atividade oxidante e antioxidante plasmática e vascular;
- Expressão vascular de marcadores de estresse oxidativo e inflamação;
- A produção de ânion superóxido ($O_2^{\cdot -}$) vascular.

PARTE II

Paper published at Neurotox Res. 31(1): 20-30, 2017. IF: 2, 9420. Qualis CBII: B1

Neurotox Res
DOI 10.1007/s12640-016-9656-y

ORIGINAL ARTICLE

Aluminum Exposure at Human Dietary Levels for 60 Days Reaches a Threshold Sufficient to Promote Memory Impairment in Rats

Caroline S. Martinez[1] · Caroline D. C. Alterman[1] · Franck M. Peçanha[1] · Dalton V. Vassallo[2] · Pâmela B. Mello-Carpes[1] · Marta Miguel[3] · Giulia A. Wiggers[1]

Received: 3 March 2016 / Revised: 23 July 2016 / Accepted: 26 July 2016

Abstract Aluminum (Al) is a significant environmental contaminant. While a good deal of research has been conducted on the acute neurotoxic effects of Al, little is known about the effects of longer-term exposure at human dietary Al levels. Therefore, the purpose of this study was to investigate the effects of 60-day Al exposure at low doses for comparison with a model of exposure known to produce neurotoxicity in rats. Three-month-old male *Wistar* rats were divided into two major groups: (1) low aluminum levels, and (2) a high aluminum level. Group 1 rats were treated orally by drinking water for 60 days as follows: (a) control—received ultrapure drinking water; (b) aluminum at 1.5 mg/kg b.w., and (c) aluminum at 8.3 mg/kg b.w. Group 2 rats were treated through oral gavages for 42 days as follows: (a) control—received ultrapure water; (b) aluminum at 100 mg/kg b.w. We analyzed cognitive parameters, biomarkers of oxidative stress and acetylcholinesterase (*AChE)* activity in hippocampus and prefrontal cortex. Al treatment even at low doses promoted recognition memory impairment seen in object recognition memory testing. Moreover, Al increased hippocampal reactive oxygen species and lipid peroxidation, reduced antioxidant capacity, and decreased *AChE* activity. Our data demonstrate that 60-day subchronic exposure to low doses of Al from feed and added to the water, which reflect human dietary Al intake, reaches a threshold sufficient to promote memory impairment and neurotoxicity. The elevation of oxidative stress and cholinergic dysfunction highlight pathways of toxic actions for this metal.

Keywords Aluminum · Cognitive dysfunction · Cholinergic dysfunction · Oxidative stress

✉ Caroline S. Martinez
caroline.s.martinez@gmail.com

✉ Giulia A. Wiggers
giuliawp@gmail.com

[1] Graduate Program in Biochemistry, Universidade Federal do Pampa, BR 472-Km 592, PO box 118, Uruguaiana, RS 97500-970, Brazil

[2] Department of Physiological Sciences, School of Medicine of Santa Casa de Misericórdia (EMESCAM), Av. Nossa Senhora da Penha 2190, Vitória, ES 29045402, Brazil

[3] Instituto de Investigación en Ciencias de la Alimentación, Campus Universitario de Cantoblanco, Nicolás Cabrera, 9, 28049 Madrid, Spain

Introduction

Aluminum (Al), a metal which accounts for about 8 % of the earth's crust, is currently a significant environmental contaminant (Exley 2012). Industrialized societies, the burning of fossil fuels, and the versatile properties of Al compounds have all contributed to increased biological availability of this nonessential metal (Exley 2013).

Oral uptake is an important route by which humans are exposed to Al. Al salts are added to commercially prepared foods for numerous reasons, such as food coloring, as anticaking agents, for pH adjusting, as emulsifiers, as a stabilizing agent, to thicken gravies and sauces, as meat-binders, as a rising agent, for pickling vegetables and candying fruits, as buffers, and as neutralizing agents. Al is also added to urban water supplies and some bottled waters as a clarifying agent (Walton 2014). Nondietary sources of Al include vaccines, where Al salts are used as adjuvants to promote immune activation, and some topical applications, such as sunscreens and deodorants. Considerable amounts

Published online: 29 July 2016

of Al are also contained in buffered aspirins and antacids (Bondy 2015).

The Joint FAO/WHO Expert Committee on Food Additives (Food and Agriculture Organization of the United Nations/World Health Organization) adjusted a tolerable weekly intake of Al for humans to 1 mg Al/Kg body weight (b.w.) (FAO/WHO 2007), which is routinely exceeded by humans. Greger (1993) estimates that Americans consume from 1 to 95 mg/Al/day in the form of Al additives and up to 10 mg/Al/day from natural sources. Moreover, the use of pharmaceutical elements containing Al considerably increases the human Al exposure. Antacids may provide doses of 50–1000 mg/day (Reinke et al. 2003).

The rate of Al absorption of the gastrointestinal tract is around 0.2 % (Priest et al. 1998). Upon reaching the blood, most of the metal is bound to transferrin which facilitates Al transport across the blood–brain barrier and into neurons and glia bearing transferrin receptors (Roskams and Connor 1990; Shirley and Lote 2005). Al can accumulate in different brain regions and its neurotoxicity has been demonstrated in cell culture, animal models, and humans (Bondy 2015; Walton 2014). Al accumulation in neurons has been related to learning and memory impairments, as well as motor incoordination and decrease in locomotor activity (Kasbe et al. 2015; Lakshmi et al. 2015). Altered cholinergic functions in pups and adulthood have also been reported (Ravi et al. 2000; Yellamma et al. 2010). Most Al studies have entailed doses of Al higher than the ones commonly found among human populations. However, human studies suggest that Al is a potential contributor to the onset, progression, or aggressiveness of neurodegenerative diseases (Walton 2014; Bondy 2015). Most epidemiological studies have shown a positive relationship between Al exposure from drinking water or occupational exposure and Alzheimer Disease (AD) (Wang et al. 2016; Walton 2014; Flaten 2001). Moreover, AD patients showed an increasing Al concentration in cerebral arteries that supply blood to the hippocampus (Bhattacharjee et al. 2013). Experimental evidence has also shown that Al can cause neurochemical and neuropathological changes as observed in AD patients (Walton 2014). However, there are still many inconsistencies in the epidemiological and toxicological data concerning Al and neurodegenerative diseases (Chin-Chan et al. 2015).

The full scale of mechanisms underlying Al neurotoxicity remains to be elucidated. For instance, Al^{3+} is a pro-oxidant acting on its own by binding to superoxide ion and promoting the formation of an aluminum superoxide radical ion, a species which is potentially more reactive than the superoxide radical (Exley 2004). The pro-oxidant activity of Al might also be explained by its synergistic action with iron promoting the Fenton reaction by reducing Fe(III) to Fe(II) (Ruipérez et al. 2012).

While a good deal of research has been conducted on the neurotoxic effects of Al, little is known about the effects of long-term exposure at human dietary Al levels. Moreover, with Al being a widespread environmental contaminant, it is crucial to explore the human Al intake threshold sufficient to promote adverse effects. Therefore, the purpose of this study was to investigate the effects of a 60-day Al exposure at low doses similar to human dietary levels on long-term object recognition memory, prefrontal cortex, and hippocampal reactive oxygen species levels, lipid peroxidation, total antioxidant capacity, and then compare these results with a model of exposure known to produce neurotoxicity in rats.

Materials and Methods

Animals

Three-month-old male *Wistar* rats (252–300 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light–dark), giving free access to water and fed with a standard chow ad libitum. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups: (1) low aluminum levels, and (2) high aluminum level. For group 1, low aluminum levels, 18 rats were subdivided ($N = 6$/each) and treated for 60 days as follows: (a) control—received ultrapure drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA, USA); (b) aluminum at 1.5 mg/kg b.w./day based on human dietary levels according to a published protocol described by Walton (2007), at the reduced Al exposure for 60 days, and (c) aluminum at 8.3 mg/kg b.w./day, which corresponds to the same human dietary aluminum levels (1.5 mg/kg b.w.) when translated to an animal dose, based on a body surface area normalization method (Reagan-Shaw et al. 2008). For group 2, high aluminum level, 12 rats were subdivided ($N = 6$/each) and treated for 42 days as follows: (a) control—received ultrapure water through oral gavages; (b) aluminum at 100 mg/kg b.w./day, a protocol known to promote cognitive impairment in rats (Prakash and Kumar 2009).

Rat body weights were measured weekly. After 60 or 42 days of treatment, the animals were submitted to behavioral tests, as follows: control behavioral experiments (open field, plus maze, and hot plate (day 01) and object recognition test (days 02–06). At the end of the treatments and tests, animals were euthanized by decapitation, the brain was removed, and bilateral hippocampi and prefrontal cortex were quickly dissected out and homogenized in 50 mM Tris HCl, pH 7.4, (1/10, w/v). Afterward, samples were centrifuged at 2400*g* for 10 min at 4 °C and the resulting supernatant fraction was frozen at −80 °C for further assay. These brain structures were investigated because of their importance for consolidation of object recognition memory. They also seem to be a target for Al deposition in AD patients (Izquierdo and Medina 1997; Andrási et al. 2005; Rusina et al. 2011).

$AlCl_3 \cdot 6\ H_2O$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water (Milli-Q © 2012 EMD Millipore, Billerica, MA). Salts and reagents were of analytical grade obtained from Sigma and Merck (Darmstadt, Germany).

Aluminum Content in the Rats' Feed

The Al content of rat chow was determined using an established method (House et al. 2012). Briefly, approximately 0.2 g of chow was digested in a 1:1 mixture of 15.8 M HNO_3 and 30 % w/v H_2O_2 in a microwave oven, and the Al content of digests was measured by TH GFAAS (Transversely Heated Graphite Furnace Atomic Absorption Spectrometry).

Behavioral Testing

Open Field, Plus Maze, and Hot Plate

Exploratory and locomotor activities were analyzed to test whether any of the procedures impaired the rat behaviors, altering the memory tests results. Each rat was placed in the left quadrant of a 50 cm × 50 cm × 39 cm open field made with wood painted white, with a frontal glass wall. Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossing and rearing, as measures for locomotor activity and exploration, respectively, were measured over 5 min (Bonini et al. 2006). To evaluate thereby anxiety state, rats were exposed to an elevated plus maze (Pellow et al. 1985). The maze had a central platform (5 × 5 cm), two open arms (50 cm long × 10 cm wide, with 0.5-cm-high borders), and two enclosed arms (50 cm deep × 10 cm wide, with 10-cm-high walls), elevated 50 cm above the ground. An animal was placed in the center of the apparatus facing the open arm and its locomotion was monitored by infrared sensors for 5 min. The time spent in the open arms was recorded. The pain threshold was measured using a hot plate test (Jacob et al. 1974) to ensure that treatment did not impair nociception. In the hot plate test, the animal is placed on the metal plate heated to 55 °C surrounded by a glass cylinder (13 × 17 cm). The latency (in seconds) of the hind paw-licking or jumping was measured. A cut-off time of 45 s was applied. Data from these tests were compared between the groups to ensure all rats presented any impairment in behavior that could affect the variables of interest in our study (i.e., memory measurements).

Object Recognition Memory Test

Chronic Al intake has been related with AD-related symptoms and disruption of object recognition as one of the earliest signs of AD (Walton 2014); thus, we proposed to investigate object recognition memory. After the treatments were completed, training and testing for the object recognition (OR) task were performed in an open-field arena (50 cm × 50 cm × 50 cm) built with polyvinyl chloride plastic, plywood, and transparent acrylic (Ennaceur and Delacour 1988; Mello-Carpes and Izquierdo 2013). Rats were first habituated to the apparatus during 20 min of free exploration for 4 consecutive days. For training, two different objects (a and b) were placed in the apparatus, and rats were allowed to freely explore the objects for 5 min. The objects were made of metal, glass, or glazed ceramic. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects were not considered as exploratory behaviors. A video camera was positioned over the OR arena, and the behavior was recorded using a video tracking system for offline analyses. After 24 h, in the test phase, one of the objects was randomly exchanged for a novel object (c), and the rats were reintroduced into the apparatus to freely explore the objects (familiar and new ones) for 5 min. To avoid confounding by lingering olfactory stimuli and preferences, the objects and the arena were cleaned with 70 % ethanol after each animal was tested. The time spent exploring the familiar and the novel object was recorded.

Biochemical Assay

Reactive Oxygen Species Levels

The levels of ROS in hippocampus and prefrontal cortex were determined by a spectrofluorometric method, as described by Loetchutinat et al. (2005). The supernatant fraction of the sample was diluted (1:10) in 50 mM Tris–Hcl (pH 7.4), and 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; 1 mM) was added to the medium. DCFH-DA

is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2́,7́-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence unit.

Lipid Peroxidation

The levels of lipid peroxidation in hippocampus and prefrontal cortex were measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. (1979), with modifications. An aliquot of each tissue was incubated with thiobarbituric acid 0.8 % (TBA), phosphoric acid buffer 1 % (H_3PO_4), and sodium dodecyl sulfate 0.8 % (SDS) at 100 °C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as nanomoles of MDA per mg of protein.

Ferric Reducing/Antioxidant Power (FRAP) Assay

The total antioxidant capacity was measured in hippocampus and prefrontal cortex by FRAP assay (Benzie and Strain 1996). This method is based on the ability of sample to decrease ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 µL of the supernatant fraction of each tissue was added to 1 mL freshly prepared and prewarmed (37 °C) FRAP reagent (300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37°C for 10 min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 µL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose–response curve of Trolox (50–1000 µM—water soluble analog of vitamin E) was prepared, and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

Acetylcholinesterase (AChE) Activity

AChE is a marker of the loss of cholinergic neurons in the forebrain. The AChE activity was assessed by the Ellman et al. (1961). The reaction mixture was composed of 100 mM phosphate buffer pH (7.4) and 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The method is based on the formation of a yellow anion, 4,4′-dithio-bis-acid nitrobenzoic after adding 0.8 mM acetylthiocholine iodide. The change in absorbance was measured for 2 min at 30 s intervals at 412 nm (SpectraMax M5 Molecular Devices, CA, USA). Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed/min/mg of protein. Proteins were measured according to Bradford (1976) using bovine serum albumin as a standard.

Statistical Analysis

Data are expressed as mean ± SEM. The OR task results were converted to a percentage of total exploration time and were analyzed using a one-sample *t* test based on a theoretical mean of 50 %. Additional data of group 1 were analyzed by ANOVA followed by Bonferroni post hoc tests when appropriate. Data of group 2 were analyzed by Student́s *t* test. Values of $p < 0.05$ were considered significant and the significance level is indicated by asterisk ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Results

Animal Weight, Fluid and Feed Intakes, and Al Content in the Rats' Feed

Individual weights of rats were similar between groups at the start and end of interventions. Water and feed intakes were also similar between the groups (Table 1).

The total Al content in rats' feed was 86.03 ± 4.76 µg Al/g/feed, which equated to a daily intake of Al from feed of 1.88 mg/Al/rat.

Behavioral Results

Open Field, Plus Maze, and Hot Plate

Rats were exposed to an open-field arena, a plus maze, and a hot plate after 42 or 60 days of treatment to verify exploratory and locomotor activity, anxiety, and pain threshold, respectively. Subchronic exposure to Al at different doses did not affect the number of crossings and rearings during the 5-min long free exploration session in the open field test (Table 2—open field). Similarly, $AlCl_3$ had no effect on the time spent in the open arms during the plus maze session (Table 2—plus maze) and in time latency to reaction on the hot plate (Table 2—hot plate).

Object Recognition Memory Test

To investigate the effect of $AlCl_3$ on object recognition long-term memory (LTM) consolidation, group 1 rats were treated for 60 days with Al at 1.5 or 8.3 mg/kg b.w./day

Table 1 Daily feed and drink intakes between the groups lack significant difference ($p > 0.05$)

Feed/fluid intakes	Group 1			Group 2	
	Control	1.5 mg Al/kg b.w./day	8.3 mg Al/kg b.w./day	Control	100 mg Al/kg b.w./day
Feed intakes	21.63 ± 0.27 g	23.34 ± 0.24 g	21.98 ± 0.61 g	21.17 ± 0.48 g	20.87 ± 0.43 g
Fluid intakes	36.17 ± 0.95 ml	35.95 ± 0.37 ml	36.24 ± 0.67 ml	32.34 ± 1.39 ml	32.97 ± 0.62 ml

Data are expressed as mean ± SEM. $p > 0.05$ (ANOVA or Student's t test)

Table 2 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on exploratory and locomotor behavior (open field), anxiety (elevated plus maze), and pain threshold (hot plate) in rats

Behavioral tasks	Group 1			Group 2	
	Control	1.5 mg Al/kg b.w./day	8.3 mg Al/kg b.w./day	Control	100 mg Al/kg b.w./day
Open field					
Crossings (n)	74.60 ± 6.90	67.67 ± 9.30	77.17 ± 7.68	49.67 ± 7.64	58.33 ± 5.53
Rearing (n)	20.67 ± 2.02	17.50 ± 2.23	21.50 ± 1.36	19.33 ± 6.42	18.67 ± 1.58
Elevated plus maze—time spent in open arm (s)	21.26 ± 6.65	32.51 ± 8.31	20.43 ± 8.70	7.81 ± 2.75	11.32 ± 4.34
Hot plate—latency (s)	7.83 ± 0.74	8.83 ± 0.40	9.16 ± 0.54	7.50 ± 0.84	8.66 ± 0.49

Data are expressed as mean ± SEM. $p > 0.05$ (ANOVA or Student's t test)

and group 2 rats were exposed to Al at 100 mg/kg b.w./day for 42 days, as the control rats treated with ultrapure water, were trained in the OR learning task. All rats explored the two new objects (a and b) for a similar percentage of the total exploration time (about 50 % each, Fig. 1a, b) in the training session. 24 h after training, in the LTM testing session, control rats explored the novel object (c) significantly more than 50 % of the total exploration time ($p = 0.0028$ for control of group 1, Fig. 1a, LTM test; $p = 0.0001$ for control of group 2, Fig. 1b, LTM test). However, in all the Al-treated groups, animals spent about 50 % of the total exploration time exploring each object (a and c), without differences between the time spent for exploring the familiar (a) and the novel objects (c).

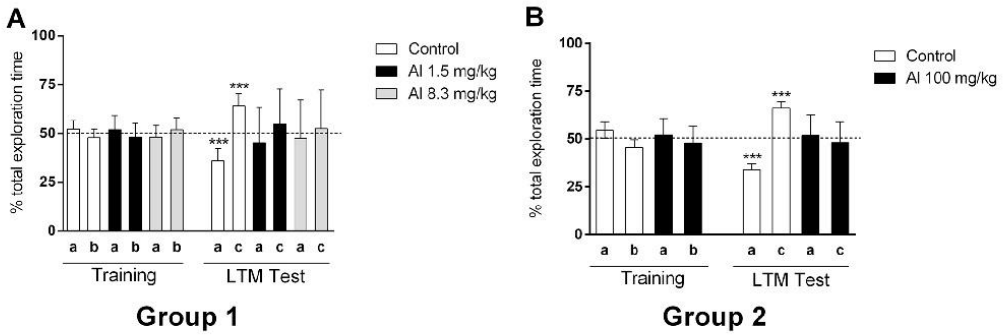

Fig. 1 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on object recognition memory at the conclusion of the treatment periods. The two groups of rats were trained on the OR task for 4 days and were tested 24 h later to evaluate their long-term recognition memory (LTM) after 60 or 42 days of treatment for groups 1 and 2, respectively. In the training session, the animals were exposed to objects "*a*" and "*b*." In the test session, the rats were exposed to a familiar object (*a*) and to a novel object (*c*). Data are expressed as mean ± SEM of the percentage of total exploration time. ***$p < 0.001$ in one-sample t-test, considering a theoretical mean of 50 % ($N = 6$)

($p = 0.55$ for Al 1.5 mg/kg and $p = 0.76$ for Al 8.3 mg/kg of group 1, Fig. 1a, LTM test; $p = 0.69$ for Al 100 mg/kg of group 2, Fig. 1b, LTM test).

Biochemical Results

Reactive Oxygen Species and Lipid Peroxidation Levels

Al treatment at different doses increased ROS levels in hippocampus ($p = 0.0251$ for control vs. Al 1.5 mg/kg and $p = 0.0041$ for control vs. Al 8.3 mg/kg, group 1, Fig. 2a; $p = 0.0241$ for control vs. Al 100 mg/kg, group 2, Fig. 2b). In prefrontal cortex, only Al at 8.3 mg/kg b.w. increased ROS levels ($p = 0.0054$ for control vs. Al 8.3 mg/kg, group 1, Fig. 2c).

There was a significant increase on lipid peroxidation in hippocampus of Al-treated rats at major doses when compared with the controls groups ($p = 0.0307$ for control vs. Al 8.3 mg/kg, group 1, Fig. 3a; $p = 0.0002$ for control vs. Al 100 mg/kg, group 2, Fig. 3b) and no differences in MDA levels after Al exposure at 1.5 mg/kg b.w. (Figure 3a). No alteration on prefrontal cortex lipid peroxidation was observed in any group (Figs. 3c, d).

Total Antioxidant Capacity—Ferric Reducing/Antioxidant Power (FRAP)

Al decreased the hippocampal total antioxidant capacity in all exposed groups, even at low levels ($p = 0.0193$ for control vs. Al 1.5 mg/kg and $p = 0.0035$ for control vs. Al 8.3 mg/kg, group 1, Fig. 4a; $p = 0.0495$ for control vs. Al 100 mg/kg, group 2, Fig. 4b). The antioxidant capacity of prefrontal cortex was decreased only in rats exposed to 100 mg/kg b.w. Al for 42 days ($p = 0.0339$ for control vs. Al 100 mg/kg, group 2, Fig. 4d).

Acetylcholinesterase (AChE) Activity

Aluminum exposure for 60 days at low levels or for 42 days at high levels decreased the enzymatic activity of

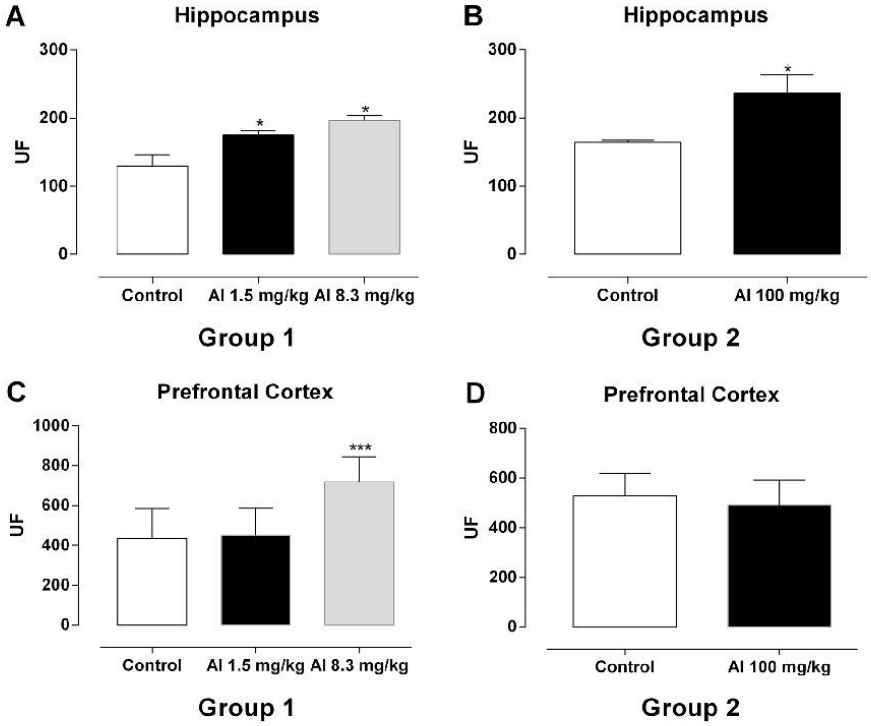

Fig. 2 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on levels of reactive oxygen species (ROS). Values of ROS on hippocampus (**a**, **b**) and prefrontal cortex (**c**, **d**). Data are expressed as mean ± SEM ($N = 6$). $^{*}p < 0.05$, $^{***}p < 0.001$ (ANOVA followed by Bonferroni or Student's t test). *UF* units of fluorescence

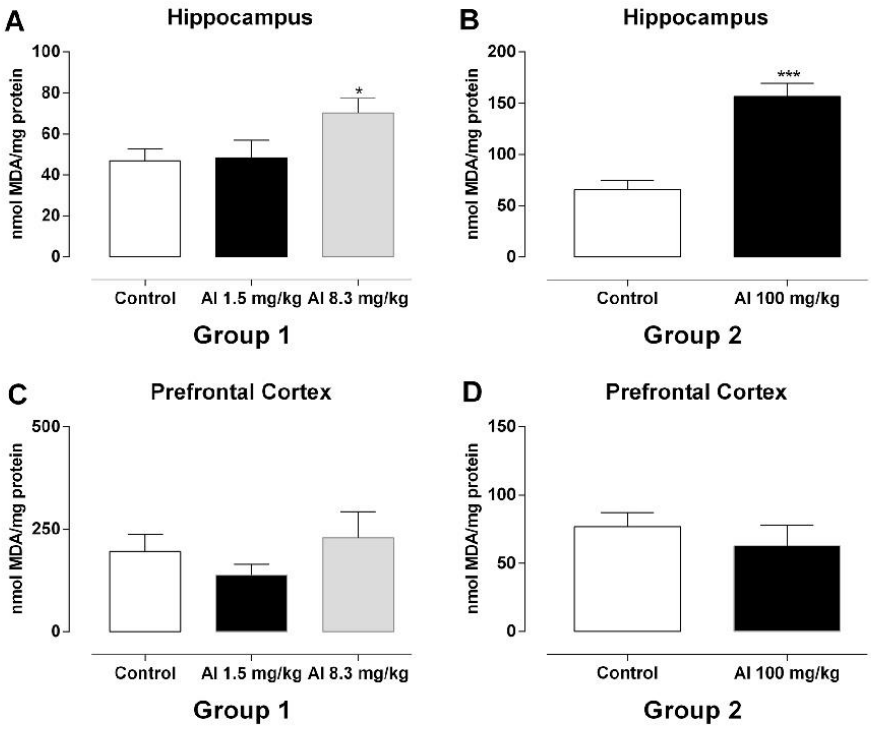

Fig. 3 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on lipid peroxidation measurements. Values of MDA (malondialdehyde) on hippocampus (**a**, **b**) and prefrontal cortex (**c**, **d**). Data are expressed as mean ± SEM (n = 6). $*p < 0.05$, $***p < 0.001$ (ANOVA followed by Bonferroni or Student's *t* test)

AChE in hippocampus ($p = 0.0116$ for control vs. Al at 1.5 mg/kg and $p = 0.0006$ for control vs. Al at 8.3 mg/kg, group 1, Fig. 5a; $p = 0.0467$ for control vs. Al at 100 mg/kg, group 2, Fig. 5b). No change was observed in AChE activity in prefrontal cortex of aluminum-treated rats (Fig. 5c, d).

Discussion

Our study aimed to investigate if Al exposure at human dietary levels could promote similar toxic effects found in a model for neurotoxicity induced by Al. Our results suggest that this metal reaches a threshold sufficient to promote neurotoxicity even at low doses. Here, we show that rats exposed to low doses of Al for 60 days could not recognize familiar objects as control rats do; this memory impairment was the same observed in rats treated with Al at a dose 66 times higher. This cognitive dysfunction in Al-treated rats came together with a marked hippocampal oxidative stress condition, with increased ROS production, lipid peroxidation, and decreased antioxidant capacity, as well as decreased AChE activity.

Human exposure to Al is practically inevitable and, due to the omnipresence of this metal in our daily life, it is difficult to maintain a "safe" Al intake level. The tolerable weekly intake of Al for humans has been set at 1 mg Al/Kg b.w. dose (FAO/World Health, Organization 2007), which is routinely exceeded by humans. Total human Al intake is difficult to determine due to its high bioavailability and ubiquity. Greger (1993) estimates that Americans consume 1–10 mg/Al/day from fresh food. In addition, 50 % of Americans consume up to 24 mg/day, 45 % between 24 and 95 mg, and about 5 % ingest more than 95 mg/Al/day in the form of Al additives. The above-mentioned study is one of the most accurate, taking into account the Al amounts in manufactured food. Moreover, the Al intake through the gastrointestinal tract can reach 126–5000 mg/day from ingested pharmaceuticals, antacids in particular (Reinke et al. 2003; Shaw and Tomljenovic 2013).

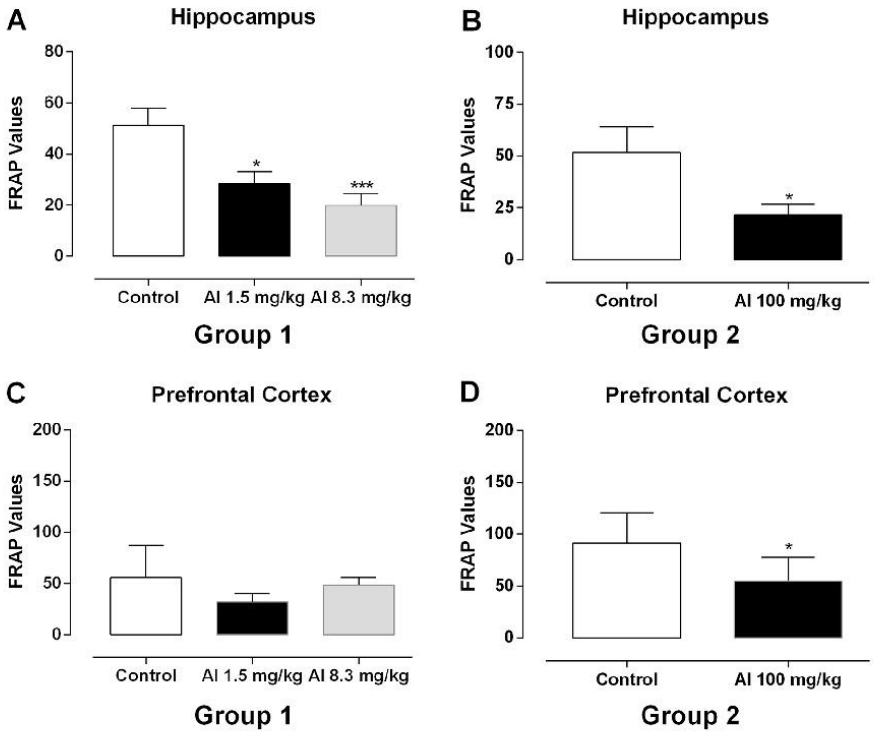

Fig. 4 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on total antioxidant capacity. Values of ferric reducing/antioxidant power (FRAP) on hippocampus (**a**, **b**) and prefrontal cortex (**c**, **d**). Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{***}p < 0.001$ (ANOVA followed by Bonferroni or Student's *t* test)

In our study, all rats including controls received 1.88 mg/Al/day from their standard feed; considering the rats mean body weights of 300 g, this amount represents 6.26 mg/Al/kg/b.w./day from feed. For the experimental groups, taking into account their mean body weights of 300 g, the total amount of Al exposure was for group 1, low aluminum levels: (a) 1.5 mg/Al/kg b.w.–2.33 mg/Al/day (0.45 mg/Al from water plus 1.88 mg/Al from feed); (b) 8.3 mg/Al/kg b.w.–4.37 mg/Al/day (2.49 mg/Al from water plus 1.88 mg/Al from feed), and for group 2, high aluminum level: (c) 100 mg/Al/kg b.w–31.88 mg/Al/day (30 mg/Al from gavage plus 1.88 mg/Al from feed). Considering that all rats consumed approximately the same amount of Al in their feed, the presence of Al in their drinking water points to this additional Al via a different exposure route as a critical issue for achieving the threshold responsible for the development of the behavioral dysfunction in Al-treated rats.

Aluminum chloride (100 mg/kg/day, p.o., 6 weeks) is a well-known model for dementia resulting in progressive deterioration of spatial memory in Morris water maze associated with oxidative damage and mitochondria impairment (Prakash and Kumar 2009, 2013). Here, we showed that this dose level of Al also promotes recognition memory dysfunction and oxidative stress in rats. It was not surprising, considering that this high dose of Al is usually used as a dementia model (Prakash and Kumar 2009, 2013) and previous studies demonstrated that it promotes cognitive deficits (Kasbe et al. 2015; Lakshmi et al. 2015). However, according to our best knowledge, the present study has been the only one to evaluate the effects of 60-day low Al exposure, similar to human dietary Al exposure, on memory. We developed the same behavioral evaluations in rats exposed to low Al doses and, surprisingly, the neurotoxicity effects are practically the same as those induced by the 100 mg/kg/day dose level. Namely, Al exposure for 60 days at 1.5 or 8.3 mg/kg/day in adult life could cause recognition memory deficits in rats.

Walton (2009) developed a longitudinal study exposing rats to Al at an equivalent-human dietary level. Rats treated

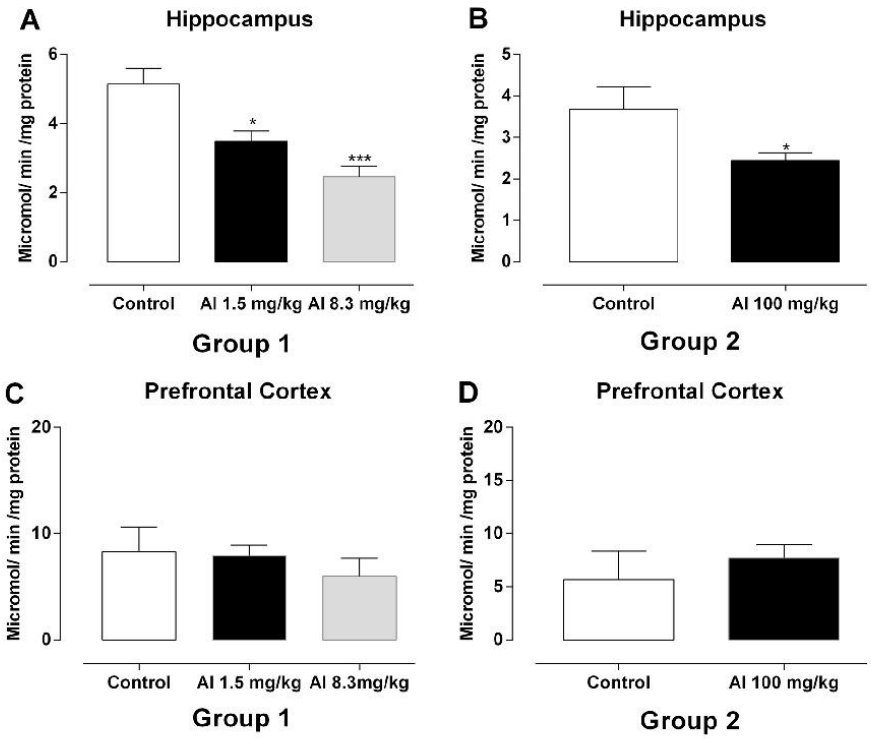

Fig. 5 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on acetylcholinesterase (AChE) activity. Values of AChE activity on hippocampus (**a**, **b**) and prefrontal cortex (**c**, **d**). Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$, $^{***}p < 0.001$ (ANOVA followed by Bonferroni or Student's *t* test)

from 12 months of age until their deaths developed memory impairment and displayed AD-like behaviors during old age. The present study found that Al at the same dose used by Walton (1.5 mg/kg/day) but, for a much shorter time, 60 days (approximating 70 months for humans or 6 years) (Everitt 1991), was sufficient to start the neurotoxicity process of this metal. Moreover, Al at 8.3 mg/kg/day, the amount equivalent to human Al exposure translated to the animal species used in this study, also impairs memory consolidation.

The neurotoxicity mechanisms induced by Al require intensive investigation. Oxidative stress seems to be one possible pathway by which this nonessential metal acts (Exley 2004). The brain, especially the hippocampus, is very sensitive to oxidative stress, mainly by its low antioxidant capacity and high level of lipid content (Kumar and Gill 2014; Erfani et al. 2015). Our results show that Al exposure caused redox imbalance in the hippocampus, as evident from an increase in ROS generation, independent of the dose. Additionally, elevation of MDA levels and a significant decline of total antioxidant capacity were observed in response to Al exposure at all Al doses investigated. On the other hand, in the prefrontal cortex, only the intermediate Al dose investigated here (8.3 mg/kg b.w.) increased ROS generation and only the high dose (100 mg/kg/b.w.) impaired the total antioxidant capacity. These results suggest that the specific toxic effects of this metal are dependent on the contamination threshold that is achieved, and the duration of exposure, but that a low dose is able to promote hippocampal toxicity and memory impairments.

Another way by which Al impairs memory is by its interference with the cholinergic system (Ravi et al. 2000; Yellamma et al. 2010). Our data reveal hippocampal

dysfunction of AChE activity induced by Al at high and low doses of exposure, without altering the prefrontal cortex enzyme activity. AChE activity is a marker for loss of cholinergic neurons, and memory dysfunction is associated mainly with cholinergic neuron loss in several regions of the brain (Whitehouse et al. 1982; Fraser and MacVicar 1996). The current study shows that the $AlCl_3$ exposure, even at low doses, decreases the activity of AChE in rat hippocampus. However, the available literature has shown that Al exposure can both stimulate and inhibit enzymatic function (Prakash and Kumar 2009; Lakshmi et al. 2015; Noremberg et al. 2016). Al has been shown to produce a biphasic effect on AChE, stimulating AChE at low levels or short exposures and inhibiting AChE at high doses and/or long exposures periods. Among the suggested hypothesis, the biphasic effect of Al on the AChE activity may be due to the direct effect of the metal or due to the peroxidation-induced changes in the structure of membrane following Al exposure (Kumar 1999). Therefore, it is likely that peroxidation of membrane structures may be responsible for the inhibited hippocampal AChE found after Al exposure.

The mechanism responsible for the formation and consolidation of long-term memory has been extensively studied (Izquierdo and Medina 1997; Alarcon et al. 2004), while the mechanism of action of Al in promoting memory dysfunction is unclear. However, considering that the hippocampus is the main structure required for memory formation and consolidation, we can affirm, based on our results, that the hippocampal oxidative stress and damage, as well as the AChE impairment induced by Al exposure may be at least partially responsible for the memory impairments.

Conclusions

Our results demonstrate that a 60-day subchronic exposure to low doses of Al from feed and added to the water, which reflect human dietary Al intake, reaches a threshold sufficient to promote memory impairment. These effects are similar to a known model of Al-induced neurotoxicity at high levels. Moreover, the hippocampal oxidative stress and the cholinergic dysfunction found in the three doses investigated highlight a pathway of action of this metal.

Acknowledgments The study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Programa Nacional de Cooperação Acadêmica; Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq 406715/2013-0]; Fundação de Amparo a Pesquisa do Espírito Santo; Fundo Estadual de Ciência e Tecnologia [39767531/07]; and Pró-reitoria de Pesquisa - Universidade Federal do Pampa [N° 10.134.14]. The authors would like to thank Professor Christopher Exley from Keele University, Staffordshire, UK, for the support on GFAAS.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alarcon JM, Malleret G, Touzani K, Vronskaya S, Ishii S, Kandel ER, Barco A (2004) Chromatin acetylation, memory, and LTP are impaired in CBP± mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42:947–959

Andrási E, Páli N, Molnár Z, Kösel S (2005) Brain aluminum, magnesium and phosphorus contents of control and Alzheimer-diseased patients. J Alzheimers Dis 7(4):273–284

Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': the FRAP assay. Anal Biochem 239:70–76

Bhattacharjee S, Zhao Y, Hill JM, Culicchia F, Kruck TP, Percy ME, Pogue AI, Walton JR, Lukiw WJ (2013) Selective accumulation of aluminum in cerebral arteries in Alzheimer's disease (AD). J Inorg Biochem 126:35–37

Bondy SC (2015) Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration. Neurotoxicology 52:222–229

Bonini JS, Bevilaqua LR, Zinn CG, Kerr DS, Medina JH, Izquierdo I et al (2006) Angiotensin II disrupts inhibitory avoidance memory retrieval. Horm Behav 50:308–313

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein–dye binding. Anal Biochem 72:248–254

Chin-Chan M, Navarro-Yepes J, Quintanilla-Vega B (2015) Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. Front Cell Neurosci 10(9):124. doi:10.3389/fncel.2015.00124

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95

Ennaceur A, Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. Behav Brain Res 31:47–59

Erfani S, Khaksari M, Oryan S, Shamsaei N, Aboutaleb N, Nikbakht F, Jamali-Raeufy N, Gorjipour F (2015) Visfatin reduces hippocampal CA1 cells death and improves learning and memory deficits after transient global ischemia/reperfusion. Neuropeptides 49:63–68. doi:10.1016/j.npep.2014.12.004

Everitt AV (1991) Ageing rat colonies at the University of Sydney. Proc Aust Assoc Gerontol 26:79–82

Exley C (2004) The pro-oxidant activity of aluminum. Free Radic Biol Med 3:380–387

Exley C (2012) Elucidating aluminiuḿs exposome. Curr Inorg Chem 2:3–7

Exley C (2013) Human exposure to aluminium. Environ Sci Process Impacts 10:1807–1816

Flaten TP (2001) Aluminium as a risk factor in Alzheimer's disease, with emphasis on drinking water. Brain Res Bull 2:187–196

Fraser DD, MacVicar BA (1996) Cholinergic-dependent plateau potential in hippocampal CA1 pyramidal neurons. J Neurosci 16:4113–4128

Greger JL (1993) Aluminum metabolism. Annu Rev Nutr 13:42–63

House E, Esiri M, Forster G, Ince PG, Exley C (2012) Aluminium, iron and copper in human brain tissues donated to the medical research council's cognitive function and ageing study. Metallomics 4:56–65

Izquierdo I, Medina JH (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol Learn Mem 68:285–316

Jacob JJ, Tremblay EC, Colombel MC (1974) Enhancement of nociceptive reactions by naloxone in mice and rats. Psychopharmacologia 37:217–223

Kasbe P, Jangra A, Lahkar M (2015) Mangiferin ameliorates aluminium chloride-induced cognitive dysfunction via alleviation of hippocampal oxido-nitrosative stress, proinflammatory cytokines and acetylcholinesterase level. J Trace Elem Med Biol 31:107–112

Kumar S (1999) Aluminium-induced biphasic effect. Med Hypotheses 52:557–559

Kumar V, Gill KD (2014) Oxidative stress and mitochondrial dysfunction in aluminium neurotoxicity and its amelioration: a review. Neurotoxicology 41:154–166

Lakshmi BV, Sudhakar M, Prakash KS (2015) Protective effect of selenium against aluminum chloride-induced Alzheimer's disease: behavioral and biochemical alterations in rats. Biol Trace Elem Res 1:67–74

Loetchutinat C, Kothan S, Dechsupa S, Meesungnoen J, Jay-Gerin J, Mankhetkorn S (2005) Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. Rad Phys Chem 72:323–331

Mello-Carpes PB, Izquierdo I (2013) The nucleus of the solitary tract → nucleus paragigantocellularis → locus coeruleus → CA1 region of dorsal hippocampus pathway is important for consolidation of object recognition memory. Neurobiol Learn Mem 100:56–63

Noremberg S, Bohrer D, Schetinger MR, Bairros AV, Gutierres J, Gonçalves JF et al (2016) Silicon reverses lipid peroxidation but not acetylcholinesterase activity induced by long-term exposure to low aluminum levels in rat brain regions. Biol Trace Elem Res 1:77–85

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167

Prakash A, Kumar A (2009) Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats. Basic Clin Pharmacol Toxicol 2:98–104

Prakash A, Kumar A (2013) Mitoprotective effect of *Centella asiatica* against aluminum-induced neurotoxicity in rats: possible relevance to its anti-oxidant and anti-apoptosis mechanism. Neurol Sci 8:1403–1409

Priest ND, Talbot RJ, Newton D, Day JP, King SJ, Fifield LK (1998) Uptake by man of aluminium in a public water supply. Hum Exp Toxicol 6:296–301

Ravi SM, Prabhu BM, Raju TR, Bindu PN (2000) Long-term effects of postnatal aluminium exposure on acetylcholinesterase activity and biogenic amine neurotransmitters in rat brain. Indian J Physiol Pharmacol 4:473–478

Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. FASEB J 3:659–661

Reinke CM, Breitkreutz J, Leuenberger H (2003) Aluminium in over-the-counter drugs: risks outweigh benefits? Drug Saf 14:1011–1025

Roskams AJ, Connor JR (1990) Aluminum access to the brain: a role for transferrin and its receptor. Proc Natl Acad Sci 87:9024–9027

Ruipérez F, Mujika JI, Ugalde JM, Exley C, Lopez X (2012) Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II). J Inorg Biochem 117:118–123

Rusina R, Matěj R, Kašparová L, Kukal J, Urban P (2011) Higher aluminum concentration in Alzheimer's disease after box-cox data transformation. Neurotox Res 4:329–333

Shaw CA, Tomljenovic L (2013) Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity. Immunol Res 2–3:304–316

Shirley DG, Lote CJ (2005) Renal handling of aluminium. Nephron Physiol 101:99–103

Walton JR (2007) A longitudinal study of rats chronically exposed to aluminum at human dietary levels. Neurosci Lett 1:29–33

Walton JR (2009) Functional impairment in aged rats chronically exposed to human range dietary aluminum equivalents. Neurotoxicology 30:182–193

Walton JR (2014) Chronic aluminum intake causes Alzheimer's disease: applying Sir Austin Bradford Hill's causality criteria. J Alzheimers Dis 4:765–838. doi:10.3233/JAD-132204

Wang Z, Wei X, Yang J, Suo J, Chen J, Liu X, Zhao X (2016) Chronic exposure to aluminum and risk of Alzheimer's disease: a meta-analysis. Neurosci Lett 610:200–206

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239

World Health Organization (2007) Safety evaluation of certain food additives and contaminants. Food Additive Series: 58. http://whqlibdoc.who.int/trs/WHO TRS 940 eng.pdf

Yellamma K, Saraswathamma S, Nirmala Kumari B (2010) Cholinergic system under aluminium toxicity in rat brain. Toxicol Int 2:106–112

PARTE III

Paper published at J Inorg Biochem. pii: S0162-0134(17)30469-5. IF: 3, 348. Qualis CBII: B1

ARTICLE IN PRESS

Journal of Inorganic Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Aluminum exposure for 60 days at an equivalent human dietary level promotes peripheral dysfunction in rats

Caroline Silveira Martinez[a], Gema Vera[b], José Antonio Uranga Ocio[b], Franck Maciel Peçanha[a], Dalton Valentim Vassallo[c], Marta Miguel[d], Giulia Alessandra Wiggers[a,*]

[a] Postgraduate Program in Biochemistry, Universidade Federal do Pampa, BR 472, Km 592, PO box 118, 97500-970 Uruguaiana, Rio Grande do Sul, Brazil
[b] Department of Ciencias Básicas de la Salud, Universidad Rey Juan Carlos, Avda. de Atenas s/n, Alcorcón, Spain
[c] Departments of Physiological Sciences, Universidade Federal do Espírito Santo, and School of Medicine of Santa Casa de Misericórdia (EMESCAM), Av. Marechal Campos 1468, 29040-090 Vitória, Espírito Santo, Brazil
[d] Bioactivity and Food Analysis Laboratory, Instituto de Investigación en Ciencias de la Alimentación, Nicolás Cabrera, 9, Campus Universitario de Cantoblanco, Madrid, Spain

ARTICLE INFO

Keywords:
Metal
Peripheral neuropathy
Oxidative stress
Inflammation

ABSTRACT

Aluminum (Al) is a neurotoxic associated with a number of chronic human diseases. We investigated the effects of Al exposure at doses similar to human dietary levels and at a high level exposure to Al on the peripheral nervous system. *Wistar* male rats were divided into two major groups and received orally: 1) First group – Low level - rats were subdivided and treated for 60 days: a) Control – received ultrapure water; b) $AlCl_3$ – received Al at 8.3 mg/kg body weight (bw) for 60 days; and 2) Second group – High level - rats were subdivided and treated for 42 days: C) Control – received ultrapure water through oral gavage; d) $AlCl_3$ – received Al at 100 mg/kg bw for 42 days. Von Frey hair test, plantar test, the presence of catalepsy and the spontaneous motor activity were investigated. Reactive oxygen species, lipid peroxidation and total antioxidant capacity, immunohistochemistry to investigate the nerve inflammation and, the specific presence of Al in the sciatic nerve fibers were investigated. Al exposure at a representative human dietary level promotes the development of mechanical allodynia, catalepsy, increased inflammation in the sciatic nerve, systemic oxidative stress and, is able to be retained in the sciatic nerve. The effects of low-dose Al were similar to those found in rats exposed to Al at a dose much higher (100 mg/kg). Our findings suggest that Al may be considered toxic for the peripheral nervous system, thus inducing peripheral dysfunction.

1. Introduction

Aluminum (Al) has no known biological function and is potentially toxic [1]. Humans are exposed to Al through dietary and non-dietary sources, and its real consequence perhaps not entire clear [2,3]. The Provisional Tolerable Weekly Intake (PTWI) of Al for humans has been adjusted to 1 mg Al/kg body weight (bw) by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) and European Food Safety Authority (EFSA) [4,5]. However, due to myriad sources of Al exposure, this health-based guidance value may be exceeded by humans [6–8].

The increased body burden of Al has been reported with several health conditions [2]. The body presence of this non-essential metal seems to trigger the development of neurological disorders [9], reproductive dysfunction [10], Autoimmune/inflammatory Syndrome Induced by Adjuvants (ASIA) such as macrophagic myofascitis [11], microcytic anemia [12], atherosclerosis plaques formation [13], osteopenia [14] and breast cancer [15].

Al is a neurotoxin able to be accumulated and retained in the brain, contributing towards both the onset and the aggressive progression of all forms of Alzheimer Disease (AD) [16,17]. Al accumulation in neurons has been related to cognitive and motor impairments, mostly related with neurodegenerative diseases [18,19] and functional impairment at low doses [20]. In the peripheral nervous system (PNS) there are evidences of Al-induced neuropathy. People exposed to Al through contaminated water (13.17 to 15.70 ppm) in the Kirazli region (Biga

Abbreviations: Al, aluminum; PTWI, provisional tolerable weekly intake; ASIA, Autoimmune/inflammatory Syndrome Induced by Adjuvants; CNS, central nervous system; PNS, peripheral nervous system; AD, Alzheimer Disease; ROS, reactive oxygen species; RNS, reactive nitrogen species; MDA, malondialdehyde; TBA, thiobarbituric acid; FRAP, ferric reducing/antioxidant power; AChE, acetylcholinesterase

* Corresponding author at: PPGBioq, UNIPAMPA, BR 472, Km 592, PO box 118, 97500-970 Uruguaiana, Rio Grande do Sul, Brazil.
E-mail address: giuliapecanha@unipampa.edu.br (G.A. Wiggers).

http://dx.doi.org/10.1016/j.jinorgbio.2017.08.011
Received 30 June 2017; Received in revised form 4 August 2017; Accepted 22 August 2017

Please cite this article as: Martinez, C.S., Journal of Inorganic Biochemistry (2017), http://dx.doi.org/10.1016/j.jinorgbio.2017.08.011

Peninsula, NW Turkey) showed a history of peripheral neuropathy [21]. More recently, Al accumulation in the dorsal root ganglion in the course of oxaliplatin treatment of cancer exacerbated the neuropathic pain [22]. In agreement, the suggested Al chelation by glutathione, known as an antioxidant and a metal chelator, decreases the concentrations of Al in the dorsal root ganglion and alleviates the neuropathic pain induced by oxaliplatin in mice [23].

However, even with the well - known toxicity of Al in the Central Nervous System (CNS), the effects of Al exposure on the PNS remain poorly understood. Moreover, most Al studies have entailed unrealistic high doses of Al which cannot be used as a common level found among human populations. Herein, we addressed the effects of Al in the PNS and motor behavior in rats exposed to both a high level of Al and also one that better represents human exposure to Al through the diet.

2. Materials and methods

2.1. Animals

Three-month-old male *Wistar* rats (360 ± 11.2 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light-dark), giving free access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups, according to Martinez et al. [24] and treated orally: 1) First group - Low aluminum level - rats were subdivided (N = 8/each) and treated for 60 days as follows: a) Control – received ultrapure drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) $AlCl_3$ – received Al at 8.3 mg/kg bw *per day* in the drinking water (changed 4 times/week), a dose based on human dietary levels translated to an animal dose according to the body surface area normalization method [25]; and 2) Second group - High aluminum level - rats were subdivided (N = 8/each) and treated for 42 days as follows: a) Control – received ultrapure water through oral gavage; b) $AlCl_3$ – received Al at 100 mg/kg bw *per day* through oral gavage once a day, representing a high level of human exposure to Al [26].

$AlCl_3{\cdot}6H_2O$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water. The concentration of each stock solution was 0.034 M and 0.331 M, respectively from 8.3 and 100 mg/kg bw. Salts and reagents were of analytical grade obtained from Sigma-Aldrich and Merck (Darmstadt, Germany).

2.2. Tactile sensitivity: Von Frey hair test

Mechanical sensitivity was assessed by measuring the withdrawal threshold to calibrated von Frey hairs (Bioseb Instruments, USA) [27]. The test was conducted at the start (0 day), middle (21 and 30 days) and end (42 or 60 days) of the treatments. Rats were placed individually on an elevated iron mesh in a clear plastic cage and were allowed to adapt to the testing environment for at least 10 min. Habituation to this environment was also performed two days before assessment. The filaments were applied to the plantar aspect of each hind paw, from below the mesh floor. Each stimulus was applied for approximately 1 s with an interstimulus interval of approximately 3 s. A significant decrease in von Frey hair threshold evoked by mechanical stimulus was defined as presence of mechanical allodynia.

2.3. Thermal sensitivity: plantar test

Responses to thermal stimuli were evaluated after mechanical sensitivity using a plantar test apparatus (Ugo Basile, Comerio VA, Italy) [28]. During the testing days rats were placed within a plastic compartment on a glass floor and a light source beneath the floor was aimed at the mid plantar surface of the hind paw. So, the withdrawal reflex interrupts the light and automatically turns-off the light and a timer. The withdrawal latency of each paw was measured during three trials at 2 min intervals and the mean of the three readings was used for data analysis.

2.4. Catalepsy

Catalepsy was measured using a modification of the “ring test” [29]. Rats were hung by their front paws from a rubber-coated metal ring fixed horizontally at a height that allowed their hind paws to just touch the bench. The time taken for the rat to move-off the ring was measured with a cut-off limit of 30 s.

2.5. Spontaneous locomotor activity

Spontaneous locomotor activity was evaluated in the middle (21 or 30 days) and end (42 and 60 days) of the treatments using individual photocell activity chambers (Cibertec S.A., Madrid, Spain) [27]. For this, rats were placed in the recording chambers and the number of interruptions of photocell beams was recorded over a 30-min period. Total number of activity counts throughout the 30 min of test duration was recorded. The mean number of crossings of the photocell beams was used for comparison.

2.6. Blood and tissue collection

At the end of the treatment period, after the behavioral assessment, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, intraperitoneal injection), and after loss of the righting reflex they were submitted to an aorta artery puncture and blood was subsequently collected to obtain plasma for the biochemical experiments. Thereafter, rats were euthanized by decapitation, and the sciatic nerve of the right hind paw was carefully removed for immunohistochemistry analysis.

2.7. Reactive oxygen species levels

Biochemical studies of oxidative stress biomarkers were performed in plasma. For that, blood was centrifuged at 2400*g* for 10 min at 4 °C and plasma was obtained for the measurements.

Levels of reactive species were determined by the spectrofluorometric method described by [30]. This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The plasma was diluted in 50 mM Tris HCl (pH 7.4) and 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence units.

2.8. Lipid peroxidation

Lipid peroxidation was measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. [31], with modifications [24]. An aliquot of plasma was incubated with thiobarbituric acid 0.6% (TBA) and phosphoric acid buffer 1% (H_3PO_4) at 100 °C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The

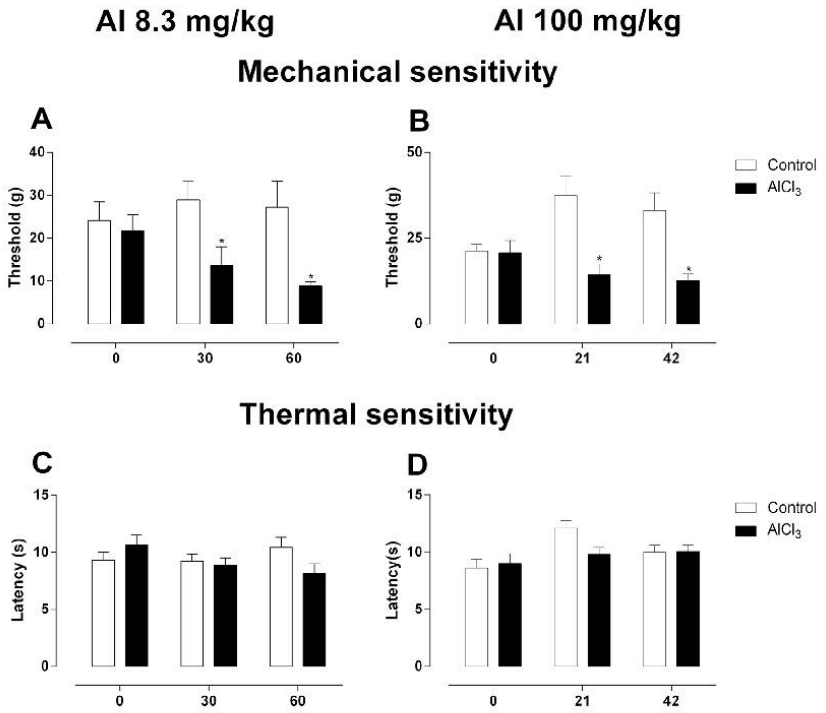

Fig. 1. Effect of chronic aluminum exposure on mechanical and thermal sensitivities. Mechanical sensitivity was measured using the Von Frey hair test and thermal sensitivity was measured using the plantar test (see text for details) before (day 0), in the middle (30 or 21 days) and end of the treatments (60 or 42 days). Respective values of mechanical and thermal sensitivities of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* – A, C) or 42 days (100 mg/kg bw *per day* – B, D). Data are expressed as mean ± SEM, n = 8, *P < 0.05 compared with their corresponding controls (Student's *t*-test).

results were expressed as nanomoles of MDA per mL of plasma.

2.9. Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant capacity was measured by FRAP assay [32], with modifications [24]. This method is based on the ability of samples to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s-triazine (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 µL of plasma was added to 1 mL freshly prepared and pre-warmed (37 °C) FRAP reagent (300 mM acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 µL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50–1000 µM – water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

2.10. Immunohistochemistry

Sciatic nerve immunohistochemistry was performed on paraffin-embedded sections of 5 µm thickness. De-paraffined slides were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3% (*v/v*) hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal bovine serum for 30 min to minimize nonspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with a monoclonal antibody against macrophage-associated antigen (CD163, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to quantify the number of activated macrophages, which is consistent with the presence of inflammation. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining the preparations were incubated without the primary antibody.

2.11. Lumogallion staining for presence of aluminum

Lumogallion staining was performed in formalin-fixed sciatic nerve using a recent validated method to identify the presence of Al in tissues [9,33]. Briefly, re-hydrated tissues sections were immediately placed into either 1 mM lumogallion (TCI Europe N.V. Belgium) buffered in 50 mM PIPES, pH 7.4 or the PIPES-buffer alone for auto-fluorescence analyses for 45 min. Slides were carefully washed 6 times with PIPES-buffer, after rinsed in ultra-pure water for 30 s, finally mounted using an aqueous mounting media and stored horizontally at 4 °C overnight prior to imaging. Sections of tissues were imaged using a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6.

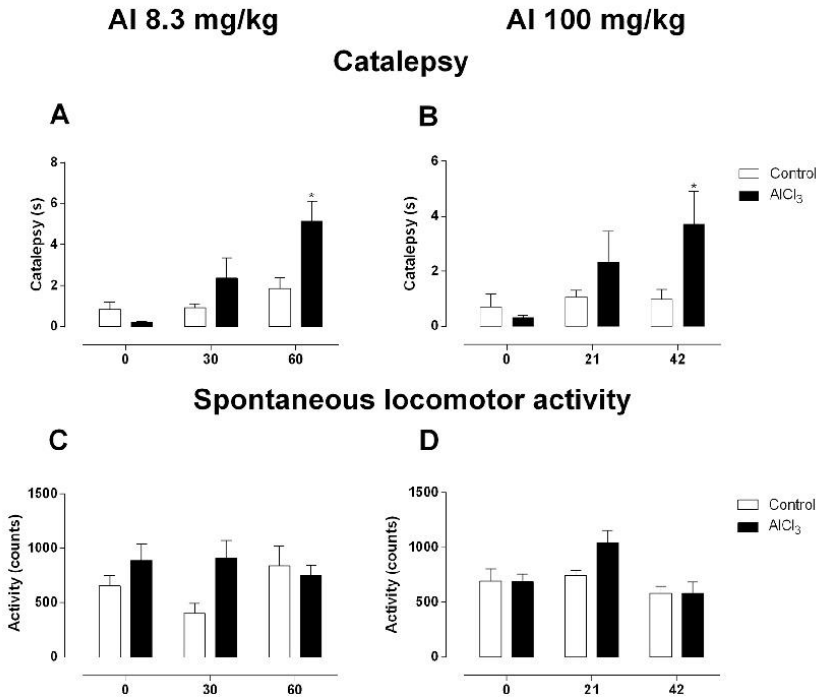

Fig. 2. Effect of chronic aluminum exposure on catalepsy and spontaneous locomotor activity. Both behavioral analysis were performed before (day 0), in the middle (30 or 21 days) and end of the treatments (60 or 42 days). Respective values of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* – A, C) or 42 days (100 mg/kg bw *per day* – B, D). Data are expressed as mean ± SEM, n = 8, *P < 0.05 compared with their corresponding controls (Student's *t*-test).

2.12. Statistical analysis

Data are expressed as mean ± SEM. Results were analyzed using unpaired Student's *t*-test for comparison between groups in the pre, middle and in the end of the treatments. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Body weight, fluid and feed intakes

Body weight of rats was similar between groups at the beginning and end of treatments. Water or Al intakes as well as the feed intake were not different between groups [24].

3.2. Aluminum promotes the development of peripheral neuropathy

Exposure to Al at low (8.3 mg/kg bw for 60 days) or at high level (100 mg/kg bw for 42 days) decreased the mechanical sensitivity threshold in the middle (30 and 21 days) and end of the treatments (60 and 42 days - Fig. 1A, B). The threshold for mechanical sensitivity before treatment was 21.92 ± 1.20 g (N = 64) without differences between groups. In control groups, this threshold did not significantly change with the treatment, whereas for Al-treated rats the mean threshold value after treatments decreased 61.2% and 40.9% for Al exposure at low and high doses, respectively (Fig. 1A, B). The thermal sensitivity tested by the plantar test was unaffected by Al (Fig. 1C, D). However, exposure to Al induced development of catalepsy. Rats exposed to low or high levels of Al showed an increased latency time for reaction in the catalepsy test, being necessary from 2 to 4 times more, respectively when compared to their respective control group, suggesting the presence of motor behavioral disorders (Fig. 2A, B). Spontaneous motor activity was not modified in the different experimental conditions (Fig. 2C, D). Taken together, these results suggest the presence of mechanical allodynia and motor behavioral disorders after Al exposure even at low doses.

3.3. Aluminum induces systemic oxidative stress and peripheral neuroinflammation

Al treatment at low and high doses promoted an imbalance between pro-oxidant and antioxidant systemic biomarkers, as observed by the raised on ROS levels, lipid peroxidation and total antioxidant capacity in plasma (Fig. 3).

Immunohistochemical analysis showed an increase in the number of activated macrophages in sciatic nerve of rats treated with Al at the low dose of 8.3 mg/kg and of rats treated with the higher dose of 100 mg/kg, when compared with the respective control groups (ranging from 10 to 34 in the control group and from 25 to 67 in the Al-treated rats – Fig. 4).

3.4. Aluminum seems to be deposited in the sciatic nerve fibers

The presence of Al in the sciatic nerve was confirmed using lumogallion and fluorescence microscopy. The nerve showed green autofluorescence in the absence of lumogallion (Fig. 5A) and no specific fluorescence in the control rats (Fig. 5B). Lumogallion fluorescence identified Al in the sciatic nerve of Al-treated rats as evidenced by

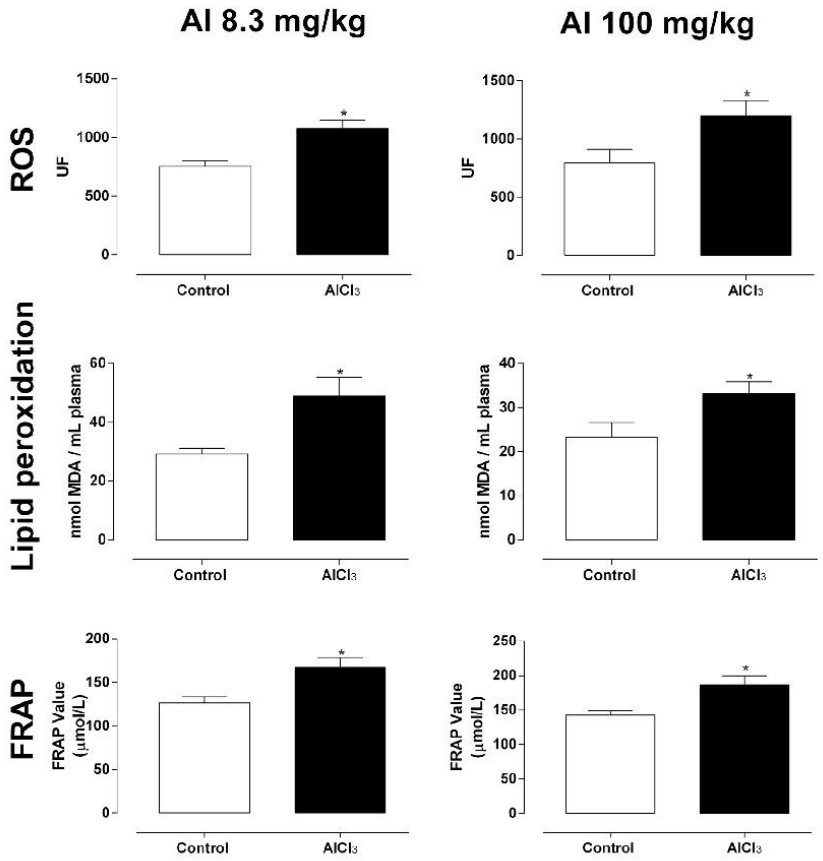

Fig. 3. Effect of chronic aluminum exposure on oxidative stress assessments. Reactive oxygen species (ROS), lipid peroxidation and total antioxidant capacity (FRAP- Ferric Reducing/ Antioxidant Power) levels of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day*) or 42 days (100 mg/kg bw *per day*). Data are expressed as mean ± SEM, n = 8, *P < 0.05 compared with their corresponding controls (Student's *t*-test).

bright orange fluorescence (Fig. 5C, D).

4. Discussion

Aluminum is neurotoxic, and evidence now points to Al as a primary etiological factor in AD [16]. Recently, we have observed that Al reaches a threshold sufficient to promote cognitive dysfunction and memory impairment even at low doses [24]. Here, we show that Al at human dietary levels could affect the PNS inducing peripheral neuropathy. Rats exposed to Al for 60 days at a representative human dietary Al level (8.3 mg/kg) have developed mechanical allodynia, peripheral nerve inflammation with increased number of activated macrophages in the sciatic nerve and, all of this submerged in an increased systemic oxidative stress. Interestingly, and in agreement with our previous findings [24], these effects are similar to those found in rats exposed to Al at a dose much higher (100 mg/kg), suggesting a nonclassical toxicological pattern. Exposure conditions, individual characteristics and, consequent distribution and bioavailability through the body seem to be more determinant for Al effects. Moreover, our study shows for the first time the specific presence of Al in the sciatic nerve fibers.

The present study supports the concern regarding safety values for human exposure to Al. Herein, rats exposed to Al in a model that aims to mimic human exposure to this metal develop peripheral dysfunction similar as those found in Al exposure at a high level. In our model of exposure, considering the amount of Al present in the animal's feed, the total Al exposure was 4.37 mg/Al/day (2.49 mg from water plus 1.88 mg from feed) while, for Al exposure at a high level, rats were treated with a total of 31.88 mg/Al/day (30 mg from gavage plus 1.88 mg from feed). Our model of exposure together with previous reports in the literature suggest that once achieved a toxic Al threshold or burden in any compartment or tissue, Al could begin its cascade of toxicity in the body, which seems occur at a similar human exposure level [24,34,35].

Data regarding Al and peripheral nerve dysfunction are scarce. Injection of Al in the subepineurial space of the sciatic nerve was used as a model for neurodegeneration in rabbits [36]. Recently, the presence of Al in the dorsal root ganglion of oxaliplatin-treated mice, a platinum-based anticancer drug, was associated with severe side effects in the peripheral nervous system (PNS) [22]. It was further confirmed by the use of the antioxidant glutathione as a suggested metal chelator which in turn decreases the concentration of Al in the dorsal root ganglion and the neuropathic pain induced by oxaliplatin [23]. The

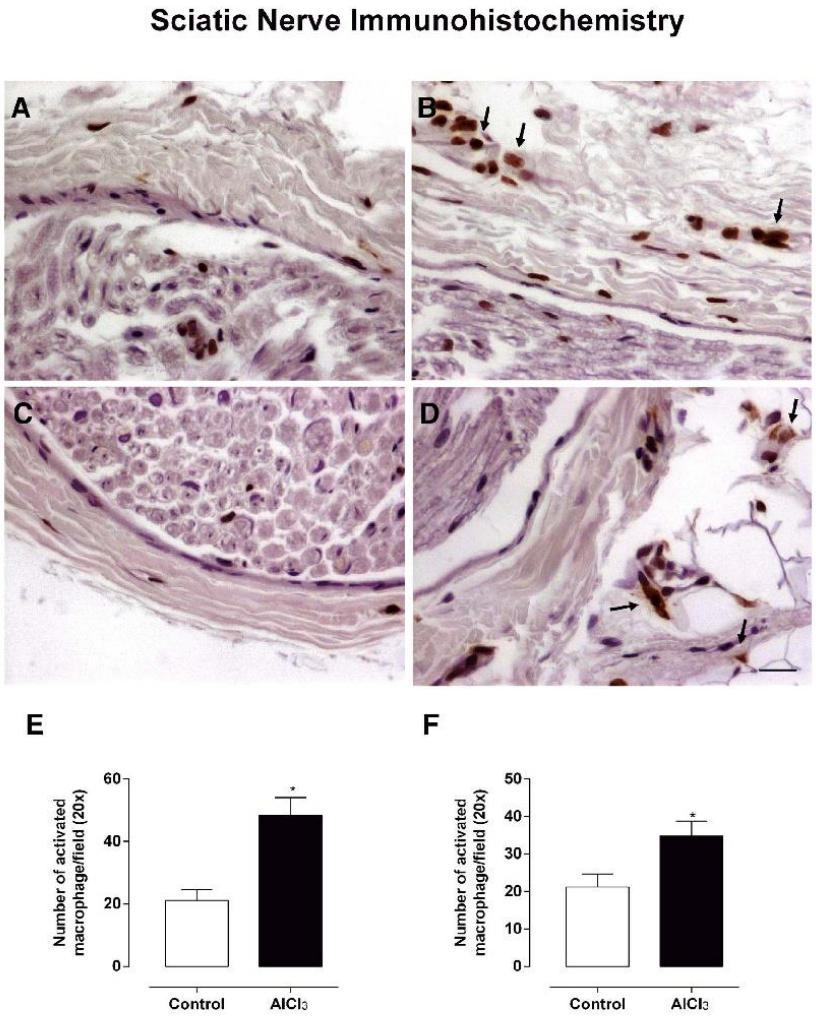

Fig. 4. Effect of chronic aluminum exposure on sciatic nerve immunohistochemistry. Activated macrophages (arrows) in sciatic nerve of controls group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D) detected by immunohistochemistry. Scale bars 20 μm and objective × 40. Average numbers of activate macrophages per field (objective × 20) of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* - E) or 42 days (100 mg/kg bw *per day* - F). Data are expressed as mean ± SEM, n = 8, *P < 0.05 compared with their corresponding controls (Student's *t*-test).

effect of high Al in water resources (140 times higher than the maximum allowable limits) on human health was evaluated, and a total of 273 people living in the Kirazli region, Turkey were interviewed and compared with a control population from Ciplak-Halileli region. The neuropathy history was about 36% higher in the region, creating a statistically significant difference [21]. Important to highlight that serum Al may have toxic effects on hemodialysis patients even when it is in the "acceptable" range (below 20 μg/L), being associated with increased mortality and, disorders of the PNS are among the various neurologic conditions that have been reported in such population [37,38]. In the present study, Al exposure induced no alteration in the sensitivity to noxious stimuli. The effect of metals and toxics in this parameter remains controversial. Mercury treatment induces heat hyperalgesia [39] and cold allodynia was observed in mice treated with Al [22]. Further work is needed to better define the effect of Al on thermal sensitivity.

In the current study Al-treated rats have developed catalepsy, which is an extreme form of immobility manifested in some Parkinson disease patients and resembles the extrapyramidal side effects that occur in humans exposed to various antipsychotic drugs, some of which acting as acetylcholinesterase (AChE) inhibitors [40]. In experimental animals, the inhibition of AChE has been related with the catalepsy development [41,42]. The catalepsy behavior found in Al-treated rats is in agreement with our previous findings. We have seen that Al exposure at an equivalent human dietary level inhibits the AChE activity in hippocampus of rats [24]. Indeed, Al exposure has a large history on

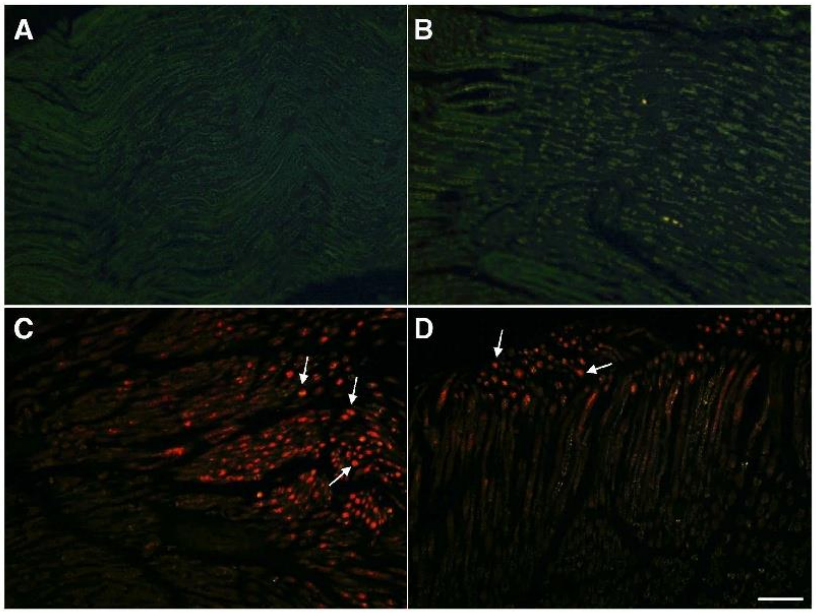

Fig. 5. Aluminum presence in peripheral nerve. Representative images of aluminum in the sciatic nerve: autofluorescence control (A), lumogallion fluorescence for aluminum in control group (B) and in rats treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* - C) or 42 days (100 mg/kg bw *per day* - D). The specific presence of Al is indicated by arrows. Scale bars: 50 μm.

cholinergic system, acting either stimulating or inhibiting AChE activity [19,26,43]. However, the neurotoxicity mechanisms of Al need to be better understood.

Al^{3+} is a potent pro-oxidant due to formation of superoxide radical ion [44] or by promoting the Fenton reaction by reducing Fe (III) to Fe (II) [45]. In the present study, rats exposed to Al have shown the features of mechanical allodynia followed by increased oxidative stress in plasma and peripheral inflammation in the sciatic nerve, suggesting the development of peripheral neuropathy in rats. While there are several lines to explain the pathophysiological basis of neuropathic dysfunction in other diseases, oxidative stress and inflammation seem to play an important role in neuronal damage, contributing towards to demyelination of peripheral nerves thus altering the normal conduction [46,47]. Overall, our findings suggest that the increased ROS, lipid peroxidation and antioxidant capacity in plasma together with the peripheral inflammation with large number of activated macrophages in the sciatic nerve could underlie the pathogenesis of peripheral impairment in Al-treated rats. Moreover, by lumogallion staining and fluorescence microscopy, we have seen for the first time the intracellular presence of Al in the sciatic nerve highlighting its interference on the neuropathic dysfunction observed after Al exposure. However, further detailed study will be necessary to verify the precise locations of deposits of Al in the nerve.

5. Conclusions

Our study provides evidence that 60-day exposures to low doses of Al, which aimed to mimic human exposure to Al by dietary source, are able to impair the PNS and, those effects are almost the same as observed after Al exposure at a high level. Here we show for the first time the specific presence of Al in the sciatic nerve fibers, which suggest that Al may trigger the onset of the peripheral dysfunction. The elevation of oxidative stress and inflammation highlight pathways of toxic actions for Al in the PNS. Moreover, our findings raise the concern regarding safety values of human exposure to Al and suggest an action of Al in the development of the neuropathic dysfunction.

Conflict of interest

None.

Author contributions

Conception and design of the study: CSM FMP GAW. Acquisition of data: CSM GV JAUO. Analysis and interpretation of data: CSM GAW GV JAUO DVV MMC. Drafting the article: CSM GAW. Revising the article and approval of the final version to be submitted: GAW MMC DVV GV JAUO FMP CSM.

Acknowledgments

The authors would like to acknowledge Professor Dr. Chris Exley for the scientific support and, Dr. Matthew Mold for the technical support on lumogallion staining, both from Keele University, Staffordshire, UK. We would like to thank to Antonio Márquez, Raquel Franco and Julio Paredes from the Laboratory of Histology of the Universidad Rey Juan Carlos for their technical assistance in tissue preparation. This work was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* [CNPq 406715/2013-0, 203503/2015-5]; the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*; *Programa Nacional de Cooperação Acadêmica*; *Pró-reitoria de Pesquisa - Universidade Federal do Pampa* [No 10.134.14] and by the Spanish Government [MINECO - AGL2012-32387, CSIC – Intramural 201570I028 and SAF2015-69294-R].

References

[1] C. Exley, Darwin, natural selection and the biological essentiality of aluminium and silicon, Trends Biochem. Sci. 12 (2009) 589–593.
[2] C. Exley, Elucidating aluminium's exposome, Curr. Inorg. Chem. 2 (2012) 3–7.
[3] C. Exley, Human exposure to aluminium, Environ. Sci.: Processes Impacts 10 (2013) 1807–1816.
[4] EFSA, Safety of aluminium from dietary intake, EFSA J. 754 (2008) 1–34.
[5] World Health Organization, Safety Evaluation of Certain Food Additives and Contaminants, Food Additive Series 58 (2007) http://whqlibdoc.who.int/trs/WHOTRS 940 eng.pdf.
[6] V. Fekete, S. Vandevijvere, F. Bolle, J. Van Loco, Estimation of dietary aluminum exposure of the Belgian adult population: evaluation of contribution of food and kitchenware, Food Chem. Toxicol. 55 (2013) 602–608.
[7] D. Gonzalez-Weller, A.J. Gutiérrez, C. Rubio, C. Revert, A. Hardisson, Dietary intake of aluminum in a Spanish population (Canary Islands), J. Agric. Food Chem. 58 (19) (2010) 10452–10457.
[8] M. Yang, L. Jiang, H. Huang, S. Zeng, F. Qiu, M. Yu, et al., Dietary exposure to aluminium and health risk assessment in the residents of Shenzhen, China, PLoS One 9 (3) (2014) e89715, , http://dx.doi.org/10.1371/journal.pone.0089715.
[9] A. Mirza, A. King, C. Troakes, C. Exley, The identification of aluminum in human brain tissue using lumogallion and fluorescence microscopy, J. Alzheimers Dis. 54 (4) (2016) 1333–1338.
[10] J.P. Klein, M. Mold, L. Mery, M. Cottier, C. Exley, Aluminum content of human semen: implications for semen quality, Reprod. Toxicol. 50 (2014) 43–48.
[11] R.K. Gherardi, J. Aouizerate, J. Cadusseau, S. Yara, F.J. Authier, Aluminum adjuvants of vaccines injected into the muscle: normal fate, pathology and associated disease, Morphology 100 (329) (2016) 85–94.
[12] J.D. Barata, P.C. D'Haese, C. Pires, L.V. Lamberts, J. Simoes, M.E. De Broe, Low-dose (5 mg/kg) desferrioxamine treatment in acutely aluminium-intoxicated haemodialysis patients using two drug administration schedules, Nephrol. Dial. Transplant. 11 (1996) 125–132.
[13] P.M. Lind, L. Olsen, L. Lind, Circulating levels of metals are related to carotid atherosclerosis in elderly, Sci. Total Environ. 416 (2012) 80–88.
[14] X. Li, C. Hu, Y. Zhu, H. Sun, Y. Li, Z. Zhang, Effects of aluminum exposure on bone mineral density, mineral, and trace elements in rats, Biol. Trace Elem. Res. 143 (2011) 378–385.
[15] C. Linhart, H. Talasz, E.M. Morandi, C. Exley, H.H. Lindner, S. Taucher, D. Egle, M. Hubalek, N. Concin, H. Ulmer, Use of underarm cosmetic products in relation to risk of breast cancer: a case-control study, EBioMedicine (17) (2017) 30233–30235, http://dx.doi.org/10.1016/j.ebiom.2017.06.005 (pii: S2352-3964).
[16] C. Exley, Aluminum should now be considered a primary etiological factor in Alzheimer's disease, J. Alzheimers Dis. Rep. (2017), http://dx.doi.org/10.3233/ADR-170010.
[17] A. Mirza, A. King, C. Troakes, C. Exley, Aluminium in brain tissue in familial Alzheimer's disease, J. Trace Elem. Med. Biol. 40 (2017) 30–36.
[18] P. Kasbe, A. Jangra, M. Lahkar, Mangiferin ameliorates aluminium chloride-induced cognitive dysfunction via alleviation of hippocampal oxido-nitrosative stress, proinflammatory cytokines and acetylcholinesterase level, J. Trace Elem. Med. Biol. 31 (2015) 107–112.
[19] B.V. Lakshmi, M. Sudhakar, K.S. Prakash, Protective effect of selenium against aluminum chloride-induced Alzheimer's disease: behavioral and biochemical alterations in rats, Biol. Trace Elem. Res. 1 (2015) 67–74.
[20] J.R. Walton, Functional impairment in aged rats chronically exposed to human range dietary aluminum equivalents, Neurotoxicology 30 (2009) 182–193.
[21] C. Bakar, H. Karaman, A. Baba, F. Sengünalp, Effect of high aluminum concentration in water resources on human health, case study: Biga Peninsula, northwest part of Turkey, Arch. Environ. Contam. Toxicol. 58 (4) (2010) 935–944.
[22] J.-H. Park, J. Chae, K. Roh, E.-J. Kil, M. Lee, C.-K. Auh, et al., Oxaliplatin-induced peripheral neuropathy via TRPA1 stimulation in mice dorsal root ganglion is correlated with aluminum accumulation, PLoS One 10 (4) (2015) e0124875, , http://dx.doi.org/10.1371/journal.pone.0124875.
[23] M. Lee, S. Cho, K. Roh, J. Chae, J.H. Park, J. Park, M.A. Lee, J. Kim, C.K. Auh, C.H. Yeom, S. Lee, Glutathione alleviated peripheral neuropathy in oxaliplatin-treated mice by removing aluminum from dorsal root ganglia, Am. J. Transl. Res. 9 (3) (2017) 926–939.
[24] C.S. Martinez, C.D. Alterman, F.M. Peçanha, D.V. Vassallo, P.B. Mello-Carpes, M. Miguel, et al., Aluminum exposure at human dietary levels for 60 days reaches a threshold sufficient to promote memory impairment in rats, Neurotox. Res. 31 (1) (2017) 20–30.
[25] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 3 (2008) 659–661.
[26] A. Prakash, A. Kumar, Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats, Basic Clin. Pharmacol. Toxicol. 2 (2009) 98–104.
[27] G. Vera, A. Chiarlone, P.A. Cabezos, D. Pascual, M.I. Martin, R. Abalo, Win 55,212-2 prevents mechanical allodynia but not alterations in feeding behaviour induced by chronic cisplatin in the rat, Life Sci. 81 (2007) 468–479.
[28] G.J. Bennett, Y.K. Xie, A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man, Pain 33 (1988) 87–107.
[29] A. Fox, A. Kesingland, C. Gentry, K. Mcnair, S. Patel, L. Urban, et al., The role of central and peripheral cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain, Pain 92 (2001) 91–100.
[30] C. Loetchutinat, S. Kothan, S. Dechsupa, J. Meesungnoen, J. Jay-Gerin, S. Mankhetkorn, Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay, Radiat. Phys. Chem. 72 (2005) 323–331.
[31] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[32] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[33] M. Mold, H. Eriksson, P. Siesjö, A. Darabi, E. Shardlow, C. Exley, Unequivocal identification of intracellular aluminium adjuvant in a monocytic THP-1 cell line, Sci Rep 5 (4) (2014) 6287, http://dx.doi.org/10.1038/srep06287.
[34] G. Crépeaux, H. Eidi, M.O. David, Y. Baba-Amer, E. Tzavara, B. Giros, et al., Non-linear dose-response of aluminium hydroxide adjuvant particles: selective low dose neurotoxicity, Toxicology 375 (2017) 48–57.
[35] C. Exley, Why industry propaganda and political interference cannot disguise the inevitable role played by human exposure to aluminum in neurodegenerative diseases, including Alzheimer's disease, Front. Neurol. 27 (2014) 5–212, http://dx.doi.org/10.3389/fneur.2014.00212.
[36] T. Kihira, S. Yoshida, J. Komoto, I. Wakayama, Y. Yase, Aluminum-induced model of motor neuron degeneration: subperineurial injection of aluminum in rabbits, Neurotoxicology 16 (3) (1995) 413–424.
[37] V.K. Bansal, S. Bansal, Nervous system disorders in dialysis patients, Handb. Clin. Neurol. 119 (2014) 395–404, http://dx.doi.org/10.1016/B978-0-7020-4086-3.00025-4.
[38] C.W. Hsu, C.H. Weng, C.C. Lee, D.T. Lin-Tan, K.H. Chen, T.H. Yen, W.H. Huang, Association of low serum aluminum level with mortality in hemodialysis patients, Ther. Clin. Risk Manag. 14 (12) (2016) 1417–1424.
[39] D.A. Rizzetti, F. Fernandez, S. Moreno, J.A. Uranga Ocio, F.M. Peçanha, G. Vera, D.V. Vassallo, M.M. Castro, G.A. Wiggers, Egg white hydrolysate promotes neuroprotection for neuropathic disorders induced by chronic exposure to low concentrations of mercury, Brain Res. 1646 (2016) 482–489.
[40] M. De Ryck, T. Schallert, P. Teitelbaum, Morphine versus haloperidol catalepsy in the rat: a behavioral analysis of postural support mechanisms, Brain Res. 201 (1980) 143–172.
[41] M.E. Castelló, B. Bolioli, F. Dajas, Catalepsy induced by striatal acetylcholinesterase inhibition with fasciculin in rats, Pharmacol. Biochem. Behav. 41 (3) (1992) 547–550.
[42] E.H. Sklan, A. Berson, K.R. Birikh, A. Gutnick, O. Shahar, S. Shoham, H. Soreq, Acetylcholinesterase modulates stress-induced motor responses through catalytic and noncatalytic properties, Biol. Psychiatry 60 (7) (2006) 741–751.
[43] S. Kumar, Aluminium-induced biphasic effect, Med. Hypotheses 52 (1999) 557–559.
[44] C. Exley, The pro-oxidant activity of aluminum, Free Radic. Biol. Med. 3 (2004) 380–387.
[45] F. Ruipérez, J.I. Mujika, J.M. Ugalde, C. Exley, X. Lopez, Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II), J. Inorg. Biochem. 117 (2012) 118–123.
[46] A. Ellis, D. Bennett, Neuroinflammation and the generation of neuropathic pain, Br. J. Anaesth. 111 (1) (2013) 26–37.
[47] R. Sandireddy, V.G. Yerra, A. Areti, P. Komirishetty, A. Kumar, Neuroinflammation and oxidative stress in diabetic neuropathy: futuristic strategies based on these targets, Int. J. Endocrinol. 2014 (2014) 674987, http://dx.doi.org/10.1155/2014/674987.

PARTE IV

Paper published at Reprod Toxicol.18;73:128-141, 2017.IF: 2, 3410. Qualis CBII: B1

Reproductive Toxicology 73 (2017) 128–141

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Aluminum exposure for 60 days at human dietary levels impairs spermatogenesis and sperm quality in rats

Caroline Silveira Martinez[a], Alyne Gourlart Escobar[a], José Antonio Uranga-Ocio[b], Franck Maciel Peçanha[a], Dalton Valentim Vassallo[c], Christopher Exley[d], Marta Miguel[e], Giulia Alessandra Wiggers[a,*]

[a] Graduate Program in Biochemistry, Universidade Federal do Pampa, BR 472, Km 592, PO Box 118, Zip Code: 97500-970, Uruguaiana, Rio Grande do Sul, Brazil
[b] Department of Basic Health Sciences, Universidad Rey Juan Carlos, Antenas s/n, Alcorcón, Spain
[c] Departments of Physiological Sciences, Universidade Federal do Espírito Santo and School of Medicine of Santa Casa de Misericórdia (EMESCAM), Av. Marechal Campos 1468, Zip Code: 29040-090, Vitória, Espírito Santo, Brazil
[d] The Birchall Centre, Lennard-Jones Laboratories, Keele University, Staffordshire, ST5 5BG, UK
[e] Bioactivity and Food Analysis Laboratory, Instituto de Investigación en Ciencias de la Alimentación, Nicolás Cabrera, 9, Campus Universitario de Cantoblanco, Madrid, Spain

ARTICLE INFO

Article history:
Received 5 May 2017
Received in revised form 3 August 2017
Accepted 9 August 2017
Available online 18 August 2017

Keywords:
Metal
Reproductive adverse effects
Sperm quality

ABSTRACT

Concerns about environmental aluminum (Al) and reproductive health have been raised. We investigated the effects of Al exposure at a human relevant dietary level and a high level exposure to Al. Experiment 1 (Lower level) rats were treated orally for 60 days: a) controls – ultrapure water; b) aluminum at 1.5 mg/kg bw/day and c) aluminum at 8.3 mg/kg bw/day. Experiment 2 (High level) rats were treated for 42 days: a) controls – ultrapure water; b) aluminum at 100 mg/kg bw/day. Al decreased sperm count, daily sperm production, sperm motility, normal morphological sperm, impaired testis histology; increased oxidative stress in reproductive organs and inflammation in testis. Our study shows the specific presence of Al in the germinative cells and, that low concentrations of Al in testes (3.35 μg/g) are sufficient to impair spermatogenesis and sperm quality. Our findings provide a better understanding of the reproductive health risk of Al.

1. Introduction

Human exposure to aluminum (Al) is inevitable, and its real consequence is largely unknown. After oxygen and silicon, Al is the third most abundant element in the Earth's crust and the increased biological availability of this metal is due to natural and anthropogenic actions over the years [1,2].

People are exposed to Al through dietary and non-dietary sources. Al salts are added to various commercially-available foods, are used as a flocculants in the treatment of drinking water and in packaging and storage of food products [3]. Humans are also exposed to considerable amounts of Al by non-dietary sources such as Al adjuvant in vaccines, medicines, cosmetics, sunscreens, deodorants and make up products [4].

In 2007, the tolerable weekly intake of Al for humans was adjusted to 1 mg Al/kg body weight (b.w.) [5]. However, it is known that humans may exceed health-based guidance values [3,6,7].

Even with a low rate of Al absorption through the gastrointestinal tract [8], taking account the overall sources of Al exposure, humans are continuously exposed to considerable and partly estimated amounts of Al every single day. Benefits are lacking between the interaction of this non-essential metal with normal biomolecules, making this body burden of Al potentially toxic [2].

Over the last years, concerns have increased about Al exposure and its relationship to reproductive health [9–11]. The decline of sperm quality and increases in infertility have been observed over recent decades [12–14], which suggests the involvement of environmental contributors to this phenomenon. Sperm health after Al exposure has been investigated; however, the findings, to date, are inconsistent [9,15]. Recently, Al content in human sperm was

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; DCF, dichlorofluorescein; MRA, mesenteric resistance arteries; MDA, malondialdehyde; TBA, thiobarbituric acid.

* Corresponding author at: PPGBioq, UNIPAMPA, BR 472, Km 592, PO Box 118, Zip Code: 97500-970, Uruguaiana, Rio Grande do Sul, Brazil.
E-mail addresses: caroline.s.martinez@gmail.com (C.S. Martinez), giuliawp@gmail.com (G.A. Wiggers).

http://dx.doi.org/10.1016/j.reprotox.2017.08.008
0890-6238/

related to reduction in sperm quality. Specifically, patients with oligozoospermia had higher Al concentration than others [16]. Experimental studies in animal models of Al intoxication support the human studies and show that Al exposure seems to be related to hormonal imbalance, decreases in sperm quality, histological abnormalities in reproductive organs and infertility [17,18].

However, studies addressing reproductive effects of Al have been conducted with doses of Al higher than might commonly be found among human populations [19–21]. Moreover, due to the suggested biphasic effect of Al [22], it is urgent to investigate the effects of Al exposure at human dietary levels and then to compare with Al effects at high levels. Herein we investigated the effects of Al exposure at three different doses: two low doses representing human Al exposure through the diet and, one model of exposure at a high Al level known to produce toxicity.

2. Material and methods

2.1. Animals

Three-month-old male *Wistar* rats (362.5 ± 11.7 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light-dark), giving free access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups, according to Martinez et al. [23]: Experiment 1 – low aluminum levels, and Experiment 2 – high aluminum level. For group 1, 18 rats were subdivided (in groups of six animals) and treated for 60 days as follows: a) the control groups received ultrapure drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) the second group received aluminum at 1.5 mg/kg bw/day based on human dietary levels according to a published protocol described by Walton [24], at the reduced Al exposure for 60 days, and c) the third group drank aluminum at 8.3 mg/kg bw/day which corresponds to the same aluminum human dietary levels (1.5 mg/kg) when translated to an animal dose based on body surface area normalization method [25]. For experiment 2, (the high aluminum level), 12 rats were subdivided (N = 6/each) and treated for 42 days as follows: a) the control group received ultrapure water through oral gavages; b) aluminum at 100 mg/kg bw/day [26].

Rat body weights, feed, water and Al intakes were measured weekly. At the end of the treatments, animals were euthanized by decapitation and the weights of testis, epididymis, prostate, vas deferens and seminal vesicle (empty, without coagulation gland), were determined. The right testis, epididymis and left vas deferens were used for sperm parameter analysis. Left testis and epididymis were divided in two segments, one of each was processed for histological and or immunohistochemical studies and the other part together with the prostate were quickly homogenized in 50 mM Tris HCl, pH 7.4, (5/10, w/v) for biochemical determinations. Afterwards, samples were centrifuged at 2400*g* for 10 min at 4 °C and the resulting supernatant fraction was frozen at −80 °C for further assay.

$AlCl_3 \cdot 6H_2O$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water (Milli-Q © 2012 EMD Millipore, Billerica, MA). The concentration of each stock solution was 0.008 mol/L, 0.034 mol/L and 0.331 mol/L, respectively from Al 1.5, 8.3 and 100 mg/kg bw. Salts and reagents were of analytical grade obtained from Sigma and Merck (Darmstadt, Germany).

2.2. Sperm parameters analysis

2.2.1. Daily sperm production per testis, sperm number and transit time in epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were counted as described by Robb et al. [27]. To calculate daily sperm production, the number of spermatids at stage 19 was divided by 6.1, which is the number of days these spermatids are present in the seminiferous epithelium. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the daily sperm production [27].

2.2.2. Sperm morphology

Sperm were obtained from the vas deferens and stored with 1 mL of 10% formal-saline until analysis. For the analysis, smears were prepared on histological slides and 200 spermatozoa per animal were evaluated under 400× magnification (Binocular, Olympus CX31). Morphological abnormalities were classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail), according to Filler [28].

2.2.3. Sperm motility

Sperm were removed from the vas deferens by internal rising with 1 mL of Human Tubular Fluid (DMPBS-Nutricell-SP-Brazil) pre-warmed to 34 °C. Then, a 10 μL aliquot was transferred to a histological slide. Under a light microscope (20× magnification, Binocular, Olympus CX31, Tokyo, Japan), 100 spermatozoa were analyzed and classified as type A: motile with progressive movement, type B: motile without progressive movement and type C: immotile. Sperm motility was expressed as% of total sperm [29].

2.3. Biochemical assay

2.3.1. Reactive oxygen species levels

The levels of reactive species (RS) in testis, epididymis and prostate were determined by a spectrofluorometric method, as described by Loetchutinat et al. [30]. This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The supernatant fraction of the sample was diluted (1:10) in 50 mM Tris–Hcl (pH 7.4) and 2′, 7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence units.

2.3.2. Lipid peroxidation

The levels of lipid peroxidation in testis, epididymis and prostate were measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. [31], with modifications. An aliquot of each tissue was incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4), and sodium dodecil sulphate 0.8% (SDS) at 100 °C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as nanomoles of MDA per mg of protein.

2.3.3. Ferric Reducing/Antioxidant Power (FRAP) assay

The total antioxidant capacity was measured in testis, epididymis and prostate by FRAP assay [32]. This method is based on the ability of the sample to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s- triazina (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 μL of the supernatant fraction of each tissue was added to 1 mL freshly prepared and pre-warmed (37 °C) FRAP reagent (500 μL of 300 mM acetate buffer (pH = 3.6), 250 μL of 10 mM TPTZ in 40 mM HCl, and 250 μL of 20 mM $FeCl_3$) in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50–1000 μM – water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

2.4. Testis and epididymis histology

To carry out the histological studies. Epididymis tissues were dehydrated, fixed in 10% formaldehyde and testis in Bouin's solution for 1–2 days. After several intensive washings, tissues embedded in paraffin, sectioned at 5 μm and stained with hematoxylin/eosin. Tissues were studied under a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6 to evaluate the morphometric parameters in testis: thickness of the seminiferous epithelium (μm) and the average number of empty seminiferous tubules/field as well as in the epididymis the average number of efferent ducts/field. The analysis was made in 10 random fields of 8 samples for each group, analysing approximately 7 seminiferous tubules per field and 5 efferent ducts per field of epididymis, in 20× magnification per section.

2.5. Testis immunohistochemistry

Testis immunohistochemistry was performed on paraffin-embedded sections of 5 μm thickness. De-paraffined slides were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter, sections were incubated for 10 min in 3% (*v/v*) hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal bovine serum for 30 min to minimize nonspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with a monoclonal antibody against macrophage-associated antigen (CD163, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to quantify the number of activated macrophages, which is consistent with the presence of inflammation. As a negative control, preparations were incubated without the primary antibody. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany).

2.6. Aluminum content in testis and epididymis

The Al content of testis and epididymis were determined using an established method [33]. Briefly, approximately 0.5 g and 0.3 g of testis and epididymis, were dried to a constant weight at 37 °C. Dried and weighed tissues were digested in a 1:1 mixture of 15.8 M HNO_3 and 30% *w/v* H_2O_2 in a microwave oven (MARS Xpress CEM Microwave Technology Ltd). Upon cooling each digest was diluted to a total volume of 5 mL with ultrapure water (cond < 0.067 < μS/cm) and the Al content of digests measured by TH GFAAS (Transversley Heated Graphite Furnace Atomic Absorption Spectrometry) using matrix-matched standards and an established analytical programme (House et al., 2012). Briefly, the TH GFAAS was calibrated by automated serial dilution of a 60 μg L^{-1} solution of Al with 1% HNO_3. Non-linear zero intercept WinLab 32-generated fits were applied (Perkin Elmer, UK). Instrument detection limits (IDL) were estimated from three times the standard deviation on the 1% HNO_3 calibration blank absorbance (n = 3 injections) divided by the Winlab32 generated calibration slope. Mean IDL for Al was 0.13 μg L^{-1} (SD 0.13 μg L^{-1}, n = 62). Concentrations of Al in NIST SRM1566B oyster tissue and IAEA-407 fish homogenate were used as spike samples and standard reference material. Results were expressed as μg Al/g tissue dry weight. Each determination was the arithmetic mean of a triplicate analysis.

2.7. Lumogallion staining

Lumogallion staining was performed in bouin and formalin-fixed testis and epididymis using a recent validated method to identify the presence of Al in tissues [34,35]. Briefly, re-hydrated tissues sections were immediately placed into either 1 mM lumogallion (TCI Europe N.V. Belgium) buffered in 50 mM PIPES, pH 7.4 or the PIPES-buffer alone for auto-fluorescence analyses for 45 min. Slides were carefully washed 6 times with PIPES-buffer, after rinsed in ultra-pure water for 30 s, finally mounted using an aqueous mounting media and stored horizontally at 4 °C overnight prior to imaging. Sections of tissues were imaged using a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6.

2.8. Statistical analysis

Data are expressed as mean ± SEM. Data of group 1 were analysed by ANOVA followed Bonferroni *post hoc* tests when appropriate and for sperm motility analysis Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data of group 2 were analysed by Student's *t*-test and Mann-Whitney test for motility data. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Body and organs weights, fluid and feed intake

Body weight of rats was similar between groups at the start and end of treatments (362.2 ± 11.7; 434.7 ± 11.1 g means at the start and end, respectively). The quantity of water, Al intakes and

Table 1
Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on daily feed and drink intakes ($p > 0.05$).

Feed/fluid intakes	Group 1			Group 2	
	Control	1.5 mg Al/kg bw/d	8.3 mg Al/kg bw/d	Control	100 mg Al/kg bw/d
Feed intakes	21.54 ± 0.27 g	22.16 ± 0.34 g	22.89 ± 0.41 g	22.23 ± 0.43 g	21.98 ± 0.34 g
Fluid intakes	35.24 ± 0.76 mL	34.99 ± 0.59 mL	35.67 ± 0.47 mL	34.32 ± 0.69 mL	35.67 ± 0.57 mL

Data are expressed as mean ± SEM. $p > 0.05$ (ANOVA or Student's *t*-test).

Table 2
Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on body weight, absolute and relative weights of reproductive organs.

Parameters	Group 1			Group 2	
	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Initial body weight (g)	360.10 ± 10.29	391.9 ± 14.87	396.4 ± 9.56	301.7 ± 9.86	315. 6 ± 14.01
Final body weight (g)	424.6 ± 9.54	450.7 ± 15.91	462.7 ± 10.58	410.1 ± 7.58	415.4 ± 11.78
Testis (g)	1.7 ± 0.13	2.01 ± 0.05	2.07 ± 0.14	1.9 ± 0.05	1.9 ± 0.06
Testis (g/100 g)	0.4 ± 0.03	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01
Epididymis (mg)	653.8 ± 23.15	703.2 ± 34.08	690.7 ± 25.86	662.2 ± 34.99	616.2 ± 35.13
Epididymis (mg/100 g)	151.6 ± 5.14	148.7 ± 5.36	142.1 ± 6.59	144.0 ± 4.71	141.7 ± 5.63
Ventral prostate (mg)	482.7 ± 42.88	429.8 ± 33.60	458.8 ± 58.61	415.8 ± 21.44	351.1 ± 21.79*
Ventral prostate (mg/100 g)	111.4 ± 9.09	91.4 ± 8.31	92.1 ± 8.16	104.3 ± 8.95	77 ± 5.31*
Full seminal vesicle (g)	1.6 ± 0.11	1.6 ± 0.21	1.6 ± 0.20	1.2 ± 0.15	1.3 ± 0.12
Full seminal vesicle (g/100 g)	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.03	0.2 ± 0.04	0.3 ± 0.02
Empty seminal vesicle (g)	0.5 ± 0.10	0.6 ± 0.11	0.6 ± 0.19	0.4 ± 0.05	0.4 ± 0.05
Empty seminal vesicle (g/100 g)	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.01
Vesicular secretion (g)	0.9 ± 0.14	0.9 ± 0.13	1.1 ± 0.13	0.7 ± 0.17	0.9 ± 0.14
Vas deferens (mg)	112 ± 14.7	97.2 ± 13.74	113.8 ± 10.44	99.6 ± 12.65	89.1 ± 9.4
Vas deferens (mg/100 g)	26.1 ± 3.56	20.1 ± 2.33	23.6 ± 2.69	21 ± 2.93	20.4 ± 1.84

Data are expressed as mean ± SEM. The relative organ weight was calculated by use of the formula: organ weight/body weight × 100. Units: g: gram, mg: milligram.
* $p < 0.05$ compared with controls from the corresponding group 2 (Student's *t*-test).

Table 3
Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm counts in testis and epididymis of rats.

Parameters	Group 1			Group 2	
Sperm count	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Testis					
Sperm number (×10⁶)	142.7 ± 8.42	104.8 ± 2.60**	93.43 ± 6.89**	148.1 ± 8.72	115.8 ± 11.84*
Sperm number (×10⁶/g)	86.13 ± 5.43	60.58 ± 0.88**	54.48 ± 5.44**	97.81 ± 6.76	65.79 ± 5.95**
DSP (×10⁶/testis/day)	23.40 ± 1.38	17.19 ± 0.42**	15.32 ± 1.13**	24.30 ± 1.21	18.98 ± 1.64*
DSPr (×10⁶/testis/day/g)	14.12 ± 0.89	9.92 ± 0.14**	8.93 ± 0.89**	16.04 ± 1.10	10.79 ± 0.97**
Epididymis					
Caput/Corpus					
Sperm number (×10⁶)	140.2 ± 12.16	132.7 ± 4.61	129.7 ± 7.58	142 ± 5.97	133.7 ± 7.53
Sperm number (×10⁶/g)	402.5 ± 28.82	351.9 ± 12.69	354.7 ± 20.10	416.0 ± 18.41	369.2 ± 10.97
Sperm transit time (days)	6.03 ± 0.45	7.74 ± 0.34*	9.77 ± 0.77*	6.21 ± 0.46	7.33 ± 0.67
Cauda					
Sperm number (×10⁶)	178.6 ± 17.81	139.6 ± 9.29	150.0 ± 11.89	166.3 ± 10.48	139.5 ± 14.88
Sperm number (×10⁶/g)	823.7 ± 62.56	642.1 ± 49.22	701.3 ± 31.66	737.7 ± 26.43	645.4 ± 35.91
Sperm transit time (days)	7.61 ± 0.62	8.11 ± 0.46	10.03 ± 1.09	7.03 ± 0.81	7.51 ± 0.81

DSP: daily sperm production; DSPr: daily sperm production relative to testis weight. Data are expressed as mean ± SEM. Units: g: gram. * $p < 0.05$, ** $p < 0.01$ compared with their corresponding controls (ANOVA or Student's *t*-test).

feed intake were not different between groups (P > 0.05; one-way ANOVA/*t*-test – Table 1). Al exposure at low levels (group 1) did not change the absolute and relative reproductive organ weights. However, Al at 100 mg/kg bw/day decreased the weight of the ventral prostate (control: 415.8 ± 21.4 *vs* Al 100 mg/kg bw/day: 351.1 ± 21.7 mg, *P < 0.05 – Table 2).

3.2. Daily sperm production per testis, sperm number and transit time in epididymis

To investigate the effect of Al on sperm count, group 1 rats were treated for 60 days with Al at 1.5 or 8.3 mg/kg bw/day and group 2 rats were exposed to Al at 100 mg/kg bw/day for 42 days, and the

Table 4
Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm morphology of rats.

Parameters	Group 1			Group 2	
Sperm morphology	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Normal	92.5 (92–94.3)	89.2 (85.6–92.2)*	83 (74.8–88)**	94 (89.63–96.13)	84 (81.38–87.75)**
Head Abnormalities					
Amorphous	2 (1.6–2.5)	3.5 (1.3–8.1)	6 (3.8–10) **	1.5 (0.8–2.5)	7.2 (6.8–11.1) **
Banana Head	0.5 (0–0.6)	1 (0–2.2)	3 (1.6–4.8) *	1.5 (1–2)	0 (0–0.6)
Detached Head	1 (0.5–3)	1.2 (0.5–2.5)	1.5 (0.8–2.3)	1.7 (0.5–4.2)	3.2 (1.2–6)*
Total of Head Abnormalities	3.7 (2.8–5.3)	6.7 (3–12.8)	10.7 (9–16.1)**	5.5 (3.5–9.6)	11.7 (9.3–15.1)*
Tail Abnormalities					
Bent Tail	1 (0.5–1.8)	1 (0.5–2.3)	2.5 (2–3)**	0.0 (0.0–0.0)	1 (0.5–1.5)**
Broken Tail	0 (0.0–0.5)	0.2 (0–0.75)	0.5 (0.3–1)	0.2 (0.0–0.6)	1.2 (0.3–4.8)
Total of Tail Abnormalities	1.5 (1.2–3.2)	2.5 (1.6–4.2)	3 (2.2–4.2)	0.2 (0.0–0.6)	2 (1.5–2.7)**

Data are expressed as median (Q1–Q3). *$p < 0.05$, **$p < 0.01$ compared with their corresponding controls (Kruskal-Wallis test followed by Dunn's or Mann–Whitney).

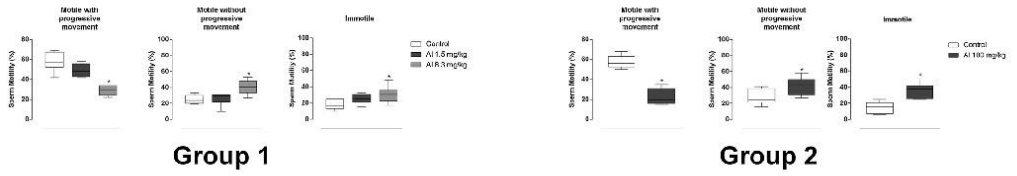

Fig. 1. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm motility: motile with progressive movement, motile without progressive movement and immotile. Data are expressed as median (Q1–Q3), n = 6, * $p < 0.05$ compared with their corresponding controls (Kruskal-Wallis test followed by Dunn's or Mann–Whitney).

control rats were treated with ultrapure water. Chronic exposure to Al at different doses altered sperm parameters in testis, there was a reduction in daily sperm production per testis and in sperm count (Table 3). In the epididymis of group 1 rats, Al increased the sperm transit time in the caput/corpus and there was an apparent decrease in sperm number, which was not statistically significant (mean of total sperm in epididymis for group 1 control: 318.8, Al 1.5 mg/kg bw/day: 272.3, Al 8.3 mg/kg bw/day: 279.7×10^6; group 2 control: 308.3, Al 100 mg/kg bw/day: 273.2×10^6, $P > 0.05$, see more details in – Table 3).

3.3. Sperm morphology and motility

Sperm analysis revealed a significant decrease in sperm with normal morphology in rats exposed to Al when compared with the control group (group 1: control: 92.5 (92–94.3), Al 1.5 mg/kg bw/day: 89.2 (85.6–92.2)* Al 8.3 mg/kg bw/day: 83 (74.8–88)*; group 2: control: 94 (89.63–96.13), Al 100 mg/kg bw/day: 84 (81.38–87.75)*, – Table 4). Group 1 rats treated for 60 days with Al 8.3 mg/kg bw/day and group 2 rats exposed to Al at 100 mg/kg bw/day, for 42 days, showed specific abnormalities. Within head phenotypes, amorphous, banana and detached head were observed; concerning tail morphology, the bent tail was the most frequency abnormality in rats exposed to Al at major doses (mean of total sperm abnormalities for group 1 control: 6.18, Al 1.5 mg/kg bw/day: 10.58, Al 8.3 mg/kg bw/day: 15.33; group 2 control: 6.58, Al 100 mg/kg bw/day: 14.41% *$P < 0.05$, see more details in – Table 4).

Regarding sperm motility, for group 1, Al exposure at the lowest dose of 1.5 mg/kg bw/day did not affect the motility (Fig. 1A). On contrast, Al exposure at 8.3 mg/kg bw/day, for 60 days, and rats exposed to Al at 100 mg/kg bw/day, for 42 days, decreased type A sperm (motile with progressive movement) accompanied by an increase in type B (motile without progressive movement) and type C sperm (immotile) (mean of total motile sperm for group 1 control: 85.66, Al 1.5 mg/kg bw/day: 75, Al 8.3 mg/kg bw/day: 59.67; group 2 control: 85.16, Al 100 mg/kg bw/day: 64% *$P < 0.05$, see more details in – Fig. 1A and B).

3.4. Reactive species and lipid peroxidation levels

Al treatment at different doses increased the levels of reactive species (RS) in epididymis (Fig. 2C and D) and in prostate (Fig. 2E and F), while in testis only Al at 8.3 mg/kg bw/day and 100 mg/kg bw/day altered this oxidative stress parameter (Fig. 2A and B).

There was a significant increase in lipid peroxidation in testis of Al treated rats at all doses evaluated (Fig. 3A and B). In epididymis and prostate, the major doses of Al increased MDA levels (Fig. 3C–F) and no differences were observed in epididymis and prostate lipid peroxidation after Al exposure at 1.5 mg/kg bw/day (Fig. 3C and E).

3.5. Total antioxidant capacity–Ferric Reducing/Antioxidant Power (FRAP)

Al at 1.5 mg/kg bw/day decreased the total antioxidant capacity in testis, while at the highest dose of 100 mg/kg bw/day there was the opposite effect (Fig. 4A and B). In the epididymis, only Al at the middle dose of 8.3 mg/kg bw/day decreased the antioxidant capacity (Fig. 4C) and, the prostate total antioxidant capacity was reduced after Al exposure at minor and major doses (Fig. 4E and F).

3.6. Testis and epididymis histology

Histopathological studies of testes showed that aluminum exposure for 60 days at the lower levels (Gp.1) or for 42 days at higher levels (Gp.2) impaired testis architecture. In Al-treated rats the thickness of the seminiferous tubules were reduced from 70.56 μm in the control group to 53.96 μm after Al exposure at 8.3 mg/kg and 52.04 μm after Al exposure at the highest dose. There was a decrease in the number of spermatogenic cells in the lumen of the seminiferous tubules in Al-treated rats, which was observed by the increased seminiferous tubules with less or absence of mature spermatogenic cells, classified as empty seminiferous tubules. For Al exposure at 8.3 mg/kg bw/day the average number of empty seminiferous tubules was almost three times the number found in the control group (Fig. 5B, D–F). However, Al exposure at the higher dose of 100 mg/kg bw/day did not decrease the number of spermatogenic cells (Fig. 5G and H). In the control groups, the structure of seminiferous tubules was normal (Fig. 5A and C). The epididymis histology revealed no differences between the structure of epididymis from control and Al-groups. Both showed similar number of empty efferent ducts with the means varying from 7.4 to 9.5 per field (Fig. 6).

3.7. Testis immunohistochemistry

Immunohistochemical analysis showed an increase in the number of activated macrophages in testes of rats treated with Al at the low dose of 8.3 mg/kg bw/day when compared with the control group (ranging from 5 to 15 in the control group and from 21 to 40 in the Al-treated rats – Fig. 7A, B and E). Al exposure at the higher dose did not stimulate inflammation in testes (Fig. 7C, D and F).

3.8. Aluminum content and lumogallion staining in testis and epididymis

We investigated the Al content in testis and epididymis of rats exposed to Al at the low dose of 8.3 mg/kg bw/day. The mean Al concentration in testis of Al-exposed rats was found to be almost twice the amount found in the control group (control 1.79 ± 0.41 *vs* Al 3.35 ± 0.47 μ/g * $p < 0.05$ Student's *t*-test). While, the Al content in the epididymis was not statistically different between groups (control 6.38 ± 0.75 *vs* Al 6.10 ± 1.13 μ/g – n = 5)

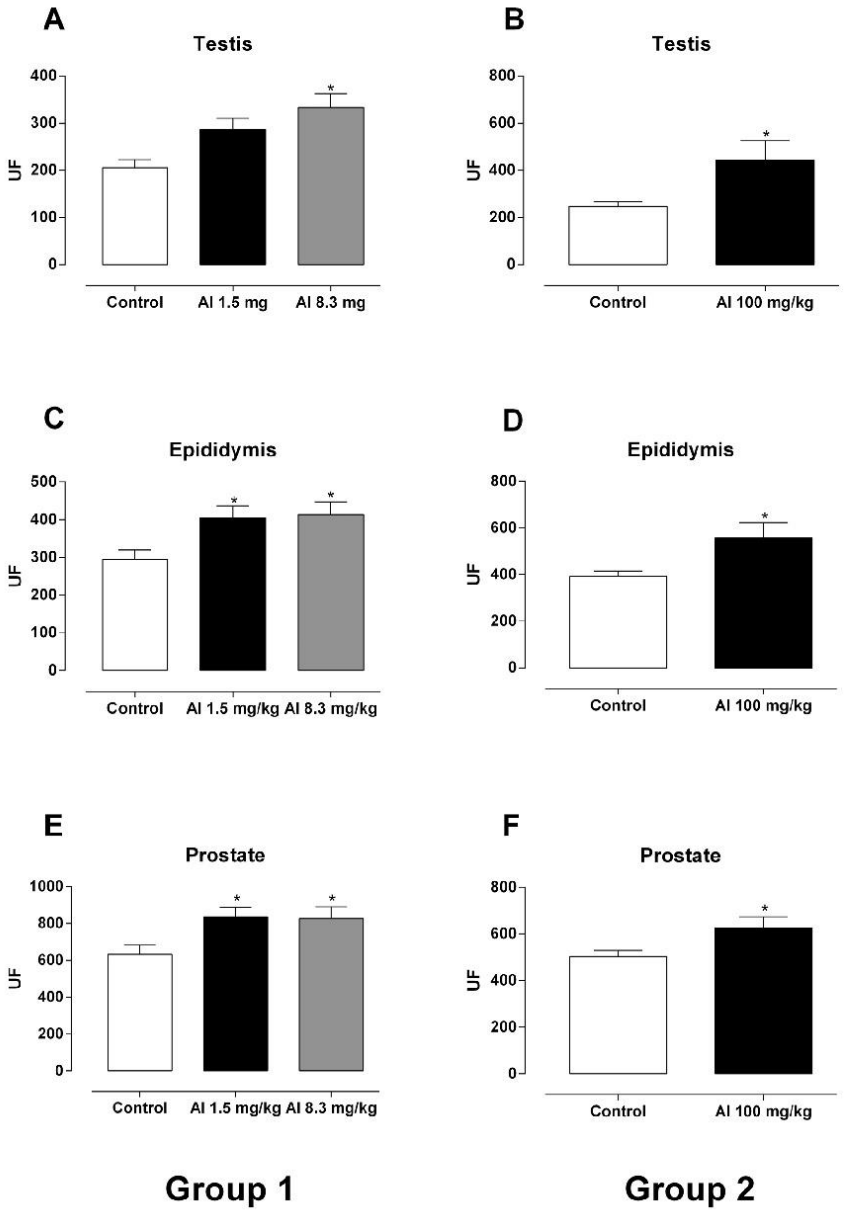

Fig. 2. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on reactive oxygen species levels (ROS). Values of ROS on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's *t*-test). UF: Units of fluorescence.

The presence of Al was confirmed using lumogallion and fluorescence microscopy. Testis and epididymis showed green autofluorescence in the absence of lumogallion (Fig. 8A, C, E and G). Lumogallion fluorescence identified Al in the germinative cells in the seminiferous tubules as evidenced by bright orange fluorescence (Fig. 8D). In the epididymis Al seemed associated with

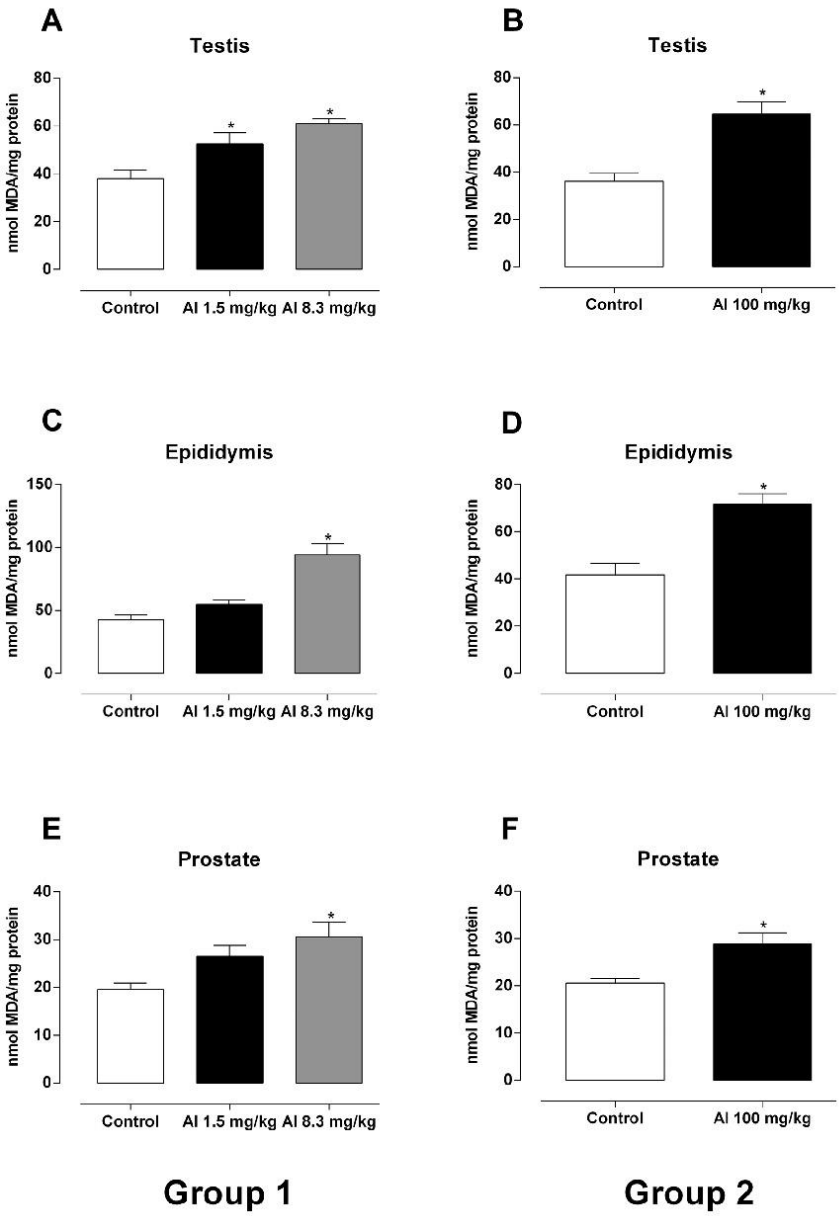

Fig. 3. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on lipid peroxidation measurements. Values of MDA (malondialdehyde) on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's *t*-test).

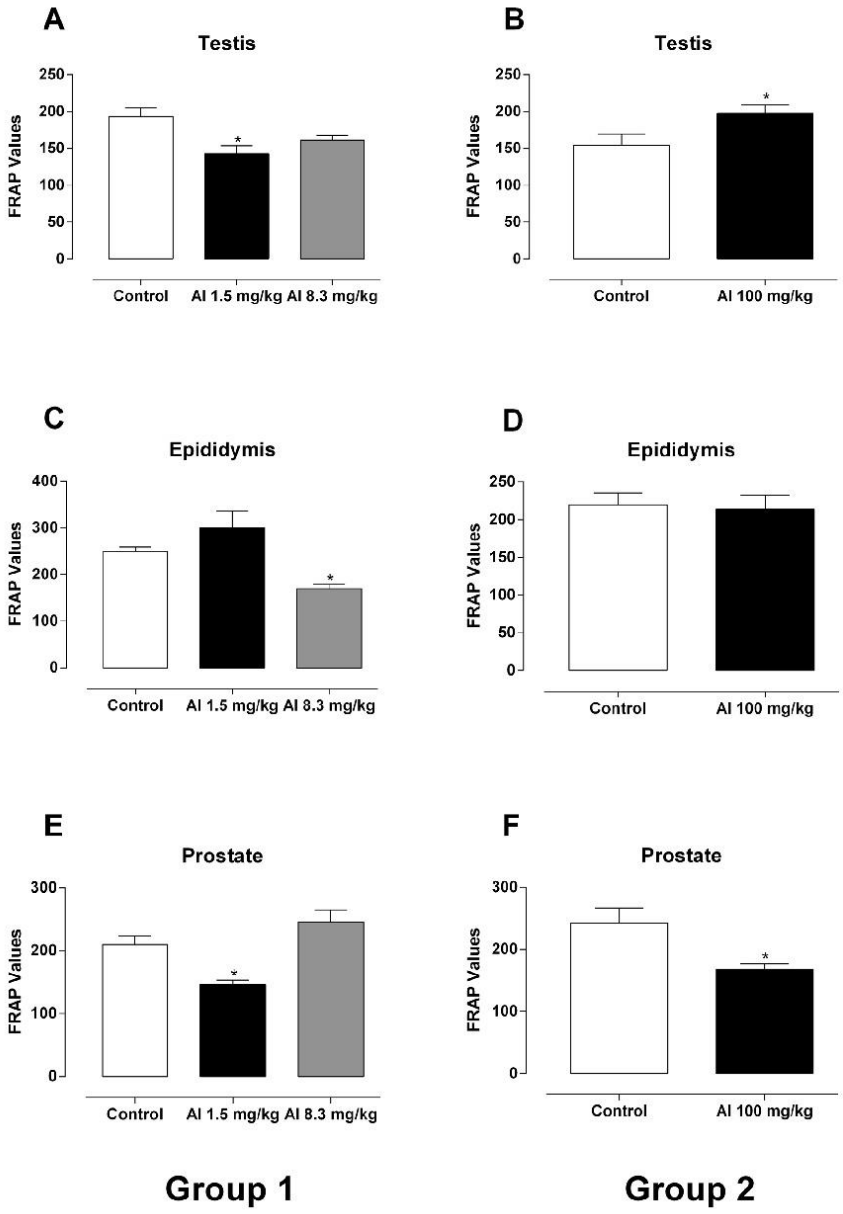

Fig. 4. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on total antioxidant capacity. Values of FRAP (Ferric Reducing/Antioxidant Power) on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's *t*-test).

blood cells. In this organ we are not able to identify differences between control and Al-treated rats, which is in accordance with the quantification of Al by TH GFAAS (Fig. 8F and H).

4. Discussion

The decline in semen quality, including in countries that previously boasted good sperm characteristics, highlights the male

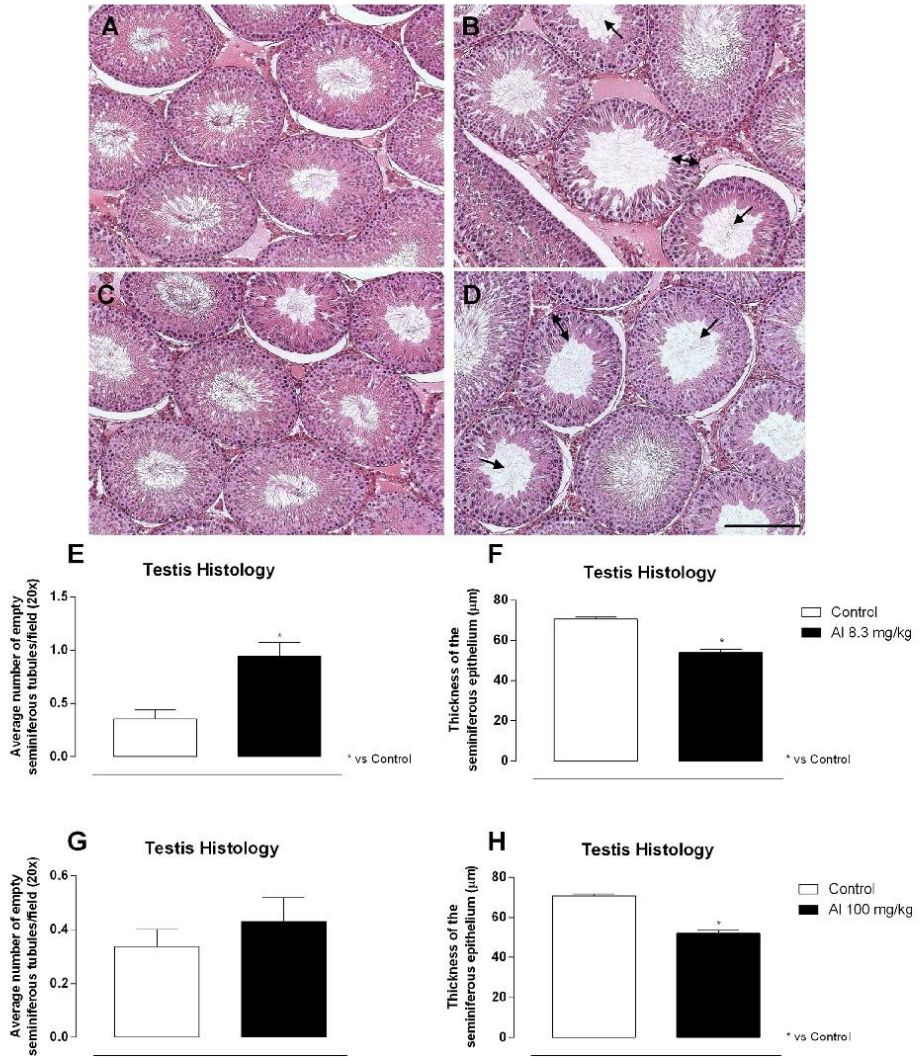

Fig. 5. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis histopathology. Control group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D). Average number of empty seminiferous tubules per field (X20) for group 1 (E) and for group 2 (F) in absolute numerical values. Testes sections of Al-treated rats showing reduction of spermatozoa in the lumen of the seminiferous tubules (arrows). Thickness of the seminiferous epithelium (µm) for group 1 (G) and for group 2 (H), showing a reduced thickness in testes of Al-treated rats (double arrows). Scale bars: 50 µm. Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (Student's *t*-test).

reproductive system as one of the major targets of environmental toxicants [36]. It seems likely that the cumulative effects of various low-dose exposures to environmental contaminants are responsible for male reproductive effects. Synergistically, the continuous increase in human exposure to Al challenged us to investigate the male reproductive effects regarding Al exposure at human dietary levels. Our results suggest that Al should be considered as a hazard to the male reproductive system even at low Al doses. Here we show that Al exposure for 60 days at human dietary levels impairs sperm quality, as observed by suppression of sperm production and count reduction followed by motility and morphological abnormalities in rats. This functional impairment appears together with a redox imbalance, with increased ROS production, lipid peroxidation and altered antioxidant capacity in reproductive organs. Surprisingly, these effects are similar to those found in rats exposed to Al at a dose more than 60 times higher. Based on these first findings, we decided

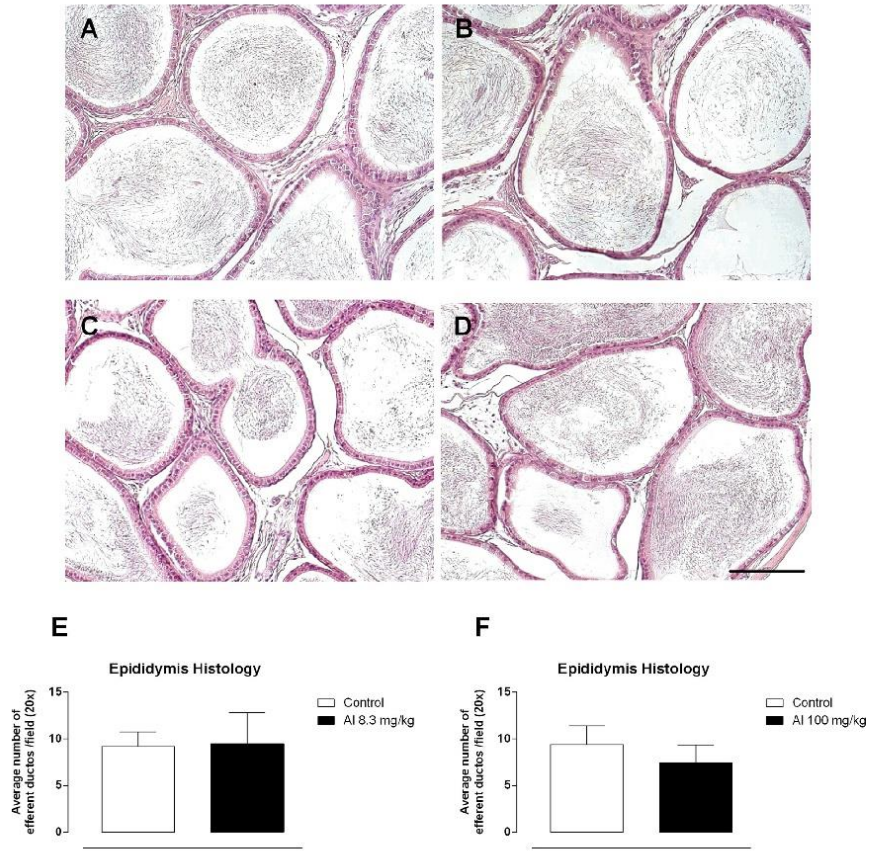

Fig. 6. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on epididymis histopathology. Control group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D). Average number of empty efferent ducts per field (X20) for group 1 (E) and for group 2 (F). Scale bars: 50 μm. Data are expressed as mean ± SEM (n = 6).

to go further to better understand the effects of Al on the male reproductive system. For this, we have chosen a dose of Al exposure at a lower level, one that better characterized the reproductive dysfunction, and then we have compared with Al at a higher dose. Unexpectedly, but in accordance with recent discoveries about Al neurotoxicity [37], Al at the lower dose of 8.3 mg/kg bw/day had worse effects on the reproductive system. Specifically, the testis histoarchitecture of rats exposed to Al at 100 mg/kg bw/day was better organized with a larger number of sperm cells and without concomitant inflammation. However, further studies are necessary to go further and better understand such discoveries.

Recently, using the same model of Al exposure at low levels, we showed that once Al achieved a threshold its toxicity is almost the same. We developed the same behavioral evaluations in rats exposed to low Al doses and the neurotoxicity effects were practically the same as those induced by the highest dose [23].

Crépeaux et al. [37], by investigating the effects of the adjuvant aluminium oxyhydroxide (Alhydrogel®) in female mice, only found neurocognitive impairments at the lowest dose of 0.2 mg Al/kg and not at 0.4 or 0.8 mg Al/kg. In the current study, we have found adverse effects after Al exposure at the higher dose. However, Al at 8.3 mg/kg, the amount equivalent to human Al exposure, showed worse effects. This may seem as though the dose is not the most important issue regarding Al toxicity, but the exposure conditions, intrinsic and individual characteristics and, consequent distribution and bioavailability through the body. Our results suggest that current safety limits (*e.g.* WHO) relating to human exposure should be reviewed.

The male reproductive system, especially the testes and spermatozoa, are very susceptible to oxidative damage, mainly because of their high content of polyunsaturated fatty acids in membranes, their limited antioxidant capacity and the ability of spermatozoa to generate reactive oxygen species [38]. Overproduction of reactive oxygen species, however, can be detrimental to sperm and, appears to be a common feature underlying male infertility [39]. Al^{3+} toxicity has correlates with pro-oxidant activity in several organs and tissues [40,26,41,42], and more recently in male reproductive toxicity [11,18,19]. In the present study, Al exposure increased oxidative stress in testis, epididymis and prostate, as evident from an increase in RS generation and MDA levels. The oxidative stress

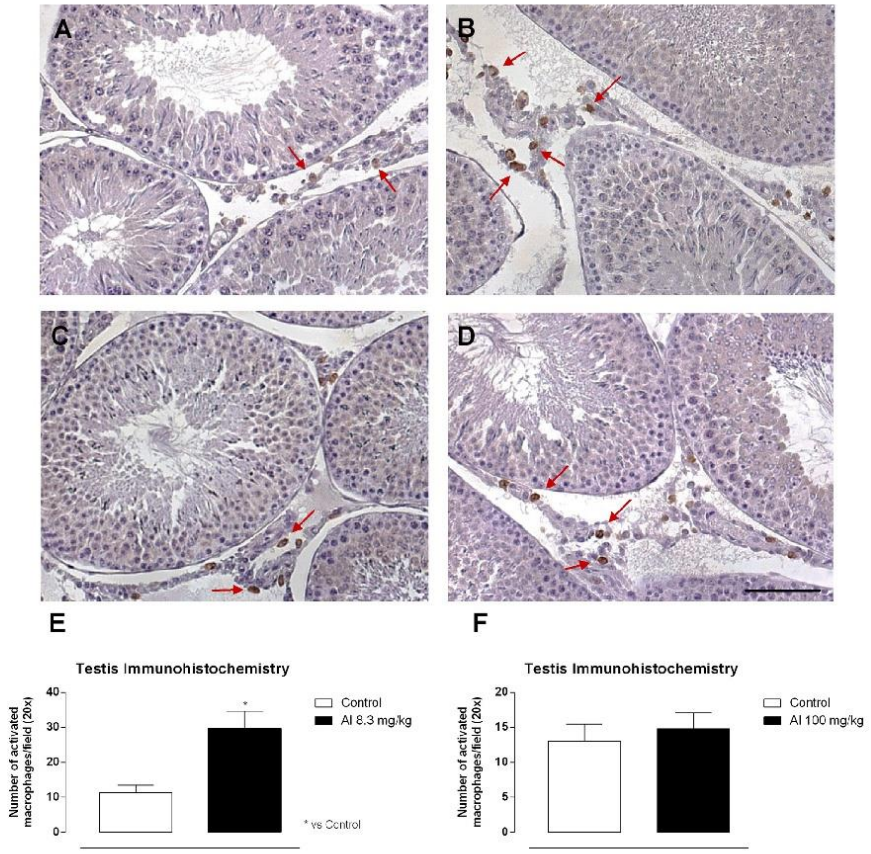

Fig. 7. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis immunohistochemistry. Activate macrophages (arrows) in testis of controls group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D) detected by immunohistochemistry. Scale bars: 50 μm. Average numbers of activated macrophages per field (objective X20) for group 1 (E) and for group 2 (F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (Student's *t*-test).

came together with an inflammatory process with large number of macrophage activated in testis of rats exposed to Al at 8.3 mg/kg bw/day. The suppression of spermatogenesis and sperm impairments as well as the histopathological changes observed, could be partially attributed to peroxidation of polyunsaturated fatty acids in the sperm membrane, needed for sperm viability [43], and, to inflammation within the testis.

Regarding the cell's defense and protection against increased oxidative stress, the total antioxidant capacity was contrastingly changed among Al exposure models and according to the organ evaluated. For example, Al exposure at the low doses of 1.5 and 8.3 mg/kg bw/day decreased the antioxidant capacity in testis while at the highest dose an increase in the antioxidant profile was observed. This suggests that Al does not have a classical toxicological pattern in that the adverse effects of this metal are dependent on the duration of exposure, contamination threshold and bioavailability that is achieved, making a low Al dose able to promote male reproductive dysfunction.

Data regarding Al and human semen quality are scarce. Studies of Hovatta [10] and Dawson [9] showed relationships between Al in seminal plasma and sperm motility. More recently, this association was also found in human sperm samples exposed to $AlCl_3$, cadmium or lead, in which Al showed the worst effects [11]. In a recent study by Klein et al. [16], semen of 62 patients were investigated and revealed high concentration of Al in individuals with low sperm count.

Experimental animal studies addressing Al exposure and the male reproductive system are more numerous. A single intraperitoneal injection of $AlCl_3$ at 25 mg/kg in mice was associated with germ cell degeneration, tubular atrophy, apoptotic cell death of spermatogonia and primary spermatocytes and, mitochondrial damage in Leydig cells [44]. $AlCl_3$ intragastrically for 4 weeks at 100 mg/kg bw/day induced histopathological alterations in testes and epididymis, increased MDA levels and promoted a reduction in glutathione levels in rats [19]. $AlCl_3$ administration at doses ranging from 34 mg/kg bw/day to 256.72 mg/kg bw/day have been related with a reduction in reproductive organs weights, sperm count and motility, decreased libido and ejaculate volume, increased sperm abnormalities and hormonal imbalance such as decrease in plasma

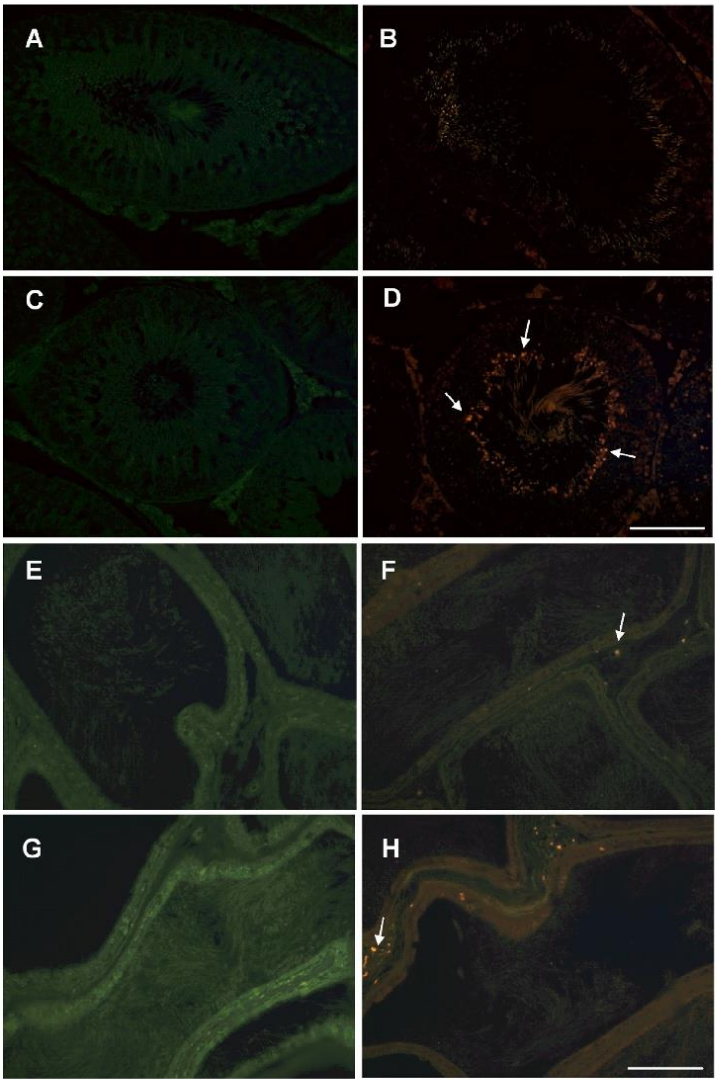

Fig. 8. Aluminum presence in reproductive tissues. Representative images of aluminum in testis and epididymis: autofluorescence in control groups (A and E) and in Al-treated rats (C and G); lumogallion fluorescence for aluminum in control group (B and F) and in Al-treated rats (D and H). The specific presence of Al is indicated by arrows. Scale bars: 50 μm.

testosterone, luteinizing hormone and follicular stimulating hormone in rats and rabbits [17,20,21].

However, these studies have been addressing the effects of Al on male reproductive system at considerable high levels of Al exposure. Also these studies failed to consider the amount of Al from the animalís feed. In our experimental model, we have measured the amount of Al from the feed [23] and, all rats including controls received 1.88 mg/Al/day from their standard feed. Therefore, taking into account the animals mean body weights of 300 g, the total amount of Al exposure for experiment 1, low aluminum levels, was: a) 1.5 mg/Al/kg bw/day – 2.33 mg/Al/day (0.45 mg/Al from water plus 1.88 mg/Al from feed); b) 8.3 mg/Al/kg bw/day – 4.37 mg/Al/day (2.49 mg/Al from water plus 1.88 mg/Al from feed), and for group 2, High Aluminum Level: c) 100 mg/Al/kg bw/day –31.88 mg/Al/day (30 mg/Al from gavage plus 1.88 mg/Al from feed).

In the current study, Al exposure for 60 days at relevant human dietary levels was able to impair sperm quality and spermato-

genesis and the Al induced oxidative stress and inflammation in the testis. Relating to our findings about Al concentrations, it is shown for the first time that concentrations of Al around 3 μg/g in testis are sufficient to induce male reproductive dysfunction. Previous studies showing male reproductive toxicity were performed with unrealistic high doses of Al (from 34 mg/kg to 400 mg/kg/bw), showing higher Al concentration in testes, between 35 μg/g and 140 μg/g [45,46,18].

The identification of Al in tissues or cells using lumogallion and fluorescence microscopy was shown to be specific for Al with no interference from any other metals and no issues relating to autofluorescence [34,35]. We have used lumogallion staining to show the presence of Al in testes of rats and, we are the first to show Al associated with unidentified structures and among germinative cells, which could reinforce its interference on the spermatogenesis process.

5. Conclusions

Our study shows that 60-day exposure to low doses of Al, which aimed to mimic human exposure to Al by the dietary route, are able to impair male reproductive health. Strikingly, the reproductive impairment was, sometimes, less-marked at the higher dose of Al, suggesting a non-linear effect of Al in this system. The current study shows, for the first time, the specific presence of Al in the germinative cells and, that low concentrations of Al in testes are sufficient to impair spermatogenesis and sperm quality. The elevation of oxidative stress and inflammation highlight pathways of toxic actions for this metal on the male reproductive system. Our findings provide a better understanding of the reproductive health risk after Al exposure.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* [CNPq 406715/2013-0, 203503/2015-5]; the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*; *Programa Nacional de Cooperação Acadêmica; Pró-reitoria de Pesquisa–Universidade Federal do Pampa* [N° 10.134.14] and by the Spanish Goverment [MINECO – AGL2012-32387, CSIC – Intramural 201570I028]. The authors would like to acknowledge Dr. Matthew Mold from Keele University, Staffordshire, UK, for the support on lumogallion staining and, Antonio Márquez, Raquel Franco and Julio Paredes from the Laboratory of Histology of the Universidad Rey Juan Carlos for their technical assistance in tissue preparation.

References

[1] C. Exley, Elucidating aluminiumís exposome, Curr. Inorg. Chem. 2 (2012) 3–7.
[2] C. Exley, Human exposure to aluminium, Environ. Sci. Process. Impacts 10 (2013) 1807–1816.
[3] V. Fekete, S. Vandevijvere, F. Bolle, J. Van Loco, Estimation of dietary aluminum exposure of the Belgian adult population: evaluation of contribution of food and kitchenware, Food Chem. Toxicol. 55 (2013) 602–608.
[4] S.C. Bondy, Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration, Neurotoxicology 52 (2015) 222–229.
[5] World Health Organization, Safety Evaluation of Certain Food Additives and Contaminants. Food Additive Series: 58, 2007 http://whqlibdoc.who.int/trs/WHOTRS940eng.pdf.
[6] D. Gonzalez-Weller, A.J. Gutiérrez, C. Rubio, C. Revert, A. Hardisson, Dietary intake of aluminum in a Spanish population (Canary Islands), J. Agric. Food Chem. 58 (19) (2010) 10452–10457.
[7] M. Yang, L. Jiang, H. Huang, S. Zeng, F. Qiu, M. Yu, et al., Dietary exposure to aluminium and health risk assessment in the residents of Shenzhen, China, PLoS One 9 (3) (2014) e89715, http://dx.doi.org/10.1371/journal.pone.0089715.
[8] J.J. Powell, R.P. Thompson, The chemistry of aluminium in thegastrointestinal lumen and its uptake and absorption, Proc. Nutr. Soc. 52 (1) (1993) 241–253.
[9] E.B. Dawson, S. Ritter, W.A. Harris, D.R. Evans, L.C. Powell, Comparison of sperm viability with seminal plasma metal levels, Biol. Trace Elem. Res. 64 (1998) 215–219.
[10] O. Hovatta, E.R. Venäläinen, L. Kuusimäki, J. Heikkilä, T. Hirvi, I. Reima, Aluminium, lead and cadmium concentrations in seminal plasma and spermatozoa, and semen quality in Finnish men, Hum. Reprod. Oxf. Engl. 13 (1998) 115–119.
[11] M. Jamalan, M.A. Ghaffari, P. Hoseinzadeh, M. Hashemitabar, M. Zeinali, Human sperm quality and metal toxicants: protective effects of some flavonoids on male reproductive function, Int. J. Fertil. Steril. 10 (2) (2016) 215–223.
[12] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Evidence for decreasing quality of semen during past 50 years, Br. Med. J. 305 (1992) 609–613.
[13] C.M. Nelson, R.G. Bunge, Semen analysis: evidence for changing parameters of male fertility potential, Fertil. Steril. 25 (1974) 503–507.
[14] E.K. Sheiner, E. Sheiner, R.D. Hammel, G. Potashnik, R. Carel, Effect of occupational exposures on male fertility: literature review, Ind. Health 41 (2003) 55–62.
[15] J.M. Mur, P. Wild, R. Rapp, J.P. Vautrin, J.P. Coulon, Demographic evaluation of the fertility of aluminium industry workers: influence of exposure to heat and static magnetic fields, Hum. Reprod. 13 (7) (1998) 2016–2019.
[16] J.P. Klein, M. Mold, L. Mery, M. Cottier, C. Exley, Aluminum content of human semen: implications for semen quality, Reprod. Toxicol. 50 (2014) 43–48.
[17] S.F. Ige, R.E. Akhigbe, The role of Allium cepa on aluminum-induced reproductive dysfunction in experimental male rat models, J. Hum. Reprod. Sci. 5 (2) (2012) 200–205.
[18] N.S. Mohammad, M.H. Arafa, H.H. Atteia, Coenzyme Q10 and fish oil synergistically alleviate aluminum chloride-induced suppression of testicular steroidogenesis and antioxidant defense, Free Radic. Res. 49 (11) (2015) 1319–1334.
[19] S.S. Oda, The influence of Omega3 fatty acids supplementation against aluminum-induced toxicity in male albino rats, Environ. Sci. Pollut. Res. Int. 23 (14) (2016) 14354–14361, http://dx.doi.org/10.1007/s11356-016-6578-4.
[20] H. Sun, C. Hu, L. Jia, Y. Zhu, H. Zhao, B. Shao, et al., Effects of aluminum exposure on serum sex hormones and androgen receptor expression in male rats, Biol. Trace Elem. Res. 144 (2011) 1050–1058.
[21] Y.Z. Zhu, H. Sun, Y. Fu, J. Wang, M. Song, M. Li, et al., Effects of sub-chronic aluminum chloride on spermatogenesis and testicular enzymatic activity in male rats, Life Sci. 102 (1) (2014) 36–40.
[22] C. Exley, J.D. Birchall, The cellular toxicity of aluminium, J. Theor. Biol. 159 (1) (1992) 83–98.
[23] C.S. Martinez, C.D. Alterman, F.M. Peçanha, D.V. Vassallo, P.B. Mello-Carpes, M. Miguel, et al., Aluminum exposure at human dietary levels for 60 days reaches a threshold sufficient to promote memory impairment in rats, Neurotox. Res. 31 (1) (2017) 20–30.
[24] J.R. Walton, A longitudinal study of rats chronically exposed to aluminum at human dietary levels, Neurosci. Lett. 1 (2007) 29–33.
[25] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 3 (2008) 659–661.
[26] A. Prakash, A. Kumar, Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats, Basic Clin. Pharmacol. Toxicol. 2 (2009) 98–104.
[27] G.W. Robb, R.P. Amman, G.J. Killian, Daily sperm production and epididymal sperm reserves of puberal and adult rats, J. Reprod. Fertil. 54 (1978) 103–107.
[28] R. Filler, Methods for evaluation of rats epididymal sperm morphology, in: R.E. Chapin, J.H. Heindel (Eds.), Male Reproductive Toxicology, Academic Press, California, 1993, pp. 334–343.
[29] C.S. Martinez, A.G. Escobar, J.G.D. Torres, D.S. Brum, F.W. Santos, M.J. Alonso, et al., Chronic exposure to low doses of mercury impairs sperm quality and induces oxidative stress in rats, J. Toxicol. Environ. Health Part A 77 (2014) 143–154.
[30] C. Loetchutinat, S. Kothan, S. Dechsupa, J. Meesungnoen, J. Jay-Gerin, S. Mankhetkorn, Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2',7'-dichlorofluorescein diacetate assay, Radiat. Phys. Chem. 72 (2005) 323–331.
[31] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[32] I.F.F. Benzie, J.J. Strain, The Ferric Reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[33] E. House, M. Esiri, G. Forster, P.G. Ince, C. Exley, Aluminium, iron and copper in human brain tissues donated to the medical research council's cognitive function and ageing study, Metallomics 4 (2012) 56–65.
[34] A. Mirza, A. King, C. Troakes, C. Exley, The identification of aluminum in human brain tissue using lumogallion and fluorescence microscopy, J. Alzheimers Dis. 54 (4) (2016) 1333–1338.
[35] M. Mold, H. Eriksson, P. Siesjö, A. Darabi, E. Shardlow, C. Exley, Unequivocal identification of intracellular aluminium adjuvant in a monocytic THP-1 cell line, Sci. Rep. 5 (4) (2014) 6287, http://dx.doi.org/10.1038/srep06287.

[36] L. Nordkap, U.N. Joensen, M. Blomberg Jensen, N. Jørgensen, Regional differences and temporal trends in male reproductive health disorders: semen quality may be a sensitive marker of environmental exposures, Mol. Cell. Endocrinol. 355 (2012) 221–230.
[37] G. Crépeaux, H. Eidi, M.O. David, Y. Baba-Amer, E. Tzavara, B. Giros, et al., Non-linear dose-response of aluminium hydroxide adjuvant particles: selective low dose neurotoxicity, Toxicology 375 (2017) 48–57.
[38] R.J. Aitken, Free radicals, lipid peroxidation and sperm function, Reprod. Fertil. Dev. 7 (1995) 659–668.
[39] T.T. Turner, J.J. Lysiak, Oxidative stress: a common factor in testicular dysfunction, Rev. J. Androl. 29 (5) (2008) 488–498.
[40] C. Exley, The pro-oxidant activity of aluminum, Free Radic. Biol. Med. 3 (2004) 380–387.
[41] F. Ruipérez, J.I. Mujika, J.M. Ugalde, C. Exley, X. Lopez, Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II), J. Inorg. Biochem. 117 (2012) 118–123.
[42] L. Yu, Q. Zhai, R. Yin, P. Li, F. Tian, X. Liu, et al., Lactobacillus plantarum CCFM639 alleviate trace element imbalance-related oxidative stress in liver and kidney of chronic aluminum exposure mice, Biol. Trace Elem. Res. 176 (2) (2016) 342–349.
[43] E. Kistanova, Y. Marchev, R. Nedeva, D. Kacheva, K. Shumkov, B. Georgiev, et al., Effect of the Spirulina platensis included in the main diet on the boar sperm quality, Biotechnol. Anim. Husb. 25 (2009) 547–557.
[44] A.M. Abdel-Moneim, Effects of taurine against histomorphological and ultrastructural changes in the testes of mice exposed to aluminium chloride, Arh. Hig. Rada Toksikol. 64 (3) (2013) 405–414.
[45] C.H. Guo, Y.F. Lu, G.S.W. Hsu, The influence of aluminum exposure on male reproduction and offspring in mice, Environ. Toxicol. Pharmacol. 20 (2005) 135–141.
[46] C.H. Guo, C.J. Huang, S.T. Chen, G.S. Wang Hsu, Serum and testicular testosterone and nitric oxide products in aluminum-treated mice, Environ. Toxicol. Pharmacol. 10 (2001) 53–60.

PARTE V

Paper published at Toxicology, 390:10-21, 2017. IF: 3, 5820. Qualis CBII: A2.

Toxicology 390 (2017) 10–21

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Full length article

Aluminum exposure at human dietary levels promotes vascular dysfunction and increases blood pressure in rats: A concerted action of NAD(P)H oxidase and COX-2

Caroline Silveira Martinez[a], Janaina Trindade Piagette[a], Alyne Gourlart Escobar[a], Ángela Martín[b,c], Roberto Palacios[b,c], Franck Maciel Peçanha[a], Dalton Valentim Vassallo[d], Christopher Exley[e], María Jesús Alonso[b,c], Marta Miguel[f], Mercedes Salaices[c,g], Giulia Alessandra Wiggers[a,*]

[a] Postgraduate Program in Biochemistry, Universidade Federal do Pampa, BR 472 – Km 592 – PO box 118, Zip Code: 97500-970, Uruguaiana, Rio Grande do Sul, Brazil
[b] Department of Ciencias Básicas de la Salud, Universidad Rey Juan Carlos, Avda. de Atenas s/n, Alcorcón, Spain
[c] Ciber de Enfermedades Cardiovasculares, Spain
[d] Departments of Physiological Sciences, Universidade Federal do Espírito Santo and School of Medicine of Santa Casa de Misericórdia (EMESCAM), Av. Marechal Campos 1468, Zip Code: 29040-090, Vitória, Espírito Santo, Brazil
[e] The Birchall Centre, Lennard-Jones Laboratories, Keele University, Staffordshire ST5 5BG, UK
[f] Bioactivity and Food Analysis Laboratory, Instituto de Investigación en Ciencias de la Alimentación, Nicolás Cabrera, 9, Campus Universitario de Cantoblanco, Madrid, Spain
[g] Department of Pharmacology, Universidad Autónoma de Madrid, Instituto de Investigación Hospital La Paz (IdiPaz), C/ Arzobispo Morcillo 4, 28029, Madrid, Spain

ARTICLE INFO

Keywords:
Metal
Cardiovascular risk
Vascular impairment
Oxidative stress

ABSTRACT

Aluminum (Al) is a non-essential metal and a significant environmental contaminant and is associated with a number of human diseases including cardiovascular disease. We investigated the effects of Al exposure at doses similar to human dietary levels on the cardiovascular system over a 60 day period. *Wistar* male rats were divided into two major groups and received orally: 1) Low aluminum level – rats were subdivided and treated for 60 days as follows: a) Untreated – ultrapure water; b) $AlCl_3$ at a dose of 8.3 mg/kg bw for 60 days, representing human Al exposure by diet; and 2) High aluminum level – rats were subdivided and treated for 42 days as follows: C) Untreated – ultrapure water; d) $AlCl_3$ at 100 mg/kg bw for 42 days, representing a high level of human exposure to Al. Effects on systolic blood pressure (SBP) and vascular function of aortic and mesenteric resistance arteries (MRA) were studied. Endothelium and smooth muscle integrity were evaluated by concentration-response curves to acetylcholine (ACh) and sodium nitroprusside. Vasoconstrictor responses to phenylephrine (Phe) in the presence and absence of endothelium and in the presence of the NOS inhibitor L-NAME, the potassium channels blocker TEA, the NAD(P)H oxidase inhibitor apocynin, superoxide dismutase (SOD), the non-selective COX inhibitor indomethacin and the selective COX-2 inhibitor NS 398 were analyzed. Vascular reactive oxygen species (ROS), lipid peroxidation and total antioxidant capacity, were measured. The mRNA expressions of eNOS, NAD(P)H oxidase 1 and 2, SOD1, COX-2 and thromboxane A2 receptor (TXA-2 R) were also investigated. Al exposure at human dietary levels impaired the cardiovascular system and these effects were almost the same as Al exposure at much higher levels. Al increased SBP, decreased ACh-induced relaxation, increased response to Phe, decreased endothelial modulation of vasoconstrictor responses, the bioavailability of nitric oxide (NO), the involvement of potassium channels on vascular responses, as well as increased ROS production from NAD(P)H oxidase and contractile prostanoids mainly from COX-2 in both aorta and mesenteric arteries. Al exposure increased vascular ROS production and lipid peroxidation as well as altered the antioxidant status in aorta and MRA. Al decreased vascular eNOS and SOD1 mRNA levels and increased the NAD(P)H oxidase 1, COX-2 and TXA-2 R mRNA levels. Our results point to an excess of ROS mainly from NAD(P)H oxidase after Al exposure and the increased vascular prostanoids from COX-2 acting in concert to decrease NO bioavailability, thus inducing vascular dysfunction and

Abbreviations: MRA, mesenteric resistance arteries; ACh, acetylcholine; Phe, phenylephrine; SOD, superoxide dismutase; TXA-2 R, thromboxane A2 receptor; NOS, nitric oxide synthase; L-NAME, Nω-nitro-L-arginine methyl ester, inhibitor of NOS; TEA, tetraethylammonium potassium channels blocker; NS 398, selective COX-2 inhibitor; SNP, sodium nitroprusside

* Corresponding author at: PPGBioq - UNIPAMPA, BR 472 – Km 592 – PO box 118, Zip Code: 97500-970, Uruguaiana – Rio Grande do Sul, Brazil. Tel.: +55 55 3413 4321; fax: +55 55 3413 4321.
E-mail address: giuliawp@gmail.com (G.A. Wiggers).

http://dx.doi.org/10.1016/j.tox.2017.08.004
Received 16 June 2017; Received in revised form 28 July 2017; Accepted 14 August 2017
Available online 19 August 2017
0300-483X/

increasing blood pressure. Therefore, 60-day chronic exposure to Al, which reflects common human dietary Al intake, appears to pose a risk for the cardiovascular system.

1. Introduction

Aluminum (Al) is the most common metal in the Earth's crust and its versatile properties as several Al compounds bring us in direct contact with this non-essential metal (Exley, 2012). Human exposure to Al through myriad ways is inevitable (Exley, 2013). In 2007, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) followed in 2008 by the European Food Safety Authority (EFSA), have adjusted the Provisional Tolerable Weekly Intake (PTWI) of Al for humans to 1 mg Al/kg body weight (b.w.) (EFSA, 2008; WHO 2007). However, it is known that the PTWI of Al may be exceeded by humans (Fekete et al., 2013; Gonzalez-Weller et al., 2010; Yang et al., 2014). Despite its ubiquity Al has no known biological function, it is not beneficial and it is only toxic (Exley, 2009). Therefore, the burgeoning increase in human exposure to Al has been concomitant with an increased development of Al-related adverse effects (Exley 2012; Klein et al., 2014). The accumulation of Al compounds in the human body has been associated with several conditions such as neurological disorders (Crépeaux et al., 2017; Mirza et al., 2016), macrophagic myofascitis (Gherardi et al., 2016), microcytic anemia (Barata et al., 1996) and osteopenia (Li et al., 2011).

Previous studies have payed attention to the relationship between Al exposure and cardiovascular risk (Korchazhkina et al., 1999; Lind et al., 2012; Subrahmanyam et al., 2016). In the last years, several cohort studies have supported a possible role for Al in cardiovascular disease (Costello et al., 2014; Neophytou et al., 2016; Subrahmanyam et al., 2016). For example, research has shown that exposure to airborne particles of Al with an aerodynamic diameter < 2.5 μm are a strong predictor of ischemic heart disease risk in Al industry workers (Costello et al., 2014; Neophytou et al., 2016). Al appears to have high affinity to human endothelial cells showing an ability to be accumulated in several human arteries (Bhattacharjee et al., 2013; Minami et al., 2001). Moreover, the circulating presence of Al seems to be a contributor for arterial stiffness in healthy individuals (Subrahmanyam et al., 2016) and to atherosclerosis plaques formation in the elderly (Lind et al., 2012).

These epidemiological studies are supported by a number of experimental studies. Animals exposed to up to 256 mg Al/kg bw over 120 days showed an increase of blood pressure and impairments in erythrocyte membranes (Zhang et al., 2016). Moreover, a single intraperitoneal injection of Al at 0.5 mg/kg promoted up-regulation of renin expression in rats (Ezomo et al., 2009). At the vascular bed, our group has recently reported that an acute exposure to Al was sufficient to promote vascular dysfunction with a reduction of vascular reactivity (Schmidt et al., 2016). Data thus far in this area have been obtained in studies where the exposure to Al might be considered as unrealistically high. Therefore we have looked to address this discrepancy by studying cardiovascular function in rats exposed to both a high level of Al and also one that better mimics human exposure to Al in the diet.

2. Materials and methods

2.1. Animals

Three-month-old male *Wistar* rats (360 ± 11.2 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12 h light-dark), giving free access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups, according to Martinez et al. (2017) and treated orally: 1) Low aluminum level − rats were subdivided (N = 10/each) and treated for 60 days as follows: a) Untreated − received ultrapure water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA) by drinking water; b) $AlCl_3$ at 8.3 mg/kg bw per day, a dose based on human dietary levels translated to an animal dose according to the body surface area normalization method (Reagan-Shaw et al., 2008); and 2) High aluminum level − rats were subdivided (N = 10/each) and treated for 42 days as follows: a) Untreated − received ultrapure water through oral gavage; b) $AlCl_3$ at 100 mg/kg bw per day through oral gavage once a day, representing a high level of human exposure to Al (Prakash and Kumar, 2009).

$AlCl_3.6\ H_2O$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water. The concentration of each stock solution was 0.034 M and 0.331 M, respectively from 8.3 and 100 mg/kg bw. Salts and reagents were of analytical grade obtained from Sigma-Aldrich and Merck (Darmstadt, Germany).

2.2. Systolic blood pressure

Indirect systolic blood pressure (SBP) was measured weekly, before the start of the treatment and during all the treatment period, using non-invasive tail-cuff plethysmography according to Wiggers et al. (2008) (AD Instruments Pty Ltd, Bella Vista, NSW, Australia).

2.3. Vascular reactivity experiments

Rat body weight, feed and water or Al intakes were measured weekly. At the end of the treatments, animals were anaesthetized with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *ip*) and euthanized. Thereafter, the thoracic aorta and the third-order MRA were carefully dissected out and cleaned of fat and connective tissues. For vascular reactivity experiments, in the day of the euthanasia, the arteries were divided into cylindrical segments of 2 mm in length and placed into Krebs-Henseleit solution (in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 23; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O_2 and 5% CO_2 (pH 7.4). The remaining aorta and mesenteric resistance arteries (MRA) were kept at −80 °C for further biochemical/biological assays.

Segments of aorta were mounted in an isolated tissue chamber and maintained at a resting tension of 1.5 g at 37 °C. Isometric tension was recorded using an isometric force transducer (TSD125BX8, Biopac Systems, Inc, Santa Barbara, CA, USA) connected to an acquisition system (MP150WSW-SYS, Biopac Systems). MRA segments were mounted in a small-vessel dual chamber myograph (Multi Wire Myograph System, DMT620, ADInstruments, Australia) for measurement of isometric tension according to Wiggers et al. (2008). Segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-to-wall tension ratio of the segments by setting their internal circumference, Lo, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg.

After a 45-min equilibration period, aortic and MRA segments were respectively exposed twice to 75 and 120 mM KCl, first to check their

functional integrity and again to assess the maximal tension developed. Afterwards, endothelial integrity was tested with acetylcholine (ACh, 10 μM) in segments that were previously contracted with phenylephrine (Phe) at a concentration that produced close to 50% of the contraction induced by KCl. After 60 min of washout, a single concentration response curve to Phe (0.01 nM – 300 μM) was performed.

To evaluate the role of the endothelium in the vasoconstrictor responses to Phe, this vascular component was mechanically removed, and its absence was confirmed by the inability of ACh to induce relaxation greater than 10% of the previous contraction due to Phe. To evaluate the participation of nitric oxide (NO), potassium channels, ROS, prostanoids or AT1 receptors on Phe responses, the effects of the nonspecific nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME 100 μM), the potassium channels blocker tetraethylammonium (TEA, 2 mM), the NADPH oxidase inhibitor apocynin (0.3 μM), the superoxide dismutase (SOD 150 U/ml), the nonselective COX inhibitor indomethacin (1 μM), the selective COX-2 inhibitor NS 398 (1 μM) were investigated by their addition 30 min before Phe in vessels with intact endothelium.

To evaluate the endothelial dependent and independent relaxations, concentration-response curves with ACh (0.01 nM − 300 μM) and sodium nitroprusside (SNP, 0.01 nM − 300 μM), respectively, were performed in segments previously contracted with Phe.

2.4. Aluminum content in blood and liver

The Al content of blood and liver was determined using an established method (House et al., 2012). Briefly, approximately 0.5 g of liver was dried to a constant weight at 37 °C. Dried and weighed tissues and 1 mL of blood, were digested in a 1:1 mixture of 15.8 M HNO_3 and 30% *w/v* H_2O_2 in a microwave oven (MARS Xpress CEM Microwave Technology Ltd) and the Al content of digests measured by TH GFAAS (Transversley Heated Graphite Furnace Atomic Absorption Spectrometry). Results were expressed as μg Al/L of blood or μg Al/g dry tissue. Each determination was the arithmetic mean of a triplicate analysis.

2.5. Reactive oxygen species levels

Biochemical studies of oxidative stress biomarkers were performed in aorta and MRA. For that, vessels were homogenized in 50 mM Tris HCl, pH 7.4, centrifuged at 2400*g* for 10 min at 4 °C and the resulting supernatant fraction was used for the measurements.

Levels of reactive species were determined by the spectrofluorometric method described by Loetchutinat et al. (2005). This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The supernatant fraction of the sample was diluted (1:10) in 50 mM Tris HCl (pH 7.4) and 2′, 7′-dichlorofluorescein diacetate (DCHF-DA; 1 mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence units.

2.6. Lipid peroxidation

Lipid peroxidation was measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. (1979), with modifications (Martinez et al., 2017). An aliquot of each tissue was incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4), and sodium dodecyl sulphate 0.8% (SDS) at 100 °C for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as nanomoles of MDA per mg of protein.

2.7. Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant capacity was measured by FRAP assay (Benzie and Strain, 1996), with modifications (Martinez et al., 2017). This method is based on the ability of samples to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s- triazine (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 μL of the supernatant fraction of each tissue was added to 1 mL freshly prepared and pre-warmed (37°C) FRAP reagent (300 mM acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50–1000 μM − water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

2.8. In situ detection of vascular $O_2\cdot^-$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* superoxide radical anion ($O_2\cdot^-$) production in both aortic and mesenteric segments, as previously described (Briones et al., 2009). Hydroethidine freely permeates cells and is oxidized in the presence of $O_2\cdot^-$ to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum at 600–700 nm. Frozen tissue segments were cut into 10 μm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37 °C in Krebs-HEPES buffer (in mM/L: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, 11 glucose, pH = 7.4). Fresh buffer containing DHE (2×10^{-6} M/L) was applied topically into each tissue section, coverslipped, incubated for 30 min in a light protected humidified chamber at 37 °C and then viewed with a fluorescent microscope (Zeiss Axioskop 2 microscope − Zeiss, Jena, Germany- Leica TCS SP2 equipped with a krypton/argon laser, ×20 objective, zoom 4×), using the same imaging settings in control and arteries from Al-treated rats. Fluorescence was detected with a 568 nm long-pass filter. For quantification, five rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated using NIH Image J software version 1.46r (http://rsbweb.nih.gov/ij/), using the same imaging settings in each case.

2.9. Lucigenin chemiluminescence assay

A lucigenin-enhanced chemiluminescence assay was used to determine NAD(P)H (NOX) activity. Aortas were homogenized in lysis buffer (50 mM KH_2PO_4, 1 mM EGTA, and 150 mM sucrose, pH 7.4). The reaction was started by the addition of NADPH (0.1 mM) to the suspension containing the sample, lucigenin (5 μM), and assay phosphate buffer. Luminescence was measured in a plate luminometer (Auto-Lumat LB 953, Berthold Technologies, Bad Wildbad, Germany). The buffer blank was subtracted from each reading. Activity was expressed as relative light units per microgram of protein.

2.10. Quantitative real time PCR assay

The mRNA expression levels were determined by quantitative real-time PCR. Total RNA was obtained using TRIzol (Invitrogen Life Technologies). A total of 1 μg of DNAse I-treated RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 10-μl reaction. qRT-PCR was performed in duplicate for each sample using 0.5 μL cDNA as template, 1× iTaq™ Universal Probes Supermix (Biorad), and 20× Taqman Gene Expression Assays (COX-2: Rn00568225_m1, NOX-1: Rn00586652_m1, SOD-1: Rn00566938_m1, TXA_2 receptor: Rn00690601_m1, Applied

Biosystems) in a 10-µl reaction using the following conditions: 2 min at 50 °C and 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. qRT-PCR for eNOS (Fd: GAGAGTGAGCTGGTGTTTGG; Rv: GGTGAACATTTCCTGTGCTGT) and NOX-2 (Fd: CCAGTGTGTCGG AATCTCCT; Rv: ATGTGCAATGGTGTGAATGG), were performed using the fluorescent dye SyBRGreen (iTaq FAST SyBRGreen Supermix with ROX, Bio-Rad, USA). For quantification, quantitative real-time PCR was carried out in a 7500 Fast (Applied Biosystems) adding a dissociation stage to the conditions already described above to show PCR product specificity. As a normalizing internal control, we amplified cyclophilin D (Rn01458749_g1). To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta Ct}$ method using untreated samples as a calibrator (Livak and Schmittgen, 2001). mRNA levels of the housekeeping gene were not modified by any of the treatments used.

2.11. Statistical analysis

Data are expressed as mean ± SEM. In the vascular reactivity experiments, vasoconstrictor responses of aorta and MRA were expressed as a percentage of the contraction induced by 75 mM and 120 mM KCl, respectively.Vasodilator responses were expressed as a percentage of the previous contraction to Phe. To compare the effect of L-NAME, TEA, apocynin, SOD, indomethacin and NS-398 −on the response to Phe in segments from each group, some results were expressed as 'differences of area under the concentration-response curves' (dAUC) in control and experimental situations AUCs were calculated from the individual concentration response curve plots; differences were expressed as the percentage of the AUC of the corresponding control situation. Results were analyzed using unpaired Student's *t*-test or two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual means. Results of biochemical experiments were analyzed using Student's *t*-test or Mann-Whitney test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Body weight, fluid and feed intake and aluminum content

Body weight of rats was similar between groups at the beginning and end of treatments. Water or Al intakes as well as the feed intake were not different between groups (Martinez et al., 2017). The whole blood Al content was similar in rats treated with the low dose of Al (Untreated: 73.43 ± 1.7 *vs* Al 8.3: 77.1 ± 6.6 µg Al/L/blood, $P > 0.05$) while that of the rats treated with the high dose was greater that the corresponding control (Untreated: 78.9 ± 5.7 *vs* Al 100: 121.1 ± 11.2 µg Al/L/blood, $P < 0.05$). However, liver Al contents were greater both in the low (Untreated: 0.25 ± 0.01 *vs* Al 8.3: 0.44 ± 0.04 µg Al/g/dry tissue, $P < 0.05$) and also in the high Al-treated groups (Untreated: 0.26 ± 0.01 *vs* Al 100: 0.72 ± 0.01 µg Al/g/dry tissue, $P < 0.05$).

3.2. Aluminum raises systolic blood pressure

Exposure to Al at low (8.3 mg/kg bw for 60 days) or at high level (100 mg/kg bw for 42 days) increased SBP after the 3rd week of treatment (Untreated: 119.0 ± 1.4 *vs* Al 8.3: 129.2 ± 1.0 mmHg, n = 8, $P < 0.05$; Untreated: 120.5 ± 1.5 *vs* Al 100: 131.5 ± 3.7 mmHg, n = 8, $P < 0.05$). However, at the end of treatments only Al at the higher dose maintained this increase (Fig. 1A, B).

3.3. Aluminum increases vascular reactivity and induces vascular dysfunction

Al exposure did not affect the response to KCl either in aorta (Untreated: 3.8 ± 0.3 *vs* Al 8.3: 4.1 ± 0.4 mN/mm; Untreated: 3.9 ± 0.2 *vs* Al 100: 4.1 ± 0.3 mN/mm; n = 15 to 20, $P > 0.05$) or MRA (Untreated: 3.6 ± 0.2 *vs* Al 8.3: 3.5 ± 0.3 mN/mm; Untreated: 4.1 ± 0.3 *vs* Al 100: 4.5 ± 0.2 mN/mm, n = 15 to 20, $P > 0.05$). However, Al treatment at both low and high levels increased the vasoconstrictor responses to Phe in aorta and MRA while decreased the endothelium-dependent responses induced by ACh only in MRA (Fig. 2A, B, Table 1). The vasodilator response induced by SNP were unaffected by Al exposure (data not shown). These results suggest that Al affects vasoconstrictor responses in both conductance and resistance arteries and impairs endothelial function only in resistance arteries.

3.4. Aluminum decreases the endothelial modulation of the vasoconstrictor response − involvement of endothelial NO synthase

To investigate whether Al exposure alters NO modulation on vasoconstrictor responses, the effect of endothelium removal and incubation with the NOS inhibitor L-NAME (100 µM), were investigated. Both endothelium removal and NOS inhibitor addition left-shifted the concentration–response curves to Phe in aorta and MRA segments from the four groups, but this effect was smaller in preparations from rats treated with either 8.3 mg/kg or 100 mg/kg of Al than in those from untreated rats, as shown by the dAUC values (Figs. 3, 4). The endothelial modulation was impaired after Al exposure at an equivalent human dietary level and this effect was almost the same as observed after Al treatment at 12 times higher. Therefore, we decided to investigate the underling mechanisms of Al toxicity by using the human relevant dose level and, Al exposure at 8.3 mg/kg decreased eNOS gene expression in aorta (Untreated: 0.85 ± 0.1 *vs* Al 8.3: 0.60 ± 0.1 relative expression,

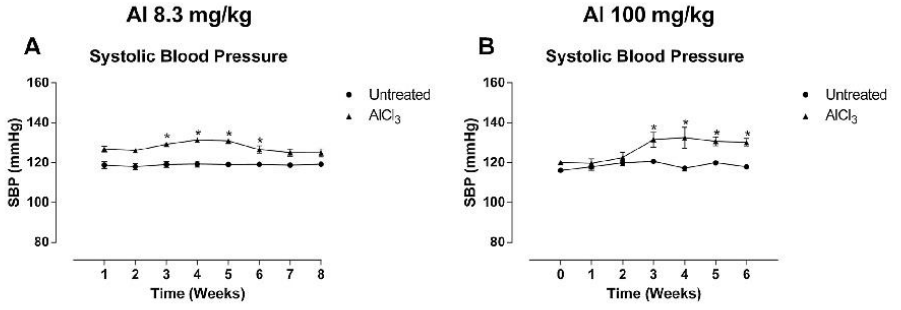

Fig. 1. Effect of chronic aluminum exposure on non-invasive systolic blood pressure. Values of systolic blood pressure (mmHg) of untreated rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* − A) or 42 days (100 mg/kg bw *per day* − B). Data are expressed as mean ± SEM, n = 8, * $P < 0.05$ compared with their corresponding controls (untreated rats) (Two-Way ANOVA followed by Bonferroni).

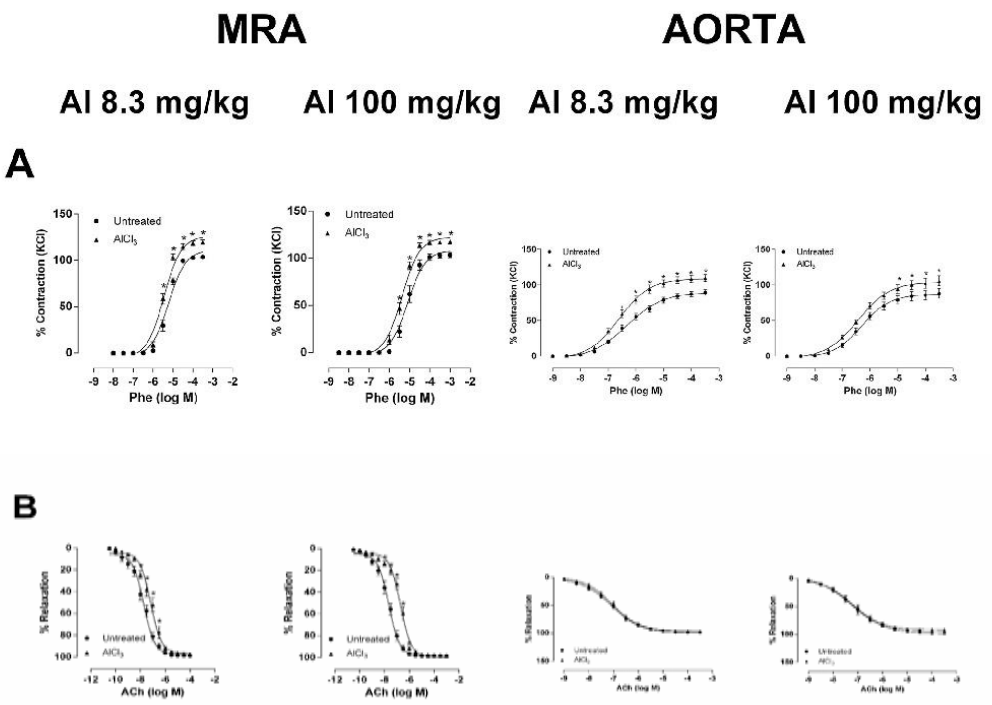

Fig. 2. Effect of chronic aluminum exposure on vascular reactivity. Concentration–response curves to (A) phenylephrine and (B) acetylcholine in aorta and MRA segments. Data are expressed as mean ± SEM, n = 8–15, * P < 0.05 compared with their corresponding Untreated groups (Two-Way ANOVA followed by Bonferroni).

Table 1
Effects of aluminum exposure to low and high doses on maximum response (R_{max}) and sensitivity (pD_2).

	Untreated		Al 8.3 mg/kg		Untreated		Al 100 mg/kg	
	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2
Aorta								
Control	89.7 ± 4.3	6.3 ± 0.1	111.1 ± 5.1#	6.5 ± 0.1	86.7 ± 6.5	6.3 ± 0.1	107.2 ± 7.4#	6.4 ± 0.1
E-	110.6 ± 6.2*	6.7 ± 0.1*	109.1 ± 2.4	7.1 ± 0.1*	116.6 ± 5.5*	7.1 ± 0.1*	108.5 ± 6.1	7.1 ± 0.2*
L-NAME	129.5 ± 5.1*	6.9 ± 0.1*	126 ± 5.5	7.2 ± 0.1*	127.3 ± 4.6*	7.1 ± 0.2*	129.2 ± 5.5*	7.1 ± 0.1*
TEA	126.9 ± 4.2*	7.0 ± 0.0*	123.5 ± 5.1	7.1 ± 0.1*	118.1 ± 5.8*	7.2 ± 0.3*	112.1 ± 7.1	7.0 ± 0.2*
Apocynin	44.6 ± 5.4*	6.3 ± 0.1	46.6 ± 4.2*	6.4 ± 0.1	50.3 ± 6.3*	6.2 ± 0.1	41.3 ± 4.0*	5.9 ± 0.1*
SOD	89.2 ± 11.2	6.0 ± 0.1	89.7 ± 8.3*	5.8 ± 0.1*	74.9 ± 13.3	6.0 ± 0.1	81.6 ± 9.8	5.7 ± 0.1*
Indomethacin	55.1 ± 3.3*	6.4 ± 0.1	62.9 ± 6.6*	6.5 ± 0.1	56.9 ± 7.3*	6.1 ± 0.1	54.3 ± 6.4*	6.2 ± 0.18
NS 398	51.3 ± 5.9*	6.1 ± 0.1	55.1 ± 4.6*	6.3 ± 0.1	58.2 ± 8.3*	6.3 ± 0.1	50.8 ± 6.5*	6.1 ± 0.1*
MRA								
Control	111 ± 1.8	4.2 ± 0.2	123 ± 3.4#	5.1 ± 0.2#	109.1 ± 3.1	3.7 ± 0.2	120 ± 2.7#	4.7 ± 0.3#
E-	120 ± 3*	5.1 ± 0.4	117.1 ± 2.8	4.1 ± 0.3	123.3 ± 4.5*	5.4 ± 0.4*	114.4 ± 2.8	4.3 ± 0.3
L-NAME	130.4 ± 4.7*	5.3 ± 0.4*	126.9 ± 5.1	5.1 ± 0.4	135.2 ± 6.2*	5.1 ± 0.6	122.4 ± 3.6	5.1 ± 0.3
Apocynin	116.2 ± 3.9	3.9 ± 0.3	114.3 ± 3.4*	4.3 ± 0.1*	119.5 ± 4.2	4.9 ± 0.2*	115.5 ± 3.2	4.4 ± 0.3
SOD	129.1 ± 3.5*	5.2 ± 0.3*	125 ± 4.6	4.9 ± 0.5	135 ± 5.8	5.3 ± 0.1*	123.5 ± 3.6	4.8 ± 0.2
Indomethacin	103.3 ± 3.1*	3.1 ± 0.3*	105.3 ± 3.1*	2.9 ± 0.3*	111.1 ± 7.6	4.1 ± 0.5	114.8 ± 5.2	4.1 ± 0.3
NS 398	125.2 ± 2.7*	4.7 ± 0.2	118.4 ± 3.3*	4.4 ± 0.2	129.7 ± 0.3*	4.7 ± 0.3	128.8 ± 2.4	5.2 ± 0.7

Parameters of maximal response (R_{max}) and sensitivity (pD_2) of the concentration-response curves to phenylephrine in both aorta and MRA before (control) and after endothelial damage (E-), L-NAME (100 μM), TEA (2 mM), apocynin (0.3 μM), SOD (150 U/ml), Indomethacin (1 μM) and NS 398 (1 μM) incubations. Results are expressed as mean ± SEM. R_{max}: maximal effect (expressed as a percentage of maximal response induced by KCl) and pD_2 expressed as a −log one-half R_{max}. * P < 0.05 compared to the corresponding control in each group # P < 0.05 compared with the Untreated group (Student's *t*-test).

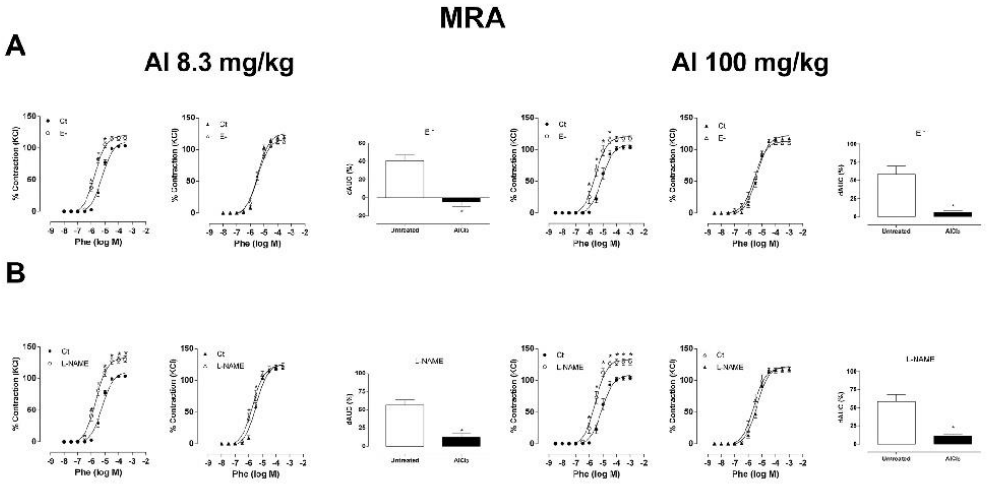

Fig. 3. Effect of chronic aluminum exposure on NO-mediated vascular response in MRA. Effects of (A) endothelium removal (E−) and (B) L-NAME (100 μM) on the concentration-response curve to phenylephrine from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) endothelium-denuded and intact segments and (B) in the presence and absence of L-NAME. Data are expressed as mean ± SEM, n = 8 to 15, * $P < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ *vs* Untreated (Student's *t*-test) in dAUC graphs.

$P < 0.05$). To verify the influence of K^+ channels on the vasoconstrictor responses, aortic segments were incubated with TEA (2 mM), a K^+ channel blocker. The presence of TEA increased the contractile response to Phe in all groups, but this enhancement was smaller in aorta from Al-treated rats, as demonstrated by the dAUC values (Fig. 5). All these findings suggest that the production and/or bioavailability of NO are impaired by Al exposure at both high and low level. The reduced participation of K^+ channels on vasoconstrictor responses follow the results of lower NO bioavailability, since NO can direct (Bolotina et al., 1994; Mistry and Garland 1998) or indirectly by cyclic GMPdependent (Carrier et al., 1997) promote the activation of K^+ channel in MRA.

3.5. Aluminum increases oxidative stress − role of NAD(P)H oxidase

The participation of oxidative stress in the vascular responses was evaluated *in vitro* using the non selective NAD(P)H oxidase inhibitor

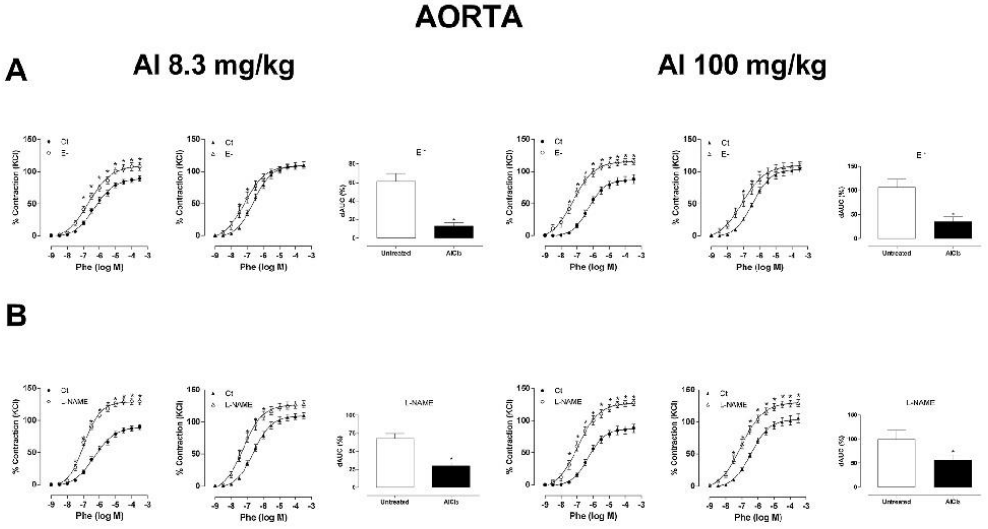

Fig. 4. Effect of chronic aluminum exposure on NO-mediated vascular response in aorta. Effects of (A) endothelium removal (E−) and (B) L-NAME (100 μM) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) endothelium-denuded and intact segments and (B) in the presence and absence of L-NAME. Data are expressed as mean ± SEM, n = 8 to 15, * $P < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ *vs* Untreated (Student's *t*-test) in dAUC graphs.

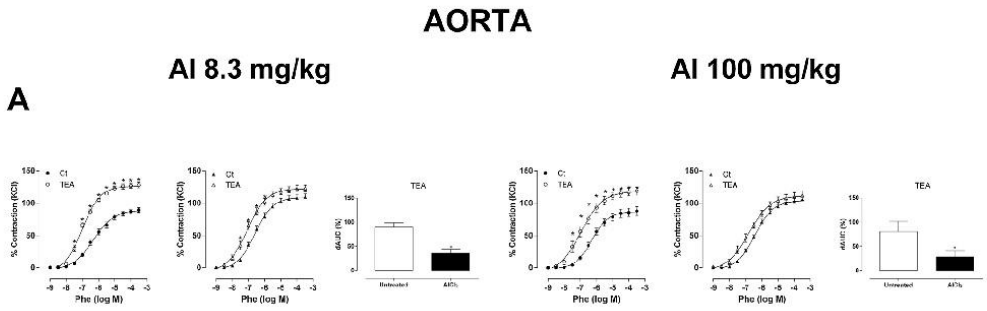

Fig. 5. Effect of chronic aluminum exposure on K+ channels action on the vascular response in aorta. Effects of (A) the K+ channels blocker TEA (2 mM) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of TEA. Data are expressed as mean ± SEM, n = 8 to 15, * P < 0.05 *vs* control curve (Two-Way ANOVA followed by Bonferroni) * P < 0.05 *vs* Untreated (Student's *t*-test) in dAUC graphs.

apocynin as well as SOD. Apocynin (0.3 μM) and SOD (150 U/ml) reduced the vascular response to Phe in MRA from rats treated with both doses of Al, while those drugs did not affect the response in untreated animals (Fig. 6); in aorta, both apocynin and SOD reduced the Phe-induced responses, being this effect greater in segments from Al-treated rats than from untreated rats, as demonstrated by the dAUC values (Fig. 7). Al exposure either at low doses or at high doses increased vascular ROS and lipid peroxidation levels (ROS (UF): Aorta − Untreated: 98.4 ± 15.3 *vs* Al 8.3: 152.9 ± 9.7; Untreated: 98.4 ± 15.3 *vs* Al 100: 201.2 ± 24.1; MRA − Untreated: 129.2 ± 12.3 *vs* Al 8.3: 231.7 ± 22.7, Untreated: 157.3 ± 13.2 *vs* Al 100: 228.5 ± 15.6 − n = 8, P < 0.05; Lipid peroxidation (nM MDA/mg protein): Aorta − Untreated: 37.1 ± 2.1 *vs* Al 8.3: 50.3 ± 4.1; Untreated: 18.3 ± 0.6 *vs* Al 100: 30.6 ± 5.4; MRA − Untreated: 8.3 ± 1.3 *vs* Al 8.3: 15.7 ± 2.7; Untreated: 7.2 ± 1.0 *vs* Al 100: 20.3 ± 2.3 − n = 8, P < 0.05). Al at both doses increased the MRA total antioxidant capacity (FRAP value (mM): Untreated: 32.1 ± 3.5 *vs* Al 8.3: 78.8 ± 4.3; Untreated: 35.9 ± 4.1 *vs* Al 100: 53.5 ± 5.9 − n = 8, P < 0.05), while in aorta the antioxidant capacity was reduced after Al exposure at 8.3 mg/kg (FRAP value (mM): Untreated: 64.1 ± 9.1 *vs* Al 8.3: 26.6 ± 4.0 P < 0.05, Untreated: 45.2 ± 2.5 *vs* Al 100: 42.9 ± 2.9 − n = 8, P > 0.05). Taking all together, these results suggest that the reduced NO bioavailability could be related with the increased oxidative stress after Al exposure.

NAD(P)H oxidase complex plays an important role on $O_2\cdot^-$ production (Martyn et al., 2006). Therefore, we investigated its possible role as source of ROS production after Al exposure. The basal $O_2\cdot^-$ production increased in both conductance and resistance arteries from rats treated with 8.3 mg/kg (Fig. 8). The NAD(P)H oxidase activity and the NOX1 gene expression were both increased in aorta from the Al-treated group compared to untreated group (Fig. 9A,B); however, NOX2 mRNA was similar in treated and untreated animals (Fig. 9C). Moreover, Al treatment inhibited the SOD1 gene expression in aorta from Al-treated animals (Fig. 9D).

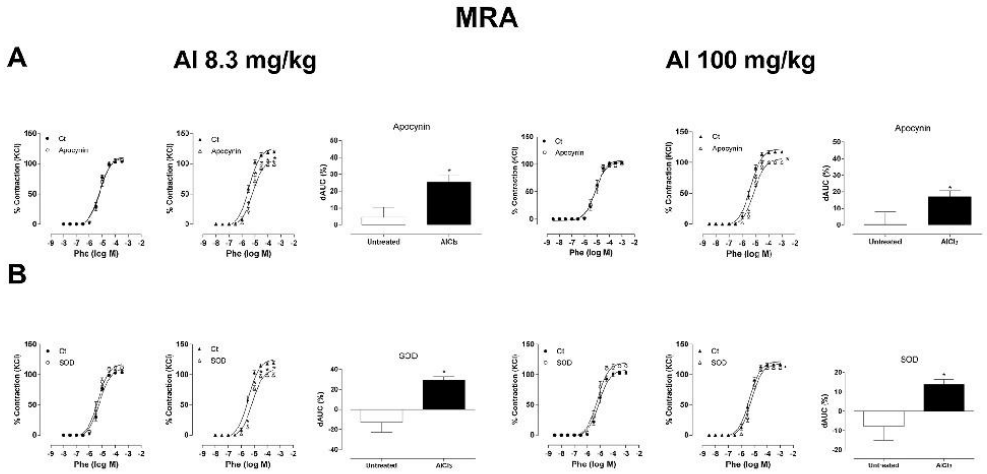

Fig. 6. Effect of chronic aluminum exposure on ROS-mediated vascular response in MRA. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μM) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine in MRA from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of apocynin and (B) in the presence and absence of SOD. Data are expressed as mean ± SEM, n = 8–15, * P < 0.05 *vs* control curve (Two-Way ANOVA followed by Bonferroni) * P < 0.05 *vs* Untreated (Student's *t*-test) in dAUC graphs.

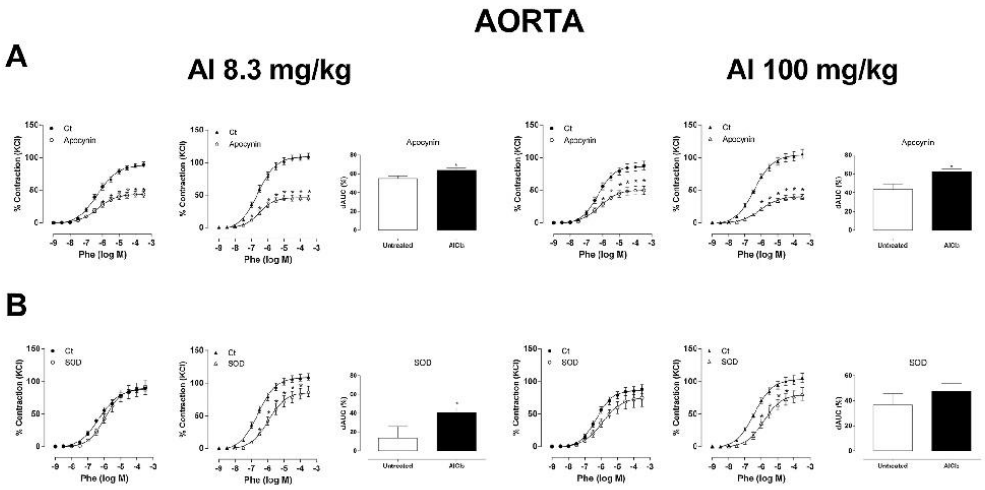

Fig. 7. Effect of chronic aluminum exposure on ROS-mediated vascular response in aorta. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μM) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of apocynin and (B) in the presence and absence of SOD. Data are expressed as mean ± SEM, n = 8–15, * $P < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ *vs* Untreated (Student's *t*-test) in dAUC graphs.

3.6. Aluminum vascular impairment – participation of COX-2-derived prostanoids

To investigate the role of prostanoids on the increased response to Phe in Al-treated rats, the cyclooxygenase inhibitor indomethacin and the selective COX-2 inhibitor NS 398 were used. Indomethacin (1 μM) and NS 398 (1 μM) reduced the vasoconstrictor response to Phe in both arteries from all groups. However, as observed by the dAUC values, this reduction was greater in segments from both Al-treated groups when compared to the untreated groups (Figs. 10, 11), suggesting an increased participation of contractile prostanoids from COX-2 on Phe-induced responses. Al exposure at the human relevant level of 8.3 mg/kg increased the mRNA levels of both COX-2 and TXA-2 R in aorta (Fig. 12). These results suggest that COX-2-derived prostanoids, probably acting on TP receptors, play a role on the vascular impairment observed after Al exposure at human dietary relevant level.

4. Discussion

The present study provides evidence that Al should be considered as a risk to the cardiovascular system even at low doses. Here, we show that Al exposure for 60 days at human dietary levels increased blood pressure and vascular reactivity in both conductance and resistance vessels through endothelium-dependent mechanisms. Moreover, these effects were almost the same when we treated rats with Al at a dose 12 times higher (100 mg/kg), representing a high level of human exposure to Al. We demonstrate that ROS and COX-2-derived prostanoids are important mediators of aorta and mesenteric vascular dysfunction observed after Al exposure. The excess of ROS mainly from NAD(P)H oxidase and the increased vascular COX-2 act in concert to decrease NO bioavailability, thus inducing vascular dysfunction and increased blood pressure.

Due to the omnipresence of Al in our daily life, human exposure to Al is still underestimated (Exley, 2013). The tolerable weekly intake of Al for humans has been set at 1 mg Al/kg bw (EFSA, 2008; WHO, 2007), which may be easily exceed by humans due to myriad sources of Al exposure (Exley 2013; Fekete et al., 2013; Gonzalez-Weller et al., 2010; Yang et al., 2014). We have recently established an experimental model of exposure to Al that mimics human exposure to this metal through diet in which rats were exposed to Al at a dose of 8.3 mg/kg bw *per day* during 60 days. Considering the amount of Al present in the animals feed, the total Al exposure was 4.37 mg/Al/day (2.49 mg from water plus 1.88 mg from feed). In addition, we have compared this low dose with a high level of human exposure to Al (100 mg/kg bw), in which rats were treated with a total of 31.88 mg/Al/day (30 mg from gavage plus 1.88 mg from feed) (Martinez et al., 2017). In the present study, Al exposure at low dose did not change the whole blood Al content between groups; however it promoted an increased Al accumulation in the liver. The lack of differences for blood contents after Al exposure at a low dose is expected since blood is only a temporal storage organ for Al (Beardmore and Exley, 2009). The high deposition of Al in the liver even without differences in the blood level supports these findings. Of interest, the human blood Al content is extremely variable in the available literature, ranging from less than 1 to 901 μg Al/L of blood (Chen et al., 2013; Lind et al., 2012)

Cardiovascular risk and human body burden of Al share the characteristic that both rise with increase of average lifetime (Assmann et al., 2017; Exley 2013). Therefore, considering the postulated mechanisms of action of Al and the underling ones regarding vascular disease, for example increased oxidative stress, finding more relations between them would not be a surprise. In previous years, studies have analyzed the possible cardiovascular adverse effects of Al exposure. Isolated rat hearts exposure to Al at 100 μM showed a reduction of both coronary blood flow and isovolumetric systolic pressure (Gomes et al., 1994). The effects of Al on heart function was further analyzed and the perfusion of hearts with Al at 40 μM caused an increase in coronary flow and both reduction in heart rate and increases in pulsatile power (Korchazhkina et al., 1998).

More recently, Al was related to the development of hypertension in experimental animals, these effects were related to an increase in renin expression or erythrocyte membrane impairments after acute or chronic Al administration up to 256 mg/kg (Ezomo et al., 2009; Zhang et al., 2016). In humans, this possible relationship was also seen, where circulating plasma Al levels were related to arterial hypertension (Granadillo et al., 1995). In a recent study, our group showed that one hour of Al exposure at 100 mg/kg is sufficient to promote vascular

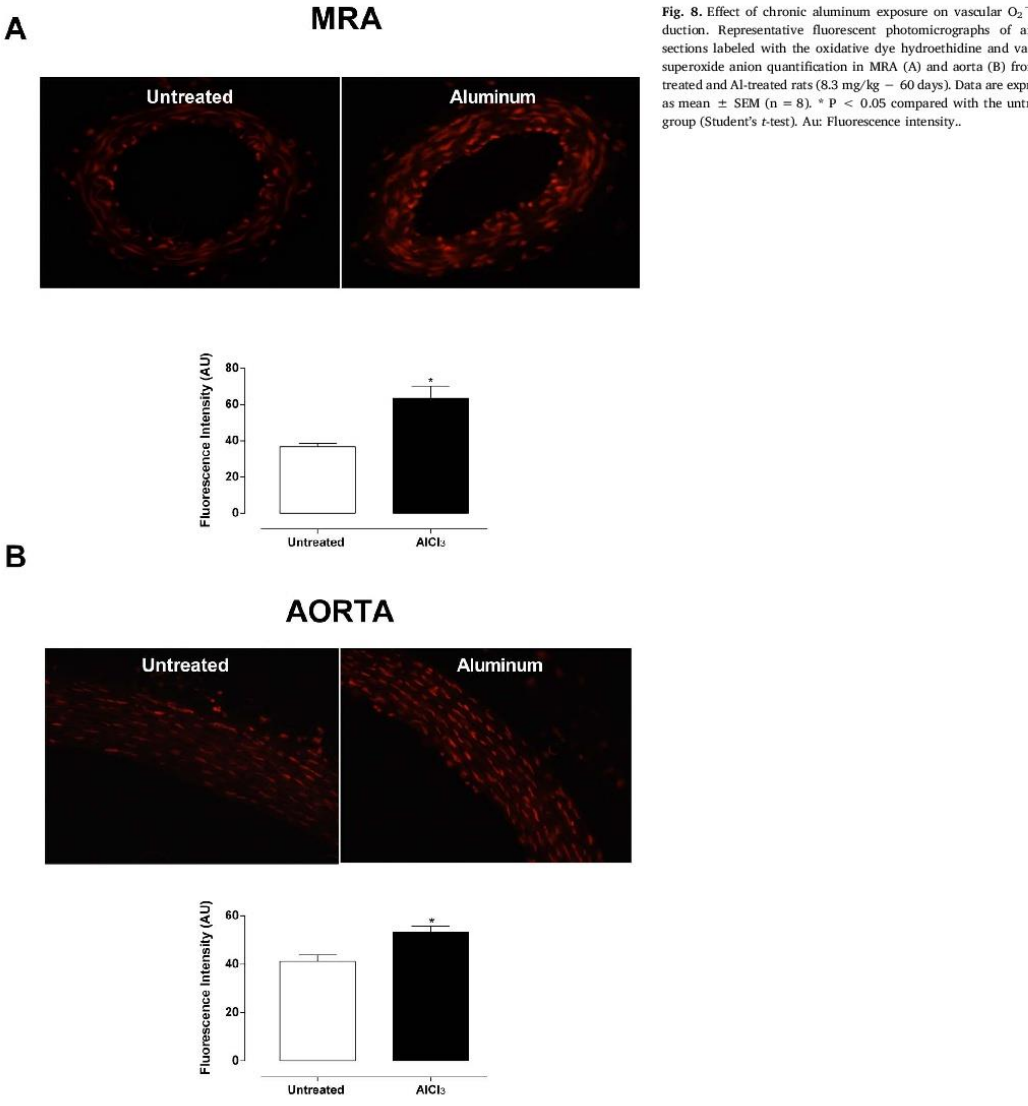

Fig. 8. Effect of chronic aluminum exposure on vascular O_2^- production. Representative fluorescent photomicrographs of arterial sections labeled with the oxidative dye hydroethidine and vascular superoxide anion quantification in MRA (A) and aorta (B) from untreated and Al-treated rats (8.3 mg/kg − 60 days). Data are expressed as mean ± SEM (n = 8). * P < 0.05 compared with the untreated group (Student's *t*-test). Au: Fluorescence intensity..

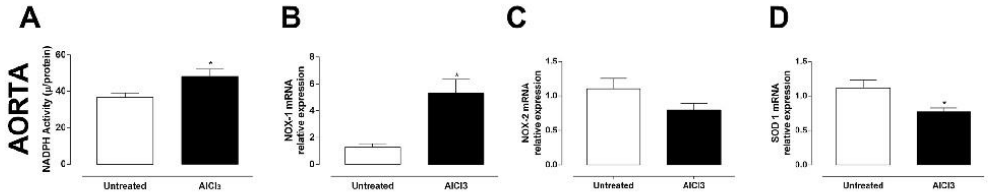

Fig. 9. Effect of chronic aluminum exposure on NOX oxidase enzyme activity, NOX-1, NOX-2 and SOD 1 gene expressions. NOX oxidase activity (A) and mRNA levels for NOX-1 (B), NOX-2 (C) and SOD 1 (D) in aortas from untreated and Al-treated rats (8.3 mg/kg − 60 days). Data are expressed as mean ± SEM (n = 8). * P < 0.05 compared with the untreated group (Student's *t*-test).

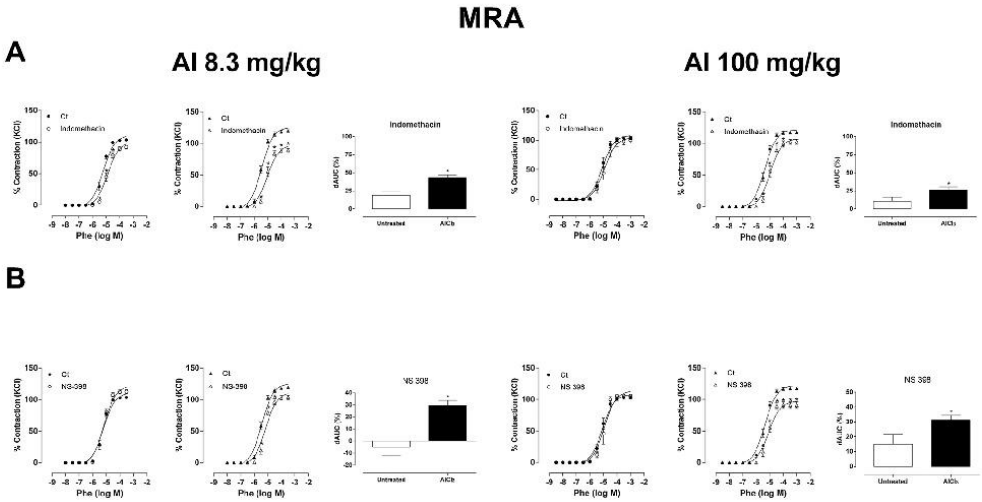

Fig. 10. Effect of chronic aluminum exposure on COX-derived prostanoids role on vascular response in MRA. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μM) and (B) selective COX-2 inhibitor NS 398 (1 μM) on the concentration– response curve to phenylephrine in MRA from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of indomethacin and (B) in the presence and absence of NS 398. Data are expressed as mean ± SEM, n = 8–15, * $P < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ *vs* Untreated (Student's *t*-test) in dAUC graphs.

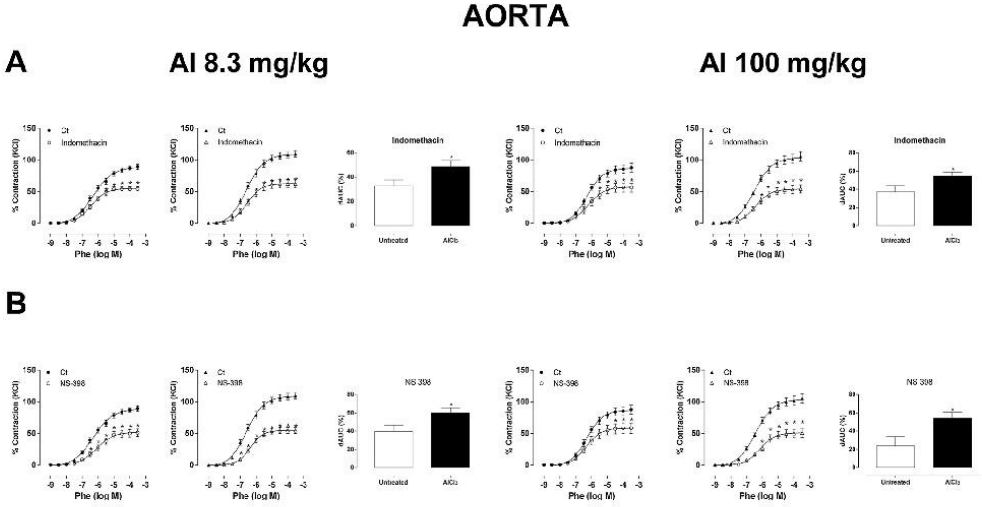

Fig. 11. Effect of chronic aluminum exposure on COX-derived prostanoids role on vascular response in aorta. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μM) and (B) selective COX-2 inhibitor NS 398 (1 μM) on the concentration – response curve to phenylephrine in aorta from untreated and Al-treated rats. The inset shows differences in the area under the concentration–response curves (dAUC) in (A) in the presence and absence of indomethacin and (B) in the presence and absence of NS 398. Data are expressed as mean ± SEM, n = 8–15, * $P < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) * $P < 0.05$ *vs* Untreated (Student's *t*-test) in dAUC graphs.

changes with reduction in vascular reactivity and increase in vascular ROS production (Schmidt et al., 2016). To our knowledge, our study is the first experimental one investigating and showing cardiovascular effects after Al exposure at human relevant dietary levels. The current study supports the possible cardiovascular risk of Al. Thus, the main results show that Al exposure: 1) increases systolic blood pressure; 2) produced endothelial dysfunction, as shown by the impaired the vasodilator response to ACh and the increased vasoconstrictor responses to Phe and 3) reduced the endothelial NO bioavailability.

Al^{3+} toxicity has been related with its pro-oxidant activity in different target organs and systems in experimental animals (Prakash and Kumar 2009; Yu et al., 2016). The pro-oxidant effects of Al are well documented and are possible due to formation of superoxide radical ion (Exley, 2004) or by promoting the Fenton reaction by reducing Fe(III)

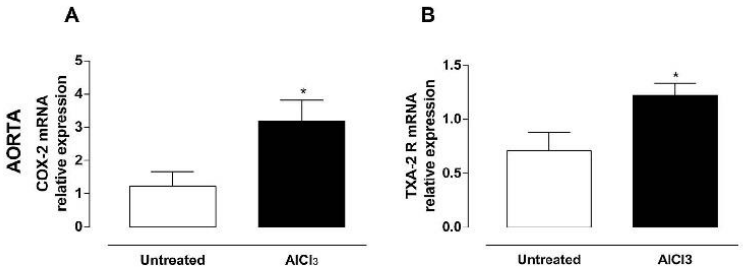

Fig. 12. Effect of chronic aluminum exposure on COX-2 and TXA-2 R gene expressions. COX-2 mRNA levels (A) and TXA-2 R mRNA levels (B) in aortas from untreated and Al-treated rats (8.3 mg/kg – 60 days). Data are expressed as mean ± SEM (n = 8). * P < 0.05 compared with the untreated group (Student's *t*-test).

to Fe(II) (Ruipérez et al., 2012). It is well known that oxidative stress alters vascular reactivity through several ways such as its effects on the NO pathway, by counteracting NO effects or by reducing its bioavailability (Hernanz et al., 2014). NO can rapidly react with $O_2\cdot^-$, promoting the formation of the powerful oxidant $ONOO^-$, leading to the loss of the vasodilator effects of NO (Álvarez et al., 2008; Zou 2007). In the present study, the suggested reduced NO bioavailability could be due to the inhibited eNOS gene expression or due to a direct effect of the increased oxidative stress.

NADPH oxidases play a central role in the production of vascular superoxide radical anion and hydrogen peroxidase (Konior et al., 2014). The isoforms NOX-1 and NOX-2 expressed in mammals seem to trigger the development of vascular pathologies such as atherosclerosis, hypertension, neurological disorders, inflammation and cancer (Konior et al., 2014; Schramm et al. 2012). In our study, the in vitro exposure to apocynin and to the antioxidant SOD promoted a higher reduction in the vasoconstrictor response to Phe in segments from Al-treated rats, suggesting the involvement of the superoxide radical anion, presumably from NADPH oxidase, on vascular dysfunction induced by Al. This was further support by the fact that the basal $O_2\cdot^-$ production, the mRNA levels of NOX-1 subunit and the NADPH oxidase activity were increased while the mRNA levels of cytosolic Cu/Zn SOD was decreased in Al-treated rats. Moreover, the vascular redox imbalance suggested by the increased ROS and lipid peroxidation as well as by the antioxidant capacity alterations, support that Al induced pro-oxidant mechanisms likely explained the observed vascular alterations.

Vascular tone is critically modulated by COX-derived prostanoids and its vascular effects in physiological and pathological conditions are depending on the activation of specific receptors (Hernanz et al., 2014; Avendaño et al., 2016). Recently, a circuitous relationship between COX-2 products and ROS acting to induce vascular dysfunction in hypertension was shown (Martínez-Revelles et al., 2013). The toxicity effects of Al have been strongly related with its pro-oxidant capacity (Exley, 2004; Ruipérez et al., 2012; Yu et al., 2016) and, recently an involvement of COX-2 pathway in the neuro-pathological effect of Al has been suggested (Yu et al., 2014; Wang et al., 2015). In the present study we found that the respective unspecific and specific inhibitors of COX-2, indomethacin and NS 398, promoted a reduction in the vasoconstrictor response to Phe mainly in aortic and MRA segments of Al-treated rats. These results suggests the participation of COX-2 derived prostanoids in the vascular dysfunction observed after Al exposure. In agreement, aortas from Al-treated rats showed an increase on mRNA levels of COX-2 and TXA-2 R. The TXA-2 R is involved in critical regulations of the vascular wall such as platelet aggregation and smooth muscle contraction and vascular changes of hypertension (Félétou et al., 2011; Nakahata, 2008). Here, its activation seems to play a role in vascular alterations found in Al-treated rats, however further experiments are necessary to better address this hypothesis.

5. Conclusions

Our study provides evidence that 60-day exposures to low doses of Al, which aimed to mimic human exposure to Al by dietary source, are able to compromise cardiovascular health. The current study shows that Al increases systolic blood pressure and vascular reactivity through endothelium-dependent mechanisms. Here, we demonstrate that ROS and COX-2-derived prostanoids are important mediators of vascular dysfunction after Al exposure. The excess of ROS mainly from NAD(P)H oxidase and increased vascular COX-2 seem to participate in the vascular alterations after Al exposure. Both act in concert to decrease NO bioavailability, which in turn induces vascular dysfunction and increases blood pressure. Therefore, our findings provide a better understanding of the cardiovascular risk of human exposure to Al.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors would like to acknowledge Dr. Ana Briones, Dr. Ana García Redondo, Dr. Raquel Rodrigues and MsC María González from Universidad Autónoma de Madrid for the scientific helping. This work was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* [CNPq 406715/2013-0, 203503/2015-5]; the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*; *Programa Nacional de Cooperação Acadêmica; Pró-reitoria de Pesquisa – Universidade Federal do Pampa* [No. 10.134.14] and by the Spanish Goverment [MINECO – AGL2012-32387, CSIC – Intramural 201570I028 and SAF2015-69294-R].

References

Álvarez, Y., Briones, A.M., Hernanz, R., Pérez-Girón, J.V., Alonso, M.J., Salaices, M., 2008. Role of NADPH oxidase and iNOS in vasoconstrictor responses of vessels from hypertensive and normotensive rats. Br. J. Pharmacol. 153, 926–935.

Assmann, K.E., Adjibade, M., Andreeva, V.A., Hercberg, S., Galan, P., Kesse-Guyot, E., 2017. Association between adherence to the mediterranean diet at midlife and healthy aging in a cohort of french adults. J. Gerontol. A Biol. Sci. Med. Sci. http://dx.doi.org/10.1093/gerona/glx066.

Avendaño, M.S., Martínez-Revelles, S., Aguado, A., Simões, M.R., González-Amor, M., Palacios, R., Guillem-Llobat, P., Vassallo, D.V., Vila, L., García-Puig, J., Beltrán, L.M., Alonso, M.J., Cachofeiro, M.V., Salaices, M., Briones, A.M., 2016. Role of COX-2-derived PGE2 on vascular stiffness and function in hypertension. Br. J. Pharmacol. 173 (9), 1541–1555.

Barata, J.D., D'Haese, P.C., Pires, C., Lamberts, L.V., Simoes, J., De Broe, M.E., 1996. Low-dose (5 mg/kg) desferrioxamine treatment in acutely aluminium-intoxicated haemodialysis patients using two drug administration schedules. Nephrol. Dial. Transplant. 11, 125–132.

Beardmore, J., Exley, C., 2009. Towards a model of non-equilibrium binding of metal ions in biological systems. J. Inorg. Biochem. 103 (2), 205–209.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power": the FRAP Assay. Anal. biochem. 239, 70–76.

Bhattacharjee, S., Zhao, Y., Hill, J.M., Culicchia, F., Kruck, T.P., Percy, M.E., et al., 2013. Selective accumulation of aluminum in cerebral arteries in Alzheimer's disease (AD). J. Inorg. Biochem. 126, 35–37.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J., Cohen, R.A., 1994. Nitric oxide directly activates calcium dependent potassium channels in vascular smooth muscle. Nature 368, 850–853.

Briones, A.M., Rodríguez-Criado, N., Hernanz, R., García-Redondo, A.B., Rodrigues-Díez,

R.R., Alonso, M.J., Egido, J., Ruiz-Ortega, M., Salaices, M., 2009. Atorvastatin prevents angiotensin II–induced vascular remodeling and oxidative stress. Hypertension 54, 142–149.
Carrier, G.O., Fuchs, L.C., Winecoff, A.P., Giulumian, A.D., White, R.E., 1997. Nitrovasodilators relax mesenteric microvessels by cGMP induced stimulation of Ca activated K channels. Am. J. Physiol. 273, H76–84.
Chen, H., Tan, C., Lin, Z., Wu, T., Diao, Y., 2013. A feasibility study of diagnosing cardiovascular diseases based on blood/urine element analysis and consensus models. Comput. Biol. Med. 43 (7), 865–869 (1).
Costello, S., Brown, D.M., Noth, E.M., Cantley, L., Slade, M.D., Tessier-Sherman, B., Hammond, S.K., Eisen, E.A., Cullen, M.R., 2014. Incident ischemic heart disease and recent occupational exposure to particulate matter in an aluminum cohort. J. Expo. Sci. Environ. Epidemiol. 24 (1), 82–88.
Crépeaux, G., Eidi, H., David, M.O., Baba-Amer, Y., Tzavara, E., Giros, B., et al., 2017. Non-linear dose-response of aluminium hydroxide adjuvant particles: selective low dose neurotoxicity. Toxicology 375, 48–57.
EFSA, 2008. Safety of aluminium from dietary intake. EFSA J. 754, 1–34.
Exley, C., 2004. The pro-oxidant activity of aluminum. Free Radic. Biol. Med. 3, 380–387.
Exley, C., 2009. Darwin, natural selection and the biological essentiality of aluminium and silicon. Trends Biochem. Sci. 12, 589–593.
Exley, C., 2012. Elucidating aluminiuṁs exposome. Curr Inorg Chem. 2, 3–7.
Exley, C., 2013. Human exposure to aluminium. Environ. Sci. Process Impacts 10, 1807–1816.
Ezomo, O.F., Matsushima, F., Meshitsuka, S., 2009. Up-regulation in the expression of renin gene by the influence of aluminium. J. Inorg. Biochem. 103 (11), 1563–1570.
Félétou, M., Huang, Y., Vanhoutte, P.M., 2011. Endothelium mediated control of vascular tone: COX-1 and COX-2 products. Br. J. Pharmacol. 164, 894–912.
Fekete, V., Vandevijvere, S., Bolle, F., Van Loco, J., 2013. Estimation of dietary aluminum exposure of the Belgian adult population: evaluation of contribution of food and kitchenware. Food Chem. Toxicol. 55, 602–608.
Gherardi, R.K., Aouizerate, J., Cadusseau, J., Yara, S., Authier, F.J., 2016. Aluminum adjuvants of vaccines injected into the muscle: normal fate, pathology and associated disease. Morphologie 100 (329), 85–94.
Gomes, M.G., Moreira, C.A., Mill, J.G., Massaroni, L., Oliveira, E.M., Stefanon, I., et al., 1994. Effects of aluminum on the mechanical and electrical activity of the Langendorff-perfused rat heart. Braz. J. Med. Biol. Res. 27, 95–100.
Gonzalez-Weller, D., Gutiérrez, A.J., Rubio, C., Revert, C., Hardisson, A., 2010. Dietary intake of aluminum in a Spanish population (Canary Islands). J. Agric. Food Chem. 58 (19), 10452–10457.
Granadillo, V.A., Tahan, J.E., Salgado, O., Elejalde, L.E., Rodriguez-Iturbe, B., Romero, G.B., et al., 1995. The influence of the blood levels of lead, aluminum and vanadium upon the arterial hypertension. Clin. Chim. Acta 233, 47–59.
Hernanz, R., Briones, A.M., Salaices, M., Alonso, M.J., 2014. New roles for old pathways? A circuitous relationship between reactive oxygen species and cyclo-oxygenase in hypertension. Clin. Sci. (Lond.) 126 (2), 111–121.
House, E., Esiri, M., Forster, G., Ince, P.G., Exley, C., 2012. Aluminium, iron and copper in human brain tissues donated to the medical research council's cognitive function and ageing study. Metallomics 4, 56–65.
Klein, J.P., Mold, M., Mery, L., Cottier, M., Exley, C., 2014. Aluminum content of human semen: implications for semen quality. Reprod. Toxicol. 50, 43–48.
Konior, A., Schramm, A., Czesnikiewicz-Guzik, M., Guzik, T.J., 2014. NADPH oxidases in vascular pathology. Antioxid. Redox Signal. 20 (17), 2794–2814.
Korchazhkina, O., Wright, G., Exley, C., 1998. Action of Al-ATP on the isolated working rat heart. J. Inorg. Biochem. 69 (3), 153–158.
Korchazhkina, O., Wright, G., Exley, C., 1999. No effect of aluminium upon the hydrolysis of ATP in the coronary circulation of the isolated working rat heart. J. Inorg. Biochem. 76 (2), 121–126.
Li, X., Hu, C., Zhu, Y., Sun, H., Li, Y., Zhang, Z., 2011. Effects of aluminum exposure on bone mineral density, mineral, and trace elements in rats. Biol. Trace Elem. Res. 143, 378–385.
Lind, P.M., Olsen, L., Lind, L., 2012. Circulating levels of metals are related to carotid atherosclerosis in elderly. Sci. Total Environ. 416, 80–88.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta C(T)) method. Methods 25 (4), 402–408.
Loetchutinat, C., Kothan, S., Dechsupa, S., Meesungnoen, J., Jay-Gerin, J., Mankhetkorn, S., 2005. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichloro-fluorescein diacetate assay. Rad. Phys. Chem. 72, 323–331.
Martínez-Revelles, S., Avendaño, M.S., García-Redondo, A.B., Alvarez, Y., Aguado, A., Pérez-Girón, J.V., García-Redondo, L., Esteban, V., Redondo, J.M., Alonso, M.J., et al., 2013. Reciprocal relationship between reactive oxygen species and cyclooxygenase-2 and vascular dysfunction in hypertension. Antioxid. Redox Signal. 18, 51–65.
Martinez, C.S., Alterman, C.D., Peçanha, F.M., Vassallo, D.V., Mello-Carpes, P.B., Miguel, M., et al., 2017. Aluminum exposure at human dietary levels for 60 days reaches a threshold sufficient to promote memory impairment in rats. Neurotox. Res. 31 (1), 20–30.
Martyn, K.D., Frederick, L.M., von, L.K., Dinauer, M.C., Knaus, U.G., 2006. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. Cell. Signal. 18, 69–82.
Minami, T., Tohno, S., Utsumi, M., Moriwake, Y., Yamada, M.O., Tohno, Y., 2001. Selective accumulations of aluminum in five human arteries. Biol. Trace Elem. Res. 79 (1), 29–38.
Mirza, A., King, A., Troakes, C., Exley, C., 2016. The identification of aluminum in human brain tissue using lumogallion and fluorescence microscopy. J. Alzheimers Dis. 54 (4), 1333–1338.
Mistry, D.K., Garland, C.J., 1998. Nitric oxide (NO) induced activation of large conductance Ca dependent K + channels (BKCa) in smooth muscle cells isolated from the rat mesenteric artery. Br. J. Pharmacol. 124, 1131–1140.
Nakahata, N., 2008. Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. Pharmacol. Ther. 118, 18–35.
Neophytou, A.M., Noth, E.M., Liu, S., Costello, S., Hammond, S.K., Cullen, M.R., et al., 2016. Ischemic heart disease incidence in relation to fine versus total particulate matter exposure in a U.S. aluminum industry cohort. PLoS One 11 (6), e0156613. http://dx.doi.org/10.1371/journal.pone.0156613.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Prakash, A., Kumar, A., 2009. Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats. Basic Clin. Pharmacol. Toxicol. 2, 98–104.
Reagan-Shaw, S., Nihal, M., Ahmad, N., 2008. Dose translation from animal to human studies revisited. FASEB J. 3, 659–661.
Ruipérez, F., Mujika, J.I., Ugalde, J.M., Exley, C., Lopez, X., 2012. Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II). J. Inorg. Biochem. 117, 118–123.
Schmidt, P.M., Escobar, A.G., Torres, J.G.D., Martinez, C.S., Rizzetti, D.A., Kunz, S.N., Vassallo, D.V., Alonso, M.J., Peçanha, F.M., Wiggers, G.A., 2016. Aluminum exposure for one hour decreases vascular reactivity in conductance and resistance arteries in rats. Toxicol. Appl. Pharmacol. 313, 109–118.
Schramm, A., Matusik, P., Osmenda, G., Guzik, T.J., 2012. Targeting NADPH oxidases in vascular pharmacology. Vascul. Pharmacol. 56 (5–6), 216–231.
Subrahmanyam, G., Pathapati, R.M., Ramalingam, K., Indira, S.A., Kantha, K., Soren, B., 2016. Arterial stiffness and trace elements in apparently healthy population- a cross-sectional study. J. Clin. Diagn. Res. 10 (9), LC12–LC15.
World Health Organization 2007. Safety Evaluation of Certain Food Additives and Contaminants. Food Additive Series: 58: http://whqlibdoc.who.int/trs/WHOTRS940eng.pdf.
Wang, H., Ye, M., Yu, L., Wang, J., Guo, Y., Lei, W., et al., 2015. Hippocampal neuronal cyclooxygenase-2 downstream signaling imbalance in a rat model of chronic aluminium gluconate administration. Behav. Brain Funct. 18, 11–18. http://dx.doi.org/10.1186/s12993-015-0054-z.
Wiggers, G.A., Pecanha, F.M., Briones, A.M., Perez-Giron, J.V., Miguel, M., Vassallo, D.V., et al., 2008. Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries. Am. J. Physiol. Heart Circ. Physiol. 295, H1033–H1043.
Yang, M., Jiang, L., Huang, H., Zeng, S., Qiu, F., Yu, M., et al., 2014. Dietary exposure to aluminium and health risk assessment in the residents of Shenzhen, China. PLoS One 9 (3), e89715. http://dx.doi.org/10.1371/journal.pone.0089715. (3).
Yu, L., Jiang, R., Su, Q., Yu, H., Yang, J., 2014. Hippocampal neuronal metal ion imbalance related oxidative stress in a rat model of chronic aluminum exposure and neuroprotection of meloxicam. Behav. Brain Funct. 11, 10–16.
Yu, L., Zhai, Q., Yin, R., Li, P., Tian, F., Liu, X., et al., 2016. Lactobacillus plantarum CCFM639 alleviate trace element imbalance-related oxidative stress in liver and kidney of chronic aluminum exposure mice. Biol. Trace Elem. Res. 176 (2), 342–349.
Zhang, Q., Cao, Z., Sun, X., Zuang, C., Huang, W., Li, Y., 2016. Aluminum trichloride induces hypertension and disturbs the function of erythrocyte membrane in male rats. Biol. Trace Elem. Res. 171 (1), 116–123.
Zou, M.H., 2007. Peroxynitrite and protein tyrosine nitration of prostacyclin synthase. Prostagland. Other Lipid Mediat. 82 (1–4), 119–127.

PARTE VI

DISCUSSÃO

Resultados deste trabalho demonstram que a exposição ao Al em uma dose equivalente de exposição humana é o suficiente para desencadear alterações fisiológicas e bioquímicas em diferentes sistemas e órgãos. A exposição ao metal por 60 dias comprometeu o aprendizado e a formação de memória de longa duração, desencadeou o comportamento cataléptico e, perifericamente, o desenvolvimento de alodínea mecânica em ratos. No sistema reprodutor masculino, a exposição ao Al prejudicou a qualidade espermática com redução da produção diária espermática (PDE), redução no número de espermatozoides no testículo e epidídimo, redução na motilidade e aumento das anormalidades morfológicas espermáticas. Ainda, o Al foi capaz de depositar-se entre as células da linhagem espermatogênica nos túbulos seminíferos desencadeando alterações estruturais como redução do epitélio germinativo. No sistema cardiovascular, a exposição ao Al em dose de exposição humana ao metal foi capaz de aumentar a pressão arterial sistólica dos animais a partir da 3ª semana de exposição e, promover alterações vasculares em artérias de resistência e condutância, causando disfunção endotelial com aumento da resposta contrátil vascular e redução da resposta vasodilatadora. Ainda, ao comparar o efeito do Al em dose semelhante a exposição humana pela dieta com uma dose alta de exposição, constatamos que os efeitos do metal em baixas doses são praticamente os mesmos aos encontrados em uma dose de exposição 12 ou 66 vezes maior. Sugerindo que, tratando-se do Al, a quantidade do metal biologicamente ativo parece ser mais importante que a dose de exposição, além disso, nossos resultados sugerem que ao alcançar um limiar de toxicidade os efeitos advindos dessa exposição são similares e, que esse limiar pode ser alcançado em dose de exposição semelhante aos níveis de exposição humana através da dieta.

O Al é atualmente o maior contaminate ambiental e doméstico e, devido as inúmeras formas e fontes de exposição ao metal, a exposição humana é praticamente inevitável e imensurável (EXLEY, 2013). A ingesta semanal máxima pela dieta foi recentemente ajustada de 7 para 1 mg Al/kg de peso corporal (EFSA 2008; WHO 2007), no entanto esses valores podem ser facilmente excedidos por uma importante parcela da população mundial (FEKETE *et al*., 2013; GONZALEZ-WELLER *et al*., 2010; YANG *et al*., 2014). Além disso, não há uma normativa internacional que estabeleça valores seguros de exposição ao Al tendo como base as diferentes fontes de exposição humana. Greger (1993), levando em consideração os níveis de

Al dos alimentos industrializados, estimou que os norte americanos consomem de 1 a 10 mg/Al/dia advindos de alimentos naturais. Além disso, 50% dos norte americanos consomem até 24 mg/dia, 45% entre 24 e 95 mg e cerca de 5% ingerem mais de 95 mg/Al/dia na forma de aditivos alimentares. Ainda, o consumo de medicamentos como antiácidos ou analgésicos tamponados expõem a população a altos níveis de exposição ao metal via TGI, podendo alcançar de 125 a 5000 mg/Al/dia (REINKE *et al*., 2003; SHAW; TOMLJENOVIC, 2013).

Objetivamos por meio deste trabalho estabelecer um modelo animal de exposição ao Al em níveis equivalentes a exposição humana ao metal pela dieta e, comparar com os efeitos de uma dose alta de exposição ao metal. Para isso, utilizamos um protocolo de exposição em baixas doses de 1,5 mg/kg/dia de acordo com o descrito por Walton (2007), porém por 60 dias de exposição. Ainda realizamos outro tratamento com Al na dose de 8,3 mg/kg/dia durante 60 dias, que representa a mesma dose de exposição de 1,5 mg/kg/dia porém ajustada para o modelo animal de exposição utilizado, baseado no método de normalização de superfície corporal (REAGAN-SHAW *et al*., 2008). Ainda, utilizamos um modelo de exposição em altas doses do metal (100 mg/Al//kg de peso corporal), com neurotoxicidade conhecida (PRAKASH; KUMAR, 2009).

Devido a ubiquidade do Al, além dos cuidados para evitar a contaminação ambiente pelo metal, realizamos a medida de Al presente na ração padrão dos animais, garantindo assim um melhor controle e a obtenção de valores de exposição mais próximos da realidade. Dessa forma, todos os animais receberam 1,88 mg/Al por dia advindos da ração, considerando a média de peso corporal dos animais de 300g isto representa 6,26 mg/Al/kg de peso corporal por dia. Assim, considerando o peso dos animais, a quantidade total de exposição ao Al para os grupos experimentais foi de: a) 1,5 mg/Al/kg – 2,33 mg/Al por dia (0,45 mg/Al da água de beber mais 1,88 mg/Al da ração); b) 8,3 mg/Al/kg – 4,37 mg/Al por dia (2,49 mg/Al da água de beber mais 1,88 mg/Al da ração); c) 100 mg/Al/kg - 31,88 mg/Al por dia (30 mg/Al pela gavagem mais 1,88 mg/Al da ração). Uma vez que todos os ratos consumiram aproximadamente a mesma quantidade de ração e consequentemente de Al, a presença de Al na água dos animais aponta para essa via adicional de exposição ao metal como um ponto crítico para o alcance do limiar de exposição responsável pelo desencadeamento das alterações tóxicas.

Walton (2007) desenvolveu um estudo longitudinal ao qual ratos eram expostos ao Al na dose de 1,5 mg/kg por dia desde os 12 meses de idade até a morte natural (velhice). Os animais expostos ao Al desenvolveram na idade avançada alterações cognitivas como déficit de memória e alterações de comportamento similares ao encontrado em pacientes com DA.

Nosso estudo demonstra que a mesma dose utilizada por Walton (1,5 mg/kg/dia) entretanto, por um período de tempo reduzido, 60 dias (aproximadamente 70 meses para humanos ou 6 anos) (EVERITT, 1991), é o suficiente para desencadear a cascata de neurotoxicidade do metal. Além disso, observamos que a exposição ao Al na dose de 8,3 mg/kg/dia, o equivalente a exposição humana através da dieta e adaptada a espécie animal utilizada no estudo, também é capaz de comprometer a consolidação da memória de longa duração além de desencadear alterações comportamentais como catalepsia e danos ao SNP observado pela presença de alodínea mecânica. Somado a isso, demonstramos pela primeira vez que os sistemas reprodutor e cardiovascular também são afetados pela exposição ao Al em baixas doses.

Nosso estudo demonstra que a exposição ao Al em altas doses também compromete o funcionamento cognitivo dos animais com danos a memória de reconhecimento de objetos. No entanto, esses resultados são esperados uma vez que a exposição ao Al na dose de 100 mg/kg/dia via oral por 6 semanas, é um modelo conhecido de neurotoxicidade e demência induzido pelo Al, ao qual desencadeia deterioração progressiva da memória espacial e dano oxidativo em ratos (PRAKASH; KUMAR, 2013; 2009). Porém, o nosso estudo permite observar alterações em outros sistemas como o cardiovascular e SNP utilizando esse modelo de exposição em altas doses.

Nosso estudo sugere um padrão toxicológico não convencional para o Al, uma vez que ao compararmos os efeitos da exposição ao Al em dose similar a exposição humana com uma dose de exposição alta ao metal não observamos diferenças nos efeitos tóxicos em nenhum sistema avaliado. Ao contrário, no sistema reprodutor masculino a dose de 8,3 mg/kg, equivalente a exposição humana pela dieta, promoveu as maiores alterações reprodutivas. Esse padrão não convencional de toxicidade foi recentemente observado por Crépeaux et al. (2017), ao investigar os efeitos do Al oxyhydroxide (Alhydrogel®), utilizado como adjuvante em vacinas, em camundongos fêmeas. Os autores encontraram alterações cognitivas e ativação da imunidade inata apenas após a exposição ao Al na menor dose de 0,2 mg Al/kg e não nas doses de 0,4 ou 0,8 mg Al/kg. Os nossos achados juntamente com os resultados prévios da literatura apontam que a dose de exposição ao metal talvez não seja o fator mais importante em relação a toxicidade do Al, mas sim características da exposição, fatores individuais de susceptibilidade, a forma química do metal e consequentemente distribuição e disponibilidade biológica do Al nos diferentes compartimentos corporais (CRÉPEAUX *et al*., 2017; EXLEY, 2013). Assim, nossos resultados sugerem que os atuais níveis de exposição humana ao Al considerados seguros deveriam ser revisados.

Corroborando com o padrão não convencional de toxicocinética do Al, no presente estudo não houve diferença entre a concentração de Al no sangue do grupo tratado com Al em baixa dose quando comparado ao grupo controle, porém houve significante acúmulo do metal no fígado e testículo. Essa ausência de diferença apenas na concentração sanguínea é esperada uma vez que o sangue é apenas um órgão temporal de estocagem e distribuição de Al (BEARDMORE; EXLEY 2009). Além disso, os valores disponíveis na literatura acerca da concentração sanguínea de Al são extremamente variáveis, partindo de valores inferiores a 1 até 901 µg Al/L de sangue (CHEN *et al*., 2013; LIND *et al*., 2012).

Ao longo das últimas décadas a hipótese do Al na DA tem sido debatida e investigada por meio de estudos experimentais e clínicos, no entanto evidências agora sugerem a DA como uma resposta aguda frente à exposição crônica ao Al (EXLEY, 2017, EXLEY, 2014). Os efeitos tóxicos do Al aparecem após o alcance de um limiar ou carga tóxica do metal (EXLEY, 2014). Os nossos resultados sugerem que níveis de exposição humana ao Al são suficientes para o alcance desse limiar de toxicidade. Ratos expostos ao Al por 60 dias não são capazes de reconhecer objetos familiares como os animais no grupo controle e, a exposição ao Al desencadeia catalepsia nos animais. Ainda, demonstramos que o metal é capaz de acumular-se perifericamente entre as fibras do nervo ciático causando neuropatia periférica.

Os efeitos do Al sobre o SNP são pouco conhecidos. O metal já foi utilizado como modelo de neurodegeneração quando injetado no nervo ciático de coelhos (KIHIRA *et al*., 1995). Recentemente, a presença de Al no gânglio da raiz dorsal de ratos tratados com cisplatina foi associada com efeitos adversos no SNP (PARK *et al*., 2015). O que foi posteriormente confirmado pelos autores ao utilizar a glutationa, antioxidante e suposto quelante, a qual reduziu o acúmulo do metal e a dor neuropática induzida pela cisplatina (LEE *et al*., 2017).

Bakar et al. (2010) entrevistaram 273 moradores da região da Kirazli, Turquia que foram expostos a altas concentrações de Al pela água tratada (140 vezes acima dos valores permitidos) e, compararam com um grupo populacional controle não-exposto. Os autores observaram que a incidência de neuropatia foi 36% maior entre os moradores da região exposta ao Al. No entanto, ainda que os efeitos do Al sobre o SNP sejam pouco conhecidos, a neurotoxicidade causada pelo metal é fortemente apontada como a principal causa de encefalopatia em pacientes renais crônicos em hemodiálise e, desordens periféricas estão entre as alterações neurológicas encontradas nesses pacientes (BANSAL *et al*., 2014; HSU *et al*., 2016). No presente estudo, o Al desencadeou alodínea mecânica sem alterar a sensibilidade térmica ou nociceptiva, enquanto que Park et al. (2015) observaram alterações de sensibilidade térmica em camundongos

expostos ao Al. No entanto, trabalhos futuros são necessários para um melhor entendimento acerca dos efeitos do Al sobre o SNP.

No presente estudo, a exposição ao Al independente da dose foi capaz de desencadear catalepsia nos ratos, a qual é uma forma extrema de imobilidade manifestada em alguns pacientes com doença de Parkinson e assemelha-se aos efeitos colaterais extrapiramidais que ocorrem em seres humanos expostos a alguns fármacos antipsicóticos, alguns dos quais atuando como inibidores da acetilcolinesterase (AChE) (DE RYCK *et al*., 1980).

Em animais experimentais a inibição da AChE tem sido relacionada com o desenvolvimento de catalepsia (CASTELLÓ *et al*., 1992; SKLAN *et al*., 2006). Assim, o desenvolvimento da catalepsia observado após a exposição ao Al vai ao encontro da inibição da atividade da AChE também observada após exposição ao metal e, apontada como um dos possíveis mecanismos de toxicidade do Al. De fato, a atividade da AChE é um marcador para a morte de neurônios colinérgicos e a disfunção cognitiva como as alterações de memória esta associada principalmente a perda de neurônios colinérgicos em diferentes regiões cerebrais (FRASER; MACVICAR, 1996; WHITEHOUSE *et al*., 1982). Nossos resultados revelam disfunção da atividade da AChE após exposição ao Al em alta ou baixa dose, com redução da atividade enzimática hipocampal. A ação do Al no sistema colinérgico tem sido caracterizada como bifásica, podendo o metal estimular ou inibir a atividade enzimática (LAKSHMI *et al*., 2015; NOREMBERG *et al*., 2016; PRAKASH; KUMAR, 2009). Sugere-se que em baixa dose e/ou curto período de exposição o Al estimule a atividade da AChE enquanto que em alta dose e/ou prolongado período de exposição o metal haja inibindo a atividade enzimática (KUMAR, 1999). Dentre as hipóteses sugeridas, acredita-se que a ação bifásica do Al sobre a AChE possa ser atribuída a um efeito direto do metal na enzima ou devido ao dano peroxidativo de membrana causado pela exposição ao Al (KUMAR, 1999). A última hipótese corrobora com os achados do presente estudo, ao qual aponta o estresse oxidativo como um dos possíveis mecanismos de toxicidade do Al.

O SNC, especialmente o hipocampo é bastante sensível ao estresse oxidativo, principalmente pela limitada capacidade antioxidante e alto conteúdo lipídico (ERFANI *et al*., 2015; KUMAR; GILL, 2014). No presente estudo, a exposição ao Al independente da dose aumentou os níveis de estresse oxidativo sistêmico e local demonstrado pelo aumento dos níveis de espécies reativas de oxigênio (EROs), peroxidação lipídica e redução da capacidade antioxidante total no plasma e hipocampo dos animais, enquanto que no córtex pré-frontal esse aumento ocorreu apenas nas doses mais altas de exposição ao metal. De fato, o Al^{3+} é um

potente pró-oxidante podendo agir via formação do complexo Al-radical ânion superóxido (EXLEY, 2004) ou promovendo a reação de Fenton através da redução do Fe (III) a Fe (II) (RUIPÉREZ *et al*., 2012). Ainda que os mecanismos de ação do Al requeiram profunda investigação, considerando que o hipocampo é a principal estrutura requerida para a formação e consolidação da memória, podemos afirmar, baseado nos nossos resultados, que o dano oxidativo e a disfunção colinérgica hipocampal induzidos pelo Al são ao menos parcialmente responsáveis pela disfunção cognitiva encontrada após exposição ao Al.

Os danos periféricos induzidos pelo Al também podem ser atribuídos, ainda que parcialmente, ao aumento do estresse oxidativo sistêmico, uma vez que o estresse oxidativo e a inflamação estão entre as bases fisiopatológicas da neuropatia periférica, contribuindo para a desmielinização de nervos periféricos, alterando assim a condução do estímulo elétrico e promovendo dano neuronal (ELLIS *et al*., 2013; SANDIREDDY *et al*., 2014). Somado a isso, a presença de alodínea mecânica veio acompanhada da presença e acúmulo intracelular de Al e, de um aumento no número de macrófagos ativados no nervo ciático. Dessa forma, nossos achados sugerem o envolvimento do estresse oxidativo e a ativação inflamatória no desencadeamento da neuropatia periférica após exposição ao Al.

O tripé acúmulo do metal, estresse oxidativo e inflamação também podem ser apontados como causadores da disfunção reprodutiva masculina observada após exposição ao Al em baixa ou alta dose. A preocupante queda de 50% na qualidade espermática nos últimos 40 anos entre homens de diferentes regiões do planeta e, o sinérgico aumento da exposição humana a contaminantes ambientais como o Al, impulsionou o desenvolvimento desse estudo (EXLEY, 2013; LEVINE *et al*., 2017). Nossos resultados sugerem que o Al em nível de exposição humana doméstica é o suficiente para comprometer o funcionamento do sistema reprodutor e, consequentemente, a fertilidade masculina. Especificamente, animais expostos ao metal por 60 dias apresentaram redução da qualidade espermática com redução na produção e no estoque de espermatozoides, queda na motilidade e aumento das anormalidades morfológicas de cabeça e cauda. Essas alterações funcionais refletiram na histoarquitetura testicular, com redução e/ou atrofia do epitélio germinativo. De forma semelhante ao encontrado no SNC, os efeitos da exposição ao Al em dose equivalente a exposição humana não diferiram significativamente dos efeitos da exposição ao Al em alta dose.

Considerando as características pró-oxidantes do Al (EXLEY, 2004) e, a capacidade de promover o estresse oxidativo em diferentes sistemas e órgãos, como recentemente observado no sistema reprodutor (JAMALAN *et al*., 2016; MOHAMMAD *et al*., 2015; ODA, 2016),

nosso estudo avaliou a presença de estresse oxidativo nos órgãos reprodutores após exposição ao metal. O Al aumentou os níveis de EROs e de peroxidação lipídica no testículo, epidídimo e próstata dos animais. Além do aumento do estresse oxidativo, a exposição ao metal estimulou respostas inflamatórias com aumento no número de macrófagos ativados no testículo após exposição ao Al na dose de 8,3 mg/kg. O sistema reprodutor, especialmente testículos e células espermáticas, são bastante susceptíveis ao dano oxidativo principalmente pelo alto teor de ácidos graxos poliinsaturados de suas membranas e, limitada capacidade antioxidante (AITKEN, 1995). Somado a isso, a superprodução de espécies reativas parece ser extremamente prejudicial ao gameta masculino e, um fato comun observado na infertilidade masculina (TURNER; LYSIAK, 2008). Assim, a aparente supressão da espermatogênese, o comprometimento da qualidade espermática e as alterações histopatológicas, podem ser, ao menos parcialmente, atribuídas ao aumento do estresse oxidativo levando a peroxidação de membranas e ao comprometimento da viabilidade espermática (KISTANOVA *et al*., 2009) e, devido a presença de inflamação no testículo.

Quanto à defesa e proteção celular contra o dano oxidativo, cada órgão respondeu de forma diferenciada frente ao aumento do estresse oxidativo e, de acordo com o nível de exposição ao metal. Por exemplo, a exposição ao Al nas doses de 1,5 e 8,3 mg/kg de peso corporal por dia diminuiu a capacidade antioxidante no testículo, enquanto que o inverso foi observado na dose mais alta de exposição (100 mg/kg). Ressaltando novamente, a ausência de um padrão toxicológico convencional após exposição ao Al, uma vez que os efeitos adversos deste metal dependem da duração da exposição, do limiar de contaminação e da disponibilidade biológica alcançada.

Estudos avaliando diretamente a relação do Al com a qualidade do sêmen humano são escassos. Hovatta (1998) e Dawson (1998) demonstraram uma associação entre a presença de Al no líquido seminal e a motilidade espermática. Mais recentemente, essa associação foi novamente encontrada em amostras de sêmen humano expostos ao $AlCl_3$, cádmio ou chumbo, onde a exposição ao Al foi responsável pelo maior comprometimento da qualidade espermática (JAMALAN *et al*., 2016). No estudo de Klein *et al.* (2014), amostras de sêmen de 62 pacientes foram avaliadas revelando uma alta concentração de Al no sêmen de indivíduos com história de oligozoospermia.

Estudos experimentais sobre os efeitos do Al no sistema reprodutor estão em maior número. Uma única injeção i.p. de cloreto de alumínio ($AlCl_3$) a 25 mg/kg em camundongos foi associada a alterações testículares e espermatogênicas como degeneração de células

germinativas, atrofia dos túbulos seminíferos, apoptose de espermatogônias e espermatócitos primários e, danos as células de Leydig (ABDEL-MONEIM, 2013). A administração intragástrica de 100 mg/kg de peso corporal de $AlCl_3$ durante 4 semanas promoveu danos histopatológicos em testículo e epidídimo, aumentou o estresse oxidativo observado pelo aumento nos níveis de malondialdeído (MDA) e redução nos níveis de glutationa em ratos (ODA, 2016). Ainda, estudos verificando os efeitos da administração de $AlCl_3$ em doses que variam de 34 mg/kg a 256,72 mg/kg de peso corporal por dia relacionaram a exposição ao Al com redução do peso dos órgãos reprodutores, redução na contagem e motilidade espermática, redução na libido e no volume de sêmen ejaculado, aumento das anormalidades morfológicas e desequilíbrio hormonal com diminuição dos níveis plasmáticos de testosterona, hormônio luteinizante (LH) e hormônio folículo estimulante (FSH) em ratos e coelhos (IGE; AKHIGBE, 2012; SUN *et al*., 2011; ZHU *et al*., 2014).

No entanto, os estudos supracitados avaliaram os efeitos da exposição ao Al em altas doses de exposição ao metal e, não consideraram a quantidade do metal presente na ração padrão dos animais, o que aumentaria ainda mais o total de Al aos quais os animais foram expostos. Em nosso modelo experimental, mensuramos a quantidade de Al presente na ração comercial dos roedores, a quantidade do metal acumulada nos órgãos reprodutores testículo e epididímo e, a específica presença do Al nos órgãos por fluorescência. Nossos resultados demonstram que uma concentração de Al ao redor de 3 μg/g, após exposição ao Al em dose equivalente a contaminação doméstica humana, é o suficiente para comprometer o funcionamento do sistema reprodutor masculino. Estudos prévios têm demonstrado disfunções reprodutivas após exposição ao Al em doses irrealistas e altas (por exemplo de 34 mg/kg a 400 mg/kg), encontrando assim, altas concentrações do metal nos testículos, variando de 35 μg/g a 140 μg/g (GUO *et al*., 2001; GUO *et al*., 2005; MOHAMMAD *et al*., 2015).

Nosso estudo, além de demonstrar o acúmulo do metal por espectrometria de absorção atômica em forno de grafite, identificou pela primeira vez a específica presença do metal nos túbulos seminíferos utilizando *lumogallion* e microscopia de fluorecência. Sabemos que mais estudos são necessários para melhor identificar a localização do metal no testículo, no entanto, a presença do Al aparentemente entre as células da linhagem germinativa reforçam a possível interferência do metal no processo espermatogênico.

Sabendo que o estresse oxidativo é um dos principais mecanismos de ação do Al e, que o dano oxidativo é um dos principais mecanismos subjacentes na doença vascular (ASSMANN *et al*., 2017; EXLEY, 2013), este estudo também investigou o efeito do Al sobre o sistema

cardiovascular. Demonstramos que a exposição ao Al em dose equivalente a contaminação humana através da dieta pode representar um risco a saúde cardiovascular. Especificamente, a exposição ao Al aumentou a pressão arterial sistólica e a reatividade vascular em artérias de resistência e condutância possivelmente via mecanismos endoteliais. Demonstramos ainda que as EROs e os prostanoides derivados da via da COX-2 são importantes mediadores da disfunção vascular observada após exposição ao Al. Nossos resultados sugerem que as EROs principalmente advindas da NAD(P)H oxidase e os prostanoides contráteis da via da COX-2 atuam reduzindo a biodisponibilidade de óxido nítrico (NO), induzindo assim a disfunção vascular e o aumento da pressão arterial.

O possível efeito do Al sobre o sistema cardiovascular foi previamente investigado em coração isolado de rato onde o metal na concentração de 100 μM reduziu o fluxo coronariano e a pressão sistólica isovolumétrica (GOMES *et al*., 1994). Posteriormente, observou-se que a perfusão cardíaca com Al na concentração de 40 μM promovia um aumento no fluxo coronariano e redução na frequência cardíaca (KORCHAZHKINA *et al*., 1998).

Recentemente, a administração aguda ou crônica de Al na dose de até 256 mg/kg foi associada ao desenvolvimento de hipertensão em ratos e, esses efeitos foram relacionados ao aumento da expressão de renina ou ao dano de membranas eritrocitárias (EZOMO *et al*., 2009; ZHANG *et al*., 2016). Em humanos, essa possível associação também foi postulada, onde a circulação plasmática de Al foi relacionada ao desenvolvimento de hipertensão arterial (GRANADILLO *et al*., 1995). Em um estudo recente, nosso grupo demonstrou que uma hora de exposição ao Al na dose de 100 mg/kg é suficiente para promover alterações vasculares com redução da reatividade vascular e aumento do estresse oxidativo (SCHMIDT *et al*., 2016). De acordo com nosso conhecimento, este estudo é o primeiro experimental investigando e demonstrando os efeitos cardiovasculares após a exposição ao Al em dose de equivalência humana através da dieta. Nosso estudo dá suporte a hipótese do aumento do risco cardiovascular induzido pelo Al e, dentre os nossos principais achados podemos destacar que a exposição ao Al: 1) aumentou a pressão arterial sistólica; 2) promoveu disfunção endotelial, demonstrado pelo comprometimento da resposta vasodilatadora à acetilcolina (ACh) e pelo aumento das respostas vasoconstritoras a fenilefrina (Phe) e, 3) reduziu a biodisponibilidade endotelial de NO.

Já bastante citado ao longo desse trabalho, a toxicidade do Al^{3+} está parcialmente relacionada com a sua capacidade pró-oxidante (EXLEY, 2004). É sabido que o estresse oxidativo altera a reatividade vascular por diferentes vias, como por exemplo, ao atuar na via

do NO se opondo aos efeitos do NO ou reduzindo sua biodisponibilidade (HERNANZ *et al*., 2014). Na presença de espécies reativas como o O2•-, o NO pode rapidamente reagir com o O2•-, formando um radical ainda mais reativo que o O2•-, o radical peroxinitrito (ONOO−), reduzindo assim os efeitos vasodilatadores do NO (ÄLVAREZ *et al*., 2008; ZOU, 2007). Em nosso estudo, a redução da biodisponibilidade do NO pode ser atribuída a inibição da expressão gênica da eNOS ou ao efeito direto do aumento do estresse oxidativo. O complexo enzimático NADPH oxidase desempenha um papel central na produção vascular do O2•- e peróxido de hidrogênio (H_2O_2) (KONIOR *et al*., 2014). As isoformas NOX-1 e NOX-2 expressas em mamíferos parecem desempenhar um papel fundamental na patogênese de doenças vasculares como aterosclerose, hipertensão, desordens vasculares neurológicas, inflamação e câncer (KONIOR *et al*., 2014; SCHRAMM *et al*., 2012). No nosso estudo, a exposição *in vitro* da apocinina (inibidor não seletivo da NOX) e do antioxidante superóxido dismutase (SOD) promoveu uma redução na resposta vascular a Phe de maior magnitude nos segmentos arteriais de ratos expostos ao Al, sugerindo assim o envolvimento do O2•-, provavelmente advindo da NOX, na disfunção vascular induzida pela exposição ao metal. A nossa hipótese foi ainda fortificada pelo aumento na produção basal de O2•- vascular, aumento na expressão de mRNA da subunidade NOX-1, aumento na atividade enzimática da NOX e pela redução dos níveis de mRNA da Cu/Zn SOD observados após exposição ao Al. Somado a isso, o desequilíbrio redox sugerido pelo aumento dos níveis de EROs e peroxidação lipídica bem como pelas alterações da capacidade antioxidante vascular, também sugere a participação dos mecanismos pró-oxidantes do Al no desencadeamento das alterações vasculares após exposição ao metal.

O tônus vascular também é fortemente modulado por prostanoides derivados da COX-2, cujos efeitos vasculares serão dependentes da ativação de receptores específicos (AVENDAÑO *et al*., 2016; HERNANZ *et al*., 2014). Recentemente, foi demonstrada uma relação em forma de circuito entre os derivados da COX-2 e as EROs, atuando em conjunto para promover a disfunção vascular na hipertensão (MARTINEZ-REVELLES *et al*., 2013). Os efeitos tóxicos do Al têm sido fortemente associados com a sua capacidade pró-oxidante (EXLEY, 2004; RUIPÉREZ *et al*., 2012; YU *et al*., 2016) entretanto, tem-se sugerido um envolvimento da via da COX-2 nas disfunções neuropatológicas induzidas pelo metal (YU *et al*., 2014; WANG *et al*., 2015). No presente estudo, o uso do inibidor não específico e específico da COX-2, indometacina e NS 398, *in vitro* reduziu a resposta vasoconstritora a Phe principalmente nas artérias aorta e mesentérica dos animais expostos ao Al em baixa ou alta dose. Esses resultados sugerem uma maior participação de prostanoides derivados da via da

COX-2 na disfunção vascular após exposição ao Al. Corroborando, as aortas de animais expostos ao Al apresentaram um aumento nos níveis de mRNA da COX-2 e dos receptores TXA-2 R. Os receptores TXA-2 R regulam importantes funções na parede vascular como agregação plaquetária, contração muscular e estão relacionados ao desencadeamento de mudanças vasculares na hipertensão (FÉLÉTOU *et al*., 2011; NAKAHATA, 2008). No nosso trabalho, a ativação dos TXA-2 R sugere a sua participação nas alterações vasculares encontradas após exposição ao Al, entretanto estudos adicionais são necessários para um melhor entendimento dessa participação.

CONCLUSÕES

Resultados deste estudo demonstram que a exposição subcrônica ao Al por 60 dias em dose equivalente a exposição humana pela dieta é o suficiente para desencadear efeitos tóxicos no sistema nervoso central e periférico, reprodutor e cardiovascular em ratos. Demonstramos ainda, que os efeitos do Al em nível doméstico de exposição são similares aos encontrados em uma exposição 12 ou 66 vezes maior, sugerindo que uma vez alcançada uma carga corporal tóxica do metal os efeitos advindos da presença corporal do Al são similares e, que o limiar para o desencadeamento da cascata de toxicidade do Al pode ser alcançado em dose de exposição humana ao metal.

Nossos resultados apontam o sistema nervoso, reprodutor e cardiovascular como alvos da toxicidade do Al. Especificamente, a exposição ao Al em dose que mimetiza a contaminação humana ao metal através da dieta promove alterações cognitivas e comportamentais com comprometimento da memória de longa duração e desenvolvimento de catalepsia, induz neuropatia periférica, promove disfunção no sistema reprodutor masculino com redução da qualidade espermática e danos a espermatogênese e, compromete o funcionamento do sistema cardiovascular ao aumentar a pressão arterial sistólica e promover disfunção endotelial com redução da biodisponibilidade de NO.

Nossos resultados demonstram a capacidade do Al em depositar-se em diferentes órgãos, desencadeando assim uma cascata de eventos pró-oxidantes e inflamatórios. Dados deste estudo apontam o aumento do estresse oxidativo com maior liberação/produção de EROs principalmente via NAD(P)H oxidase e ativação da cascata inflamatória do ácido araquidônico, via aumento da expressão gênica da COX-2, como possíveis mecanismos de ação do Al. Estes mecanismos parecem ser responsáveis ao menos parcialmente pelo desencadeamento das alterações sistêmicas observadas após exposição ao metal.

Resultados deste estudo fornecem informações valiosas acerca do risco neurológico, reprodutor e cardiovascular após exposição ao Al e, devem servir de alerta em relação aos níveis atualmente considerados seguros de exposição humana ao metal.

REFERÊNCIAS

ABDEL-MONEIM, A.M. Effects of taurine against histomorphological and ultrastructural changes in the testes of mice exposed to aluminium chloride. **Arh Hig Rada Toksikol** 64: 405-14, 2013.

AITKEN, R.J. Free radicals, lipid peroxidation and sperm function. **Reprod Fertil Dev** 7: 659-668, 1995.

ALDRIDGE, S., DOWNS, A.J. The Group 13 Metals Aluminium, Gallium, Indium and Thallium: Chemical Patterns and Peculiarities. **Ed. John Wiley & Sons Ltd**., 2011.

ÄLVAREZ, Y., BRIONES, A.M., HERNANZ, R., PÉREZ-GIRÓN, J.V., ALONSO, M.J., SALAICES, M. Role of NADPH oxidase and iNOS in vasoconstrictor responses of vessels from hypertensive and normotensive rats. **Br J Pharmacol** 153: 926–935, 2008.

ASSMANN, K.E., ADJIBADE, M., ANDREEVA, V.A., HERCBERG, S., GALAN, P., KESSE-GUYOT, E. Association Between Adherence to the Mediterranean Diet at Midlife and Healthy Aging in a Cohort of French Adults. **J Gerontol A Biol Sci Med Sci** doi: 10.1093/gerona/glx066, 2017.

AVENDAÑO, M.S., MARTÍNEZ-REVELLES, S., AGUADO, A., SIMÕES, M.R., GONZÁLEZ-AMOR, M., PALACIOS, R., GUILLEM-LLOBAT, P., VASSALLO, D.V., VILA, L., GARCÍA-PUIG, J., BELTRÁN, L.M., ALONSO, M.J., CACHOFEIRO, M.V., SALAICES, M., BRIONES, A.M. Role of COX-2-derived PGE2 on vascular stiffness and function in hypertension. **Br J Pharmacol** 173: 1541-55, 2016.

AZEVEDO, F.A., CHASIN, A.A.M. Metais: gerenciamento da toxicidade. São Paulo: Editora Atheneu, 2003.

BAKAR, C., KARAMAN, H., BABA, A., SENGÜNALP, F. Effect of high aluminum concentration in water resources on human health, case study: Biga Peninsula, northwest part of Turkey. **Arch Environ Contam Toxicol** 58: 935-44, 2010.

BANASIK, A., LANKOFF, A., PISKULAK, A., ADAMOWSKA, K., LISOWSKA, H., WOJCIK, A. Aluminum-induced micronuclei and apoptosis in human peripheral-blood lymphocytes treated during different phases of the cell cycle. **Environ Toxicol** 20: 402-406, 2005.

BANSAL, V.K., BANSAL, S. Nervous system disorders in dialysis patients. **Handb Clin Neurol** 119: 395-404, 2014.

BEARDMORE, J., EXLEY, C. Towards a model of non-equilibrium binding of metal ions in biological systems. **J Inorg Biochem** 103: 205-209, 2009.

BEARDMORE, J., RUGG, G., EXLEY, C. A systems biology approach to the blood-aluminium problem: the application and testing of a computational model. **J Inorg Biochem** 101: 1187–1191, 2007.

BHATTACHARJEE, S., ZHAO, Y., HILL, J.M., CULICCHIA, F., KRUCK, T.P., PERCY, M.E., POGUE, A.I., WALTON, J.R., LUKIW, W.J. Selective accumulation of aluminum in cerebral arteries in Alzheimer's disease. **J Inorg Biochem** 126: 35–37, 2013.

BONDY, S.C. Low levels of aluminum can lead to behavioral and morphological changes associated with Alzheimer's disease and age-related neurodegeneration. **Neurotoxicology** 52: 222-229, 2015.

BRASIL, Ministério da Saúde. Estabelece os procedimentos e responsabilidades relativos ao controle e vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Portaria n. 2914/2011. [Publicado no Diário Oficial da União; 2011; Dezembro 12].

BRASIL, Ministério do Desenvolvimento, Indústria e Comércio Exterior. Alumínio: A indústria Brasileira em 2012. Disponível em: <http://www.mdic.gov.br>. Acesso em 1 de novembro de 2017.

CAMPBELL, A., BONDY, S.C. Aluminum induced oxidative events and its relation to inflammation: a role for the metal in Alzheimer's disease. **Cell Mol Biol** (Noisy-le-grand), 46: 721-30, 2000.

CARLSEN, E., GIWERCMAN, A., KEIDING, N., SKAKKEBAEK, N.E. Evidence for decreasing quality of semen during past 50 years. **Br Med J** 305: 609–613, 1992.

CASTELLÓ, M.E., BOLIOLI, B., DAJAS, F. Catalepsy induced by striatal acetylcholinesterase inhibition with fasciculin in rats. **Pharmacol Biochem Behav** 41: 547-50, 1992.

CHEN, H., TAN, C., LIN, Z., WU, T., DIAO, Y. A feasibility study of diagnosing cardiovascular diseases based on blood/urine element analysis and consensus models. **Comput Biol Med** 43: 865-9, 2013.

CHIN-CHAN, M., NAVARRO-YEPES, J., QUINTANILLA-VEGA, B. Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. **Front Cell Neurosci** 9:124. doi: 10.3389/fncel.2015.00124, 2015.

CNI (CONFEDERAÇÃO NACIONAL DA INDÚSTRIA). A sustentabilidade da indústria brasileira do alumínio. Brasília: CNI, 2012.

COCHRAN, M., GODDARD, G., RAMM, G., LUDWIGSON, N., MARSHALL, J., HALLIDAY, J. Absorbed aluminium is found with two cytosolic protein fractions, other than ferritin, in the rat duodenum. **Gut** 34: 643–646, 1993.

COSTELLO, S., BROWN, D.M., NOTH, E.M., CANTLEY, L., SLADE, M.D., TESSIER-SHERMAN, B., HAMMOND, S.K., EISEN, E.A., CULLEN, M.R. Incident ischemic heart disease and recent occupational exposure to particulate matter in an aluminum cohort. **J Expo Sci Environ Epidemiol** 24: 82–88, 2014.

CRAPPER, D.R., KRISHNAN, S.S., DALTON, A.J. Brain aluminum distribution in Alzheimer's disease and experimental neurofibrillary degeneration. **Science** 180: 511–513, 1973.

CRÉPEAUX, G., EIDI, H., DAVID, M.O., BABA-AMER, Y., TZAVARA, E., GIROS, B., AUTHIER, F.J., EXLEY, C., SHAW, C.A., CADUSSEAU, J., GHERARDI, R.K. Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective low dose neurotoxicity. **Toxicology** 375: 48-57, 2017.

DAWSON, E.B., RITTER, S., HARRIS, W.A., EVANS, D.R., POWELL, L.C. Comparison of sperm viability with seminal plasma metal levels. **Biol Trace Elem Res** 64: 215–219, 1998.

DE RYCK, M., SCHALLERT, T., TEITELBAUM, P. Morphine versus haloperidol catalepsy in the rat: A behavioral analysis of postural support mechanisms. **Brain Res** 201: 143–172, 1980.

DIVINE, K.K., LEWIS, J.L., GRANT, P.G., BENCH, G. Quantitative particle-induced X-ray emission imaging of rat olfactory epithelium applied to the permeability of rat epithelium to inhaled aluminum. **Chem Res Toxicol** 12: 575–581, 1999.

EFSA. Safety of aluminium from dietary intake. **EFSA J** 754: 1–34, 2008.

ELLIS, A., BENNETT, D. Neuroinflammation and the generation of neuropathic pain. **Br J Anaesth** 111: 26-37, 2013.

ENNACEUR, A., DELACOUR, J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data **Behav Brain Res** 31: 47-59, 1988.

ERFANI, S., KHAKSARI, M., ORYAN, S., SHAMSAEI, N., ABOUTALEB, N., NIKBAKHT, F., JAMALI-RAEUFY, N., GORJIPOUR, F. Visfatin reduces hippocampal CA1 cells death and improves learning and memory deficits after transient global ischemia/reperfusion. **Neuropeptides** 49: 63-8, 2015.

EVERITT, A.V. Ageing rat colonies at the University of Sydney. **Proc Aust Assoc Gerontol** 26: 79-82, 1991.

EXLEY, C. A molecular mechanism of aluminium-induced Alzheimer's disease? **Journal of Inorganic Biochemistry** 76: 133-140, 1999.

EXLEY, C. Aluminum Should Now Be Considered a Primary Etiological Factor in Alzheimer's Disease. **Journal of Alzheimer's Disease Reports** DOI 10.3233/ADR-170010, 2017.

EXLEY, C. Does antiperspirant use increase the risk of aluminium-related disease, including Alzheimer's disease? **Mol Med Today** 4: 107–109, 1998.

EXLEY, C. Elucidating aluminium´s exposome. **Curr Inorg Chem** 2: 3-7, 2012.

EXLEY, C. Human exposure to aluminium. **Environ Sci Process Impacts** 10: 1807-16, 2013.

EXLEY, C. Reflections upon and recent insight into the mechanism of formation of hydroxyaluminosilicates and the therapeutic potential of silicic acid. **Coordination Chemistry Reviews** 256: 82-88, 2012.

EXLEY, C. The pro-oxidant activity of aluminum. **Free Radic Biol Med** 3: 380- 7, 2004.

EXLEY, C. Why industry propaganda and political interference cannot disguise the inevitable role played by human exposure to aluminum in neurodegenerative diseases, including Alzheimer's disease. **Front Neurol** 27: 5-212. doi: 10.3389/fneur.2014.00212, 2014.

EXLEY, C., BEARDMORE, J., RUGG, G. A computational approach to the blood aluminium problem? **Int J Quantum Chem** 107: 275-278, 2007.

EXLEY, C., BIRCHALL, J.D. The cellular toxicity of aluminium. **J Theoret Biol** 159:83–98, 1992.

EXLEY, C., BURGESS, E., DAY, J.P., JEFFERY, E.H., MELETHIL, S., YOKEL, R.A. Aluminum Toxicokinetics **J Toxicol Environ Health** 6: 569-584, 1996.

EXLEY, C., CHAPPELL, J.S., BIRCHALL, J.D. A mechanism for acute aluminium toxicity in fish. **J Theoret Biol** 151: 417–428, 1991.

EZOMO, O.F., MATSUSHIMA, F., MESHITSUKA, S. Up-regulation in the expression of renin gene by the influence of aluminium. **J Inorg Biochem** 103: 1563–1570, 2009.

FEKETE, V., VANDEVIJVERE, S., BOLLE, F., VAN LOCO, J. Estimation of dietary aluminum exposure of the Belgian adult population: evaluation of contribution of food and kitchenware. **Food Chem Toxicol** 55: 602-8, 2013.

FÉLÉTOU, M., HUANG, Y., VANHOUTTE, P.M. Endothelium mediated control of vascular tone: COX-1 and COX-2 products. **Br J Pharmacol**. 164: 894–912, 2011.

FLAREND, R., BIN, T., ELMORE, D., HEM, S.L. A preliminary study of the dermal absorption of aluminium from antiperspirants using aluminium-26. **Food Chem Toxicol** 39: 163–168, 2001.

FLATEN, T.P. Aluminium as a risk factor in Alzheimer's disease, with emphasis on drinking water. **Brain Res Bull** 2: 187-96, 2001.

FRASER, D.D., MACVICAR, B.A. Cholinergic-dependent plateau potential in hippocampal CA1 pyramidal neurons. **J Neurosci** 16: 4113–4128, 1996.

FROMENT, D.H., MOLITORIS, B.A., BUDDINGTON, B., MILLER, N.L., ALFREY, A.C. Site and mechanism of enhanced gastrointestinal absorption of aluminum by citrate. **Kidney Int** 36: 978–984, 1989.

GENUIS, S.J., BIRKHOLZ, D., RODUSHKIN, I., BEESOON, S. Blood, urine, and sweat (BUS) study: monitoring and elimination of bioaccumulated toxic elements. **Arch Environ Contam Toxicol** 61: 344–357, 2011.

GHERARDI, R.K., COQUET, M., CHERIN, P., BELEC, L., MORETTO, P., DREYFUS, P.A., PELLISSIER, J.F., CHARIOT, P., AUTHIER, F.J. Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. **Brain** 124: 1821–1831, 2001.

GHERARDI, R.K., EIDI, H., CRÉPEAUX, G., AUTHIER, F.J., CADUSSEAU, J. Biopersistence and brain translocation of aluminum adjuvants of vaccines. **Front Neurol** 5: 6 - 4, 2015.

GOMES, M.G., MOREIRA, C.A., MILL, J.G., MASSARONI, L., OLIVEIRA, E.M., STEFANON, I., VASSALLO, D.V. Effects of aluminum on the mechanical and electrical activity of the Langendorff-perfused rat heart. **Braz J Med Biol Res** 27: 95–100, 1994.

GONZALEZ-WELLER, D., GUTIÉRREZ, A.J., RUBIO, C., REVERT, C., HARDISSON, A. Dietary intake of aluminum in a Spanish population (Canary Islands). **J Agric Food Chem** 58: 10452-7, 2010.

GORADEL, N.H., SHOOR, F.G., NEGAHDARI, B., MALEKSHAHI, Z.V., HASHEMZEHI, M., MASOUDIFAR, A., MIRZAEI, H. Stem cell therapy: A new therapeutic option for Cardiovascular Diseases. **J Cell Biochem** doi: 10.1002/jcb.26169, 2017.

GRANADILLO, V.A., TAHAN, J.E., SALGADO, O., ELEJALDE, L.E., RODRIGUEZ-ITURBE, B., ROMERO, G.B., ROMERO, R.A. The influence of the blood levels of lead, aluminum and vanadium upon the arterial hypertension. **Clin Chim Acta** 233: 47–59, 1995.

GREGER, J.L. Aluminum metabolism. **Annu Rev Nutr** 13: 42-63, 1993.

GREGER, J.L., BAIER, M.J. Excretion and retention of low or moderate levels of aluminium by human subjects. **Food Chem. Toxicol** 21: 473–477, 1983.

GUO, C., HUANG, C., CHEN, S., WANG HSU, G. Serum and testicular testosterone and nitric oxide products in aluminum-treated mice. **Environ Toxicol Pharmacol** 10: 53–60, 2001.

GUO, C.H., LU, Y.F., HSU, G.S.W. The influence of aluminum exposure on male reproduction and offspring in mice. **Environ Toxicol Pharmacol** 20: 135–41, 2005.

HALLIWELL, B., GUTTERIDGE, J.M.C. Free Radicals in Biology and Medicine. 3ª ed. Editora Oxford. Estados Unidos, cap.8, p. 592, 1999.

HARRIS, W.R., BERTHON, G., DAY, J.P., EXLEY, C., FLATEN, T.P., FORBES, W.F., KISS, T., ORVIG, C., ZATTA, P.F. Speciation of aluminum in biological systems. **J Toxicol Environ Health** 48: 543-568, 1996.

HERNANZ, R., BRIONES, A.M., SALAICES, M., ALONSO, M.J. New roles for old pathways? A circuitous relationship between reactive oxygen species and cyclooxygenase in hypertension. **Clin Sci (Lond)** 126: 111-21, 2014.

HOVATTA, O., VENÄLÄINEN, E.R., KUUSIMÄKI, L., HEIKKILÄ, J., HIRVI, T., REIMA, I. Aluminium, lead and cadmium concentrations in seminal plasma and spermatozoa, and semen quality in Finnish men. **Hum Reprod** 13: 115–119, 1998.

HSU, C.W., WENG, C.H., LEE, C.C., LIN-TAN, D.T., CHEN, K.H., YEN, T.H., HUANG, W.H. Association of low serum aluminum level with mortality in hemodialysis patients. **Ther Clin Risk Manag** 14: 1417-1424, 2016.

IAI (THE INTERNATIONAL ALUMINIUM INSTITUTE). Global Aluminium Recycling: A Cornerstone of Sustainable Development. Londres: International Aluminium Institute, 2009.

IGE, S.F., AKHIGBE, R.E. The role of Allium cepa on aluminum-induced reproductive dysfunction in experimental male rat models. **J Hum Reprod Sci** 5: 200–205,

JAMALAN, M., GHAFFARI, M.A., HOSEINZADEH, P., HASHEMITABAR, M., ZEINALI, M. Human sperm quality and metal toxicants: protective effects of someflavonoids on male reproductive function. **Int J Fertil Steril** 10: 215–223, 2016.

JOHNSON, V.J., SHARMA, R.P. Aluminum disrupts the pro-inflammatory cytokine/neurotrophin balance in primary brain rotation-mediated aggregate cultures: possible role in neurodegeneration. **Neurotoxicol** 24: 261–268, 2003.

KASBE, P., JANGRA, A., LAHKAR, M. Mangiferin ameliorates aluminium chloride-induced cognitive dysfunction via alleviation of hippocampal oxido-nitrosative stress, proinflammatory cytokines and acetylcholinesterase level. **J Trace Elem Med Biol** 31: 107-12, 2015.

KHACHATURIAN, Z.S. Calcium hypothesis of Alzheimer's disease and brain aging. **Ann N Y Acad Sci** 747: 1-11, 1994.

KHAN, Z., COMBADIÈRE, C., AUTHIER, F.J., ITIER, V., LUX, F., EXLEY, C., MAHROUF-YORGOV, M., DECROUY, X., MORETTO, P., TILLEMENT, O., GHERARDI, R.K., CADUSSEAU, J. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. **BMC Med** 11: 99. doi: 10.1186/1741-7015-11-99, 2013.

KIHIRA, T., YOSHIDA, S., KOMOTO, J., WAKAYAMA, I., YASE, Y. Aluminum-induced model of motor neuron degeneration: subperineurial injection of aluminum in rabbits. **Neurotoxicol** 16: 413-24, 1995.

KING, S.J., TEMPLAR, J., MILLER, R.V., DAY, J.P., DOBSON, C.B., ITZHAKI, R.F., FIFIELD, L.K., ALLAN, G.L. Aluminium and Alzheimer's disease: Sites of aluminium binding in human neuroblastoma cells determined using 26Al and accelerator mass spectrometry. **Nucl. Instr. Methods Phys Res** B92: 469–472, 1994.

KISTANOVA, E., MARCHEV, Y., NEDEVA, R., KACHEVA, D., SHUMKOV, K., GEORGIEV, B. et al. Effect of the Spirulina platensis included in the main diet on the boar sperm quality. **Biotechnology in Animal Husbandry** 25: 547–57, 2009.

KLEIN, J.P., MOLD, M., MERY, L., COTTIER, M., EXLEY, C. Aluminum content of humansemen: implications for semen quality. **Reprod Toxicol** 50: 43–48, 2014.

KONIOR, A., SCHRAMM, A., CZESNIKIEWICZ-GUZIK, M., GUZIK, T.J. NADPH oxidases in vascular pathology. **Antioxid Redox Signal** 20: 2794-814, 2014.

KORCHAZHKINA, O., WRIGHT, G., EXLEY, C. Action of Al-ATP on the isolated working rat heart. **J Inorg Biochem** 69: 153-8, 1998.

KORCHAZHKINA, O., WRIGHT, G., EXLEY, C. No effect of aluminium upon the hydrolysis of ATP in the coronary circulation of the isolated working rat heart. **J Inorg Biochem** 76: 121–126, 1999.

KUMAR, S. Aluminium-induced biphasic effect. **Medical Hypotheses** 52: 557–559, 1999.

KUMAR, V., GILL, K.D. Oxidative stress and mitochondrial dysfunction in aluminium neurotoxicity and its amelioration: a review. **Neurotoxicol** 41: 154-66, 2014.

LAKSHMI, B.V., SUDHAKAR, M., PRAKASH, K.S. Protective effect of selenium against aluminum chloride-induced Alzheimer's disease: behavioral and biochemical alterations in rats. **Biol Trace Elem Res** 1: 67-74, 2015.

LEE, M., CHO, S., ROH, K., CHAE, J., PARK, J.H., PARK, J., LEE, M.A., KIM, J., AUH, C.K., YEOM, C.H., LEE, S. Glutathione alleviated peripheral neuropathy in oxaliplatin-treated mice by removing aluminum from dorsal root ganglia. **Am J Transl Res** 15: 926-939, 2017.

LEVINE, H., JØRGENSEN, N., MARTINO-ANDRADE, A., MENDIOLA, J., WEKSLER-DERRI, D., MINDLIS, I., PINOTTI, R., SWAN, S.H. Temporal trends in sperm count: a systematic review and meta-regression analysis. **Human Reprod Update** 23: 646-659, 2017.

LI, X., HU, C., ZHU, Y., SUN, H., LI, Y., ZHANG, Z. Effects of aluminum exposure on bone mineral density, mineral, and trace elements in rats. **Biol Trace Elem Res** 143: 378-385, 2011.

LIND, P.M., OLSEN, L., LIND, L. Circulating levels of metals are related to carotid atherosclerosis in elderly. **Sci Total Environ** 416: 80-8, 2012.

LINHART, C., TALASZ, H., MORANDI, E.M., EXLEY, C., LINDNER, H.H., TAUCHER, S., EGLE, D., HUBALEK, M., CONCIN, N., ULMER, H. Use of Underarm Cosmetic Products in Relation to Risk of Breast Cancer: A Case-Control Study. **EBioMedicine** pii: S2352-3964(17) 30233-5. doi: 10.1016/j.ebiom.2017.06.005, 2017.

LOTE, C.J., WOOD, J.A., SAUNDERS, H.C., SPENCER, A.J. Aluminium deposition in liver following aluminium citrate or chloride administration in conscious rats. **J Physiol Lond** 459: 374P, 1993.

LUKIW, W.J., PERCY, M.E., KRUCK, T.P. Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture. **J Inorg Biochem** 99: 1895–1898, 2005.

MANDRIOTA, S.J. A Case-control Study Adds a New Piece to the Aluminium/Breast Cancer Puzzle. **EBioMedicine** 22: 22-23, 2017.

MANDRIOTA, S.J., TENAN, M., FERRARI, P., SAPPINO, A.P. Aluminium chloride promotes tumorigenesis and metastasis in normal murine mammary gland epithelial cells. **Int J Cancer** 139: 2781-2790, 2016.

MARTÍNEZ-REVELLES, S., AVENDAÑO, M.S., GARCÍA-REDONDO, A.B., ALVAREZ, Y., AGUADO, A., PÉREZ-GIRÓN, J.V., GARCÍA-REDONDO, L., ESTEBAN, V., REDONDO, J.M., ALONSO, M.J., BRIONES, A.M., SALAICES, M. Reciprocal relationship between reactive oxygen species and cyclooxygenase-2 and vascular dysfunction in hypertension. **Antioxid Redox Signaling** 18: 51–65, 2013.

MINAMI, T., TOHNO, S., UTSUMI, M., MORIWAKE, Y., YAMADA, M.O., TOHNO, Y. Selective accumulations of aluminum in five human arteries. **Biol Trace Elem Res** 79: 29–38, 2001.

MIRZA, A., KING, A., TROAKES, C., EXLEY, C. Aluminium in brain tissue in familial Alzheimer's disease. **J Trace Elem Med Biol** 40: 30-36, 2017.

MOHAMMAD, N.S., ARAFA, M.H., ATTEIA, H.H. Coenzyme Q10 and fish oil synergistically alleviate aluminum chloride-induced suppression of testicular steroidogenesis and antioxidant defense. **Free Radic Res** 49: 1319–1334, 2015.

NAKAHATA, N. Thromboxane A2: physiology/ pathophysiology, cellular signal transduction and pharmacology. **Pharmacol Ther** 118: 18–35, 2008.

NELSON, C.M., BUNGE, R.G. Semen analysis: evidence for changing parameters of male fertility potential. **Fertil Steril** 25: 503–507, 1974.

NEOPHYTOU, A.M., NOTH, E.M., LIU, S., COSTELLO, S., HAMMOND, S.K., CULLEN, M.R., EISEN, E.A. Ischemic heart disease incidence in relation to fine versus total particulate matter exposure in a U.S. aluminum industry cohort. **PLoS One** 11 (6): e0156613, 2016.

NICHOLSON, J.W., CZARNECKA, B. Review paper: Role of aluminum in glass-ionomer dental cements and its biological effects. **J Biomater Appl** 24: 293–308, 2009.

NICHOLSON, S., EXLEY. C. Aluminum: a potential pro-oxidant in sunscreens/sunblocks? **Free Radical Biol Med** 43: 1216–1217, 2007.

NOREMBERG, S., BOHRER, D., SCHETINGER, M.R., BAIRROS, A.V., GUTIERRES, J., GONÇALVES, J.F., VEIGA, M., SANTOS, F.W. Silicon Reverses Lipid Peroxidation but not Acetylcholinesterase Activity Induced by Long-Term Exposure to Low Aluminum Levels in Rat Brain Regions. **Biol Trace Elem Res** 169: 77-85, 2016.

ODA, S.S. The influence of Omega3 fatty acids supplementation against aluminum-induced toxicity in male albino rats. **Environ Sci Pollut Res Int** 23: 14354–14361, 2016.

OECD (ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT). Aluminium factsheet. In: Material Resources, Productivity and the Environment. Mechelen: OECD Publishing, 2015.

PARK, J.H., CHAE, J., ROH, K., KIL, E.J., LEE, M., AUH, C.K., LEE, M.A., YEOM, C.H., LEE, S. Oxaliplatin-Induced Peripheral Neuropathy via TRPA1 Stimulation in Mice Dorsal Root Ganglion Is Correlated with Aluminum Accumulation. **PLoS ONE** 10(4): e0124875. doi:10.1371/journal.pone.0124875, 2015.

PERL, D.P., FOGARTY, U., HARPAZ, N., SACHAR, D.B. Bacterial-metal interactions: the potential role of aluminum and other trace elements in the etiology of Crohn's disease. **Inflammatory Bowel Dis** 10: 881–883, 2004.

PERL, D.P., GOOD, P.F. Uptake of aluminium into central nervous system along nasal-olfactory pathways. **Lancet** 1: 1028, 1987.

PINEAU, A., GUILLARD, O., FAVREAU, F., MARRAULD, A., FAUCONNEAU, B. In vitro study of percutaneous absorption of aluminum from antiperspirants through human skin in the Franz™ diffusion cell. **J Inorg Biochem** 110: 21–26, 2012.

POWELL, J.J., AINLEY, C.C., EVANS, R., THOMPSON, R.P.H. Intestinal perfusion of dietary levels of aluminium: Association with the mucosa. **Gut** 35: 1053–1057, 1994.

POWELL, J.J., JUGDAOHSINGH, R., THOMPSON, R.P. The regulation of mineral absorption in the gastrointestinal tract. **Proc Nutr Soc** 58: 147–153, 1999.

PRAKASH, A., KUMAR, A. Effect of N-acetyl cysteine against aluminium-induced cognitive dysfunction and oxidative damage in rats. **Basic Clin Pharmacol Toxicol** 2: 98-104, 2009.

PRAKASH, A., KUMAR, A. Mitoprotective effect of Centella asiatica against aluminum-induced neurotoxicity in rats: possible relevance to its anti-oxidant and anti-apoptosis mechanism. **Neurol Sci** 8: 1403-9, 2013.

QIU, C., KIVIPELTO, M., VON, STRAUSS, E. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. **Dialogues Clin Neurosci** 11: 111–128, 2009.

REAGAN-SHAW, S., NIHAL, M., AHMAD, N. Dose translation from animal to human studies revisited. **FASEB J** 3: 659-61, 2008.

REINKE, C.M., BREITKREUTZ, J., LEUENBERGER, H. Aluminium in over-the-counter drugs: risks outweigh benefits? **Drug Saf** 14: 1011-25, 2003.

RIIHIMÄKI, V., AITIO, A. Occupational exposure to aluminum and its biomonitoring in perspective. **Crit Rev Toxicol** 42: 827– 853, 2012.

ROMANSKI, S. A., MCCARTHY, J.T., KLUGE, K., FITZPATRICK, L.A. Detection of subtle aluminum- related renal osteodystrophy. **Mayo Clin Proc** 68: 419–426, 1993.

RUIPÉREZ, F., MUJIKA, J.I., UGALDE, J.M., EXLEY, C., LOPEZ, X. Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II). **J Inorg Biochem** 117: 118-23, 2012.

SANDIREDDY, R., YERRA, V.G., ARETI, A., KOMIRISHETTY, P., KUMAR, A. Neuroinflammation and oxidative stress in diabetic neuropathy: futuristic strategies based on these targets. **Int J Endocrinol** doi: 10.1155/2014/674987, 2014.

SAPPINO, A.P., BUSER, R., LESNE, L., GIMELLI, S., BÉNA, F., BELIN, D., MANDRIOTA, S.J. Aluminium chloride promotes anchorage-independent growth in human mammary epithelial cells. **J Appl Toxicol** 32: 233-243, 2012.

SASS, J.B., ANG, L.C. JUURLINK, B.H. Aluminum pretreatment impairs the ability of astrocytes to protect neurons from glutamate mediated toxicity. **Brain Res** 621: 207–214, 1993.

SCHMIDT, P.M., ESCOBAR, A.G., TORRES, J.G.D., MARTINEZ, C.S., RIZZETTI, D.A., KUNZ, S.N., VASSALLO, D.V., ALONSO, M.J., PEÇANHA, F.M., WIGGERS, G.A. Aluminum exposure for one hour decreases vascular reactivity in conductance and resistance arteries in rats. **Toxicol Appl Pharmacol** 313: 109–118: 2016.

SCHRAMM, A., MATUSIK, P., OSMENDA, G., GUZIK, T.J. Targeting NADPH oxidases in vascular pharmacology. **Vascul Pharmacol** 56: 216-31, 2012.

SHEINER, E.K., SHEINER, E., HAMMEL, R.D., POTASHNIK, G., CAREL, R. Effect of occupational exposures on male fertility: literature review. **Ind Health** 41: 55–62, 2003.

SKLAN, E.H., BERSON, A., BIRIKH, K.R., GUTNICK, A., SHAHAR, O., SHOHAM, S., SOREQ, H. Acetylcholinesterase modulates stress-induced motor responses through catalytic and noncatalytic properties. **Biol Psychiatry** 60: 741-51, 2006.

SPENCER, A. J., WOOD, J. A., FREEMAN, M. S., SAUNDERS, H. C., LOTE, C. J. Deposition of aluminium in kidney following aluminium chloride or citrate administration in conscious rats. **J Physiol London** 483: 171–172, 1995.

SUBRAHMANYAM, G., PATHAPATI, R.M., RAMALINGAM, K., INDIRA, S.A., KANTHA, K., SOREN, B. Arterial stiffness and trace elements in apparently healthy population- a cross sectional study. **J Clin Diagn Res** 10: LC12–LC15, 2016.

SUN, H,. HU, C., JIA, L., ZHU, Y., ZHAO, H., SHAO, B., WANG, N., ZHANG, Z., LI, Y. Effects of aluminum exposure on serum sex hormones and androgen receptor expression in male rats. **Biol Trace Elem Res** 144: 1050–1058, 2011.

TOMLJENOVIC, L, SHAW, C.A. Do aluminum vaccine adjuvants contribute to the rising prevalence of autism? **J Inorg Biochem** 105: 1489–1499, 2011.

TURNER, T.T., LYSIAK, J.J. Oxidative stress: a common factor in testicular dysfunction. Review. **J Androl** 29: 488-98, 2008.

WALKER, V.R., SUTTON, R.A.L., MEIRAV, O., SOSSI, V., JOHNSON, R., KLEIN, J., FINK, D., MIDDLETON, R. Tissue disposition of 26Al in rats measured by accelerator mass spectrometry. **Clin Invest Med** 17: 420–425, 1994.

WALTON, J.R. A longitudinal study of rats chronically exposed to aluminum at human dietary levels. **Neurosci Lett** 1: 29-33, 2007.

WALTON, J.R. Chronic aluminum intake causes Alzheimer's disease: applying Sir Austin Bradford Hill's causality criteria. **J Alzheimers Dis** 4: 765-838, 2014.

WANG, H., YE, M., YU, L., WANG, J., GUO, Y., LEI, W., YANG, J. Hippocampal neuronal cyclooxygenase-2 downstream signaling imbalance in a rat model of chronic aluminium gluconate administration. **Behav Brain Funct** 18: 11-8, 2015.

WANG, Z., WEI, X., YANG, J., SUO, J., CHEN, J., LIU, X., ZHAO, X. Chronic exposure to aluminum and risk of Alzheimer's disease: A meta-analysis. **Neurosci Lett** 610: 200-6, 2016.

WHITEHOUSE, P.J., PRICE, D.L., STRUBLE, R.G., CLARK, A.W., COYLE, J.T., DELON, M.R. Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain. **Science** 215: 1237–1239, 1982.

WIMO, A., PRINCE, M. World Alzheimer Report 2010: The Global Economic Impact of Dementia. **Alzheimer's Disease International.** http://www. alz.co.uk/research/world-report-2010, 2010.

WORLD HEALTH ORGANIZATION. Safety Evaluation of Certain Food Additives and Contaminants. Food Additive Series: 58: http://whqlibdoc.who.int/trs/WHO TRS 940 eng.pdf, 2007.

XU, Z.X., TANG, J.P., BADR, M., MELETHIL, S. Kinetics of aluminum in rats III: Effect of route of administration. **J Pharm Sci** 81: 160–163, 1992.

YANAGISHITA, T., TAMADA, Y., OHSHIMA, Y., ITO, K., AKITA, Y., WATANABE, D. Histological localization of aluminum in topical aluminum chloride treatment for palmar hyperhidrosis. **J Dermatol Sci** 67: 69-71, 2012

YANG, M., JIANG, L., HUANG, H., ZENG, S., QIU, F., YU, M., LI, X., WEI, S. Dietary exposure to aluminium and health risk assessment in the residents of Shenzhen, China. **PLoS One** 9(3): e89715, 2014.

YOKEL, R.A., ACKRILL, P., BURGESS, E., DAY, J.P., DOMINGO, J.L., FLATEN, T.P., SAVORY, J. Prevention and treatment of aluminum toxicity including chelation therapy: Status and research needs**. J Toxicol Environ Health** 48: 667–683, 1996.

YU, L., ZHAI, Q., YIN, R., LI, P., TIAN, F., LIU, X., ZHAO, J., GONG, J., ZHANG, H., CHEN, W. Lactobacillus plantarum CCFM639 Alleviate Trace Element Imbalance-Related Oxidative Stress in Liver and Kidney of Chronic Aluminum Exposure Mice. **Biol Trace Elem Res**. 176: 342-349, 2016.

ZAFFE, D., BERTOLDI, C., CONSOLO, U. Accumulation of aluminium in lamellar bone after implantation of titanium plates, Ti-6Al-4V screws, hydroxyapatite granules. **Biomaterials** 25: 3837–3844, 2004.

ZHANG, Q., CAO, Z., SUN, X., ZUANG, C., HUANG, W., LI, Y. Aluminum trichloride induces hypertension and disturbs the function of erythrocyte membrane in male rats. **Biol Trace Elem Res** 171: 116–123, 2016.

ZHU, Y.Z., SUN, H., FU, Y., WANG, J., SONG, M., LI, M., LI, Y.F., MIAO, L.G. Effects of sub-chronic aluminum chloride on spermatogenesis and testicular enzymatic activity in male rats. **Life Sci** 102: 36–40, 2014.

ZOU, M.H. Peroxynitrite and protein tyrosine nitration of prostacyclin synthase. **Prostaglandins Other Lipid Mediat** 82: 119–127, 2007.

ANEXO I – Certificado de aprovação de protocolo na CEUA / UNIPAMPA.

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **028/2014**

Título: **Efeitos da exposição crônica ao cloreto de alumínio (AlCl3) sobre parâmetros cardiovasculares, reprodutores e bioquímicos de ratos**

Data da aprovação: **31/10/2014**

Período de vigência do projeto: De: **10/2014** Até: **10/2017**

Pesquisador: **GIULIA ALESSANDRA WIGGERS PEÇANHA**

Campus: **URUGUAIANA**

Telefone: **(55) 9914-7174**

E-mail: **giullawp@gmail.com**

Digitally signed by ALESSANDRA SAYURI KIKUCHI TAMAJUSUKU
NEIS:98256009004
DN: cn=ALESSANDRA SAYURI KIKUCHI TAMAJUSUKU NEIS:98256009004, c=BR, o=ICP-Brasil, ou=RFB e-CPF A3, email=alessandratamajusuku@unipampa.edu.br

Professor Adjunto
Coordenadora da CEUA/UNIPAMPA