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Gisele Gomes de Andrade

EFEITO DO ENVELHECIMENTO SOBRE AS VESÍCULAS EXTRACELULARES CIRCULANTES E CENTRAIS

Porto Alegre

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Dissertação apresentada ao Programa de Pós Graduação em Ciências Biológicas: Farmacologia e Terapêutica, da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas.

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Dedicatória

Dedico essa dissertação aos meus pais Marilene Gomes de Andrade e a Aldo Gomes de Andrade, pelo amor e apoio incondicional durante toda a minha vida.

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

José de Alencar

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À Universidade Federal do Rio Grande do Sul, ao programa de Pós Graduação em Ciências Biológicas: Farmacologia e Terapêutica pela oportunidade de realização do Mestrado.

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Lista de Abreviaturas

AChE Acetilcolinestrease

BHE Barreira hematoencefálica

BHL Barreira hematoliquórico

DNA Ácido Desoxirribonucleico

IL-4 Interleucina-4

IL-6 Interleucina-6

IL-1β Interleucina-1beta

LCR Liquido cefalorraquidiano

LPS Lipopolissacarídeo

miRNA microRNA

NTA Análise de Rastreamento de Nanopartículas

RNA Ácido ribonucleico

SNC Sistema Nervoso Central

TNF-α Fator de Necrose tumoral-α

TGF-β Fator de transformação do crescimento-β

VEs Vesículas Extracelulares

Resumo

O envelhecimento é um processo complexo caracterizado pelo declínio progressivo de diferentes tecidos e sistemas, acarretando susceptibilidades ao desenvolvimento de doenças neurodegenerativas que estão diretamente associadas ao avanço da idade. O objetivo desse estudo foi investigar o perfil exossomal e a carga inflamatória de vesículas extracelulares em plasma e líquido cefalorraquidiano (LCR) no processo de envelhecimento. Para tanto foram usados ratos machos Wistar de 3 e 21 meses de idade (n = 12). O LCR e o plasma desses animais foram coletados e o isolamento de vesículas extracelulares foi realizado usando um kit comercial e a concentração de proteína total, a atividade de acetilcolinesterase (AChE), os níveis de CD63 e interleucina-1 beta (IL-1β) foram avaliados. Os resultados obtidos mostraram que os níveis circulantes de IL-1β foram significativamente menores nas vesículas extracelulares em comparação com jovens adultos, enquanto que, o envelhecimento não alterou os níveis de IL-1β no LCR; houve uma redução dos níveis de CD63, um marcador exossomal, em plasma de ratos envelhecidos, enquanto que um aumento relacionado à idade nos níveis de CD63 no LCR foi observado; e houve um aumento da atividade de AChE em plasma e LCR do grupo envelhecido. Nossos dados sugerem que o aumento dos níveis de IL-1β nas vesículas extracelulares circulantes pode ser associado, pelo menos em parte, a condições inflamatórias relacionadas ao envelhecimento e a alteração nas vesículas extracelulares do LCR, analisada pelos níveis de CD63, em ratos envelhecidos podem estar envolvidos na susceptibilidade a distúrbios neurodegenerativos.

Palavras-chave: Envelhecimento; vesículas extracelulares; inflamação; líquido cefalorraquidiano; plasma

Abstract

Aging is a complex process characterized by the progressive decline of different tissues and systems, increasing the susceptibility to neurodegenerative diseases. The aim of this study was to investigate exosomal markers and inflammatory cargo of extracellular vesicles obtained from cerebrospinal fluid and plasma in aging process. Male Wistar rats of 3-month-old and 21-month-old were used (n= 12). The cerebrospinal fluid and plasma of animals were collected and isolation of extracellular vesicles was performed using a commercial kit. Total protein concentration, acetylcholinesterase **(**AChE) activity, CD63, an exosomal marker, and interleukin-1β (IL-1β) levels were evaluated. The results obtained showed a decrease in circulating IL-1β levels in extracellular vesicles in aged rats, whereas, aging did not alter exosomal IL-1β levels in cerebrospinal fluid; an age-related increase was observed in CD63 levels in extracellular vesicles from cerebrospinal fluid, while its content was decreased in plasma extracellular vesicles of aged group; and there was an increase in AChE activity in plasma and cerebrospinal fluid of the aged group. Our data suggest that IL-1β levels in circulating extracellular vesicles can be linked at least in part to aging-related inflammatory conditions and a disruption on cerebrospinal fluid, evaluated by CD63 levels, in aged rats can be involved to susceptibility to neurodegenerative disorders.

Keywords: aging; extracellular vesicles; inflammation; cerebrospinal fluid, plasma

Introdução

O envelhecimento é um processo dinâmico e progressivo, caracterizado por alterações morfológicas, funcionais, bioquímicas e psicológicas que culminam com a redução da funcionalidade e maior vulnerabilidade e incidência de doenças. Entre as modificações associadas ao envelhecimento, destaca-se o declínio das funções motoras e cognitivas (Jurgens & Johnson, 2010; Minciullo et al., 2015; Paradies et al., 2011). Neste contexto, o envelhecimento é amplamente reconhecido como um fator de risco no desenvolvimento de várias patologias como doenças neurodegenerativas, cardiovasculares, diabetes e câncer, as quais representam as principais causas de morte nos idosos (Ballard et al., 2011; Xu et al, 2013).

O prejuízo cognitivo associado ao envelhecimento está relacionado, entre outros fatores, a níveis elevados de citocinas inflamatórias tanto em roedores quanto em humanos (Griffin et al., 2006; Kohman et al., 2011; Speisman et al., 2012). O processo inflamatório crônico e progressivo no envelhecimento também é chamado de "inflammaging", além de uma alteração das funções do sistema imune, denominada imunossenescência (Franceschi et al. 2000a; Fulop et al., 2015; Panickar & Jewell, 2015).

Alterações nos níveis de citocinas pró e anti-inflamatórias já foram descritas durante o processo de envelhecimento. Por exemplo, um estudo realizado pelo nosso grupo observou menores níveis de interleucina-4 (IL-4), uma citocina anti-inflamatória, em hipocampos de ratos Wistar de 20 meses de idade, os quais apresentaram também níveis elevados de citocinas inflamatórias, IL-1β e fator de necrose tumoral-α (TNF-α) (Lovatel et al., 2013). Além disso, alguns autores demonstraram que os níveis séricos de fator de transformação do crescimento-β (TGF-β), o qual possui um papel essencial na

manutenção da homeostase da resposta imune, estavam diminuídos em indivíduos com idade avançada (Gorelik & Flavell, 2002; Lin et al., 2009).

É interessante comentar que evidências epidemiológicas sugerem que homens e mulheres envelhecidos e de meia-idade com altos níveis circulantes de mediadores inflamatórios, como IL-1β e Interleucina-6 (IL-6), apresentam maior risco de desenvolvimento de resistência à insulina e de diabetes (Barzilay et al.,2001; Han et al.,2002; Pradhan et al.,2003; Pradhan et al.,2001). É amplamente conhecido que o envelhecimento, independente da obesidade, pode aumentar o risco de doenças metabólicas crônicas que envolvam vias inflamatórias (Licastro et al.,2005; Stout et al.,2014; Tchkonia et al.,2010).

Embora as vesículas extracelulares tenham sido recentemente relacionadas a eventos inflamatórios, do nosso conhecimento, o envolvimento do conteúdo dessas vesículas no processo do envelhecimento nunca foi estudado.

As vesículas extracelulares incluem os exossomos, microvesículas e outras estruturas vesiculares liberadas pelas células (Figura 1).

Os exossomos são pequenas vesículas originadas pela invaginação da membrana de corpos multivesiculares, são mantidas em corpos multivesiculares e secretadas para o meio extracelular. São vesículas, medindo entre 30 e 1000 nm, que contém o perfil das células de origem, incluindo RNA (Ácido ribonucleico), DNA (Ácido Desoxirribonucleico) e microRNAs (miRNA), e proteínas (Andaloussi et al.,2013; Mizrak et a., 2013; Valadi et al., 2007). Já as microvesículas são vesículas maiores produzidas pela evaginação da membrana plasmática (Figura 1).

Há descrição de produção e liberação de vesículas extracelulares, tanto por células saudáveis, quanto por células relacionadas a doenças, como células tumorais de diferentes tecidos; sendo reconhecidas como importantes mediadores na comunicação

intercelular pela transferência de sua carga entre células, tanto localmente como sistemicamente (Akers et al.,2013; Skog et al., 2008).

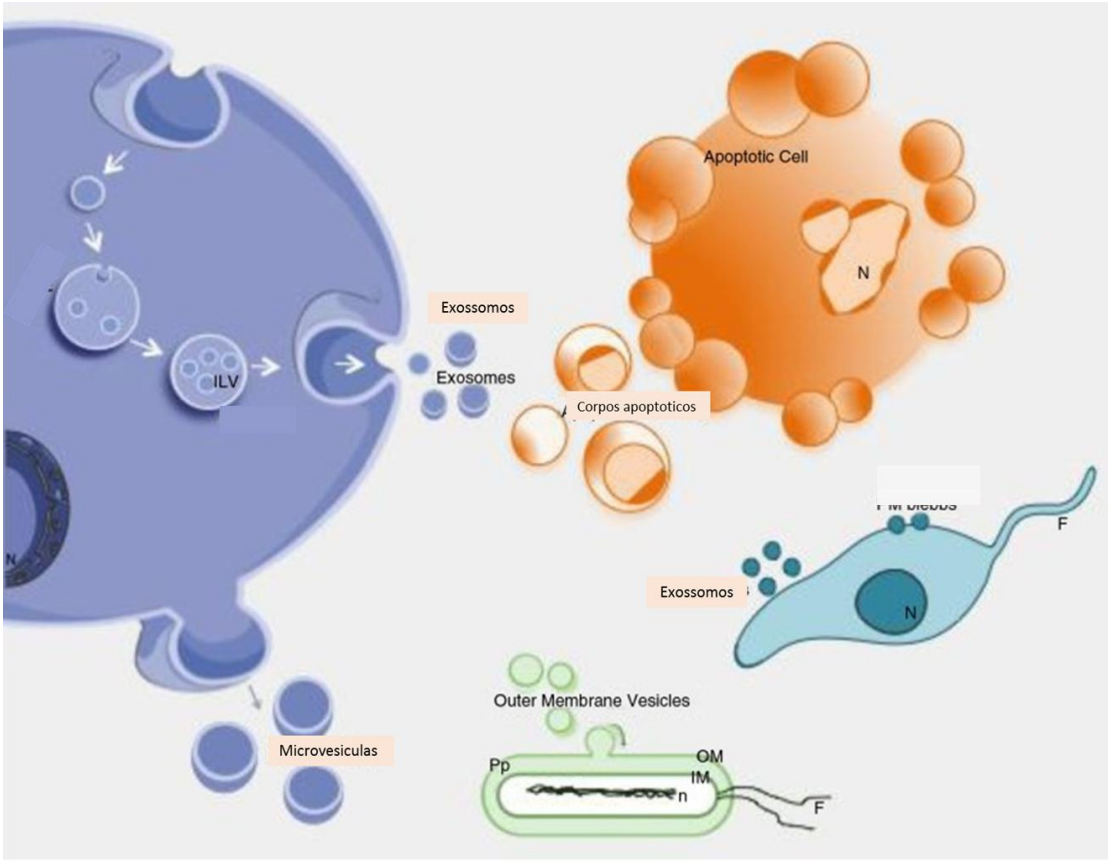

Figura 1 – Biogênese e liberação de vesículas extracelulares (Adaptada de Yanez-Mo M, et al,2015). Os exossomos originados pela invaginação da membrana de corpos multivesiculares, mantidas em corpos multivesiculares e secretadas para o meio extracelular. As microvesículas são vesículas maiores produzidas pela evaginação da membrana plasmática.

As vesículas extracelulares foram identificadas em diferentes fluidos corporais. Embora não haja um amplo consenso sobre algumas características, por exemplo, o tamanho, o que dificulta a determinação do tipo específico de vesícula isolada de cultura de células e amostras de fluidos corporais (Lotvall et al.,2014) por métodos que observem a distribuição de tamanho das partículas em amostras em suspensão líquida, como a Análise de Rastreamento de Nanopartículas (NTA).

Foi descrito que as vesículas extracelulares, como os exossomos, podem carrear citocinas especialmente a IL-1β, assim como outros componentes que regulam a sinalização de vias inflamatórias e a própria produção de IL-1β (Figura 2) (Haneklaus et al. 2013; Vaccari et al., 2016, Buzas et al., 2014). Boilard e colegas (2010) demonstraram que as vesículas extracelulares derivadas de plaquetas carregam IL-1β no líquido sinovial de pacientes com artrite reumatóide e essas vesículas induzem a secreção de IL-8 por fibroblastos sinoviais, contribuindo para a inflamação das articulações.

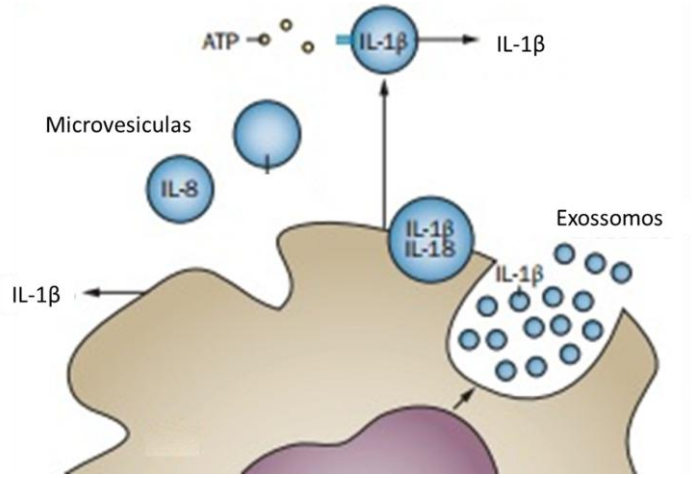

Figura 2 – Associação das citocinas inflamatórias com as vesículas extracelulares (Adaptada de Buzas et. al, 2014). As vesículas extracelulares, microvesículas e exossomos, envolvidos na liberação de citocinas, como a IL-1β.

Ainda, Smith e colegas (2014) sugerem que as vesículas extracelulares desempenham papéis críticos em uma variedade de doenças crônicas relacionadas com idade, incluindo doenças neurodegenerativas (como por exemplo, doença de Alzheimer), doenças metabólicas, e doença cardiovasculares. No entanto, apesar de alguns estudos demonstrarem um papel das vesículas extracelulares na fisiopatologia de

doenças relacionadas à idade, poucos estudos investigaram o papel das vesículas extracelulares durante o processo de envelhecimento fisiológico.

No entanto, diferentes tipos de vesículas extracelulares possuem em sua estrutura tipos distintos de proteínas e outros componentes. As tetraspaninas, como o CD63 e o CD81 (Figura 3), são amplamente utilizadas como marcadores de exossomos. Além disso, alguns autores sugerem a enzima AChE como marcadora de exossomos (Bellingham et al.,2012; Perez-Gonzalez et al.,2012; Vaccari et al.,2015). Nosso grupo demonstrou, recentemente, que o processo de envelhecimento alterou o perfil de exossomos circulantes, especificamente em soro de ratos Wistar, com uma redução dos níveis de CD63, um marcador exossomal, e um aumento da atividade da AChE. Nesse trabalho, foi demonstrado também um aumento nos níveis de espécies reativas e uma redução da atividade da enzima antioxidante, superóxido dismutase, nas vesículas extracelulares obtidas de soro de ratos envelhecidos, comparados aos animais adultos jovens (Bertoldi et al., 2017).

Eitan e colaboradores (2017) descreveram que o envelhecimento altera o tamanho e a quantidade de exossomos circulantes em humanos. Ainda, descrevem que houve uma maior internalização dos exossomos por células B. Ainda em modelos *in vitro*, Mitsuhashi e colaboradores (2013) demonstraram que o envelhecimento aumentou a liberação exossomal de RNAm de citocinas, como a IL-6 e o TNF-alfa, por macrófagos estimulados pelo peptídeo B-amilóide. Assim, há evidências de que o envelhecimento possa alterar o perfil periférico das vesículas exossomais, incluindo o conteúdo de moléculas inflamatórias.

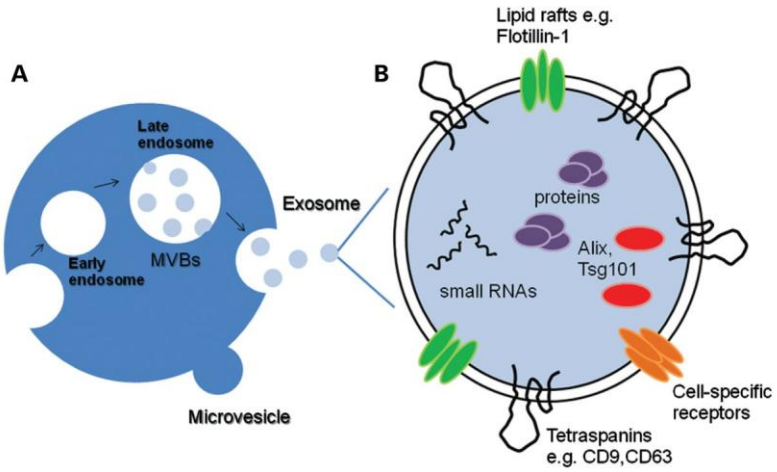

Figura 3- Exossomos e suas proteínas de superfície, como as tetraespaninas, CD9 e CD63, marcadores internos, como Alix e Tsg101, outras proteínas e microRNAs (Lee et al., 2012).

Gupta e Pulliam (2014) sugerem que os exossomos circulantes, assim como as células mononucleares, podem atravessar a barreira hematoencefálica e alterar a função de células no SNC, como os neurônios, e que os exossomos derivados de monócitos/macrófagos podem ter um papel de "cavalo de tróia". Segundo os autores, esses exossomos que atravessariam a barreira hematoencefálica podem ser internalizados por neurônios, alterando inclusive a liberação exossomal destas células (Alvarez-Erviti et al.,2011).

Alguns autores sugerem uma comunicação bidirecional entre o sistema imune periférico e o SNC (Wrona, 2006; Deleidi et al., 2015). Alguns autores assumiram que as vesículas extracelulares de células periféricas podem atravessar a barreira hematoencefálica e distribuir sua carga no SNC e *vice-versa* (Balusu et al., 2016; Lopez-Ramirez et al., 2013). Shi e colegas (2014) mostraram que a α-sinucleína

radiomarcada pelo LCR foi facilmente transportada para o sangue com envolvimento dos exossomos.

Nos últimos anos, a Barreira Hematoencefálica (BHE) também tem sido alvo de diversos estudos para compreender o seu papel em doenças inflamatórias e neurodegenerativas (Vandenbroucke et al, 2012; Brkic et al, 2015; Demeestere et al., 2015; Gorle et al., 2016). A BHE, localizada nos capilares do cérebro, sendo uma estrutura que atua protegendo o cérebro de passagens de substancias do sangue para o SNC, como fatores de coagulação e anticorpos, que podem interferir na neurotransmissão (Reese et al.,1967).

Além da BHE, a Barreira Hematoliquórico (BHL) é formada por uma camada de células epiteliais no plexo coroide, com uma função importante de controlar a entrada de substancias no LCR (Davson et al.,1987). O LCR desempenha diferentes funções, entre as reguladoras estão a de distribuição de fatores neurotróficos, estabilização do pH do cérebro e gradientes químicos, além de fornecer uma via excretora do SNC para solutos que não podem atravessar facilmente a barreira (Lehtinen et al, 2011). Recentemente, Balusu e colegas (2016) demonstraram que um modelo de inflamação sistêmica induzido por lipopolissacarídeo (LPS) é capaz de aumentar o número de vesículas extracelulares no líquido cefalorraquidiano de camundongos.

Considerando que estudos avaliando o papel das vesículas extracelulares no processo inflamatório associado ao envelhecimento fisiológico são escassos, nossa hipótese de trabalho é que os marcadores de exossomos obtidos de líquido cefalorraquidiano e plasma e sua carga inflamatória são alterados, de forma diferente, pelo processo de envelhecimento.

1. Objetivos

1.1 Objetivo Geral

- Investigar o perfil exossomal e a carga inflamatória de vesículas extracelulares em plasma e líquido cefalorraquidiano no processo de envelhecimento de ratos Wistar.

1.2 Objetivos Específicos

- Estudar o perfil exossomal, através do marcador de exossomos CD63, conteúdo de proteínas totais e a atividade da AChE, em plasma e líquido cefalorraquidiano de ratos Wistar de 3 e 21 meses.

- Quantificar a interleucina pró-inflamatória, IL-1β, em vesículas extracelulares extraídas de plasma e líquido cefalorraquidiano de ratos Wistar de 3 e 21 meses.

2. Artigo

Neuroimmunomodulation

Aging process alters IL-1β and CD63 levels differently in extracellular vesicles obtained from plasma and CSF

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Running Head: Aging process alters IL-1β and CD63 in extracellular vesicles

Keywords: aging; extracellular vesicles; inflammation; cerebrospinal fluid, plasma

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Abstract

Objective(s): The aim of this study was to investigate exosomal markers and inflammatory cargo of extracellular vesicles obtained from cerebrospinal fluid and plasma in aging process. Moreover, we also studied the inflammatory cargo quantifying IL-1β levels. **Methods**: Male Wistar rats of 3-month-old and 21-month-old were used (n= 12 group). The cerebrospinal fluid and plasma of animals were collected and isolation of extracellular vesicles was performed using a commercial kit. Total protein concentration, acetylcholinesterase (AChE) activity, CD63 and IL-1β levels were evaluated. **Results**: AChE activity in extracellular vesicles was increased in both samples, specifically circulating and cerebrospinal fluid, of aged group. An age-related increase was observed in CD63 levels in extracellular vesicles from cerebrospinal fluid, while its content was decreased in plasma extracellular vesicles of aged group. Student's t-test showed that aged rats had significant higher circulating IL-1β levels in extracellular vesicles compared to young adult, without any effect on central content. **Conclusion**: Our data suggest that the normal aging process can change differently central and circulating profiles of extracellular vesicles; increased IL-1β levels in circulating extracellular vesicles can be linked at least in part to aging-related inflammatory conditions and a disruption on cerebrospinal fluid exosomes, evaluated by CD63 levels, in aged rats can be involved to susceptibility to neurodegenerative disorders.

Introduction

A chronic inflammatory state has been described during the physiological aging process which has been called as "inflammaging" [1,2,3,4] characterized by increased pro-inflammatory cytokines levels including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) [5].

The contribution of extracellular vesicles (EVs) to peripheral inflammation has been recently suggested [6]. These vesicles, such as exosomes, carry a specific cargo of proteins, lipids and nucleic acids and are currently consider one of most complex and physiologically relevant messengers between cells [7,6]. Boilard and colleagues (2010) demonstrated that platelet-derived EVs carry IL-1β in synovial fluid from patients with rheumatoid arthritis. Interestingly, these vesicles induce IL-8 secretion by synovial fibroblasts, contributing to joint inflammation [8]. However, to our knowledge, there are no reports evaluating exosomal inflammatory cytokines in aging process.

Although a growing body of evidence shows that peripheral inflammation induces the production of brain cytokines [9] with a critical role in the induction of inflammatory disease-related sickness symptoms [10,11] and that healthy aging can change circulating EVs profile in rodents, impacting serum CD63 levels, an exosomal marker, and acetylcholinesterase (AChE) activity [12] there is a lack of studies comparing the impact of aging process on exosomal pro-inflammatory cargo in plasma and cerebrospinal fluid (CSF).

It is important to consider that the role of EVS, including exosomes, in inflammatory cross-talk between the periphery and central nervous system (CNS) remains unclear. Several authors have assumed that EVs from peripheral cells can cross the blood-brain barrier (BBB) and distribute their cargo into the CNS and *vice-versa* [13, 14]. For

example, Shi and colleagues (2014) showed that CSF radiolabeled α-synuclein was readily transported to blood from mouse with a modest involvement of exosomes [15].

Our working hypothesis was that exosome markers and their inflammatory cargo are altered differently in CSF and circulating extracellular vesicles by the aging process. Thus, the aim of this study was to investigate the exosomal profile, specifically protein concentration, CD63 content and AChE activity in plasma and CSF from 3 and 21-months-old Wistar rats. Moreover, we also quantified their inflammatory cargo by IL-1β levels.

1. Material and methods

1.1. Animals

Male Wistar rats of different ages, 3-month-old and 21-month-old were used (n=12). The animals were provided by the Centro de Reprodução Animal de Laboratório (CREAL) and maintained under standard conditions (12-h light/dark, 22 ± 2 ºC) with food and water *ad libitum*. The Local Ethics Committee (CEUA – Comissão de Ética no Uso de Animais – UFRGS; nr.29818) approved all animals procedures and experimental conditions.

1.2. Samples collection

Rats were anesthetized for CSF collection with a mixture of ketamine and xylazine (75 and 10 mg/kg, respectively). The animals were decapitated and the trunk blood was collected and the samples containing plasma were stored at -80°C for further analysis.

1.3. Exosome isolation

The isolation was performed using a commercial kit based on vesicles precipitation (ExoQuick^{TM}– System Bioscience) following the manufacturer's instructions. Samples were thawed on ice and centrifuged at 3000xg for 15 min at 4°C. The supernatant was collected and then incubated with ExoQuick^{TM} for 60 minutes at 4°C. The mixture of ExoQuick^{TM}/samples was centrifuged twice at 1,500xg for 30 and 5 minutes, respectively, in order to remove the supernatant. The remained pellet was resuspended in PBS and store at -20°C. In order to evaluate CD63 and IL-1β levels, the exosomes were lysate with specific detergent, Triton X-100, at a final concentration of 0.1% [20].

1.4. IL-1β levels

The levels of IL-1β were determined using Rat ELISA Assay kits (Colorimetric Detection, Catalog Number: DY501, R&D Systems, USA) according to the manufacturer's instructions. Briefly, 96-well plate was coated with capture antibody overnight. The plate was then blocked for non-specific binding using Reagent Diluent. The exosomes and standards curve were incubated with the detection antibody. The plate was incubated with Streptavidin-HRP followed by Substrate Solution. The Stop Solution was added and the absorbance was measured on a microplate reader (450 nm). The cytokines levels were expressed as pg/ml.

1.5. CD63 levels

The CD63 levels were measured using a specific kit (ExoELISA kit, System Biosciences) following the manufacturer's instructions. In a 96-well plate, 50 µl of prepared protein standards or sample were added and incubated overnight at 4°C. After incubation, 50 µl of the specific primary antibody CD63 was added and incubate at

room temperature for 1 hour followed by incubation with secondary antibody. Finally, 50 µl of super-sensitive TMB ELISA substrate was added and the absorbance was read at 450 nm. The CD63 levels were expressed in exosomes particles/mg protein.

1.6. Acetylcholinesterase (AChE) activity

The AChE activity was evaluated by slight modifications in the colorimetric method described by Ellman and co-workers using acetylthiocholine iodide as a substrate. The hydrolysis rate of acetylthiocholine iodide (Sigma, USA) was measured at 412 nm through the release of the thiol compound that reacts with 5, 5-dithiobis-(2-nitrobenzoic acid) DTNB producing the color-forming compound TNB. The AChE activity was normalized for total protein content.

1.7. Quantification of total proteins

Protein was measured by the Coomassie blue method using bovine serum albumin as standard (Bradford, 1976).

1.8. Statistical analysis

For data analysis we used GraphPad Prism v.6. Statistical analysis was performed using Student's t-test. Differences with p-values less than 0.05 were considered significant.

2. Results

2.1 Effect of aging process on CSF extracellular vesicles profile

CSF obtained of young adult rats had significant higher total protein content in extracellular vesicles compared to aged ones (Student's t-test; p=0.005, Fig. 1a). There

was no significant difference between IL-1β levels in CSF extracellular vesicles obtained of young adult and aged rats (Student's t-test, p= 0.16; Fig. 1b).

Increased CD63 levels, an exosomal marker, was found in aged CSF compared to young adult ones (Student's t-test; $p<0.05$, Fig. 1c). AChE activity was raised in aged group compared to young adult in CSF extracellular vesicles (Student's t-test, $p<0.001$; Fig.1d).

2.2 Effect of aging process on plasma extracellular vesicles profile

There was no significant difference between the total protein levels in plasma extracellular vesicles obtained of young adult and aged rats (Student's t-test, p= 0.29; Fig. 2a). Student's t-test showed differences between IL-1β levels in plasma extracellular vesicles comparing the tested ages. Young adult rats had significant higher IL-1β levels compared to aged ones (Student's t-test; p=0.04, Fig. 2b).

Aged rats had decreased CD63 levels, an exosomal marker, in plasma (Student's t-test; $p<0.05$, Fig. 2c). There was increased plasma exosomal AChE activity in aged groups compared to young adult (Student's t-test; p=0.014, Fig.2d).

Discussion

The present study supports the idea that extracellular vesicles profile is affected by the aging process. To our knowledge, this is the first study evaluating the impact of healthy aging process on central and circulating extracellular vesicles, respectively obtained from CSF and plasma, in a rodent model.

Previously reports propose that exosomes can carry cytokines such as IL-1β and inflammasome components, for example in synovial fluid from patients with

rheumatoid arthritis [8]. Consistent with these data, our work was able to detect IL-1β in both tested samples, extracellular vesicles obtained of CSF and plasma.

Interestingly, reduced IL-1β levels of extracellular vesicles isolated from plasma were found in aged rats. It is possible to suggest that our data can be related at least in part to systemic inflammation widely described in aging process, as well to susceptibility to age-related diseases, as atherosclerosis and diabetes [16,17,18]. Additionally, systemic inflammation is involved in the pathogenesis of endothelial dysfunction, leading to structural and functional changes in the endothelium [19,20]. Although exosomes can be uptake via endocytosis in several tissues, our data can indicate increases on free IL-1β instead of package into exosomes which could indicate different biological functions.

It has been recognized that circulating exosomes and their cargos can be critically involved in cardiovascular pathophysiology, such as cardiomyocyte hypertrophy, apoptosis, and angiogenesis. Moreover, these exosomes may undergo changes in both number and cargo, contributing to the pathogenesis of cardiovascular diseases [21]. Interestingly, IL-1β degradation occurs more slowly in serum of aged when compared to young and middle-aged animals [22]. Our finding can be related to those about degradation of IL-1β in aging process. ~~Taken that higher levels of exosomal IL-1β were found in plasma of aged rats,~~ It is possible to suggest that IL-1β would be protected from degradation and consequently exosomes and their inflammatory cargo could be uptake through endocytosis in several tissues [23, 10]. Besides, we can infer that exosomal IL-1β would increase the "inflammaging" process.

At this moment, it is impossible to determine the cellular origin of extracellular vesicles here observed. However, Li and colleagues (2017) reported higher expression of

proinflammatory cytokines, IL-1β and TNF-α, in the aorta of old rats indicating as a central molecular mechanism responsible for endothelial dysfunction in aging [24].

It is interesting to note that higher IL-1β levels of CSF exosomes could be expected. However, McLay and colleagues (2000) found that passage of IL-1β across the BBB was significantly decreased in old mice as compared with young or middle-aged animals [22] that can be related to a physiological adaptation avoiding an exacerbation of inflammation in CNS with additional IL-1β from the peripheral circulation.

Besides, our work opened new perspectives; conditions with compromised blood-CSF-barrier allowing leakage blood-brain can exacerbate inflammatory status in brain due IL-1β transport across the BBB. Considering that BBB remains intact in aging only with slight changes in the passage of specific molecules [5], we could suggest the involvement of exosomes with this selectivity.

Other remarkable data is that AChE activity of CSF exosomes increased in aging process, as well in circulating ones, it is reasonable to suppose that neural secreted exosome can carry the AChE, indicating peripheral and central cholinergic system loss in aged animals. Besides, although AChE has been used as exosomal marker [2] our results agree with our previous work [12], suggesting that this parameter is not adequate to study exosomes/EVs in aging process.

In addition, it was possible to observe a decreased CD63 levels, an exosomal marker, in plasma, while in CSF samples was increased in aged compared to young adult group. Thus, it is possible to suggest that the aging process changes the profile of EVs in different body fluids. Our finding about plasma CD63 levels agrees with our previous report, since aging altered the serum CD63 levels [12], corroborating the hypothesis that there is a decrease in circulating exosomes in aging process.

Exosomes play an intercellular communication network in the brain and based on previous studies, it has been hypothesized that this network represents the substrate for the spread in the CNS of pathogenic proteins that cause neurodegenerative diseases [14, 25]. Therefore, Laulagnier and colleagues (2017) showed that neuronal exosomes can actually act as vehicles for the intercellular transport of Amyloid precursor protein (APP) and its catabolites [26]. Taken that CD63 levels were centrally increased, it is possible to infer that exosomes and their toxic cargo are less able to cross the BBB to be removed.

In conclusion, our data suggest that the normal aging process can change differently the central and circulating profiles of extracellular vesicles; altered IL-1β in circulating extracellular vesicles can be linked at least in part to aging-related inflammatory conditions and a disruption on CSF exosomes, evaluated by CD63 levels, in aged rats can be involved to susceptibility to neurodegenerative disorders.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1A - Total protein content in CSF extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, p=0.005 (n=12).

Figure 1B - IL-1β levels in CSF extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD, Student's t-test, p= 0.16 (n=12).

Figure 1C - CD63 levels in CSF extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, $p<0.05$ (n=12).

Figure 1D - AChE activity in CSF extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, $p<0.001$ (n=12).

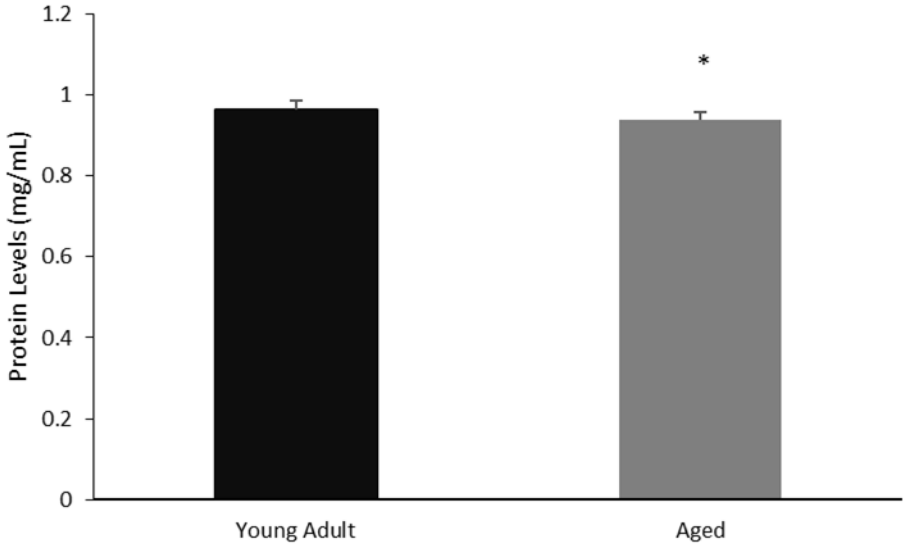
Figure 2A - Total protein content in Plasma extracellular vesicles from young adult and aged rats. Extracellular vesicles isolated from Plasma. The columns represent the mean ± SD, Student's t-test, p= 0.29 (n=12).

Figure 2B - IL-1β levels in Plasma extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, p=0.04 (n=12).

Figure 2C - CD63 levels in Plasma extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, $p<0.05$ (n=12).

Figure 2D - AChE activity in Plasma extracellular vesicles from young adult and aged rats. The columns represent the mean ± SD. Student's t-test; *significant differently from young adult, p=0.014.

Figure 1A



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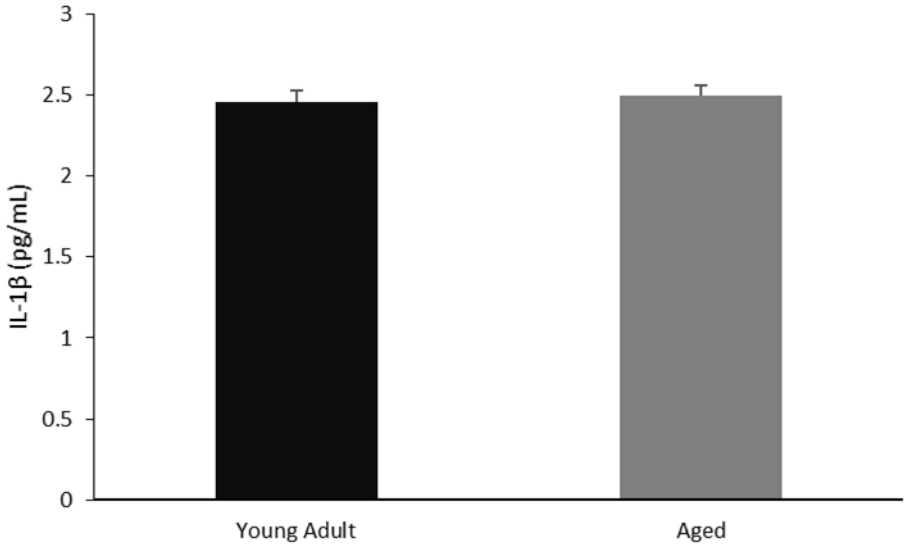

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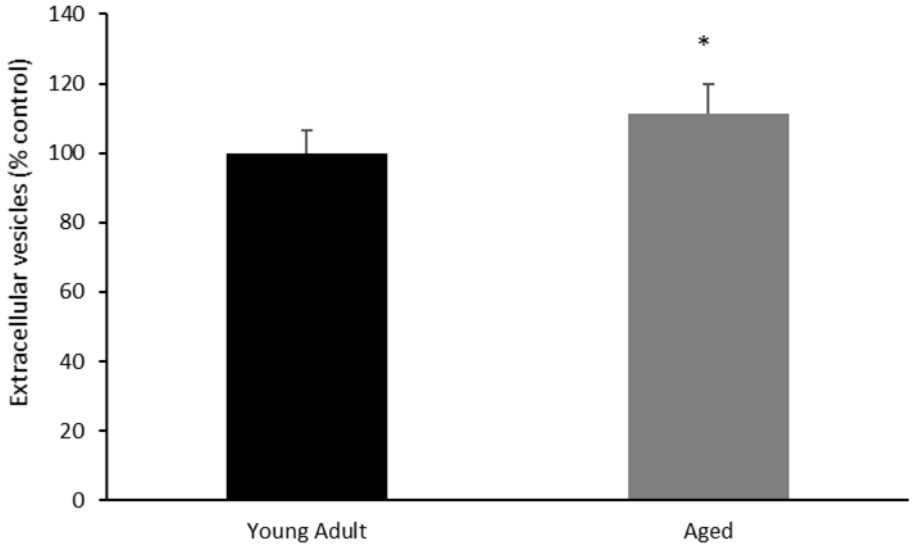

Extracellular vesicles (% control)
140
120
100
80
60
40
20
0
*
Young Adult
Aged

Figure 1D

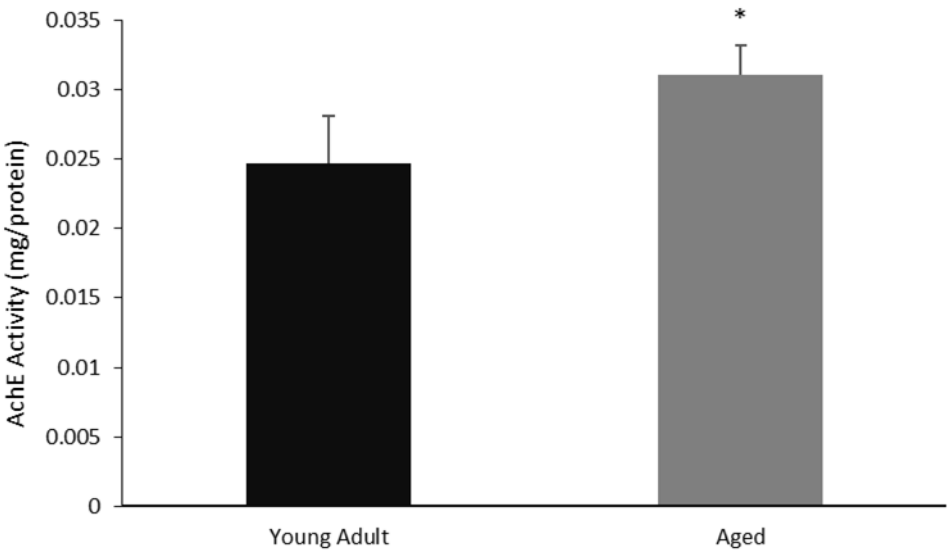

0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
AchE Activity (mg/protein)
*
Young Adult
Aged

Figure 2A

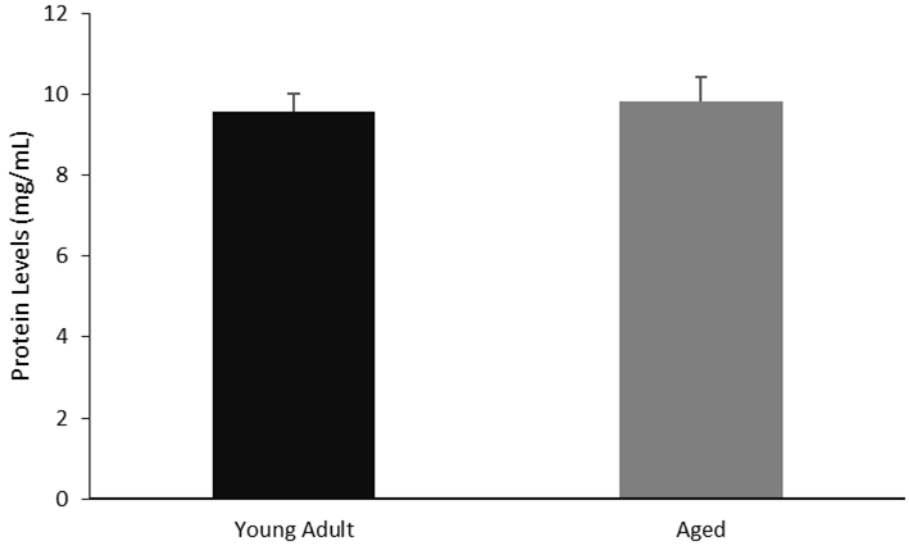


Figura 2B

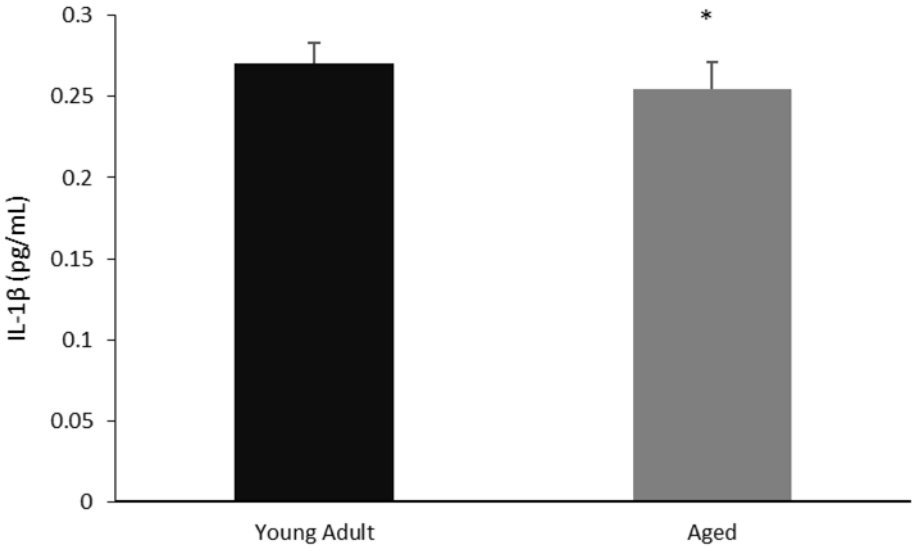


Figura 2C

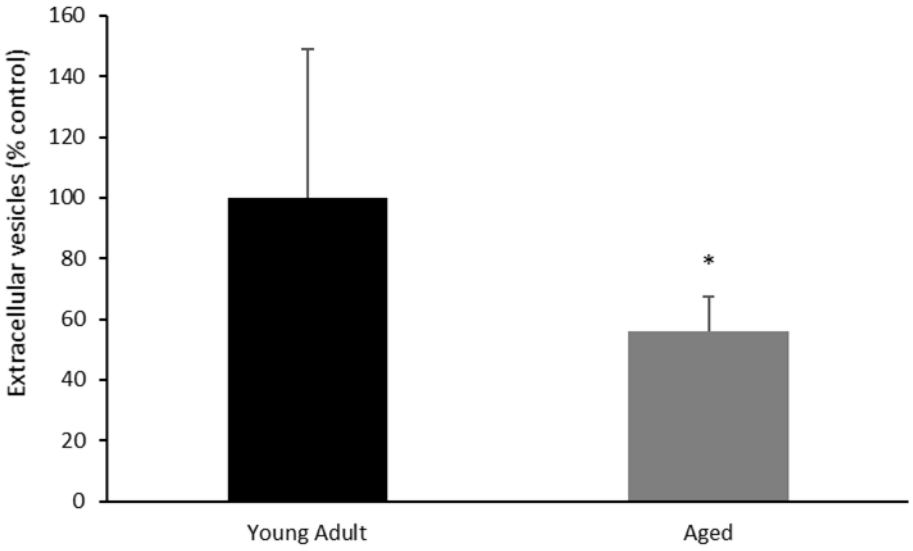
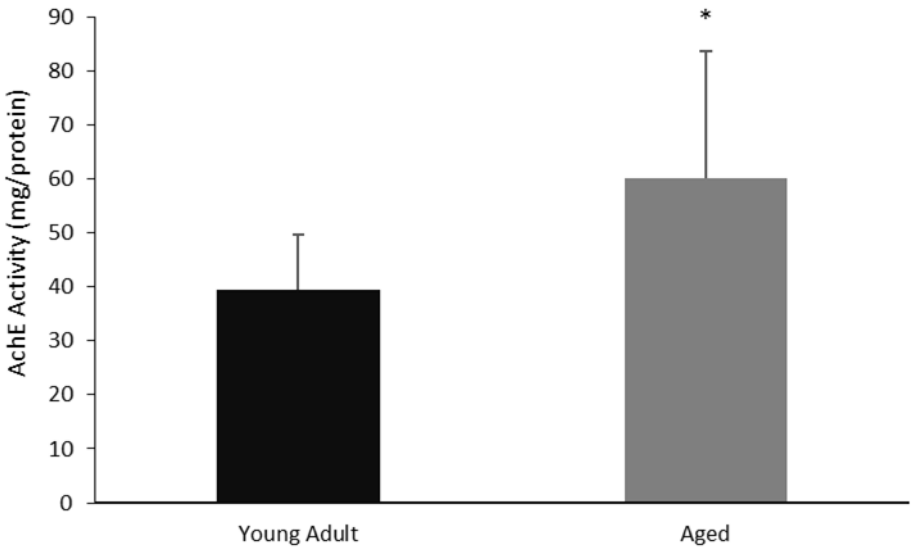

Extracellular vesicles (% control)
160
140
120
100
80
60
40
20
0
*
Young Adult
Aged

Figura 2D


90
80
70
60
50
40
30
20
10
0
AchE Activity (mg/protein)
*
Young Adult
Aged

3. Discussão e Conclusões

Neste trabalho, comparamos o efeito do envelhecimento sobre o perfil exossomal e a carga inflamatória de vesículas extracelulares em plasma e líquido cefalorraquidiano utilizando ratos Wistar.

O envelhecimento normal pode mudar de forma diferente os perfis centrais e circulantes das vesículas extracelulares, uma vez que observamos:

- níveis circulantes de IL-1β significativamente menores nas vesículas extracelulares em comparação com jovens adultos, enquanto que, o envelhecimento não alterou os níveis de IL-1β no líquido cefalorraquidiano;

- uma redução dos níveis de CD63, um marcador exossomal, em plasma de ratos envelhecidos (concordando com os dados anteriores do grupo com soro de ratos Wistar), enquanto que houve um aumento relacionado à idade nos níveis de CD63 no líquido cefalorraquidiano;

Nossos dados sugerem que, no envelhecimento, ocorre uma redução na passagem de vesículas extracelulares pela barreira hematoencefálica, uma vez que: (1) o impacto do envelhecimento sobre IL-1β em vesículas, perifericamente observado, não foi encontrado no líquido cefalorraquidiano, podendo refletir uma alteração adaptativa, protegendo o SNC da inflamação periférica; e (2) o aumento nos níveis de CD63 no líquido cefalorraquidiano pode representar que os exossomos produzidos centralmente, inclusive com moléculas a serem depuradas, estão “aprisionados” no SNC.

Ainda, nossos resultados indicam um aumento da atividade de acetilcolinesterase tanto em plasma (concordando com nossos dados de soro), quanto no líquido cefalorraquidiano do grupo envelhecido.

Podemos concluir que os níveis de IL-1β nas vesículas extracelulares circulantes em animais envelhecidos podem estar associados, pelo menos em parte, a condições

inflamatórias, ainda que, uma alteração nas vesículas extracelulares do líquido cefalorraquidiano, avaliada pelos níveis de CD63, em ratos envelhecidos podem estar envolvidos na susceptibilidade a doenças neurodegenerativas.

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