UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

AVALIAÇÃO DO EFEITO DA RUTINA EM UM MODELO *IN VITRO* DE PRIVAÇÃO DE OXIGÊNIO E GLICOSE

Aline Matté

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Orientadora: Profª Drª Christianne Gazzana Salbego

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“É preciso que eu suporte duas ou três larvas

se quiser conhecer as borboletas”

Antoine de Saint-Exupéry

“Aprender é a única coisa de que a mente

nunca se cansa, nunca tem medo e

nunca se arrepende”

Leonardo da Vinci

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A minha querida irmã Cris, pela atenção, carinho, paciência, preocupação e enorme ajuda.

Índice

APRESENTAÇÃO

Esta dissertação está organizada em seções dispostas da seguinte forma: introdução, objetivos, artigo científico a ser submetido, discussão, conclusões, perspectivas, referências bibliográficas e anexo.

A **introdução** apresenta o embasamento teórico, que nos levou a formular a proposta de trabalho.

O **objetivo geral e os objetivos específicos** estão dispostos no corpo da dissertação.

Os métodos utilizados e resultados obtidos, assim como as referências bibliográficas específicas, encontram-se no tópico resultados, o qual está apresentado na forma de **artigo científico** a ser submetido à publicação. Esse trabalho foi desenvolvido no Laboratório de Neuroproteção e Sinalização Celular do Departamento de Bioquímica-ICBS-UFRGS, em colaboração com o grupo coordenado pela professora Drª. Cristiane Matté, da mesma Instituição.

A **discussão** contém uma interpretação geral dos resultados obtidos no trabalho.

A seção **conclusões** aborda as conclusões gerais obtidas.

A seção **perspectivas** aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos.

A seção **referências bibliográficas** lista as referências utilizadas na introdução e discussão da dissertação.

RESUMO

A isquemia cerebral está entre as principais causas de mortalidade e morbidade em países desenvolvidos e, apesar dos constantes estudos, ainda não existe um tratamento realmente eficaz para a recuperação tecidual e funcional do cérebro após o episódio isquêmico. Frente a isso, nossos objetivos foram investigar o efeito neuroprotetor da rutina, um flavonoide que apresenta como principais propriedades as atividades antioxidante e hipolipidêmica, e avaliar os possíveis mecanismos de ação em um modelo *in vitro* de isquemia cerebral em culturas organotípicas de hipocampo de ratos. O tratamento com rutina diminuiu significativamente a morte celular induzida pela privação de oxigênio e glicose. Nas culturas submetidas à isquemia foi observado um aumento na produção de espécies reativas, que foi diminuída com o tratamento com rutina no período de recuperação de 24 horas. Não observamos nenhuma alteração significativa na massa mitocondrial e no potencial de membrana mitocondrial. A análise por *Western blotting* revelou que a neuroproteção exercida pela rutina está associada à modulação da via de sobrevivência PI3K/Akt e um de seus substratos, a GSK-3β. Em conjunto, nossos resultados sugerem um efeito neuroprotetor promissor da rutina contra os danos causados pela privação de oxigênio e glicose, dando suporte à ideia de que esse flavonoide poderia ser considerado como uma estratégia terapêutica no tratamento dos danos causados pela isquemia cerebral.

ABSTRACT

Cerebral ischemia is one of the major causes of mortality and morbidity in developed countries and, in spite of constant studies, there is still no accurately effective treatment for tissue and functional recovery of the brain after the ischemic episode. In view of this, our objectives were to investigate the neuroprotective effect of rutin, a flavonoid that presents antioxidant and hypolipidemic activities, on an *in vitro* model of cerebral ischemia, performed in organotypic cultures of rat hippocampus. We also verified some of the possible mechanisms of rutin action. Treatment with rutin significantly decreased cell death induced by oxygen and glucose deprivation. In cultures subjected to ischemia, it was observed an increase in production of reactive species, which was decreased by rutin treatment in the recovery period of 24 hours. No significant alterations were observed in mitochondrial mass and mitochondrial membrane potential. Analysis by Western blotting revealed that the neuroprotection exerted by rutin is associated with modulation of the PI3K/Akt pathway for survival and its substrates GSK-3β. In conjunction, our results suggest that rutin has promising neuroprotective effect against damage caused by deprivation of oxygen and glucose, supporting the idea that this flavonoid could be considered as a therapeutic strategy in the treatment of damage caused by cerebral ischemia.

LISTA DE ABREVIATURAS

4-VO: oclusão dos 4 vasos

Akt/PKB: proteína cinase B

AVC: acidente vascular cerebral

AVE: acidente vascular encefálico

BAD: promotor de morte associado a Bcl-2

CA1: corno de Ammon 1 (**C**ornus **A**mmonis)

CA3: corno de Ammon 3 (**C**ornus **A**mmonis)

DG: giro denteado (**D**entate **G**yrus)

ERN: espécies reativas de nitrogênio

EROs: espécies reativas de oxigênio

FDA: Food and Drug Administration

FKHR: fator de transcrição do receptor Fas

GSK-3β: glicogênio sintase cinase 3 beta

MAPK: proteína cinase ativada por mitógenos

MCA: artéria cerebral média

mTOR: proteína alvo da rapamicina em mamíferos

PDK1 e PDK2: proteínas cinases dependentes de fosfoinositóis 1 e 2

PKA: proteína cinase A

PKC: proteína cinase C

PI: iodeto de propídio

PI3K: fosfatidilinositol 3-cinase

PIP_2: fosfatidilinositol bifosfato

PIP_3: fosfatidilinositol trifosfato

POG: privação de oxigênio e glicose

Rut: rutina

SNC: sistema nervoso central

TBARS: substâncias reativas ao ácido tiobarbitúrico

tPA: ativador de plasminogênio tecidual

1. INTRODUÇÃO

1.1. Isquemia Cerebral

A isquemia cerebral, conhecida clinicamente como acidente vascular encefálico (AVE) e popularmente como derrame, é a segunda causa de mortalidade e a que mais leva à invalidez em todo o mundo (Donnan, Fisher, Macleod et al., 2008). No Brasil, a doença está em primeiro lugar no ranking geral de óbitos e incapacidades, gerando grande impacto econômico e social. De acordo com dados do Ministério da Saúde, em 2010 foram registrados quase 100 mil óbitos por acidente vascular cerebral (AVC) (Ministério da Saúde, 2012).

Essa doença está intimamente ligada a vários fatores de risco como a hipertensão, hipercolesterolemia, o diabetes e o tabagismo. Os sintomas clínicos mais comuns são a perda de força muscular de um lado do corpo, problemas de visão e de fala, desvio da boca para um lado do rosto, sensação de formigamento no braço, dores de cabeça súbita ou intensa sem causa conhecida, tonturas e perda de equilíbrio ou coordenação (Ministério da Saúde, 2012; American Stroke Association, 2013).

A isquemia cerebral é caracterizada por uma redução grave ou por um bloqueio do fluxo sanguíneo ao cérebro. Esse órgão é altamente dependente do fluxo sanguíneo contínuo para o suprimento de oxigênio e glicose, sendo mais vulnerável ao dano isquêmico do que os outros tecidos. Isto porque a bioenergética cerebral normal possui características especiais, que incluem uma taxa metabólica alta, reservas energéticas limitadas e uma grande dependência do metabolismo aeróbico da glicose. Além disso, o cérebro possui uma capacidade limitada de reparo a determinados danos, fazendo com que mudanças mínimas na disponibilização de oxigênio e de glicose a esse órgão possam afetar gravemente sua função, ocasionando alterações

bioquímicas e moleculares (Dirnagl, Iadecola e Moskowitz, 1999; Lipton, 1999; Rodrigo, Fernandez, Serrano et al., 2005).

A isquemia focal, o tipo mais frequente de isquemia cerebral em humanos, é definida como uma interrupção do fluxo sanguíneo para uma parte do cérebro. O outro tipo é a isquemia global, que resulta da interrupção transitória do fluxo sanguíneo para todo o cérebro, como ocorre durante uma parada cardíaca, por exemplo. Além dos danos causados pela falta de oxigênio e metabólitos durante a isquemia, a volta da circulação sanguínea pode aumentar ainda mais a morte neuronal, especificamente nas áreas mais vulneráveis do cérebro, como a região do Corno de Ammon 1 (CA1) do hipocampo (Schmidt-Kastner e Freund, 1991; Taylor, Obrenovitch e Symon, 1996; Lipton, 1999).

Apesar dos constantes estudos que nos levam a compreender melhor o complexo funcionamento fisiológico e patológico do cérebro, assim como os mecanismos associados à morte celular cerebral na isquemia, existem até o momento poucas opções farmacológicas tanto para prevenir como para tratar a isquemia cerebral. Agentes antiplaquetários e anticoagulantes são utilizados como medida profilática em indivíduos com fatores de risco para AVC. Além disso, o tratamento utilizado na prática clínica, e o único aprovado pelo *Food and Drug Administration* (FDA), é a terapia trombolítica com a administração da alteplase ou ativador de plasminogênio tecidual (tPA). Entretanto, esse tratamento possui diversas limitações, como o tempo de administração ao paciente que deverá ser no máximo 3 horas após a isquemia (e até 4,5 horas em alguns pacientes elegíveis) e apenas 5% dos pacientes são aptos a recebê-la (Donnan, Fisher, Macleod et al., 2008; Hall, Leonardo, Collier et al., 2009; American Stroke Association, 2013).

Para o desenvolvimento de terapias clinicamente efetivas e consequente diminuição da morte cerebral é essencial o entendimento dos eventos moleculares

associados à morte celular desencadeada pela isquemia, bem como daqueles envolvidos nas estratégias celulares de sobrevivência a estímulos nocivos.

1.1.1. Eventos envolvidos na morte celular após a isquemia

No cérebro exposto a um episódio isquêmico ocorrem diversos eventos complexos e intrincados, os quais conjuntamente são denominados de “cascata neurotóxica isquêmica”. Uma oclusão vascular com duração de poucos minutos, leva a uma sequência de eventos fisiopatológicos que ocorrem em determinada ordem, os quais apresentam relevantes inter-relações entre si e perduram por horas ou até dias (Durukan e Tatlisumak, 2007). Devido à falha energética sucedida pela privação de oxigênio e glicose, ocorre excessiva liberação e falha na recaptação do neurotransmissor glutamato, levando à despolarização neuronal, aumento dos níveis intracelulares de Ca^{2+}, produção excessiva de espécies reativas de oxigênio, depleção dos níveis de enzimas antioxidantes, produção de mediadores inflamatórios, além da ativação de segundos-mensageiros envolvidos na sinalização da morte celular programada. Essa sequência de eventos e a ativação de enzimas que danificam a estrutura das membranas celulares leva à perda da compartimentalização, abalo da homeostase e, finalmente, à morte celular (Figura 1) (Dirnagl, Iadecola e Moskowitz, 1999; Lo, Dalkara e Moskowitz, 2003).

1.1.2. Vulnerabilidade seletiva do hipocampo e morte celular tardia

Ao comparar populações de células dentro da mesma região do cérebro, observa-se que estas distintas populações celulares apresentam diferente vulnerabilidade frente a um episódio isquêmico. A esta característica, observada principalmente na região hipocampal, dá-se o nome de vulnerabilidade seletiva do hipocampo. Enquanto as células piramidais de CA1 parecem ser as mais sensíveis à isquemia, as células piramidais da região vizinha Corno de Ammon 3 (CA3) e as células granulares do giro denteado (DG) são resistentes. Já na região do hilo, apenas

uma subpopulação de células parece ser vulnerável (Schreiber e Baudry, 1995; Yang, Kitagawa, Ohtsuki et al., 2000). Diversos estudos tanto *in vitro* (Bernaudin, Nouvelot, MacKenzie et al., 1998; Cimarosti, Rodnight, Tavares et al., 2001; Valentim, Rodnight, Geyer et al., 2003; Horn, Gerhardt, Geyer et al., 2005*)* quanto *in vivo* (Ordy, Wengenack, Bialobok et al., 1993; Hsu, Sik, Gallyas et al., 1994; Valentim, Geyer, Tavares et al., 2001) já demostraram essa vulnerabilidade diferenciada.

Além disso, na isquemia cerebral global (ICG) observa-se um fenômeno conhecido como “morte neuronal tardia”, caracterizada pela detecção de sinais de morte celular apenas três ou quatro dias após o insulto. No hipocampo, as duas populações de células vulneráveis sofrem degeneração de forma distinta, as do hilo degeneram no período de 24 horas, ao passo que as células piramidais de CA1 não morrem até 3-4 dias após a exposição às condições isquêmicas (Kirino, 1982; Ordy, Wengenack, Bialobok et al., 1993; Bartus, Dean, Mennerick et al., 1998).

Após a isquemia cerebral, o hipocampo apresenta duas áreas com respostas diferenciadas: a área do infarto, onde ocorre a redução do fluxo sanguíneo e a área da penumbra, ao redor da área do infarto, onde se observa a morte tardia. Essas características do hipocampo abordadas sugerem a presença de alguns neurônios ainda viáveis que podem estar comprometidos, mas ainda deixam uma janela terapêutica para intervenção farmacológica após a isquemia (Ordy, Wengenack, Bialobok et al., 1993; Dirnagl, Iadecola e Moskowitz, 1999).

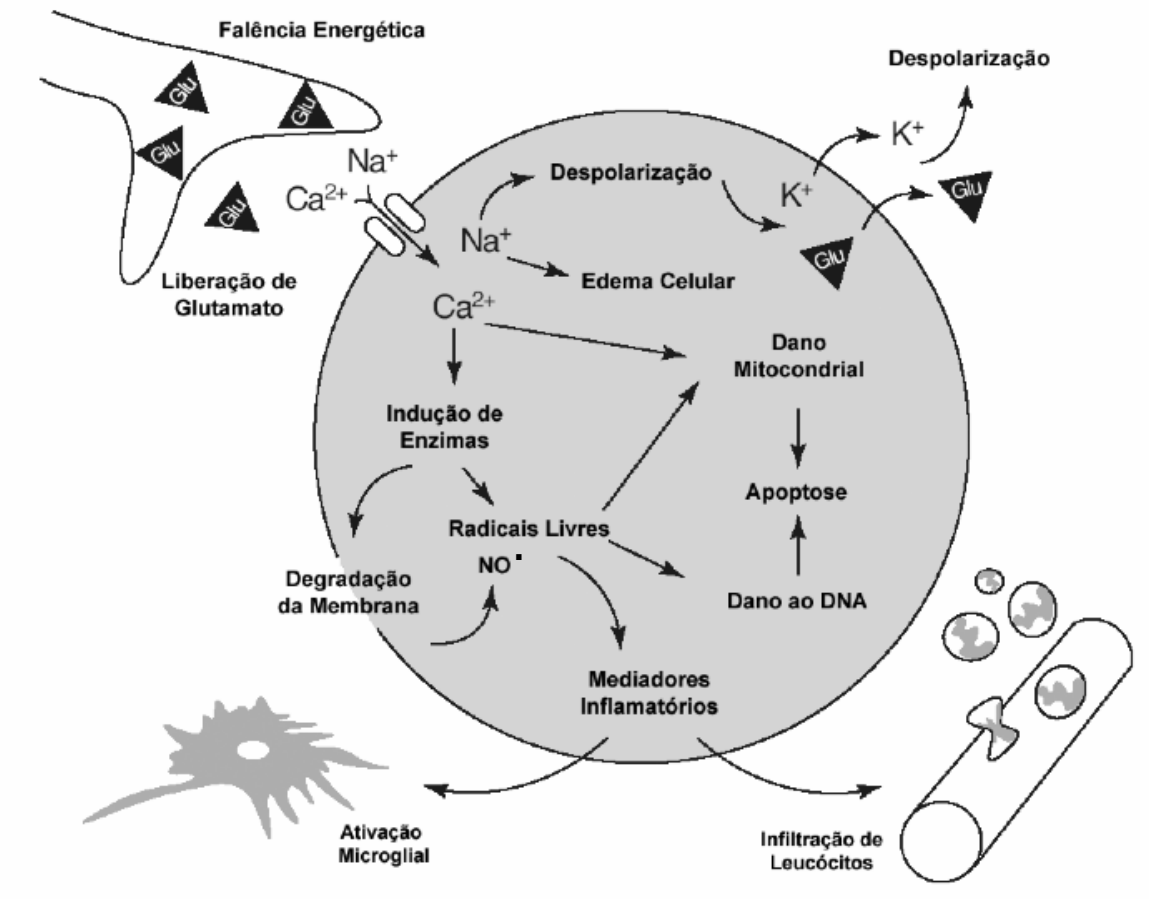

Figura 1. Visão geral simplificada dos mecanismos fisiopatológicos no cérebro isquêmico (Modificada de Dirgnal, Iadecola e Moskowitz, 1999).

1.1.3. Modelos experimentais de isquemia

1.1.3.1. Modelos *in vivo*

Nos modelos de ICG, os quais afetam todo o cérebro, são interrompidos os grandes vasos extracranianos, simulando um ataque cardíaco ou forte hipotensão. O modelo experimental mais utilizado é o da oclusão dos 4 vasos (4-VO) (Pulsinelli e Brierley, 1979; Schmidt-Kastner e Freund, 1991).

Nos modelos de isquemia cerebral focal, quando afetam uma pequena região do cérebro, são interrompidas artérias cerebrais específicas, sendo que o modelo mais usado é a oclusão da artéria cerebral média (MCA) (Ginsberg e Busto, 1989; Loetscher, Niederhauser, Kemp et al., 2001).

1.1.3.2. Modelos *in vitro*

Os modelos *in vitro* são ferramentas importantes para o estudo de diversas doenças, uma vez que constituem uma técnica mais simples e controlada para os estudos moleculares e celulares do que os *in vivo* (Pringle, Angunawela, Wilde et al., 1997). A técnica de culturas organotípicas vem sendo utilizada em nosso grupo para a investigação da morte neuronal induzida pela privação de oxigênio e glicose (POG) (Valentim, Rodnight, Geyer et al., 2003; Horn, Gerhardt, Geyer et al., 2005), toxicidade induzida pelo peptídeo β-amiloide (Nassif, Hoppe, Santin et al., 2007; Frozza, Horn, Hoppe et al., 2009) e estudo de compostos com possível atividade neuroprotetora frente a essas lesões (Cimarosti, Zamin, Frozza et al., 2005; Zamin, Dillenburg-Pilla, Argenta-Comiran et al., 2006; Simao, Zamin, Frozza et al., 2009; Hoppe, Frozza, Pires et al., 2013).

O modelo de culturas organotípicas de hipocampo foi desenvolvido em 1981 por Gähwiller e modificado por Stoppini e colaboradores (1991). Basicamente, trata-se de um método que mantém fatias de um tecido em cultivo, sobre uma interface entre o ar e o meio de cultivo, podendo assim permanecer por diversas semanas. A multiplicidade celular original do tecido cerebral (incluindo microglia e astrócitos) e as conexões intraneurais que estão presentes *in vivo* são mantidas, sendo essas características importantes para a investigação dos fatores fisiológicos e compostos farmacológicos que possam contribuir para a plasticidade sináptica e sobrevivência neuronal (Gahwiler, 1981; Stoppini, Buchs e Muller, 1991; Gahwiler, Capogna, Debanne et al., 1997; Clapp-Lilly, Smith, Perry et al., 2001; Holopainen, 2005).

A POG é um modelo experimental que reproduz *in vitro* parte das condições de uma isquemia cerebral *in vivo*. As culturas são expostas a um meio sem glicose e a uma atmosfera saturada de nitrogênio, simulando a interrupção do fluxo sanguíneo que ocorre durante um episódio isquêmico, observando-se as mesmas características com relação à vulnerabilidade seletiva e à morte neuronal tardia vistas nos modelos *in vivo*. Desta forma, essa metodologia constitui uma ótima ferramenta para o estudo dos mecanismos associados à isquemia cerebral e para a triagem de potenciais drogas neuroprotetoras (Strasser e Fischer, 1995; Laake, Haug, Wieloch et al., 1999; Sundstrom, Morrison, Bradley et al., 2005).

1.2. Estresse oxidativo

O estresse oxidativo é caracterizado pelo desequilíbrio entre sistemas pró-oxidantes, que levam à geração de espécies reativas de oxigênio (EROs) e de nitrogênio (ERN) e os sistemas antioxidantes, responsáveis pela remoção dessas espécies reativas. Em condições fisiológicas, as espécies reativas são formadas no organismo e controladas pelas defesas antioxidantes. Porém em estados patológicos pode ocorrer uma diminuição nas defesas antioxidantes endógenas e/ou um acréscimo na produção de radicais livres, promovendo o estresse oxidativo (Halliwell e Whiteman, 2004; Salvador e Henriques, 2004).

O tecido cerebral é particularmente sensível ao estresse oxidativo, pois utiliza grandes quantidades de O_2, possui baixas concentrações de defesas antioxidantes e alto conteúdo de lipídios poli-insaturados, os quais são mais suscetíveis à oxidação por espécies reativas (Sayre, Perry e Smith, 2008).

O estresse oxidativo tem sido associado a doenças neurológicas e neurodegerativas, tais como as doenças de Parkinson e Alzheimer e também à isquemia cerebral, tendo uma participação importante nos processos de morte celular

observados nessas patologias (Valko, Leibfritz, Moncol et al., 2007; Sayre, Perry e Smith, 2008). Durante a isquemia cerebral o fluxo sanguíneo é significativamente reduzido e na reperfusão a disponibilidade de oxigênio aumenta bruscamente, paralelamente às defesas antioxidantes endógenas estão depletadas devido ao excesso de produção de EROs por enzimas citosólicas pró-oxidantes, bem como pelo metabolismo mitocondrial, inativação de sistemas de detoxificação, consumo de antioxidantes e falha no reabastecimento de antioxidantes do tecido cerebral isquêmico (Fujimura, Tominaga e Chan, 2005).

1.3. Mitocôndria

As mitocôndrias são organelas intracelulares com dimensões e forma distintas, as quais dependem do estado metabólico e tecido em que se encontram. São responsáveis pela maior parte da energia gerada e utilizada pelas células eucarióticas, desempenhando assim, um papel fundamental na sobrevivência celular (Daum, 1985; Nicholls, 2002). A fosforilação oxidativa, acoplada à cadeia transportadora de elétrons, fornece a maior parte do ATP formado na célula. A cadeia respiratória é um complexo enzimático responsável pela transferência de elétrons vindos do metabolismo intermediário para a redução do oxigênio e síntese de ATP. Durante este processo, os elétrons oriundos das coenzimas NADH e $FADH_2$, reduzidas durante o ciclo do ácido cítrico, são transferidos para os complexos I e II e destes para os complexos III e IV, até o aceptor final de elétrons, o oxigênio molecular. A passagem de elétrons através dos complexos I, III e IV é acompanhada pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Este gradiente eletroquímico, responsável pela formação do potencial de membrana mitocondrial, dirige o fluxo de prótons de volta à matriz mitocondrial, que utiliza esta energia para a síntese de ATP (Huttemann, Lee, Pecinova et al., 2008). Em condições fisiológicas, em torno de 1-

4% dos elétrons são desviados do fluxo pelos complexos da cadeia transportadora de elétrons, causando a redução incompleta do oxigênio molecular, formando espécies reativas tóxicas, tornando a mitocôndria uma importante fonte de espécies reativas de oxigênio (Koopman, Nijtmans, Dieteren et al., 2010).

As mitocôndrias cerebrais são relativamente sensíveis à lesão isquêmica e sabe-se que a disfunção mitocondrial desempenha um papel importante no mecanismo fisiopatológico da neurodegeneração aguda causada por esse insulto (Fiskum, Murphy e Beal, 1999). Durante o período isquêmico, a oxidação de proteínas de membrana leva ao aumento na permeabilidade da membrana mitocondrial interna devido à abertura do poro de transição de permeabilidade mitocondrial, desencadeando o inchamento mitocondrial e a ruptura da membrana mitocondrial externa. Deste modo, proteínas pró-apoptóticas, como o fator indutor de apoptose e citocromo *c*, são liberados no citosol desencadeando a morte celular (Moro, Almeida, Bolanos et al., 2005; Galluzzi, Blomgren e Kroemer, 2009; Reddy e Reddy, 2011).

No período de reperfusão há um aumento da geração de EROs, oxidação dos lipídios de membrana e das proteínas de transporte de elétrons, contribuindo para a indução de uma cascata de eventos que, assim como durante a isquemia, leva à morte celular (Fiskum, Murphy e Beal, 1999).

1.4. Rutina

Os flavonoides são um grupo de substâncias naturais encontrados principalmente em frutas e vegetais. A propriedade mais descrita é sua capacidade antioxidante, mas possuem também atividades anticarcinogênica, anti-inflamatória, antialérgica, antiaterosclerótica, entre outras. A rutina (Rut) foi isolada a partir de laranjas em 1930, no início acreditava-se ser um composto pertencente ao grupo das vitaminas, sendo na época denominada vitamina P. Após descobriu-se que essa

substância era na verdade um flavonoide e a partir disso muitas pesquisas começaram a ser realizadas na tentativa de isolar a molécula e estudar o mecanismo de ação pela qual atua (Nijveldt, van Nood, van Hoorn et al., 2001). Sua estrutura química pode ser visualizada na Figura 2.

A Rut é uma substância de cor amarela, encontrada em diversas fontes alimentares como frutas cítricas, uva, maçãs, cebola, tomates e bebidas como chá preto e vinho tinto. Entre os vegetais, as principais fontes são: *Sophora japonica* L., *Ginkgo biloba*, *Faopyrum esculentum* Moech e *Dimorphandra mollis*, sendo essa uma árvore encontrada na flora brasileira e perfaz cerca de 50% da produção mundial (Gomes e Gomes, 2000; Nijveldt, van Nood, van Hoorn et al., 2001; Lao, Lin, Kuo et al., 2005; Chan, Xia e Fu, 2007).

Tradicionalmente é utilizada como agente antimicrobiano, antifúngico e antialérgico (Sharma, Ali, Ali et al., 2013). Além dessas, estudos demonstram que esse flavonoide apresenta atividades antiviral, anticarcinogênica, anti-inflamatória, antiagregante plaquetária, anti-hipertensiva, hipolipidêmica, e possui ação no fortalecimento dos capilares, que é resultado da sua elevada capacidade de sequestrar radicais livres e atividade antioxidante (Guo, Wei e Liu, 2007; Guerrero, Castillo, Quinones et al., 2012; Sharma, Ali, Ali et al., 2013).

Linhagens de células humanas não tumorais, TIG-1 e HUVE, foram incubadas com concentrações crescentes de Rut, não apresentando citotoxicidade (Matsuo, Sasaki, Saga et al., 2005). A administração de concentrações relativamente altas de Rut em ratos, não causou dano ao DNA das células da medula óssea (da Silva, Herrmann, Heuser et al., 2002) e também não apresentou atividade mutagênica (Sahu, Basu e Sharma, 1981; Das, Wang e Lien, 1994).

A Rut foi detectada no plasma no período de 2 a 8 h após sua administração intraperitoneal em ratos, sendo o pico entre 2 – 4 h (Rodrigues, Marcilio, Frazao Muzitano et al., 2013).

Em modelos *in vitro* foi sugerido que a Rut consegue transpassar o endotélio cerebral e atravessar a barreira hematoencefálica, se tornando uma alternativa para o estudo de sua ação em doenças que afetam o Sistema Nervoso Central (SNC) (Youdim, Dobbie, Kuhnle et al., 2003). Tem sido demonstrado o potencial neuroprotetor da Rut em modelos animais de isquemia cerebral global e focal. Dentre eles foi visto, em modelo *in vivo,* que esse flavonoide melhorou o decréscimo da memória espacial e a morte neuronal na região de CA1 do hipocampo induzida pela isquemia cerebral repetida em ratos (Pu, Mishima, Irie et al., 2007).

Quando a Rut foi administrada em camundongos antes da isquemia cerebral global, induziu uma redução acentuada no tamanho da área do infarto, atenuou a deficiência na memória de curto prazo e coordenação motora e diminuiu os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), ensaio empregado como um índice de estresse oxidativo (Gupta, Singh e Sharma, 2003). Outros autores demonstraram que essa substância também reduziu o tamanho da área do infarto, os déficits neurológicos comportamentais, as espécies reativas e a perda neuronal por apoptose devido à redução da expressão da p53 em isquemia cerebral focal em ratos (Khan, Ahmad, Ishrat et al., 2009). Entretanto, não há estudos em isquemia cerebral que relacionem a Rut e as vias de sobrevivência, como a via da fosfatidilinositol 3-cinase (PI3K).

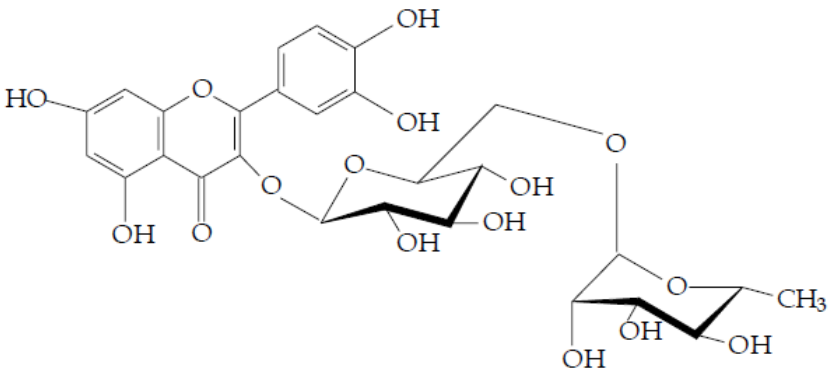

Figura 2. Estrutura química da rutina.

1.5. Vias de sinalização avaliadas nesse estudo

1.5.1. A via da PI3K e a proteína cinase B (Akt/PKB)

A PI3K é uma enzima amplamente expressa, que regula processos celulares, como proliferação, crescimento e apoptose (Nieto-Sampedro, Valle-Argos, Gomez-Nicola et al., 2011; Toker, 2012). Essa proteína consiste de um complexo heterodimérico, formado pela subunidade p85 (reguladora) e pela p110 (catalítica), as quais estão dissociadas e agrupam-se no momento da ativação, a qual é desencadeada através de um estímulo, que frequentemente é a interação de um fator de crescimento com um receptor tirosina cinase. Uma vez ativado, o receptor recruta a PI3K para perto da membrana plasmática, onde age sobre as moléculas de fosfatidilinositol bifosfato (PIP_2), fosforilando à fosfatidilinositol trifosfato (PIP_3) (Datta, Brunet e Greenberg, 1999; Cohen e Frame, 2001).

O aumento dos níveis intracelulares de PIP_3 ativa as proteínas cinases dependentes de fosfoinositóis (PDK1 e PDK2), levando ao deslocamento da Akt/PKB para a membrana plasmática. As PDKs 1 e 2 possuem ativação constitutiva e são capazes de fosforilar a Akt em dois diferentes sítios: a PDK1 fosforila a proteína na treonina 308, enquanto a PDK2 fosforila na serina 473, sendo que a fosforilação nos dois sítios é necessária para a total ativação da Akt/PKB. Essa possui um papel central nessa via, podendo interagir com diversos substratos (Coffer, Jin e Woodgett, 1998; Datta, Brunet e Greenberg, 1999; Vivanco e Sawyers, 2002; Song, Ouyang e Bao, 2005). O mecanismo de ativação da Akt pode ser melhor visualizado na figura 3.

A Akt está altamente expressa no SNC, é responsável pela fosforilação de vários substratos nucleares e citosólicos que regulam o crescimento celular e o metabolismo. Promove a proliferação, sobrevivência e proteção neuronal através da fosforilação de proteínas que regulam a apoptose como o promotor de morte associado à Bcl-2 (BAD), caspase-9, fator de transcrição do receptor Fas (FKHR), IκB

cinase, glicogênio sintase cinase 3 beta (GSK-3β) e a proteína alvo da rapamicina em mamíferos (mTOR) (Datta, Brunet e Greenberg, 1999; Yuan e Yankner, 2000) (Figura 3).

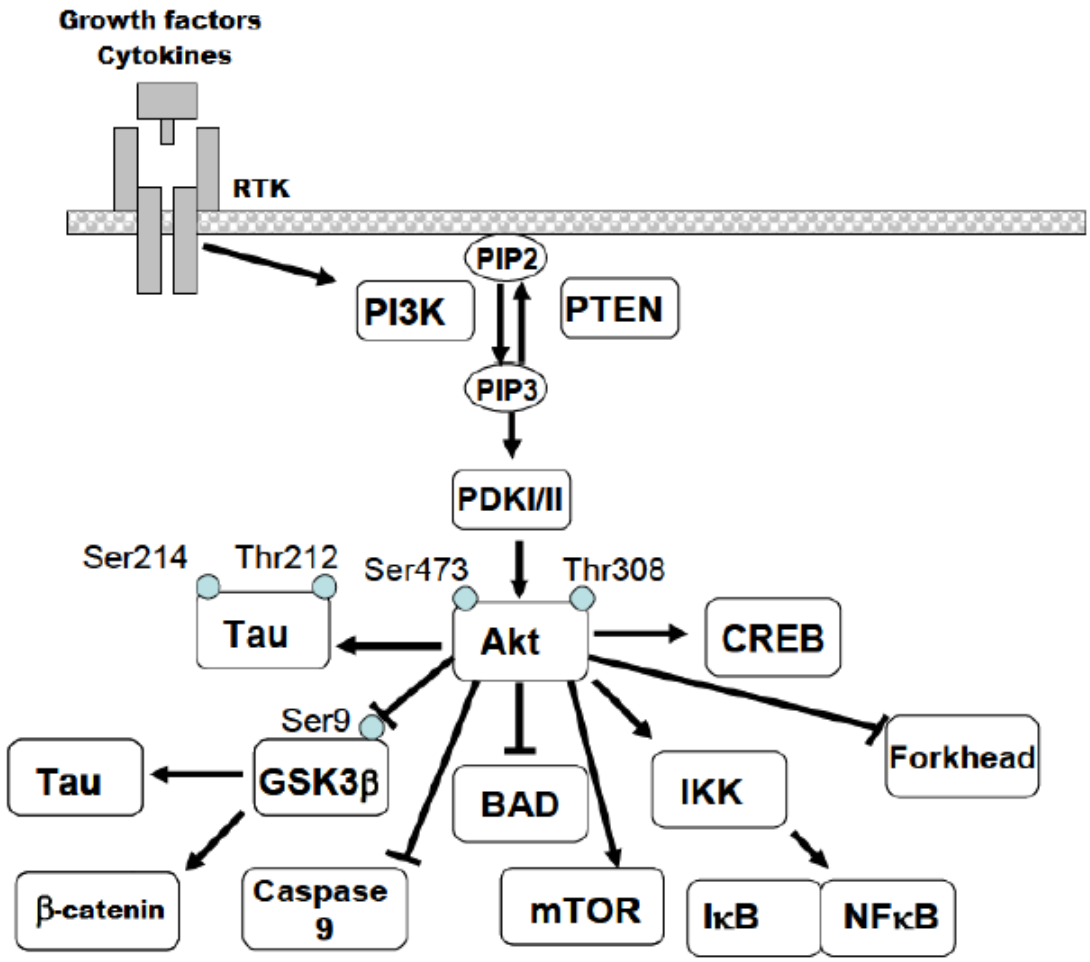

Figura 3. Via de sinalização celular mediada por PI3K/Akt simplificada. Detalhe da ativação da Akt e a regulação de seus substratos (Adaptado de Vivanco e Sawyers, 2002).

1.5.2. A Proteína GSK-3β

Um dos principais alvos da Akt é a GSK-3β. Quando a Akt está ativa, pode fosforilar a GSK-3β no resíduo de Serina 9, inativando-a. Outros inibidores dessa proteína são a proteína cinase ativada por mitógenos (MAPK), a proteína cinase A

(PKA), a proteína cinase C (PKC), entre outras (Cohen e Frame, 2001; Wang, Tsai, Peng et al., 2012).

A GSK-3β é um importante modulador de apoptose. Estudos apontam a associação entre essa proteína e a sobrevivência celular, mostrando que a sua ativação regula a atividade de diversos fatores de transcrição necessários para a sobrevivência celular. Sua inibição pela ativação da via PI3K/Akt pode proteger os neurônios de uma ampla variedade de lesões potencialmente fatais, demostrando que a GSK-3β pode ser um alvo inicial na morte celular (Pap e Cooper, 1998; Cohen e Frame, 2001; Grimes e Jope, 2001; Li, Bijur e Jope, 2002).

2. OBJETIVOS

2.1. Objetivo geral

Investigar o efeito neuroprotetor da Rut e os seus possíveis mecanismos de ação em um modelo *in vitro* de isquemia cerebral.

2.2. Objetivos Específicos

- Avaliar o efeito neuroprotetor da Rut, em diferentes concentrações, no modelo de POG em culturas organotípicas de hipocampo.
- Avaliar o efeito da Rut como antioxidante.
- Avaliar parâmetros de biogênese mitocondrial.
- Verificar o efeito da rutina sobre a via de sinalização celular da PI3K.

3. RESULTADOS

3.1. Artigo Científico

NEUROPROTECTIVE EFFECT OF RUTIN AGAINST OXYGEN AND GLUCOSE DEPRIVATION IN HIPPOCAMPAL ORGANOTYPIC SLICE CULTURES

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NEUROPROTECTIVE EFFECT OF RUTIN AGAINST OXYGEN AND GLUCOSE DEPRIVATION IN HIPPOCAMPAL ORGANOTYPIC SLICE CULTURES

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List to abbreviations:

DMSO - dimethyl sulfoxide

GSK-3β - glycogen synthase kinase-3β

H_2DCFDA - 2',7'-dichlorofluorescein diacetate

HBSS - Hank's balanced salt solution

MEM - minimum essential medium

OGD - oxygen and glucose deprivation

pAkt - phospho-Akt

pGSK-3β - phospho-GSK-3β

PI - propidium iodide

PI3K - phosphoinositide 3-kinase

RNS - reactive nitrogen species

ROS - reactive oxygen species

Rut - Rutin

SDS - sodium dodecylsulfate

TBARS - thiobarbituric acid reactive substances

Abstract

The reduction in the supply of glucose and oxygen to the brain that occurs in cerebral ischemia leads to a complex cascade of cell events that result in cellular death. Rutin (Rut) has applications in several diseases due to its wide biological activities, high safety margins and lower cost. Our objective was to investigate the neuroprotective effect of Rut, and the possible mechanisms involved, on organotypic hippocampal slices culture exposed to oxygen and glucose deprivation (OGD) as a model of ischemia. Cultures were exposed to 1 hour of OGD and then treated for 24 hours with Rut (recovery period). Our results showed that Rut treatment significantly decreased cell death induced by OGD. In cultures subjected to ischemia, we verified an increase in reactive species formation, which was decreased by Rut treatment. We also evaluated mitochondrial parameters, and no alterations were observed in mitochondrial mass and mitochondrial membrane potential. The phosphoinositide 3-kinase (PI3K) pathway and proteins Akt and glycogen synthase kinase-3β (GSK-3β) are involved in Rut-mediated neuroprotection against ischemic injury. Our results suggest a promising neuroprotective effect of Rut against damage caused by OGD.

Keywords: Oxygen and glucose deprivation; organotypic hippocampal culture; rutin; neuroprotection; oxidative stress; mitochondrial biogenesis; Akt/GSK-3β.

1. Introduction

Ischemic stroke is an important cause of death and morbidity worldwide (Donnan et al., 2008). It results from a transient or permanent reduction in cerebral blood flow that leads to a complex cascade of cellular events, resulting in severe neuronal degeneration and, consequently, in loss brain function (Dirnagl et al., 1999; Lipton, 1999; White et al., 2000). Despite the incessant studies to understand the mechanisms associated with ischemic cell death, few pharmacological options are currently available for the prevention and treatment of patients who underwent cerebral ischemia (Donnan et al., 2008; Hall et al., 2009).

Several *in vitro* and *in vivo* models are used to elucidate the mechanisms involved in ischemic damage and to evaluate potential neuroprotective action of drugs. The model of organotypic hippocampal slice cultures, combined with the oxygen and glucose deprivation (OGD), an *in vitro* system, is used to study the ischemia-induced injury. This model offers great advantages because it mimics closely the *in vivo* condition (Cimarosti et al., 2001; Pringle et al., 1997; Tavares et al., 2001), the interneuron connections and cellular architecture and also, neurons survive during long-term culture and physiologically mature during this period, allowing an extend survival study (Cho et al., 2004; Muller et al., 1993; Xiang et al., 2000).

Rutin (Rut) is a flavonoid found in many dietary sources, such as citrus fruits, grapes, apples, onions, black tea and red wine. It is extracted from various plants, but it is found in highest concentration in *Saphora japonica* L., *Faopyrum esculentum* Moech and *Dimorphandra mollis* (Chan et al., 2007; Lao et al., 2005; Nijveldt et al., 2001). Traditionally it is used as an antiallergic, antimicrobial and antifungal agent (Sharma et al., 2013). Besides these, some studies have shown that this flavonoid has anticarcinogenic, antiviral, hypolipidemic activities, and has role in strengthening the

capillaries, due to its antioxidant activity (Guo et al., 2007; Sharma et al., 2013). A potential neuroprotective effect of rutin has been shown in animal models of global and focal cerebral ischemia (Gupta et al., 2003; Khan et al., 2009; Pu et al., 2007).

Under physiological conditions, reactive species are formed in the organism in concentrations controlled by antioxidant defenses. However, an increase in reactive species production and/or a decrease in endogenous antioxidant defenses in disease states may occur, promoting redox unbalance (Halliwell and Whiteman, 2004). Brain tissue presents a high intake of oxygen associated with a low concentration of antioxidant defenses, and high content of polyunsaturated lipids, which makes this tissue very susceptible to oxidative stress (Sayre et al., 2008). The oxidative stress has been linked to neurological and neurodegerative disorders, such as Alzheimer's and Parkinson's disease, as well as cerebral ischemia, contributing to the neurotoxicity triggered by the ischemic episode and culminating in cell death (Sayre et al., 2008; Valko et al., 2007).

Brain ischemia is associated with high susceptibility of mitochondrial damage and it is known that mitochondrial dysfunction plays an important role in the pathophysiological mechanism in acute neurodegeneration caused by ischemic insult. Mitochondria participate in several intracellular processes, such as calcium homeostasis and the control of life and death through apoptosis (Fiskum et al., 1999; Koopman et al., 2010; Nicholls, 2002).

Histological and biochemical evidence suggests that cell death following ischemic insult may occur by apoptosis and necrosis (Lipton, 1999). It is believed that this death may occur due to an imbalance in signaling events. PI3K regulates essential cellular processes such as proliferation, growth and apoptosis. Akt is a downstream kinase of PI3K, full activated by phosphorylation on Thr308 and Ser473 residues (Coffer et al., 1998; Datta et al., 1999). One way by which active Akt mediates its anti-

apoptotic effects is by phosphorylating and inactivating glycogen synthase kinase-3β (GSK-3β), which is an important modulator of apoptosis (Pap and Cooper, 1998).

The aim of this study was to investigate the neuroprotective effect of Rut in organotypic hippocampal slice culture exposed to OGD, an *in vitro* model of neuronal death. We also investigated whether the neuroprotective action of the Rut was related to PI3K signaling pathway by activating Akt and inactivated GSK-3β, as well as its activity as a scavenger of reactive species and possible relationship with mitochondrial biogenesis.

2. Experimental procedures

2.1. Organotypic hippocampal slice cultures

All animal procedures were approved by local Animal Care Committee and are in accordance with the NIH Guide for the care and use of laboratory animals. Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991), with some modifications (Valentim et al., 2003). Briefly, 400 µm thick hippocampal slices were prepared from 6–8-day-old male *Wistar* rats using a Mcllwain tissue chopper and separated in ice-cold Hank's balanced salt solution (HBSS) (Gibco) supplemented with 25 mM HEPES, 1% Fungizone (Gibco) and 36 µL/100 mL gentamicin (Schering®), pH 7.2. Six slices were placed on one Millicell culture insert (Millicell®- CM, 0.4 µm) and the inserts were transferred to a six-well culture plate (Cell Culture Cluster, TPP). Each well contained 1 mL of tissue culture medium consisting of 50% minimum essential medium (MEM) (Gibco), 25% heat inactivated horse serum (Gibco) and 25% HBSS (Gibco), supplemented with glucose 36 mM, HEPES 25 mM and $NaHCO_3$ 4 mM (final concentrations). Fungizone 1% and

gentamicin 36 µL/100 mL were added to the medium. The pH was adjusted to 7.3 and immediately after the solution was filtered (Millex-GS, Millipore®). Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37ºC. The medium was changed every 3 days and experiments were carried out after 14 days *in vitro*.

2.2. OGD

OGD was achieved by combining hypoxia with aglycemia, according to the method described by Strasser and Fischer (1995), with some modifications (Cimarosti et al., 2001). After 14 days *in vitro*, the inserts were transferred to a sterilized 6-well plate, and incubated with 1 mL of OGD medium consisting of HBSS lacking glucose for 15 min to deplete glucose from intracellular stores and extracellular space. After that, medium was replaced by one with the same composition but previously bubbled with nitrogen for 30 min. The cultures were immediately transferred to an anaerobic chamber at 37°C in which the oxygen was replaced by nitrogen, and left in these conditions for 60 min. During this process control slices were maintained in an incubator with 5% CO_2 atmosphere at 37ºC. After the deprivation period, slices were washed three times with HBSS and incubated in culture medium under normoxic conditions for 24h, corresponding to the recovery period.

2.3. Drug exposition

Rut was obtained from Sigma Chemical (St. Louis, MO, USA) and was dissolved in 0.01% dimethyl sulfoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA). For the first set of experiments we tested the effect of different Rut concentrations: 0.25, 0.50, 1.00 and 1.5 mM. The drug and/or DMSO were added to the medium

immediately after OGD and maintained during the recovery period (24h). After these we choose the concentration of 1.5 mM Rut to the remaining experiments.

2.4. Quantification of Cellular Death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999). After a recovery period of 23 h, 5 µM PI (Sigma Chemical) was added to the cultures and incubated for 1 h. PI uptake is indicative of significant membrane injury (Macklis and Madison, 1990). Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and analyzed using Scion Image software (www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the ‘‘density slice’’ option of Scion Image software and compared to the total slice area to obtain the percentage of damage (Valentim et al., 2003).

2.5. Western blot analysis

After obtaining fluorescent images, slices were homogenized on lyses buffer [4% sodium dodecylsulfate (SDS), 2.1 mM EDTA, 50 mM Tris], aliquots were taken for protein determination (Peterson, 1983) and β-mercaptoethanol (Sigma Chemical) was added to a final concentration of 5%. Proteins were separated (40 µg per lane) on 12% SDS-polyacrylamide gel electrophoresis (Sigma Chemical). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD, CA. USA). Membranes were incubated for 60 min at 4ºC in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20) and further incubated with the appropriate primary antibody dissolved in the

blocking solution overnight at 4ºC. Primary antibodies anti-phospho-Akt (Ser473) (pAkt, 1:1000; Cell Signaling Technology), anti-Akt (1:1000; Cell Signaling Technology), anti-phospho-GSK-3β (Ser9) (pGSK-3β ,1:1000; Cell Signaling Technology) and anti-GSK-3β (1:1000; Cell Signaling Technology) were used. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, Piscataway, NI, USA) for 2 h. Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech) was detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with Optiquant software (Packard Instruments). For each experiment, the test groups were referred to vehicle treated control cultures not exposed to OGD, which were considered 100%, thus assuring the same signal intensity for control and test groups. The data are expressed as percentage of phosphorylated protein, which was obtained by the ratio of the immunocontent of phosphoprotein (pAkt or pGSK-3β) to the whole amount of the protein (Akt or GSK-3β) provided by the immunodetection assay with the total antibodies.

2.6. Flow cytometry assay

Mitochondrial mass and membrane potential were evaluated using MitoTracker® Green and Red (Invitrogen, Molecular Probes, Eugene, OR, USA), respectively, in a FACScalibur flow cytometer (BD Biosciences). The slices (100 mg) were dissociated with 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 1 mg% of collagenase IV, filtered and incubated with the probs. One hundred microliters of each sample was incubated in a bath at 37ºC, in the dark, for 45 min in a final concentration of 100µM MitoTracker® green+red. After that, 10.000 cells were evaluated per sample. Data were analyzed using the software FlowJo.

2.7. Reactive species measurement (DCF assay)

The reactive oxygen species (ROS) and reactive nitrogen species (RNS) production were detected using an oxidant-sensing fluorescent probe, 2',7'-dichlorofluorescein diacetate (H_2DCFDA) (Sigma Chemical). After 24 hours of OGD recovery, hippocampal slices were washed with PBS pH 7.4 and incubated with 30 μM H_2DCFDA in PBS, at 37°C in the dark for 30 minutes. H_2DCFDA is cleaved by cellular esterases and form H_2DCF that is oxidized by ROS and RNS which are present in the sample, producing a fluorescent compound, DCF (LeBel et al., 1992). After the incubation time, the intensity of DCF fluorescence was observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and analyzed using Scion Image software (www.scioncorp.com). The area where DCF fluorescence was detectable above background levels was determined using the ''density slice'' option of Scion Image software and compared to the total slice area to obtain the percentage of damage.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey's test (GraphPad Prism software 5.0). Differences between mean values were considered significant when $p < 0.05$.

3. Results

3.1. Rut protects against cell death induced by OGD

The exposure of the cultures to 60 min of OGD and 24 h of recovery caused a marked fluorescence in the hippocampus, indicating a high incorporation of PI, as presented in the photomicrograph in Fig. 1A (OGD group). Quantification of PI fluorescence showed that OGD caused damage in about 23% of the hippocampus area, a significant increase compared to control cultures with a basal hippocampus damage of 0.3% (Fig. 1B). Treatment with 0.25, 0.50, 1.00 and 1.50 mM of Rut significantly reduced the PI incorporation in the hippocampus to 6.3, 6.2, 7.2, and 1.7%, respectively (Fig. 1B) which means decrease of cell injury. There were no statistical difference among the tested doses, thus we chose the concentrations of 1.5 mM to the next experiments for investigate the mechanisms by which Rut exerts its neuroprotective action. Also no difference was detectable between the treatments in the control slices, indicating that rutin had no toxic effect in basal conditions (Fig. 1B).

3.2. Rut-mediated neuroprotection is related to the lower reactive species production

To determine whether Rut protected cultured hippocampal slices from OGD-induced cell death by decreasing the reactive species formation, ROS e RNS were evaluated with the fluorescent probe H_2DCFDA, since this is one of the most prominent markers to reflect the oxidative state of cells in general (Possel et al., 1997). As shown in Fig. 2(A,B), slices that undergone OGD for 1 h followed by 24 h of recovery exhibited an increase in reactive species production when compared with control slices. The treatment with Rut induced a decrease of the fluorescence induced by reactive species.

3.3. Evaluation of mitochondrial mass and membrane potential

To investigate the involvement of mitochondrial biogenesis on the neuroprotective effect of Rut, we evaluated the mitochondrial mass and membrane potential in a flow cytometer by MitoTracker® Green and Red probes, respectively. As we can see in figure 3, there are no statistical differences in the mitochondrial mass and membrane potential among any of the groups, so we cannot say whether one of the mechanisms of neuroprotection of Rut is through mitochondrial biogenesis.

3.4. Phosphorylation of Akt and GSK-3β is involved with Rut neuroprotective effect

To investigate the involvement of PI3K pathway on the neuroprotective effect of Rut, we examined the phosphorylation status of Akt and GSK-3β, two downstream proteins of this pathway. Considering that Akt is a master protein kinase that promotes neuronal survival after global brain ischemia (Zhang et al., 2004), the status of Akt phosphorylation was examined after 24 h of recovery by Western blotting. Rut-treated cultures, previously submitted to OGD, showed an increase on Akt phosphorylation compared to control and OGD treated with DMSO (Fig. 4B). The percentage of Akt phosphorylated from OGD culture was significantly decreased when compared to control culture treated with Rut (Fig. 4B). There was no significant difference in the intensity of pAkt when compared control and OGD.

When Akt is activated, it phosphorylates and inactivates GSK-3β, preventing apoptosis (Cohen and Frame, 2001; Pap and Cooper, 1998). The level of GSK-3β phosphorylation also was examined, by Western blotting, after 24 h of recovery. Rut-treated cultures and submitted to OGD showed an increase in the intensity of pGSK-3β compared to the remaining groups (Fig. 5B). However, OGD did not significantly change the levels of pGSK-3β after 24 h of recovery. No alteration in the total amount of Akt and GSK-3β was observed (representative Western blotting in Fig. 4A and 5A).

4. Discussion

In the present study, we tested whether Rut has a neuroprotective effect in ischemic injury and proposed underlying mechanisms by which this neuroprotection occurs. For this purpose, we used organotypic hippocampal slice cultures subjected to OGD, an *in vitro* model of ischemic injury, which mimics *in vivo* ischemia. This model is widely used by our research group to study compounds with neuroprotective activity, allowing the evaluation of the action mechanisms associated.

We show that Rut at concentrations of 0.25, 0.50, 1.00 and 1.50 mM protects organotypic hippocampal cultures against OGD. There was no statistical difference between the treatments, but the concentration of 1.5 mM appeared to be more effective, so it was used for other experiments. These results collaborated with data obtained by *in vivo* models of cerebral ischemia. Pu et al. (2007) suggest that Rut is a potent neuroprotective agent in repeated cerebral ischemia and was able to reduce the death in the CA1 region of the hippocampus. Other studies also reported that this flavonoid reduced the size of the infarct and neuronal loss caused by cerebral ischemia (Gupta et al., 2003; Khan et al., 2009).

Excessive calcium influx, oxidative stress, mitochondrial damage and cellular edema are involved in ischemic cell death. Once we confirmed the efficiency of Rut as a neuroprotective strategy, we decided to investigate the mechanisms by which it exerts this effect. First, we hypothesized that Rut could protect neurons against cell death induced by OGD by its antioxidative properties. Antioxidant activity of Rut is already well described, considering that this substance has powerful antioxidant capacity against various oxidant systems *in vitro* and strong radical-scavenging activity (Sharma et al., 2013). We have already demonstrated that cerebral ischemia induces a decrease of antioxidant defenses and therefore increases neuronal damage in cerebral

cortex and hippocampus of rats (Simao et al., 2011). Weis et al. (2011) reported that perinatal hypoxia-ischemia induced oxidative damage in the same brain structures of newborn rats, and these events may contribute to the later morphological damage in the brain. Therefore it is very important, particularly in pharmaceutical research, identifying drugs with neuroprotective property that can act against oxidative stress, especially very soon after an ischemic episode. To test our hypothesis that the antioxidant property Rut's could be one of the mechanisms by which Rut protects the neurons against OGD, we used the probe H_2DCFDA to evaluate reactive species synthesis. Our results showed a large increase in fluorescence produced by oxidation of DCF in cultures subjected to OGD and Rut significantly decreased this elevation. It has been reported that Rut was able to decrease the levels of thiobarbituric acid reactive substances (TBARS) in rats subjected to global cerebral ischemia (Gupta et al., 2003). It also prevents the decrease of antioxidant enzymes and increased levels of reactive species in rats subjected to ischemic injury (Annapurna et al., 2013; Khan et al., 2009). Since neuronal damage in cerebral ischemia is also induced by free radicals (Chan, 2001) and Rut of neuroprotection is associated with antioxidant capacity, this flavonoid may be a viable agent to be used in diseases in which cellular injury is caused by oxidative stress.

Whereas in cultures subjected to OGD the production of reactive species increased and considering that mitochondria is the major source of ROS and are relatively sensitive to ischemic damage, we decided to evaluate mitochondrial parameters. We used the probes MitoTracker ® Green and Red to measure mitochondrial mass and membrane potential, respectively. We found no statistical differences between the groups in these two parameters. According Siesjö et al. (1999) mitochondrial dysfunction is observed during ischemia, which depends on the duration of the ischemic event. After short periods of ischemia, mitochondrial function is usually normalized during reperfusion. However, after a longer duration of ischemia,

reperfusion could be accompanied by a secondary mitochondrial failure. The potential protective effect of rutin against methylmercury-induced mitochondrial dysfunction *in vitro* was investigated by Franco et al. (2010). When mouse brain mitochondria were incubated with methylmercury, it was observed a decrease in mitochondrial function, however when co-incubated with Rut, this drug was not able to protect the mitochondrial toxicity induced by methylmercury. In the literature only other models of cerebral ischemia were used for the assessment of mitochondrial function (Fiskum et al., 1999; Liang et al., 2013; Schwarzkopf et al., 2013; Weis et al., 2012). Thus, we suppose that due to the injury model used here we cannot observe changes in mitochondrial parameters, and by this way we may not say whether the neuroprotective effect of Rut is related to mitochondrial biogenesis.

The PI3K pathway has protective effects over cells, that are mediated primarily by Akt, one of its downstream targets (Franke et al., 1997). Several authors have suggested that apoptosis is one of death processes activated in cerebral ischemia, knowing the potential anti-apoptotic effect of PI3K pathway, substances that activate this pathway present potential to prevent ischemic lesions (MacManus et al., 1993; Nitatori et al., 1995). Therefore, we hypothesized that Rut could protect neurons against cell death induced by OGD by activating PI3K/Akt pathway. Our results indicated that Rut increased Akt phosphorylation after OGD, and consequently their activation. Rut increased phosphorylation of Akt and its protective effect was inhibited by LY294002 in H9c2 cardiac cells after induction of cell death by H_2O_2, indicating that the protective action of Rut involves the activation of Akt (Jeong et al., 2009). Friguls et al. (2001) demonstrated that after 24 hours the protein Akt returns to baseline values. Results of our research group, using organotypic hippocampal slice cultures and OGD, demonstrating that there was no change in the phosphorylation of this protein after 24h of recovery (Horn et al., 2005). These results support our present work, in which we

also did not observe any increase on Akt phosphorylation 24 h after OGD in cultures treated with vehicle only.

Following the PI3K/Akt pathway, we investigated whether Rut had effect on GSK-3β phosphorylation. The Akt has been recognized as the major protein kinases that promote neuronal survival at least in part by phosphorylation and inactivation of targets downstream, such as GSK-3β (Song et al., 2005; Yano et al., 2001; Zhang et al., 2004). This inhibition can protect neurons from wide variety of life-threatening injuries. Showing that GSK-3β may be a factor in the initial cell death (Cohen and Frame, 2001; Cross et al., 1995; Pap and Cooper, 1998). The GSK-3β inhibitor reduced the infarct volume after transient focal cerebral ischemia, suggesting that the inhibition of this protein is neuroprotective against ischemic injury (Kelly et al., 2004). We have shown that Rut increased phosphorylation of GSK-3β, such as Akt, but in this case the protein was inactivated, thereby preventing cell death by apoptosis. Jover-Mengual et al. (2010) did not observe significant changes in the levels of pGSK-3β 24 h after global cerebral ischemia, corroborating with our results, where we did not observe difference in phosphorylation of GSK-3β at 24 h after OGD in cultures that received only the vehicle.

In conclusion, here it was demonstrated the involvement of PI3K pathway by measuring the phosphorylation of GSK-3β and Akt proteins after treatment with Rut hippocampal organotypic culture following a model of cerebral ischemia in vitro. To our knowledge this work was the first to show that the PI3K pathway and proteins Akt and GSK-3β are involved in the neuroprotection of Rut against ischemic injury. We also showed that Rut plays its neuroprotective role due to its antioxidant activity. Our study provides evidence for the beneficial early administration of Rut and its underling mechanisms and interrelated pathways that may rescue neuronal cells after global brain ischemia.

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Fig.1. Effect of Rutin (Rut) on cell damage induced by OGD for 60 min in organotypic hippocampal cultures. (A) Representative photomicrographs of slices stained with PI after treatment with 0.25, 0.50, 1.00 and 1.50 mM of Rut and after 24 h recovery (magnification: 40x). (B) PI incorporation quantification from pictures showed in (A). Bars represent the mean ± S.E.M., n = 8-11. *** Significantly different from control cultures. # Significantly different from cultures exposed to OGD alone; $p < 0.001$ (one-way ANOVA followed by Tukey's test).

Fig.2. Effect of Rut on dichlorofluorescein oxidation in organotypic hippocampal cultures. (A) Representative photomicrographs of slices stained with DCF after treatment with Rut and after 24 h recovery (magnification: 40x). (B) DCF quantification from pictures showed in (A). Bars represent the mean ± S.E.M., n = 6. *** Significantly different from control cultures. # Significantly different from cultures exposed to OGD alone; $p < 0.01$ (one-way ANOVA followed by Tukey's test).

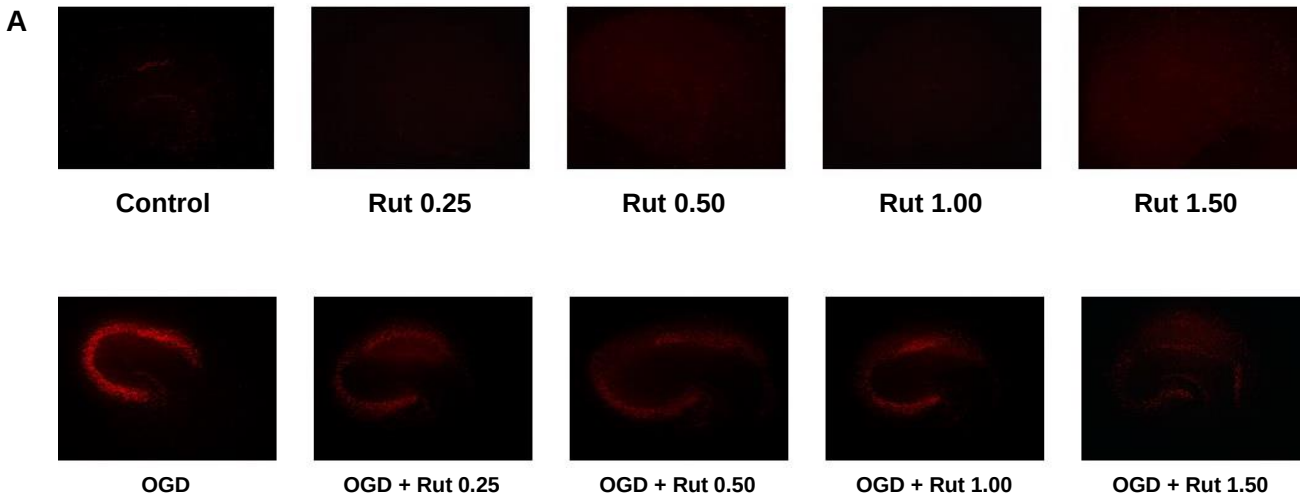
Fig.3. Effect of Rut on mitochondrial mass and mitochondrial membrane potential, measured by MitoTracker® Green and Red probes, respectively, in organotypic hippocampal cultures. (A) MitoTracker® Green Low. (B) MitoTracker® Green High. (C) MitoTracker® Red Low. (D) MitoTracker® Red High. Bars represent the mean ± S.E.M., n = 8.

Fig.4. Effect of Rut on the percentage of phosphorylated Akt in organotypic hippocampal cultures. (A) Representative western blots showing levels of pAkt and Akt. (B)The average level of pAkt was increased on OGD Rut-treated culture after 24 h recovery. Bars represent the mean ± S.E.M., n = 7. *** Significantly different from

control cultures. # Significantly different from cultures exposed to OGD alone; $p < 0.001$ (one-way ANOVA followed by Tukey's test).

Fig.5. Effect of Rut on the percentage of phosphorylated GSK-3β in organotypic hippocampal cultures. (A) Representative western blots showing levels of p GSK-3β and GSK-3β. (B)The average level of p GSK-3β was increased on OGD Rut-treated culture after 24 h recovery. Bars represent the mean ± S.E.M., n = 7. *** Significantly different from control cultures. # Significantly different from cultures exposed to OGD alone; $p < 0.001$ (one-way ANOVA followed by Tukey's test).

Fig.1.


A
Control
Rut 0.25
Rut 0.50
Rut 1.00
Rut 1.50
OGD
OGD + Rut 0.25
OGD + Rut 0.50
OGD + Rut 1.00
OGD + Rut 1.50

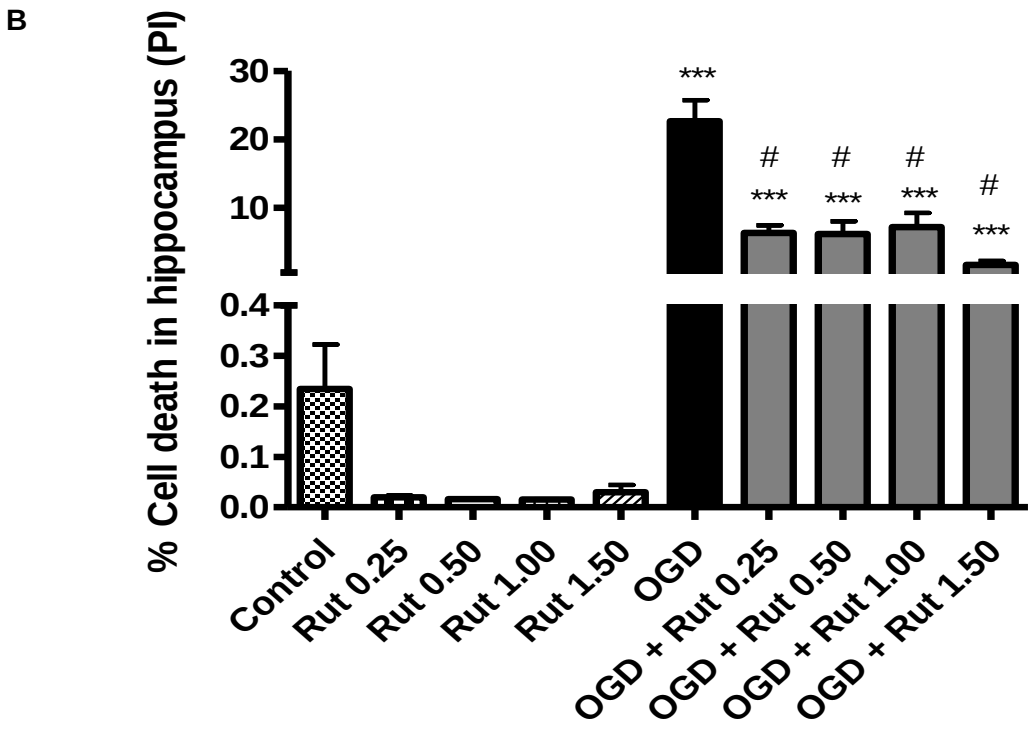

B
% Cell death in hippocampus (PI)
30
20
10
0.4
0.3
0.2
0.1
0.0
***
#
***
#
***
#
***
#
***
Control
Rut 0.25
Rut 0.50
Rut 1.00
Rut 1.50
OGD
OGD + Rut 0.25
OGD + Rut 0.50
OGD + Rut 1.00
OGD + Rut 1.50

Fig.2.

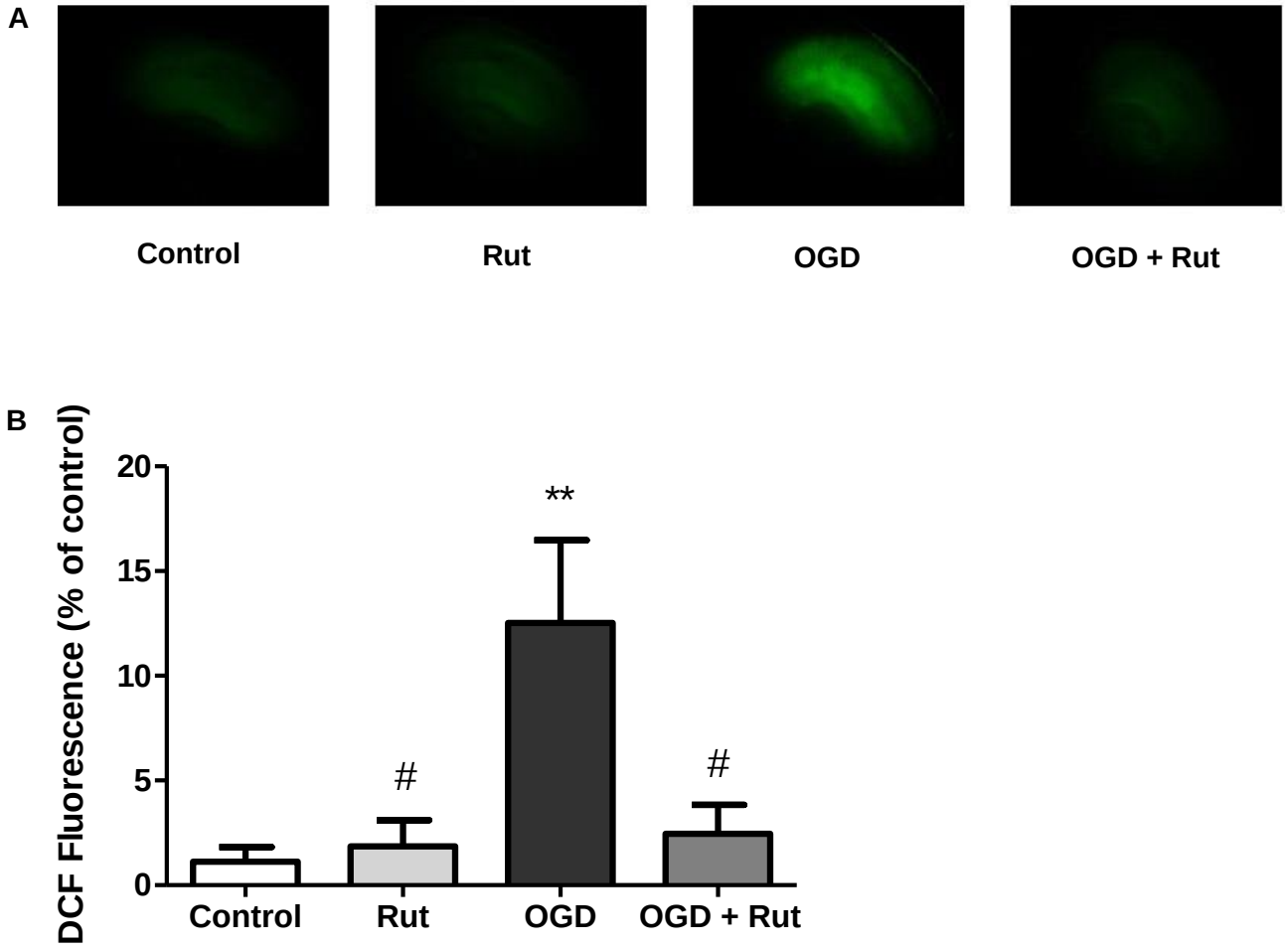

A
Control
Rut
OGD
OGD + Rut
B
DCF Fluorescence (% of control)
20
15
10
5
0
**
#
#
Control
Rut
OGD
OGD + Rut

Fig.3.

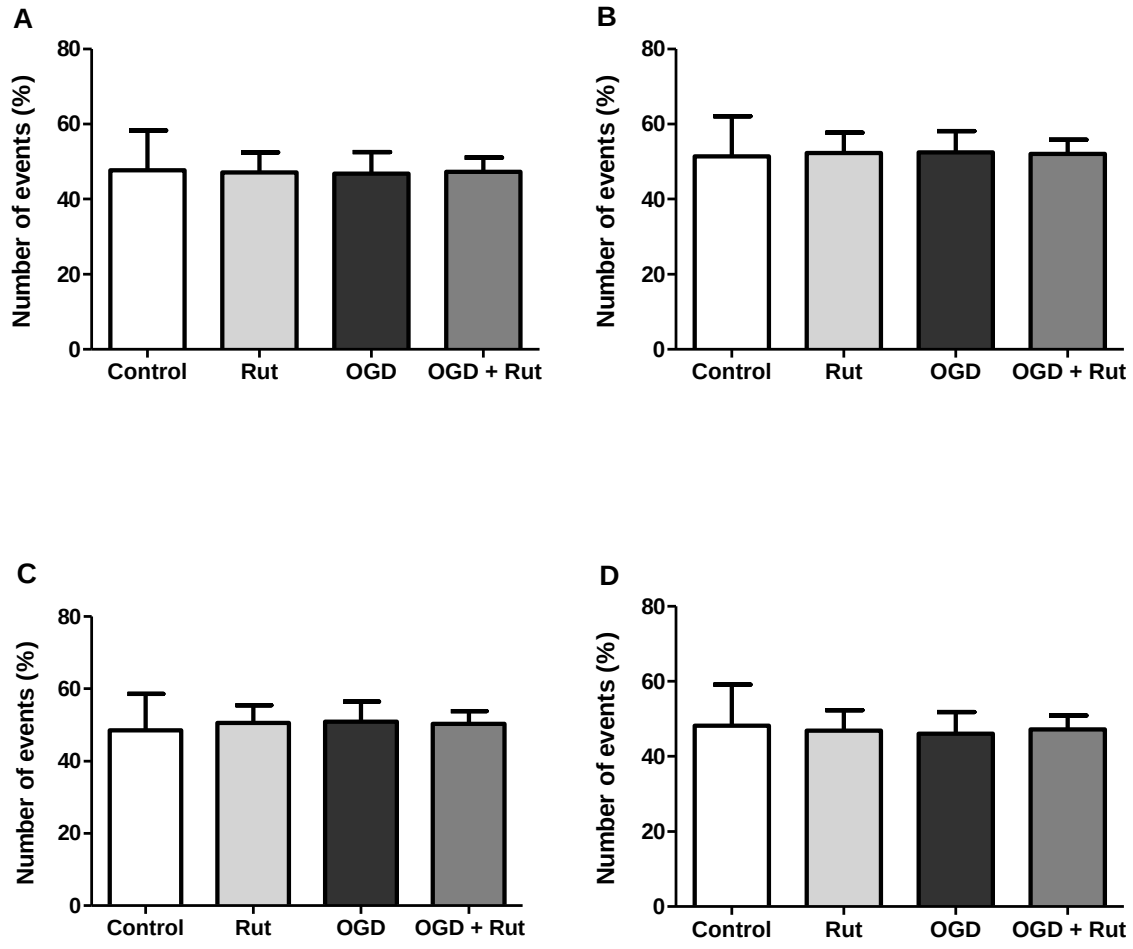


Fig.4.

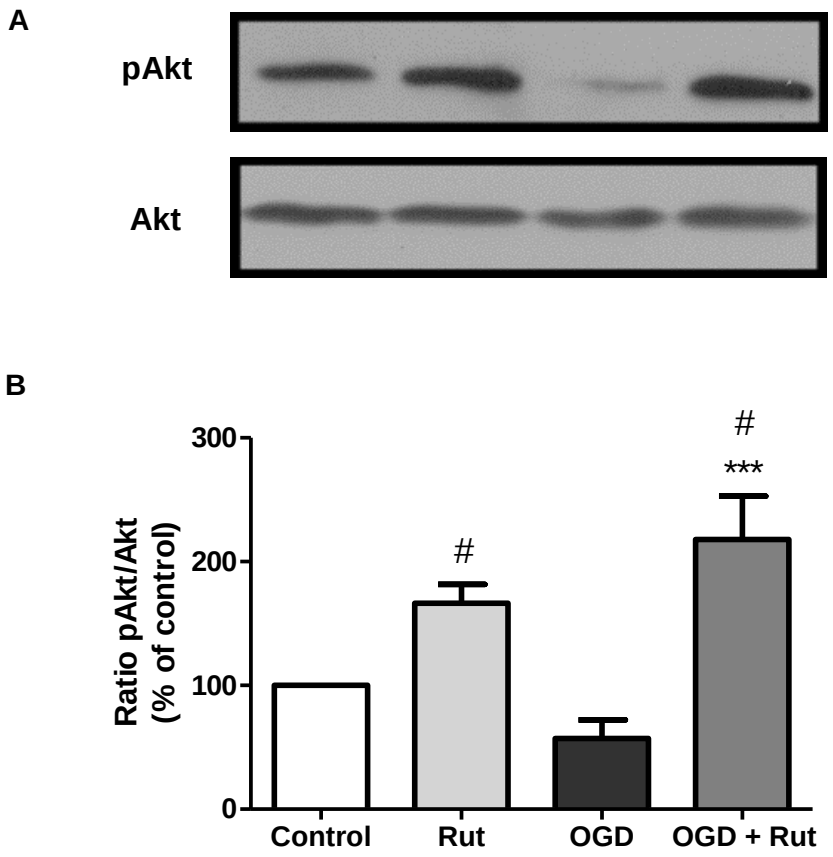
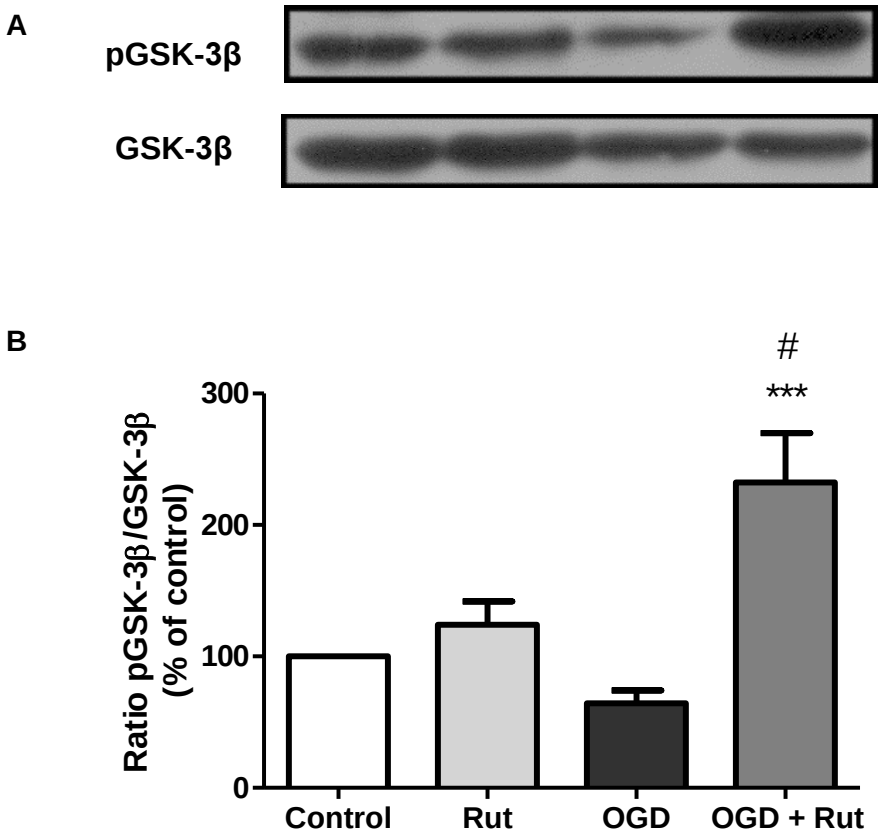

A
pAkt
Akt
B
Ratio pAkt/Akt
(% of control)
300
200
100
0
#
#
***
Control
Rut
OGD
OGD + Rut

Fig.5.


A
pGSK-3β
GSK-3β
B
#
***
300
200
100
0
Ratio pGSK-3β/GSK-3β
(% of control)
Control
Rut
OGD
OGD + Rut

4. DISCUSSÃO

Com o aumento da expectativa de vida, tem sido observada uma consequente elevação na incidência de isquemia cerebral. Essa possui grande relevância social, devido à alta frequência e altos níveis de morbidade, bem como os elevados custos de manutenção de pacientes com sequelas. A terapia trombolítica com a administração de alteplase é o tratamento utilizado atualmente, mas apenas um pequeno percentual de pacientes é aprovado para sua utilização (American Stroke Association, 2013). Devido a isso, é fundamental a pesquisa de novas drogas com potencial neuroprotetor, visando novas estratégias terapêuticas para o tratamento da isquemia cerebral, sendo este um dos grandes desafios da pesquisa biomédica nas próximas décadas.

Devido à relevância do tema, foi estudado o potencial neuroprotetor da Rut em modelo experimental de IC. Para isso, foram utilizadas culturas organotípicas de hipocampo expostas à POG como modelo *in vitro* de lesão isquêmica. Esse modelo possui como principal característica a manutenção da organização do tecido tal qual ocorre *in vivo* (Stoppini, Buchs e Muller, 1991) e é rotineiramente utilizado por nosso grupo para investigar os mecanismos de morte celular, neuroproteção e sinalização celular (Tavares, Cimarosti, Valentim et al., 2001; Valentim, Rodnight, Geyer et al., 2003; Zamin, Dillenburg-Pilla, Argenta-Comiran et al., 2006; Simao, Zamin, Frozza et al., 2009).

Entre os metabólitos isolados de plantas, os flavonoides possuem uma grande diversidade estrutural e um amplo espectro de atividades biológicas, fazendo deles um grupo de importante interesse terapêutico. O flavonoide Rut é tradicionalmente utilizado como agente antimicrobiano, antifúngico e antialérgico. No entanto, trabalhos atuais demonstram que esse possui benefícios farmacológicos multi-espectro para o

tratamento de várias doenças crônicas, tais como a diabetes, hipercolesterolemia e hipertensão (Guerrero, Castillo, Quinones et al., 2012; Sharma, Ali, Ali et al., 2013). Trabalhos *in vitro* e *in vivo* vêm despertando grande interesse sobre os efeitos da Rut no organismo. Já foram demonstrados efeitos benéficos em doenças neurodegenerativas e neuropsiquiátricas tais como a doença de Alzheimer (Javed, Khan, Ahmad et al., 2012), a doença de Parkinson (Khan, Raza, Javed et al., 2012), a isquemia cerebral (Gupta, Singh e Sharma, 2003; Khan, Ahmad, Ishrat et al., 2009) a depressão (Machado, Bettio, Cunha et al., 2008) e em gliomas (Santos, Silva, Pitanga et al., 2011). Considerando que o tratamento com Rut contra os danos causados pela isquemia ainda possui poucos estudos, nosso objetivo, nesse trabalho, foi avaliar o efeito neuroprotetor da Rut sobre a morte induzida pela POG em culturas organotípicas de hipocampo de ratos, bem como investigar possíveis mecanismos de ação envolvidos.

Como primeiro passo, foi realizada uma curva de concentração de Rut e avaliada a morte celular através da incorporação do corante iodeto de propídio (PI). O PI é uma molécula excluída por células sadias, mas capaz de permear células com a membrana danificada, ligando-se aos ácidos nucleicos, gerando fluorescência frente à luz UV, sendo utilizado como indicativo de dano celular (Macklis e Madison, 1990). A Rut e/ou DMSO foram adicionados ao meio imediatamente após a POG e mantidos durante o período de recuperação de 24h. As concentrações da droga utilizadas foram de 0,25; 0,50; 1,00 e 1,50 mM. Todos os tratamentos foram capazes de diminuir significativamente a incorporação de PI causada pela POG, demonstrando um relevante efeito neuroprotetor. Não houve diferença estatistica entre os tratamentos e a concentração de 1,5 mM foi utilizada para os demais experimentos. O potencial neuroprotetor da Rut em ratos submetidos à isquemia cerebral repetida foi demonstrado por Pu e colaboradores (2007), sendo que os animais tratados apresentaram uma menor morte na região de CA1. Outros estudos também relataram

que esse flavonoide reduziu o tamanho da área de infarto e a perda neuronal provocado pela isquemia cerebral (Gupta, Singh e Sharma, 2003; Khan, Ahmad, Ishrat et al., 2009). Em condições basais, nenhuma das concentrações testadas aumentou a incorporação de PI, se comparada ao controle tratado com DMSO, mostrando que a Rut não foi tóxica para as culturas. Nesse sentido, essa substância não apresentou citotoxicidade em células humanas normais, não causou dano ao DNA de células de medula óssea e não demonstrou atividade mutagênica (Sahu, Basu e Sharma, 1981; Das, Wang e Lien, 1994; da Silva, Herrmann, Heuser et al., 2002; Matsuo, Sasaki, Saga et al., 2005).

Diversos eventos estão envolvidos com a morte celular isquêmica, como o aumento do cálcio intracelular, o estresse oxidativo, o dano mitocondrial e o edema celular. Diante da efetiva ação neuroprotetora da Rut e uma vez que os mecanismos pelo qual ela exerce esse efeito não estão bem esclarecidos, nosso segundo passo foi investigar os possíveis mecanismos de ação envolvidos.

A ação antioxidante da Rut já é bem descrita. Noroozi e colaboradores (1998) mostraram que a Rut apresentou melhores resultados que os da vitamina C na inibição dos danos oxidativos induzidos pelo H_2O_2 em DNA de linfócitos humanos e, Hanasaki e colaboradores (1994) revelaram que a Rut apresentou efeito de eliminação do radical hidroxila 100-300 vezes superior ao manitol. O cérebro é particularmente sensível ao estresse oxidativo, assim é de grande importância o estudo de drogas com propriedades antioxidantes. Em trabalho anterior, demonstramos que a isquemia cerebral diminuiu as defesas antioxidantes e, em consequência, aumenta o dano neuronal no córtex cerebral e hipocampo de ratos (Simao, Matte, Matte et al., 2011). Outros autores relataram que a hipóxia-isquemia neonatal induziu danos oxidativos no hipocampo e no córtex cerebral de ratos Wistar recém-nascidos, que esses eventos podem contribuir para o dano morfológico cerebral e destacam a importância da pesquisa de estratégias neuroprotetoras visando combater o estresse oxidativo o mais

rápido possível após o episódio isquêmico (Weis, Schunck, Pettenuzzo et al., 2011). Gupta e colaboradores (2003) revelaram que a Rut foi capaz de diminuir os níveis de TBARS em ratos submetidos à isquemia cerebral global. Outros autores mostraram que essa droga evitou a diminuição das enzimas antioxidantes e o aumento dos níveis de espécies reativas em ratos que sofreram lesão isquêmica (Khan, Ahmad, Ishrat et al., 2009; Annapurna, Ansari e Manjunath, 2013). Nossos resultados mostraram um aumento das espécies reativas nas culturas submetidas à POG e a Rut diminuiu significativamente essa elevação. Sabendo que na isquemia cerebral há um dano neuronal induzido por radicais livres e drogas antioxidantes são relatadas como tendo atividade neuroprotetora, nossos resultados sugerem que a Rut pode ser um agente viável a ser usado em doenças em que um dano celular é causado por estresse oxidativo.

Tendo em vista que houve um aumento das espécies reativas nas fatias submetidas à POG, e uma vez que as mitocôndrias são as principais fontes de EROs e que são relativamente sensíveis ao dano isquêmico, foram avaliados parâmetros relacionados a função mitocondrial. A massa mitocondrial e o potencial de membrana foram mensurados, em citômetro de fluxo, utilizando as sondas MitoTracker® Green e Red, respectivamente. Não foram observadas diferenças estatisticamente significativas entre os grupos nesses dois parâmetros avaliados. Weis e colaboradores (2012) mostraram que houve inibição da atividade dos complexos da cadeia transportadora de elétrons e uma diminuição da massa e potencial de membrana em ratos recém-nascidos submetidos à hipóxia-isquemia. Segundo Siesjö e colaboradores (1999) a isquemia é acompanhada por uma disfunção mitocondrial. Após breves períodos de isquemia, a função mitocondrial é geralmente normalizada durante a reperfusão. Porém, após uma isquemia de duração mais longa, a reperfusão pode ser acompanhada por uma falha mitocondrial secundária. Essa falha é observada em áreas vulneráveis em isquemias de curta duração, mas está presente também em

outras áreas em isquemias mais longas. Em experimento com mitocôndrias cerebrais isoladas de ratos adultos, a Rut foi ineficaz para protegê-las da toxicidade do metilmercúrio, o qual induziu uma significante diminuição da função mitocondrial, monstrando que não é capaz de atenuar a disfunção mitocondrial nesse modelo (Franco, Posser, Missau et al., 2010). Em pesquisa na literatura, somente outros modelos de isquemia cerebral foram utilizados para a avaliação da função mitocondrial (Fiskum, Murphy e Beal, 1999; Weis, Pettenuzzo, Krolow et al., 2012; Liang, Xu, Zhang et al., 2013; Schwarzkopf, Hagl, Eckert et al., 2013). Desta forma, podemos supor que com o modelo de lesão utilizado em nosso trabalho não foi possível verificar alterações na massa e no potencial de membrana. Assim, não podemos afirmar se um dos mecanismos de neuroproteção da Rut envolve a biogênese mitocondrial.

Continuando com a investigação dos mecanismos envolvidos na neuroproteção induzida pela Rut, foi analisado o possível envolvimento da via de sinalização da PI3K. Essa via possui um papel importante na sobrevivência celular e é possível avaliar sua ativação através da medida do estado de fosforilação de duas proteínas centrais: a Akt e a GSK-3β (Pap e Cooper, 1998; Datta, Brunet e Greenberg, 1999). A via da PI3K pode determinar o destino de uma célula durante o estresse oxidativo, uma vez que alvos dessa via regulam mecanismos pró-apoptóticos. A Akt é um desses alvos, e o aumento de sua fosforilação ocorre após várias condições que resultam em estresse oxidativo, tal como ocorre durante a isquemia cerebral, o que pode conferir resistência à lesão neuronal e ao estresse oxidativo (Chong, Kang e Maiese, 2004).

Diversos autores apontam que a apoptose é um dos processos de morte ativados pela isquemia/reperfusão, assim, a via da PI3K, que possui efeito anti-apoptótico, pode estar relacionada com mecanismos de prevenção em lesões isquêmicas (MacManus, Buchan, Hill et al., 1993; Nitatori, Sato, Waguri et al., 1995). A Akt tem sido reconhecida como uma das principais proteínas cinases que promovem a sobrevivência neuronal (Yano, Morioka, Fukunaga et al., 2001; Zhang, Yin e Chen,

2004). Nossos resultados mostraram que a Rut foi capaz de aumentar a fosforilação da Akt, e consequentemente a sua ativação. Jeong e colaboradores (2009) utilizaram células cardíacas H9c2, induziram morte celular por H_2O_2 e trataram com Rut. Seus resultados demonstraram que a Rut aumentou a fosforilação da Akt e seu efeito protetor foi inibido pelo LY294002, um inibidor da via da PI3K/Akt, indicando que a ação protetora da Rut envolve a ativação da Akt neste modelo experimental.

Vários estudos mostram a ativação da proteína Akt após um evento isquêmico (Namura, Nagata, Kikuchi et al., 2000; Janelidze, Hu, Siesjo et al., 2001; Kawano, Morioka, Yano et al., 2002). Por outro lado, também foi demonstrado que após 24h a proteína Akt retorna aos valores do controle (Friguls, Justicia, Pallas et al., 2001). Resultados do nosso grupo de pesquisa utilizando culturas organotípicas de hipocampo e POG demonstram não haver mudança na fosforilação dessa proteína após 24 h de recuperação (Horn, Gerhardt, Geyer et al., 2005). Esses resultados corroboram com o presente trabalho, no qual também não observamos aumento da fosforilação da Akt 24 h após a POG nas culturas que receberam somente o veículo.

Seguindo a via da PI3K/Akt, investigou-se o efeito da Rut sobre a proteína pró-apoptótica GSK-3β. A Akt promove a sobrevivência celular, pelo menos em parte, por fosforilação e inativação de alvos pró-apoptóticos, sendo um destes a proteína GSK-3β (Cross, Alessi, Cohen et al., 1995; Endo, Nito, Kamada et al., 2006). A utilização do inibidor da GSK-3β reduziu o volume do infarto após isquemia cerebral focal transitória, sugerindo que a inibição dessa proteína possui efeito neuroprotetor contra a lesão isquêmica (Kelly, Zhao, Hua Sun et al., 2004). Assim como a Akt, foi observado que a Rut aumentou a fosforilação da GSK-3β, o que leva a inativação da proteína, diminuindo assim a morte celular por apoptose. Jover-Mengual e colaboradores (2010) não observaram mudanças significativas nos níveis de pGSK-3β 24 h após isquemia cerebral global. Assim como nesse estudo, também não foi

observado em nosso trabalho, diferença na fosforilação da GSK-3β nas amostras expostas à POG e tratadas apenas com o veículo.

Já foi demonstrado que a Akt pode bloquear a geração de EROs (Chong, Kang e Maiese, 2004), sugerindo que os mecanismos de neuroproteção da Rut encontrados nesse trabalho podem estar relacionados a essa propriedade.

Nossos resultados sugerem que a Rut apresenta um forte potencial neuroprotetor em caso de lesão isquêmica e mostra pela primeira vez na literatura que a via da PI3K e as proteínas Akt e GSK-3β estão envolvidas na neuroproteção da Rut contra a lesão isquêmica.

5. CONCLUSÕES

Os resultados obtidos nesta dissertação nos permitem concluir que o tratamento com Rut após uma hora de exposição à POG e recuperação de 24 horas:

- ✓ Conferiu menor morte celular, principalmente na região de CA1, verificada pela diminuição da incorporação de PI;
- ✓ Não houve alteração na massa mitocondrial e no potencial de membrana, medidos em citômetro de fluxo com as sondas MitoTracker Green e Red, respectivamente;
- ✓ Reduziu a produção de espécies reativas, observada pela diminuição da fluorescência do DCF;
- ✓ Ativou a via de sobrevivência celular PI3K, observada pelo aumento da fosforilação das proteínas Akt e GSK-3β.

6. PERSPECTIVAS

Como continuação deste trabalho, pretende-se trabalhar com os seguintes objetivos:

- Avaliar o envolvimento de outras vias de sinalização, através da avaliação das proteínas ERK, JNK e NFκB;
- Investigar o efeito da administração de Rut *in vivo* após a isquemia cerebral global;
- Verificar o efeito da Rut na produção de EROs pelos ensaios de TBARS e TRAP, enzimas anti e pró-oxidantes, como iNOS, SOD e catalase no modelo *in vivo*;
- Avaliar *in vivo* os parâmetros analisados *in vitro*, e relacionar com o modelo utilizado nesse estudo.

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