



**SERVIÇO PÚBLICO FEDERAL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE UBERLÂNDIA**

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA



Efeito do tratamento com *Vochysia rufa* Mart. e glibenclamida sobre o estresse oxidativo e a expressão de proteínas motoras e de ancoragem em cérebro de ratos diabéticos

Aluna: Alice Vieira da Costa

Orientador: Dr. Foued Salmen Espindola

Co-Orientadora: Dra. Luciana Karen Calábria

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APRESENTAÇÃO

O formato desta tese obedece as normas do Programa de Pós-graduação em Genética e Bioquímica. Ela é composta de três capítulos, sendo o capítulo 1 referente a fundamentação teórica, que embasa os outros dois capítulos.

Capítulo 1 - Fundamentação Teórica.

Capítulo 2 - Tratamento com glibenclamida modula a expressão e localização de miosina-II β no cérebro de ratos diabéticos.

Capítulo 3 - Extrato aquoso de *Vochysia rufa* promove expressão diferencial de proteínas envolvidas na formação e tráfico de vesículas no cérebro de ratos diabéticos.

Os capítulos 2 e 3 foram escritos no formato de um artigo científico, em inglês, revisado por nativo. Cada artigo representa o estudo na íntegra, está formatado dentro das normas das revistas citadas nos seus referentes capítulos, e aguardam as considerações dos membros da banca e a defesa para submissão.

RESUMO

O diabetes aumenta o estresse oxidativo e provoca várias alterações no transporte de proteínas e de ancoragem das vesículas sinápticas. Os extratos de plantas têm sido muito utilizados para tratar a hiperglicemia e diabetes. Extratos aquosos de *Vochysia rufa*, uma espécie do Cerrado brasileiro, têm sido prescritos e utilizados como tratamento complementar ou alternativo. Foram analisados os efeitos do extrato aquoso de *Vochysia rufa* sobre marcadores de estresse oxidativo e na expressão e localização de proteínas de transporte e ancoragem das vesículas sinápticas no cérebro de ratos diabéticos. Trinta e dois ratos Wistar machos foram divididos aleatoriamente em 4 grupos: não-diabéticos, diabéticos, diabéticos tratados com glibenclamida (6 mg/kg de peso corporal por via oral) e diabéticos tratados com extrato de *Vochysia rufa* (500 mg/kg de peso corporal por via oral) por 43 dias. Após os tratamentos, os cérebros foram coletados e os seguintes parâmetros foram avaliados: atividade enzimática da glutathione peroxidase e glutathione S-transferase, a concentração de superóxido dismutase, sulfidril total, a peroxidação lipídica e de glutathione reduzida. Os níveis e localização de proteínas, como CaMKII, miosina - Va, sinapsina -1, SNAP -25 e GLUT4 também foram analisados. O extrato de *Vochysia rufa* diminuiu SOD, GSH, TBARS e os níveis totais de sulfidril e aumentou os níveis de GST. Além disso, a miosina -Va e sinapsina -1 apresentaram os níveis de expressão diminuídas, ao contrário de CaMKII e SNAP-25 que aumentaram. Estes resultados foram confirmados por imunohistoquímica. Os resultados sugerem que o extrato aquoso de *Vochysia rufa* tem um efeito neuroprotetor em cérebros de ratos diabéticos, reduzindo o estresse oxidativo e controlando as mudanças na miosina -Va e sinapsina -1.

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LISTA DE ABREVIATURAS

ATP – Adenosina tri-fosfato

CaMKII- proteína quinase-II dependente de Ca^{2+} /calmodulina

CEUA – Comitê de Ética na Utilização de Animais

CuSOD – Superóxido dismutase citosólica

D – Diabético

DG – Diabético tratado com glibenclamida

DV- Diabético tratado com *Vochysia rufa*

dl – Decilitro

DNA – Ácido Desoxirribonucleico

DTNB – Ácido dinitrobenzóico

g – Gramas

GLUT- proteínas transportadoras de glicose

GPx – Glutathione peroxidase

GR – Glutathione reductase

GSH – Glutathione reduzida

GSSG – Glutathione oxidada

GST – Glutathione S-transferase

h - Horas

H.E. – Hematoxilina e Eosina

L - Litros

MDA – Malondialdeído

mRNA - ácido ribonucleico mensageiro

mg - Miligramas

mL - Mililitros

MnSOD – Superóxido dismutase mitocondrial

NADH – Nicotinamida adenina dinucleotídio

NADPH - Nicotinamida adenina dinucleotídio fosfato

ND – Não diabético

pH – Potencial hidrogeniônico

RPM – Rotações por minuto

s - Segundos

SOD – Superóxido dismutase

SNAP 25- proteína associada ao sinaptossomo

SNARE- proteínas de fusão sensível ao receptor de ligação ao N-etilmaleimido solúvel

TBARS – Método para avaliação de peroxidação lipídica

TNF- α - Factores de necrose tumoral alfa

ug - Microgramas

uL - Microlitros

uM - Micromolar

umol – Micromol

VGlut-1 - Vesicular transportador de glutamato 1

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Capítulo 1

1. Fundamentação teórica

1.1 *Diabetes mellitus*

Diabetes mellitus é uma síndrome metabólica que afeta cerca de 171 milhões de pessoas no mundo (Mccrimmon, Ryan *et al.*, 2012) e está associada com alterações de secreção e/ou ação da insulina, o processo patológico é agravado por danos acumulados ao longo do tempo, aumentando os riscos macro e microvasculares, e trazendo complicações para vários órgãos, como rins, coração e cérebro (Gispen e Biessels, 2000; Association, 2012; Mccrimmon, Ryan *et al.*, 2012). No sistema nervoso central, o quadro hiperglicêmico característico do *diabetes mellitus* altera os níveis de neurotransmissores, causando morte celular e reduzindo a capacidade de aprendizagem e memória (Mooradian, 1988; Mccall, 1992; Biessels, Kappelle *et al.*, 1994; Di Mario, Morano *et al.*, 1995; Helkala, Niskanen *et al.*, 1995; Mccrimmon, Ryan *et al.*, 2012; Jing, Chen *et al.*, 2013).

A insulina é um hormônio anabólico que desempenha funções de controle de glicemia, síntese proteica e controle do peso corporal (Haber, Curi *et al.*, 2001; Wendt, 2005), além de atuar na modulação cognitiva do sistema nervoso central, na memória, aprendizado e plasticidade sináptica (Zhao, Koslovsky *et al.*, 1996; Park, 2001). Este hormônio é responsável pela ativação de uma cascata de sinalização que regula a translocação dos transportadores de glicose para a membrana da célula (Schaan, Rabelo *et al.*, 2004) (Figura 01). Alterando esta sinalização, a captação de glicose fica prejudicada e a hiperglicemia é instalada desencadeando distúrbios no metabolismo e alterando o balanço entre pró- e antioxidantes (Halliwell; Gutteridge, 2003).

O *diabetes mellitus* é classificado em quatro categorias: diabetes do tipo 1, causado pela deficiência absoluta da secreção de insulina, por conta da destruição das células beta do pâncreas por mecanismos auto-imunes que podem ser acionados por fatores ambientais ou por causas idiopáticas; diabetes tipo 2, caracterizada pela resistência insulínica, apesar da secreção normal pelas células do pâncreas, sendo o mais comum e estando intimamente ligado à obesidade e ao sedentarismo; diabetes por outras causas que não se enquadram nos tipos 1 e 2, como as doenças do pâncreas exócrino e drogas; e diabetes gestacional,

diagnosticado na gestação, podendo ou não persistir após o parto (Association, 2012; 2013).

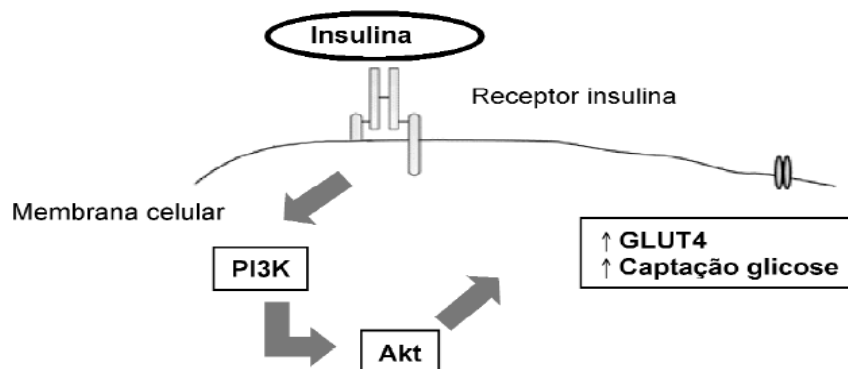


Figura 01: Visão esquemática das vias de sinalização da insulina que facilitam a captação de glicose (Adaptado de Schaan, Rabelo *et al.*, 2004).

1.2 Diabetes mellitus, cérebro e estresse oxidativo

A hiperglicemia no estado diabético aumenta as desordens fisiológicas, evidenciando as lesões celulares causadas pelos radicais livres e aumentando as complicações decorrentes da cronicidade da doença (Reis, Veloso *et al.*, 2008), bem como é responsável por alterações celulares decorrentes dos processos de glicação não enzimática e de glico-oxidação, que está relacionada com a produção de espécies reativas do oxigênio e a formação de produtos finais de glicação avançada. Estas alterações contribuem para o aumento da peroxidação lipídica, modificação irreversível de proteínas, DNA e lipídios (Jennings, Jones *et al.*, 1987; Baynes e Thorpe, 1999; Rosen, Nawroth *et al.*, 2001; Genet, Kale *et al.*, 2002; Siddiqui, Taha *et al.*, 2005).

O estresse oxidativo é uma condição biológica de desequilíbrio entre a produção de espécies reativas de oxigênio e a capacidade do organismo em neutralizar estas espécies, criando um ambiente neurotóxico (Taniyama e Griendling, 2003; Gerecke, Kolobova *et al.*, 2013).

As espécies reativas de oxigênio são íons, átomos ou moléculas que possuem um oxigênio com capacidade altamente oxidativa. Estas espécies apresentam uma grande instabilidade e elevada reatividade, podendo ser radicalares como o superóxido e o radical hidroxil, e não radicalar como o peróxido de hidrogênio (Kohen e Nyska, 2002; Reis, Veloso *et al.*, 2008).

O balanço redox tem mostrado ser um fator importante na atividade neuronal do sistema nervoso central e este reflete nos outros sistemas que estão sob seu controle, como o cardiovascular (Cardoso, Colombari *et al.*, 2006). Um exemplo é o peróxido de hidrogênio, uma espécie reativa do oxigênio, que está relacionada com a modulação da transmissão (Chen, Avshalumov *et al.*, 2001) e da plasticidade sináptica (Auerbach e Segal, 1997). Em decorrência deste desequilíbrio e da relação entre a atividade neuronal e os agentes oxidantes, algumas complicações são observadas em pacientes diabéticos, como demências, convulsões, comprometimento cognitivo e alterações no aprendizado e memória. Porém, os mecanismos bioquímicos responsáveis por estas alterações ainda são desconhecidos (Coleman, Judd *et al.*, 2004).

1.3 Defesas antioxidantes

A célula desenvolveu uma série de mecanismos de defesa contra a exposição a radicais livres que vai desde mecanismos de reparo até defesas antioxidantes enzimáticas e não enzimáticas (Cadenas, 1997a; b). As defesas antioxidantes enzimáticas incluem a glutathione peroxidase, a superóxido dismutase e a catalase (Figura 02); enquanto as defesas antioxidantes não enzimáticas são representadas pelo ácido ascórbico (vitamina C), glutathione, α -tocoferol (vitamina E), flavonóides e carotenóides (Valko, Leibfritz *et al.*, 2007). Em condições normais, existe um equilíbrio entre a produção de espécies reativas do oxigênio e a defesa antioxidante. Como o diabetes está associado com o aumento do estresse oxidativo, este balanço fica comprometido e induz modificações em lipídeos, carboidratos, proteínas e DNA (Du, Edelstein *et al.*, 2000; Wen, Skidmore *et al.*, 2002; Ceriello e Motz, 2004).

A glutathione peroxidase é uma enzima antioxidante que converte peróxido de hidrogênio em água em uma reação de oxidação da glutathione reduzida no seu correspondente dissulfeto (GSSG), posteriormente regenerado pela glutathione redutase por intermédio da oxidação de NADPH, o qual é utilizado como cofator doador de ânions de hidrogênio (Cardoso, Colombari *et al.*, 2006; Huber e Almeida, 2008). A enzima superóxido dismutase (SOD) age transformando dois ânions radicais superóxidos em um peróxido de hidrogênio. Existem três isoformas de SOD que se diferenciam pelo metal associado e a sua localização celular: SOD1

(CuZnSOD) no citosol e mitocôndria, SOD2 (MnSOD) na matriz mitocondrial e SOD3 (CuZnSOD) na matriz extracelular (Fukai e Ushio-Fukai, 2011; Kawamata e Manfredi, 2010).

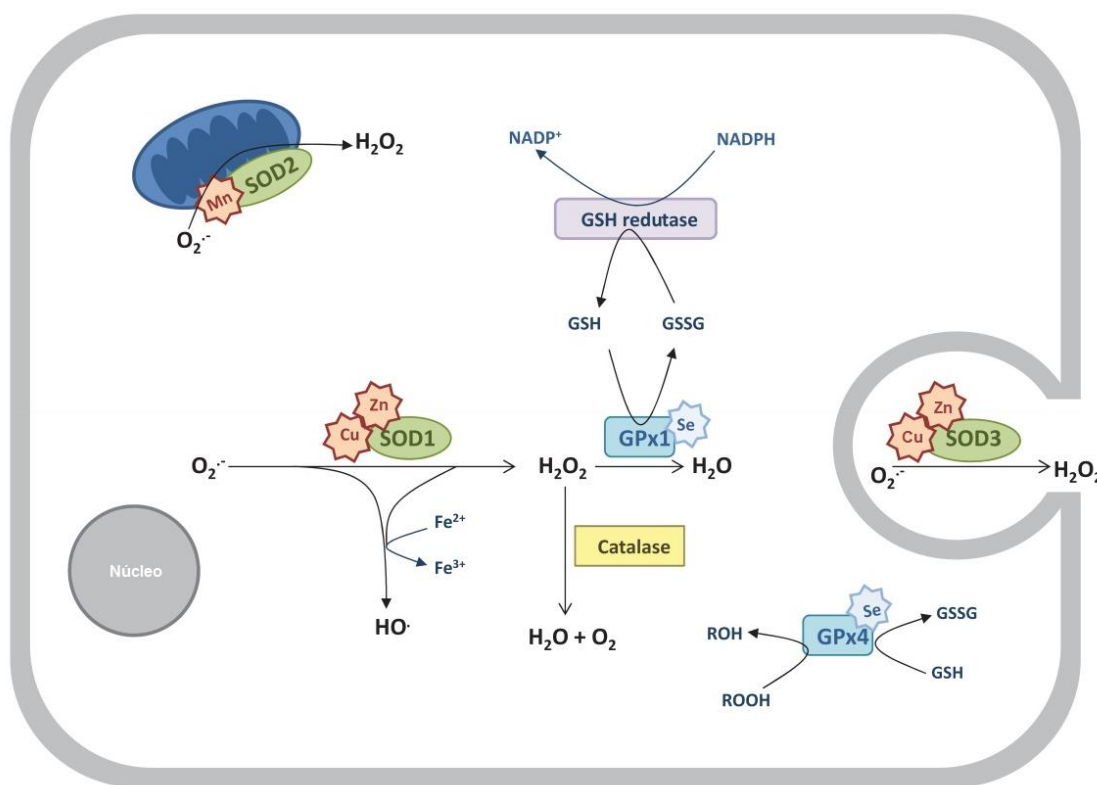


Figura 02: Principais defesas antioxidantes. Abreviações: SOD, superóxido dismutase; GSH, glutathiona reduzida; GSSG, glutathiona oxidada; GPx, glutathiona peroxidase (Adaptado de Sousa, Afonso *et al.*, 2012).

Outro antioxidante enzimático é a catalase, uma enzima encontrada no interior dos peroxissomos, que possui duas isoformas (selênio-independente e selênio-dependente) e exerce a função de decompor o peróxido de hidrogênio em água e oxigênio (Mates, Perez-Gomez *et al.*, 1999).

Por outro lado, as enzimas glutathionas transferases geralmente apresentam-se em meio biológico como complexos homo ou heterodímeros e com dois sítios catalíticos, um específico para a glutathiona reduzida e outro para eletrófilos (Huber e Almeida, 2008). A glutathiona reduzida é um tripeptídeo de ácido glutâmico, cisteína e glicina que exerce a função de cosubstrato da família das enzimas glutathiona peroxidase (Vasconcelos, Goulart *et al.*, 2007).

Estudos comprovam que o maior número de espécies reativas do oxigênio durante a hiperglicemia contribui para o aumento de dano neuronal e alterações na transmissão sináptica. Calábria (2010) avaliou os efeitos do estresse oxidativo e os níveis de expressão de motores moleculares no cérebro de ratos diabetizados com estreptozotocina, e revelou que as miosinas das classes –II e –V estão associadas ao diabetes e que a suplementação antioxidante não altera a atividade das enzimas antioxidantes, apesar de restaurar os níveis proteicos de miosina-Va.

1.4 Miosinas

As miosinas compõem uma superfamília com 20 classes, baseado em análise do domínio motor (Krendel e Mooseker, 2005). As miosinas são motores moleculares responsáveis pelo tráfego de vesículas e transporte de organelas sobre o filamento de actina, bem como desempenham vários papéis fundamentais nas células eucarióticas, como o rastreamento celular, a citocinese, a fagocitose, a contração muscular e ainda a extensão do cone de crescimento e manutenção da forma celular (Hirokawa, Niwa *et al.*, 2010). Esses motores moleculares se caracterizam por possuírem uma cadeia pesada dividida em três domínios funcionais (Figura 03), e no caso da miosina-V, da seguinte forma: um domínio motor N-terminal ou domínio “cabeça”, que apresenta as regiões ligantes de actina; um domínio “pescoço” regulatório contendo seis repetições de uma sequência coesa de aminoácidos: isoleucina, glutamina, arginina, glicina e tirosina, denominada “motivo IQ”, além de sítios de ligação para cálcio e calmodulina; e um domínio C-terminal ou domínio “cauda,” que se divide em três subdomínios: proximal, formada por segmentos curtos de alfa-hélice dupla, responsáveis pela dimerização das duas cadeias pesadas; medial, com estrutura variável contendo segmentos globulares alternados com segmentos em alfa-hélice duplos; globular, região menos conservada, onde são ancorados diferentes tipos de cargas a serem transportadas (Benashski, Harrison *et al.*, 1997; Reck-Peterson, Provance *et al.*, 2000; Berg, Powell *et al.*, 2001; Krendel e Mooseker, 2005; Loubéry e Coudrier, 2008). Nesta grande família de miosinas, somente as classes -I, -II, -V e -VI estão envolvidas nos processos de endocitose e exocitose (Hodge e Cope, 2000; Buss, Luzio *et al.*, 2001; Foth, Goedecke *et al.*, 2006).

As miosinas da classe II estão presentes em células musculares e não musculares, e desempenham papel importante na migração de neurônios e nos cones de crescimento (Vallee, Seale *et al.*, 2009), plasticidade sináptica (Rex, Gavin *et al.*, 2010) e liberação do neurotransmissor nas zonas ativas (Mochida, Kobayashi *et al.*, 1994), podendo ser detectadas nos dendritos pós-sinápticos (Miyazaki, Watanabe *et al.*, 2000; Cheng, Hoogenraad *et al.*, 2006) no desenvolvimento de espinhos dendríticos (Ryu, Liu *et al.*, 2006). A miosina-II apresenta mais de quinze isoformas de cadeia pesada em vertebrados (Conti e Adelstein, 2008), sendo três isoformas não muscular presentes em mamíferos: -IIA, -IIB e -IIC. A miosina-IIB é a mais frequentemente encontrada em cérebro (Murakami, Mehta *et al.*, 1991), com localização cortical no corpo e axônio de neurônios (Cheng, Murakami *et al.*, 1992; Rochlin, Itoh *et al.*, 1995).

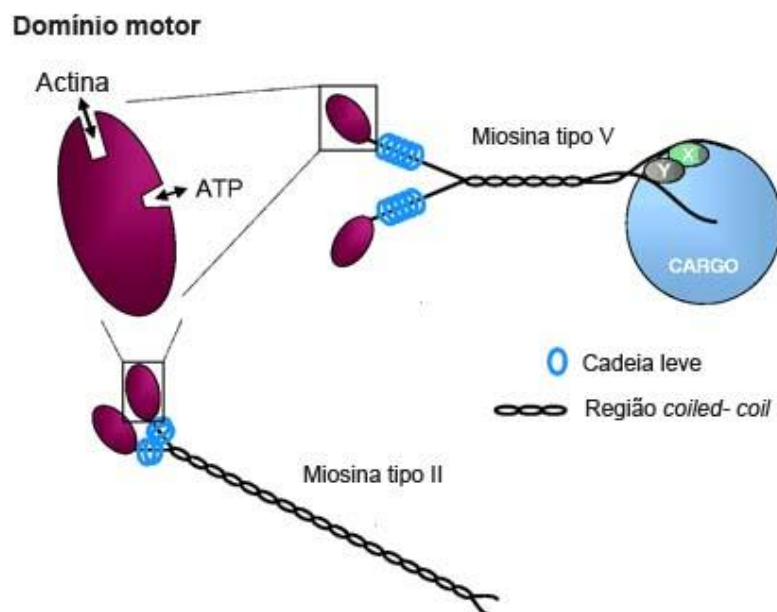


Figura 03: Estruturas básicas das miosinas –II e –V e a distinção pelo domínio cauda: tipo II, com cauda em espiral que medeia a dimerização e montagem em estruturas filamentosas, e tipo V com cadeias pesadas que dimerizam por meio de serpentinas enroladas, mas não em filamentos. As proteínas X e Y representam proteínas de ligação que podem mediar interações com carga (Adaptado de Hutagalung, Landsverk *et al.*, 2002).

A miosina-V está subdividida em três subclasses em vertebrados: miosina-Va, presente em cérebro e glândulas (Espindola, Espreafico *et al.*, 1992; Cheney, O'shea *et al.*, 1993; Nascimento, Cheney *et al.*, 1996; Espindola, Banzi *et al.*, 2008);

miosina-Vb, presente principalmente no hipocampo, giro denteado, amígdala e córtex cerebral, e no tecido epitelial (Zhao, Koslovsky *et al.*, 1996; Lapierre, Kumar *et al.*, 2001); e miosina-Vc, presente em tecidos epiteliais, glandulares e cerebelo (Bridgman e Elkin, 2000; Rodriguez e Cheney, 2002; Marchelletta, Jacobs *et al.*, 2008; Jacobs, Weigert *et al.*, 2009).

A miosina-Va está localizada na densidade pós-sináptica e em vesículas, e tem sido relacionada com função neuronal e de cognição (Hirokawa, Niwa *et al.*, 2010), apresentando a característica de se ligar diretamente a várias proteínas citoplasmáticas, incluindo sinaptobrevina, sinaptofisina, syntaxina e CaMKII (Prekeris e Terrian, 1997; Evans, Lee *et al.*, 1998; Costa, Mani *et al.*, 1999; Ohyama, Komiya *et al.*, 2001).

O cérebro possui grande quantidade de miosina-Va e dados de imunodeteção em cérebro e cerebelo de ratos, bem como análises de imunocitoquímica, mostram intensa marcação nas extensões dendríticas das células de Purkinje e na região perinuclear (Espindola, Espreafico *et al.*, 1992; Tilelli, Martins *et al.*, 2003), sendo as vesículas e as organelas as principais cargas transportadas por esta miosina nos neurônios e em outros tipos celulares (Langford e Molyneaux, 1998; Depina e Langford, 1999).

Além de todas as patologias envolvendo as miosinas –II e –V, estudos mostram que o diabetes altera a expressão destas miosinas no cérebro de ratos, apresentando uma diminuição na expressão de miosina-Va (Da Costa, Calabria *et al.*, 2011; Da Costa, Calabria *et al.*, 2013) e um aumento na expressão de miosina-IIB (Calabria, Da Cruz *et al.*, 2011). Entretanto, os mecanismos de regulação da glicemia e os seus efeitos sobre a expressão de motores moleculares ainda são desconhecidos e aguardam maiores investigações.

1.5 Proteínas que se associam a vesículas sinápticas

A sinapsina-I é uma fosfoproteína encontrada nos terminais pré-sinápticos neuronais onde se associa com vesículas sinápticas. É uma proteína de membrana periférica, que está associada com o citoplasma de praticamente todas as pequenas vesículas sinápticas e amplamente distribuída em terminais pré-sinápticos dos sistemas nervosos central e periférico (Navone, Di Gioia *et al.*, 1989; Parfitt, Hoffer *et al.*, 1991; Nayak, Moore *et al.*, 1996). Estudos sugerem que a

sinapsina-I desempenha um papel chave na libertação de neurotransmissores, regulando a disponibilidade de vesículas sinápticas (Llinas, McGuinness *et al.*, 1985; Ceccaldi, Grohovaz *et al.*, 1995). Além disso, a sinapsina-I é um substrato importante para a proteína quinase-II dependente de cálcio e calmodulina (CaMKII) (Figura 04), sendo que o seu estado de fosforilação regula a liberação de neurotransmissores pela disponibilidade de vesículas sinápticas (Huttner, Schiebler *et al.*, 1983; Nestler e Greengard, 1983; Llinas, McGuinness *et al.*, 1985; Schiebler, Jahn *et al.*, 1986).

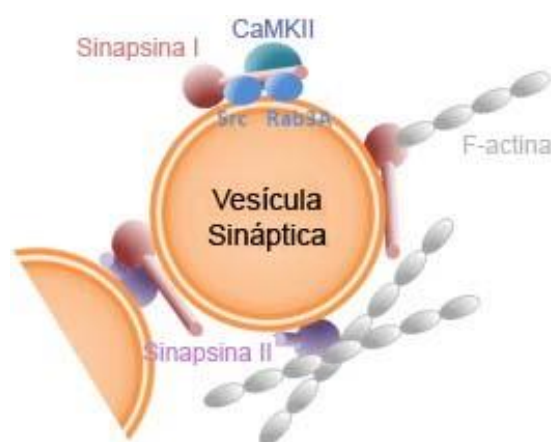


Figura 04: Esquema da interação entre vesícula sináptica, sinapsina-I e CaMKII (Adaptado de Humeau, Candiani *et al.*, 2011).

A CaMKII compreende uma família de isoformas semelhantes (α , β , γ e δ). As isoformas α e β são as predominantes no cérebro e cada isoforma consiste de um domínio catalítico com um domínio autoinibitório, um segmento variável e um domínio de auto-associação. O domínio catalítico contém os sítios de ligação ATP e substrato, bem como locais para a interação com as proteínas de ancoragem (Hudmon e Schulman, 2002; Lisman, Schulman *et al.*, 2002). Nayak, Moore *et al.*, 1996 evidenciaram que CaMKII e sinapsina-I estão envolvidas na formação do potencial de longa duração e, conseqüentemente, na memória e aprendizado. Em ratos diabéticos induzidos por estreptozotocina, (Liao, Xiang *et al.*, 2013) revelaram que há uma diminuição na fosforilação de CaMKII no hipocampo, o que poderia ocasionar um processo de disfunção cognitiva.

deste estado patológico, pode alterar os níveis de expressão das proteínas pré-sináptica, como sinapsina I, sinaptofisina e SNAP-25 (Van Guilder, Brucklacher *et al.*, 2008).

1.6 Transportador de glicose

A captação da glicose no sistema nervoso dos mamíferos é mediada pela família de proteínas transportadoras de glicose (GLUT). Treze membros fazem parte da família GLUT, sendo que vários deles estão presentes no cérebro (Wood e Trayhurn, 2003; Uldry e Thorens, 2004), como o GLUT1, também expresso na barreira hematoencefálica (Wood e Trayhurn, 2003); o GLUT3, distribuído no corpo celular de neurônios por todo o sistema nervoso central (Nagamatsu, Sawa *et al.*, 1993; Maher, Vannucci *et al.*, 1994; Choeiri, Staines *et al.*, 2002); o GLUT4, expresso numa localização somatodendrítico citoplasmática (El Messari, Leloup *et al.*, 1998; Choeiri, Staines *et al.*, 2002; El Messari, Ait-Ikhlef *et al.*, 2002); e o GLUT8, presente nos compartimentos intracelulares de neurônios (Piroli, Grillo *et al.*, 2002).

O GLUT4, um transportador de glicose insulino-sensível, pode estar distribuído no cérebro (Rayner, Thomas *et al.*, 1994), mas também no tecido adiposo, coração e músculo esquelético, onde é responsável pela redução dos níveis de glicose no plasma na condição pós-prandial. Neste caso, a insulina age estimulando a translocação de vesículas contendo GLUT4 para a membrana plasmática (Bryant, Govers *et al.*, 2002; Wood e Trayhurn, 2003).

Alguns estudos, investigando a expressão de GLUTs no diabetes mostram uma discrepância na compreensão dos efeitos da concentração de glicose no cérebro (Shah, Desilva *et al.*, 2012). Um exemplo disso é a detecção de níveis aumentados de GLUT4 no cerebelo de ratos db/db e diminuídos em ratos com diabetes induzido por estreptozotocina (Vannucci, Koehler-Stec *et al.*, 1998).

1. 7 *Vochysia rufa* e as plantas no tratamento do *diabetes mellitus*

A hiperglicemia crônica em consequência do diabetes implica em um dano neuronal direto que leva à alteração da função dos neurotransmissores (Saller, 1984; Bitar, Koulu *et al.*, 1986; Trulson, Jacoby *et al.*, 1986), redução da atividade motora (Marshall, Friedman *et al.*, 1976; Marshall, 1978) e disfunção cognitiva (Mccrimmon, Ryan *et al.*, 2012). Estudos recentes têm sido conduzidos para avaliar

os efeitos da administração de extrato bruto ou princípio ativo de diferentes plantas no controle da glicemia, como também analisar o seu efeito no cérebro. Nesse sentido, tem sido mostrada a ação antioxidante do extrato de chá branco (*Camellia sinensis*) (Espinosa, Lopez-Jimenez *et al.*, 2013); os efeitos sobre a disfunção cognitiva do extrato de raiz de ginseng (*Panax ginseng*) (Al-Hazmi, Rawi *et al.*, 2013); a inibição de radicais livres e aumento da atividade de enzimas antioxidantes do ácido cafeíco de *Abacopteris penangiana* (Fu, Du *et al.*, 2013); a redução na liberação de espécies reativas do oxigênio, TNF- α e óxido nítrico como um efeito protetor de *Rehmannia glutinosa* (Tian, An *et al.*, 2006); a atenuação do aumento da expressão de produtos finais de glicação avançada e NF κ B após o uso de semente de uva (*Vitis vinifera*) (Xu, Li *et al.*, 2008); como também, o efeito contra danos oxidativos, alteração da expressão de miosina-Va e a perda neuronal no hipocampo com a administração de *Pouteria ramiflora* (Da Costa, Calabria *et al.*, 2013).

As plantas têm um papel importante no tratamento do diabetes, particularmente em países em desenvolvimento, onde o acesso a medicamentos tradicionais é geralmente restrito à populações de maior poder aquisitivo. Portanto, estudo de plantas no desenvolvimento de tratamentos alternativos para o controle do *diabetes mellitus* e da obesidade é crucial (Manzan, Carneiro *et al.*, 2002; Ali, Houghton *et al.*, 2006).

No Cerrado brasileiro, a espécie *Vochysia rufa*, conhecida popularmente como “quina doce” (Figura 06), tem sido utilizada por pacientes diabéticos para o controle da glicemia. Frente a isso, o nosso estudo foi baseado no relato de experiência do médico Dr. Ricardo Rodrigues que atende pacientes com *diabetes mellitus* do tipo 1 em uma Unidade de Saúde da Prefeitura de Uberlândia, Minas Gerais, que relatam o uso de um macerado aquoso da casca de *V. rufa*. Apesar da ausência de estudos científicos, a prescrição de insulina a estes pacientes foi reduzida em 50%.



Figura 06: Espécie *Vochysia rufa* fotografada em Abadia dos Dourados, Minas Gerais (Gouveia, 2012).

A família Vochysiaceae abrange seis gêneros e cerca de 200 espécies neotropicais, com poucos representantes em regiões subtropicais. Entretanto, o gênero *Vochysia* compreende cerca de 100 espécies características por serem grandes árvores e arbustos que ocorrem ao longo da América Tropical, do México ao Peru. Várias destas espécies são utilizadas por comunidades indígenas na América do Sul para uma variedade de aplicações terapêuticas relacionadas com a inflamação (Schultes e Raffauf, 1990), devido à presença do ácido betulínico (Weniger, Lobstein et al., 2005). As espécies desse gênero também são utilizadas no tratamento de feridas da pele e para aliviar doenças respiratórias, como asma e congestão pulmonar (Schultes e Raffauf, 1990); apresentam atividade antiulcerogênica gástrica devido à presença de triterpenos pentacíclicos, como o ácido betulínico, ácido betulínico epi, eritrodiol e misturas de derivados ursólicos e oleanólicos (Gomes Rde, Bonamin et al., 2009); além do tratamento de resfriados e como vermífugos (Silva, 2009).

Administração de extratos aquosos de *Vochysia rufa* a ratos tratados com estreptozotocina para indução de diabetes (ratos diabetizados) e tratados com extrato aquoso de *Vochysia rufa* levou a uma diminuição da glicemia. No pâncreas, os efeitos do tratamento com o extrato da planta revelaram um controle da atividade antioxidante, especialmente em relação aos níveis das enzimas glutathione peroxidase e glutathione S-transferase, além de alguns parâmetros bioquímicos no soro (Gouveia, 2012). No fígado, Moraes, (2013) determinou que o tratamento com

o extrato de *Vochysia rufa* teve um efeito antioxidante, diminuindo os níveis de peroxidação lipídica e aumentando os níveis de algumas enzimas, além de conferir resistência à apoptose. Em estudos com células somáticas de *Drosophila melanogaster*, Moraes (2010) avaliou diferentes doses do extrato aquoso de *Vochysia rufa* quanto ao seu potencial mutagênico, sua ação anti-mutagênica e anti-recombinogênica contra os efeitos recombinogênicos do cloridrato de doxorrubicina. Na glândula parótida, análises morfométricas mostraram que o diabetes altera o diâmetro de células acinares, porém o tratamento com o extrato de *Vochysia rufa* não modifica este quadro (Caixeta, Moura *et al.*, 2012); resultado observado também em análises feitas em ilhotas de Langerhans no pâncreas (Rodvalho, 2013).

O estudo apresentado nos seguintes capítulos desta dissertação avaliaram o potencial hipoglicemiante e antioxidante dos extratos de *Vochysia rufa* no cérebro de ratos diabetizados.

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Capítulo 2

Tratamento com glibenclamida modula a expressão e localização de miosina-II β no cérebro de ratos diabéticos

1. ARTIGO CIENTÍFICO

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GLIBENCLAMIDE TREATMENT MODULATES EXPRESSION AND LOCALIZATION OF MYOSIN-II β IN DIABETIC RAT BRAIN

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Abstract

Myosin-IIB is a non-muscle isoform in the brain with increased expression in the brains of diabetic rats. Chronic hyperglycemia caused by diabetes can impair learning and memory. Oral hypoglycemic agents such as glibenclamide have been used to control hyperglycemia. We report changes in the expression and distribution of myosin-IIB in the frontal cortex and hippocampus of diabetic rats treated with glibenclamide. To establish this, brains were harvested after 43 days of treatment with glibenclamide (6 mg/kg bw orally), homogenized and analyzed by Western blotting, qRT-PCR and immunohistochemistry. As expected, myosin-IIB expression increased in the brains of diabetic rats. However, protein levels returned to normalcy after treatment with glibenclamide. In addition, *MYH10* gene expression decreased in diabetic rats treated with glibenclamide. Moreover, we found weak myosin-IIB labeling in the hippocampus and frontal cortex of rats treated with glibenclamide. Therefore, the expression of myosin-IIB is affected by diabetes mellitus and may be modulated by glibenclamide treatment in rats. Structural changes in the hippocampus and prefrontal cortex are reversible, and glibenclamide treatment may reduce patho-physiological changes in the brain. Our findings suggest a possible correlation between glibenclamide effects and myosin-IIB function in the brain of diabetised rats.

Keywords

Diabetes mellitus; Streptozotocin; Glibenclamide; Brain; Myosin-IIB; Hippocampus.

Introduction

Myosins are a superfamily of diverse proteins that are localized in different cell types and specialized areas. These proteins are involved in various pathways such as motility, mechanoenzymology, interactions with loads and the endomembrane system (Hartman & Spudich 2012). Some myosins are linked to vesicle trafficking (myosin-Va), endosome and AMPA receptor recruitment (myosin-Vb), endocytic trafficking in neurons (myosin-VI), GLUT4 traffic (myosin-Ic) and actin dynamics in neurons (myosin-IIb) (Hartman & Spudich 2012, Hirokawa *et al.* 2010, Rex *et al.*, Rex *et al.* 2010).

Myosin-II exist in three non-muscle isoforms: IIA, IIB and IIC. The motor activity of these proteins is regulated by the phosphorylation of a common pair of regulatory myosin light chains (Vicente-Manzanares *et al.* 2009, Yu *et al.* 2012). Myosin-IIb is the predominant form in the brain and is involved in vesicle transport in ganglion neurons, retrograde flow of growth cones, the phosphorylation status of the dendritic spine, cell migration and cytokinesis during cell division (Lo *et al.* 2004, Yang *et al.* 2012, Hodges *et al.* 2011, Takagishi *et al.* 2005, Golomb *et al.* 2004).

Recently, we showed the differential expression of myosins such as myosin-IIb (Calabria *et al.* 2011) and myosin-Va (da Costa *et al.* 2011, da Costa *et al.* 2013) in the brains of rats with streptozotocin-induced diabetes. Studies have shown that adults with type 1 and type 2 diabetes have compromised memory and executive function, which are associated with an increased risk of cognitive dysfunction. This damage is associated with chronic hyperglycemia and the long duration of diabetes (for reviews, see McCrimmon *et al.* 2012)

Several hypoglycemi-inducing drugs have been used by diabetics, such as metformin, gliclazide and glibenclamide (Association 2012). Glibenclamide is a sulphonylurea, that acts on pancreatic beta cells by depolarizing cell membranes and releasing insulin to control diabetes (Serrano-Martin *et al.* 2006, Sokolovska *et al.* 2012). There is evidence that glibenclamide protects against changes caused by diabetes by reducing glomerular hypertrophy and tubular degeneration (Akbar *et al.* 2012), decreasing glycosylated hemoglobin, increasing high density lipoprotein cholesterol (Wang *et al.* 2012) and reducing beta-thromboglobulin levels (Asad *et al.* 2011). In this study, we evaluated the effects of glibenclamide, administered

orally over 43 days for the treatment of hypoglycemia, on the expression and localization of myosin-IIb in the brains of rats with streptozotocin-induced diabetes.

Materials and methods

Animals

Male Wistar rats (200 – 220 g) were housed under standard conditions (22±1°C, humidity 60±5% and 12 h light/12 h dark cycle) with food and water *ad libitum*. Procedures for animal handling and use followed the resolutions proposed by the Brazilian Society of Science on Laboratory Animals and by the Ethics Committee on Animal Research of the Federal University of Uberlândia, Brazil (CEUA/UFU 060/10).

Induction of diabetes mellitus

The rats were let to fast for 24-h, anesthetized by intraperitoneal injection of xylazine (10 mg/kg)/ ketamine (75 mg/kg) and injected with streptozotocin (40 mg/kg bw, 0.01 M citrate buffer, pH 4.5; Sigma-Aldrich, USA) via the penile vein (2 mL/kg bw). Non-diabetic rats were injected with the same volume of citrate buffer. Fasting was maintained for another 90 min post injection. Fasting blood glucose levels above 250 mg/dL were scored as diabetic. Glycemic levels were monitored for an additional 10 days with Biocheck Glucose Test Strips (Bioeasy, Brazil).

Groups and glibenclamide treatments

Animals were randomly separated into three groups ($n = 10$ rats/group): non-diabetic (ND), diabetic (D) and diabetic treated with glibenclamide (6 mg/kg bw orally) (DG). Water and glibenclamide were administered by oral gavage for 43 days. Body weight was recorded weekly. After this period, the animals were fasted for 12-h, anesthetized and then had their brains surgically removed.

Western blotting

The brains were homogenized in a homogenization buffer (0.2 M phosphate buffer, pH 7.4, 2 mM dithiothreitol, 1 mM benzamide, 0.5 mM phenylmethane sulfonyl-fluoride, 0.1 M aprotinin, 0.5 mM pepabloc) and centrifuged at 5,000×g for 10 min at 4 °C. The protein content of the supernatants was estimated using the (Bradford

1976) assay. The expression of myosin-IIb relative to beta-actin was analyzed by western blot as described previously by Calábria *et al.* (2011). The intensity of the protein bands was analyzed and compared using Scion Image software, version Alpha 4.0.3.2 (Scion Corporation, USA) and the results were expressed as a percentage of total protein content.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from the brains using TRIzol® reagent following the manufacturer's instructions (Invitrogen, USA). RNA samples (2 µg) were reverse-transcribed using MMLV-RT (Amersham Biosciences, Sweden). Standards were prepared by cloning PCR products of MYH10 and housekeeping gene β -2-microglobulin (B2M) fragments using a TOPOTA Cloning Dual Promoter Kit (Invitrogen, USA). Plasmid concentration was measured and the copy numbers calculated according to Yin *et al.* [26]. A MYO2 and B2M construct at a concentration of 7 copies per µL was serially diluted to create a standard curve ranging from 1 to 10. The efficiency of reaction was calculated using $E = (10^{-1/\text{slope}} - 1) \times 100$, where the log of each dilution was plotted against ΔCT (see below) of the housekeeping and target genes. To determine MYO2 cellular expression, quantitative RT-PCR analysis was performed using a 7300 Real Time PCR System (PE Applied Biosystems, CA) and SybrGreen PCR Core Reagent (PE Applied Biosystems, CA). PCR Primers were designed using the Primer Express 3.0 Program (PE Applied Biosystems, CA). The set of primers used for the B2M fragment amplification was: 5'-CGTCGTGCTTGCCATTCA-3' and 5'-TCCTCAACTGCTACGTGTCTCAG-3'. The MYO2 forward and reverse primers were: 5'-CCATGCCGGAGAACACAGT-3' and 5'-AAGCCCAGACCAAAGAGCAG-3', respectively. The relative expression of each specific product was calculated by $2^{-\Delta\Delta CT}$ (CT = the fluorescence threshold value; ΔCT = CT of the target gene - CT of the reference gene (B2M); $\Delta\Delta CT$ = ΔCT of the target sample - ΔCT of the calibrator sample).

Immunohistochemistry

The brains were fixed with 10% formaldehyde solution in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h, dehydrated in ethanol, rinsed in xylene and embedded in

paraffin at room temperature. Five micrometer sections were pretreated in a microwave for 5 min with 4 mM citrate buffer (pH 6.0) containing 0.025% Tween 20. The sections were then incubated with anti-myosin-IIB primary antibody for 16 h followed by incubation with a Post Primary Block NovoLink™ Max Polymer Detection System (Novo castra Laboratories Ltd., United Kingdom). After three washes with TBS-T, the sections were incubated with the NovoLink polymer for 30 min at 37 °C. Chromogen was developed using 3, 3'-diaminobenzidine and then the material was counterstained with hematoxylin, dehydrated, mounted with ERV-Mount (EasyPath, Brazil) and analyzed with the 40x and 100x objective lenses of a Leica DM500 microscope (Microsystems, Germany). Negative controls were prepared by omitting the primary antibody from the reaction.

Statistical analysis

All values obtained were expressed as mean±SEM (standard error of the mean). Statistical analysis was performed with the Student t-test and data were analyzed using SigmaStat 3.5 software (Systat Software Inc., USA). A *p*-value <0.05 was considered significant.

Results

In this study, correlation between myosin-IIB expression and oral administration of glibenclamide in the brain of diabetic rats. Figure 2 shows differential expression of the myosin-IIB protein was analyzed by Western blotting. Immunoreactivity of myosin-IIB increased by ~20% in diabetic compared to non-diabetic rat brains. Interestingly, these levels reached normalcy after glibenclamide treatment.

Real time PCR was performed to measure changes in myosin-IIB gene (*MYH10*) levels (Fig. 1). These changes were calculated using beta-2-microglobulin (*B2M*) as an endogenous control. *MYH10* mRNA levels in diabetic brains increased by ~140% compared to non-diabetic brains. However, *MYH10* mRNA concomitant glibenclamide treatment reduced *MYH10* levels by ~65%.

Morphology was evaluated by hematoxylin and eosin staining and distribution of the myosin-IIB protein was analyzed by immunohistochemistry (Figs. 3 and 4). The nuclear diameter of neurons in diabetic rats appeared to be smaller

than in non-diabetic rats. The same was not observed in the cortex of diabetic rats treated with glibenclamide (Fig. 3). Immunostaining in diabetic rats revealed intense myosin-IIB labeling in the frontal cortex, mainly in pyramidal neurons and their projections (Fig. 3). Although the distribution of myosin-IIB in the brain was similar for all groups, different staining patterns may be linked to the function of myosin-IIB. The distribution of myosin-IIB in other regions of the brain was the same for non-diabetic and diabetic rats. Myosin-IIB staining was more intense in the dentate gyrus of the hippocampus of diabetic rats than it was in non-diabetic rats (Fig. 4). Otherwise, immunolabeling was faint in the cortex and hippocampus of diabetic rats treated with glibenclamide. Moreover, immunolabeling was more intense in the granule cell layer and polymorphic layers the diabetic group compared to non-diabetic and glibenclamide-treated groups. In mossy cells of the dentate gyrus, myosin-IIB was distributed mainly in the cytoplasm.

Discussion

In this study, we demonstrated that diabetic rats treated with glibenclamide for 43 days had lower levels of myosin-IIB protein and mRNA expression in the brain and weaker immunolabeling in the frontal cortex and hippocampus compared to a diabetic control group.

All forms of diabetes are characterized by chronic hyperglycaemia that may be linked to neurodegeneration (de la Monte 2009), learning and memory deficit (McCrimmon *et al.* 2012), and neurophysiological and structural changes in the brain (Biessels *et al.* 2002). These changes are especially apparent in certain regions such as the hippocampus and cerebral cortex (Baydas *et al.* 2003, Biessels *et al.* 1996, Hasanein & Shahidi 2010). These regions are affected particularly because they are susceptible to metabolic (McCrimmon *et al.* 2012) and mitochondrial changes (Ceretta *et al.* 2010). Our results confirm that diabetes alters myosin-IIB gene and protein expression in diabetic rat brains, and reveal that glibenclamide treatment reestablishes normal levels of this protein and significantly reduces gene expression. Nevertheless, the aim of this study was not to disclose the accuracy of glibenclamide as an antidiabetic, but to link the effects of this hypoglycemic to myosin-IIB expression in the brain.

Glibenclamide is one of the most widely used antidiabetic drugs and the most popular sulfonylurea. It produces effects consistent with ATP-K⁺ channel inhibition (Schmid-Antomarchi *et al.* 1987, Sturgess *et al.* 1988, Zunkler *et al.* 1988, Zunkler *et al.* 1989) that stimulate insulin secretion (Serrano-Martin *et al.* 2006, Sturgess *et al.* 1988) and consequent hypoglycaemic activity. Sulfonylurea biochemically increased myosin ATPase activity (Mozaffari *et al.* 1988), which was discovered by studying the mechanochemical cycle of myosin-IIb (Rosenfeld *et al.* 2003). However, the precise molecular mechanism for drug-mediated activity modulation is still unknown.

Glibenclamide also regulates the expression of GLUT-1 in the kidneys, heart and liver of diabetic rats (Sokolovska *et al.* 2012). It has anti-mutagenic and antioxidant properties (Rabbani *et al.* 2010) and can interfere with mitochondrial bioenergetics in nonpancreatic cells by inducing changes in membrane ion permeability (Fernandes *et al.* 2004). Some classes of myosins, such as I (Bose *et al.* 2002), II (Fulcher *et al.* 2008, Steimle *et al.* 2005) and V (Yoshizaki *et al.* 2007) are involved in insulin-induced GLUT4-vesicle fusion/translocation and/or directly in diabetes.

Recently, we demonstrated that members of the myosin family such as myosin-IIb (Calabria *et al.* 2011) and myosin-Va (da Costa *et al.* 2013, da Costa *et al.* 2011) are differentially expressed in the brains of rats with streptozotocin-induced diabetes. Indeed, these molecular motors have been identified in pancreatic islets (Espindola *et al.* 2008, Wilson *et al.* 1998) and identified by their involvement in the migration of insulin secretory granules (Lajoix *et al.* 2006, Lida *et al.* 1997). Myosin-II is abundantly expressed in the brain by three distinct isoforms present in isolated post-synaptic densities of mature forebrain synapses (Cheng *et al.* 2006, Cheng *et al.* 2000, Miyazaki *et al.* 2000). Myosin-IIb is a necessary component of memory formation and synaptic plasticity in the mature nervous system (Rex *et al.* 2010).

Brain morphology was evaluated by H.E. staining, which revealed differences in the nuclear diameters of neurons. The neurons of diabetic rats appear to be smaller and have more intense hematoxylin coloration than the neurons of non-diabetic rats. However, this difference did not exist between the neurons of diabetic rats and those treated with glibenclamide. These alterations may be linked to apoptosis and aggregated chromatin (Ye *et al.* 2011) or could be a reflection of

increased metabolism and protein synthesis in response to increased neuronal activity (da Costa et al. 2013).

In our conditions, no change in protein distribution were observed either in the frontal cortex, in accordance with Calabria et al (2011), or in the hippocampus. Although the distribution of myosin-IIB was similar in the brains of diabetic, control and treated animals, differences in the staining pattern may be linked to myosin-IIB function. This difference may be associated with myosin-IIB function in these regions (Calabria *et al.* 2011, Rochlin *et al.* 1995).

Myosin-IIB plays important roles in migrating neurons and growth cones (Vallee *et al.* 2009) and has been detected in postsynaptic dendrites (Cheng et al. 2006, Miyazaki et al. 2000), where it modulates development of these structures (Ryu *et al.* 2006). Myosin-IIB is widely distributed within the neurons where it provides a structural mechanism for synaptic plasticity (Cheng et al. 2000). We showed that myosin-IIB is mainly distributed in the projections of pyramidal neurons in the cerebral cortex of diabetic rats and that staining decreases after glibenclamide treatment.

Myosin-IIB was localized in the hippocampus, mainly in mossy cells, and in neurons of the granule and polymorphic cell layers. Immunolabeling was more intense in diabetic rat brains than in non-diabetic ones. Many studies have found changes in the hippocampus of diabetics, such as decreased spine density on pyramidal neurons (Martinez-Tellez *et al.* 2005), synaptic reorganization (Grillo *et al.* 2005), increased neuronal vulnerability (Revsin *et al.* 2005, Saravia *et al.* 2002), reduced cell proliferation and neurogenesis (Alvarez *et al.* 2009, Beauquis *et al.* 2006), neuronal apoptosis (Li *et al.* 2002), and increased nuclear diameters of neurons (da Costa et al. 2013). A known function of myosin-II in CA1 pyramidal neurons is to contribute to an actin-dependent process that underlies memory consolidation in response to synaptic stimulation (Rex et al. 2010). It is possible that myosin-II has unknown systems level functions that account for disruption in hippocampus-dependent memory consolidation (Rex et al. 2010).

We showed that myosin-IIB levels in the hippocampus recover in diabetic rats treated with glibenclamide. In this region of the brain, ATP-K⁺ channels are probably located on presynaptic mossy fibers and there is evidence that sulphonylurea increase the glutamate released from these fibers (Ben-Ari 1990). Moreover, high-

affinity glibenclamide binding sites were found in the brain, especially in the cerebral cortex and hippocampus (Treherne & Ashford 1991). These discoveries are important because they indicate a possible regulation mechanism specific to myosin-IIb. This is also significant because of a recent study showing that acarbose treatment, an inhibitor of α -glycosidase used for glycemia control (Van de Laar *et al.* 2006), is incapable of restoring myosin-Va levels in the hippocampus (da Costa *et al.* 2013).

In summary, myosin-IIb expression is affected by diabetes mellitus and may be modulated by glibenclamide treatment in rats. Structural changes in the hippocampus (Luine *et al.* 1994) and prefrontal cortex (Radley *et al.* 2005) are reversible. Additionally, glibenclamide can reverse damage caused by diabetes on myosin-IIb expression and reduce patho-physiological changes in the brain. Finally, these results contribute to the knowledge base on myosin regulation in the brains of diabetic rats.

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Conflict of interest

The authors are not having any conflict of interest related to this paper.

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Figures

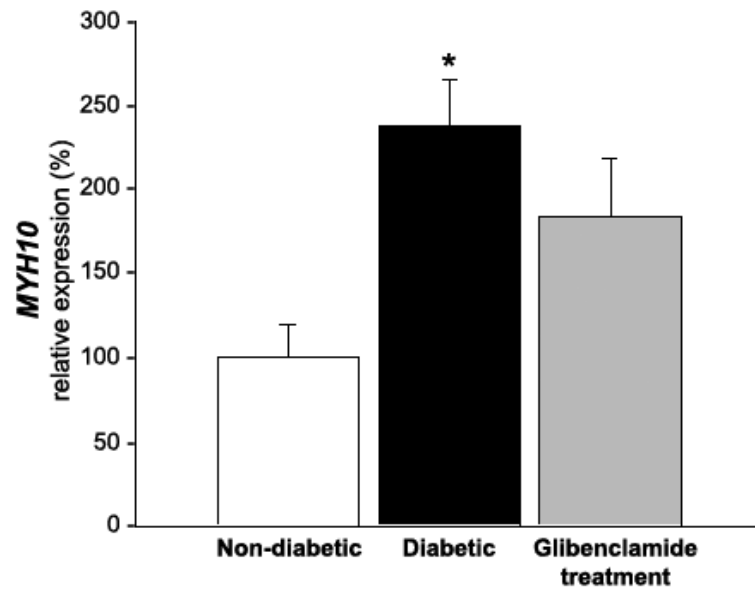


Fig. 1 *MYH10* expression in the brains of non-diabetic, diabetic and diabetic rats treated with glibenclamide. The histograms show significantly higher upregulated *MYH10* mRNA expression in the diabetic brain samples than in the non-diabetic brain samples. (*) $p < 0.05$, $n = 4$ rats/group.

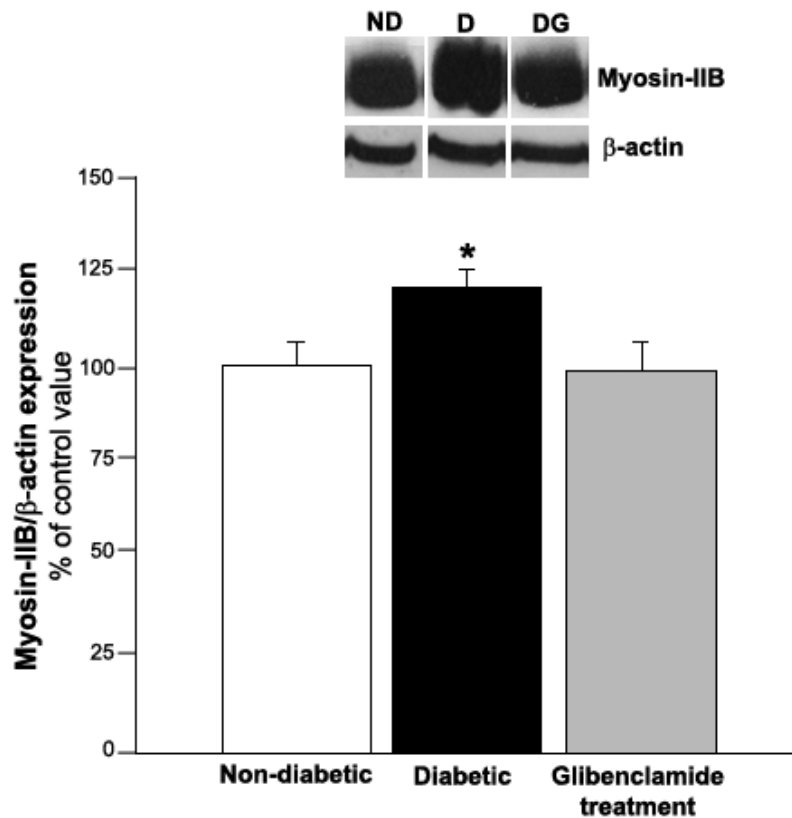


Fig. 2 Western blot and quantification of myosin-IIB expression from supernatant brain samples of non-diabetic (ND), diabetic (D) and diabetic rats treated with glibenclamide (DG) after 43 days. Myosin-IIB protein levels on the immunoblot were determined by densitometric quantification and expressed as a percentage within groups. Values represent mean $\pm$ SEM. *Represents significant mean differences between D vs. other groups ($p<0.05$), $n= 4$ rats/group.

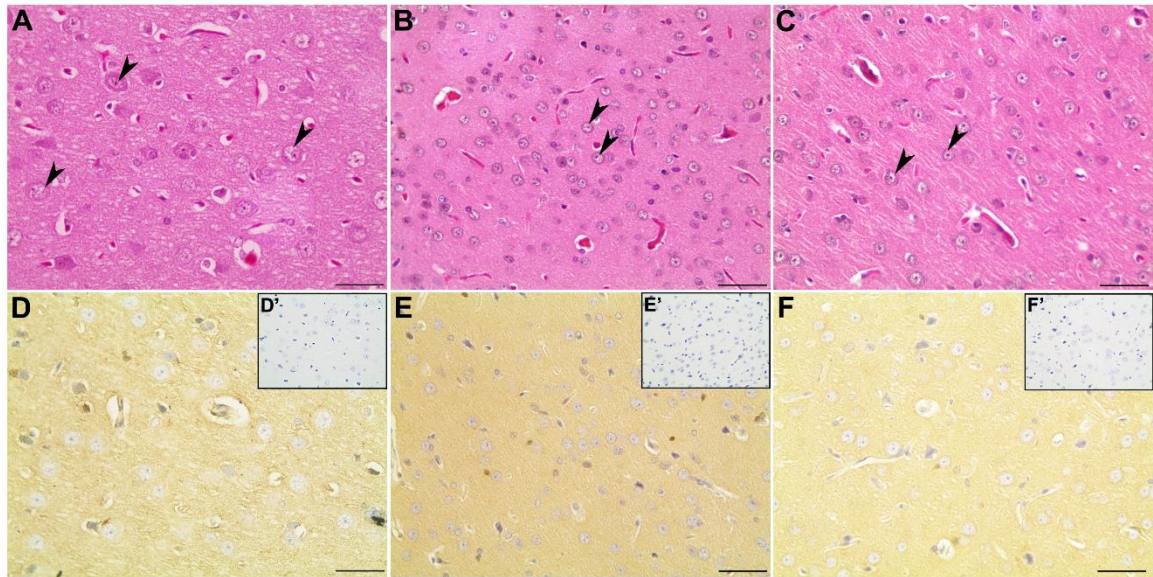


Fig. 3 Myosin-IIB hematoxylin and immunolocalization and eosin stain in sections of the frontal cortex of rat brains: non-diabetic (A; D; D'), diabetic (B; E; E') and diabetic treated with glibenclamide (C; F; F'). Neuronal cell diameter (arrowhead) differed among groups (A; B; C). Myosin-IIB (brown immunostaining) is mainly distributed in the projections of pyramidal neurons. All experiments were repeated twice with similar results. Negative control: D'; E'; F'. Bar: 25 μ m.

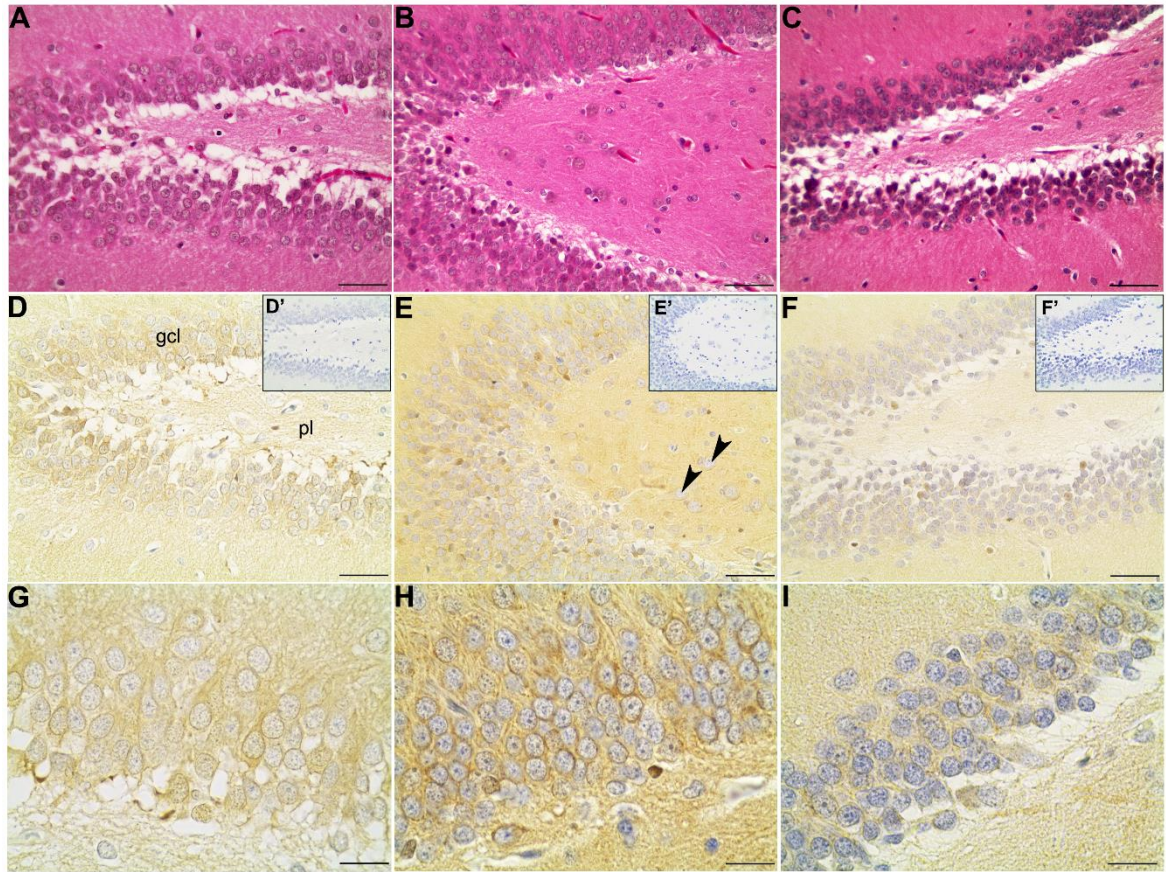


Fig. 4 Myosin-IIB distribution and hematoxylin and eosin staining in the hippocampus of non-diabetic (A; D; D'; G), diabetic (B; E; E'; H) and diabetic rats treated with glibenclamide (C; F; F'; I). Myosin-IIB staining (brown immunostaining) differed in regions of the dentate gyrus. Staining was more intense in the granule cell layer (gcl) and polymorphic layer (pl) in the diabetic group than in the same layers in the non-diabetic and diabetic treated with glibenclamide groups. Myosin-IIB was mainly distributed in the cytoplasm of the mossy cells (arrowhead) in dentate gyrus. All experiments were repeated twice with similar results. Detail of the granule cell layer: G; H; I. Negative control: D'; E'; F'. Bar: 25 µm (A-F), and 50 µm (G-I).

Capítulo 3

Extrato aquoso de *Vochysia rufa* promove expressão diferencial de proteínas envolvidas na formação e tráfico de vesículas no cérebro de ratos diabéticos

1. ARTIGO CIENTÍFICO

Journal of Diabetes and its Complications (fator de impacto: 2,032)

AQUEOUS EXTRACT OF *Vochysia rufa* PROMOTES DIFFERENTIAL EXPRESSION OF PROTEINS IMPLICATED IN VESICULAR TRAFFICKING IN DIABETIC RAT BRAIN

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Abstract

Introduction: Diabetes increases oxidative stress and causes several changes in protein transport and docking of synaptic vesicles. Plant extracts have long been used to treat hyperglycemia and diabetes. Aqueous extracts of *Vochysia rufa*, a species endemic to the Brazilian Cerrado ecosystem, have increasingly been prescribed and used as a complementary or alternative treatment. **Objective:** We examined the effects of an aqueous extract of *Vochysia rufa* on oxidative stress markers and on the expression and localization of transport proteins and synaptic vesicle docking in the diabetic rat brain. **Design/Methods:** Thirty-two male Wistar rats were randomly divided into 4 groups: non-diabetic, diabetic, diabetic treated with glibenclamide (6 mg / kg bw orally) and diabetic treated with *Vochysia rufa* extract (500 mg/kg bw orally) for forty-three days. After the treatments, brains were collected and the following parameters were evaluated: enzymatic activity of glutathione peroxidase and glutathione S-transferase, superoxide dismutase concentration, total sulfhydryl, lipid peroxidation and reduced glutathione. The levels and localization of proteins such as CaMKII, myosin-Va, synapsin-1, SNAP-25 and GLUT4 were also analyzed. **Results:** *Vochysia rufa* extract decreased SOD, GSH, TBARS and total sulfhydryl levels and increased GST levels. Additionally, myosin-Va and synapsin-1 expression decreased and levels proteins such as CaMKII and SNAP-25 increased. These results were confirmed by immunolocalization. **Conclusion:** The results suggest that an aqueous extract of *Vochysia rufa* has a neuroprotective effect on diabetic rat brains by reducing oxidative stress and controlling changes in myosin-Va and synapsin-1 expression.

Keywords

Diabetes mellitus; Oxidative stress; Myosin-Va; CaMKII; SNAP-25; Synapsin-1; GLUT4.

Introduction

Diabetes is characterized by persistent hyperglycemia, which leads to increasing reactive oxygen species and consequent changes tissue damage (see review, Forbes and Cooper, 2013; Maritim et al., 2003; Ugochukwu and Cobourne, 2003). Diabetes causes memory and learning impairment (Biessels et al., 1994; McCrimmon et al., 2012) as well as morphological changes such as decreased nuclear diameter of pyramidal neurons of the hippocampus (da Costa et al., 2013). Hyperglycemia leads to imbalance in the oxidative status of the neural tissue, causing an increase in free radical production via auto-oxidation of glucose, glycation of proteins, and low production of antioxidant defenses (see review, Maritim et al., 2003). The concentration of these reactive oxygen species is controlled by antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Robertson et al., 2004).

Hyperglycemia alters cellular redox balance and protein trafficking involved in synaptic vesicle docking. This may be due to changes in expression of myosins (Calabria et al., 2011; da Costa et al., 2011; da Costa et al., 2013), GLUT4 (Vannucci et al., 1998), presynaptic proteins, such as synapsin-1, synaptophysin, and SNAP-25 (VanGuilder et al., 2008). Hyperglycemia also decreases CaMKII phosphorylation in the hippocampus, which may be associated with cognitive dysfunction (Liao et al., 2013).

Many plants have hypoglycemic properties (Negri, 2005) and are less toxic than oral hypoglycemic agents, such as sulfonylureas and metformins (Chattopadhyay, 1993; Ponnachan et al., 1993). Thus, several studies have been conducted on using plant extracts to alleviate oxidative stress (see review, Chang et al., 2013; Taleb-Senouci et al., 2009). The present study was designed to evaluate the effect of *Vochysia rufa* treatment on oxidative stress and the expression of proteins implicated in vesicular trafficking such as myosin-Va, CaMKII, synapsin-1, SNAP-25, GLUT4, and the distribution of these proteins in the cortex and hippocampal regions of the diabetic rat brain.

Material and methods

Plant and chemical material

Stem bark from *Vochysia rufa* Mart. was collected from the Cerrado (Abadia dos Dourados, MG, Brazil). The material was botanically identified at the Institute of Biology of the Federal University of Uberlandia, (voucher specimen, Herbarium Uberlandense, HUFU, 58,888).

Aqueous extract preparation

Stem barks were dried in an oven at 35 °C for 14 days/hours and then ground to a powder using an electric mill. The powder (200 g) was macerated in distilled water for 24 h (1:5 w/v) and the corresponding extract was filtered and centrifuged at 4,400xg for 15 min at 4°C. The supernatant was frozen at -20°C and then lyophilized to yield approximately 6% (w/w). The extract was stored at -20°C.

Animals

Male Wistar rats (200-220 g) were housed under standard conditions (22±1 °C, humidity 60±5 %, 12 h light/12 h dark cycle) with food and water *ad libitum*. All procedures for handling, using and euthanizing these animals followed the guidelines proposed by the Brazilian Society of Laboratory Animal Science and the Ethics Committee for Animal Research at the Federal University of Uberlandia, Brazil (CEUA/UFU 060/10).

Induction of diabetes mellitus

Rats were fasted for 24 h, anesthetized via intraperitoneal injection of xylazine (10 mg/kg body weight, bw)/ketamine (75 mg/kg bw) and injected with streptozotocin (40 mg/kg bw, 0.01 M citrate buffer, pH 4.5; Sigma- Aldrich) via the penile vein (2 mL/kg bw). Ten days after injection, rats with fasting blood glucose levels above 250 mg/dL were scored as diabetic. Body weight was recorded and blood glucose was assessed using reactive strips (Contour Glucose Test Strip) (Bayer, Mishawaka, USA).

Groups and treatments

Rats were randomly divided into 4 groups (n=8 rats/group): non-diabetic (ND), diabetic (D), diabetic treated with glibenclamide (6 mg/kg bw orally) (DG), and diabetic treated with *Vochysia rufa* extract (500 mg/kg bw orally) (DV). Four rats per group were used for biochemical analysis and four rats per group were used for histology analysis. The treated groups received glibenclamide or aqueous extract of *Vochysia rufa* for 43 days, while the control groups received water by gavage.

Sample collection

Animals (n=32) were sacrificed on day 43 and their brains were quickly transferred to a fixation buffer containing 10% formaldehyde solution in 0.1 M phosphate-buffered saline (pH 7.4). At the same time, blood was collected from the portal vein to confirm blood glucose levels. For biochemical analysis, each brain was homogenized separately on ice in homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethanesulfonyl-fluoride, 0.1 M aprotinin, 0.1 mM pefabloc). The homogenates were then centrifuged at $15,000 \times g$ for 30 min at 4 °C, and the total protein concentration in the supernatants was measured by the Bradford assay (Bradford, 1976).

Oxidative stress biomarkers

Antioxidant enzyme assays: Superoxide dismutase (SOD) activity was determined by adenocromo inhibition during adrenaline oxidation. The results were expressed as SOD/ μ g of protein (Misra and Fridovich, 1972). Glutathione peroxidase (GPx) activity was quantified by the oxidation of NADPH in the presence of glutathione reductase (GR), measured at 340 nm and expressed in μ mol.min⁻¹.g⁻¹ (Flohe and Gunzler, 1984). GST activity was measured at 340 nm using CDNB as substrate and a 0.15 M GSH (Habig et al., 1974).

Reduced glutathione assay (non-protein thiols): Whole blood levels of GSH were determined using DTNB (2-dithionitrobenzoic acid) (Beutler et al., 1963).

Lipid peroxidation: Brain tissue levels of thiobarbituric acid reactive substances (TBARS) were estimated colorimetrically (Hermes-Lima et al., 1995). The results were expressed in nmol.g⁻¹ tissue.

Sulfhydryl levels: The level of sulfhydryl was determined using 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), as described by Faureand, (1995).

Antibodies

Rabbit affinity-purified polyclonal antibodies were used in this study. Anti-chicken brain myosin-Va (anti-myosin-Va) head domain recombinant protein (Espreafico et al., 1992; Suter et al., 2000) and anti-glucose transporter GLUT4 (α -GLUT4) by Abcam Inc.. Mouse monoclonal antibodies included anti-calcium/calmodulin-dependent protein kinase II α (anti- α CaMKII), synaptosomal-associated protein 25 (anti-SNAP25), and anti-synapsin-Ia (anti-synapsin) by BD Transduction and anti- β -actin by Sigma. Secondary antibodies (anti-mouse IgG and anti-rabbit IgG) conjugated to peroxidase were obtained from GE Healthcare.

Western blotting

Aliquots of supernatant samples were mixed with a small volume of electrophoresis sample buffer containing 100 mM Tris-HCl, pH 8.0, and 25 % glycerol. Supernatant samples containing 30 μ g of protein were analyzed by SDS-PAGE on 5-22 % polyacrylamide gradients and electroblotted onto nitrocellulose membranes in Tris-glycine buffer (Towbin et al., 1992). Blots were incubated with 5 % dried defatted milk in TBS-T (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.05 % Tween 20) and probed with anti-myosin-Va (Espindola et al., 2008), anti- α CaMKII, anti-synapsin, anti-SNAP-25 and anti-GLUT4 primary antibodies. A β -actin antibody was used as a control. Blots were incubated with peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG and washed several times with TBS-T. Antibodies bound to the membranes were visualized by chemiluminescence after treatment with ECLTM (GE Healthcare), followed by exposure to HyperfilmTM (GE Healthcare). The detection time was the same for all samples. Band intensities were analyzed with Scion Image software, version Alpha 4.0.3.2 (Scion Corporation), and the results were expressed as percentages of total content.

Immunohistochemistry

For the histology analysis, brains were dissected and fixed with 10 % formaldehyde solution in 0.1 M phosphate-buffered saline (pH7.4) for 24 h, dehydrated in ethanol,

rinsed in xylene and embedded in paraffin. Seven-micrometer sections were pretreated with 4 mM citrate buffer (pHn 6.0) containing 0.025% Tween 20 in a microwave for 5 min. The sections were then incubated with anti-myosin-Va (Espindola et al., 2008), anti- α CaMKII (BD Biosciences), anti-synapsin (BD Biosciences), anti-SNAP-25 (BD Biosciences) and anti-GLUT4 (Abcam Inc.) primary antibodies for 16 h, followed by incubation with the Post Primary Block NovoLink™ Max Polymer Detection System (Novocastra Laboratories Ltd.). After three washes with TBS-T, the sections were incubated with the NovoLink polymer for 30 min at 37 °C. Chromogen development was performed with 3,3'-diaminobenzidine and the material was counterstained with hematoxylin, dehydrated, mounted with ERV-Mount (EasyPath, Brazil) and analyzed using a Leica DM500 microscope (Microsystems) with a 40× objective lens. Negative controls were prepared by omitting the primary antibody from the reaction.

Statistical analysis

All values were expressed as means $\pm$ SEM. Data were initially analyzed by one-way analysis of variance (ANOVA) using SigmaStat 3.5 software (Systat Software Inc.). Differences between means were detected by ANOVA and tested for statistical significance using the Student's t-test ($p < 0.05$).

Results and discussion

Increased oxidative stress in the central nervous system has frequently been observed in diabetic patients and animal models (Biessels et al., 1994; McCrimmon et al., 2012). This oxidative stress is thought to attack cellular proteins, lipid membranes, nucleic acids and consequently causes cell death (Maritim et al., 2003). Our study aimed to evaluate oxidative stress markers and antioxidant enzyme levels in diabetic rats treated with an aqueous extract of *Vochysia rufa*.

Figure 1 shows GPx, SOD, GST, and GSH activity, antioxidative enzyme concentration, total sulfhydryl and TBARS in the brain. GPx activity decreased in the diabetic as compared to non-diabetic rats. Conversely, SOD, TBARS, GSH and total sulfhydryl levels increased. This result is attributable to a high rate of oxygen consumption and high polyunsaturated fatty acid content especially pronounced in diabetic brains (Valko et al., 2007). It is believed that plants with antioxidant

properties can prevent or protect tissues against the damaging effect of free radicals (Yazdanparast et al., 2007). Treatment with *Vochysia rufa* extract significantly decreased SOD, TBARS and total sulfhydryl and increased GST activity. Zucaro et al. (2000) showed that the genera *Vochysia* has abundant polyphenols and triterpenes, with antidiabetic properties (Sabu et al., 2002). Several studies have shown that plant extracts can reverse changes in the brain caused by diabetes. Curcumin supplements, for example, decrease lipid peroxidation levels in rat brains (Belviranli et al., 2013). *Bacopa monnieri* not only affects lipid peroxidation and GSH levels, but also modulates SOD and GPx activity in different regions of the brain (Kapoor et al., 2009). *Pouteria ramiflora* extract increases GPx and SOD levels and decreases lipid peroxidation in rat brains (da Costa et al., 2013). Glibenclamide, used as a positive control, increased GPx and decreased SOD activity, similar to *Vochysia rufa* treatment. This sulfonylurea affects beta cells by increasing insulin secretion (Serrano-Martin et al., 2006; Sokolovska et al., 2012), which probably reduces oxidative stress by decreasing hyperglycemia.

Diabetes is linked to changes in neurotransmission that may cause cognitive dysfunction (McCrimmon et al., 2012). Neurotransmission is dependent on the formation of a complex in which proteins of the synaptic vesicles and the plasma membrane. However, if one side of this pair is missing, the complex will be unstructured (Jahn and Sudhof, 1999; Lin and Scheller, 2000). Proteins such as myosin-Va, CaMKII, SNAP-25 and synapsin are involved in neurotransmission, whereas GLUT4 participates as an insulin-sensitive glucose transporter. CaMKII is an important enzyme in the regulation of glutamatergic synapses (Lisman et al., 2002), myosin-Va is a molecular motor associated with transport vesicles in neurons (Hirokawa et al., 2010), synapsin is involved in the regulation of exocytotic release vesicles (Ceccaldi et al., 1995) and SNAP-25 participates in SNARE complex assembly (Greaves et al., 2009; Washbourne et al., 2001). Figure 2 shows the immunoreactivities of myosin-Va (190 kDa), α -CaMKII (52 kDa), synapsin-1 (80 kDa), SNAP-25 (25 kDa), GLUT4 (56 kDa) measured densitometrically. Western blot analysis revealed that diabetic rats had decreased myosin-Va protein levels in the brain (~13 %), as compared to the non-diabetic group, whereas treatment with *Vochysia rufa* extract increased myosin-Va (~8%) in controls, as compared to the diabetic group. We have shown that myosin-Va expression changes during

treatment with aqueous *Pouteria ramiflora* extract , likely as a response to the plant's antioxidant and neuroprotective properties (da Costa et al., 2013). Similarly, it is possible that the antioxidant properties of the *Vochysia* genus had the same effect (Gomes Rde et al., 2009).

Treatment with *Vochysia rufa* extract and glibenclamide increased α -CaMKII (~10 %) compared to the diabetic group. *Vochysia rufa* treatment also increased Synapsin-1 (~11 %) relative to the diabetic group. However, there were no differences in SNAP-25 and GLUT4 expression between groups. Oxidative stress associated with the expression of protein vesicles that may affect synaptic function and plasticity (Ansari et al., 2008) or alter the release of vesicles, appears to be a presynaptic action of the reactive oxygen species (Giniatullin et al., 2006). We suggest that, by decreasing oxidative stress, *Vochysia rufa* extract improved the levels of proteins associated with vesicle trafficking and anchoring.

Immunohistochemistry analysis showed that myosin-Va was distributed throughout the frontal (Fig. 3) and occipital cortex (Fig. 5) in non-diabetics with greater concentrations in the cytoplasmic soma and extensions of pyramidal neurons as well as intense perinuclear labeling in the pattern described by Takagishi et al. (2007). Lower levels were observed in the diabetic group, as described by da Costa et al. (2011, 2013), but treatment with either *Vochysia rufa* or glibenclamide reversed this effect. CaMKII distribution was uniform in the frontal cortexes of non-diabetics and diabetics but limited marking in the soma and perinuclear region. Glibenclamide treatments enhanced this labeling in the soma and neuron nucleus compared to non-diabetic, diabetic and diabetic rats treated with *Vochysia rufa* extract. Rats treated with *Vochysia rufa* extract had strong perinuclear distribution of CaMKII, as compared to non-diabetic and diabetic groups, but reduced levels were observed in the soma of glibenclamide-treated groups. (Fig. 3).

Synapsin-1 distribution in the frontal (Fig. 4) and occipital (Fig. 6) cortex of non-diabetics was more intense in the soma and extension of pyramidal neurons and around the cell body, dendrites and axons compared to diabetics. Diabetic rats treated with *Vochysia rufa* extract and glibenclamide showed an increase in labeling similar to non-diabetic rats. SNAP-25 distribution in the frontal (Fig. 4) and occipital (Fig. 6) cortex of the non-diabetics was similar to diabetics with labeling in the soma and extension of pyramidal neurons, and around the dendrites, axons and fibers.

Diabetic rats treated with *Vochysia rufa* extract and glibenclamide had greater labeling intensity, but in locations similar to non-diabetic and diabetic rats. Staining was greater in the neuron nucleus of treated or untreated diabetic rats and staining was more intense in the group treated with *Vochysia rufa* extract (Figs. 4 and 6). GLUT4 distribution in the frontal (Fig. 4) and occipital (Fig. 6) cortex did not differ among groups and was weak and diffuse in the cytoplasm and fibers.

There was no difference between groups in the immunolocalization of α -CaMKII, synapsin-1, SNAP-25 and GLUT4 in the dentate gyrus of the hippocampus (Fig. 7). α -CaMKII and GLUT4 produced low expression levels in all groups and especially in the neuronal cytoplasm of the granular layer, fibers and neuronal cytoplasm of the polymorphic layer. Synapsin-1 and SNAP-25 have strong marking in the neuronal cytoplasm of the granular layer, fibers and cytoplasm of neurons in polymorphic layer (Fig. 7). Therefore, aqueous *Vochysia rufa* extract enhances myosin-Va, synapsin-1 and SNAP-25 expression. These proteins are related to synaptic transmission given that synapsin-1 binds to synaptic vesicles and cytoskeletal components to prevent their migration (Ceccaldi et al., 1995). The loss of synapsin-1 reduced vesicle numbers and could have induced structural changes in nerve terminals (Takei et al. 1995). In our study, synapsin-1 decreased the expression and may have induced plants are constantly used to improve hyperglycemia and thereby improve oxidative stress (Chang et al., 2013; Panickar, 2013; Pari and Latha, 2004).

One study showed that ethanolic and aqueous plant extracts had no effect on the levels of binding protein for complex formation. *Albizzia julibrissin* extract has a high concentration of polyphenols that interfere with and decrease interaction between vesicle proteins (Riley et al., 2006). It was confirmed that the aqueous *Vochysia rufa* extract increase expression levels of the myosin-Va and synapsin-1 and reducing oxidative stress, thus, protects the brain from damage caused by hyperglycemia. Future studies are needed to understand the mechanism of action of *Vochysia rufa* extract in brain of diabetic rats.

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Conflict of interest

The authors are not having any conflict of interest related to this paper.

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Figures

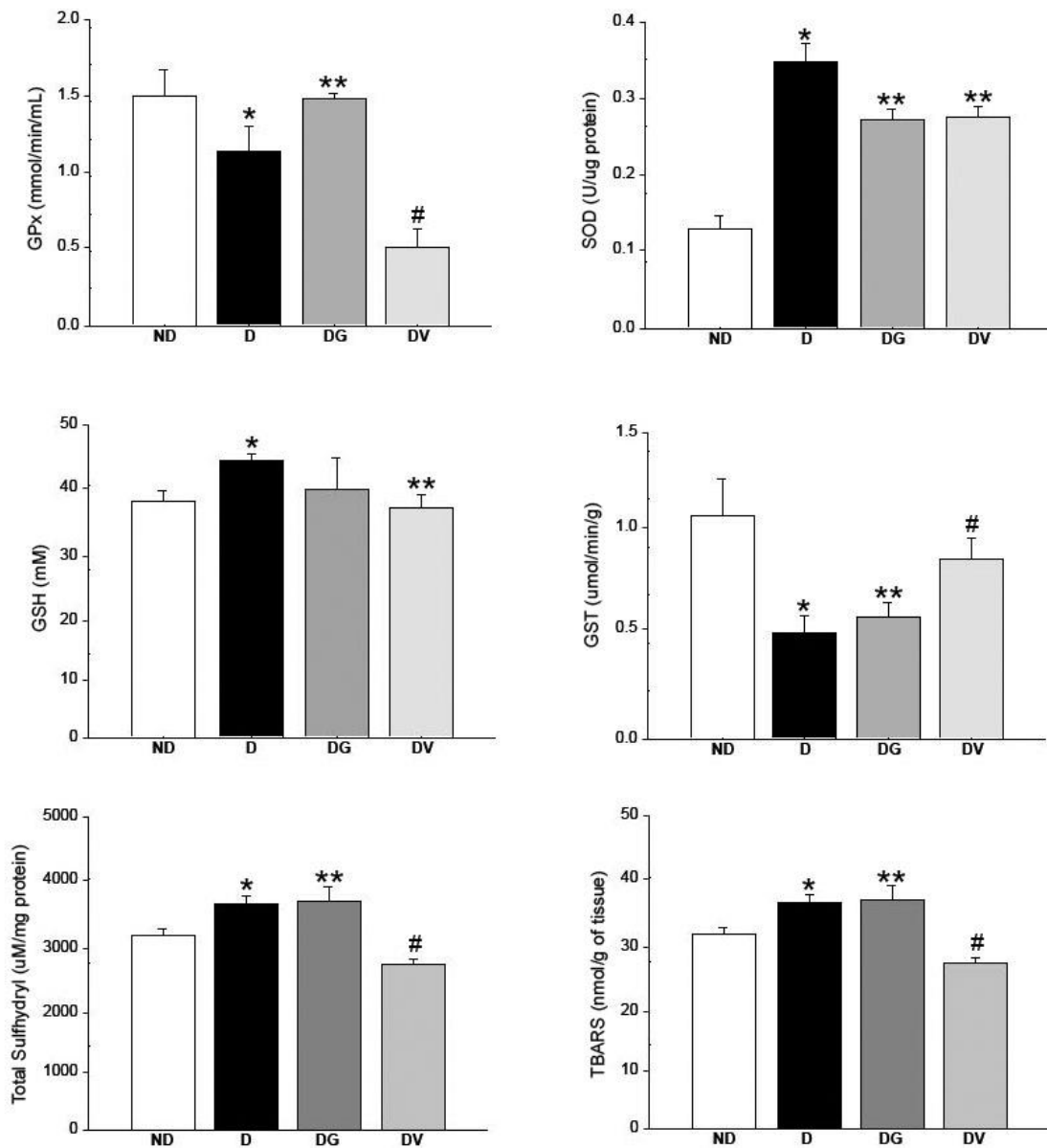


Fig. 01 - Effect of *Vochysia rufa* extract on glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione S-transferase (GST) activity and reduced glutathione (GSH), total sulphydryl and malondialdehyde (TBARS) concentrations in rat brains. Values are given as the mean $\pm$ SEM for groups (n = 4 rats/group). Significant differences are marked: * when compared with non-diabetics (ND) ($p < 0.05$), ** when compared with diabetics (D) ($p < 0.05$), # when compared with diabetics treated with glibenclamide (DG) ($p < 0.05$).

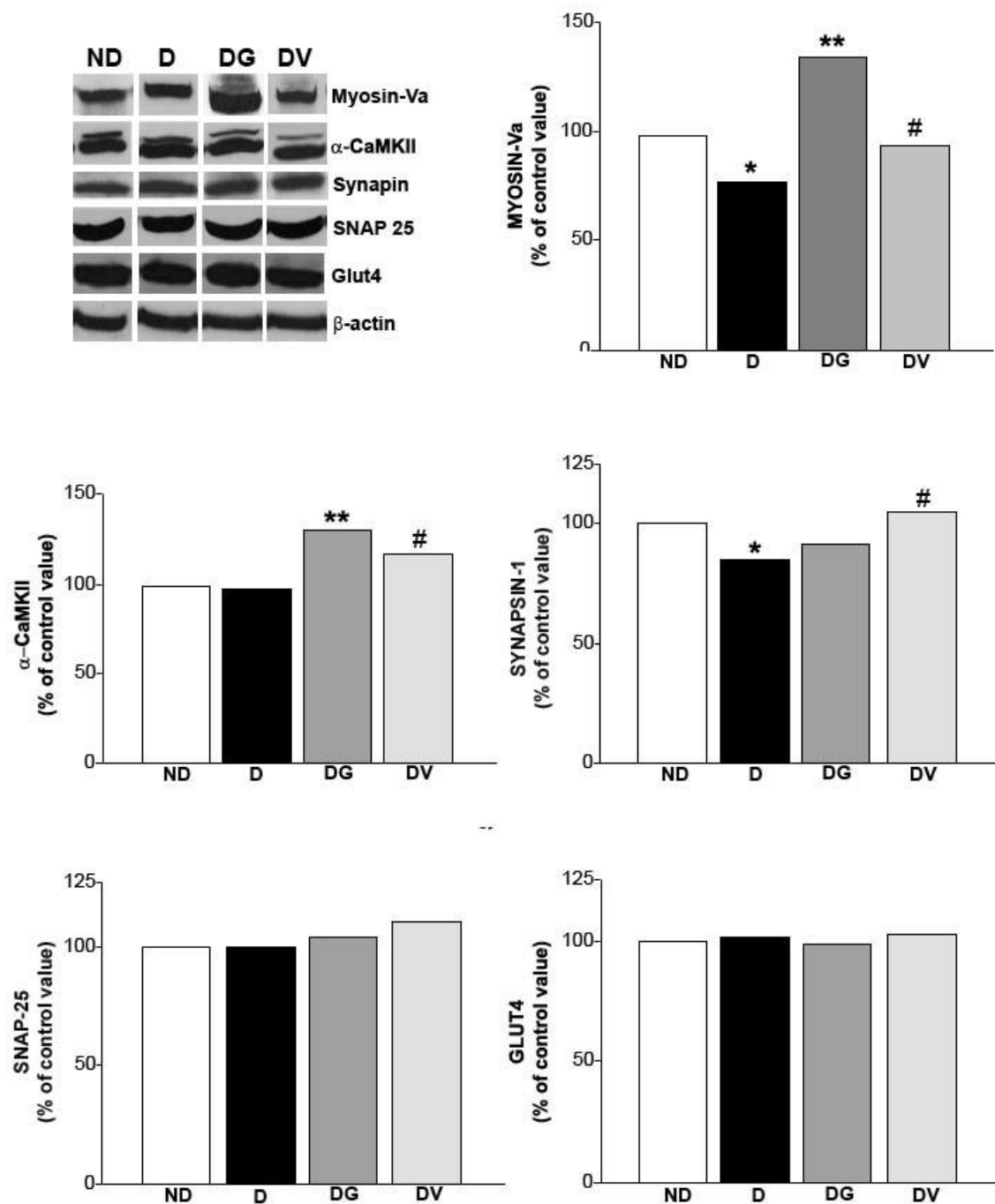


Fig. 02 - Effect of diabetes mellitus on myosin-Va, α-CaMKII, synapsin-1, SNAP-25 and GLUT4 protein levels in rat brains. Western blots and quantification from supernatant brain samples of non-diabetics (ND), diabetics (D), diabetics treated with glibenclamide (DG) and diabetics treated with *Vochysia rufa* extract (DV). The protein level in the immunoblot was determined by densitometric readings and expressed as a percentage within groups (n=4 rats/group). Values represent means and differences are marked: * when compared with non-diabetics (ND) ($p < 0.05$) and ** when compared with diabetics (D) ($p < 0.05$).

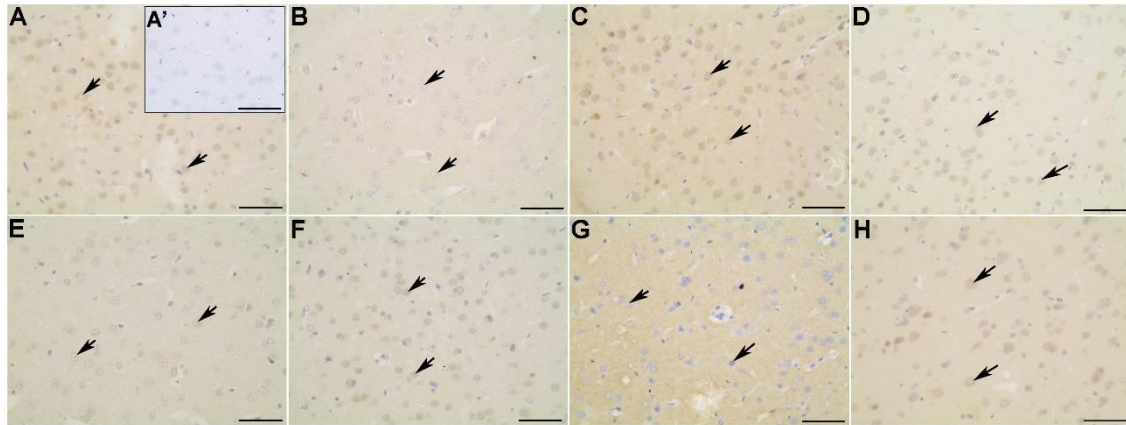


Fig. 03 - Myosin-Va (A-D) and α -CaMKII (E-H) distribution in the frontal cortex of non-diabetic (A; A'; E), diabetic (B; F), diabetic treated with glibenclamide (C; G), and diabetic treated with *Vochysia rufa* extract (D; H) rat brains. Myosin-Va distribution in non-diabetic is more intense in the cytoplasmic soma and extension of pyramidal neurons (arrow) and produces intense perinuclear labeling. α -CaMKII distribution was uniform in non-diabetic and diabetic rat brains and produced less intense marking in the cytoplasm soma and perinuclear region. The nucleus of neurons in rat brains treated with glibenclamide is stained more intensely than in non-diabetic, diabetic and diabetic treated with *Vochysia rufa* extract. Negative control (A'). Bar: 25 μ m (A-H) and 50 μ m (A').

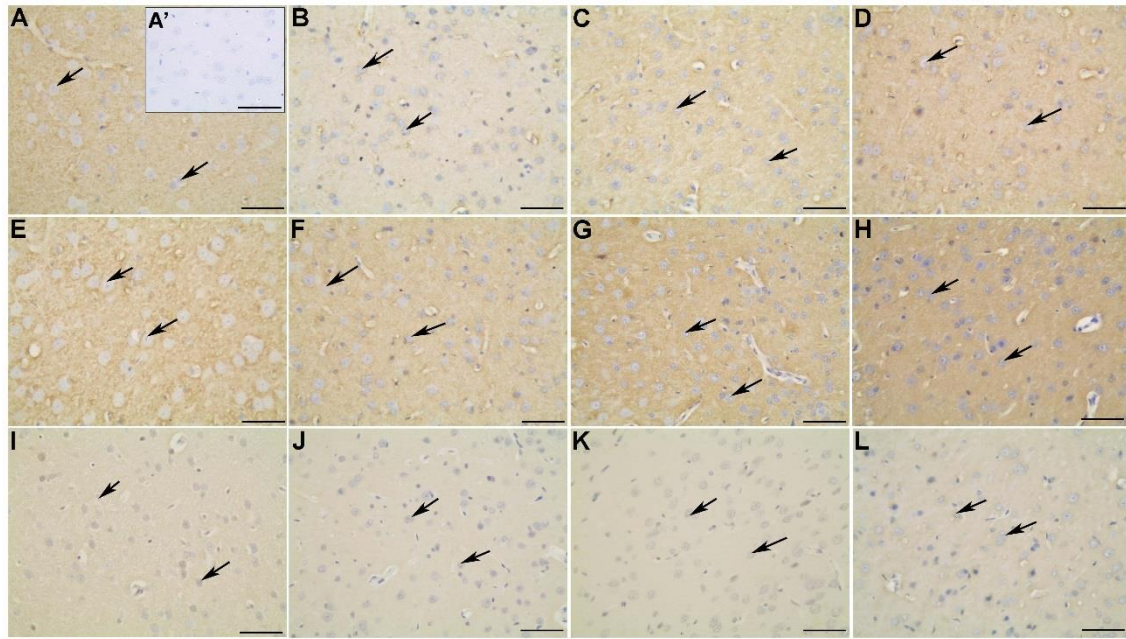


Fig. 04 - Synapsin-1 (A-D), SNAP-25 (E-H) and GLUT4 (I-L) distribution in the frontal cortex of non-diabetic (A; A'; E; I), diabetic (B; F; J), diabetic treated with glibenclamide (C; G; K), and diabetic treated with *Vochysia rufa* extract (D; H; L) rat brains. Synapsin-1 distribution in the frontal cortex of the non-diabetics shows stronger intensity in the soma and extension of pyramidal neurons (arrow) and immunolabeling around the cell body, dendrites and axons compared to diabetic. SNAP-25 distribution in the frontal cortex of the non-diabetics is similar to diabetics with labeling in the soma and extension of pyramidal neurons (arrow) and immunolabeling around the dendrites, axons and fibers. Staining is greater in the nucleus of neurons of treated or non-treated diabetic rats and more intense in the group treated with *Vochysia rufa* extract. GLUT4 distribution in the frontal cortex does not differ among the groups. This labeling is weak and diffuse in the cytoplasm and fibers. Negative control (A'). Bar: 25 μ m (A-L) and 50 μ m (A').

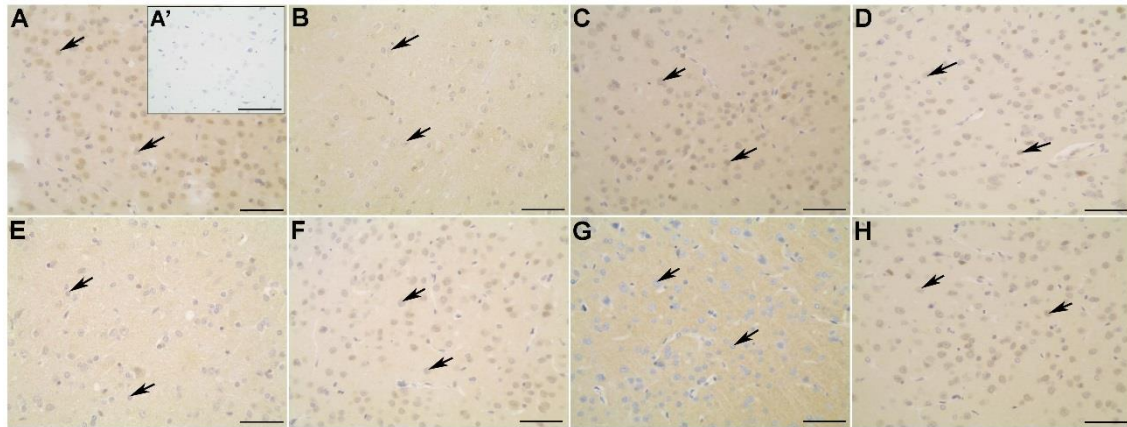


Fig. 05 - Myosin-Va (A-D) and α -CaMKII (E-H) distribution in the occipital cortex of non-diabetic (A; A'; E), diabetic (B; F), diabetic treated with glibenclamide (C; G), and diabetic treated with *Vochysia rufa* extract (D; H) rat brains. Myosin-Va distribution in non-diabetic is more intense in the cytoplasmic soma and extension of pyramidal neurons (arrow) and produces intense perinuclear labeling. α -CaMKII distribution is uniform in non-diabetic and light marking can be seen in the cytoplasm soma and perinuclear region of the diabetic. The nucleus of neurons is stained more in rats treated with glibenclamide than in non-diabetic, diabetic and diabetic treated with *Vochysia rufa* extract. Negative control (A'). Bar: 25 μ m (A-H), and 50 μ m (A').

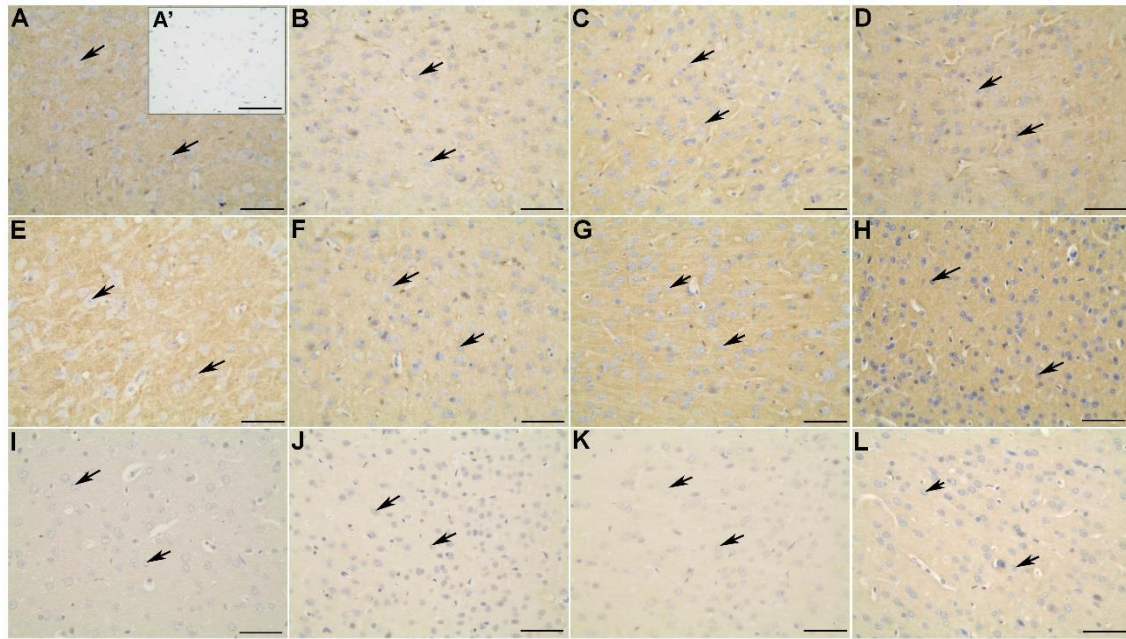


Fig. 06 - Synapsin-1 (A-D), SNAP 25 (E-H) and GLUT4 (I-L) distribution in the occipital cortex of the non-diabetic (A; A'; E; I), diabetic (B; F; J), diabetic treated with glibenclamide (C; G; K), and diabetic treated with *Vochysia rufa* extract (D; H; L) rat brains. Synapsin-1 distribution in the frontal cortex of non-diabetics is more intense in the soma and extension of pyramidal neurons (arrow), immunolabeling around the cell body, dendrites and axons, compared to diabetic. SNAP-25 distribution in the frontal cortex of the non-diabetics is similar to diabetics with labeling in the soma and extension of pyramidal neurons (arrow) and immunolabeling around the dendrites, axons and fibers. Staining increased in the nuclei of neurons of treated and non-treated diabetic rats, and intensified in the group treated with *Vochysia rufa* extract. There were no differences in frontal-cortex GLUT4 distribution among the groups. This labeling is weak and diffuse in cytoplasm and fibers. Negative control (A'). Bar: 25 μ m (A-L), and 50 μ m (A').

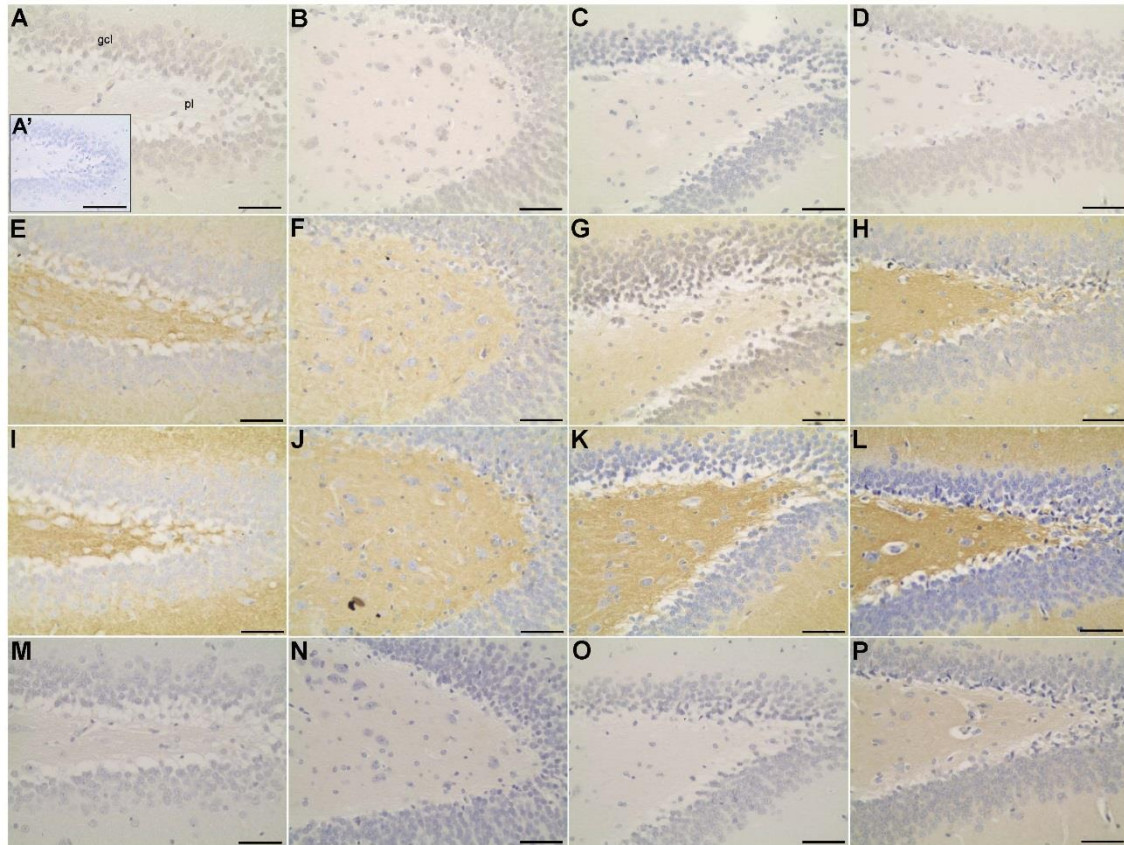


Fig. 07 Immunolocalization of α -CaMKII (A-D), synapsin-1 (E-H), SNAP 25 (I-L) and GLUT4 (M-P) in dentate gyrus of the hippocampus of non-diabetic (A; A'; E; I; M), diabetic (B; F; J; N), diabetic treated with glibenclamide (C; G; K; O), and diabetic treated with *Vochysia rufa* extract (D; H; L; P) rat brains. α -CaMKII and GLUT4 labeling was weak in all groups. This marking is found in the cytoplasm of neurons of the layer (gcl), fibers and cytoplasm of neurons of the polymorphic layer (pl). Synapsin-1 and SNAP-25 have strong marking in the cytoplasm of neurons of the granular layer (gcl), fibers and cytoplasm of neurons in the polymorphic layer (pl). Negative control (A'). Bar: 25 μ m (A-P), and 50 μ m (A').