Universidade Federal do Rio Grande do Sul

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MECANISMOS DE TOXICIDADE DA PROLINA E EFEITOS DA ADMINISTRAÇÃO DE CREATINA E PIRUVATO EM MODELO DE HIPERPROLINEMIA MATERNA DE RATAS

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Orientador: Prof. Dr. Clovis Milton Duval Wannmacher

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Índice

RESUMO

Prolina (Pro) é metabolizada por suas próprias enzimas especializadas com seus próprios mecanismos de regulação e localizações teciduais e subcelulares. Foram descritos erros inatos no metabolismo da Pro em seres humanos. A Hiperprolinemia tipo II é uma doença hereditária causada por uma deficiência de Δ1-pirrolino-5-carboxilato desidrogenase, cuja característica bioquímica é a acumulação de Pro no plasma e tecidos causando diversos problemas, incluindo dano cerebral em alguns pacientes. Vários investigadores demonstram a Pro como uma fonte de espécies reativas de oxigênio (EROs) e uma relação entre uma concentração elevada de Pro e sintomas neurológicos tem sido demonstrada em pacientes com HPII. Uma vez que as mitocôndrias constituem uma fonte importante de EROs, investigou-se a geração de EROs mediada por Pro em mitocôndrias de fígado de rato. Os resultados sugeriram que uma função normal do complexo III da cadeia transportadora de elétrons (CTE) parece ser essencial para a toxicidade da Pro, enquanto que a atividade dos complexos I e IV, e presença Ca^{+2}, aparentemente, não são necessárias. Como existe uma escassez de informações sobre as funções da Pro no crescimento e desenvolvimento do feto e recém-nascido, bem como uma falta de novas estratégias terapêuticas, também investigamos a ação do piruvato e de creatina sobre os efeitos desencadeados pela administração crônica de Pro em ratas durante a gravidez e lactação sobre o cérebro da prole. A administração de Pro nas mães induziu estresse oxidativo e diminuiu as atividades da piruvato quinase e creatina quinase no cérebro da prole. No entanto, a co-administração de creatina e piruvato preveniram apenas parcialmente as alterações causadas pela administração de Pro.

ABSTRACT

Proline (Pro) is metabolized by its own specialized enzymes with their own tissue and subcellular localizations and mechanisms of regulation. Inborn errors of Pro metabolism have been described. Type II Hyperprolinemia is an inherited disorder caused by a deficiency of Δ1-pyrroline- 5-carboxilic acid dehydrogenase, whose biochemical hallmark is Pro accumulation in plasma and tissues causing several problems including cerebral damage in some affected patients. Several investigators have shown that Pro is a source of reactive oxygen species (ROS) and a relationship between a high concentration of Pro and neurological symptoms has been demonstrated in patients with HPII. Since mitochondria constitute a major source of ROS, we investigated the generation of Pro-mediated ROS by Pro in rat liver mitochondria. We found that a normal function of complex III of the electron transport chain (ETC) seems to be essential for Pro toxicity, whereas activity of complexes I and IV and Ca^{+2} presence apparently are not required. Because there is a paucity of information about roles for Pro in growth and development of the fetus and neonate, as well as a lack on new therapeutic strategies, we also investigated the action of pyruvate and creatine on the effects elicited by chronic Pro administration to female rats during pregnancy and lactation on the offspring brain. We found that Pro administration to the mothers induced oxidative stress and diminished the pyruvate kinase and creatine kinase activities in the brain of the offspring. However, co-administration of creatine plus pyruvate did not fully prevented the alterations caused by Pro administration.

LISTA DE ABREVIATURAS

ADP: adenosina difosfato

ATP: adenosina trifosfato

CK: creatina quinase

CR: creatina

Cu-Zn-SOD: superóxido dismutase com cobre e zinco

DCFH-DA: diacetato de diclorofluoresceína

DNA: ácido desoxirribonucleico

EIM: erros inatos do metabolismo

ERNs: espécies reativas de nitrogênio

EROs: espécies reativas de oxigênio

FAD: flavina-adenina dinucleótido

FADH2: flavina-adenina dinucleótido reduzido

FBP: frutose-1,6-bisfosfato

GPx: glutationa peroxidase

GSH: glutationa

HPI: hiperprolinemia tipo 1

HPII: hiperprolinemia tipo 2

NAD+: nicotinamida adenina dinucleotídeo

NADH: nicotinamida adenina dinucleotídeo reduzido

NADPH: nicotinamida adenina dinucleótido fosfato reduzido

OAT: ornitina-gama-aminotransferase

P5C: delta-1-pirrolina-5-carboxilato

P5CDH: delta-1-pirrolina-5-carboxilato desidrogenase

P5CR: delta-1-pirrolina-5-carboxilato redutase

P5CS: delta-1-pirrolina-5-carboxilato sintetase

PCR: fosfocreatina

PEP: fosfoenolpiruvato

PK: piruvato quinase

POX: prolina oxidase

Pro: prolina

PRODH: prolina desidrogenase

RNA: ácido ribonucleico

SNC: sistema nervoso central

TBARS: substâncias reativas ao ácido tiobarbitúrico

TCA: ciclo do ácido tricarboxílico

TRAP: capacidade antioxidante total

INTRODUÇÃO

1. L-Prolina

Com a adição de dois novos membros, selenocisteína (Bock et al. 1991) e pirrolisina (Hao et al. 2002, Srinivasan et al. 2002), existem hoje vinte e dois aminoácidos proteogênicos naturais, codificados geneticamente em organismos vivos. A Prolina (Pro) é um desses vinte e dois aminoácidos e é tradicionalmente classificada como um dos aminoácidos não essenciais em mamíferos, porque existe um conjunto específico de enzimas designado para sintetizar Pro a partir dos seus precursores (glutamato e ornitina) em células de mamíferos (Hiramatsu et al. 1994, Young and El-Khoury 1995).

Pro e seu metabólito (hidroxiprolina) são aminoácidos únicos química e bioquimicamente (Hu et al. 2008, Kaul et al. 2008). Pro não possui uma grupo amino primário, mas sim um grupo amino secundário (imino). Logo, por causa do nitrogênio alfa estar contido dentro de um anel de pirrolidina, a Pro não pode ser metabolizada por enzimas de aminoácidos genéricas, isto é, aminotransferases, decarboxilases, e Racemases (Adams 1970, Phang 1985). Em vez disso, uma família especial de enzimas evoluiu com suas próprias localizações subcelulares e mecanismos de regulação.

Dado que o metabolismo da Pro é distinto do metabolismo de aminoácidos primários, isso pode desempenhar um papel regulador ou, alternativamente, o seu metabolismo pode ser reservado para situações fisiológicas/fisiopatológicas especiais (Phang 1985). Por isso, a Pro tem um papel central no metabolismo e está cada vez mais sendo reconhecida como um aminoácido crítico em bioenergética, controle redox celular, apoptose e

câncer (Phang 1985, Donald et al. 2001, Phang et al. 2001, Rivera and Maxwell 2005, Pandhare et al. 2006).

1.1 Biossíntese e degradação da L-Prolina

As vias metabólicas da Pro foram primeiramente caracterizados por Elijah Adams e Harold Strecker, em meados de 1960 (Adams 1970, Phang 1985, Phang et al. 2001). A rota de biossíntese de Pro começa pelos precursores ornitina ou glutamato, com delta-1-pirrolina-5-carboxilato (P5C) como o intermediário comum, o qual está em equilíbrio tautomérico com o semialdeído-gama-glutâmico (Adams 1970, Ross et al. 1978, Smith and Phang 1979, Strecker 1957). A enzima delta-1-pirrolina-5-carboxilato sintetase (P5CS; uma enzima bifuncional, EC 1.2.1.41/2.7.2.11) é responsável pela conversão de glutamato em P5C dependente de ATP e NADPH. Por outro lado, a enzima ornitina-gama-aminotransferase (OAT; EC 2.6.1.13) catalisa a conversão de ornitina a P5C, com um alfa-cetoácido como aceptor amino (Phang et al. 2001, Flynn et al. 1989).

P5C é um intermediário chave, não só porque é o precursor da Pro, mas também é o produto imediato de sua degradação, estando presente tanto intracelularmente como no plasma. Também é importante ressaltar que o P5C é um intermediário obrigatório que abrange o ciclo da ureia e o ciclo do ácido tricarboxílico (TCA) e pode desempenhar um papel anaplerótico para ambos os ciclos metabólicos (Adams 1970, Phang 1985).

Uma vez formado, o P5C é libertado da mitocôndria e é convertido para Pro pela delta-1-pirrolina-5-carboxilato redutase (P5CR; EC 1.5.1.2) citosólica, uma enzima encontrada em concentrações baixas em todos os tecidos e que

utiliza quer NADH ou NADPH como cofator, porém tem uma maior afinidade para o NADPH (Phang et al. 2001).

O primeiro passo na rota de degradação da Pro começa pela enzima prolina oxidase (POX), também chamada de prolina desidrogenase (PRODH; EC 1.5.99.8), uma flavoenzima localizada na membrana mitocondrial interna que converte a Pro a P5C. Nesta reação, a transferência de elétrons ocorre a partir da Pro para o FAD (flavina-adenina-dinucleotideo) e gera FADH2, que fornece os seus elétrons para o complexo II da cadeia de transporte de elétrons e ATP é formado por fosforilação oxidativa através da transferência subsequente desses elétrons via citocromo *c*.

O segundo passo não-enzimático envolve a conversão de P5C para semialdeído -gama-glutâmico, que é convertido para a ornitina através da reação reversível catalisada pela OAT ou ao glutamato pela enzima delta-1-pirrolina-5-carboxilato desidrogenase (P5CDH; EC 1.5.1.12), a qual usa NAD+ (nicotinamida adenina dinucleotídeo) gerando NADH para entrega de elétrons na respiração mitocondrial.

Uma característica interessante do metabolismo da Pro é a interconversão de Pro em P5C formando um ciclo, o “Ciclo da Prolina”. A POX tem um papel central nesse ciclo porque está ligada fortemente à membrana interna mitocondrial e os elétrons da oxidação da Pro são passados para a cadeia de transporte de elétrons no sítio II com o citocromo *c* como o aceptor de elétrons (Adams 1970, Phang 1985). P5C pode ser convertido a glutamato e alfa-cetoglutarato para contribuir anapleroticamente para o ciclo dos ácidos tricarboxílicos (TCA) (Phang 1985). No entanto, também é convertido de volta a

Pro por P5CR no citosol para formar um ciclo metabólico. O Ciclo da Prolina forma uma interligação metabólica com a via das pentoses-fosfato e serve para converter o potencial redutor da via das pentoses-fosfato em um sistema gerador de ATP nas mitocôndrias (Phang et al. 1980, Hagedorn and Phang 1983, Hagedorn and Phang 1986).

No entanto, trabalhos recentes têm demonstrado que o ciclo da Pro, além de ATP pode formar também superóxido (O_2^-) através da oxidação da Pro pela POX. Isso por que o FAD, cofator utilizado pela POX, tem acesso direto ao oxigênio dissolvido, logo os elétrons derivados da Pro podem diretamente reduzir o oxigênio formando superóxido (White et al. 2007).

2. Erros Inatos do Metabolismo (EIM)

Os Erros Inatos do Metabolismo (EIM) são distúrbios hereditários, geralmente devidos a deficiências enzimáticas, ocasionando um bloqueio nas rotas metabólicas, acumulando substâncias tóxicas e/ou reduzindo a síntese de substâncias essenciais. A frequência isolada de cada EIM é baixa, mas no conjunto, os mais de 500 distúrbios conhecidos atingem aproximadamente 0,1% da população (Scriver et al. 2001, Giugliani 1988).

Um dos aspectos clínicos mais comuns consiste na alteração do desenvolvimento do sistema nervoso central (SNC). Por outro lado, os EIM podem ser completamente assintomáticos (Holtzman 1978). Até o momento, não se sabe a patogenia completa que ligue o defeito bioquímico primário à função anormal do SNC. A principal dificuldade consiste em estabelecer a hierarquia das causas, distinguir entre causas e efeitos e relacionar alterações estruturais com alterações funcionais.

Os EIM dos aminoácidos como a fenilcetonúria, a homocistinúria e a hiperprolinemia têm sido os mais pesquisados, devido à frequência relativamente alta na população, possibilidade de detecção precoce em recém-nascidos e eventual prevenção e tratamento através de dietas específicas. Embora nenhum modelo animal reproduza integralmente a doença humana, as informações acumuladas permitem a correlação entre os dados obtidos em modelos animais com aqueles obtidos em pacientes, contribuindo para a investigação da patologia destas doenças.

2.1 Erros Inatos no Metabolismo da Pro – Hiperprolinemia

Os erros inatos no metabolismo da L-Prolina são conhecidos como: Hiperprolinemias Tipos I e II, deficiência de delta-1-pirrolino-5-carboxilato sintetase, deficiência de ornitina aminotransferase (OAT), hidroxiprolinemia e iminoglicinúria.

O primeiro estudo do efeito direto do envolvimento da Pro em doença humana foi relatada por Schafer e colegas (Schafer et al. 1962) em uma família com hiperprolinemia, disfunção cerebral, anomalias renais, nefropatia hereditária e surdez.

Hiperprolinemia (HP) pode se apresentar como qualquer uma das duas desordens metabólicas hereditárias. Estas duas desordens, tipo I ou II (HPI ou HPII), são definidas por distintas deficiências bioquímicas e genéticas na via catabólica da prolina.

HPI é uma doença rara hereditária autossômica recessiva caracterizada pela deficiência hepática de prolina oxidase (também chamada prolina desidrogenase). HPI é bioquimicamente diagnosticada por níveis plasmáticos

elevados de Pro sem excreção urinária de P5C, os níveis de Pro podem variar de cinco a dez vezes (700 a 2400 µM) acima dos valores normais (51-271 µM). Alguns estudos mostram que hiperprolinemia leve (500 a 1000 µM) podem ser observados em HPI heterozigotos (Phang et al. 2001).

As manifestações clínicas em pacientes com HPI ainda não estão bem caracterizadas. Alguns fenótipos encontrados em pacientes com HPI são defeitos neurológicos, renais e auditivos; alterações oculares; retardo mental e outras alterações neurológicas, enquanto na maioria são assintomáticos (Mitsubuchi et al. 2008, Phang et al. 2001). Como a HPI não está necessariamente associada com manifestações clínicas, este transtorno tem sido considerado uma condição benigna na maioria dos indivíduos sob a maioria das circunstâncias (Phang et al. 2001).

Já a HPII também é uma doença rara hereditária autossômica recessiva, porém se caracteriza pela deficiência da atividade da delta-1-pirrolino-5-carboxilato desidrogenase. Esta doença é caracterizada bioquimicamente pelo acúmulo de Pro e P5C no plasma, urina e fluído cefalorraquidiano. Quantitativamente, o principal metabolito que se acumula no tecido de pacientes com HPII é a Pro e não P5C (Fleming et al. 1984, Flynn et al. 1989, Phang et al. 2001). As concentrações plasmáticas de Pro em HPII são maiores do que aqueles de HPI e podem variar de 10 a quinze vezes (500-3700 µM) acima dos valores normais (51 a 271 µM), onde em homozigotos, os níveis plasmáticos de Pro quase sempre exceder 1500 µM.

Apesar de irmãos hiperprolinêmicos assintomáticos terem sido identificados em algumas genealogias (Pavone et al. 1975), vários pacientes

hiperprolinêmicos, até agora detectados, apresentam manifestações neurológica incluindo convulsões e retardo mental (Di Rosa et al. 2008, Phang et al. 2001). Neste contexto, uma relação entre elevada concentração de Pro e sintomas neurológicos tem sido demonstrada em pacientes com HPII (Flynn et al. 1989). Em contraste com HPI, há evidências persuasivas de que HPII é causalmente associada com manifestações neurológicas (Phang et al. 2001).

2.1.1 Tratamento

As terapias empregadas atualmente, como restrição dietética de Pro, apenas alcançam modesta queda na concentração plasmática de Pro sem qualquer impacto no estado clinico dos pacientes (Mitsubuchi et al. 2008). Alguns autores sugerem que a terapia dietética é até mesmo desnecessária (Phang et al., 2001).

3. Estresse Oxidativo

O termo estresse oxidativo é vagamente definido. Em essência, refere-se a um grave desequilíbrio entre a produção de espécies reativas e de defesa antioxidante. Estresse oxidativo tem sido definido como um distúrbio no equilíbrio pró-oxidante-antioxidante em favor do primeiro, levando a potenciais danos (Sies 1997).

Estresse oxidativo pode resultar de níveis diminuídos de antioxidantes, por exemplo, as mutações que afetam as atividades de enzimas de defesa antioxidantes tais como CuZnSOD, GPx, ou toxinas que destroem as defesas antioxidantes. Por exemplo, muitos xenobióticos são metabolizados por conjugação com a GSH; altas doses podem esgotar GSH e causar estresse

oxidativo. Deficiências em minerais dietéticos (por exemplo, Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Se) e / ou antioxidantes também podem causar estresse oxidativo.

Além disso, o estresse oxidativo também se dá pelo aumento da produção de espécies reativas tanto de oxigênio (EROs) como de nitrogênio (ERNs). EROs é um termo coletivo que inclui radicais de oxigênio (superóxido, hidroxila, hidroxiperoxila) e alguns não-radicais que são agentes de oxidação e / ou são facilmente convertidos em radicais como o ácido hipocloroso (HOCl), o ácido hipobromoso (HOBr), o ozônio (O_3), o peróxidonitrito ($ONOO^-$) e o peróxido de hidrogênio (H_2O_2). O radical livre possui um elétron desemparelhado, que o torna muito instável e reativo, com grande capacidade de se combinar com moléculas integrantes da estrutura celular como lipídios, proteínas e DNA (Halliwell and Gutteridge 1986, Halliwell and Gutteridge 2007).

A cadeia de transporte de elétrons mitocondrial é a grande e contínua fonte de espécies reativas de oxigênio (ERO) celulares. Os principais locais para a redução monoeletrônica do oxigênio e vazamento de elétrons são nos complexos I e III, resultando em produção de radicais superóxido (O_2^-) na cadeia respiratória (Cadenas et al. 1977).

O radical O_2^- é citotóxico e também pode permitir a geração de outros radicais livres, quando sofre reação de dismutação. O O_2^- origina H_2O_2 que é pouco reativo a nível fisiológico, mas que pode atacar diretamente algumas enzimas, e o O_2^- pode reagir com H_2O_2 e formar o radical hidroxila (OH^-). O radical OH^-, é o mais danoso dos radicais livres, danifica proteínas, lipídios, membranas celulares do núcleo e da mitocôndria, RNA e DNA. No DNA, a OH^-

ataca as bases nitrogenadas e a desoxirribose, levando à ruptura da molécula. (Halliwell and Gutteridge 2007).

Muitos estudos têm mostrado aumento de dano oxidativo a todas as principais classes de biomoléculas nos cérebros dos pacientes com Alzheimer (Halliwell 2001, Butterfield 2002, Liu et al. 2003). Outras doenças em que o estresse oxidativo tem sido implicado incluem o câncer, aterosclerose, doenças neurodegenerativas e diabetes (Hagen et al. 1994, Chowienczyk et al. 2000, Halliwell 2000, Halliwell 2001, Halliwell 2002b, Halliwell 2002a, Parthasarathy et al. 2000).

4. Metabolismo Energético

As mitocôndrias são a principal fonte de espécies reativas porque são responsáveis pela oferta de energia das células. Na execução das principais vias metabólicas para produção de ATP, essas organelas consomem a maior quantidade (85-95%) de oxigênio das células para permitir a fosforilação oxidativa, o que depende da cadeia de transporte de elétrons através da ação de vários complexos respiratórios localizados na membrana mitocondrial interna. Qualquer comprometimento na cadeia de transporte de elétrons, por sua vez, leva à diminuição da produção de ATP e aumento da formação de radicais livres tóxicos. Entretanto, outros sistemas podem formar ATP.

4.1 Creatina quinase

A creatina quinase (CK; EC 2.7.3.2) catalisa a transferência reversível de um grupo N-fosforil da fosfocreatina (PCR) para a adenosina difosfato (ADP), formando adenosina trifosfato (ATP) e creatina (Cr) em uma reação dependente de magnésio. Essa enzima é especialmente fundamental nos

tecidos com alto e flutuante consumo de ATP, como músculo esquelético e cardíaco, cérebro e retina, onde a fosfocreatina serve como reservatório de energia para regeneração rápida de ATP. A CK consiste de duas subunidades, B (tipo cérebro) e M (tipo muscular), compartimentalizadas especificamente nos locais onde a energia é liberada ou utilizada (Wallimann et al. 1992).

São conhecidas cinco isoenzimas da creatina quinase, dois tipos de subunidades compõem as três isoformas citosólicas, a M-CK e a B-CK (Wallimann et al. 1992). Essas subunidades se combinam *in vivo*, formando as isoenzimas citosólicas diméricas MM-, BB-CK e MB. A isoenzima MM-CK é encontrada predominantemente no músculo esquelético, a BB-CK no cérebro e a MB-CK no músculo cardíaco (Eppenberger et al. 1967). Há também duas isoformas mitocondriais encontradas no espaço intermembranas, a forma Mi-CK ubíqua (Mi_a-CK) expressa predominantemente no cérebro, e a forma Mi-CK sarcomérica (Mi_b-CK) no músculo estriado. Ambas formam homodímeros ou homooctâmeros, sendo a última a forma oligomérica predominante *in vivo* (Wallimann et al. 2011).

Geralmente as isoenzimas citosólica e mitocondrial são co-expressas em tecidos específicos dentro de compartimentos subcelulares onde a energia liberada é captada (glicólise e mitocôndria) ou utilizada (ATPases e cinases no citosol) sendo ligadas funcional ou estruturalmente pelo circuito de PCr (Wallimann et al. 1992). Assim, esse sistema de ligação das diferentes isoenzimas de CK com ATP/ADP, Cr/PCr permite a distribuição intracelular de energia, confirmando seu papel chave no metabolismo energético.

O cérebro possui uma alta demanda energética e o sistema da CK exerce uma grande contribuição na manutenção dos níveis de ATP. Portanto, condições que levem a alterações no seu funcionamento podem estar envolvidas na via de neurodegeneração com consequente perda neuronal (Tomimoto et al. 1993). Nesse contexto, autores têm mostrado que a diminuição da atividade da CK pode ser um dos indicadores de dano celular em doenças como Alzheimer (Aksenov et al. 1997) enquanto outros trabalhos mostram que alguns aminoácidos acumulados em erros inatos do metabolismo provocam alteração energética cerebral (Tonin et al. 2009, Kessler et al. 2003b, Rech et al. 2008).

4.2 Piruvato quinase

A reação de transferência de um grupamento fosforil do fosfoenolpiruvato (PEP) para o ADP, formando piruvato e ATP, é a última etapa da via glicolítica e é catalisada pela ação da enzima piruvato quinase (PK, ATP: piruvato 2-O-fosfotransferase, EC 2.7.1.40), sendo esta uma das enzimas reguladoras dessa via (Valentini et al. 2000). A glicólise é uma via fundamental para manutenção da homeostasia e do funcionamento celular, principalmente nas células nervosas que dependem muito dessa via para suprimento de energia.

São conhecidas quatro isoenzimas (L, R, M1 e M2) da PK expressas em diferentes tecidos nos mamíferos. Cada uma delas possui propriedades regulatórias e cinéticas distintas, refletindo a sua importância no suprimento das necessidades metabólicas de cada tecido (Jurica et al. 1998). Os genes L e M codificam as quatro isoenzimas em humanos. O gene L codifica a isozima

L encontrada no fígado e R que está presente nos eritrócitos (Noguchi et al. 1987). Já o gene M codifica as isozimas M1 e M2 (Noguchi et al. 1986). M1 está predominantemente presente no músculo esquelético e no cérebro, enquanto a M2 é encontrada primariamente no tecido fetal, células proliferativas e tumorais (Dombrauckas et al. 2005, Mazurek et al. 2005). Os tipos M2, L e R apresentam regulação alostérica e são ativados heterotropicamente pela frutose-1,6-bisfosfato (FBP) (Jurica et al. 1998). Por outro lado, o tipo M1 apresenta cinética hiperbólica e não exibe regulação alostérica (Mattevi et al. 1996).

Considerando que o piruvato, produto final do último passo da via glicolítica catalisado pela PK, está envolvido em tal variedade de vias metabólicas, a PK pode ser considerada uma enzima chave não só para a via glicolítica, mas também para o metabolismo celular inteiro (Mattevi et al. 1996).

OBJETIVOS

Objetivo Geral

Avaliar o mecanismo de ação da prolina em mitocôndrias de fígado de ratos e o efeito da administração de prolina a ratas Wistar adultas durante o período de gestação e amamentação sobre alguns parâmetros bioquímicos do córtex e hipocampo dos filhotes.

Objetivos Específicos

1-Investigar o efeito da prolina sobre os complexos da fosforilação oxidativa mitocondrial em fígado de ratos Wistar adultos;

2-Desenvolver e aplicar um modelo químico experimental crônico de hiperprolinemia materna através da administração de prolina em uma concentração tóxica em ratas Wistar adultas grávidas;

3-Estudar em córtex e hipocampo da prole destes animais alguns parâmetros bioquímicos (atividade de enzimas relacionadas com a homeostasia energética e estresse oxidativo).

4-Investigar o eventual efeito preventivo da administração da associação de substâncias com propriedades energéticas e antioxidantes (creatina e piruvato) sobre os parâmetros bioquímicos alterados no cérebro da prole.

ARTIGO 1: Submetido a Cell Biochemistry and Function

Proline-induced reactive species of oxygen is related to the complex III function in mitochondria

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ABSTRACT

Hyperprolinemia type II (HPII) is caused by a deficiency in P5C dehydrogenase activity. Higher tissue accumulation of Pro is the biochemical hallmark of this condition. The central enzyme in this metabolic system is POX, a flavin adenine dinucleotide-containing enzyme which is tightly bound to mitochondrial inner membranes and participates in a metabolic cycle that shuttles redox equivalents between mitochondria and cytosol. Since mitochondria constitute a major source of reactive oxygen species (ROS), in this study we investigated the in vitro generation of ROS by pathophysiologic concentrations of proline (0.25 – 2.5 mM) in mitochondria of liver from Wistar rats. ROS formation was followed by oxidation of DFC-DA in the presence of GSH or creatine. The results suggested that the generation of proline-mediated ROS in rat liver mitochondria required a normal function of complex III of the mitochondrial electron transport chain but not the activity of complexes I and IV. The results also showed that calcium influences the ROS production Pro-mediated and that proline could not oxidize thiol groups directly. On the other hand, creatine, a direct and indict antioxidant, was capable to attenuate the Pro induction of ROS. If these effects of proline occur in hyperprolinemic patients, mitochondrial generation of ROS may be one of the pathophysiologic mechanisms of this disease, and supplementation of creatine might be considered to attenuate the tissue damage.

Keywords: proline toxicity; ROS, mitochondria; creatine; calcium

INTRODUCTION

Proline and its metabolite (hydroxyproline) are unique amino acids both chemically and biochemically [1, 2]. Because the alpha nitrogen is contained within a pyrrolidine ring, proline cannot be metabolized by generic amino acid enzymes, that is, aminotransferases, decarboxylases, and racemases [3, 4]. Instead, a special family of enzymes evolved with their own subcellular localizations and mechanisms of regulation for metabolize proline.

Currently, the interest in research on proline metabolism and nutrition have increased [5-9]. The hyperprolinemias (HP) both type I and II are rare metabolic disorders of inherited biochemical abnormalities in the pathway of proline degradation. The degradation of proline in mammals involves the conversion of proline to Δ^1-pyrroline-5-carboxylic acid (P5C) by proline oxidase (POX) and the oxidation of P5C to glutamic acid by P5C dehydrogenase [10, 11]. The cause of HPI has been shown to be a deficiency of proline oxidase (POX), the first enzyme in the proline degradative pathway [12] and the enzymatic defect in type II hyperprolinemia is due to an absence of P5C dehydrogenase activity, the second step of the pathway [13]. The plasma concentrations of Pro in HPII are greater than those of HPI, can range from ten to fifteen times (500–3700 μM) above normal values (51 to 271 μM) [14]. In addition, currently therapies, i.e., restriction of proline, are used in HP patients, but only modest control of plasma proline is achieved with no impact in clinical condition [14, 15]. Therefore, the search for new therapeutic strategies is necessary. One that can be considered is the administration of the energy substrate creatine (Cr) which prevents ROS production by an ADP-recycling system through creatine kinase activity [16]. Moreover, creatine is a potent antioxidant [17].

An interesting feature of the proline metabolism is that the interconversion between proline and pyrroline-5-carboxylate forms a proline cycle. After proline oxidation by POX, pyrroline-5-carboxylate can be converted to glutamate and α-ketoglutarate to contribute anaplerotically to the TCA cycle [4]. However, it is also converted back to proline by pyrroline-5-carboxylate reductase in the cytosol. Proline can then be transported into mitochondria where POX mediates its conversion back to P5C, accompanying the generation of ATP or ROS, completing a proline cycle [18, 19]. Obviously, the identification of a mechanism switching between ROS and ATP would be of great interest.

There is an apparent relationship between an accumulation of Pro and neurological symptoms in some patients with HPII [20]. Although neurological dysfunction is found in some hyperprolinemic patients, the exact mechanism by which it occurs remains poorly understood. Despite the different clinical and neuropathological conditions, the pathomechanisms associated with diseases that affect the central nervous system (CNS) seem to have a number of common features in their processes, such as oxidative stress. In fact, an increasing number of pathological situations involving the CNS, such as neurodegenerative disorders, seizures, ischemia/reperfusion and dementia, have been associated with oxidative stress [21]. In vivo and in vitro findings showing that Vitamins E and C (free radical scavengers), as well as GSH (thiol reducing agent) prevented the inhibitory action of Pro on the Na+,K+-ATPase activity, a crucial enzyme to maintain the ionic gradient in the CNS, suggest the involvement of reactive oxygen species and SH group modifications in this effect [22].

Since oxidative stress is defined as an imbalance between formation and scavenging (neutralizing) of free radicals, several investigators have claimed that during

proline oxidation, superoxide may be formed directly by POX during catalytic turnover or indirectly by downstream events in the mitochondrial electron pathway [23-26]. This phenomenon is also observed in plants and recent studies with Arabidopsis seedlings shown that proline caused a calcium-dependent ROS production [27], indicating that calcium treatment can increase proline toxicity.

In mammals, POX activity is developmentally regulated and tissue specific: mainly in liver and kidney, but also presented in brain and heart [28, 29]. In addition, other studies show that proline transport across the inner membrane of rat liver mitochondria is enhanced in the presence of respiratory substrates such as succinate and it is inhibited by uncouplers of oxidative phosphorylation [30].

On the basis of these findings, in order to contribute for the understanding of the mechanism of pathogenesis linked to proline in patients with HPII, we investigated the role of pathological doses of proline (0.25 – 2.5 mM) on respiration parameters and ROS production using different substrates jointly with several complex inhibitors in rat liver mitochondria. Further, we also investigated the influence of calcium in the proline-mediated ROS and the SH group modifications. Given the lack of efficacious of treatments that fully abolish proline-induced toxicity, the present study was also designed to test the potential protective effects of the antioxidant creatine.

MATERIALS AND METHODS

Chemicals

Dithiothreitol (DTT), L –cysteine, 5,5'-dithio-bis(2-nitrobenzoic acid), N-ethylmaleymide (NEM), o-phthalaldehyde (OPT), reduced glutathione (GSH),

glutathione disulfide (GSSG), dichlorofluorescin diacetate (DCFHDA), Antimycin A, Calcium chloride ($CaCl_2$), Ruthenium Red, ethylene glycol tetraacetic acid (EGTA), Ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), succinic acid and L-proline were obtained from Sigma (St. Louis, MO, USA); sodium phosphate, potassium chloride, bovine serum albumin (BSA), sodium hydroxide, potassium cyanide, sucrose, mannitol and L-glutamate were obtained from Vetec (Rio de Janeiro, RJ, Brazil).

Animals

Male Wistar rats (2–4 months) obtained from the Department of Chemistry, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil, were housed in groups of five animals and maintained under standard conditions (12-h light/dark, 22 ± 2°C) with a commercial chow (Supra®, Brazil) and water ad libitum. The ‘‘Principles of Laboratory Animal Care’’ (NIH publication no. 80-23, revised 1996) were followed in all the experiments, and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Santa Maria.

Sulfhydryl Content

This assay is based on the reduction of 5,5’-dithio-bis(2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412 nm [31]. Briefly, different concentrations of proline (0.25, 0.5 and 1 mM) were incubated from 1 up to 12 hours with different sources of thiol

group, L-cysteine (0.4 mM) or DTT (0.2mM) in PBS buffer pH 7.4, containing 1 mM of Ethylenediaminetetraacetic acid . Aliquots were taken and mixed with 50 µM of DTNB and the absorption was measured at 412nm a spectrophotometer Spectronic Genesys 8, Spectronic Instruments, Rochester, NewYork. Results are reported as percentage of control.

Glutathione levels

Glutathione (GSH) and glutathione disulfide (GSSG) were determined with fluorescence detection after reaction of the supernatant of the H3PO4/NaH2PO4–EDTA rat liver solution, with o-phthalaldehyde (OPT), pH 8.0, according to Hissin and Hilf [32]. In brief, rat liver (0.250 g) was homogeneized in 3.75 ml phosphate buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) and 1 mL of H_3PO_4 25% were centrifuged at 100,000 x g (Himac CP80MX ultracentrifuge) for 30 min at 4ºC. A pre-incubation with 2.5 mM of proline were performed at room temperature for 1 hour. For GSH determination 100 µl of the supernatant was added to 1.8 ml phosphate buffer and 100 µl of OPT (1mg/mL). After mixing and incubating at room temperature for 15 min, the fluorescence was measured at 420 nm emission and 350 nm excitation wavelengths. For GSSG determination 250 µl of the supernatant was added to 100 µl of N-ethylmaleymide (NEM) and incubated at room temperature for 30 min. After the incubation, 140 µl of the mixture was added to 1.76 ml of NaOH (100 mM) buffer and 100 µl of OPT. After mixing and incubating at room temperature for 15 min, the fluorescence was measured at 420 nm and 350 nm emission and excitation wavelengths, respectively (slits 5, 5) with a fluorescence spectrophotometer Hitachi F-2000. The GSH and GSSG contents were determined from comparisons with a linear reduced or

oxidized glutathione standard curve. Results were expressed as percentage of control, with 0.471 and 0.058 µg/mg of protein as reference levels for GSH and GSSG, respectively.

Isolation of fresh rat liver mitochondria

Liver mitochondria were isolated as previously described by Brustovetsky and Dubinsky [33] with minor modifications. Wistar rats were killed by decapitation. The liver was rapidly removed and placed on ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA free fatty acid) and 10 mM HEPES pH 7.2. The tissue was then homogenized (1:10 w/v) and the resulting suspension centrifuged for 7 min at 2,000 x g. Next, the supernatant was centrifuged for 10 min at 12,000 x g. The pellet was resuspended in isolation buffer II containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 10 mM HEPES pH 7.2 and centrifuged at 12,000 x g for 10 min. The supernatant was discard and the final pellet gently washed and resuspended in 1 mL buffer containing 50µM EGTA, 100 mM sucrose, 65 mM KCl and 10 mM HEPES, pH 7.2.

Mitochondrial respiration

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode [34] connected to a suitable recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 ºC in 1.5 ml of standard respiratory medium containing 100 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 50 µM EGTA with 0.2 mg of liver mitochondria.

Respiration States 4 and 3 were initiated with 5 mM glutamate/malate or 5 mM succinate, ADP (0.3 mM) and P_i (1 mM), respectively. The respiratory state 2 (oxygen consumption in the absence of substrate), state 3 (oxygen consumption in the presence of substrate and ADP) and 4 (oxygen consumption after ADP phosphorylation) and respiratory control ratio (RCR = state 3/state 4) were calculated according to Chance and Williams 35.

Determination of ROS production in rat liver mitochondria

Experiments were carried out in a standard reaction medium containing 100 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 50 µM EGTA and 100 µg/mL of mitochondrial protein. Substrates (5 mM glutamate/malate or 5 mM succinate), ADP (0.3 mM) and P_i (1mM) were added subsequently in the medium after the reaction have started with DCF. The DCFHDA (2 µM) was added to monitor ROS formation in the presence or absence of proline (2.5 mM) with creatine (1mM), antimycin A (1 µM), $CaCl_2$ (10µM), Ruthenium Red (5 µM) and KCN (0.75mM) with Rotenone (0.01 mM). The formation of the oxidized fluorescent derivative (DCF) was monitored with a fluorescence spectrophotometer, Hitachi F-2000 using excitation and emission wavelengths of 488 and 525 nm, respectively [36].

Protein estimation

Protein concentration was measured second method described by Lowry et al. [37], using bovine serum albumin (BSA) as standard.

Statistical analysis

The results are expressed as means ± SEM. Data from DCFHDA oxidation were obtained directly from the spectrofluorimeter software and for sake of clarity part of the continuous reading data were not included in the statistical analysis. Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures followed by the Bonferroni's Comparison Test when the F value was significant. Differences were considered statistically significant at $p < 0.05$.

RESULTS

As recently demonstrated [22], GSH, a thiol-reducing agent, was able to prevent the inhibitory role of Pro on Na^+,K^+-ATPase activity, suggesting that this inhibition may be mediated by oxidation of thiol groups on the enzyme. Based on these findings we investigated in this study if proline would react directly with different thiol sources. Proline did not alter thiol groups even with long times of incubation (fig 1).

Next, we evaluated the effect of proline on the mitochondria respiration parameters (Table 1). We observed a significant interaction between proline and the different respiration states along the time supplied by the two substrates, glutamate/malate or succinate [$F(3,12)= 74.98$; $p<0.0001$; $F(3,12)= 251.86$; $p<0.0001$], respectively. Proline increased significantly the oxygen consumption in the state 2 of respiration before the addition of substrates glutamate/malate or succinate. The decreased oxygen consumption after the addition of substrates glutamate/malate or succinate and ADP indicates uncoupling between respiration and generation of ATP as

measured by the drop in oxygen consumption after addition of ADP in the respiration state 3.

One of the major damaging consequences of enhanced electron flow is an increased generation of oxygen reactive species (ROS) such as superoxide and hydrogen peroxide [38]. To assess the level of ROS production by mitochondria in the presence of proline and to investigate the extent of respiratory function necessary for it, the generation of ROS by mitochondria was evaluated by the addition of the compound DCFH-DA with several respiration inhibitors jointly with the different substrates and ADP with inorganic phosphate (P_i) (Fig 2 and 3).

We found a significant interaction between proline and the inhibitors along the time with the two substrates glutamate/malate or succinate [$F(15,36)= 7.75$; $p<0.0001$; $F(15,36)=7.68$; $p<0.0001$], respectively. As shown in Fig 2A, mitochondria using glutamate/malate as substrate in the presence of proline caused a markedly increase in the ROS production in comparison with the control and the addition of inhibitors of complex I and/or complex IV were not able to stop the ROS production proline-dependent. KCN also increased ROS production at similar magnitude than proline. This effect was slightly different with mitochondria using succinate as substrate (Fig. 2B). The presence of proline caused increase in the ROS production immediately after addition of ADP and P_i and similarly to the previously results with glutamate/malate, the inhibitors combined were not able to prevent the effect of proline. KCN alone also increased ROS production and when it was combined with proline, this production was more pronounced than with proline alone.

Next, we investigated the influence of a complex III inhibitor Antimycin A in the ROS production proline-dependent. We observed a significant interaction between

proline and Antimycin A along the time with glutamate/malate or succinate as substrate [$F(9,24)=16.57$; $p<0.0001$; $F(9,24)=24.36$; $p<0.0001$], respectively. As illustrated in Fig. 3, Antimycin A suppressed significantly the effect of Pro under the basal condition with the two substrates.

In Fig. 4 we observe the influence of calcium, an important and ubiquitous second messenger that can influence many cellular processes, including ROS generation [39]. To understand whether calcium can also affect proline toxicity, we investigated the generation of ROS by mitochondria in the presence of Pro followed by the addition of $CaCl_2$ and/ or Ca^{+2} channel blocker Ruthenium Red. We found an interaction between proline and calcium along the time with glutamate/malate [$F(20,75)= 3.77$; $p<0.0001$] or succinate [$F(20,72)= 4.29$; $p< 0.0001$] as substrate. Proline increased ROS production in the presence of calcium when comparing with the control levels for the two substrates.

Finally, we considered the effect of the addition of creatine, an antioxidant, in the reaction medium. Creatine exerts a strong antioxidant effect by significantly reducing the intra-mitochondrial production of ROS [16]. We observed a strong interaction between proline and creatine along the time with glutamate/malate [$F(18,72) = 58.80$; $p<0.0001$] or succinate [$F(18,48) = 118.69$; $p<0.0001$] as substrate. The presence of creatine was capable to abolish the increase of ROS proline-dependent under the basal levels using glutamate/malate or succinate as substrates. As can be observed, the decrease in the ROS production was more pronounced when succinate was used as substrate (fig 5).

DISCUSSION AND CONCLUSION

Proline is metabolized by its own specialized enzymes with their own tissue and subcellular localizations and mechanisms of regulation. Hyperprolinemias result from the deficiency of specific enzymes for proline catabolism, leading to tissue accumulation of this amino acid [15]. HPII is due to absence of Δ-1-pyrroline-5-carboxylic acid dehydrogenase activity [13] and the deficiency of this enzyme causes accumulation of P5C and proline. Hyperprolinemic patients can present neurological symptoms and brain abnormalities, whose etiopathogenesis is poorly understood. However, a causal relationship between high concentration of Pro and recurrent seizures has been documented in patients with type II hyperprolinemia, suggesting that high sustained tissue Pro levels may predispose to convulsions [20].

Since the brain is particularly vulnerable to oxidative damage due to the high utilization of oxygen [40], oxidative stress is an important event related to the pathogenesis of some neurodegenerative disorders [41-43]. It is known in the literature that proline-derived electrons are transferred to the enzyme-bound flavine adenine dinucleotide (FAD). The electrons can be transferred into site II of the electron transport chain through cytochrome *c*. Tanner's lab, however, has shown that the FAD is directly accessible to oxygen in *Thermus thermophilus* and the electrons can directly reduce oxygen to yield superoxide [44].

As far as we know, the present study is the first to show that 2.5 mM of proline, a concentration found in hyperprolinemic patients, is a source of measurable ROS production that can alter the redox state of the cell. Since generation of ROS by mitochondrial respiratory chain is a physiological and continuous process [38, 45], the effects of acute oxidative stress on mitochondrial electron transport chain (ETC) was

examined by measuring oxygen consumption. We found that Pro caused an increase in the oxygen consumption in state 2 of respiration, leading to an increased generation of oxygen radicals. Moreover, the decreased oxygen consumption after the addition of ADP indicates that the oxidative stress caused uncoupling between respiration and generation of ATP (Table 1). These data suggest that Pro, at concentration found in the plasma of HPII patients, can switch the respiratory chain from ATP to ROS production by mitochondria.

The direct involvement of electron transport chain in the generation of ROS can be evaluated by the addition of complex inhibitors. In this regard, we investigated the influence of complex I, III and IV inhibitors in the ROS production caused by the presence of proline (Fig. 2 and 3). Recently, it was demonstrated that Pro significantly decreases cytochrome *c* oxidase (complex IV) activity in the cerebral cortex [46]. Cytochrome *c* oxidase is a complex essential for virtually all energy production in cells. Therefore, an inhibition of this enzyme or other complex of the respiratory chain can potentially lead to incomplete reduction of oxygen and, consequently, to increased free radical formation and reduced ATP synthesis [47, 48]. Indeed, we verified an increase in the ROS production following the addition of KCN, a complex IV inhibitor. However, KCN when combined with Pro altered ROS production only when succinate was used as substrate (fig. 2B). In contrast, we also combined KCN with Rotenone (a complex I inhibitor), which reduced the ROS production caused by KCN. This may be due to the fact that complex I together with complex III is the major source of ROS in mitochondria [49]. On the basis of these data, we also investigated the role of Complex III inhibitor Antimycin A in the proline-mediated ROS generation (Fig. 3) and we found that a normal function of complex III of the ETC seems to be essential for proline effect, since Antimycin A completely reduced the levels of ROS proline-dependent. Our

results are in agreement with other reports, which claim that electrons from proline are passed into the electron transport chain at site II [3, 4].

The mitochondrial responses can be regulated by calcium influx from the cytosol. Interaction between proline and calcium indicated that calcium altered the generation of ROS proline-dependent (fig 4). Our results are in agreement with other study showing that exogenous applied proline is capable of inducing calcium-dependent generation of ROS in Arabidopsis seedlings [27].

It is already known in the literature the effectiveness of creatine against the mitochondrial ROS formation. The activation of mitochondrial creatine kinase (mt-CK) by creatine regulates the generation of reactive oxygen species through an ADP re-cycling system[16]. In fact, we obtained similar results with the use of creatine on the ROS production by Pro, which was completely abolished using the two studied substrates (Fig. 5 A and B).

Therefore, if these effects of proline also occur in the mitochondria of the hyperprolinemic patients, it is possible that the induction of ROS may contribute to the tissue damage observed in some of them. In this case, it is possible that the use of antioxidants like creatine may be beneficial to these patients.

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Table 1: Effect of 2.5 mM Proline on respiratory chain parameters of rat liver mitochondria with different substrates (5mM glutamate/malate and 5mM succinate).

	State 2	State 3	State 4	RCR
Glutamate/Malate				
Control	13.14 ±1.17	52.81 ± 2.74	8.39 ± 0.86	6.37 ± 0.80
Proline	30.73 ± 6.85*	18.50 ± 1.07*	12.03 ± 0.93	1.54 ± 0.03
Succinate				
Control	7.59 ± 0.55	60.53 ± 1.99	11.16 ± 0.65	5.45 ± 0.29
Proline	39.13 ± 5.38*	17.74 ± 1.31*	16.69 ± 2.23	1,07 ± 0,14

Data are expressed as mean ± SEM of 3 animals. States 2, 3 and 4 = nmol of O_2 per min per mg of protein; RCR = state 3/state 4; *$p<0.05$ compared to the control (Two-way ANOVA for repeated measures followed by Bonferroni's comparison test).

FIGURE CAPTIONS

Fig. 1: Effects of proline on different sources of thiol groups. In letter A and B, respectively, the product of 50 µM DTNB mixed with aliquots of different concentrations of proline (0.25, 0.5 and 1 mM) incubated with L-cysteine (0.4 mM) or DTT (0.2mM) in PBS buffer pH 7.4, containing 1 mM of EDTA. In letter C, effects of 2.5 mM Pro on reduced (GSH) and oxidaized (GSSG) glutathione levels of rat liver.

Fig. 2: Effects of proline on liver mitochondrial DCFHDA oxidation in the presence of Complex I (rotenone) and IV (KCN) inhibitors. Mitochondria were incubated in a reaction medium containing 50 µM EGTA 100 mM sucrose, 65 mM KCl, and 10 mM HEPES, pH = 7.2. The substrates glutamate/malate (5 mM), letter A, or succinate (5 mM), letter B, were added 300 s after the reaction started. ADP (0.3 mM) and Pi (1 mM) were added together after 500 s of the reaction. The symbols represent the following: (open circle) Control; (open Square) KCN 0.75 mM; (open inverted triangle) KCN 0.75 mM + Rotenone 0.01 mM; (filled triangle) Pro 2.5mM; (filled square) Pro 2.5mM plus KCN 0.75 mM; (filled inverted triangle) Pro 2.5mM plus KCN 0.75 mM plus rotenone 0.01mM. *$p<0.05$ compared to the control; # $p<0.05$ compared to the Pro exposure (two-way ANOVA for repeated measures, followed by Bonferroni's Comparison Test when appropriate).

Fig. 3: Effects of proline on liver mitochondrial DCFHDA oxidation in the presence of Complex III (Antimycin A) inhibitor. Mitochondria were incubated in a reaction medium containing 50 µM EGTA 100 mM sucrose, 65 mM KCl, and 10 mM HEPES, pH = 7.2. The substrates glutamate/malate (5 mM), letter A, or succinate (5 mM), letter B, were added 300 s after the reaction started. ADP (0.3 mM) and Pi (1 mM) were added together after 500 s of reaction. The symbols represent the following: (open circle) control; (open Square) antimycin A 1µM; (filled triangle) proline 2.5mM; (filled square) proline 2.5 mM plus antimycin A 1µM. *$p<0.05$ compared to the control (two-way ANOVA for repeated measures, followed by Bonferroni's Comparison Test when appropriate).

Fig. 4: Effects of proline on liver mitochondrial DCFHDA oxidation in the presence of calcium and Ca^{+2} channel blocker Ruthenium Red. Mitochondria were incubated in a reaction medium containing 50 µM EGTA 100 mM sucrose, 65 mM KCl, and 10 mM HEPES, pH = 7.2. The substrates glutamate/malate (5 mM), letter A, or succinate (5 mM), letter B, were added 300 s after the reaction started; ADP (0.3 mM) and Pi (1 mM) were added together after 500 s and subsequently $CaCl_2$ (10 µM) was added after 600s of reaction. The symbols represent the following: (open circle) control; (open Square) $CaCl_2$ 10 µM; (open inverted triangle) $CaCl_2$ 10µM and Ruthenium Red 5 µM, (filled triangle) proline 2.5mM, (filled square) pro 2.5 mM plus $CaCl_2$ 10 µM; (filled inverted triangle) pro 2.5mM plus $CaCl_2$ 10µM plus Ruthenium Red 5 µM. *$p<0.05$ compared to the control (two-way ANOVA for repeated measures, followed by Bonferroni's Comparison Test when appropriate).

Fig. 5: Effects of proline on liver mitochondrial DCFHDA oxidation in the presence of creatine. Mitochondria were incubated in a reaction medium containing 50 µM EGTA 100 mM sucrose, 65 mM KCl, and 10 mM HEPES, pH = 7.2. The substrates glutamate/malate (5 mM), letter A, or succinate (5 mM), letter B, besides ADP (0.3 mM) and Pi (1 mM) were added together since the initiation of reaction. The symbols represent the following: (open circle) control; (open square) creatine 1 mM; (filled triangle) proline 2.5 mM; (filled square) proline 2.5 mM and creatine 1 mM. *$p<0.05$ compared to the control (two-way ANOVA for repeated measures, followed by Bonferroni's Comparison Test when appropriate).

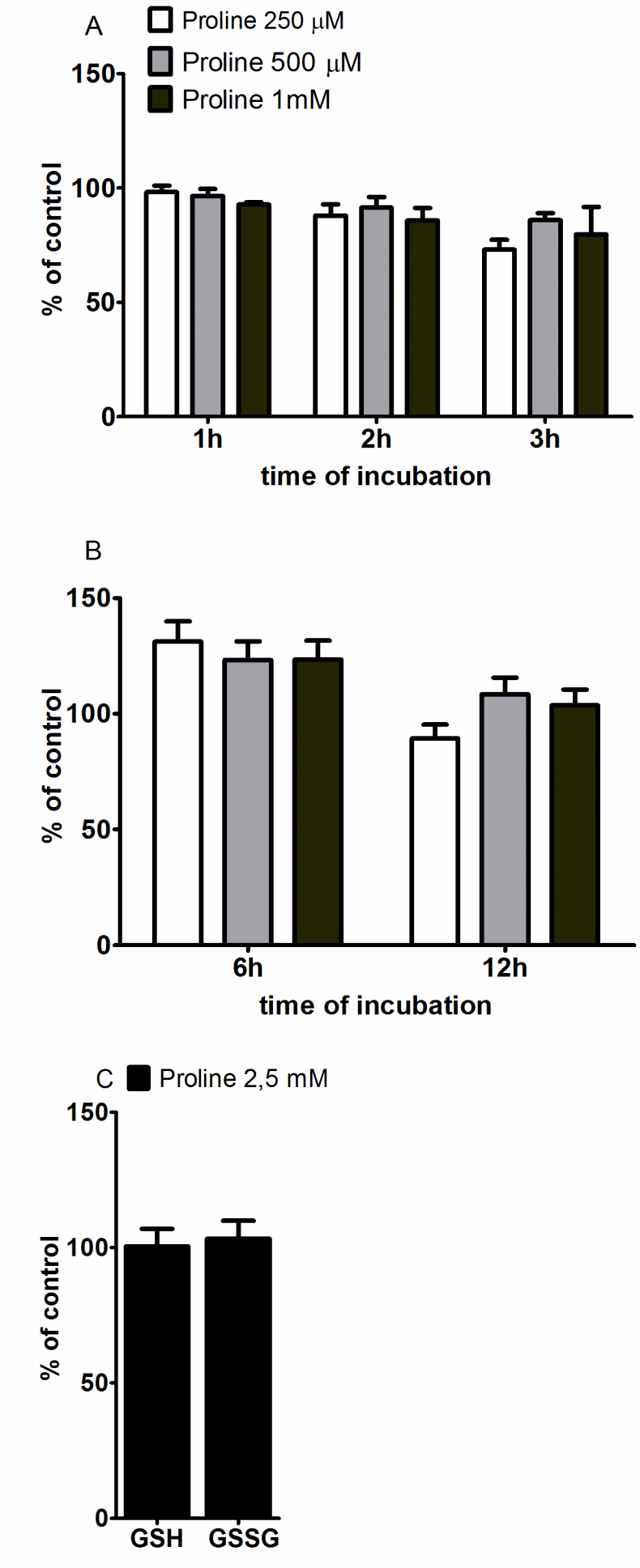
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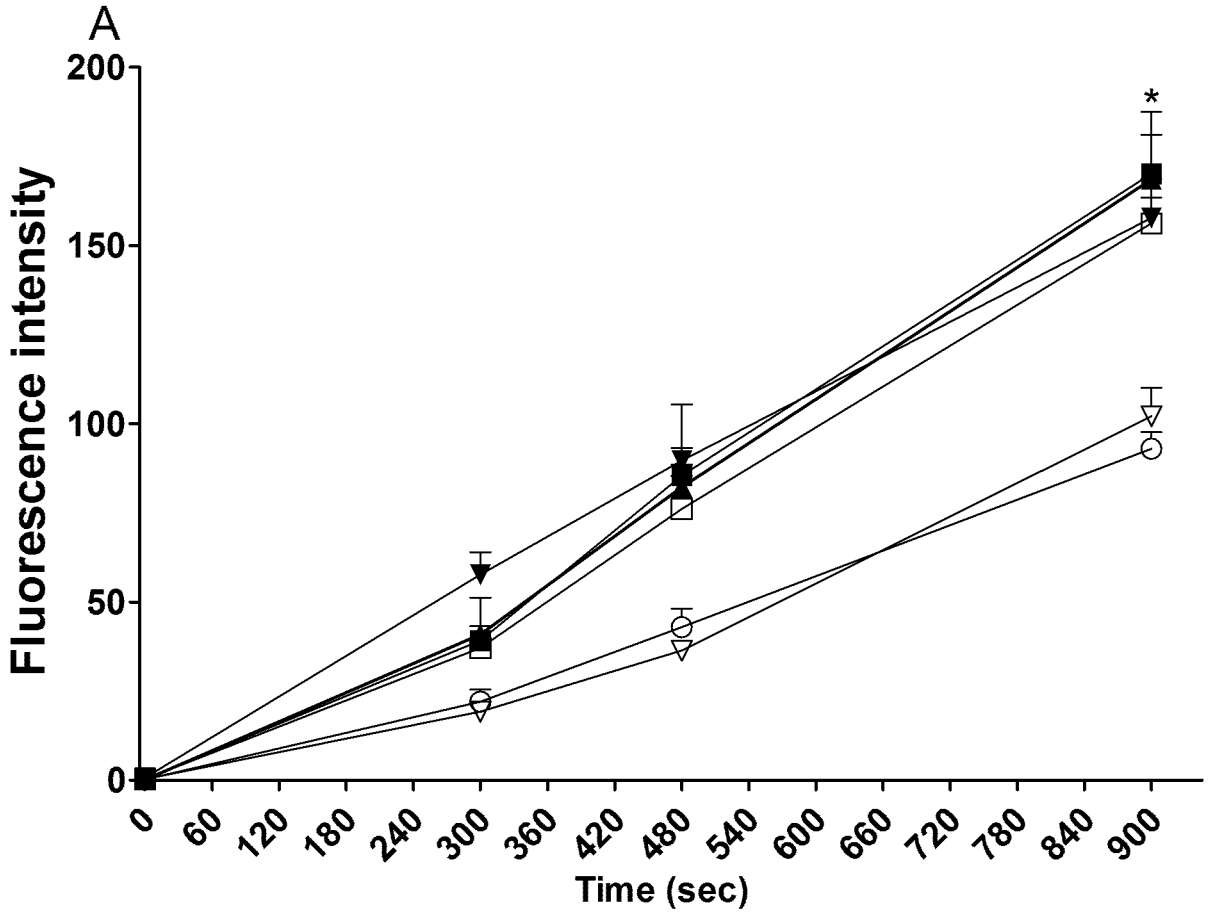
Fig. 1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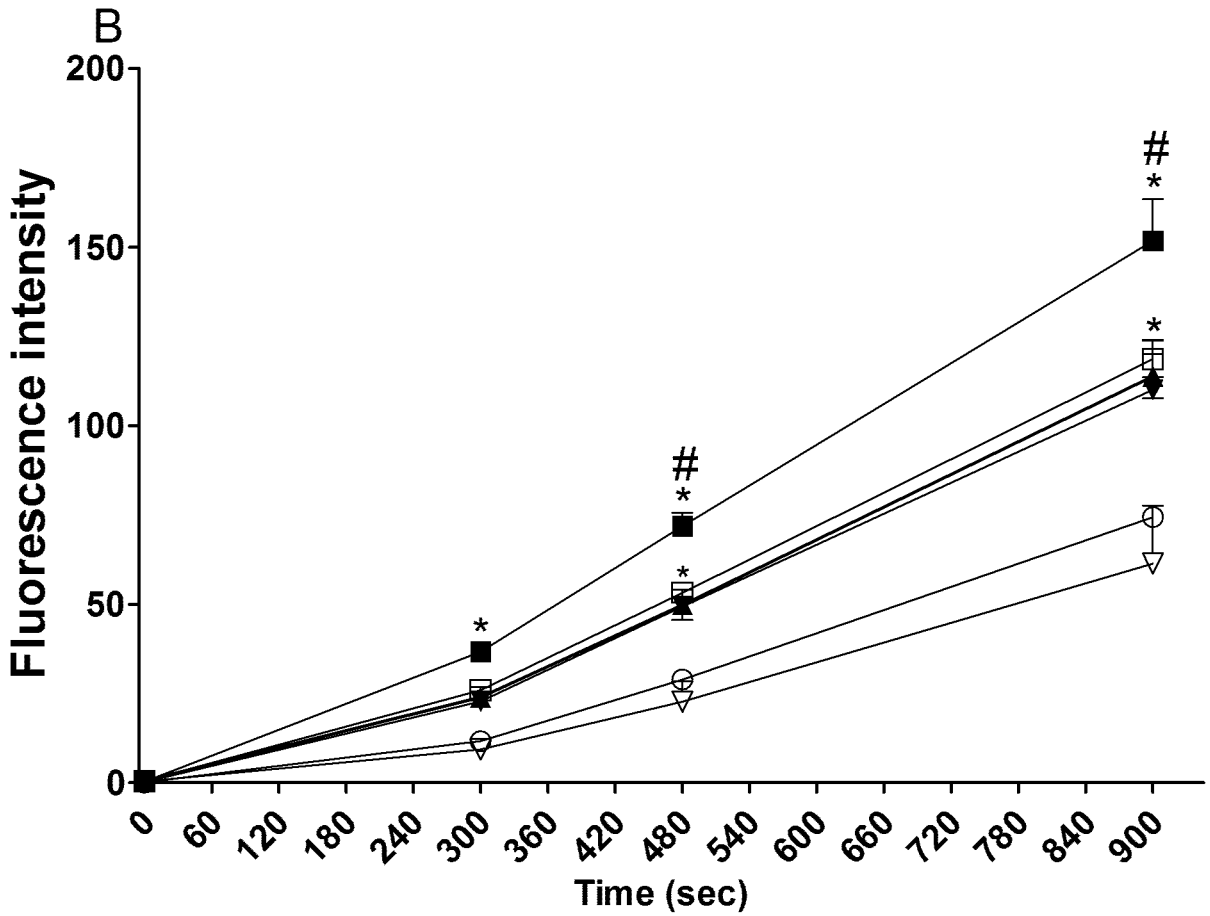
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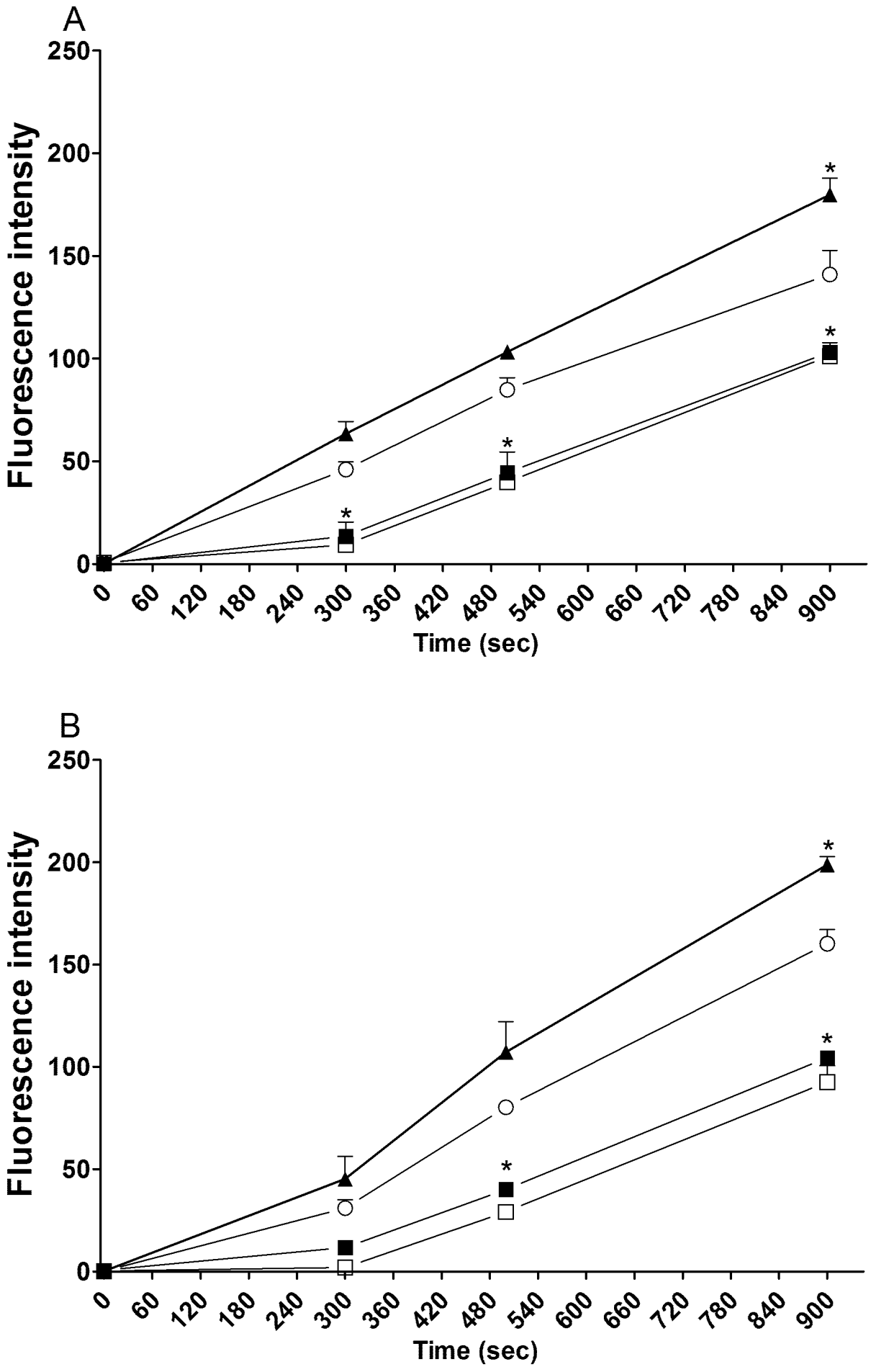
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Fig. 3

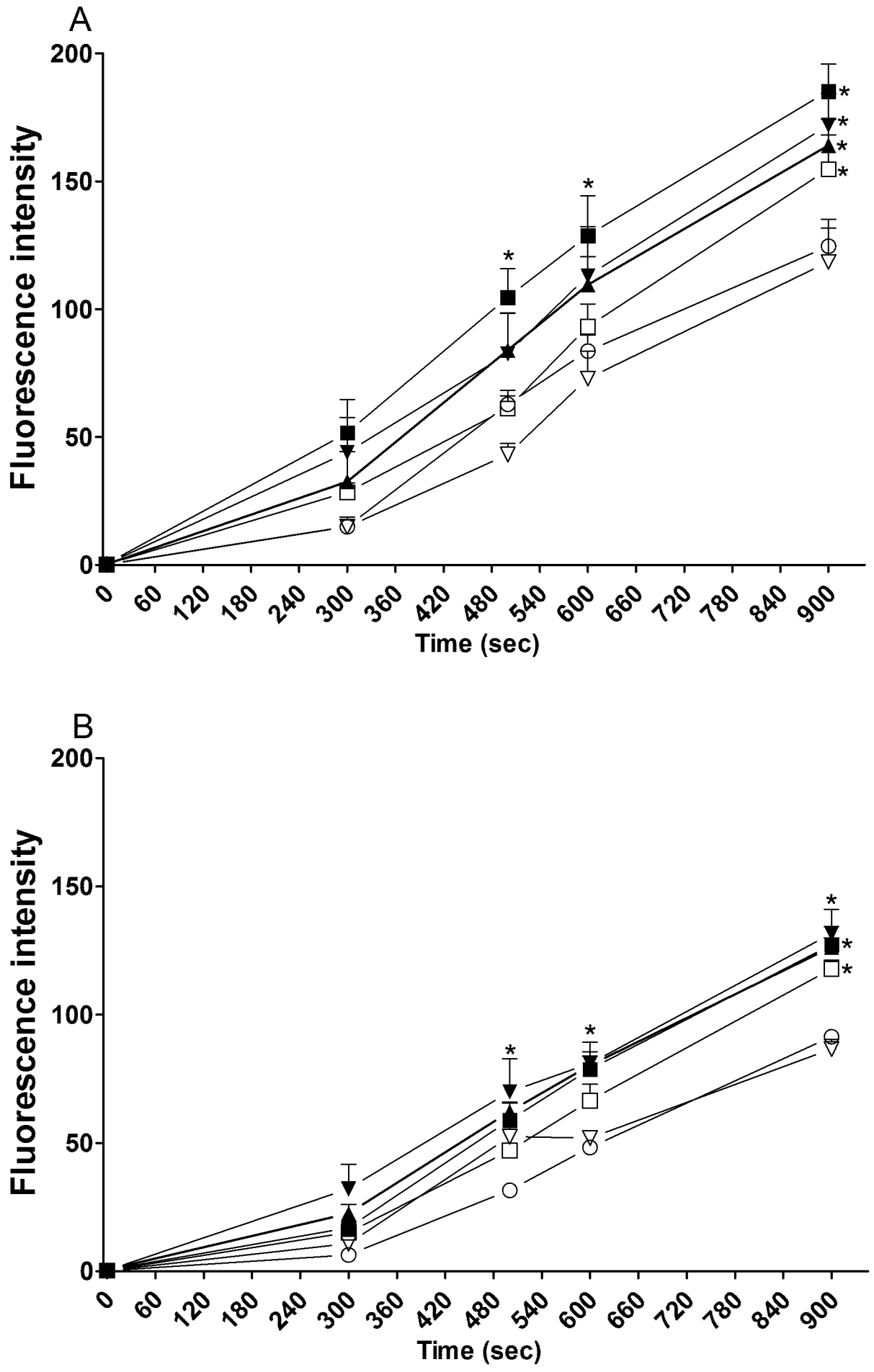
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Fig. 4

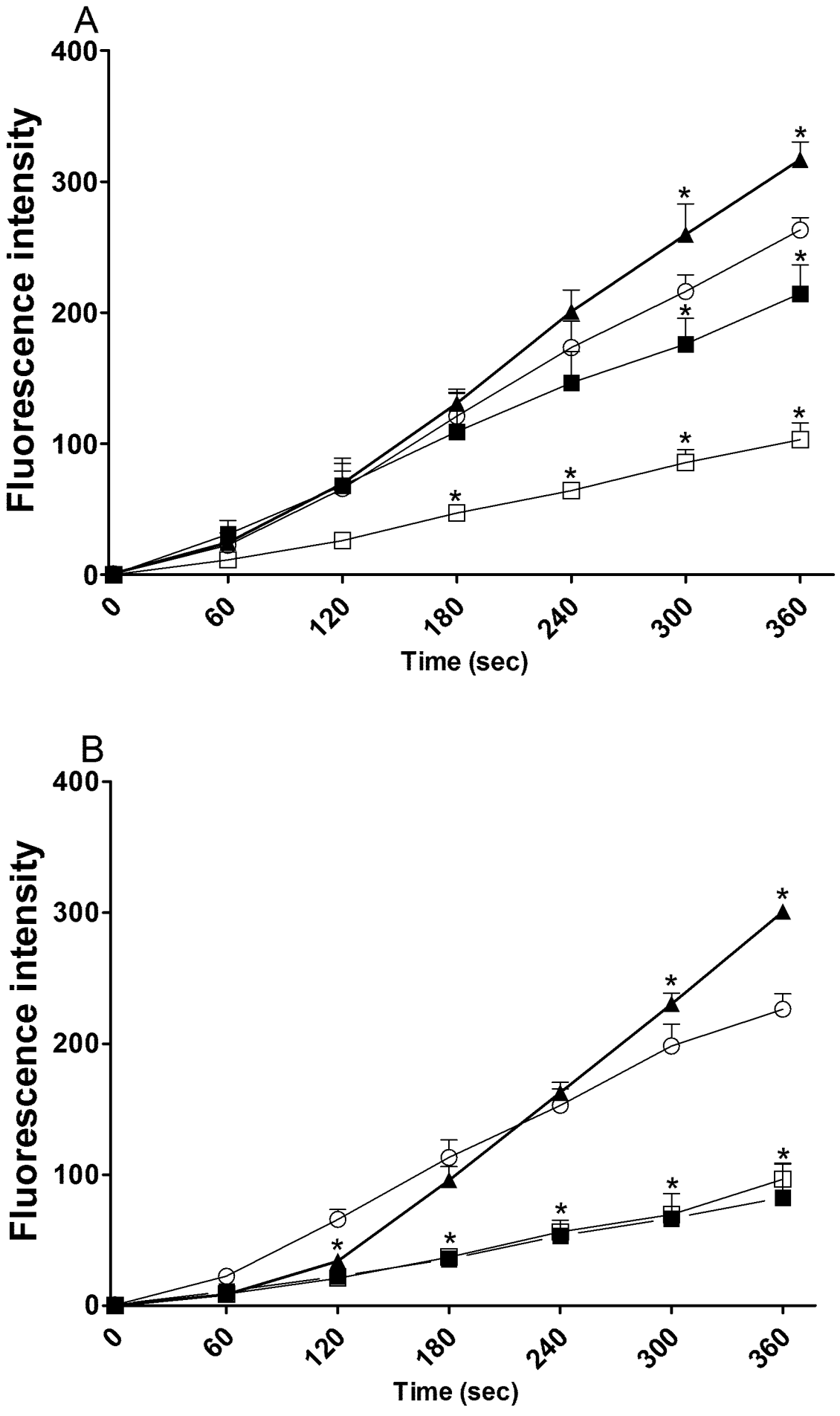
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Fig. 5

Proline administration to female rats during pregnancy and lactation induces oxidative stress in the brain of the offspring

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ABSTRACT

Type II Hyperprolinemia is an inherited disorder caused by a deficiency of Δ1-pyrroline- 5-carboxilic acid dehydrogenase, whose biochemical hallmark is proline accumulation in plasma and tissues causing several problems including cerebral damage in some affected children. In the present study we investigated the action of pyruvate and creatine on the effects elicited by chronic proline administration to female rats during pregnancy and lactation on the offspring. We evaluated some oxidative stress parameters as well as the activities of two enzymes of the energy homeostasis, creatine kinase (CK) and pyruvate kinase (PK), in brain cortex and hippocampus of the offspring. We found that proline administration to the mothers induced oxidative stress and diminished the enzymes activity in the two brain structures of the offspring. However, co-administration of creatine plus pyruvate did not fully prevent the alterations caused by proline administration. These results suggest that children born from hyperprolinemic mothers should be at risk to developing brain alterations. Besides, creatine and pyruvate supplementation in the use doses seems to have only partial benefit.

Keywords: proline; hyperprolinemia; oxidative stress; creatine kinase; pyruvate kinase; brain.

1. INTRODUCTION

Hyperprolinemia type II (HPII) is an inborn error of metabolism caused by a severe deficiency of Δ1-pyrroline-5-carboxilic acid dehydrogenase activity, resulting in tissue accumulation of proline (Pro). Hyperprolinemic patients may present neurological manifestations including seizures and mental retardation. HPII increases plasma and tissues levels of Pro and, despite many hyperprolinemic individuals remain asymptomatic, high concentrations of Pro and neurological symptoms in hyperprolinemia type II seem to be correlated (Hu et al., 2008; Phang et al., 2001).

As the mechanisms underlying hyperprolinemia symptoms are still obscure, our group has developed a chemical model for hyperprolinemia in rats mimicking plasma and tissue levels of proline found in human HPII (Moreira et al., 1989). Rats chronically treated with proline following such experimental model present a significant deficit in habituation to the open field, indicating an impairment of learning/memory. Since the brain is particularly vulnerable to oxidative damage due to the high utilization of oxygen (Butterfield and Stadtman, 1997), oxidative stress is an important event related to the pathogenesis of some neurodegenerative disorders (Andersen et al., 1997; Halliwell and Gutteridge, 1985; Reznick and Packer, 1993). On the other hand, pretreatment with vitamins E and C prevented the effects of Pro administration which inhibits cerebral cortex and hippocampal (Na+,K+)- ATPase (Ferreira et al., 2011; Franzon et al., 2003) and brain acetylcholinesterase activities in rats (Delwing et al., 2003c) both considered critical enzymes for normal CNS function (for a detailed revision, (Wyse and Netto, 2011)).

Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that alteration in creatine kinase (CK) activity

may participate in a neurodegenerative pathway leading to neuronal loss in the brain (Tomimoto et al., 1993). CK catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. This enzyme activity plays a key role in energy metabolism of tissues with intermittently high and fluctuating energy requirements, such as neuronal tissues like brain and retina (Wallimann et al., 1992). In fact, we have demonstrated that proline inhibits the *in vivo* and *in vitro* CK activity in rat brain (Kessler et al., 2003a, 2003b). Moreover, another enzyme crucial in the energy metabolism of the brain is pyruvate kinase (PK), which catalyzes the final step in the glycolytic pathway, the conversion of phosphoenolpyruvate (PEP) to pyruvate with the synthesis of one ATP molecule. Considering that PK is a key enzyme for glucose metabolism in the brain, the inhibition of brain PK by phenylalanine (Phe) may contribute to a lack on glycolysis leading to brain damage and culminating in neurological symptoms developed by patients affected by Phenylketonuria (PKU) (Glazer and Weber, 1971). In addition, we also verified that *in vivo* and *in vitro* PK activity decreases after exposure to agents promoting generation of free radicals, like tyrosine, and this inhibition *in vitro* could be reversed by the addition of GSH (de Andrade et al., 2012). Taken these data together, it is possible that proline administration to female rats may inhibit these enzymes even in the offspring.

Although much is known about proline needs in mamals (Barbul, 2008), there is a paucity of information about roles for proline in growth and development of the fetus and neonate, as well as lactation performance of mothers. In addition, currently therapies, i.e., restriction of proline, are used in HP patients, but only modest control of plasma proline is achieved with no impact in clinical condition (Mitsubuchi et al., 2008; Phang et al., 2001). Therefore, the search for new therapeutic strategies is necessary. One that can be considered is the administration of the energy substrates creatine and

pyruvate, two substances known to act as antioxidants. We have reported that these two substances prevent oxidative stress and behavioral defects in rats subjected to intra-hippocampal administration of phenylalanine (Berti et al., 2012). In this regard, in the present research we investigated the impact of proline administration to female rats during pregnancy and lactation on some parameters of energy metabolism and oxidative stress in the offspring. Besides, we investigated the possible preventive effect of pyruvate and creatine on the alterations of the same parameters.

2. MATERIALS AND METHODS

2.1 Animals and Reagents

Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were housed in four groups of three males and seven females until the day that pregnancy was observed. On this day, females were housed separately and seven male babies were kept with mother after the birth day. They were maintained on a 12:12 h light/dark cycle, in an air-conditioned constant temperature (22 ±1ºC) colony room with free access to water and a commercial chow (Supra®, Brazil). After birth, rats were weighed every day in order to accompany the growing of the offspring. The ‘‘Principles of Laboratory Animal Care’’ (NIH publication no. 80-23, revised 1996) were followed in all the experiments, and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2 Treatment

Twenty eight female rats were randomly separated into four groups and coupled with three males in each group. The females received twice a day at 12 hour intervals administration of 10 µl/g of body weight of the following buffered solutions (pH was adjusted to 7.4): saline group, 0.9% saline subcutaneously plus 0.9% saline intraperitoneally; proline group, proline (0.2 mg/g body weight) (Moreira et al., 1989) plus 0.9% saline intraperitoneally; group creatine + pyruvate: pyruvate (0.2 mg/g body weigh) (Ryu et al., 2006) + creatine (0.4 mg/ g body weigh) (Stockler et al., 1994) intraperitoneally plus 0.9% saline subcutaneously; group proline + creatine + pyruvate, the same doses through the same vias as indicated above Treatment of females began at the moment of the couple and continued until weaning of the offspring (at 21 postpartum days). Pups were killed without anesthesia at 21 days of age. For each set of biochemical measurements, seven pups from different litters of the same group were used. Some animals of the offspring were used for pilot studies and the others were used for experiments not included in this manuscript.

2.3 Tissue preparation

Rats of 21th day-old were killed by decapitation, the brains were rapidly removed and the cerebral cortex and hippocampus were immediately dissected on an ice-cooled glass plate. The cerebral structures were washed and homogenized (1:10 w/v) with a Potter-Elvehjem glass homogenizer in an ice-cold buffer or containing 20 mM sodium phosphate and 140 mM potassium chloride, pH 7.4 for oxidative stress parameters or containing 0.32 M sucrose , 1 mM EGTA and 10 mM Tris–HCl ,pH 7.4 for energy metabolism enzymes determinations. All the homogenates were centrifuged at 800 g for 10 min at 4ºC in a Sorval centrifuge, the pellets were discarded and part of supernatants were kept at -75 ºC until its use for experiments for oxidative stress determinations. The other part of supernatants, for energy enzymes activities were

centrifuged again at 10 000 xg for 15 min at 4 ºC. The mitochondria- free supernatants were used for the determination of the protein concentration and the pyruvate kinase and cytosolic creatine kinase enzyme assays. The pellets were centrifuged one more time at 10 000 xg for 15 min at 4 ºC and resuspended in a buffer containing 100mM Tris-HCl and 15 mM MgSO4 and were used for the determination of the protein concentration and mitochondrial creatine kinase enzyme assay.

2.4 DCFA oxidation

ROS formation was determined by the method that measures the conversion of 2,7-dichlorofluresceine diacetate (DCFH-DA) to dichlorofluoresceine (DCF) by ROS mediated oxidation as described by Le Bel et al. (LeBel et al., 1992). DCFH-DA was dissolved in ethanol and stored as 5 mM stock at -20°C. The samples were incubated for 30 min at 37°C with DCFH-DA (100 µM) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl (10 ml). Esterases present in the samples remove DA and DCFH formed is oxidized to DCF by ROS present in the samples. Then, fluorescence was directly measured (excitation and emission wavelengths 488 and 525 nm, respectively) with a SpectraMax® M5 / M5e Microplate Reader. A calibration curve was performed with to dichlorofluoresceine (DCF) as standard, to calculate the concentration of ROS present in the samples. ROS concentration was expressed in arbitrary units (DCF/mg total proteins).

2.5 Total Radical-Trapping Antioxidant Parameter (TRAP)

TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2'-azo-bis(2-amidinopropane) hydrochloride (ABAP) (Lissi et al., 1992). In a 96-well plate it was added 10 µL of the test samples in a reaction medium containing the free radical source

ABAP 10mM, dissolved in sodium phosphate buffer 50 mM pH 8.6, and Luminol (19µM) dissolved in NaOH 0,1M as external probe to monitor radical production. The chemiluminescence generated was detected by 1450 MicroBeta® TriLux Microplate Scintillation and Luminescence Counter. The TRAP of the samples was evaluated by inhibition of chemiluminescence along 70 minutes as area under curve (AUC). Area under curve (AUC) of 7 µg samples and radical basal production was achieved by software (GraphPad Prism Software Inc., San Diego, CA, USA – version 5.00) as described by Dresch et al 2009 (Dresch et al., 2009). The results were expressed by the ratio of area under curve of the sample (A) per area under curve of the radical basal production (A0).

2. 6 Thiobarbituric acid reactive substances (TBARS)

TBARS levels were determined according to the method described by Ohkawa et al. (Ohkawa et al., 1979). Briefly, 40 µl of 8.1% sodium dodecyl sulfate, 0.3 mL of 20% acetic acid solution adjusted to pH 3.5 and 0.3 mL of 0.8% thiobarbituric acid were added to 160 µL of tissue homogenate in a 1.5 mL eppendorf tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at 1,000 x g for 10 min, the supernatant was taken and the resulting pink-stained TBARS were determined spectrophotometrically at 532 nm. A calibration curve was performed with the polymerization of malonyldialdehyde (MDA) formed after the acid hydrolysis 1,1,3,3-tetramethoxypropane (TMP) to calculate the concentration of TBARS present in the samples. The results were reported as nmol of TBARS per mg protein.

2.7 Total sulfhydryl content

This assay is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412nm (Aksenov and Markesbery, 2001). Briefly, 14 µL of supernatant were added in 270 µL of PBS buffer pH 7.4, containing 1 mM of Ethylenediaminetetraacetic acid .Then, 16 µL of 10mM DTNB was added. This was followed by 30-min incubation at room temperature in a dark room. Absorption was measured at 412nm. Results are reported as nmol TNB/mg of protein.

2.8 Superoxide dismutase assay (SOD)

SOD activity was measured by the method of Maklund (Marklund, 1985). This method is based on the capacity of pyrogallol autoxidize, a process highly dependent on O2, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 412nm. A calibration curve was performed with purified SOD as standard, to calculate the activity of SOD present in the samples. The results are reported as U/mg of protein.

2.9 Glutathione peroxidase assay (GPx)

GPx activity was measured according to Wendel (Wendel, 1981) using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/ethylenediaminetetraacetic acid 1mM, pH 7.7, 2mM glutathione, 0.3 U/mL glutathione reductase, 0.4mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and 0,06 mg protein of supernatant . The specific activity was calculated as nmol of NADPH consumed per minute per mg of protein.

2.10 Pyruvate kinase activity assay

Pyruvate kinase activity was assayed as described by Leong et al. (Leong et al., 1981) with minor modifications. The incubation medium consisted of 0.1 M Tris /HCl buffer, pH 7.5, 10 mM MgCl, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 1.0 unit of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100 and approximately 0.03mg protein of samples. The reaction started by the addition of 1.0 mM phosphoenol-pyruvate (PEP). All assays were performed in duplicate at 25 ºC. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine and the amount of creatine already present in the enzymatic material. Results were expressed as µmol of pyruvate formed transformed per min per mg of protein.

2. 11 Creatine Kinase Activity Assay

The creatine formed was estimated according to the colorimetric method of Hughes et al. (Hughes, 1962). The reaction mixture contained 60 mM Tris-HCl buffer (pH 7.5), 7 mM phosphocreatine, 9 mM MgSO4, 0.02% triton X-100, and approximately 1 µg protein in a final volume of 0.1 mL. After 5 min of pre-incubation at 37ºC, the reaction was started by the addition of 0.3 µmol ADP. The reaction was stopped after 10 min by the addition of 1 µmol of p-hydroxymercuribenzoic acid. The color was developed by the addition of 2% α-naphtol and 0.05% diacetyl and read after 20 min at 540 nm. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine and the amount of creatine already present in the enzymatic material. Results were expressed as µmol of creatine formed per min per mg protein.

2.12 Protein Determination

The protein content was determined by the method of Lowry et al. (Lowry et al., 1951) using serum bovine albumin as the standard.

2.13 Statistical Analysis

Statistics was performed using the Statistical Package for the Social Science (SPSS) software in a PC-compatible computer. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Tukey's HSD Multiple Comparison Test when appropriate. Data were considered statistically significant when $p < 0.05$.

3. RESULTS

Tables 1 and 2 summarize the effects of the treatments of female rats during pregnancy and lactation on parameters of oxidative stress and energy homeostasis in the brain structures from the pups.

The effect of treatments on ROS formation was assessed by DCFH oxidation. We examined that the proline and creatine + pyruvate treatments had significantly interaction in this parameter for both structures, cerebral cortex and hippocampus [$F(1,28)= 94.38$, $p<0.0001$ and $F(1,26)=37.22$, $p<0.0001$; respectively]. Comparison between groups in the two tissues by the Tukey test showed that proline decreased ROS formation and the association of proline with creatine + pyruvate prevented this decrease, suggesting that the decrease of ROS could be due to a response to oxidative stress.

Two-way analysis of TRAP results showed significant interaction [$F(1,26)$ 10.71, $p<0.01$ and $F(1,25)= 17.49$, $p<0.0001$ for brain cortex and hippocampus, respectively. In the cerebral cortex TRAP increased significantly, which means that the antioxidant capacity of the samples decreased in comparison to the saline group, but co-

administration of creatine + pyruvate did not prevent the increase of TRAP. In hippocampus, although interaction was significant, proline did not increase TRAP significantly.

Lipoperoxidation of the brain structures was assessed by thiobarbituric acid reactive substances (TBARS). and we observed that although there are statistically significant interaction between the groups for both, cerebral cortex [$F(1,27)=4.70$, $p<0.05$] and hippocampus [$F(1,27)= 54.50$, $p<0.0001$], only in hippocampus Pro had main effect [$F(1,27)=13.88$, $p<0.001$]. These data demonstrate that the TBARS levels in cerebral cortex were not altered by proline administration alone, but were increased by co-administration of creatine + pyruvate. Differently, in hippocampus proline administration was able to increase significantly the TBARS levels and, as observed in the cerebral cortex, and co-administration of creatine + pyruvate prevented this increase.

Next, we investigate the total sulfhydryl content in the brain of the treated groups and we also observed on the two-way ANOVA test significant interaction between them for both cerebral cortex and hippocampus [$F(1,27)= 24.84$, $p<0.0001$ and $F(1,27)= 30.71$, $p<0.0001$, respectively. In agreement with the TRAP data there was a significant diminution in the sulphydryl content of cerebral cortex. Similarly, in the hippocampus, the sulphydryl content decreased in all groups treated with the amino acids in comparison to group that only received saline. However, co-administration of creatine + pyruvate did not prevent the effect of proline.

We investigated the effect of treatments on the antioxidant enzymes GPx and SOD in the cerebral cortex and hippocampus. As we can be observe in Table 1 and 2. SOD enzyme showed a similar activity in cerebral cortex and hippocampus. Proline administration showed main effect for cerebral cortex [$F(1,22)= 11.68$, $p<0.01$] and for

hippocampus [$F(1,21)= 13.29$, $p<0.01$]. However, significant interaction between proline and creatine + pyruvate was not observed for the two tissues, suggesting that the co-administration of proline with the two substances did not prevent the effect of proline. On the other hand, proline administration did not show main effect on GPx activity in cerebral cortex and co-administration of creatine + pyruvate did not show interaction. In hippocampus, proline administration had main effect [$F(1,23)= 11.68$, $p<0.01$], but co-administration of creatine + pyruvate did not show interaction suggesting that this association did not prevent the effect of proline.

We also investigate if the treatments could affect the activities of two important enzymes of the energy homeostasis in the brain structures. Two-way ANOVA showed significant main effect for proline on PK activity in the cerebral cortex [$F(1,23)= 18.62$, $p<0.0001$], and co-administration of creatine + pyruvate with proline prevented the decrease of PK activity. Furthermore, in the hippocampus, proline presented significant main effect [$F(1,26) = 12.35$, $p<0.01$] and co-administration of creatine + pyruvate with proline prevented the decrease of PK activity.

Finally, we investigated the effect of the treatments in the compartmentalized CK activity. Two-way ANOVA showed significant interaction between proline and the association of creatine + pyruvate on cytosolic CK activity in brain cortex [$F(1,27)= 7.76$, $p<0.01$] and in hippocampus $F(1,26)= 32.43$, $p<0.0001$]. For mitochondrial CK activity two-way ANOVA showed significant main effect for proline in brain cortex [$F(1,27) = 7.21$, $p<0.05$] and in hippocampus [$F(1,27)= 7.45$, $p<0.05$]. In addition, co-administration of creatine + pyruvate with proline prevented the decrease of PK activity in the two tissues.

4. DISCUSSION

Although many hyperprolinemic patients present a neurological syndrome consisting mainly of seizures and mental retardation, others affected individuals are clinically normal (Phang et al., 2001). However, a causal relationship between high concentration of Pro and recurrent seizures has been documented in patients with type II hyperprolinemia, suggesting that high sustained tissue Pro levels may predispose to convulsions (Flynn et al., 1989). In this regard, our research groups have developed a chemical experimental model of hyperprolinemia in rats (Moreira et al., 1989) that reproduces the tissue levels of Pro found in human HP II (Phang et al., 2001) in order to investigate the effect of high doses of this amino acid on several biochemical and behavioral parameters. Because there are no reports on maternal hyperprolinemia, we decided to investigate the effect of proline administration to female rats during pregnancy and lactation on some parameters of oxidative stress and on the activities of two enzymes related to energy homeostasis in brain cortex and hippocampus of the offspring immediately after weaning.

It has been shown that Pro administration impairs memory (Bavaresco et al., 2005), induces free radical generation and reduces antioxidants defenses in rat brain, suggesting that Pro elicits oxidative stress (Delwing et al., 2003a; Delwing et al., 2003b; Liu et al., 2005). In the present work we detected decrease in ROS content by proline in the two brain structures tested. It was previously described that during proline oxidation, the superoxide formed leads to toxic levels of superoxide anion radicals, but the co-expression of mitochondrial superoxide dismutase (SOD) diminishes this effect (Hu et al., 2007). These data are in agreement with our results showing the increase of SOD activity in the two cerebral structures of the proline group. Therefore, it is feasible that the increased SOD activity might dismutate superoxide radicals diminishing its

content. Glutathione peroxidase (GPx) was not significantly altered by proline administration. It is known that GPx is not the only enzyme which detoxifies H_2O_2. Cells are equipped with catalase, thioredoxin system and peroxiredoxins and in recent years, it has been showed that peroxiredoxins (Prx) may be the most important H_2O_2-removal systems in animals. Though their catalytic efficiency is less than that of GPx or catalase, are abundant in the cytosol, typically constituting 0.1 to 0.8% of total soluble protein, exhibiting higher affinity toward H_2O_2 (Chae et al., 1999; Rhee et al., 2005).

Since oxidative stress is defined as the imbalance between free radical production and antioxidant defenses, and several investigators have shown that proline oxidation is a source of reactive oxygen species (ROS) (Donald et al., 2001; Liu et al., 2006; Maxwell and Rivera, 2003; Pandhare et al., 2006). Our results indicate that Pro induces free radical generation even in the offspring of hyperprolinemic female rats leading to lipid peroxidation, as verified by the TBARS levels on the Hippocampus. Besides, Pro reduces antioxidant defenses in the cerebral cortex as demonstrated by the reduction of total antioxidant capacity (TRAP) and the reduction of the sulphydryl content for both brain structures. Moreover, proline increases SOD activity and decreases DCFH oxidation, suggesting that superoxide radicals were initially produced.

Because the sulfhydryl group reacts with various oxygen species, this reactivity turns thiol-containing enzymes in vulnerable targets of ROS. Our results demonstrate that CK and PK, two thiol-containing enzymes that are crucial for energy homeostasis in brain are inhibited in the offspring from mothers subjected to proline administration. In support of these findings, a decrease of CK activity is considered one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, including Alzheimer's disease (Aksenov et al., 1997). Moreover, it has been reported that pyruvate deficit as a consequence of PK inhibition may induce cell death (Valentini

et al., 2000). On the other hand, this study showed that the co-administration of creatine plus pyruvate prevented the decrease of the activity of these enzymes. In fact, creatine administration has proven to be protective for neurons in a variety of pathogenic conditions (Brustovetsky et al., 2001). In addition, we have also observed that the association of these two substances prevented alterations on some oxidative stress parameters, besides behavioral effects, caused by the intra-hippocampal administration of phenylalanine (Berti et al., 2012).

5. CONCLUSION

In summary, in this study we demonstrated that the administration of proline to female rats during pregnancy and lactation affect some metabolic parameters in the brain of the offspring, such as oxidative stress and enzymes of the energy homeostasis. Moreover, the co-administration of creatine and pyruvate was able to prevent the harmful effects of proline on the energy enzymes, CK and PK, and partially on some parameters of oxidative stress.

Although these parameters vary with the structure, we observed a tendency of hippocampus to be more sensitive than cerebral cortex to the proline effects. These findings may support other studies which show that administration of proline produces retrograde amnesia and disrupt the formation of new memories in poultry (Cherkin et al., 1976; Van Harreveld and Fifkova, 1973).

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Table 1. Effect of proline and creatine plus pyruvate administration to female rats during pregnancy and lactation on parameters of oxidative stress and energy homeostasis in brain cortex of the offspring

Groups	saline	proline	creatine+pyruvate	creatine+pyruvate+proline
Oxidative stress parameters				
DCFH oxidation	38.3±0.2	24.5±0.6[a]	47.1±0.2	40.5±0.3
TRAP	0.78±0.01[a]	0.83±0.01[b]	0.84±0.01	0.84±0.01
TBARS	2.15±0.11	1.87±0.10[c]	3.09±0.18[a]	3.49±0.20[a]
Total sulfhydryls	204±14.2[a]	118±4.2[b]	1285±4.3	120±0.3
SOD	1.99±0.31	5.24±0.86[a]	2.06±0.42	4.62±1.07
GPx	80.1±1.5	86.2±6.5	101.6±3.6[b]	90.8±4.3
Energy parameters				
Pyruvate kinase	4.14±0.08	3.52±0.09[a]	4.67±0.13	4.32±0.14
Creatine kinase (cytosol)	8.93±0.41[a]	6.97±0.52[a]	8.55±0.47	9.37±0.57
Creatine kinase (mitochondria)	5.32±0.42	3.75±0.21[a]	6.64±0.41	6.17±0.41

Data are mean ± SEM for 7 independent animals for each group. Equal letters between the groups mean no significant difference between them. (One-way ANOVA test, followed by Tukey's HSD Multiple Comparison Test). a = $p < 0.05$, different from the other groups; b= different from saline group; c= different from proline+creatine+pyruvate group. Abbreviations: DCFH: 2',7'-Dihidrochlorofluresceine oxidation, expressed as DCF formed per mg of protein; TRAP:Total Radical-Trapping Antioxidant Parameter expressed as A/A_0 ;TBARS: Thiobarbituric acid reactive substances expressed as nmol of TBARS per mg of protein; Sulfhydryl content expressed as nmol of TNB formed per mg of protein; SOD: Superoxide dismutase expressed as U per mg of protein ; GPX: Glutathione peroxidase activity expressed as nmol of NADPH consumed per minute per mg of protein; pyruvate kinase expressed as µmol of pyruvate formed per min per mg of protein; creatine kinase expressed as µmol of creatine formed per min per mg of protein.

Table 2. Effect of proline and creatine plus pyruvate administration to female rats during pregnancy and lactation on parameters of oxidative stress and energy homeostasis in brain hippocampus of the offspring

Groups	saline	proline	creatine+pyruvate	creatine+pyruvate+proline
Oxidative stress parameters				
DCFH oxidation	53.7±1.9	28.76±0.28[a]	44.16±4.05	45.06±1.40
TRAP	0.84±0.01	0.85±0.01	0.86±0.01	0.82±0.01
TBARS	2.54±0.19	4.61±0.23[a]	3.89±0.11	3.21±0.18
Total sulfhydryls	209±8.9[a]	151±6.9[b]	159±2.3	166±1.9
SOD	2.71±0.44	7.30±1.31[a]	2.90±0.61	4.73±0.59
GPx	129±5.4	106±2.4	178±14.2[a]	132±6.9
Energy enzymes				
Pyruvate kinase	5.98±0.27	3.86±0.20[b]	5.50±0.57	4.85±0.38
Creatine kinase (cytosol)	10.8±0.49	7.08±0.31[a]	9.10±0.64	10.88±0.39
Creatine kinase (mitochondria)	7.17±0.34	5.52±0.22[a]	8.02±0.45	7.48±0.52

Data are mean ± SEM for 7 independent animals for each group. Equal letters between the groups mean no significant difference between them. (One-way ANOVA test, followed by Tukey's HSD Multiple Comparison Test). a = $p < 0.05$, different from the other groups; b= different from saline group; c= different from proline+creatine+pyruvate group. Abbreviations: DCFH: 2',7'-Dihidrochlorofluresceine oxidation, expressed as DCF formed per mg of protein; TRAP:Total Radical-Trapping Antioxidant Parameter expressed as A/A_0 ;TBARS: Thiobarbituric acid reactive substances expressed as nmol of TBARS per mg of protein; Sulfhydryl content expressed as nmol of TNB formed per mg of protein; SOD: Superoxide dismutase expressed as U per mg of protein ; GPX: Glutathione peroxidase activity expressed as nmol of NADPH consumed per minute per mg of protein; pyruvate kinase expressed as µmol of pyruvate formed per min per mg of protein; creatine kinase expressed as µmol of creatine formed per min per mg of protein

DISCUSSÃO

Por ser um aminoácido secundário, a Prolina é metabolizada pelas suas próprias enzimas especializadas, com suas localizações teciduais próprias e mecanismos de regulação. A deficiência de alguma das enzimas específicas para o catabolismo da prolina resulta em hiperprolinemia levando ao acúmulo tecidual deste aminoácido (Phang et al. 2001).

Pacientes hiperprolinêmicos podem apresentar sintomas neurológicos e anormalidades cerebrais, cuja etiopatogenia é pouco compreendida. Embora vários pacientes hiperprolinêmicos apresentem uma síndrome neurológica que consiste principalmente de convulsões e retardo mental, outros indivíduos afetados são clinicamente normais (Phang et al. 2001). No entanto, uma relação causal entre a concentração elevada de Pro e convulsões recorrentes tem sido documentada em pacientes com hiperprolinemia tipo II, sugerindo que altos níveis de Pro podem predispor a convulsões. (Flynn et al. 1989). A este respeito, nosso grupo de pesquisa tem desenvolvido um modelo químico experimental de hiperprolinemia em ratos (Moreira et al. 1989) que mimetiza os níveis teciduais de Pro encontrados em seres humanos com HP II (Phang et al. 2001), a fim de investigar o efeito de Pro em vários parâmetros bioquímicos.

Os modelos animais não reproduzem completamente as doenças humanas em toda sua complexidade. No entanto, os modelos químicos em animais têm sido largamente utilizados, porque eles têm a vantagem de isolar cada substância conhecida que se acumula em doenças humanas e estudar contra o controle adequado. Portanto, os modelos animais são importantes na investigação dos mecanismos fisiopatológicos das doenças, especialmente no

metabolismo cerebral, ajudando a sugerir medidas de prevenção e novas drogas para tratamento.

Os ratos tratados cronicamente com prolina segundo nosso modelo experimental apresentaram um déficit significativo na habituação ao campo aberto, indicando uma deficiência de aprendizado / memória (Moreira et al. 1989). Além disso, nosso grupo tem demonstrado através desse modelo que concentrações elevadas de Pro induzem a peroxidação lipídica e reduzem as defesas antioxidantes não-enzimáticas e enzimáticas em cérebro de ratos (Delwing et al. 2003a). Por outro lado, o pré-tratamento com vitaminas E e C previnem os efeitos da administração de Pro, a qual inibe a atividade da enzima (Na +, K +) – ATPase do córtex cerebral e hipocampo (Ferreira et al. 2011, Franzon et al. 2003) e a atividade da acetilcolinesterase em cérebro de ratos (Delwing et al. 2003b), ambas as enzimas consideradas críticas para o funcionamento normal do SNC. Esses resultados sugerem que o estresse oxidativo está intimamente ligado à toxicidade da Pro. Portanto, parece que altos níveis de prolina podem ser neurotóxicos, ou pelo menos podem predispor ao dano cerebral.

Como o cérebro é particularmente vulnerável ao dano oxidativo devido à alta utilização de oxigênio (Butterfield and Stadtman 1997), o estresse oxidativo é considerado um importante evento relacionado com a patogênese de algumas doenças neurodegenerativas (Andersen et al. 1997, Halliwell and Gutteridge 1985, Reznick and Packer 1993). É conhecido na literatura que na oxidação da Pro a P5C pela POX, os elétrons derivados da Pro são transferidos para o FAD. Subsequentemente, os elétrons podem ser transferidos para o sítio II da cadeia de transporte de elétrons através do

citocromo *c*. Tanner e colegas, no entanto, têm mostrado que a FAD é diretamente acessível ao oxigênio; os elétrons podem reduzir diretamente oxigênio para produzir superóxido em *T. thermophilus* (White et al. 2007). Como o estresse oxidativo é definido como o desequilíbrio entre a produção de radicais livres e defesas antioxidantes, vários investigadores demonstraram que a oxidação de Pro é uma fonte de espécies reativas de oxigênio (EROs), portanto pode provocar estresse oxidativo (Pandhare et al. 2006, Maxwell and Rivera 2003, Donald et al. 2001, Liu et al. 2006). Nossos resultados *in vitro* obtidos pela exposição aguda de mitocôndrias hepáticas de ratos à concentrações clínicas de Pro corroboram os dados da literatura, mostrando que a Pro pode causar aumento na produção de EROs na mitocôndria através do complexo III. Porém, diferentemente das plantas (Chen et al. 2011), a produção de EROs mediada por Pro nas mitocôndrias hepáticas de ratos se mostrou independente da presença de cálcio.

Embora muito se saiba sobre as necessidades de Pro em mamíferos (Barbul 2008), há uma escassez de informações sobre as funções de Pro no crescimento e desenvolvimento do feto e recém-nascido, bem como o desempenho da lactação das mães. Em adição a isso, as terapias empregadas atualmente, como a restrição dietética de prolina, apenas alcançam modesta queda na concentração plasmática de prolina sem qualquer impacto no estado clinico dos pacientes (Mitsubuchi et al. 2008). No entanto, nosso grupo mostrou em estudo anterior com outro EIM de aminoácidos, a fenilcetonúria, que os substratos energéticos e antioxidantes (piruvato e creatina) se mostraram eficazes na prevenção do dano hipocampal causado pela fenilalanina (Berti et al. 2012). No presente trabalho investigamos os possíveis danos causados pela

Pro na prole de indivíduos tratados cronicamente com esse amino ácido, além do possível efeito terapêutico da associação entre creatina e piruvato.

Os resultados estão de acordo com a literatura e mostram, embora variem bastante segundo a estrutura cerebral, que a Pro induz geração de radicais livres, mesmo na prole de ratas hiperprolinêmicas levando à peroxidação lipídica, como verificado pelos níveis de TBARS no hipocampo. Além disso, reduz defesas antioxidantes no córtex cerebral, como demonstrado pela redução da capacidade antioxidante total (TRAP) e a redução do teor de sulfidrilas para ambas as estruturas cerebrais.

Essa reatividade dos radicais livres (provavelmente oriundos da oxidação da Pro) com os grupos sulfidrilas também é evidenciada através da inibição das enzimas CK e PK, as quais possuem esses grupos tiólicos em suas estruturas. Esses resultados são reforçados por outros estudos do nosso grupo nos quais a atividade *in vivo* ou *in vitro* dessas enzimas diminui após a exposição a agentes promotores de radicais livres (Kessler et al. 2003a, Kessler et al. 2003b, de Andrade et al. 2012).

Além disso, o efeito do tratamento com Pro sobre a formação de EROs também foi avaliado pela utilização da sonda fluorescente DCFH-DA. No entanto, usando DCFH-DA, detectamos reduções na produção de EROs no grupo tratado com Pro em ambas as estruturas cerebrais testadas. Apesar de parecer contraditório isso é possível porque, como anteriormente descrito pelo grupo de Phang, durante a oxidação da Pro o superóxido é formado diretamente pela POX ou indiretamente por eventos posteriores na cadeia de transporte de elétrons, o que conduz a níveis tóxicos de superóxido e leva à co-

expressão da superóxido dismutase mitocondrial (SOD) diminuindo este efeito (Hu et al. 2007). Estes dados estão reforçados pelos nossos resultados de atividade da SOD, a qual aumenta em ambas as estruturas cerebrais do grupo que recebeu Pro. Portanto, evidencia esse efeito de compensação pelo aumento da geração de radicais livres provocadas pela Pro.

Embora a administração de creatina venha sendo reportada como protetora para os neurônios em uma variedade de condições patogênicas (Brustovetsky et al. 2001) e a associação dela com o piruvato se mostrou eficaz em proteger contra os prejuízos causados pela administração intra-hipocampal de fenilalanina (Berti et al. 2012), nosso trabalho demonstrou que essa associação não foi capaz de prevenir completamente os danos oxidativos causados pelo tratamento com Pro. Porém, essa abordagem foi eficiente em relação às enzimas do metabolismo energético CK e PK, as quais foram inibidas com a administração de Pro e a administração concomitante de creatina e piruvato preveniu a inibição da atividade dessas enzimas.

CONCLUSÃO

Esse estudo nos permite concluir que o tratamento das ratas mães com Pro foi capaz de causar um desequilíbrio nos parâmetros oxidativos e energéticos no cérebro dos filhotes. Embora estes parâmetros variem conforme a estrutura cerebral, observamos uma tendência do hipocampo ser mais sensível que o córtex aos danos induzidos pela Pro, o qual apresentou peroxidação lipídica e redução do teor de tióis totais. Estes resultados foram corroborados por outros estudos que mostram que a administração de Pro produz amnésia retrógrada e interrompe a formação de novas memórias em aves (Cherkin et al. 1976, Van Harreveld and Fifkova 1973).

Em síntese, a co-administração de creatina e piruvato não foi capaz de prevenir completamente todas as alterações dos parâmetros analisados. Portanto, mais estudos visando o refinamento dessa abordagem terapêutica serão necessários.

PERSPECTIVAS

Avaliar e aperfeiçoar o modelo de utilização de substâncias neuroprotetoras e antioxidantes sobre o efeito da L-prolina no cérebro de animais. Além disso, investigar parâmetros comportamentais da prole de ratas tratadas com Pro e possíveis agentes terapêuticos.

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