UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ADMINISTRAÇÃO INTRAESTRIATAL DE HIPOXANTINA ALTERA PERFIL INFLAMATÓRIO E NEUROENERGÉTICO VIA ESTRESSE OXIDATIVO EM ESTRIADO DE RATOS

HELENA BIASIBETTI BRENDLER

ORIENTADORA

Profª. Drª. Angela Terezinha de Souza Wyse

Porto Alegre

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota".

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RESUMO

A hipoxantina, principal oxipurina envolvida na via de salvação das purinas no cérebro, acumula-se na doença de Lesch-Nyhan, um erro inato do metabolismo das purinas. Os sintomas clínicos manifestam-se precocemente na vida dos pacientes, incluindo alterações motoras e cognitivas, retardo mental e automutilação. Embora os mecanismos subjacentes da disfunção cerebral na doença de Lesch-Nyhan sejam pouco compreendidos, o acúmulo de hipoxantina parece contribuir para os danos neurológicos. O objetivo deste estudo foi investigar os efeitos da administração intraestriatal de hipoxantina em ratos infantis e adultos jovens submetidos à cirurgia estereotáxica. Neste estudo, analisamos primeiramente o efeito da hipoxantina sobre os parâmetros neuroinflamatórios e oxidativos em estriados de ratos infantis e adultos jovens. Foram avaliados também alguns parâmetros neuroenergéticos. Ratos Wistar de 21 e 60 dias de vida foram submetidos à cirurgia estereotáxica e foram divididos em dois grupos: controle (infusão de solução salina 0,9%) e hipoxantina (10 μM). A administração intraestriatal de hipoxantina aumentou os níveis de IL-6 e TNF-α e o imunoconteúdo da subunidade NF-kB / p65 nuclear em estriado de ambas as idades de ratos. A ativação microglial e astrocitária foram observadas pelo aumento do imunoconteúdo de Iba1 e GFAP, respectivamente, no estriado de ratos de 21 dias. Todos os parâmetros oxidativos foram alterados, sugerindo uma forte relação neurotóxica de hipoxantina e estresse oxidativo. Em ratos de 60 dias de vida, a hipoxantina aumentou a atividade da succinato desidrogenase e do complexo II e diminuiu a atividade da citocromo c oxidase e seu imunoconteúdo. A injeção de hipoxantina diminuiu a porcentagem marcação de membrana mitocondrial e aumentou marcação de potencial mitocondrial. A hipoxantina também diminuiu o número de células vivas e aumentou o número de células apoptóticas. Em ratos de 21 dias de vida, a hipoxantina alterou alguns parâmetros do metabolismo energético e diminui a atividade de Na^+,K^+-ATPase, provavelmente por danos às proteínas, visto pela redução de conteúdo de sulfidrilas. Nossos achados mostram que a administração de hipoxantina alterou parâmetros neuroinflamatórios e neuroenergéticos, possivelmente por meio de desequilíbrio oxidativo, sugerindo que esses processos podem estar envolvidos, pelo menos em parte, com os distúrbios neurológicos encontrados em pacientes com doença de Lesch-Nyhan.

Palavras-chave: hipoxantina; neuroinflamação; estresse oxidativo; neuroenergética; apoptose; Na^+,K^+-ATPase

ABSTRACT

Hypoxanthine, the major oxypurine metabolite involved in purine's salvage pathway in the brain, is accumulated in Lesch-Nyhan disease, an inborn error of metabolism of purine. The clinical symptoms manifest early in the patients' lives, including motor and cognitive alterations, mental retardation and self-mutilation. Although the underlying mechanisms of brain dysfunction in Lesch-Nyhan disease are poorly understood, the accumulation of hypoxanthine appears to contribute to neurological damage. The purpose of this study was to investigate the effects of hypoxanthine intrastriatal administration in infant and young adult rats submitted to stereotactic surgery. We firstly analyzed the effect of hypoxanthine on neuroinflammatory and oxidative parameters in striatum of infant and young adult rats. We also evaluated some neuroenergetic parameters. Wistar rats of 21 and 60 days of life underwent stereotactic surgery and were divided into two groups: control (infusion of saline 0.9%) and hypoxanthine (10 μM). Intrastriatal administration of hypoxanthine increased IL-6 and TNF-α levels and nuclear immunocontent of NF-κB/p65 subunit in striatum of rats of both ages. Microglial and astrocyte activation was seen by the increase in Iba1 and GFAP immunocontent, respectively, in striatum of infant rats. All oxidative parameters were altered, suggesting a strong neurotoxic hypoxanthine role on oxidative stress. In 60-day-old rats hypoxanthine increased succinate dehydrogenase and complex II activities and diminished cytochrome c oxidase activity and immunocontent. Hypoxanthine injection decreased the percentage of cells with mitochondrial membrane label and increased mitochondrial mass potential labeling. Hypoxanthine also diminished the number of live cells and increased the number of apoptotic cells. In 21-days-old rats hypoxanthine altered some energy metabolism parameters and decreased Na^+,K^+-ATPase activity probably by protein damage, seen in the reduction of sulfhydryl content. Our findings show that the administration of hypoxanthine altered neuroinflammatory and neuroenergetic parameters, possibly through oxidative imbalance, suggesting that these processes may be involved, at least in part, with the neurological disorders found in patients with Lesch-Nyhan disease.

Keywords: hypoxanthine; neuroinflammation; oxidative stress; neuroenergetic; apoptosis; Na^+,K^+-ATPase

LISTA DE ABREVIATURAS

ATP: Adenosina trifosfato
BHE: Barreira hemato-encefálica
CII: Complexo II
CAT: Catalase
CK: Creatina quinase
COX: Citocromo c oxidase
DCFDA: Diacetato de 2'7'- diclorofluoresceína
DCFH: 2'7'- diclorofluoresceína
EIM: Erros inatos do metabolismo
ERO: Espécies reativas de oxigênio
GFAP: Proteína glial fibrilar ácida
GSH: Glutationa reduzida
GPx: Glutationa peroxidase
HPRT: Hipoxantina-guanina fosforibosiltransferase
Iba1: Molécula adaptadora de ligação de cálcio ionizado 1
IL-6: Interleucina 6
iNOS : Óxido nítrico sintase induzível
NADH: Nicotinamida adenina dinucleotídeo
NF-κB: Fator nuclear kappa B
NO: Óxido nítrico
PI: Iodeto de propídeo
PK: Piruvato quinase
SDH: Succinato desidrogenase
SNC: Sistema nervoso central
SOD: Superóxido dismutase
TBARS: Substâncias reativas ao ácido tiobarbitúrico
TCA: Ácido tricarboxílico
TNF-α: Fator de necrose tumoral alfa

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1. INTRODUÇÃO

1.1. Erros inatos do metabolismo

Erros inatos do metabolismo (EIM) são doenças hereditárias, multifatoriais, caracterizadas por defeitos genéticos que promovem a síntese anômala de uma proteína, geralmente uma enzima, com atividade parcial ou totalmente reduzida, resultando na alteração da via metabólica com consequente acúmulo de substrato e diminuição da síntese de produto. Outras substâncias tóxicas podem ser originadas por esse bloqueio metabólico através de rotas metabólicas alternativas (Scriver, 2008).

Correspondendo a cerca de 10% de todas as doenças genéticas, os EIM somam aproximadamente 1000 doenças diferentes identificadas até o momento (Mak et al., 2013). As doenças decorrentes dos EIM são consideradas raras, quando analisadas individualmente. Porém, em conjunto, apresentam alta frequência, acometendo aproximadamente 1:1000 recém-nascidos vivos (Scriver, 2008). Outro aspecto importante a ressaltar é a prevalência de EIM em grupos étnicos, podendo qualquer um ser acometido (Mak et al., 2013), contudo a frequência de determinado EIM pode variar em populações específicas, especialmente devido ao número de casamentos dentro da família, uma vez que um quinto dos casais da população mundial é formado por matrimônios consanguíneos (Hamamy, 2012).

As manifestações clínicas dessas doenças são diversas e geralmente inespecíficas, como por exemplo, vômitos, odores peculiares, letargia, atraso no desenvolvimento psicomotor, acidose metabólica, convulsões e coma,

sendo que esses sintomas podem aparecer desde as primeiras horas de vida até alguns meses após o nascimento (Kasper et al., 2010). No entanto, alguns EIM podem ser absolutamente assintomáticos (Raghuveer et al., 2006). Essa diversidade sintomatológica deve-se principalmente às diferenças no espectro da deficiência enzimática, área do metabolismo e/ou tecido afetados.

A classificação dos EIM é determinada pela área do metabolismo afetada, que podem ser: de aminoácidos, de ácidos orgânicos, de glicídios, de lipídios, de glicosaminoglicanos, de glicoproteínas, de enzimas eritrocitárias, de metais, de lipoproteínas, de hormônios, de proteínas plasmáticas e de purinas e pirimidinas. Dentre os EIM das purinas, destaca-se a doença de Lesch-Nyhan, caracterizada pelo acúmulo tecidual de hipoxantina, xantina e ácido úrico (Lesch and Nyhan, 1964).

1.2. Metabolismo de purinas

As purinas são moléculas heterocíclicas nitrogenadas que ocorre mais amplamente na natureza. Além do papel essencial na estrutura do DNA e do RNA, os numerosos derivados purínicos são importantes a diversos processos metabólicos. Por exemplo, Adenosina 5'-trifosfato (ATP) é a principal molécula energética celular, Adenosina 3',5'-ciclofosfato (cAMP) é um segundo mensageiro capaz de controlar a ativação de proteínas quinases na célula; nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinucleotídeo ($FADH_2$) são coenzimas que participam nos processos de redução-oxidação celular e geração de energia. Adenina e guanina são principais bases púricas encontradas nas células, as quais podem formar nucleosídeos através da incorporação de ribose ou 2-desoxirribose ao anel purínico. Nucleotídeos

purínicos, os quais desempenham importantes papéis no metabolismo celular, resultam da adição de ésteres de fosfato nos nucleosídeos (Rosemeyer, 2004).

Paralelamente, outra classe de derivados purínicos, as oxipurinas, tais como hipoxantina e xantina, apresentam um importante papel no metabolismo celular. O fornecimento de purinas para o reservatório celular depende das vias de síntese *de novo*, recuperação ou através da degradação de nucleosídeos exógenos. A via de síntese de novo das purinas consiste em diversos passos metabólicos, utilizando grande quantidade de ATP celular para fornecimento de energia (Nelson and Cox, 2013).

A síntese *de novo* das purinas é composta por uma série de reações enzimáticas as quais requerem a hidrólise de ATP, resultando na formação de inosina monofosfato (IMP) (Henderson et al., 2008). Por outro lado, a via de recuperação utiliza hipoxantina, guanina e adenina como substrato para a ação de enzimas fosforribosiltransferases. Essas enzimas transferem a ribose fosfato do fosforribosilpirofosfato para a base purínica na presença de magnésio. Adenina fosforribosiltransferase converte adenina em adenina monofosfato (AMP), enquanto a enzima Hipoxantina–guanina fosforribosiltransferase (HPRT) age sobre hipoxantina e guanina para formar IMP e guanosina monofosfato (GMP), respectivamente. Essa rota de recuperação conserva energia e permite que as células formem nucleotídeos a partir de bases pré-existentes, tendo em vista que 90% das purinas livres geradas durante o metabolismo intracelular provêm da rota de salvação (Kelley and Andersson, 2014).

A manutenção dos níveis intracelulares de purinas também pode ser realizada através da incorporação de purinas livres e nucleosídeos produzidos

no meio extracelular. A incorporação desses compostos depende da atividade das enzimas envolvidas na rota de regeneração das purinas. A via de degradação das purinas envolve as enzimas 5´-nucleotidase, purina nucleosídeo fosforilase, xantina oxidase, guanase e adenosina desaminase, sendo ácido úrico o produto final, o qual pode ser facilmente excretado (Jinnah and Friedmann, 2001; Visser et al., 2000).

A hipoxantina é uma das maiores fontes de purinas passíveis de reutilização nos tecidos de mamíferos, ela atua como um intermediário ativo nas células, podendo ser degradada pela enzima xantina oxidase, formando xantina, ou IMP pela ação da enzima HPRT (figura 1). Esta reação é responsável pela manutenção da quantidade de hipoxantina intracelular, o qual será utilizado na conversão de produtos úteis para as células (Visser et al., 2000).

A HPRT apresenta-se amplamente distribuída nos tecidos, especialmente no sistema nervoso central (SNC), sendo que os gânglios da base, particularmente, são dependentes da atividade dessa enzima para manutenção dos níveis de purinas teciduais através da via de reutilização (Visser et al., 2000). Tendo em vista a vital importância dos compostos purínicos e da atividade da enzima HPRT para o metabolismo das purinas, defeitos nessa enzima (perda parcial ou total da atividade), reduzem a afinidade da enzima pela hipoxantina, guanina e PP-ribose-P, com consequente acúmulo desses compostos, caracterizando a doença de Lesch-Nyhan, apresentando acúmulo tecidual de oxipurinas, especialmente a hipoxantina, e pronunciadas alterações no SNC (Nyhan, 2008).

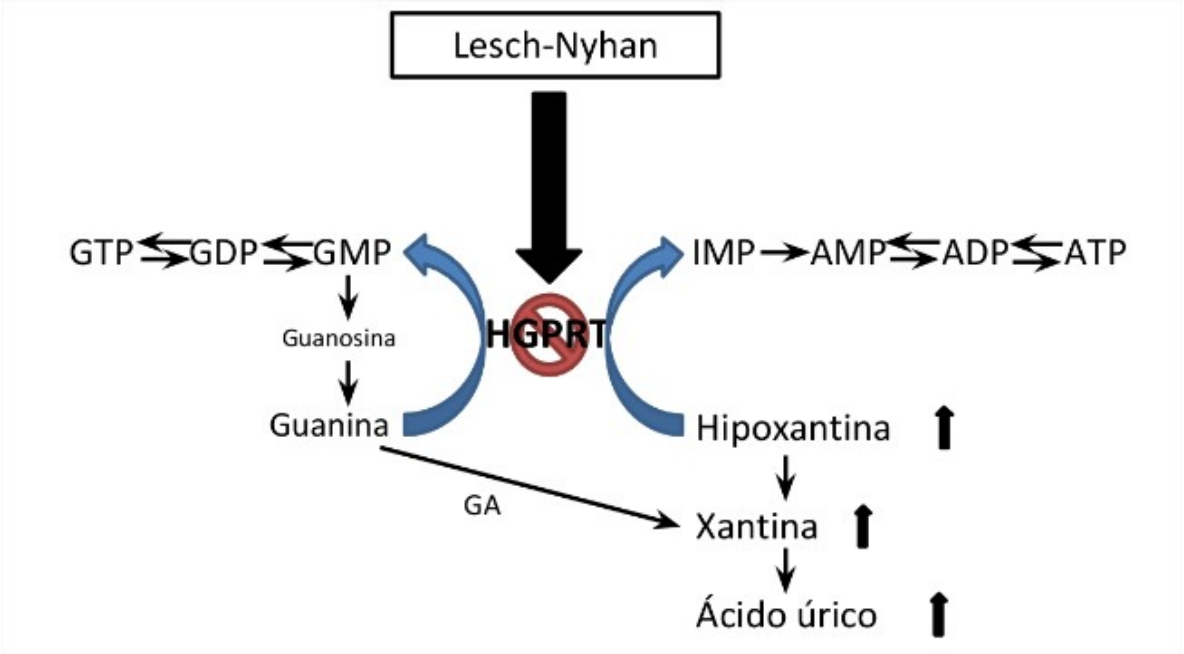


Figura 1. Metabolismo da hipoxantina na doença de Lesch-Nyhan. Abreviações utilizadas: ADP, adenosina difosfato; AMP, adenosina monofosfato; ATP, adenosina trifosfato; GDP, guanosina difosfato; GMP, guanosina monofosfato; GTP, guanosina trifosfato; GA, guanase; HGPRT, hipoxantina-guanina fosforribosiltransferase; IMP, inosina monofosfato.

1.3. Doença de Lesch-Nyhan

A doença de Lesch-Nyhan é um EIM associado à herança autossômica recessiva ligada ao sexo. Caracteriza-se pela deficiência na atividade da enzima HPRT a qual catalisa a conversão de hipoxantina e guanina em seus respectivos nucleotídeos IMP e GMP (Jinnah et al., 2013; Nyhan, 1978). Além disso, ocorre um aumento da via de síntese de novo das purinas decorrente da ineficiência na rota de regeneração (Torres and Puig, 2007). Essas alterações acarretam o acúmulo tecidual de xantina, ácido úrico e, principalmente, hipoxantina. O primeiro caso da doença foi publicado por Lesch e Nyhan (Lesch and Nyhan, 1964). Atualmente, essa doença apresenta prevalência a

nível mundial aproximada de 1/ 380.000 nascidos vivos, sendo 1/ 235.000 nascidos vivos na Espanha (Torres and Puig, 2007).

Pacientes portadores da doença de Lesch-Nyhan geralmente apresentam atividade da HPRT inferior a 1,5%. Diversos estudos apontam para as marcantes características genéticas (Torres and Puig, 2015), metabólicas (Jinnah et al., 2013), cognitivas (Schretlen et al., 2015) e comportamentais (Cauwels and Martens, 2005) associadas a esta doença. Pacientes afetados apresentam proeminentes alterações motoras e cognitivas, aliado à hiperuricemia, gota e automutilação, a qual é caracterizada por mordeduras de lábios, língua e dedos, com aparente perda tecidual (Cauwels and Martens, 2005; Jinnah et al., 1990). Indivíduos com a doença tipicamente têm um curso normal pré-natal e perinatal. A característica mais comum é o atraso no desenvolvimento durante o primeiro ano de vida, com hipotonia e atraso de habilidades motoras normalmente evidentes nos três a seis meses de idade. Nos primeiros anos de vida, os pacientes apresentam movimentos involuntários anormais indicativos de envolvimento extrapiramidal (Schretlen et al., 2005). Com a manutenção do controle dos sintomas de forma eficaz, a maioria de indivíduos sobrevive até a segunda ou terceira década de vida (Nyhan et al., 2014).

Estudos histopatológicos demonstraram que os pacientes com a doença de Lesch-Nyhan apresentam diminuição do volume cerebral da substância branca mais pronunciado do que da substância cinzenta, provavelmente causada por uma deficiência no desenvolvimento e não por uma alteração neurodegenerativa (Schretlen et al., 2015). Estudos neuroquímicos demonstraram uma redução do conteúdo dopaminérgico no estriado, similares

àqueles encontrados em pacientes portadores da doença de Parkinson (Göttle et al., 2014; Visser et al., 2002). Segundo alguns autores o estresse oxidativo poderia estar envolvido no processo de deterioração estriatal (Blesa et al., 2015; Göttle et al., 2014). Além disso, a dependência estriatal da atividade da HPRT para a manutenção dos níveis de purinas, aliada à diminuição dos prolongamentos dos neurônios dopaminérgicos poderiam contribuir para o aparecimento de disfunção dopaminérgica (Göttle et al., 2014).

A análise dos níveis séricos de hipoxantina também contribui para a determinação do diagnóstico definitivo (Harkness et al., 1988; Puig et al., 1989). Os valores obtidos para esta oxipurina em plasma de indivíduos normais correspondem a 1,7 μM para hipoxantina, entretanto, em pacientes com a doença de Lesch-Nyhan estas concentrações podem atingir valores de 10 μM. As concentrações de hipoxantina também se encontram elevadas similarmente no líquido cefalorraquidiano dos indivíduos portadores da doença (Puig et al., 1989).

O tratamento para controlar a superprodução de ácido úrico em pacientes com doença de Lesch-Nyhan é feito pela administração de alopurinol, que inibe a conversão de hipoxantina e guanina em ácido úrico pela enzima xantina oxidase. Uma vez que o alopurinol aumenta simultaneamente os metabólitos relacionados da oxipurina, é necessária uma hidratação generosa para manter um fluxo de urina ativo, eliminando continuamente todos os metabólitos de purina. Infelizmente, controlar a superprodução de ácido úrico não melhora os outros sintomas clínicos, que têm uma patogênese independente (Jinnah, 2009).

Estudos realizados em nosso grupo demonstram que a hipoxantina inibe significativamente a atividade da Na^+,K^+-ATPase e altera alguns parâmetros de estresse oxidativo em estriado de ratos de 60 dias de vida (Bavaresco et al., 2007a; Caren Serra Bavaresco et al., 2008). Entretanto, os mecanismos pelos quais a hipoxantina atua no SNC e o exato papel desta oxipurina no dano neurológico presente na doença de Lesch-Nyhan ainda permanecem desconhecidos.

1.4. Modelos experimentais da doença de Lesch-Nyhan

Os modelos experimentais são essenciais para o melhor entendimento da doença de Lesch-Nyhan uma vez que essa doença é rara, o que torna os estudos com pacientes muito desafiadores, pois mesmo se um número suficiente de pacientes fosse examinado, os tipos de estudos necessários para elucidar a base biológica para a disfunção comportamental não poderiam ser realizados com seres humanos (Jinnah and Friedmann, 2001).

O uso de camundongos geneticamente modificados é uma importante ferramenta, na obtenção de informações para a compreensão de doenças metabólicas. Porém em alguns modelos o fenótipo desenvolvido pelos animais não mimetizam plenamente a patologia humana (Elsea and Lucas, 2002). Na tentativa de estudar a causa subjacente do complexo fenótipo observado em pessoas com a doença de Lesch-Nyhan, foram criados pelo menos dois modelos com camundongos que exibem deficiência no gene Hprt que codifica a enzima HPRT, no entanto, os camundongos apresentam comportamento normal, sem fenótipo aparente (Hooper et al., 1987; Kuehn et al., 1987).

Outro modelo animal desenvolvido para abordar o significado funcional da perda de dopamina na doença de Lesch-Nyhan implica em administração da neurotoxina 6-hidroxidopamina, uma vez que pacientes afetados apresentam uma grave perda dopaminérgica e uma redução na mobilidade que se assemelha à doença de Parkinson. A principal limitação desse modelo é que não é possível explorar os mecanismos pelos quais a deficiência de HPRT pode desencadear o defeito dopaminérgico (Jinnah, 2009).

Modelos celulares de células neurais e não-neurais também foram desenvolvidos para explorar aspectos específicos do metabolismo e da biologia celular em um ambiente experimental muito controlado. Contudo, os modelos celulares também têm suas limitações. Uma limitação é que os resultados obtidos em condições artificiais *in vitro* podem não se aplicar *in vivo*. Uma solução para este problema seria tentar replicar as principais conclusões de modelos celulares usando modelos *in vivo* mais complexos ou pacientes. Outra limitação dos modelos celulares é que os resultados obtidos com um tipo de célula podem não se aplicar a outros tipos celulares (Jinnah, 2009).

1.5. Neuroinflamação

A inflamação é, essencialmente, uma resposta protetora cujo propósito final é eliminar o agente indutor de injúria. O agente danoso é primeiramente reconhecido pelo sistema imune do organismo, seguido da liberação de mediadores químicos e ativação de diversos tipos celulares que se acumulam no sítio inflamatório. Dentre os mediadores podemos citar histamina, serotonina, prostaglandinas, leucotrienos, fator de ativação plaquetário, espécies reativas de oxigênio (ERO), óxido nítrico (NO), citocinas, quimiocinas, proteínas de fase aguda, fator de transcrição nuclear kappa B (NF-κB) e o

sistema complemento. Embora a inflamação tenha uma função protetora no controle de infecções e promova a reparação tecidual, também pode causar danos nos tecidos e doenças (Abbas et al., 2010).

Neuroinflamação é inflamação associada ao tecido nervoso, incluindo cérebro e medula espinhal. Pode ter início em resposta a diversos sinais, incluindo infecção, lesão cerebral traumática, metabolitos tóxicos ou auto-imunidade. No cérebro, diferentemente dos outros tecidos, a inflamação é caracterizada pela ativação de microglia e astrócitos, principalmente a primeira (Xanthos and Sandkühler, 2014).

O SNC é tipicamente um local imunologicamente privilegiado, uma vez que as células imunes periféricas são geralmente bloqueadas pela barreira hemato-encefálica (BHE) (Das Sarma, 2014). No entanto, como pode ser visto na figura 2, a ativação microglial e astrócitária promove a liberação de uma série de fatores que modulam mediadores pró- e anti-inflamatórios (citocinas, quimiocinas, NO, fatores de crescimento e ERO), que, por sua vez, regulam positivamente moléculas de adesão, aumentando a permeabilidade da barreira hematoencefálica, facilitando a invasão de células imunes periféricas, que induzem a liberação de moléculas potencialmente tóxicas, podendo comprometer células cerebrais (Lucas et al., 2006). Assim, o SNC pode ser influenciado não só por mediadores pró- e anti-inflamatórios produzidos dentro do cérebro, mas também através das ações de mediadores oriundos da periferia (Xanthos and Sandkühler, 2014). Além disso, embora a inflamação seja uma resposta iniciada para proteger o sistema nervoso central de agentes infecciosos, o resultado pode ser uma inflamação tóxica e generalizada, bem como migração adicional de leucócitos através da BHE (Gendelman, 2002).

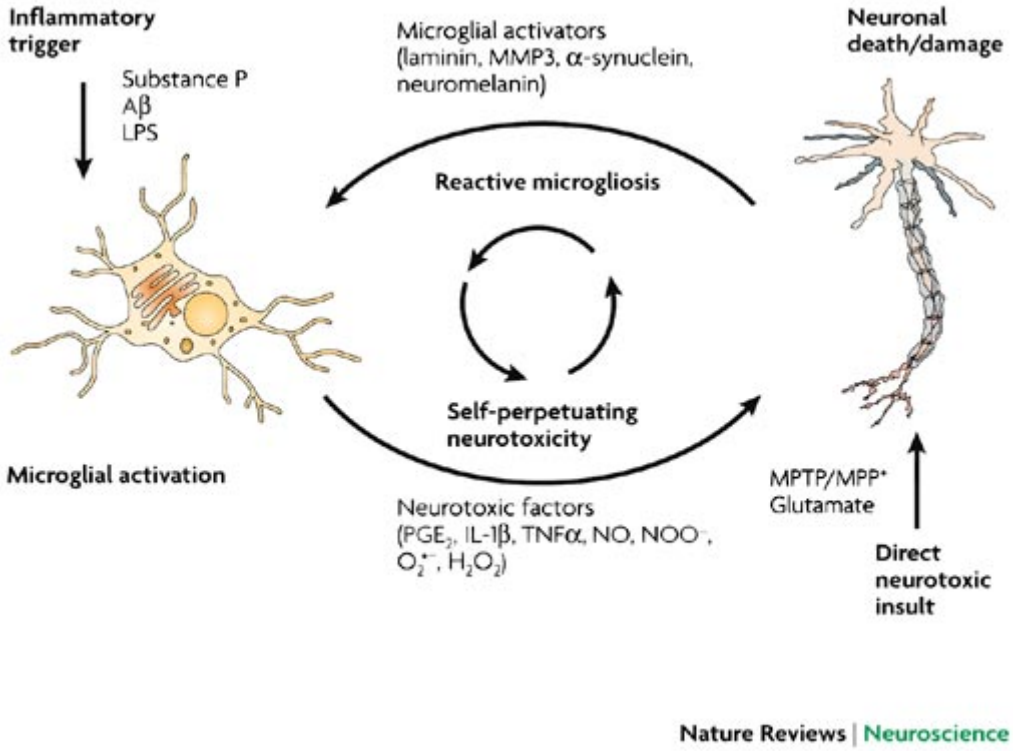


Figura 2. Participação da ativação microglial na morte neuronal (Fonte Nature Reviews).

1.6. Radicais livres e estresse oxidativo

O radical livre define-se por ser qualquer espécie química que contém um ou mais elétron(s) desemparelhado(s) no seu orbital mais externo, levando a uma elevada reatividade e instabilidade da molécula. Eles são gerados fisiologicamente nos sistemas vivos quando aproximadamente 5% do oxigênio molecular (O_2) é incompletamente reduzido à formação de água na cadeia transportadora de elétrons. Existem diversas espécies reativas, dentre as principais estão o ânion superóxido (O_2•), peróxido de hidrogênio (H_2O_2), radical hidroxila (OH•) e peroxinitrito ($ONOO^-$), o qual é formado a partir da reação do NO• com o O_2•- (Halliwell, 2012).

As espécies reativas também estão associada a muitos processos fisiológicos, executando funções biológicas importantes. Evidências apontam para a participação destas espécies reativas em processos de sinalização celular (Schieber and Chandel, 2014), síntese e regulação proteica (Dasuri et al., 2013), nos processos inflamatórios (El Assar et al., 2013) e em eventos mitogênicos (Valko et al., 2007). Contudo, em altas concentrações, as espécies reativas apresentam um papel tóxico para as células. Neste contexto, são amplamente discutidas na literatura as alterações nocivas provocadas por espécies reativas em lipídios, proteínas e também no DNA e que podem levar à perda da função celular. Além disso, enzimas, proteínas transportadoras e receptores podem ter sua estrutura alterada quando oxidadas por espécies reativas com consequente prejuízo das suas funções (Halliwell, 2012; Valko et al., 2007).

Fisiologicamente as espécies reativas são mantidas em equilíbrio devido, em grande parte, à capacidade de neutralização do sistema de defesa antioxidante enzimático e não enzimático. Dentre as defesas antioxidantes enzimáticas estão a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). Dentre os antioxidantes não enzimáticos podemos citar ácido ascórbico (vitamina C), α-tocoferol (vitamina E), glutationa, carotenóides, flavonóides, entre outros (Halliwell, 2011). A SOD é uma metaloenzima responsável pela dismutação do ânion superóxido gerando peróxido de hidrogênio e oxigênio. Nesta perspectiva, o peróxido de hidrogênio, formado pela ação da SOD, é removido pela ação das enzimas CAT e GPX, resultando em H_2O e O_2 (Abreu and Cabelli, 2010). A CAT é mais comumente encontrada nos peroxissomos da maioria dos tecidos em humanos, mas

também é encontrada na mitocôndria. A catalase mitocondrial tem um papel preventivo principalmente contra as alterações lipídicas da membrana, inativação de componentes da cadeia respiratória, mutações e rupturas do DNA mitocondrial e abertura de poros de transição de permeabilidade pelo peróxido de hidrogênio (Zamocky et al., 2008). A GPx, localizada nas membranas celulares, converte o peroxido de hidrogênio através do acoplamento de sua redução a água com concomitante oxidação da glutationa ao dissulfeto de glutationa (Margis et al., 2008).

O processo denominado como estresse oxidativo é caracterizado pelo desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes, e pode estar associado a diversas patologias. O cérebro, por sua vez, é particularmente suscetível ao estresse oxidativo. Alguns fatores que tornam o SNC altamente vulnerável às espécies reativas incluem: alto consumo de oxigênio; presença de neurotransmissores que sofrem auto-oxidação como dopamina, serotonina e noradrenalina; alta concentração de ferro; lipídios de membrana ricos em ácidos graxos insaturados. Além disso, o cérebro apresenta baixos níveis de defesas antioxidantes enzimáticas e não-enzimáticas (Halliwell, 2012). Evidências têm sugerido o envolvimento do estresse oxidativo na patogênese de doenças neurodegenerativas como Huntington, Alzheimer e Parkinson (Blesa et al., 2015; Gil-Mohapel et al., 2014; Wang et al., 2014) e erros inatos do metabolismo (Olsen et al., 2015).

1.7. Metabolismo energético

Comparada à sua elevada atividade metabólica, o cérebro possui pouca reserva energética, necessitando de um suprimento contínuo energético,

principalmente glicose. A glicólise em conjunto com o ciclo do ácido cítrico e a fosforilação oxidativa são as rotas essenciais para a produção de energia cerebral (Vaishnavi et al., 2010).

A glicólise é composta por uma série de reações que ocorrem no citosol, tendo como produto final o piruvato. O ciclo do ácido cítrico ocorre na matriz mitocondrial e consiste em uma sequência de reações em que, cada volta do ciclo, são formadas três moléculas de NADH, uma de $FADH_2$, duas de CO_2 e uma de guanosina trifosfato (GTP). O NADH e $FADH_2$ produzidos no ciclo são carreadores de elétrons e são utilizados na cadeia transportadora de elétrons para a produção de ATP na fosforilação oxidativa (Vuoristo et al., 2016).

A cadeia transportadora de elétrons é composta por quatro complexos enzimáticos e dois transportadores móveis de elétrons (coenzima Q e citocromo c) os quais realizam a transferência dos elétrons desde as coenzimas reduzidas até o aceptor final, o oxigênio. Os elétrons provenientes do NADH são entregues ao complexo I (NADH desidrogenase ou NADH: ubiquinona oxirredutase) que transfere esses elétrons para a ubiquinona, formando ubiquinol. O complexo II (ou succinato:ubiquinona oxirredutase, CII) é formado pela enzima succinato desidrogenase (SDH) e três subunidades hidrofóbicas. Esse complexo reduz a ubiquinona a ubiquinol com elétrons do $FADH_2$, proveniente da oxidação do succinato a fumarato no ciclo do ácido cítrico. O complexo III (ubiquinona-citocromo c oxirredutase) transfere elétrons do ubiquinol para o citocromo c. O complexo IV, também denominado citocromo c oxidase (COX), catalisa a transferência dos elétrons do citocromo c reduzido para o O_2, reduzindo-o a H_2O. O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de

prótons da matriz mitocondrial para o espaço intermembranas, pelos complexos I, III e IV (Figura 4). Com isso, cria-se um gradiente eletroquímico transmembrana que é utilizado como força próton-motriz pelo complexo V (ATP sintase) para síntese de ATP partir de ADP e Pi, processo denominado fosforilação oxidativa (Navarro and Boveris, 2007).

Alterações no funcionamento normal da cadeia respiratória mitocondrial levam à diminuição da síntese de ATP. Nesse contexto, dados da literatura sugerem que uma diminuição do metabolismo energético pode estar implicada na fisiopatologia de alguns distúrbios neurológicos, como demência, isquemia cerebral e as doenças de Alzheimer e Parkinson (Beal, 2008; Gibson et al., 2010; Kowaltowski et al., 2009; Schurr, 2002).

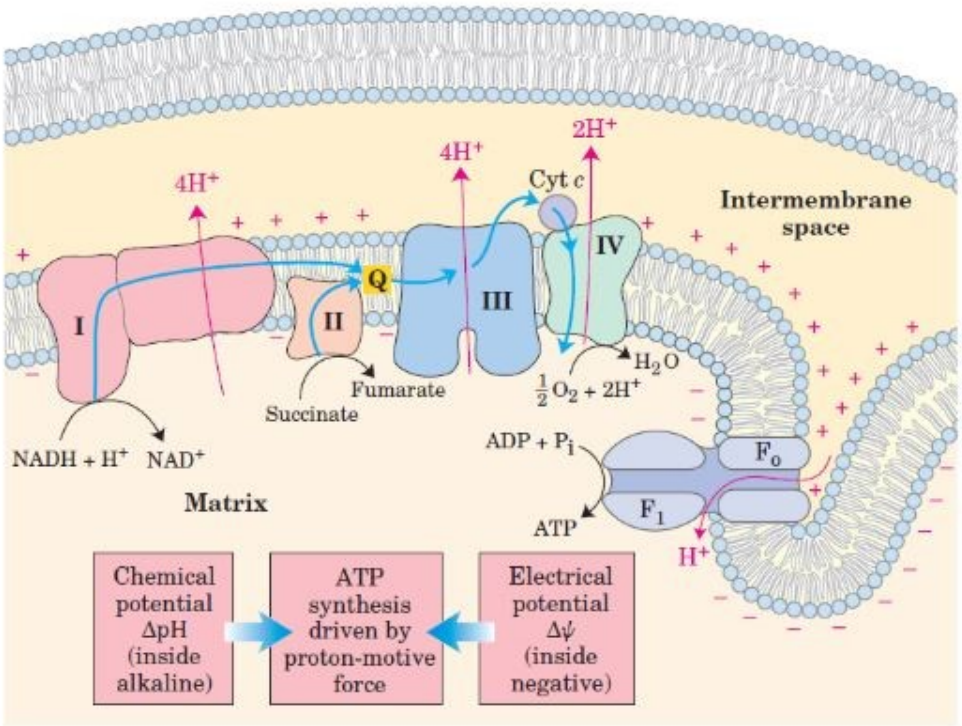


Figura 3. Fosforilação oxidativa (Nelson and Cox, 2008).

1.8. Na^+,K^+-ATPase

A Na^+,K^+-ATPase é uma proteína integral de membrana que utiliza energia da hidrólise da molécula de ATP para translocar, simultaneamente, 3 íons Na^+ para o compartimento extracelular em troca do transporte de 2 íons K^+ para dentro da célula, gerando um gradiente eletroquímico através da membrana. Diversas funções celulares estão acopladas à manutenção das concentrações intracelulares e extracelulares de Na^+ e K^+, tais como controle do volume celular, excitabilidade neuronal, atividade de enzimas citosólicas, contração muscular, além de auxiliar no movimento de outros íons e compostos através da membrana, como, por exemplo, neurotransmissores (Kaplan, 2002; Ogawa et al., 2009). Essa enzima funcional é composta por duas subunidades α e duas subunidades menores β, sendo que a subunidade α é responsável pela atividade catalítica da enzima e sofre fosforilação e mudança conformacional acoplada à hidrólise de ATP e ao transporte dos íons Na^+ e K^+. Essa subunidade também contém o sítio de ligação da ouabaína (glicosídeo cardíaco e inibidor específico da enzima). A subunidade β é uma proteína glicosilada de adesão intercelular necessária para direcionar a subunidade α para a membrana plasmática. Outra subunidade ainda menor (γ) tem sido implicada na regulação da atividade dessa enzima (Geering, 2008) (Figura 3).

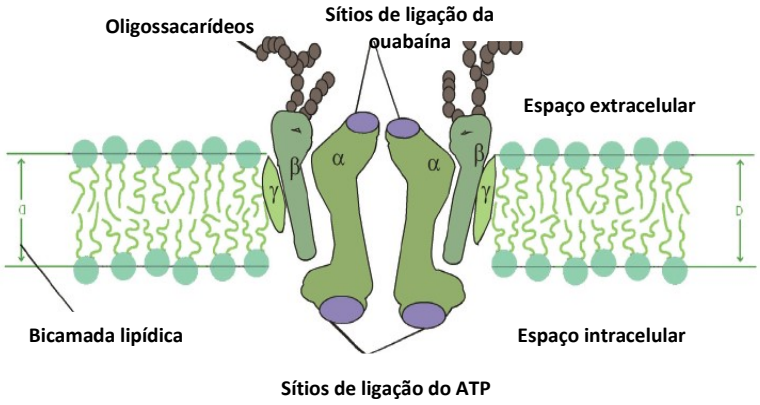


Figura 4. Estrutura da Na^+,K^+-ATPase (Modificado de Suhail, 2010).

Foram identificados em mamíferos genes que codificam quatro isoformas da subunidade catalítica α (α_1, α_2, α_3 e α_4), sendo que as isoformas α_1, α_2 e α_3 encontram-se em diferentes tipos celulares do SNC. A isoforma α_1 caracteriza-se por ser ubiquamente expressa enquanto a α_2 é expressa em pequenos subconjuntos de neurônios, porém em muitos astrócitos. A isoforma α_3 é exclusivamente expressa em neurônios de várias estruturas cerebrais (Bøttger et al., 2011). Em mamíferos existem três isoformas da subunidade β (β_1, β_2 e β_3), sendo que a isoforma β_1 é expressa em neurônios enquanto a β_2 é encontrada em astrócitos (Benarroch, 2011; Geering, 2008).

A atividade da enzima Na^+,K^+-ATPase é regulada por diversos fatores como disponibilidade de substrato, componentes da membrana celular, hormônios e fosforilação (Hatou, 2011; Poulsen et al., 2010). Além disso, essa enzima é sensível ao ataque de radicais livres (Liu et al., 2012), podendo ser inibida por metabólitos formados durante a peroxidação lipídica e por alterações na membrana plasmática (Banerjee et al., 2012).

A inibição da atividade da Na^+,K^+-ATPase está relacionada a diversas patologias do SNC tais como depressão (Goldstein et al., 2006), epilepsia (Marquezan et al., 2013), doença de Parkinson e doença de Alzheimer (Arnaiz and Ordieres, 2014; Vitvitsky et al., 2012). Estudos mostram também que a atividade da Na^+,K^+-ATPase também está alterada em EIM (Bavaresco et al., 2007a; Ferreira et al., 2011; Kolling et al., 2016; Wyse et al., 2002). No entanto, mais estudos são necessários para compreender os efeitos da hipoxantina sobre a função da Na^+,K^+-ATPase, especialmente durante o desenvolvimento cerebral.

2. OBJETIVOS

2.1 Objetivo geral

A fim de investigar e compreender as alterações cerebrais promovidas pela hipoxantina, o objetivo **geral** do presente trabalho foi avaliar o efeito dessa purina sobre o perfil neuroinflamatório e estado redox em estriado de ratos submetidos ao modelo de injeção intraestriatal de hipoxantina. Além disso, investigamos os possíveis efeitos da administração de hipoxantina sobre a bioenergética cerebral, bem como possíveis mecanismos de morte celular em diferentes idades de ratos.

2.2 Objetivos específicos

Os objetivos específicos encontram-se subdivididos, os quais serão apresentados na forma de artigos científicos, como segue:

Capítulo I

- Avaliar o efeito da hipoxantina sobre o perfil inflamatório, determinando o imunoconteúdo de NF-κB, níveis de citocinas (interleucina 6 (IL-6) e fator de necrose tumoral alfa (TNF-α)), bem como o imunoconteúdo de óxido nítrico sintase induzível (iNOS) e níveis de nitritos em estriado de ratos infantis e adultos jovens submetidos ao modelo de administração intraestriatal de hipoxantina;
- Analisar uma possível neuroinflamação, avaliando a ativação microglial e astrócitária através do imunoconteúdo de Iba1 (molécula adaptadora de ligação de cálcio ionizado 1) e GFAP (proteína glial fibrilar ácida), respectivamente, em

estriado de ratos infantis e adultos jovens submetidos à administração intraestriatal de hipoxantina;

- Avaliar alguns parâmetros de estresse oxidativo, a saber: os níveis de espécies reativas, a atividade de enzimas antioxidantes (SOD, CAT e GPx), bem como dano ao DNA em estriado de ratos infantis e adultos jovens submetidos ao modelo de administração intraestriatal de hipoxantina;

Capítulo II

- Investigar o efeito da hipoxantina sobre a bioenergética, avaliando a atividade da piruvato quinase, enzimas da fosforilação oxidativa, níveis de ATP, bem como a regulação de creatina quinase (CK) citosólica e mitocondrial em estriado de ratos de 60 dias submetidos ao modelo de administração intraestriatal de hipoxantina;

- Analisar a funcionalidade da mitocôndria pelo método de MitoTracker, determinando a massa e o potencial mitocondrial, além de investigar morte celular por Anexina-PI em estriado de ratos de 60 dias submetidos ao modelo de administração intraestriatal de hipoxantina;

Capítulo III

- Determinar a atividade da Na^+,K^+-ATPase e o imunoconteúdo das subunidades catalíticas (α_1, α_2 e α_3) em estriado de ratos de 21 dias tratados com hipoxantina;

- Investigar o efeito da hipoxantina sobre a bioenergética, avaliando a atividade da piruvato quinase, enzimas da fosforilação oxidativa, níveis de ATP e regulação de creatina quinase citosólica e mitocondrial em estriado de ratos de 21 dias submetidos ao modelo.

3. PROCEDIMENTOS EXPERIMENTAIS E RESULTADOS

Os procedimentos experimentais e resultados serão apresentados na forma de artigos científicos. Todas as informações referentes ao desenho experimental estão contidas nos capítulos I, II e III.

3.1 Capítulo I

Hypoxanthine Intrastriatal Administration Alters Neuroinflammatory Profile and Redox Status in Striatum of Infant and Young Adult Rats

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Hypoxanthine Intrastriatal Administration Alters Neuroinflammatory Profile and Redox Status in Striatum of Infant and Young Adult Rats

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Abstract Hypoxanthine, the major oxypurine metabolite involved in purine's salvage pathway in the brain, is accumulated in Lesch-Nyhan disease, an inborn error of metabolism of purine. The purpose of this study was to investigate the effects of hypoxanthine intrastriatal administration on infant and young adult rats submitted to stereotactic surgery. We analyzed the effect of hypoxanthine on neuroinflammatory parameters, such as cytokine levels, immunocontent of NF-κB/p65 subunit, iNOS immunocontent, nitrite levels, as well as IBA1 and GFAP immunocontent in striatum of infant and young adult rats. We also evaluate some oxidative parameters, including reactive species production, superoxide dismutase, catalase, glutathione peroxidase activities, as well as DNA damage. Wistar rats of 21 and 60 days of life underwent stereotactic surgery and were divided into two groups: control (infusion of saline 0.9 %) and hypoxanthine (10 μM). Intrastriatal administration of hypoxanthine increased IL-6 levels in striatum of both ages of rats tested, while TNF-α increased only in 21-day-old rats. Hypoxanthine also increased nuclear immunocontent of NF-κB/p65 subunit in striatum of both ages of rats. Nitrite levels were decreased in striatum of 21-day-old rats; however, the immunocontent of iNOS was increased in striatum of hypoxanthine groups. Microglial and astrocyte activation was seen by the increase in IBA1 and GFAP immunocontent, respectively, in striatum of infant rats. All oxidative parameters were altered, suggesting a strong neurotoxic hypoxanthine role on oxidative stress. According to our results, hypoxanthine intrastriatal administration increases neuroinflammatory parameters perhaps through oxidative misbalance, suggesting that this process may be involved, at least in part, to neurological disorders found in patients with Lesch-Nyhan disease.

Keywords Hypoxanthine · Neuroinflammation · Microglial and astrocyte markers · DNA damage · Lesch-Nyhan disease

Introduction

Purines are a class of molecules that present several functions for the cellular biology, for instance nucleic acid synthesis, cofactor reactions, energy-consuming reactions, and cellular signaling. Hypoxanthine is the major purine metabolite involved in the purine's salvage pathway in the brain. This mechanism is vital for the maintenance of physiologic purine levels. Deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8) activity leads to the inborn error of metabolism known as Lesch-Nyhan disease (LND), which is characterized by high levels of oxypurines, mainly hypoxanthine and uric acid [1–3].

HPRT is present in all tissues and distributed equally throughout the brain [4], although the symptoms presented by LND patients seem to be related more specifically to extrapyramidal effects and basal ganglia

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dysfunction [5]. LND patients present five times the normal concentration of hypoxanthine, but rather normal levels of xanthine in cerebrospinal fluid [6, 7]. However, brain tissue has relatively lower levels of xanthine oxidase; therefore, the production of uric acid from xanthine and hypoxanthine is narrow [8]. Histopathological studies showed that patients with LND present a decrease in brain volume and a white matter deficit more pronounced than gray matter [9], probably caused by a developmental deficiency rather than a degenerative one [10]. Symptomatology of LND patients is motor dysfunction (spasticity, dystonia), cerebral palsy, cognitive and behavioral disturbances, and self-mutilation behavior, as well as hyperuricemia [11, 12].

Central nervous system (CNS) has several mechanisms responsible for maintaining immune homeostasis. Microglia is the basic neuroinflammatory mediator, responding as the major brain's immune cells. Neuroinflammation is characterized by activation of microglia and astrocyte with consequent release of many factors that modulate pro- and anti-inflammatory mediators, such as cytokines, chemokines, nitric oxide (NO), prostaglandins, growth factors, superoxide species, and nuclear factor-kappa B (NF-κB) translocation [13–15].

It is well established that high levels of reactive species induce oxidative stress, promoting several cellular injury as lipid, protein, and DNA damage [16]. It has been demonstrated the hypoxanthine/xanthine oxidase action as a source of oxidative stress in the vascular system [17]. Previous works have shown hypoxanthine's capability to induce lipid peroxidation, reduce total radical-trapping antioxidant parameter, and also alter antioxidant enzymes [18, 19].

Since LND symptoms generally occur in early stage of life and the average life expectancy of patient with LND under treatment is early- to mid-20s [11], in the current research we aimed to evaluate hypoxanthine intrastriatal administration in infant and young adult rats. In order to verify whether high hypoxanthine levels could alter neuroinflammatory profile and its mechanism, in the present study we evaluated the effect of intrastriatal hypoxanthine administration on some neuroinflammatory parameters such as pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), immunocontent of NF-κB, inducible nitric oxide synthase (iNOS), nitrite levels, as well as on microglial and astrocyte markers including ionized calcium-binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP). Some oxidative parameters such as reactive species generation, antioxidant enzymes activities, and DNA damage were also evaluated.

Materials and Methods

Animals and Reagents

Male Wistar rats of 21 (infant) and 60-day-old (young adult) were obtained from the Central Animal House of the Department of Biochemistry of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. Animals were maintained under a standard dark–light cycle (lights on between 07:00 and 19:00 hours) at a room temperature of 22 ± 1 °C, with free access to a 20 % (*w*/*w*) commercial protein chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#25717).

Acrylamide, bisacrylamide, SDS, and β-mercaptoethanol used in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies were purchased from Santa Cruz Biotechnology, BD Biosciences, and Cell Signaling Technology. Anti-rabbit IgG peroxidase-conjugated and reagents to detect chemiluminescence (ECL) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond-ECL (Hybond-ECL-nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). All other chemical reagents used for analysis were obtained from Sigma Aldrich Co., St. Louis, MO, USA.

Experimental Procedure

The model developed in this experimental study was based on the infusion of hypoxanthine directly into the right striatum, using a stereotactic apparatus, which is widely described in the current scientific literature [20–22]. In this technique, the coordinates specific to the right striatum for each age of rats were based on the stereotactic atlas coordinates developed by Paxinos and Watson [23] (coordinates relative from bregma for 21- and 60-day-old rats, respectively: AP −0.6 mm, ML −3.0 mm, V −4.0 mm from the dura; AP −0.5 mm, ML −2.5 mm, V −2.5 mm from the dura). In all experiments, the intrastriatal infusions of the drug occurred 2 days after surgery, to avoid any influence of anesthetic substances on the results, since studies show neuroprotective effect for xylazine and ketamine [24]. Later, a needle (0.9 mm) infusion was adjusted within the guide cannula placed in the animal and 2 μL of a solution of hypoxanthine (20 pmol/2 μL) or vehicle (saline 0.9 %) was administered in the right striatum of the animal 1 μL per minute. Thus, the animals were divided into two groups: (1) control (saline infusion); (2) hypoxanthine (hypoxanthine infusion). Animals were sacrificed by decapitation without anesthesia 30 min after the saline or hypoxanthine

infusion, brain was dissected, and the structure analyzed was striatum. Hypoxanthine dose used in our study was chosen according to Puig and colleagues [25], being equivalent to that found in patients with Lesch-Nyhan disease. This model was used throughout all experiments described in this work.

Cytokines (IL-6 and TNF-α) Assay

Striatum was homogenized in 1:5 (*w*/*v*) saline (0.9 % NaCl). The homogenate was centrifuged at 800×*g* for 10 min at 4 °C, being the supernatant used for the technic. IL-6 and TNF-α levels in striatum were quantified by a rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (InvitrogenTM, Life Technologies, Carlsbad, CA, USA) as described by Scherer et al. [26].

Cellular Fractionation for Cytosolic and Nuclear NF-κB/p65 Subunit

Cellular fractionation for cytosolic and nuclear NF-κB/p65 subunit was obtained as described by da Cunha et al. [27]. Striatum was homogenized in 300 μL hypotonic lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 5 mM NaF, 1 mM sodium orthovanadate plus protease inhibitor cocktail. Samples homogenate were then lysed with 18 μL 10 % IGEPAL. The homogenate was centrifuged (14,000×*g*, 30 s, 4 °C), and supernatants containing the cytosolic fraction were stored at −80 °C. The nuclear pellet was resuspended in 200 μL ice-cold hypertonic extraction buffer (10 mM HEPES (pH 7.9), 0.40 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 0.25 mM EDTA, 25 % glycerol plus protease inhibitor cocktail). After 40 min of intermittent mixing, extracts were centrifuged (14,000×*g*, 10 min, 4 °C), and supernatants containing nuclear protein were secured. For electrophoresis analysis, aliquot samples were dissolved in 25 % (*v*/*v*) of a solution containing 40 % glycerol, 5 % mercaptoethanol, and 50 mM Tris–HCl, pH 6.8.

Western Blotting Analysis of NF-κB/p65 Subunit, iNOS, IBA1, and GFAP

Protein samples were separated by 10 % SDS-PAGE (30 μg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20 % methanol, and 0.25 % SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5 % bovine serum albumin (BSA)). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween-20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing the respective antibody anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology), iNOS antibody diluted 1:1000 (Sigma-Aldrich), rabbit polyclonal anti-IBA1 (1:1000, Wako), anti-GFAP (1:2000, Cell Signaling Technology), and anti-β-actin (1:1000, Cell Signaling Technology). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Nitrite Assay

For nitrite levels measurement, 100 μL of supernatant of striatum was mixed with 100 μL Griess reagent (1:1 mixture of 1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature [28, 29]. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards.

Oxidative Stress Parameters Assays

Striatum was homogenized in 10 volumes (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Then the homogenates were centrifuged at 800×*g* for 10 min at 4 °C. The pellet was discarded, and the supernatant was used for the measurements of oxidative stress parameters as described by Schmitz and colleagues [30].

2′7′-Dichlorofluorescein Fluorescence Assay

Reactive oxygen species production was measured according to LeBel and colleagues [31] which is based on oxidation of 2′7′-dichlorofluorescein (H_2DCF). Samples were incubated in a medium containing 100 μM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution. The reaction produces dichlorofluorescein (DCF), a fluorescent compound which is measured at λ_{em} = 488 nm and λ_{ex} = 525 nm. Results were represented as nanomole DCF/milligram protein.

Superoxide Dismutase Assay

Superoxide dismutase (SOD) activity assay was performed according to Marklund [32]. SOD activity is measured through pyrogallol autoxidation, a process highly dependent on superoxide, which is a substrate for this enzyme. Hence, in the presence of SOD, pyrogallol autoxidation is inhibited, and

the activity was then indirectly assayed at 420 nm. A calibration curve was performed with purified SOD as a standard. The results were represented as units per milligram of protein.

Catalase Assay

Catalase (CAT) activity was assayed based on the consumption of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL [33]. The results were represented as units per milligram of protein, being one CAT unit 1 μmol of H_2O_2 consumed per minute.

Glutathione Peroxidase Assay

Glutathione peroxidase (GPx) activity was measured utilizing tert-butyl-hydroperoxide as a substrate [34]. NADPH disappearance was monitored at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, and 0.1 mM NADPH. The specific activity is represented as units per milligram of protein, being one GPx unit 1 μmol of NADPH consumed per minute.

Single Cell Gel Electrophoresis (Comet Assay)

Alkaline comet assay was performed as described by Singh and colleagues [35] in accordance with general guidelines for the use of the comet assay [36, 37]. Striatum were homogenized in phosphate-buffered saline (PBS) and the samples were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose, which set at 4 °C for 5 min. To maintain DNA as "nucleoids," slides were incubated in ice-cold lyses solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1 % triton X-100 with 10 % DMSO) removing cell proteins. Afterwards, slides were placed on a horizontal electrophoresis system, covered with fresh solution (300 mM NaOH plus 1 mM EDTA, pH > 13) for 20 min at 4 °C allowing DNA to unwind and express of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). The slides were then neutralized, washed in bi-distilled water, and stained with silver staining protocol. Gels were analyzed in optical microscope after drying at room temperature overnight. One hundred cells (50 cells from each of the two replicate slides) were selected and evaluated. Cells received a scored according to the tail length, from 0 (no migration) to 4 (maximal migration) according to tail intensity [36]. Therefore, the damage index (DI) for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions at least by two different individuals.

Protein Determination

Protein content of samples was measured by Lowry and colleagues [38] using bovine serum albumin as standard.

Statistical Analysis

Data were analyzed by Student's *t* test was used to evaluate the different parameters. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

Initially, we investigated the effect of hypoxanthine administration on immunocontent of cytosolic and nuclear fraction of NF-κB/p65 subunit. Panels a and b of Fig. 1 show that hypoxanthine administration significantly increased the immunocontent of nuclear fraction of NF-κB/p65 subunit in striatum of 21- [$t(5) = 3.20$; $p < 0.05$] and 60-day-old rats [$t(5) = 6.39$; $p < 0.01$], but no alteration was observed in cytosolic fraction of NF-κB/p65 subunit in rat striatum of 21- [$t(5) = 1.09$; $p > 0.05$] and 60-day-old [$t(5) = 1.42$; $p > 0.05$], as compared to control.

Since NF-κB regulates the innate immune response, which is activated rapidly in response to a wide range of stimuli, including pro-inflammatory cytokines (IL-6 and TNF-α) [39], we evaluated the effect of hypoxanthine administration on cytokine levels (IL-6 and TNF-α) in striatum of 21- and 60-day-old rats. Figure 2a shows that hypoxanthine administration increased IL-6 in rat striatum of 21-day-old [$t(5) = 3.92$; $p < 0.005$] and of 60-day-old [$t(5) = 3.77$; $p < 0,005$] (Fig. 2b). TNF-α was significant increase in striatum of 21-day-old [$t(5) = 2.57$; $p < 0.05$], but not in 60-day-old rats [$t(5) = 0.84$; $p > 0.05$].

Considering that NF-κB is associated with several inflammation mediators, including iNOS [40, 41], we evaluated the iNOS immunocontent and nitrite levels in striatum of infant and young adult rats. Figure 3a, b shows a significant increase in iNOS immunocontent in striatum of rats of both ages [21-day-old: $t(6) = 2.64$, $p < 0.05$; and 60-day-old: $t(6) = 5.04$, $p < 0.001$]. Figure 3c, d shows that hypoxanthine administration was able to diminished nitrite levels in striatum of infant rats [$t(6) = 4.93$; $p < 0.001$], but not in young adult rats [$t(6) = 0.18$; $p > 0.05$].

In order to evaluate the effect of hypoxanthine on immune response of glial cells, we measured the immunocontent of IBA1 and GFAP with the aiming of analyze both microglia and astrocyte activation, respectively. Accordingly, we notice an increase in the immunocontent of IBA1 [$t(6) = 4.20$; $p < 0.01$] and GFAP [$t(6) = 2.39$; $p < 0.05$] in striatum of

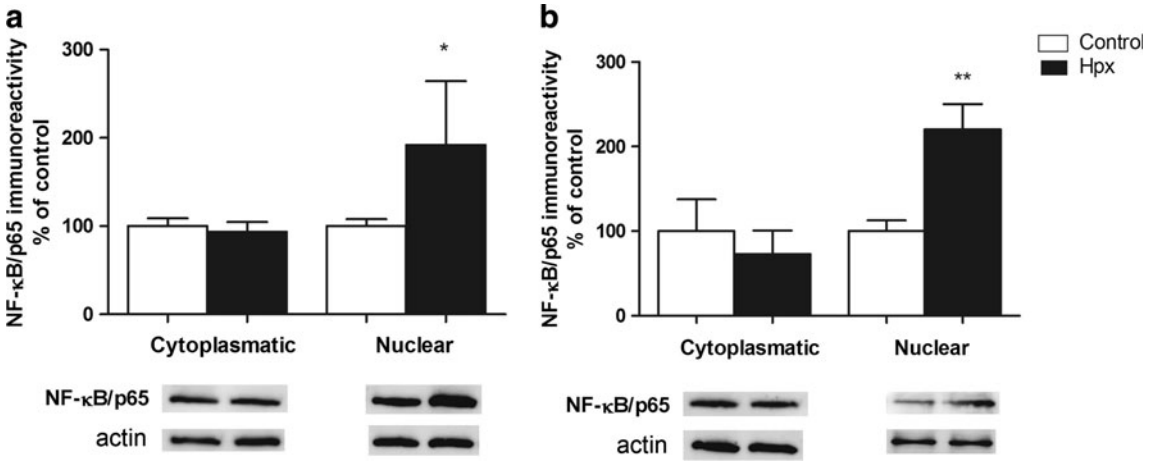


Fig. 1 Effects of hypoxanthine intrastriatal administration on cytosolic and nuclear fraction of NF-κB/p65 subunit. **a** Striatum of 21-day-old rats and **b** striatum of 60-day-old rats. Results are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for six to seven animals in each group. Different from control, $*p < 0.05$, $**p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *NF-κB*, nuclear factor-kappa B

21-day-old rats when compared to control group (Fig. 4a, c). However, there was no significant change in the immunocontent of both proteins in 60-day-old rats (Fig. 4b, d).

Since neuroinflammation is involved in oxidative stress and vice versa, we decided to evaluate hypoxanthine effect on some oxidative parameters in striatum of rats. Firstly, we measured reactive species production by oxidation of H_2DCF, which is increased in infant [$t(6) = 3.32$; $p < 0.01$] and young adult rats [$t(6) = 2.47$; $p < 0.05$] (Fig. 5a, b). Regarding antioxidant status, previous studies from our group [18, 42] are important to highlight, due to the results obtained on SOD, CAT, and GPx activities in striatum of 60-day-old rats. In the present study, this led us to evaluate the same enzymes in striatum of 21-day-old rats. Figure 5c–e shows a decrease in the activities of SOD, CAT, and GPx [$t(6) = 2.49$; $p < 0.05$; $t(6) = 2.24$; $p < 0.05$; $t(6) = 2.82$; $p < 0.05$], respectively. We also analyze DNA damage detected a massive increase in DNA damage index in striatum of infant [$t(6) = 21.08$; $p < 0.0001$]

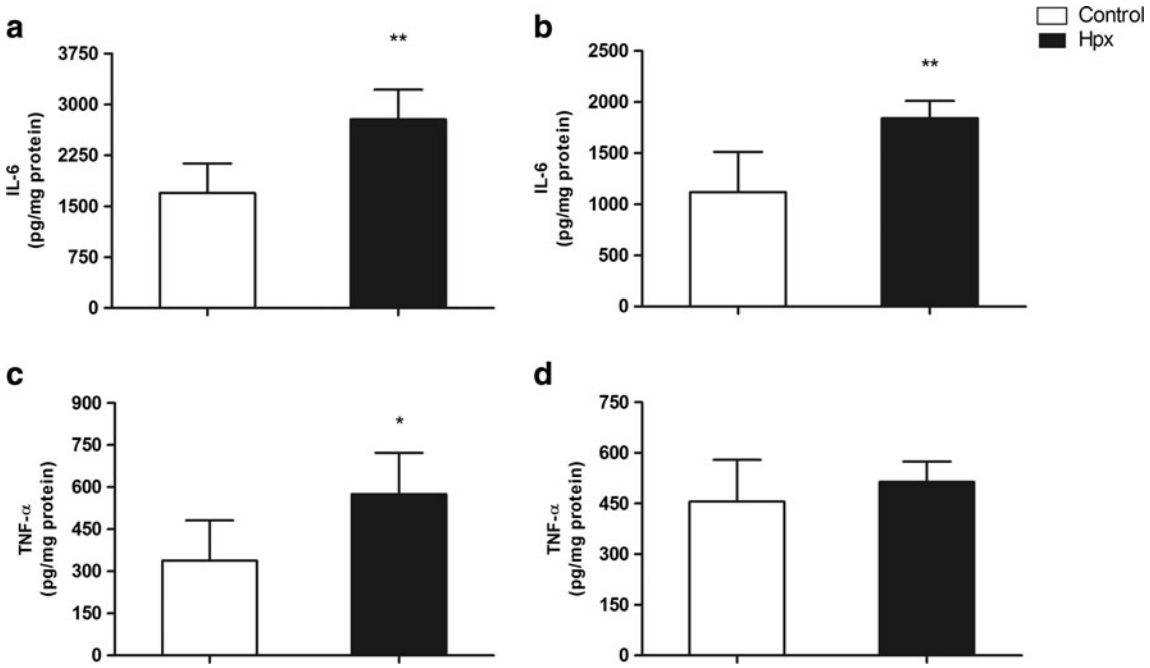


Fig. 2 Effects of hypoxanthine intrastriatal administration on cytokines levels in striatum of infant and young adult rats. **a** IL-6 in 21-day-old rats; **b** IL-6 in 60-day-old rats; **c** TNF-α in 21-day-old rats; and **d** TNF-α in 60-day-old rats. Results are expressed as picogram per milligram of protein. Data are mean ± SD for five to six animals in each group. Different from control, $*p < 0.05$, $**p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor alpha

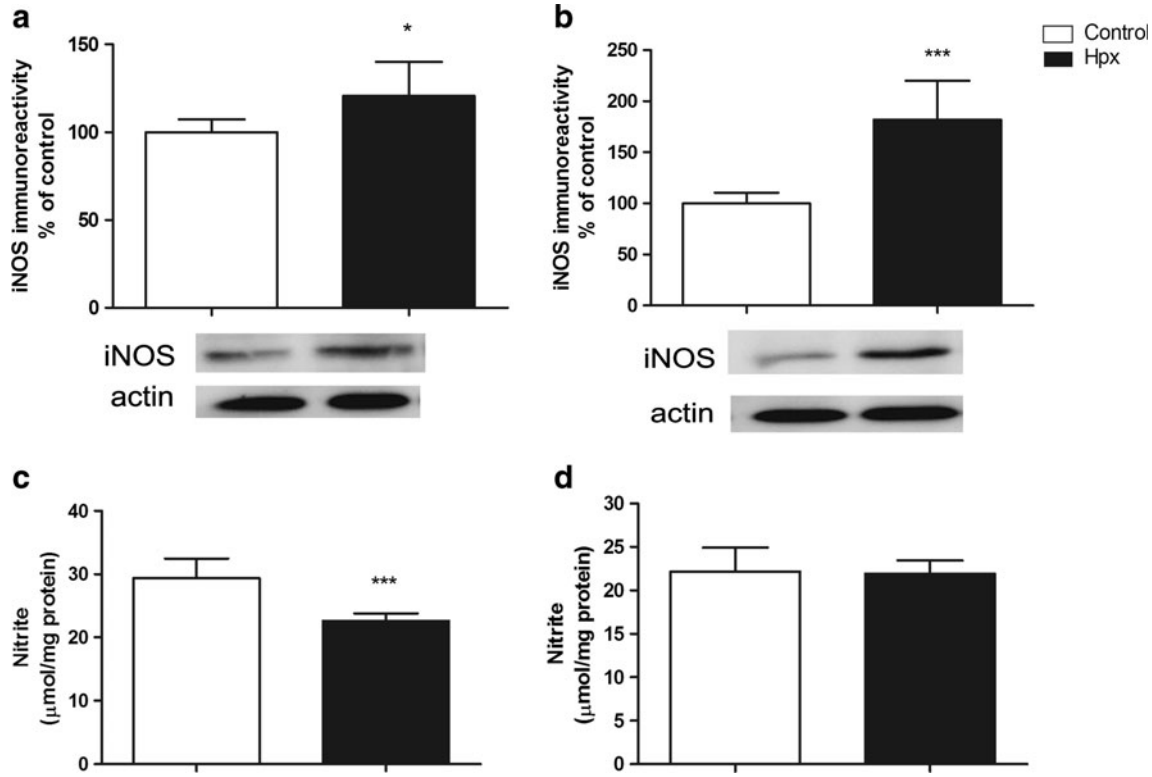


Fig. 3 Effects of hypoxanthine intrastriatal administration on inducible nitric oxide synthase immunocontent and nitrite levels. **a** iNOS immunocontent in striatum of 21-day-old rats and **b** striatum of 60-day-old rats. Results are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for six to seven animals in each group. **c** Nitrite levels in striatum of 21-day-old rats and **d** striatum of 60-day-old rats. Results are expressed as micromole per milligram of protein. Data are mean ± SD for six to seven animals in each group. Different from control, $*p < 0.05$, $***p < 0.001$ (Student's *t* test). *Hpx*, hypoxanthine; *iNOS*, inducible nitric oxide synthase

and young adult [$t(6) = 53.68$; $p < 0.0001$] rats, when compared to control group (Fig. 5f, g).

Discussion

Severe variation in purine salvage pathway as a result of HPRT activity deficiency causes LND, leading to hypoxanthine accumulation in patient's urine, plasma, and cerebrospinal fluid [3]. Although the underlying mechanisms of brain dysfunction in LND are insufficiently understood, the accumulation of oxypurines, essentially hypoxanthine, has been proposed to contribute to the neurological dysfunction present in this disease [7]. A recent study showed that high hypoxanthine levels alter various transcription factors related in early development of neuronal dopamine and in pan-neuronal differentiation with elevated expression of neurotransmitter receptors for dopamine, adenosine, and serotonin in cellular model, as seen by Torres and Puig [43]

For a long time, CNS was considered to be "immune prerogative," not being responsive or cooperate with inflammation. Nowadays, it is known that the CNS has a certain role in inflammation, and response to injury, infection or disease, resident CNS cells, mainly microglia, that generates inflammatory mediators that modulate the pro- and anti-inflammatory mediators (cytokines, chemokine, NO, PGs, growth factors, and the species of superoxide). These mediators upregulate adhesion molecules, increase permeability of the blood-brain barrier, facilitating the invasion of peripheral immune cells, inducing the release of potentially toxic molecules that can compromise brain cells [44]. A relation among neuroinflammation, oxidative stress, neurodegenerative disease, and others disorders has been suggested [45]. Based on these, the purpose of the present study was to evaluate pro-inflammatory cytokines levels, NF-κB/p65 subunit immunocontent, nitrite levels, iNOS immunocontent, as well as immunocontent of IBA1 and GFAP in striatum of 21- and 60-day-old rats subjected to intrastriatal administration of hypoxanthine. Some oxidative parameters such as reactive species generation, antioxidant enzymes activities, and DNA damage were also evaluated.

It is well known that NF-κB is activated in consequence of inflammation. Other member of the NF-κB family, NF-κB/p65, is situated in the cytoplasm bound to inhibitory proteins (IκB), hence inactivated. Activation induces nuclear translocation of NF-κB/p65, followed by transcriptional activation of

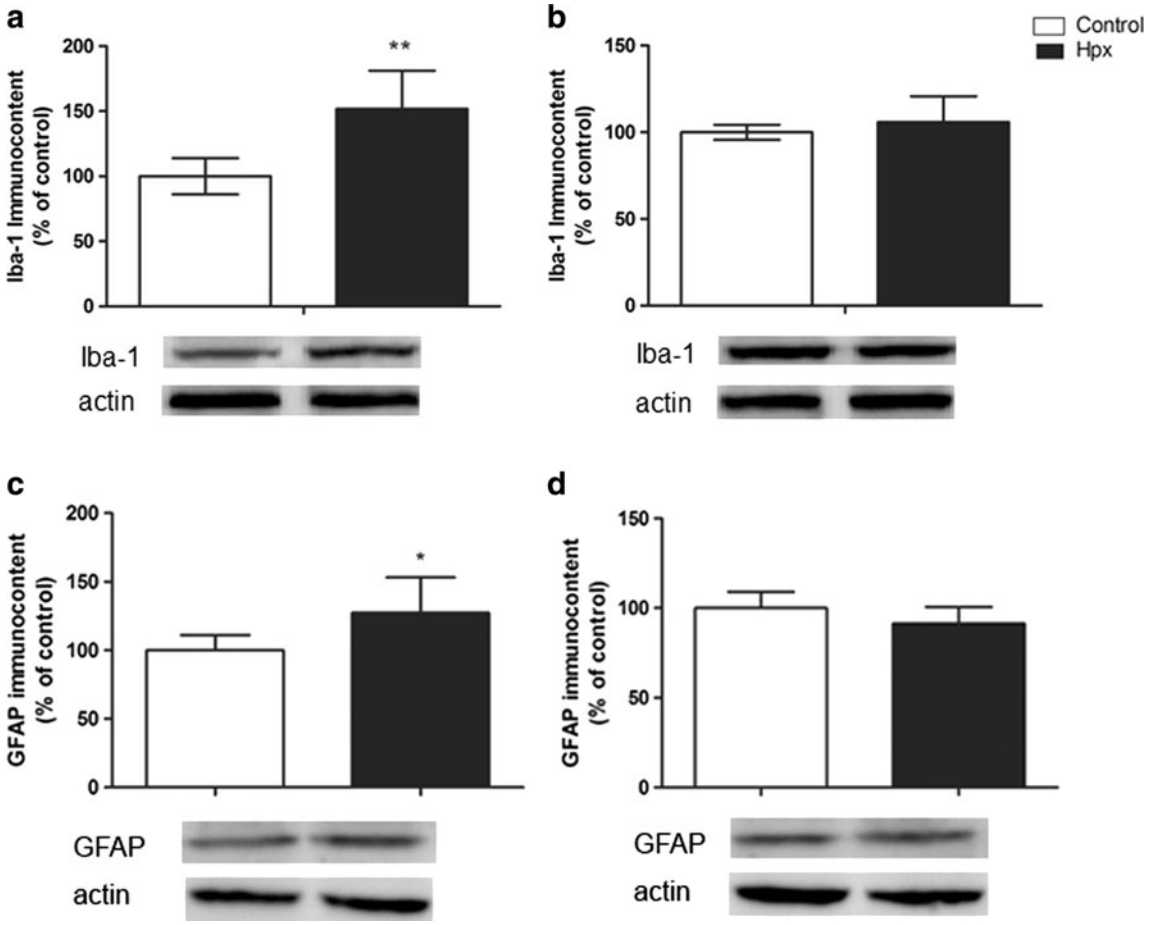


Fig. 4 Effects of hypoxanthine intrastriatal administration on microglial and astrocyte activation. **a** IBA1 immunocontent in striatum of 21-day-old rats and **b** IBA1 immunocontent in striatum of 60-day-old rats. **c** GFAP immunocontent in striatum of 21-day-old rats and **d** GFAP immunocontent in striatum of 60-day-old rats. Results are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for six to seven animals in each group. Different from control, $*p < 0.05$, $**p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *IBA1*, ionized calcium-binding adapter molecule 1; *GFAP*, glial fibrillary acidic protein

specific target genes [46]. These translocations are often frequently taken as indicators of NF-κB activation and are related with cellular response to oxidants or to the inflammatory and acute immune response [15]. NF-κB has been associated with several inflammatory mediators such as IL-6 [47–49], TNF-α [50, 51], and iNOS [40, 41, 52]. In our study, there was an increase immunocontent nuclear NF-κB/p65 subunit in striatum of infant and young adult rats subjected to intrastriatal injection of hypoxanthine (Fig. 1). Cytokines are molecules involved in signal transmission between cells during the initiation of immune responses. Results showed that the hypoxanthine intrastriatal administration provoked an increase in pro-inflammatory cytokines, including IL-6 and TNF-α, in striatum of 21-day-old rats and only IL-6 increases in 60-day-old rats (Fig. 2). In agreement with our results, Gudbjornsson and colleagues [53] showed that hypoxanthine is related to inflammation of rheumatoid arthritis, which presents enhanced purine metabolism and diffusion of oxypurines. Although the mechanism(s) by which hypoxanthine acts on inflammation are not fully understood, our findings suggest that the increase in cytokines levels could be closely related to NF-κB activation.

Another relevant inflammatory mediator is NO. Oxidation products, nitrite, and nitrate are the main metabolites that can be used as an indicator of NO production [54]. NO is produced by converting the amino acid L-arginine to L-citrulline by the action of nitric oxide synthase (NOS). Inducible nitric oxide synthase (iNOS) is an isoform that is expressed via cytokines, endotoxins, and other inflammation mediators being expressed mainly in microglial cells, neutrophils, macrophages, fibroblasts, endothelial cells, and the vascular smooth muscle and mediates the production of large amounts of NO [55, 56]. To understand hypoxanthine mechanism on inflammation, we also evaluated nitrite levels in rats subjected to hypoxanthine administration. We observed that hypoxanthine decreases nitrite levels in striatum of 21-day-old rats but do not alter in 60-day-old (Fig. 3c, d). It has been shown that there is a relation between the formation of NO and synthesis of reactive species, such as superoxide and hydrogen peroxide [57]. Hydrogen peroxide can also react with

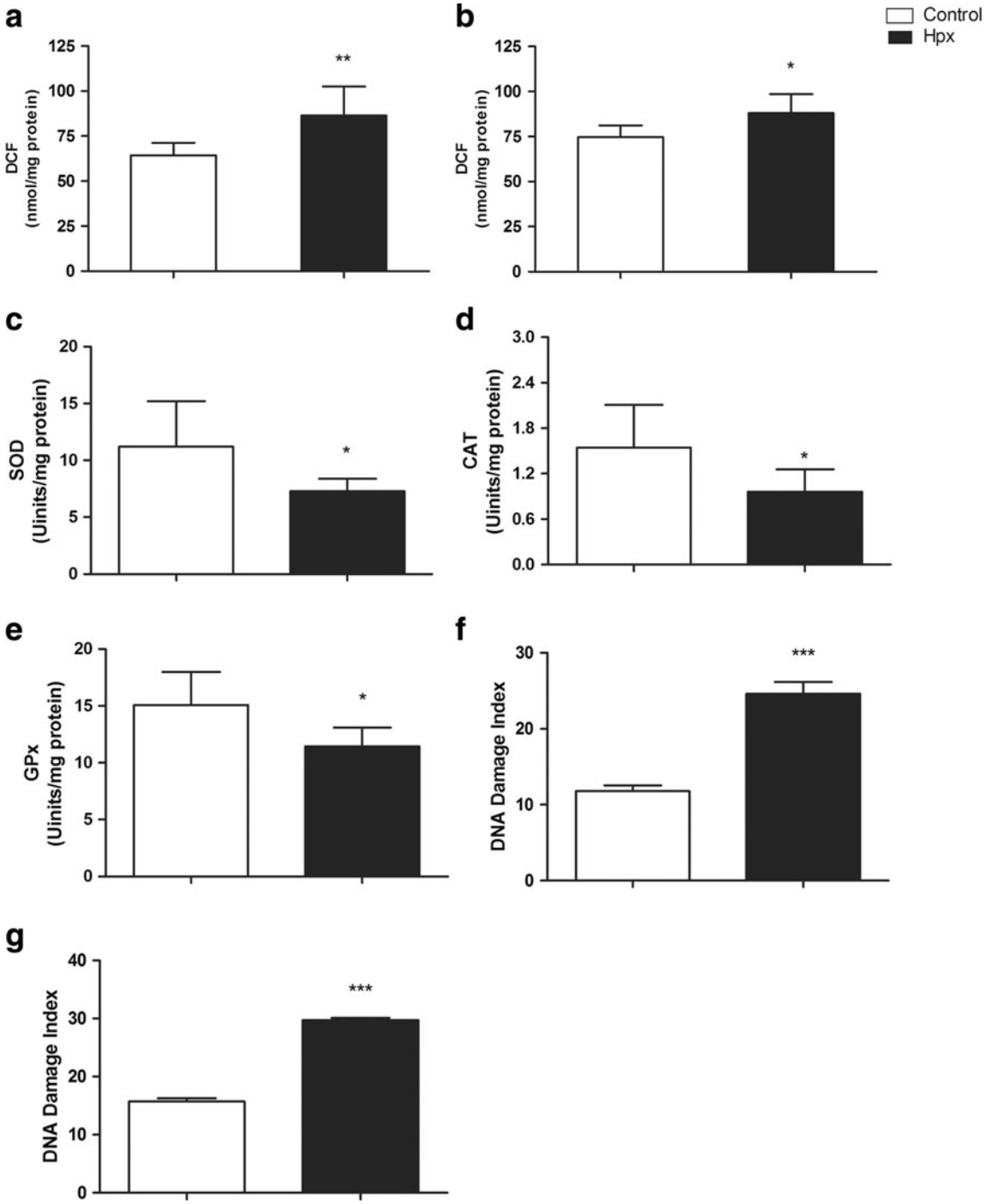


Fig. 5 Effects of hypoxanthine intrastriatal administration on oxidative parameters. **a** H_2DCF oxidation in striatum of 21-day-old rats and **b** striatum of 60-day-old rats. Results are expressed as nanomole per milligram of protein. **c** SOD activity; **d** CAT activity; and **e** GPx activity in striatum of 21-day-old rats. Results are expressed as units per milligram of protein. **f** DNA damage index in striatum of 21-day-old rats and **g** striatum of 60-day-old rats. Results are expressed as DNA damage index. Data are mean ± SD for six to seven animals in each group. Different from control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t* test). *Hpx*, hypoxanthine; *H_2DCF*, 2′7′-dichlorofluorescein; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx*, glutathione peroxidase

NO generating peroxynitrite, a powerful cytotoxic substance [58], suggesting a diminished bioavailability of NO. This possibly explains the decrease in nitrite levels in striatum of infant rats.

Since iNOS is responsible for catalyzing the formation of NO through inflammatory stimuli and closely related to NF-κB activation, we also analyzed iNOS immunocontent. Results showed an increase in this enzyme immunocontent

in striatum of 21- and 60-day-old rats (Fig. 3a, b). Increased protein expression of iNOS is an indicative of neuroinflammatory and neurodegeneration, which seems to be related to microglial activation [57, 59].

We also analyzed microglial and astrocyte activation, measuring IBA1 and GFAP immunocontent, respectively. Microglia, the major antigen presenting in CNS, can react promptly to antigens, releasing several inflammatory factors previous mentioned in this study such as NO, TNF-α, IL-6, and others. Excessive microglial activation is now known to corroborate to CNS disease by the liberation of cytotoxic factors leading to neuron damage [57, 60, 61]. Our results showed that hypoxanthine increased IBA1 immunocontent only in 21-day-old rats (Fig. 4a). Microglia can increase iNOS expression as well as NADPH oxidase, generating a great deal of NO and O^2, which could act as inflammatory agents and promote microglial activation, as well as peroxynitrite synthesis, as mentioned earlier, a major cytotoxic metabolite [57, 60]. There was no change in IBA1 immunocontent in 60-day-old rats, this could be explained by the close relation between TNF-α and microglial activation [62].

Control of synapse formation, maturation, and elimination are astrocyte function, which are essential to development and maintenance of neural circuits. Together with microglia, astrocyte contributes to regulation of the neuroinflammation, being responsible for immune role of the CNS [63]. Loss of normal functions and gain of abnormal effects are some consequences of reactive astrocytes, being feature in a variety of disease processes [64]. Upregulation of intermediate filament proteins, particularly GFAP by reactive astrocytes, is probably the finest hallmark of reactive astrocytes and reactive gliosis measure [65]. Hypoxanthine was able to increase GFAP immunoreactivity in infant rats' striatum, suggesting an astrogliosis event. No alteration was seen in young adult rats, perhaps by difference in the neurodevelopmental stage, meaning that infant rats are more bound to suffer from hypoxanthine administration and its neuroinflammatory effects.

Since one of the possible mechanism in which NF-κB may be acting as a pro-inflammatory signal is via oxidative stress [66] and hypoxanthine may have a oxidative effect [18, 19], we decide to analyze some oxidative parameters in both ages of rats. Our results showed an increase in reactive species production seen by H_2DCF oxidation in infant and young adult rats; antioxidant enzyme deregulation in striatum of 21-day-old rats. Similar alteration was seen by Bavaresco and colleagues [42] in 60-day-old rats. It has been known that reactive oxygen species can attack directly the DNA producing single strand breaks [67]; in our study, we observed DNA damage, what may link hypoxanthine strongly to oxidative stress formation. This mechanism could explain the generation of inflammatory profile caused by hypoxanthine.

In summary, our findings demonstrated that intrastriatal administration of hypoxanthine presented an increase in pro-inflammatory cytokine levels, NF-κB, and iNOS, decreased nitrite levels, as well as altered microglial and astrocyte activation, suggesting that this substance may promote a pro-inflammatory status in striatum of infant and young adult rats by generation of oxidative stress. This study provides new basis elucidating hypoxanthine toxicity mechanisms on inflammatory and oxidative parameters, suggesting that this processes may be involved, at least in part, with disorders found in patients with LND.

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Compliance with Ethical Standards

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

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3.2 Capítulo II

Hypoxanthine Induces Neuroenergetic Impairment and Cell Death in Striatum of Young Adult Wistar Rats

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Status: a ser submetido

Hypoxanthine Induces Neuroenergetic Impairment and Cell Death in Striatum of Young Adult Wistar Rats

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Abstract

Hypoxanthine is the major purine involved in the salvage pathway of purines in the brain. High levels of hypoxanthine are characteristic of Lesch-Nyhan Disease. Since hypoxanthine is a purine closely related to ATP formation, the aim of this study was to investigate the effect of intrastriatal hypoxanthine administration on neuroenergetic parameters, mitochondrial function and cell death in striatum of rats. We investigate the hypoxanthine effect on pyruvate kinase, succinate dehydrogenase and complex II activities. We also evaluated cytochrome c oxidase activity and immunoconent, as well as ATP levels, mitochondrial mass and membrane potential. Cell death parameters in striatum of rats were also evaluated. Wistar rats of 60 days of life underwent stereotactic surgery and were divided into two groups: control (infusion of saline 0.9 %) and hypoxanthine (10 μM). Intrastriatal hypoxanthine administration did not alter pyruvate kinase activity, but increased succinate dehydrogenase and complex II activities and diminished cytochrome c oxidase activity and immunocontent. Hypoxanthine injection decreased the percentage of cells with mitochondrial membrane label and increased mitochondrial membrane potential labeling. Hypoxanthine also diminished the number of live cells and increased the number of apoptotic cells. According to our results, hypoxanthine administration altered neuroenergetic parameters, leading to mitochondrial dysfunction and further cell death by apoptosis, suggesting that these processes may be involved, at least in part, with neurological symptoms found in patients with Lesch-Nyhan disease.

Keywords: Hypoxanthine; neuroenergetic; ATP; apoptosis.

Introduction

Hypoxanthine is the main purine involved in the salvage pathway of purines in the brain, being basal ganglia notably dependent on this route for the maintenance of physiologic purine levels. Lesch–Nyhan Disease (LND), an innate metabolic error of purine metabolism, caused by a severe deficiency of hypoxanthine–guanine phosphoribosyl-transferase (HPRT, EC 2.4.2.8) activity [1]. LND patients present five times the normal concentration of hypoxanthine in cerebrospinal fluid [2,3]. Symptomatology of LND patients are cerebral palsy, cognitive and behavioral disturbances, motor dysfunction (spasticity, dystonia), self-mutilation behavior, as well as hyperuricemia [4–6].

Although the brain represents only 2% of the body weight, its energy requirements are very high, and solid regulatory mechanisms operate to provide proper delivery of energy substrates for the neuronal activity. About 25% of the glucose and 20% of the oxygen consumed by the human body are dedicated to cerebral functions [7,8]. Therefore, the brain is extremely sensitive to disruption of energy resources, being the most metabolically active organ in the human body. Mitochondria have a fundamental function in cellular energy metabolism. A portion of the free energy derived from the oxidation of food is transformed to ATP inside of the mitochondria, in a depending oxygen process. When oxygen rate is restricted, glycolytic products are metabolized directly in the cytosol by the less efficient anaerobic respiration, being independent of mitochondria. The mitochondrial ATP production relies on the electron transport chain (ETC), composed of respiratory chain complexes I–IV [9–11]. Accordingly, the maintenance of ATP levels is very important for physiological neuronal activity.

Impaired energy metabolism may trigger pro-apoptotic signaling (programmed cell death), oxidative stress and damage to lipids, protein and DNA [12], leading to conditions that affect the central nervous system (CNS) [13–15].

Since the mechanisms by which hypoxanthine acts in the CNS and the exact role of this oxypurine on the neurological damage present in the LND still need to be further elucidated, in the present study we investigated the effect of the intrastriatal hypoxanthine administration on neuroenergetic parameters in striatum of 60-day-old rats. We initially analyzed some energy parameters (activities of pyruvate kinase (PK), succinate dehydrogenase (SDH), complex II (CII), cytochrome c oxidase (COX), creatine kinase (CK) and ATP levels) as well as the effect of hypoxanthine administration on mitochondrial mass and mitochondrial membrane potential. Cell death parameters in striatum of rats were also evaluated.

Material and Methods

Animals and reagents

Male Wistar rats of 60-day-old were obtained from the Central Animal House of the Department of Biochemistry of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. Animals were maintained under a standard dark–light cycle at a room temperature of 22±1°C, with free access to a commercial protein chow and water. All the chemical reagents used for analysis were obtained from Sigma Aldrich Co., St. Louis,

MO, USA. The kit for determination of ATP levels was purchased from Perkin-Elmer, Waltham, MA, USA.

Ethics statement

All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (No. 80-23, revised 1996) and a research project approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil (# 25717). All efforts were made to minimize the number of animals used and their suffering.

Experimental model

The animals were anaesthetized with ketamine and xylazine (75 and 15 mg/kg i.p., respectively). Based on stereotaxic measures, a 27 gauge 0.9 mm diameter cannula was positioned and fixed in the rat right striatum as detailed described by Biasibetti and colleagues [16]. The coordinates used for infant and young adult rats were based on Paxinos and Watson's atlas [17] (coordinates relative from bregma: AP -0.5 mm, ML -2.5 mm, V -2.5 mm from the dura). The animals were divided into two groups (n=7 animals per group): (1) control (2 µL of 0.9% saline infusion); (2) hypoxanthine (2 µL of 10 µM hypoxanthine infusion). Animals were decapitated 30 minutes after the saline or hypoxanthine infusion, brain was dissected and the structure analyzed was striatum.

Tissue preparation

For determination of pyruvate kinase activity, striatum were homogenized with a Teflon-glass homogenizer in five volumes of ice-cold SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl), pH 7.4. Afterwards, the homogenate was centrifuged at 800×g and the pellet was discarded. Supernatant was centrifuged at 10,000×g for 10 min. The supernatant solution was collected and used for enzymatic assay. For SDH, CII and COX activity determination, striatum were homogenized (1:20 w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/ml heparin), pH 7.4. The homogenates were centrifuged at 800×g for 10 min and the supernatants were used for enzyme activity determination. For creatine kinase activity, the striatum was washed in SET buffer (0.32 M sucrose/1 mM EGTA/10 mM Tris–HCl; pH7.4), chopped and homogenized in the same SET buffer (1:10, w/v). Homogenate was centrifuged at 800xg for 10 min at 4ºC, keeping the supernatant, which was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant of the second centrifugation was used to determine the activity of the CK cytosolic fraction. The sediment was resuspended in Tris–HCl buffer, pH 7.5 containing 100 mM $MgSO_4$ to determine the activity of CK mitochondrial fraction.

Pyruvate kinase activity

Pyruvate kinase activity was assayed essentially as described by Leong and colleagues [18]. The incubation medium consisted of 0.1 MTris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 unit of L-

lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 ml of the mitochondria-free supernatant in a final volume of 0.5 ml. After 30 min of preincubation, the reaction was started by the addition of 1.0 mM phosphoenolpyruvate (PEP). Results were expressed as micromoles of pyruvate formed per minute per milligram of protein.

Succinate Dehydrogenase and Complex II activities

Immediately before the assay, the samples were frozen and defrost three times. The activities of succinate:phenazine oxidoreductase (succinate dehydrogenase, SDH) and succinate-2,6-dichloroindophenol-oxidoreductase (complex II) [19]. This method is based on the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength (å=19.1 mM-1 cm-1) in the presence of phenazine methosulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 µM DCIP was pre-incubated with 40 to 80 µg homogenate protein at 30°C for 20 min. Subsequently, for complex II activity, 4 mM sodium azide, 7 µM rotenone and the reaction was initiated by addition of 40 µM DCIP and was monitored for 5 min.

Cytochrome c oxidase activity

The activity of cytochrome c oxidase (COX) was determined according to Rustin and colleagues [20]. Enzymatic activity was measured by following the decrease in absorbance due to oxidation of previously reduced cytochrome c at

550 nm with 560 nm as reference wavelength (ε=19.1 mM−1cm−1). The reaction buffer contained 10 mM potassium phosphate pH 7.0 and 0.6 mM n-dodecyl-β-D-maltoside. The reaction was initiated by the addition of 0.7 µg reduced cytochrome c and monitored for 10 min.

ATP assay

Structures were homogenized in 1 ml of 0.1 M NaOH. Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin Elmer, Waltham, MA, USA), in accordance with Ferreira and colleagues [21]. Chemiluminescence was measured using the Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as micromoles per gram of tissue.

Creatine kinase activity

The reaction mixture consisted in the following medium: 65 mM Tris–HCl buffer (pH 7.5) containing 7 mM of phosphocreatine, 9 mM of $MgSO_4$, and approximately 0.4 to 1.2 mg of protein in a final volume of 0.1 ml. The reaction was started by the addition of 3.2 mM of ADP plus 0.8 mM of reduced glutathione after 5 min of preincubation at 37ºC. After 10 min, the reaction was stopped by the addition of 1 mmol p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [22]. The color was developed by the addition of 0.1 ml of 2% a-naphthol and 0.1 ml

0.05% diacetyl in a final volume of 1 ml, and read after 20 min at 540 nm. Results are expressed as micromoles of creatine per minute per milligram of protein.

Immunocontent of Cytochrome c Oxidase by Western Blotting

Samples were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % SDS, then dissolved in 25 % (v/v) of a solution containing 40 % glycerol, 5 % mercaptoethanol, and 50 mM Tris–HCl, pH 6.8 and boiled for 5 min. Protein samples were separated by 10% SDS-PAGE (30 µg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The membranes were then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), then incubated overnight at 4 °C in blocking solution containing the respective antibody cytochrome c oxidase (1:1000) and anti-β-actin (1:1000). The blot was then washed twice for 5 min with T-TBS and incubated for 2 hours in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS and developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Mitochondrial Mass and Membrane Potential Analyses

Striata were mechanically dissociated in PBS containing collagenase. To determine the mitochondrial mass and membrane potential (Δψ), dissociated cells were incubated with 100 nm MitoTracker Green and 100 nm MitoTracker Red (MTG and MTR, respectively, diluted from 1 mM stock solutions in DMSO), for 45 min, at 37 °C (in the dark). Therefore, it was possible to establish a relationship between MTG and MTO staining for accessing the rate of mitochondrial function [23]. Immediately after staining, cell suspensions were analyzed on a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA), using red (670 nm long pass) and green (530 nm/30) filters. Controls stained with individual dyes were used to determine compensation parameters. For each sample, 10.000 events corresponding to intact cells (as gated in FSC vs. SSC plots) were analyzed.

Cell Death Analysis

Striata were mechanically dissociated in PBS containing collagenase. Cells were labeled by incubation with annexin V-FITC and propidium iodide (PI) in a binding buffer (apoptosis detection kit I-556547; BD Pharmingen) for 15 min at room temperature in the dark, according to the manufacturer's instruction. Stained cells were acquired (10,000 for gated cells) on a FACS Calibur flow cytometer Becton–Dickinson, Franklin Lakes, NJ, USA, using green (530 nm/30) and red (670 nm long pass) filters. Controls stained with individual dyes were used to determine compensation parameters.

Protein determination

Protein content of samples was determined using bovine serum albumin as standard, according to Lowry [24].

Statistical analysis

Data were analyzed by Student's t test for comparison of two means. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a compatible computer. Results are expressed as means ± SD, and differences were considered statistically significant when $p < 0.05$.

Results

To better comprehend the mechanisms involving hypoxanthine neurotoxicity in striatum of 60-day-old rats, initially we investigated the effect of hypoxanthine administration on enzymatic activity of glycolytic pathway in striatum of 60-day-old rats, performed by the measurement of PK, a crucial enzyme of glycolysis pathway. Figure 1a shows no substantial difference in PK activity between the hypoxanthine and control group ($p>0.05$).

Further on oxidative pathway, we analyzed SDH and complex II activities. As seen in Figure 1b and c hypoxanthine injection was able to significantly increase the activity of both enzymes ($p<0.01$, $p<0.05$, respectively) when compared to controls. In addition we analyzed cytochrome c oxidase, the

rate limiting enzyme of oxidative phosphorylation. Figure 2a and b exhibit significant decrease in both activity and immunocontent of this enzyme ($p<0.001$, $p<0.01$, respectively). We also analyzed ATP levels and hypoxanthine administration was able to decrease significantly ATP levels (Figure 2 c) ($p<0.05$). Other significant system that can contribute in the sustenance of ATP levels is CK cytosolic and mitochondrial isoenzymes. As illustrated in Figure 3 a and b, hypoxanthine administration decreased the activities of cytosolic ($p<0.001$) and mitochondrial ($p<0.05$) CK.

To better elucidate the effect of hypoxanthine on mitochondrial mass and membrane potential ($\Delta\psi$) we labeled striatal cells with the fluorescent dyes MTG and MTR, respectively. Hypoxanthine injection decreased the percentage of cells labeled with MTG ($p<0.01$) and increase MTR labeling ($p<0.05$). Therefore the ratio between MTG and MTR was diminished ($p<0.001$) in hypoxanthine group as compared to controls (Figure 4 a).

Given that mitochondria have a central role in cell viability and ATP can regulate cell death by apoptosis [25], we labeled striatal cells with annexin V and PI and analyzed these cell populations by flow cytometry to examine the effects of hypoxanthine administration on cell death parameters in striatum of 60-day-old rats. Hypoxanthine decreased the number of living cells (annexin V^-/PI^-) ($p<0.05$), increasing apoptotic events (annexin V^+/PI^-) in striatum ($p<0.001$) (Figure 4 b). Hypoxanthine administration did not alter the number of necrotic cells (annexin V^-/PI^+) in striatum of 60-day-old rats ($p>0.05$) (Figure 4 b).

Discussion

Hypoxanthine, the major purine metabolite involved in the purine salvage pathway in the brain, is increased more than five times that its regular levels in plasma of patients with LND. This disorder and its less severe variants are caused by mutations in the HPRT1 gene, which encodes the purines recycling enzyme HPRT [4,26,27]. Previous studies relate hypoxanthine excess to the brain damage of patients affected by by LND, since hypoxanthine diminishes adenosine transport and alters various transcription factors related in early development of neuronal dopamine and in pan-neuronal differentiation [28,29]. Considering the physiopathology of LND still need to be further elucidated, in the present study we investigated the effect of intrastriatal injection of hypoxanthine on energy parameters in order to better understand the mechanisms involved in this condition.

In the present study, initially we investigated the effect of hypoxanthine administration on pyruvate kinase activity (PK). PK is a rate-regulating glycolytic enzyme that catalyzes the irreversible conversion of PEP to pyruvate, synthesizing one molecule of ATP [30]. High concentration of hypoxanthine did not alter PK activity in striatum of 60-day-old rats (Fig. 1 a). Extending our investigation on tricarboxylic acid (TCA) cycle we evaluated the activity of SDH, an enzyme of the TCA cycle located in the inner mitochondrial membrane where it can directly transfer electrons into the electron transport chain [10]. As seen in figure 1 b, we observed a significant increase in SDH activity in striatum of 60-day-old rats subjected to administration of hypoxanthine. This condition is

also seen in other disorders such as hyperprolinemia, sarcosinemia, Huntington's disease and Alzheimer's disease [31–34]

Further on mitochondrial oxidative phosphorylation we analyzed the mitochondrial Complex II of the electron transport chain, which is an essential component of oxidative phosphorylation and is coupled to the synthesis of ATP from ADP and Pi [10]. Accordingly to SDH activity, we observed an increase in complex II activity (figure 1 c). This enzyme catalyzes the oxidation of matrix succinate to fumarate, feeding electrons to the cytochrome complex. The regulation of complex II activity is not fully comprehend, despite the involvement of this enzyme in several conditions, recently emerging as important point of investigation in cell signaling, cancer biology, immunology, cardiovascular conditions and neurodegeneration [35]. It is well known that glucose is an essential and prevalent energy substrate for the adult brain under physiological condition; however there are alternative substrates such as ketone bodies, glycogen and amino acids that may also be used under certain circumstances [10]. Since PK is not altered, one explanation to SDH and complex II increase could be by anaplerotic routes.

Considering that mitochondrial respiratory chain and enzymatic phosphoryl transfer network are crucial for brain energy metabolism and function, we further analyze cytochrome c oxidase activity and immunocontent, a rate-limiting enzyme of the mitochondrial respiratory chain. Hypoxanthine administration decreased significantly both parameters evaluated (figure 2 a and b). Our findings are similar to those found in preclinical and clinical studies showing that SDH activity was increased, whereas cytochrome c oxidase activity was decreased in cerebral cortex of mice exposed to chronic hypoxia

[36] and in brain of patients with Alzheimer's disease [34]. Additionally, some studies have shown that an increase in SDH activity can correlate with stimulation of anaerobic pathways coupled with reverse operation of TCA cycle towards the reduction of oxaloacetate to succinate when oxidative phosphorylation is impaired [37]. Also, the increase in SDH and complex II activities and inhibition of cytochrome c oxidase activity can potentially lead to incomplete reduction of oxygen and consequently increase free radical formation and reduce ATP synthesis [38–41].

Accordingly with cytochrome c oxidase results, we also measured ATP levels and observed a decrease in this parameter in striatum of hypoxanthine group when compared to control (figure 2 c). We observed a reduction of the electron transport chain flow (impaired oxidative phosphorylation) caused by hypoxanthine, resulting in lower ATP production, which is necessary to sustain energy requirement of cells. At cellular level, ATP reduction leads to neuron depolarization causing failure of membrane ion-transport. Interventions for improving mitochondrial function by maintaining ATP levels may have solid influence for improving neuronal dysfunction and loss. Intracellular ATP levels lower than 10% of the initial value result in cell death [42] and hypoxanthine was able to decrease in approximately 25%.

Coupling spatial intracellular ATP-producing and ATP-consuming is a fundamental process to cell function. CK is a major phosphotransfer system in cells with high-energy demand. CK is responsible for the relocation of ATP phosphoryl group from where it is produced (mostly in mitochondria) to where it is consumed (mainly in the cytosol) [43]. Hypoxanthine administration decreased cytosolic and mitochondrial CK activities (Figure 4). Considering that

some enzymes involved in energy metabolism require sulfhydryl groups and that previous studies of our group showed that hypoxanthine alters membrane protein thiol content and induces oxidative stress [16,44], the present results showed a decreased in ATP availability, which could lead to the accumulation of debris and ultimately cell death by oxidative stress [45].

Mitochondrial dysfunction affects metabolism function, increases radical formation contributing to oxidative stress, and induces apoptosis, which may lead to cellular and ATP imbalance [11,14]. Evidences suggest that abnormalities in mitochondria are involved not only in aging, neurodegenerative diseases, cancer, and several other mitochondrial diseases [46,47], but also in inborn errors of metabolism [31,48,49]. To better comprehend the effect of hypoxanthine effect on mitochondrial function we analyzed mitochondrial mass and membrane potential (Δψ). Hypoxanthine administration decreased the percentage of cells labeled with MTG and increased MTR labeling when compared to controls (Figure 4 a). Mitochondrial membrane potential is considered a key indicator of mitochondrial function [50], which is in accordance with the increase in SDH and complex II activities, and modifications in these parameters suggest mitochondrial dysfunction [51].

Since mitochondrial damage can initiate signaling pathways of intrinsic/extrinsic apoptosis and regulated necrosis [52], cell redox state can also collaborate to apoptosis, and reactive species produced by mitochondria are involved in cellular damage [53], we analyzed the effect of hypoxanthine on striatal cells with annexin V and PI labeling. Figure 4 b shows that hypoxanthine decreased the number of living cells (annexin V^-/PI^-), consequently increasing apoptotic events (annexin V^+/PI^-) in striatum. The number of necrotic cells

(annexin V^-/PI^+) was not altered by hypoxanthine administration in striatum of 60-day-old rats.

In summary, the present results shed light into the mechanisms of hypoxanthine-induced neurotoxicity in striatum of 60-day-old rats. Intrastriatal hypoxanthine administration caused neuroenergetic impairment resulting in ATP depletion which can lead to mitochondrial dysfunction and further cell death by apoptosis. This study provides new enlightening basis of hypoxanthine neurotoxicity mechanisms on energetic and cell viability parameters, suggesting that this processes may be involved, at least in part, with disorders found in patients with LND.

Conflict of interest

All authors declare that there are no financial/commercial conflicts of interest.

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Figure Legends

Figure 1. Effects of hypoxanthine intrastriatal administration on pyruvate kinase (a), succinate dehydrogenase (b) and complex II (c) activities in striatum of 60-day-old rats. Data are mean ± SD for six to seven animals in each group. Data from pyruvate kinase are expressed as μmol of pyruvate per mg of protein; data from succinate dehydrogenase and complex II are expressed as nmol per min per mg of protein. Different from control, *$p < 0.05$, **$p < 0.01$ (Student's t test). Hpx, hypoxanthine; SDH, succinate dehydrogenase.

Figure 2. Effects of hypoxanthine intrastriatal administration on cytochrome c oxidase activity (a), and immunocontent (b) and ATP levels (c) in striatum of 60-day-old rats. Data are mean ± SD for six to seven animals in each group. Data from cytochrome c oxidase activity are expressed as nmol per min per mg of protein; data from western blot are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data from ATP levels are expressed as μmol per gram of tissue. Different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). Hpx, hypoxanthine.

Figure 3. Effects of hypoxanthine intrastriatal administration on cytosolic creatine kinase (a) and mitochondrial creatine kinase (b) activities in striatum of 60-day-old rats. Data are mean ± SD for six to seven animals in each group. Data are expressed as μmol of creatine per minute per mg of protein. Different from control, *$p < 0.05$, ***$p < 0.001$ (Student's t test). Hpx, hypoxanthine; CK, creatine kinase.

Figure 4. Effects of hypoxanthine intrastriatal administration on mitochondrial and cell death evaluation. Mitochondrial mass and membrane potential (a) and Annexin-PI assay (b) in striatum of 60-day-old rats. Data are mean ± SD for six to seven animals in each group. Different from control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). Hpx, hypoxanthine; MTG, MitoTracker Green; MTR, MitoTracker Red.

Figure 1

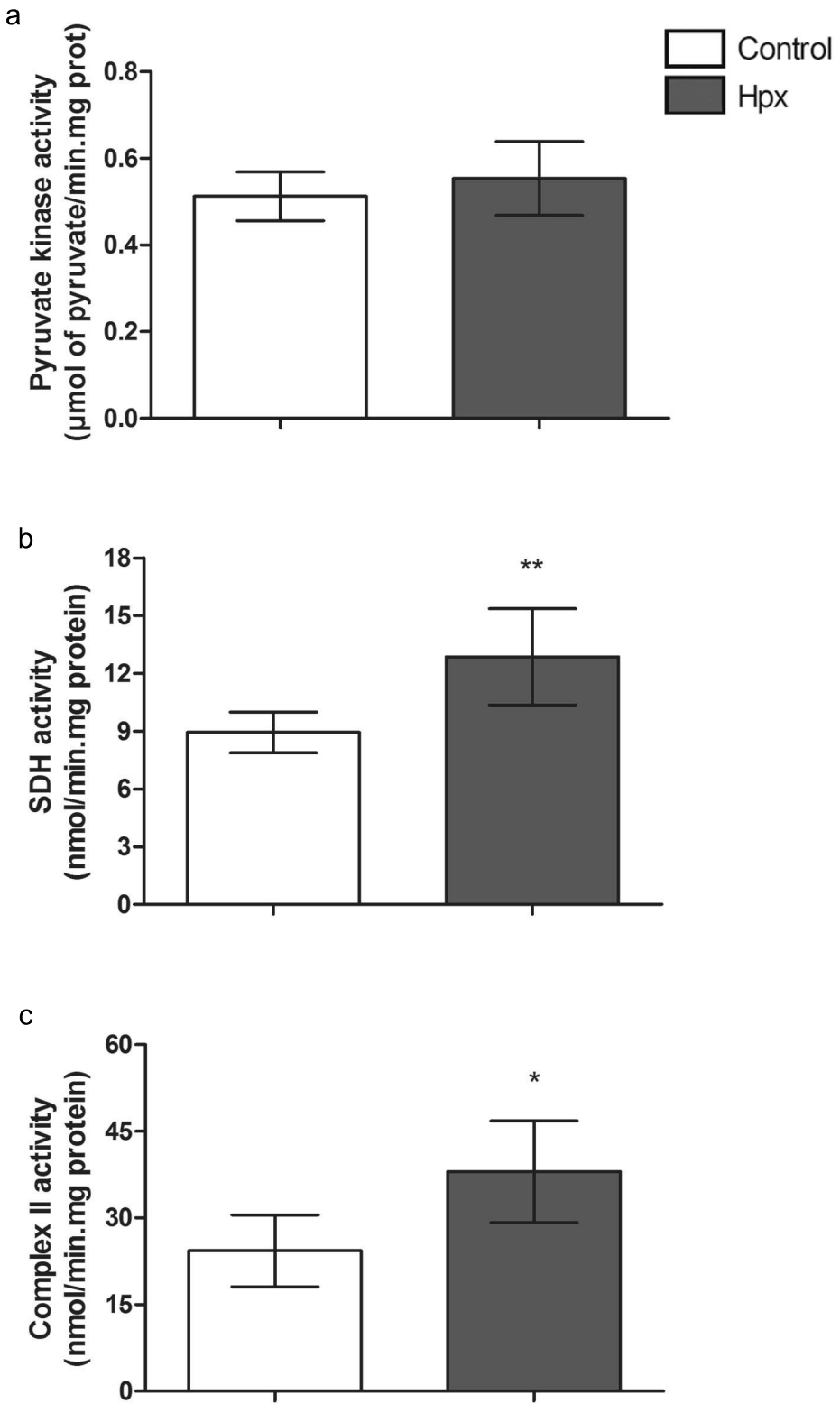

a
Control
Hpx
Pyruvate kinase activity
(µmol of pyruvate/min.mg prot)
0.8
0.6
0.4
0.2
0.0
b
SDH activity
(nmol/min.mg protein)
18
15
12
9
6
3
0
**
c
Complex II activity
(nmol/min.mg protein)
60
45
30
15
0
*

Figure 2

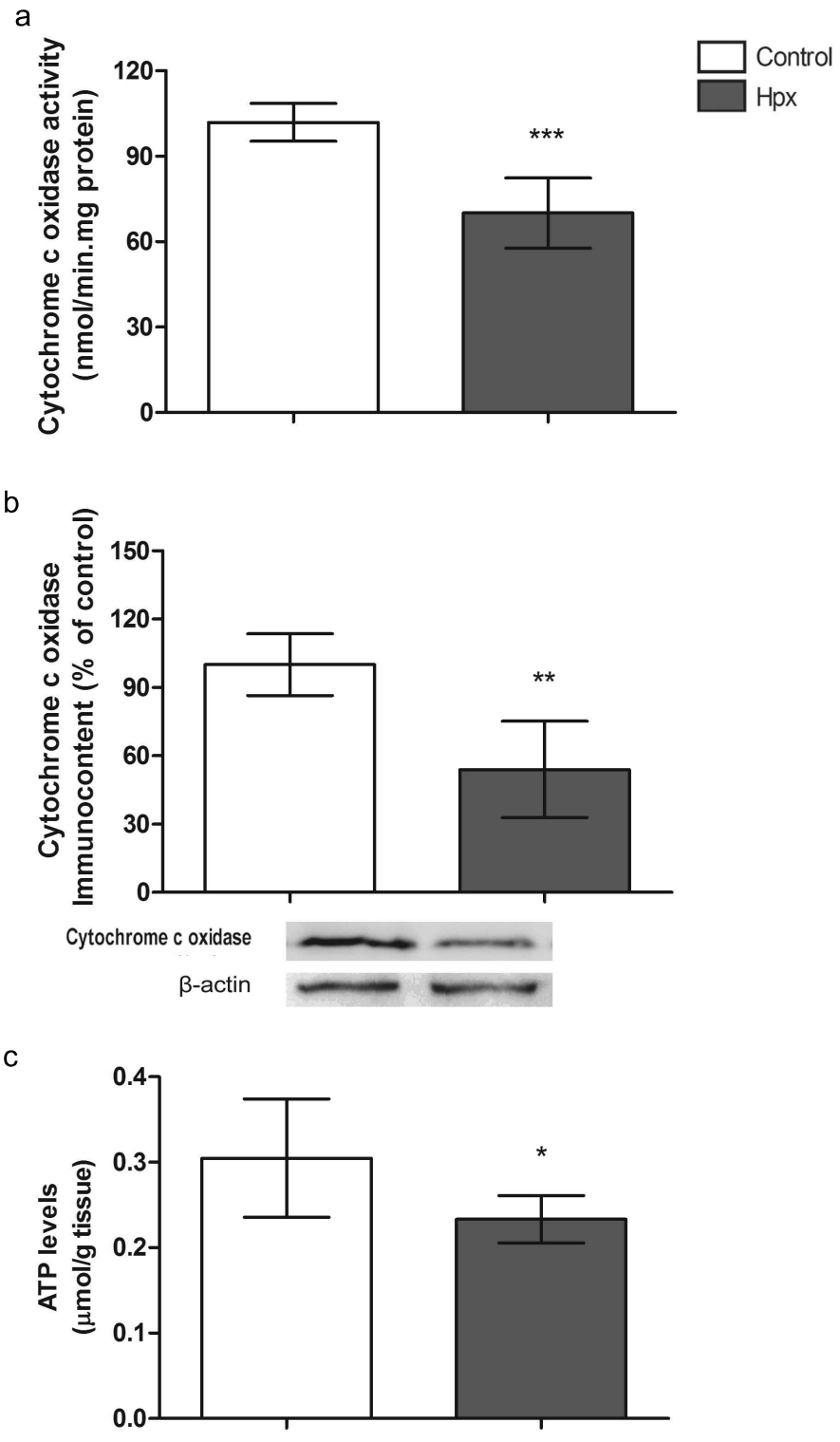

a
Control
Hpx
Cytochrome c oxidase activity (nmol/min.mg protein)
120
90
60
30
0
***
b
Cytochrome c oxidase Immunocontent (% of control)
150
120
90
60
30
0
**
Cytochrome c oxidase
β-actin
c
ATP levels (μmol/g tissue)
0.4
0.3
0.2
0.1
0.0
*

Figure 3

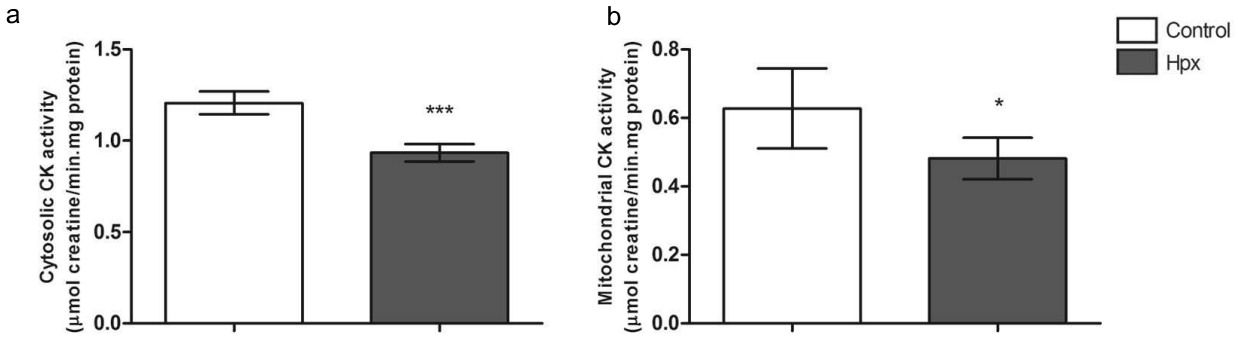
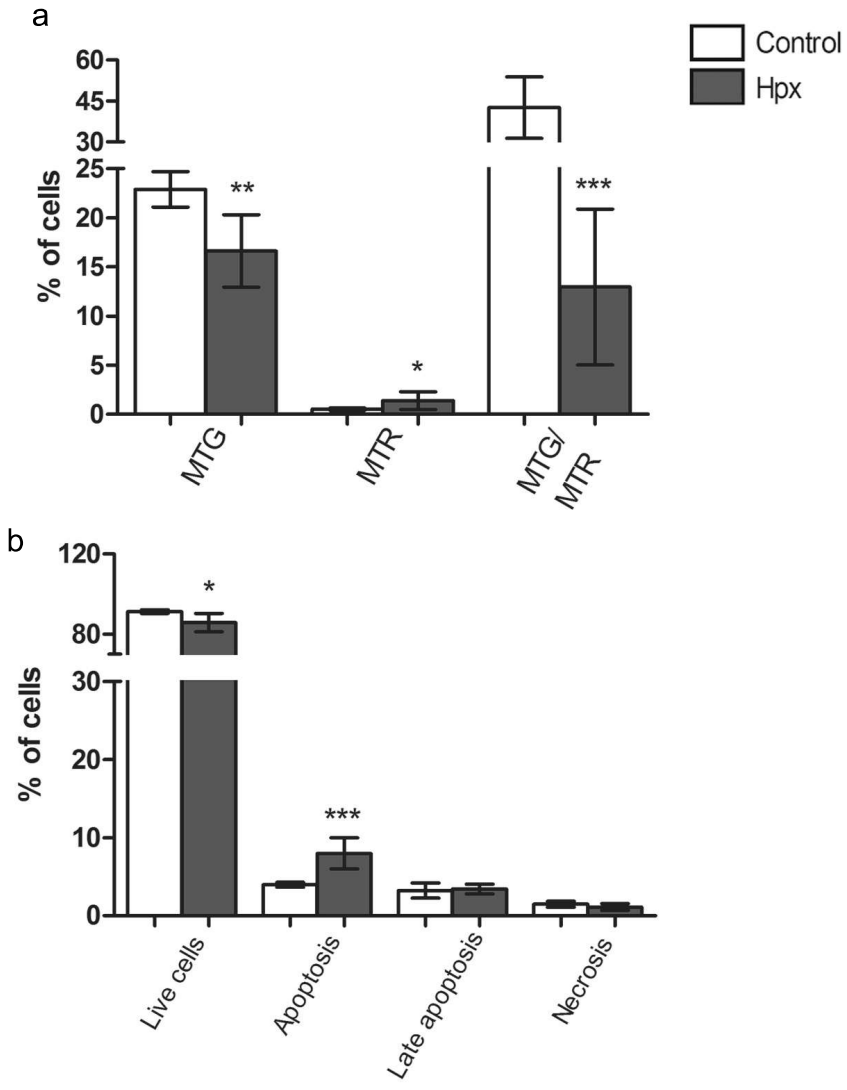


Figure 4



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3.3 Capítulo III

Intrastriatal Hypoxanthine Administration Alters Energy Metabolism, Creatine Kinase and Na^+,K^+-ATPase in Striatum of Infant Rats

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Status: a ser submetido

Intrastriatal Hypoxanthine Administration Alters Energy Metabolism, Creatine Kinase and Na^+,K^+-ATPase in Striatum of Infant Rats

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Abstract

High levels of hypoxanthine, major purine involved in the salvage pathway of purines, is distinctive of Lesch-Nyhan disease, an inborn error of metabolism of purine. Since energy metabolism is the set of processes that makes energy available for cellular activity and Na^+,K^+-ATPase is an energy consuming enzyme responsible for physiological neuronal activity, the aim of this study was to investigate the effect of intrastriatal hypoxanthine administration on neuroenergetic parameters, Na^+,K^+-ATPase and possible oxidative damage in striatum of infant rats. We investigate the hypoxanthine outcome on pyruvate kinase, succinate dehydrogenase and complex II activities. We also evaluated cytochrome c oxidase activity and immunoconent, along with ATP levels and creatine kinase activity. Na^+,K^+-ATPase activity and immunocontent of catalytic α subunits as well as lipid peroxidation and sulfhydryl content in striatum of rats were also evaluated. Wistar rats of 21 days of life underwent stereotactic surgery and were divided into two groups: (1) vehicle-injected group (control) and (2) hypoxanthine-injected group. Results showed that hypoxanthine administration decreased pyruvate kinase activity, but increased succinate dehydrogenase and complex II activities. Hypoxanthine did not alter cytochrome c oxidase activity and immunocontent as well as ATP levels, but decreased cytosolic and mitochondrial creatine kinase activities. We also observed significant decreased in Na^+,K^+-ATPase activity, but not on the immunocontent of this enzyme. As for oxidative damage we observed that hypoxanthine administration diminished the sulfhydryl content in striatum of rats. According to our results, hypoxanthine administration altered neuroenergetic parameters and Na^+,K^+-ATPase activity, probably by oxidative contribution, suggesting that this process may be involved, at least in part, with neurological symptoms found in patients with Lesch-Nyhan disease.

Keywords: Hypoxanthine; neuroenergetic; Na^+,K^+-ATPase; thiol content.

Introduction

Hypoxanthine is the main purine nucleobase involved in the salvage purine pathway in the brain, being basal ganglia particularly dependent of this route to maintain physiological purine levels (Jinnah and Friedmann, 2001; Visser et al., 2000). Tissue accumulation of hypoxanthine occurs in Lesch-Nyhan disease, a metabolic disorder caused by severe deficiency of hypoxanthine-guanine phosphoribosyltranspherase (HPRT) activity (Jinnah, 2009). HPRT is present in all tissues, distributed equally throughout the brain (Jinnah et al., 1992), however the symptoms presented by LND patients appear to be more related to extrapyramidal effects and basal ganglia dysfunction (Visser et al., 2000). Affected patients present hyperuricemia, spasticity, dystonia, cognitive deficits and self-mutilation behavior, which is characterized by biting of the lips, tongue and fingers with apparent tissue loss (Cauwels and Martens, 2005; Jinnah and Friedmann, 2001). In addition, they also present decrease in brain volume with white and gray matter deficit (Göttle et al., 2014; Schretlen et al., 2015). Although the underlying mechanisms of brain dysfunction in Lesch-Nyhan disease are poorly understood, the accumulation of oxypurines, such as hypoxanthine, has been proposed to contribute to the neurological dysfunction of this disease (Jinnah et al., 2013; Torres and Puig, 2015).

The brain is the most metabolically active organ in the human body and has high energy consumption, so regulatory mechanisms operate to afford appropriate distribution of energy substrates to the neuronal activity. Thus, the brain is extremely sensitive to energy disruption (Bélanger et al., 2011;

Magistretti and Allaman, 2015). In accordance, the conservation of ATP levels is necessary to physiological neuronal activity. Defective energy metabolism can induce pro-apoptotic signaling, oxidative stress and damage to lipids, protein and DNA (Owen and Sunram-Lea, 2011), which lead to conditions affecting the central nervous system (CNS) (Schurr, 2002; Petrozzi et al., 2007; Beal, 2008).

The maintenance of ionic gradients across the plasma membrane is the major energy-consuming process of the brain, and is crucial for excitability. Conservation of these gradients is achieved principally through the activity of ionic pumps provided by ATP, particularly Na^+,K^+-ATPase, accounting for approximately 50% of basal glucose oxidation in the CNS (Kaplan, 2002; Mobasheri et al., 2000). Na^+,K^+-ATPase is an enzyme embedded in the cell membrane, responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the CNS necessary to maintain neuronal excitability (Arnaiz and Ordieres, 2014).

It has been demonstrated that Na^+,K^+-ATPase activity is decreased in epilepsy (Grisar, 1984; Scharfman, 2007), in many neurodegenerative disorders, including Alzheimer's disease (Ohnishi et al., 2015; Zhang et al., 2013), as well as in experimental cerebral ischemia (Wyse et al., 2000); additionally, this enzyme is inhibited by free radicals (Srikanthan et al., 2016). In this context, Na^+,K^+-ATPase seems to be particularly susceptible to free radical-induced damage since its activity has been associated with plasma membrane lipid composition (Jamme et al., 1995; Roy et al., 2016) and the redox state of sulfhydryl groups (Morel et al., 1998; Petrushanko et al., 2012). Previous studies from our group showed that hypoxanthine administration was able to

decrease Na^+,K^+-ATPase activity in about 50% in striatum of 60-day-old rats (Bavaresco et al., 2007a). Disturbance in Na^+,K^+-ATPase may induce significant damage to brain function and it is found in various diseases that affect the CNS (Ferreira et al., 2011; Arnaiz and Ordieres, 2014; Kinoshita et al., 2016).

Since the effect of hypoxanthine in the CNS and the function of this purine on neurological dysfunction presented by Lesch-Nyhan patients still need to be further enlighten and mitochondrial respiratory chain and enzymatic phosphoryl transfer complex are crucial for brain energy metabolism and function, in the present study we investigate the effect of the hypoxanthine intrastriatal administration on neuroenergetic parameters and Na^+,K^+-ATPase activity in striatum of 21-day-old rats. We initially analyzed some energy parameters such as pyruvate kinase (PK), succinate dehydrogenase (SDH), complex II (CII), cytochrome c oxidase (COX), creatine kinase (CK) and ATP levels. Na^+,K^+-ATPase activity, lipid peroxidation and protein damage were also evaluated in striatum of rats.

Material and Methods

Animals and reagents

Male Wistar rats of 21-day-old were obtained from the Central Animal House of the Department of Biochemistry of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. Animals were maintained under a standard dark–light cycle at a room temperature of 22±1°C,

with free access to a commercial protein chow and water. All the chemical reagents used for analysis were obtained from Sigma Aldrich Co., St. Louis, MO, USA. The kit for determination of ATP levels was purchased from Perkin-Elmer, Waltham, MA, USA.

Ethics statement

All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (No. 80-23, revised 1996) and a research project approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil (# 25717). All efforts were made to minimize the number of animals used and their suffering.

Experimental model

The animals were anaesthetized with ketamine and xylazine (75 and 15 mg/kg i.p., respectively). Based on stereotaxic measures, a 27 gauge 0.9 mm diameter cannula was positioned and fixed in the rat right striatum as detailed described by Biasibetti and colleagues (Biasibetti et al., 2016). The coordinates used for infant and young adult rats were based on Paxinos and Watson's atlas (Paxinos and Watson, 2006) (coordinates relative from bregma: AP -0.6 mm, ML -3.0 mm, V -4.0 mm from the dura). The animals were divided into two groups (n=7 animals per group): (1) control (2 µL of 0.9% saline infusion); (2)

hypoxanthine (2 µL of 10 µM hypoxanthine infusion). Animals were decapitated 30 minutes after the infusion, brain was dissected and the striatum was analyzed.

Tissue preparation

For determination of pyruvate kinase activity, striatum were homogenized with a Teflon-glass homogenizer in five volumes of ice-cold SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl), pH 7.4. Afterwards, the homogenate was centrifuged at 800×g and the pellet was discarded. Supernatant was centrifuged at 10,000×g for 10 min. The supernatant solution was collected and used for enzymatic assay. For SDH, CII and COX activity determination, striatum were homogenized (1:20 w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/ml heparin), pH 7.4. The homogenates were centrifuged at 800×g for 10 min and the supernatants were used for enzyme activity determination. For creatine kinase activity, the striatum was washed in SET buffer (0.32 M sucrose/1 mM EGTA/10 mM Tris–HCl; pH7.4), chopped and homogenized in the same SET buffer (1:10, w/v). Homogenate was centrifuged at 800xg for 10 min at 4ºC, keeping the supernatant, which was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant of the second centrifugation was used to determine the activity of the CK cytosolic fraction. The sediment was resuspended in Tris–HCl buffer, pH 7.5 containing 100 mM $MgSO_4$ to determine the activity of CK mitochondrial fraction.

Determination of Na^+,K^+-ATPase activity

Striatum was homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. Homogenates were centrifuged at 1,000 g for 10 min at 4 °C. Supernatants were taken for Na^+,K^+-ATPase activity assay. Fourteen rats were needed for this assay (seven per group). Reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 µL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was determined by the difference between the two assays (Wyse et al., 2000). Inorganic phosphate (Pi) released was measured by the method of Chan and colleagues (Chan et al., 1986), and enzyme specific activity was expressed as nmol Pi/min/ mg protein.

Pyruvate kinase activity

Pyruvate kinase activity was assayed essentially as described by Leong and colleagues (Leong et al., 1981). The incubation medium consisted of 0.1 MTris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 unit of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 ml of the mitochondria-free supernatant in a final volume of 0.5 ml. After 30 min of

preincubation, the reaction was started by the addition of 1.0 mM phosphoenolpyruvate (PEP). Results were expressed as micromoles of pyruvate formed per minute per milligram of protein.

Succinate Dehydrogenase and Complex II activities

Immediately before the assay, the samples were frozen and defrost three times. The activities of succinate:phenazine oxidoreductase (succinate dehydrogenase, SDH) and succinate-2,6-dichloroindophenol-oxidoreductase (complex II) (Fischer et al., 1985). This method is based on the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength (å=19.1 mM-1 cm-1) in the presence of phenazine methosulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 µM DCIP was pre-incubated with 40 to 80 µg homogenate protein at 30°C for 20 min. Subsequently, for complex II activity, 4 mM sodium azide, 7 µM rotenone and the reaction was initiated by addition of 40 µM DCIP and was monitored for 5 min.

Cytochrome c oxidase activity

The activity of cytochrome c oxidase (COX) was determined according to Rustin and colleagues (Rustin et al., 1994). Enzymatic activity was measured by following the decrease in absorbance due to oxidation of previously reduced

cytochrome c at 550 nm with 560 nm as reference wavelength (ε=19.1 mM−1cm−1). The reaction buffer contained 10 mM potassium phosphate pH 7.0 and 0.6 mM n-dodecyl-β-D-maltoside. The reaction was initiated by the addition of 0.7 µg reduced cytochrome c and monitored for 10 min.

ATP assay

Structures were homogenized in 1 ml of 0.1 M NaOH. Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin Elmer, Waltham, MA,USA), in accordance with Ferreira and colleagues (Ferreira et al., 2012). Chemiluminescence was measured using the Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as micromoles per gram of tissue.

Creatine kinase activity

The reaction mixture consisted in the following medium: 65 mM Tris–HCl buffer (pH 7.5) containing 7 mM of phosphocreatine, 9 mM of $MgSO_4$, and approximately 0.4 to 1.2 mg of protein in a final volume of 0.1 ml. The reaction was started by the addition of 3.2 mM of ADP plus 0.8 mM of reduced glutathione after 5 min of preincubation at 37ºC. After 10 min, the reaction was stopped by the addition of 1 mmol p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (Hughes,

1962). The color was developed by the addition of 0.1 ml of 2% a-naphthol and 0.1 ml 0.05% diacetyl in a final volume of 1 ml, and read after 20 min at 540 nm. Results are expressed as micromoles of creatine per minute per milligram of protein.

Immunocontent of Catalytic α Subunits and Cytochrome c Oxidase by Western Blotting

Samples were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% SDS, then dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, and 50 mM Tris–HCl, pH 6.8 and boiled for 5 min. Protein samples were separated by 10% SDS-PAGE (30 µg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The membranes were then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), then incubated overnight at 4 °C in blocking solution containing the respective antibody anti-Na^+,K^+-ATPase α1, α2, α3 subunits (1:2000), cytochrome c oxidase (1:1000) and anti-β-actin (1:1000). The blot was then washed twice for 5 min with T-TBS and incubated for 2 hours in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS and developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate,

Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Thiobarbituric Acid Reactive Substances (TBARS)

TBARS is an index of lipid peroxidation and was measured in accordance with Ohkawa and colleagues (Ohkawa et al., 1979). Samples were incubated in a medium containing 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, and 0.8% thiobarbituric acid. This mixture was vortexed, and the reaction was carried out in a boiling water bath for 1 h. The resulting pink-stained TBARS were determined spectrophotometrically at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. Results were represented as nmol of TBARS/mg of protein.

Sulfhydryl Content

This assay was performed as described by Aksenov and Markesbery (Aksenov and Markesbery, 2001), which is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups, which become oxidized (disulfide), generating a yellow substance (TNB) whose absorption is measured by spectrophotometer at 412 nm. Briefly, 15 µL of homogenate were added to 275 µL of phosphate buffer saline pH 7.4 containing 1 mM EDTA. The reaction starts by the addition of 10 µL of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. The sulfhydryl content is inversely correlated

to oxidative damage to the protein. Results were expressed as nmol of TNB per milligram of protein.

Protein determination

Protein content of samples was determined using bovine serum albumin as standard, according to Lowry (Lowry et al., 1951).

Statistical analysis

Data were analyzed by Student's t test for comparison of two means. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a compatible computer. Results are expressed as means ± SD, and differences were considered statistically significant when $p < 0.05$.

Results

Initially we evaluated energy parameters, considering the importance of energy consumption ratio for cellular activity. We started by analyzing the effects of hypoxanthine intrastriatal administration on pyruvate kinase activity, a crucial enzyme of glycolysis pathway, in striatum of 21-day-old rats.

Hypoxanthine administration decreased pyruvate kinase activity as seen in figure 1 a ($p<0.05$).

Further on oxidative pathway, we investigated SDH and complex II activities. Hypoxanthine injection was able to significantly increase the two enzymes activities ($p<0.01$, $p<0.05$, respectively) when compared to controls as seen in figure 1 b and c. Additionally we analyzed the rate limiting enzyme of oxidative phosphorylation, cytochrome c oxidase. Figure 2 a and b reveal no significant alteration in both activity and immunocontent of this enzyme ($p>0.05$) in striatum of 21-day-old rats. Furthermore we measured ATP levels and hypoxanthine administration also did not significantly modify this parameter (Figure 2 c) ($p>0.05$). Another relevant system that helps maintaining ATP levels are cytosolic and mitochondrial creatine kinase isoenzymes. Figure 3 a and b show that hypoxanthine treatment decreased cytosolic and mitochondrial creatine kinase activities ($p<0.01$).

Additionally we investigated the effect of hypoxanthine administration on Na^+,K^+-ATPase activity in striatum of 21-day-old rats. Figure 4 a shows hypoxanthine decreased Na^+,K^+-ATPase activity ($p<0.05$). Further we evaluated the effect of hypoxanthine administration on immunocontent of Na^+,K^+-ATPase α1, α2, and α3 catalytic subunits. Figure 4 b shows no variation on these parameters ($p>0.05$), as compared to control.

Since Na^+,K^+-ATPase activity its associated with membrane lipid composition and the redox state of sulfhydryl groups (Jamme et al., 1995; Morel et al., 1998) we evaluated lipid peroxidation by TBARS and protein damage by sulfhydryl content to better elucidate the effect of hypoxanthine on diminished Na^+,K^+-ATPase activity. As for membrane lipid peroxidation, hypoxanthine

administration did not alter TBARS levels ($p>0.05$) as seen in figure 5 a. In a different manner, hypoxanthine intrastriatal administration decreased sulfhydryl content ($p<0.01$) in striatum of 21-day-old rats (figure 5 b).

Discussion

Hypoxanthine is the major purine metabolite involved in the purine salvage pathway in the brain, which is increased more than five times its regular levels in Lesch-Nyhan disease. Patients with this disease present several traces and symptoms, including mental retardation, self-injury behavior and dysfunction of the dopamine transmitter system of the basal ganglia (Göttle et al., 2014; Jinnah and Friedmann, 2001). Hypoxanthine accumulation is an ordinary feature of patients with Lesch-Nyhan (Harkness, 1988; Jinnah et al., 2013). However the physiopathology involved in the brain damage of Lesch-Nyhan disease remains unclear.

Cytosolic glycolysis and mitochondrial oxidative phosphorylation are two processes responsible for ATP synthesis in the CNS (Bratic and Trifunovic, 2010) and defective energy metabolism may lead to dysfunctions in CNS (Magistretti and Allaman, 2015; Petrozzi et al., 2007). Na^+,K^+-ATPase is a crucial enzyme for brain excitability, primarily fueled by oxidative phosphorylation, being responsible for the generation of the membrane potential while consuming approximately 50% of ATP generated in CNS (Kaplan, 2002; Mobasheri et al., 2000). Considering the previous, the aim of the present study was to evaluate the effect of hypoxanthine intrastriatal

administration on energy parameters and Na^+,K^+-ATPase activity in order to better understand the mechanisms involved in hypoxanthine neurotoxicity.

Under normal conditions, the brains energy requirements are met almost exclusively by the breakdown of glucose. Pyruvate, synthetized by pyruvate kinase, is the end product of glycolysis, which is converted into acetyl CoA entering the tricarboxylic acid (TCA) cycle when there is sufficient oxygen available (Liu and Vander Heiden, 2015). High concentration of hypoxanthine, equal to those found in cerebrospinal fluid of patients with Lesch-Nyhan disease, decreased pyruvate kinase activity, a thiol-containing enzyme, in striatum of 21-day-old rats (Fig. 1 a). Impairment in glucose utilization in the CNS has been proposed as a possible cause of dementia in some neurodegenerative disease (Mergenthaler et al., 2013).

Further on energy metabolism, we also investigated the activity of succinate dehydrogenase, as well as the activities of complexes of mitochondrial respiratory chain (II and IV). As for TCA cycle we measured the activity of succinate dehydrogenase, an enzyme located in the inner mitochondrial membrane, catalyzing the oxidation of succinate to fumarate and transferring electrons directly into the electron transport chain (Owen et al., 2002; Rustin et al., 2002). Results show significant increase in succinate dehydrogenase activity in striatum of 21-day-old rats subjected to hypoxanthine administration, as seen in figure 1 b. This condition could happen as a compensatory response, through anaplerotic reactions (Owen et al., 2002). Increase in succinate dehydrogenase activity is also present in others neurological disorders (Bubber et al., 2005; Chinopoulos, 2013; Ferreira et al., 2010; Naseri et al., 2016).

Since the mitochondrial electron transport chain is an essential component of oxidative phosphorylation and is coupled to the synthesis of ATP (Nelson and Cox, 2013) we continued our investigation on mitochondrial oxidative phosphorylation evaluating Complex II. In accordance with increase in succinate dehydrogenase activity, we observed as well an increase in complex II activity (figure 1 c). Complex II is a mitochondrial respiratory chain enzyme that is directly involved in the TCA cycle. This enzyme feeds electrons to the cytochrome complex catalyzing the oxidation of matrix succinate to fumarate (Hoekstra and Bayley, 2013). Studies of mitochondrial Complex II have clinical importance, considering this enzyme recent relation to cancer biology, immunology, cardiovascular conditions and neurodegeneration, although its regulation is not yet fully comprehend (Sakai et al., 2013; Stepanova et al., 2016).

Additionally on mitochondrial respiratory chain, we analyze cytochrome c oxidase activity and immunocontent, since enzymatic phosphoryl transfer network is vital to the brain energy metabolism and function. Cytochrome c oxidase is the terminal and the rate-limiting enzyme of the oxidative phosphorylation (Srinivasan and Avadhani, 2012). Results show (figure 2 a and b) that hypoxanthine administration did not alter significantly cytochrome c oxidase activity along with the immunocontent. However, increase in succinate dehydrogenase and complex II activities and unaltered cytochrome c oxidase activity may lead to incomplete reduction of oxygen and thus rise free radical formation (Stowe and Camara, 2009). Although free radicals could originate from several sources in cells, over 90% of intracellular reactive oxygen species are produced inside the mitochondria. This species are originated by incomplete

reduction of oxygen or by leak of electrons from the respiratory chain (Halliwell, 2007; Olsen et al., 2015); moreover, previous studies from our group have already associated hypoxanthine administration with reactive species production and oxidative stress (Bavaresco et al., 2008; Biasibetti et al., 2016).

Considering that ATP levels are essential for Na^+,K^+-ATPase activity as well as physiological neuronal activity, we evaluated the final energetic product of oxidative phosphorylation, determining intracellular ATP levels. As expected with cytochrome c oxidase results, hypoxanthine administration did not alter ATP levels in striatum of 21-day-old rats, when compared to control (figure 2 c). Coupling spatial intracellular ATP-producing and ATP-consuming is a fundamental process to cell function. Creatine kinase is a secondary source of ATP and is activated when the demand of energy is large and the production of ATP from oxidative phosphorylation is impaired or not enough (Andres et al., 2008). This enzyme is responsible for the reallocation of ATP phosphoryl group from mitochondria (where it is produced) to the cytosol (where it is consumed) (Dzeja and Terzic, 2003). Hypoxanthine administration deceased cytosolic and mitochondrial CK activities in striatum of 21-day-old rats (Figure 3). However, since both isoforms of the enzyme are diminished, there is no intracellular ATP imbalance (Schlattner et al., 2006).

We also investigated the influence of hypoxanthine administration on Na^+,K^+-ATPase activity in striatum of 21-day-old rats, noticing that hypoxanthine reduced this enzyme activity as seen in figure 4 a. Results confirmed the same effect from previous studies showing that intrastriatal hypoxanthine injection reduces Na^+,K^+-ATPase activity in rat striatum of 60-day-old rats (Bavaresco et al., 2007a). Na^+,K^+-ATPase is a membrane-embedded enzyme highly

susceptible to free radical insult and decreased activity has been associated with excitotoxicity and neuronal damage (Arnaiz and Ordieres, 2014; Srikanthan et al., 2016). Also, it has been demonstrated more vulnerability to glutamate neurotoxicity in striatal neurons when Na^+,K^+-ATPase activity is reduced (Calabresi et al., 1995). Furthermore, when this enzyme activity is impaired, intracellular Na^+ concentration increases, collaborating to the pathological mechanisms involved in cerebral edema (Arnaiz and Ordieres, 2014).

Further, we also measured the immunocontent of Na^+,K^+-ATPase α1, α2, and α3 catalytic subunits, to better comprehend the mechanisms involving Na^+,K^+-ATPase decrease in striatum of 21-day-old rats. Na^+,K^+-ATPase is an oligomeric enzyme consisting of α, β and ɣ subunits. Alpha is the catalytic subunit, presenting the binding sites for ATP and the inhibitor ouabain and has different isoforms: α1, α2, α3, or α4, the last identified only in testes (Bøttger et al., 2011). Subunits α1, α2, and α3 bind inhibitor ouabain with low, intermediate and high affinity, respectively. In neurons are present the last two isoforms whereas in glial cells are localized α1 and α2 isoforms (Geering, 2008). Hypoxanthine administration did not altered the immunocontent of Na^+,K^+-ATPase α1, α2, and α3 subunits when compared to control group as seen in figure 4 b.

Na^+,K^+-ATPase is a transmembrane enzyme with normal activity crucial for brain function, since decreased activity is related to selective neuron damage in the rat brain (Arnaiz and Ordieres, 2014). We also evaluated lipid peroxidation by TBARS and protein damage by sulfhydryl content to better elucidate the effect of hypoxanthine on diminished Na^+,K^+-ATPase activity, considering that there was no alteration in ATP status. Lipid peroxidation may

cause significant changes in the permeability, fluidity and integrity of biomembranes, resulting in altered cell signaling (Halliwell, 2012). Besides, lipid peroxidation appears to be implicated in several neurodegenerative disorders, for example Huntington, Alzheimer and Parkinson's disease (Reed, 2011). However we observed that intrastriatal hypoxanthine administration did not alter lipid peroxidation (figure 5 a), what may suggest that lipid membrane modifications are not involved in inhibition of Na^+,K^+-ATPase activity by hypoxanthine.

Considering that some enzymes involved in energy metabolism require sulfhydryl groups and loss of protein sulfhydryl groups are a major target for oxidative damage we analyzed this parameter (Rahal et al., 2014). In this study, hypoxanthine injection decreased protein thiol content (figure 5 b) which relates to previous studies of our group showing that hypoxanthine administration leads to oxidative stress (Bavaresco et al., 2007b; Biasibetti et al., 2016). Therefore, oxidative stress provoked by hypoxanthine may induce a loss of thiol groups which in turn could be responsible for the inhibition of Na^+,K^+-ATPase. Along the oxidative process, free radicals likely led to oxidation of the thiol groups of Na^+,K^+-ATPase, possibly by creating disulfide bridges modifying the secondary structure of this protein and inhibiting its function.

In summary, the present results shed light into the mechanisms of hypoxanthine-induced neurotoxicity in striatum of 21-day-old rats. Intrastriatal hypoxanthine administration caused neuroenergetic imbalance and Na^+,K^+-ATPase activity impairment in striatum of 21-day-old rats. Further, hypoxanthine induced a sulfhydryl content decrease which could explain reduction in Na^+,K^+-ATPase. Despite the difficulty to extrapolate our results to the human condition,

this study provides new enlightening basis of hypoxanthine on the inhibition of Na^+,K^+-ATPase activity, which is a crucial enzyme to normal CNS function, and the induction of oxidative stress might be involved, at least in part, in the pathophysiology of the neurological features present in patients with Lesch-Nyhan disease.

Conflict of interest

All authors declare that there are no financial/commercial conflicts of interest.

Acknowledgements

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Figure Legends

Figure 1. Effects of hypoxanthine intrastriatal administration on pyruvate kinase (a), succinate dehydrogenase (b) and complex II (c) activities in striatum of 21-day-old rats. Data are mean ± SD for seven animals in each group. Data from pyruvate kinase are expressed as μmol of pyruvate per mg of protein; data from succinate dehydrogenase and complex II are expressed as nmol per min per mg of protein. Different from control, $*p < 0.05$, $**p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *SDH*, succinate dehydrogenase.

Figure 2. Effects of hypoxanthine intrastriatal administration on cytochrome c oxidase activity (a), and immunocontent (b) and ATP levels (c) in striatum of 21-day-old rats. Data are mean ± SD for seven animals in each group. Data from cytochrome c oxidase activity are expressed as nmol per min per mg of protein; data from western blot are expressed as percentage of control. Uniformity of gel loading was confirmed with β-actin as standard. Data from ATP levels are expressed as μmol per gram of tissue. Different from control, $*p < 0.05$, (Student's *t* test). *Hpx*, hypoxanthine.

Figure 3. Effects of hypoxanthine intrastriatal administration on cytosolic creatine kinase (a) and mitochondrial creatine kinase (b) activities in striatum of 21-day-old rats. Data are mean ± SD for seven animals in each group. Results are expressed as μmol of creatine per minute per mg of protein. Different from control, $**p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *CK*, creatine kinase.

Figure 4. Effects of hypoxanthine intrastriatal administration on Na^+,K^+-ATPase (a) activity and (b) catalytic subunits (α_1, α_2 and α_3) immunocontent in striatum of 21-day-old rats. Results are expressed as nmol of Pi per min per mg of protein for enzymatic activity and percentage of control for Western Blot. Uniformity of gel loading was confirmed with β-actin as standard. Data are mean ± SD for seven animals in each group. Different from control, $*p < 0.05$ (Student's *t* test). *Hpx*, hypoxanthine.

Figure 5. Effects of hypoxanthine intrastriatal administration on TBARS levels (a) and sulfhydryl content (b) in striatum of 60-day-old rats. Data are

mean ± SD for seven animals in each group. Results are expressed as nmol of TBARS per mg of protein and as nmol of TNB per mg of protein for sulfhydryl content. Different from control, $^{**}p < 0.01$ (Student's *t* test). *Hpx*, hypoxanthine; *TBARS*, thiobarbituric acid reactive substances.

Figure 1

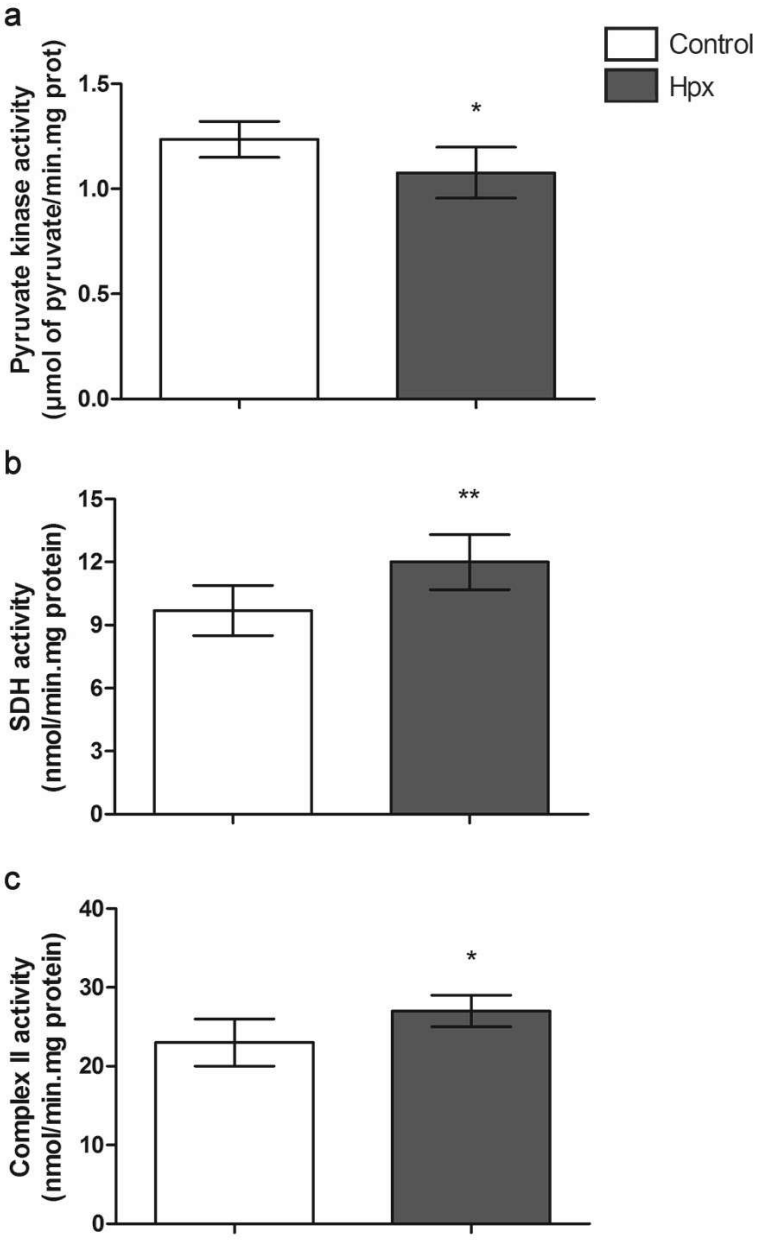

a
Pyruvate kinase activity
(µmol of pyruvate/min.mg prot)
1.5
1.0
0.5
0.0
*
Control
Hpx
b
SDH activity
(nmol/min.mg protein)
15
12
9
6
3
0
**
c
Complex II activity
(nmol/min.mg protein)
40
30
20
10
0
*

Figure 2

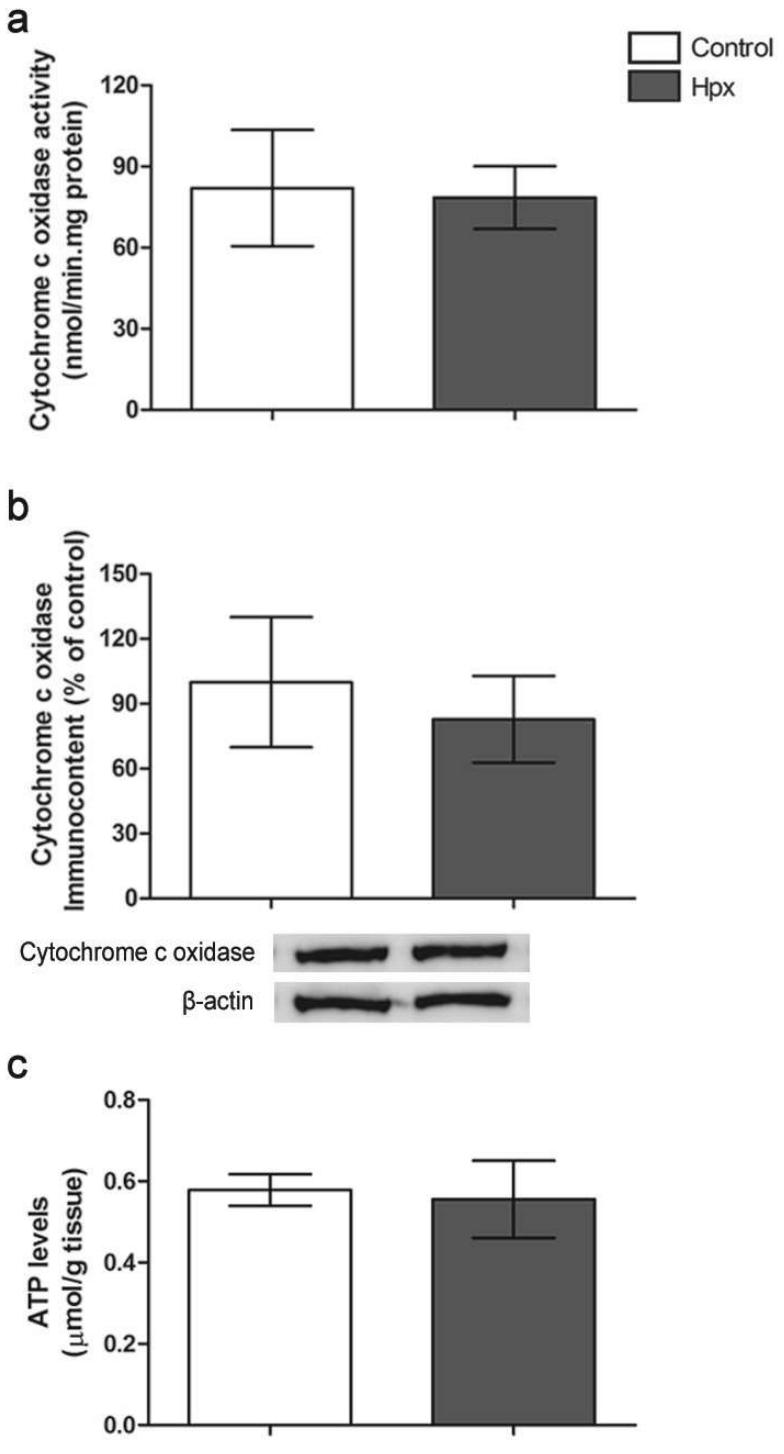

a
Control
Hpx
Cytochrome c oxidase activity (nmol/min.mg protein)
120
90
60
30
0
b
Cytochrome c oxidase Immunocontent (% of control)
150
120
90
60
30
0
Cytochrome c oxidase
β-actin
c
ATP levels (μmol/g tissue)
0.8
0.6
0.4
0.2
0.0

Figure 3

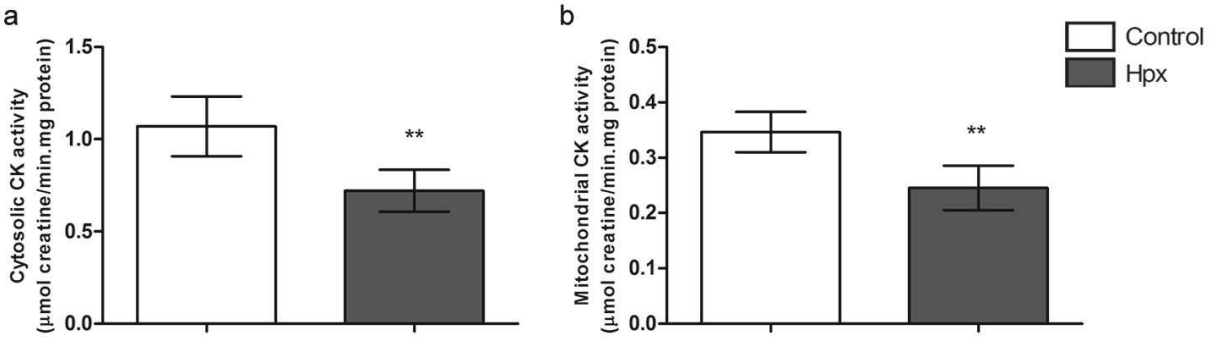


Figure 4

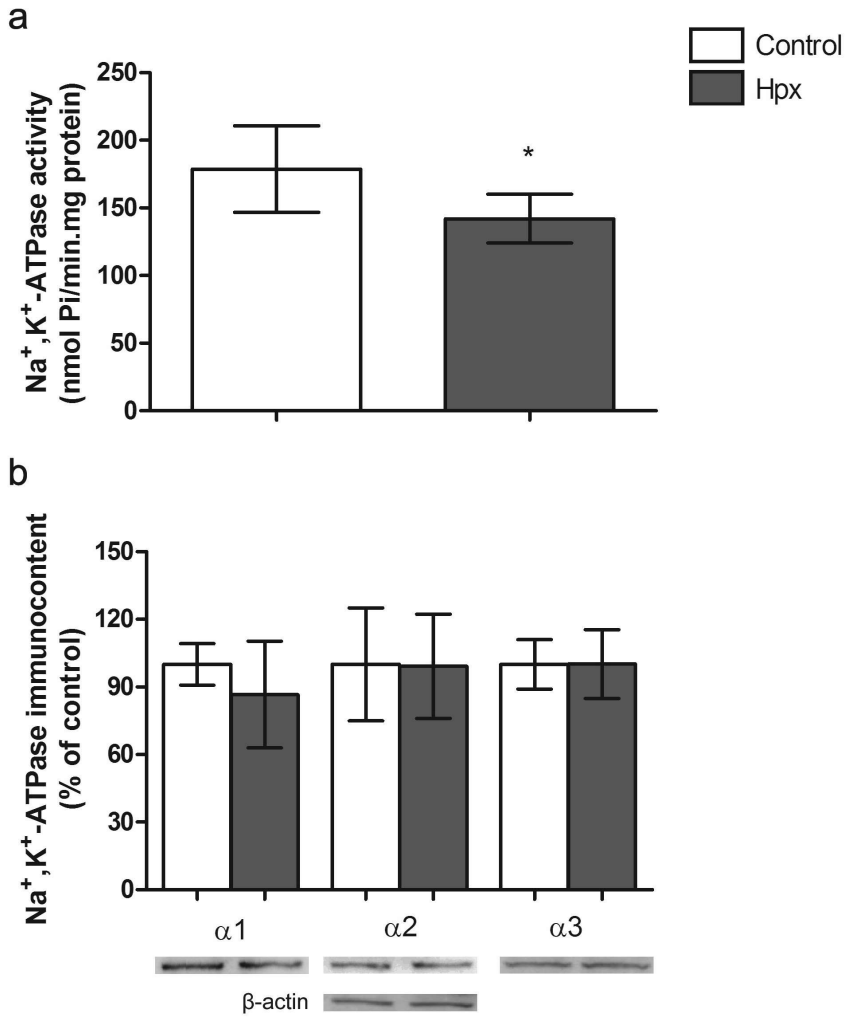
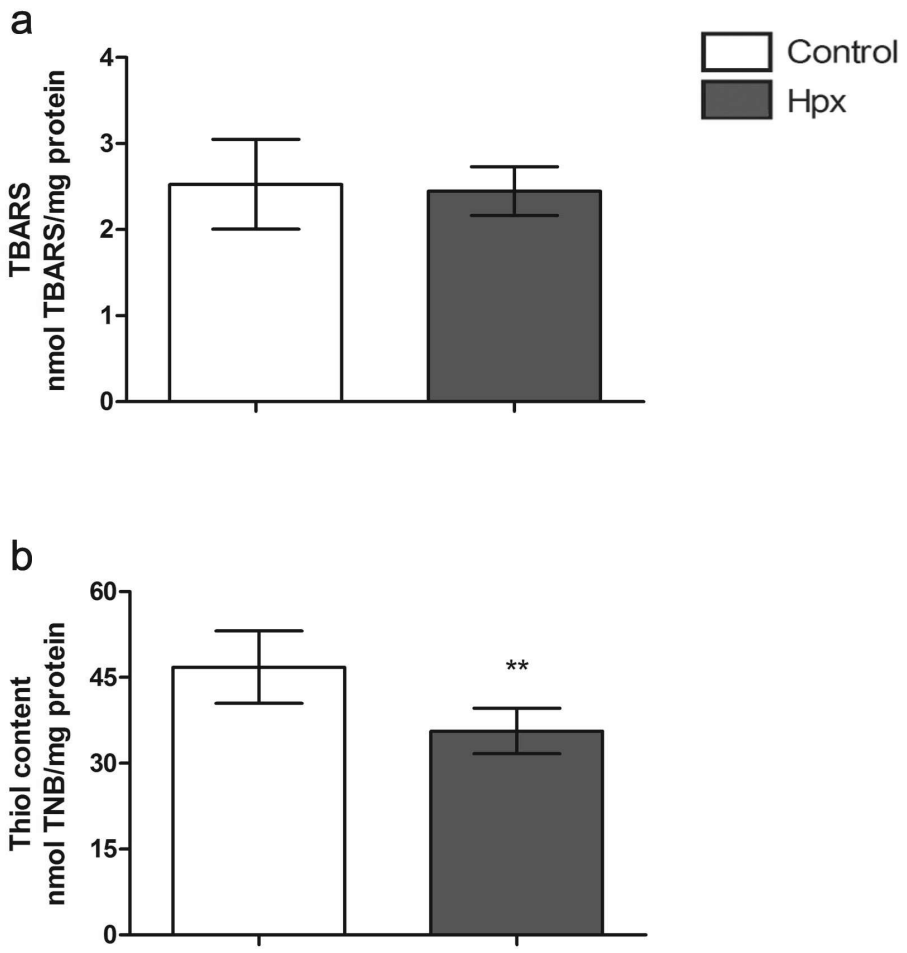


Figure 5


a
TBARS
nmol TBARS/mg protein
4
3
2
1
0
Control
Hpx
b
Thiol content
nmol TNB/mg protein
60
45
30
15
0
**

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4. DISCUSSÃO

A hipoxantina, principal metabólito de purina envolvido na via de salvação de purinas no cérebro, encontra-se aumentada no plasma de pacientes com doença de Lesch-Nyhan cinco vezes mais do que em indivíduos normais. Essa desordem e suas variantes menos severas são causadas por mutações no gene HPRT1, que codifica a enzima de reciclagem de purinas HPRT (Jinnah et al., 2013, 2004). Os pacientes com essa doença apresentam vários sinais e sintomas, incluindo retardo mental, comportamento de automutilação além de disfunção do sistema dopaminérgico nos gânglios da base (Göttle et al., 2014; Jinnah, 2009). Considerando que o acúmulo de hipoxantina está relacionado com a fisiopatologia do dano cerebral encontrado nos pacientes com a doença de Lesch-Nyhan, no presente estudo investigamos o efeito da injeção intraestriatal de hipoxantina sobre o perfil inflamatório, parâmetros energéticos e atividade da Na^+,K^+-ATPase, analisando um possível envolvimento oxidativo para melhor compreender os mecanismos envolvidos na neurotoxicidade dessa purina.

No capítulo I dessa dissertação analisamos primeiramente o efeito da hipoxantina sobre o perfil neuroinflamatório. Durante muito tempo, o SNC foi considerado imunologicamente privilegiado. Acreditava-se que não havia relação entre o SNC e processos inflamatórios. Atualmente, sabe-se que o SNC tem um papel na inflamação e na resposta a lesões, infecções ou doenças. Células do SNC, principalmente a microglia, geram mediadores inflamatórios (citocinas, quimiocinas, óxido nítrico, prostaglandinas, fatores de crescimento e as espécies reativas de oxigênio) modulando entre pró- e anti-inflamatórios (Heneka et al., 2015). Estes mediadores agregam as moléculas

de adesão, aumentam a permeabilidade da barreira hemato-encefálica, facilitando a invasão de células de resposta imune e induzindo a liberação de moléculas potencialmente tóxicas que podem comprometer as células cerebrais (Lucas et al., 2006). Estudos sugerem uma relação entre neuroinflamação, estresse oxidativo, e erros inatos do metabolismo (Donida et al., 2017; Olsen et al., 2015; Ribeiro et al., 2013; Schweinberger and Wyse, 2016). Visto isso, o objetivo do primeiro capítulo foi avaliar o imunoconteúdo da subunidade p65 de NF-κB, os níveis de citocinas pró-inflamatórias, os níveis de nitritos, o imunoconteúdo de iNOS, bem como avaliar o imunoconteúdo de Iba1 e GFAP em estriado de ratos de 21 e 60 dias de vida submetidos à administração intraestriatal de hipoxantina. Alguns parâmetros oxidativos, como geração de espécies reativas, atividades de enzimas antioxidantes e análise de dano ao DNA também foram avaliados.

Sabe-se que o NF-kB é ativado em consequência de sinalização inflamatória. O NF-κB situa-se no citoplasma ligado a proteínas inibidoras (IκB), dessa forma permanecendo inativado. A ativação induz a translocação nuclear de NF-kB, seguida pela ativação transcricional de genes alvo específicos (Hoesel and Schmid, 2013). Esse fator de transcrição tem sido associado a vários mediadores inflamatórios tais como IL-6 (Son et al., 2008), TNF-α (Zhao et al., 2015) e iNOS (Arias-Salvatierra et al., 2011). Em nosso estudo, a administração de hipoxantina aumentou o imunoconteúdo nuclear de NF-kB em estriado de ratos de 21 e 60 dias de vida.

As citocinas são moléculas envolvidas na transmissão de sinais entre as células durante o início de respostas imunes (Zhang and An, 2007). Nossos resultados mostram que a administração intraestriatal de hipoxantina provocou

um aumento nos níveis de citocinas pró-inflamatórias, incluindo IL-6 e TNF-α, em estriado de ratos de 21 dias de idade. Nos animais de 60 dias de vida há um aumento apenas de IL-6. Em acordo com nossos resultados, Gudbjörnsson e colaboradores demonstraram que a hipoxantina está relacionada à inflamação presente na artrite reumatoide, em que há maior metabolismo e difusão de purinas (Gudbjörnsson et al., 1991). Embora o mecanismo pelo qual a hipoxantina atua sobre a resposta inflamatória não está totalmente elucidado, nossos achados sugerem que o aumento nos níveis de citocinas pode estar estreitamente relacionado com a ativação do NF-kB.

O óxido nítrico (NO) é outro mediador importante para a resposta inflamatória. Os produtos da oxidação, nitritos e nitratos, são os principais metabólitos utilizados como indicadores da produção de NO (Lo Faro et al., 2014). O NO é produzido por conversão do aminoácido L-arginina em L-citrulina através da ação da enzima óxido nítrico sintase (NOS). A óxido nítrico sintase induzível (iNOS) é uma isoforma que é expressa através de citocinas, endotoxinas e outras vias inflamatórias (Habib and Ali, 2011). Para entender a influência da hipoxantina sobre a inflamação, também avaliamos os níveis de nitritos em ratos submetidos à administração de hipoxantina. Observamos que a hipoxantina diminui os níveis de nitritos no estriado de ratos de 21 dias de idade. Estudos mostram uma relação entre a formação de NO e a síntese de espécies reativas, como o ânion superóxido e peróxido de hidrogênio. O ânion superóxido também pode reagir com NO gerando peroxinitrito, uma substância citotóxica, sugerindo uma diminuição da biodisponibilidade de NO (Brown and Neher, 2010; El Assar et al., 2013).

Uma vez que a enzima iNOS é responsável por catalisar a formação de NO através de estímulos inflamatórios e a mesma está intimamente relacionada com a ativação de NF-κB, também analisamos seu imunoconteúdo. Os resultados revelam um aumento no imunoconteúdo de iNOS em estriado de ratos de 21 e 60 dias de vida submetidos à administração de hipoxantina. Estudos mostram que o aumento na expressão proteica de iNOS é um indicativo de neuroinflamação e neurodegeneração, e que parece estar relacionado também com a ativação microglial (Brown and Neher, 2010; Ribeiro et al., 2013).

Além disso, analisamos a ativação microglial e astrocitária através da medida do imunoconteúdo de Iba1 e GFAP, respectivamente. Sabe-se que a ativação microglial excessiva corrobora a doenças do SNC pela liberação de fatores citotóxicos que conduzem aos danos celulares (Magni et al., 2012; Xu et al., 2016). Nossos resultados mostram que a administração de hipoxantina aumentou o imunoconteúdo de Iba1 somente em ratos de 21 dias de idade. Não houve alteração de Iba1 em estriado de ratos de 60 dias de idade, isso pode ser explicado pela estreita relação entre TNF-α e a ativação microglial (Takeuchi et al., 2006).

Juntamente com a microglia, o astrócito contribui para a regulação da neuroinflamação, sendo também responsável pelo papel imune do SNC (Guizzetti et al., 2014). Perda de funções fisiológicas são consequências da ativação astrocitária, uma característica de diversos processos patológicos (Sofroniew, 2009). O aumento de proteínas de filamentos intermediários, particularmente a GFAP, caracteriza a ativação astrocitária, também conhecida como astrogliose (Pekny et al., 2014). No presente estudo, vimos que a

hipoxantina foi capaz de aumentar a imunorreatividade de GFAP em estriado de ratos de 21 dias, sugerindo uma astrogliose. Nenhuma alteração foi observada em ratos adultos jovens, talvez por diferença na fase de desenvolvimento neurológico, o que significa que os ratos de 21 dias de vida são mais suscetíveis a sofrer pela administração de hipoxantina e seus efeitos neuroinflamatórios.

Visto que um dos possíveis mecanismos de ativação de NF-κB é através de estresse oxidativo (Morgan and Liu, 2011) e que a hipoxantina pode ter um efeito oxidativo (Bavaresco et al., 2008), decidimos analisar alguns parâmetros oxidativos em ambas as idades de ratos. Nossos resultados mostraram um aumento na produção de espécies reativas observada pela oxidação de H_2DCF e desregulação de enzimas antioxidante em estriado de ratos de 21 dias de idade, já visto no modelo de 60 dias (Bavaresco et al., 2008). Sabendo que espécies reativas de oxigênio podem atacar diretamente biomoléculas como o DNA (Kryston et al., 2011); em nosso estudo, observamos dano ao DNA, o que pode relacionar fortemente a hipoxantina com a geração de estresse oxidativo. Esse mecanismo poderia explicar, em parte, a alteração do perfil inflamatório causado pela hipoxantina.

No capítulo II, a fim de analisar o possível envolvimento neurenergético em alterações causadas pela hipoxantina, avaliamos algumas enzimas do metabolismo energético em estriado de ratos de 60 dias. Inicialmente, investigamos o efeito da administração de hipoxantina sobre a atividade de piruvato quinase (PK). PK é uma enzima glicolítica que catalisa a conversão irreversível de fosfoenolpiruvato em piruvato, sintetizando uma molécula de ATP (Liu and Vander Heiden, 2015). Vimos que a administração de hipoxantina

não alterou a atividade de PK em estriado de ratos de 60 dias. Ampliando nossa investigação para o ciclo de ácido tricarboxílico (TCA), avaliamos a atividade da enzima succinato desidrogenase (SDH), uma enzima localizada na membrana mitocondrial interna, onde transfere elétrons diretamente para a cadeia transportadora de elétrons. Observamos um aumento significativo na atividade de SDH em estriado de ratos de 60 dias submetidos à administração de hipoxantina. Essa condição também foi observada em outros distúrbios como hiperprolinemia, sarcosinemia, doença de Huntington e doença de Alzheimer (Bubber et al., 2005; de Andrade et al., 2016; Ferreira et al., 2010; Naseri et al., 2016).

Em seguida investigamos o efeito da administração de hipoxantina sobre a atividade do complexo II mitocondrial da cadeia transportadora de elétrons, que é um componente essencial da fosforilação oxidativa e é acoplado à síntese de ATP de ADP e Pi (Hoekstra and Bayley, 2013). Observamos que a administração intraestriatal de hipoxantina aumentou a atividade do complexo II. A regulação da atividade do complexo II não está totalmente compreendida, apesar do envolvimento desta enzima a diversas condições, emergindo recentemente como ponto de investigação importante na sinalização celular, biologia do câncer, imunologia, doenças cardiovasculares e neurodegeneração (Hoekstra and Bayley, 2013; Stepanova et al., 2016). Uma possível explicação para o aumento da atividade de SDH e complexo II poderia ser por vias anapleróticas, uma vez que a enzima PK não está alterada.

Considerando que a cadeia respiratória mitocondrial e a fosforilação oxidativa são cruciais para o metabolismo e função energética cerebral, analisamos ainda a atividade e o imunoconteúdo da enzima citocromo c

oxidase, uma enzima limitante da cadeia respiratória mitocondrial. A administração de hipoxantina diminuiu significativamente ambos parâmetros avaliados. Nossos achados são semelhantes aos encontrados em estudos pré-clínicos e clínicos que mostram um aumento na atividade da SDH, e diminuição da atividade da citocromo c oxidase em córtex cerebral de ratos expostos à hipóxia crônica (Caceda et al., 2001) e no cérebro de pacientes com doença de Alzheimer (Bubber et al., 2005). Ademais, o aumento das atividades de SDH e complexo II e a inibição da atividade da citocromo c oxidase podem, potencialmente, levar à redução incompleta do oxigênio e consequentemente aumentar a formação de radicais livres, além de reduzir a síntese de ATP (Li et al., 2006; Stowe and Camara, 2009). Visto isso, no presente estudo, avaliamos os níveis de ATP e pudemos observar que a administração de hipoxantina foi capaz de diminuir os níveis de ATP intracelular.

A regulação da relação espacial intracelular entre ATP produzido e ATP consumido é um processo fundamental para a função celular. A enzima creatina quinase (CK) faz parte de um importante sistema de fosfotransferência em células com alta demanda energética. A CK é responsável pela relocalização do grupo ATP fosforil de onde é produzido (principalmente nas mitocôndrias) até onde é consumido (principalmente no citosol) (Wallimann et al., 2011). No nosso trabalho, a administração de hipoxantina diminuiu as atividades das isoformas citosólica e mitocondrial da CK. Nosso achados são um indicativo da ação oxidativa da hipoxantina, possivelmente agindo através da diminuição grupos tióis presentes nas enzimas, necessários para o funcionamento normal das mesmas.

A disfunção mitocondrial pode afetar o metabolismo através do aumento da formação de radicais livres, dessa maneira contribuindo para o estresse oxidativo e indução de apoptose (Olsen et al., 2015). Estudos sugerem que anormalidades mitocondriais estão envolvidas não só com o envelhecimento, doenças neurodegenerativas e câncer (Jain et al., 2016; Reddy and Reddy, 2011), mas também com erros inatos do metabolismo (Ferreira et al., 2010; Kolling et al., 2012; Seminotti et al., 2016). Para melhor compreender o efeito da hipoxantina sobre a função mitocondrial analisamos massa mitocondrial e potencial de membrana mitocondrial (Δψ), vistos pela marcação com MitoTracker Green e MitoTracker Red respectivamente. A administração de hipoxantina diminuiu a porcentagem de células marcadas com MitoTracker Green (MTG) e aumentou a marcação com MitoTracker Red (MTR) quando comparada com os grupos controles. O potencial da membrana mitocondrial é considerado um indicador da função mitocondrial (Distelmaier et al., 2008), e o aumento visto pela administração de hipoxantina está de acordo com o aumento das atividades de SDH e complexo II visto no mesmo modelo. Contudo modificações nesses parâmetros indicam uma disfunção mitocondrial (Iijima et al., 2006).

Considerando que disfunções mitocondriais podem iniciar vias de sinalização de apoptose intrínseca/extrínseca e necrose (Gottlieb and Carreira, 2010), analisamos o efeito da hipoxantina sobre células estriatais através da marcação com anexina V e iodeto de propídeo (PI). A administração de hipoxantina diminuiu o número de células vivas, consequentemente aumentando os eventos apoptóticos no estriado. O número de células necróticas não foi alterado em estriado de ratos de 60 dias de idade. A possível

alteração no estado redox celular, causada pela hipoxantina, pode colaborar para a apoptose, uma vez que as espécies reativas produzidas pelas mitocôndrias estão envolvidas no dano celular (Delmas et al., 2011),

No capítulo III, analisamos alterações energéticas, bem como a atividade da enzima Na^+,K^+-ATPase em estriado de ratos de 21 dias submetidos à administração intraestriatal de hipoxantina. Em condições normais, a necessidade energética cerebral é atingida quase que exclusivamente pela metabolização da glicose. O piruvato, sintetizado pela piruvato quinase, é o produto final da glicólise, que é convertido em acetil CoA entrando no ciclo do TCA quando há oxigênio suficiente disponível (Liu and Vander Heiden, 2015). A administração de hipoxantina diminuiu a atividade da piruvato quinase em estriado de ratos de 21 dias de vida. A deficiência na utilização de glicose no SNC tem sido proposta como uma possível base patofisiológica de desordens cerebrais (Mergenthaler et al., 2013).

Quanto ao ciclo de TCA, medimos a atividade da succinato desidrogenase. Os resultados mostram um aumento significativo na atividade da succinato desidrogenase em estriado de ratos de 21 dias sujeitos à administração de hipoxantina. Essa condição pode ocorrer como resposta compensatória, através de reações anapleróticas (Owen et al., 2002). O aumento da atividade da succinato desidrogenase está também presente em outras patologias, como a doença de Huntington, a doença de Alzheimer, e também em erros inatos do metabolismo como hiperprolinemia e sarcosinemia (de Andrade et al., 2016; Ferreira et al., 2010; Naseri et al., 2016).

Observamos também que a administração de hipoxantina provocou um aumento na atividade do complexo II em estriado de ratos de 21 dias de vida.

Essa enzima mitocondrial da cadeia respiratória está diretamente envolvida no ciclo TCA. O complexo II fornece elétrons para o complexo III catalisando a oxidação do succinato a fumarato (Hoekstra and Bayley, 2013). Em seguida analisamos a atividade e imunoconteúdo da enzima citocromo c oxidase, uma vez que a rede enzimática de transferência de elétrons é vital para o metabolismo e função da energia cerebral (Srinivasan and Avadhani, 2012). Os resultados mostram que a administração de hipoxantina não alterou significativamente a atividade e o imunoconteúdo da enzima citocromo c oxidase. Contudo, o aumento das atividades da succinato desidrogenase e do complexo II e a inalteração da atividade da citocromo c oxidase podem levar a uma redução incompleta de oxigênio aumentando, dessa forma, a geração de radicais livres como discutido anteriormente (Stowe and Camara, 2009). Embora os radicais livres possam ter origem em várias fontes nas células, mais de 90% das espécies reativas de oxigênio são produzidas dentro da mitocôndria. Essas espécies podem ser originadas pela redução incompleta de oxigênio ou por vazamento de elétrons da cadeia transportadora de elétrons (Halliwell, 2012; Olsen et al., 2015). Além disso, estudos prévios de nosso grupo já associaram a administração de hipoxantina à produção de espécies reativas e estresse oxidativo (Bavaresco et al., 2008; Biasibetti et al., 2016).

Uma vez que os níveis de ATP são essenciais para a atividade neuronal fisiológica, avaliamos o produto energético final da fosforilação oxidativa (Owen and Sunram-Lea, 2011), determinando os níveis intracelulares de ATP. A administração de hipoxantina não alterou os níveis de ATP em estriado de ratos de 21 dias, quando comparados com o grupo controle. Avaliando o efeito da administração de hipoxantina sobre a atividade da enzima creatina quinase,

observamos uma diminuição na atividade de ambas isoformas, citosólica e mitocondrial em estriado de ratos de 21 dias de idade.

A fim de analisar o impacto da alteração de algumas enzimas do metabolismo energético causado pela ação da hipoxantina, investigamos também a atividade da Na^+,K^+-ATPase. Essa enzima é crucial para a excitabilidade neuronal, uma vez que é responsável pela geração do potencial de membrana, consumindo nesse processo aproximadamente 50% do ATP gerado no SNC (Kaplan, 2002; Mobasheri et al., 2000). No presente trabalho observamos que a administração de hipoxantina reduziu a atividade dessa enzima. Os resultados confirmam o mesmo efeito de estudos anteriores que mostram que a injeção intraestriatal de hipoxantina reduz a atividade Na^+,K^+-ATPase em estriado de ratos de 60 dias de vida (Bavaresco et al., 2007b). Na^+,K^+-ATPase é uma enzima de membrana altamente suscetível ao insulto por espécies reativas e a diminuição de sua atividade tem sido associada à excitotoxicidade e dano neuronal (Arnaiz and Ordieres, 2014; Srikanthan et al., 2016). Avaliamos também o imunoconteúdo das subunidades catalíticas α1, α2 e α3 da Na^+,K^+-ATPase para melhor compreender os mecanismos envolvidos na diminuição da atividade enzimática em estriado de ratos de 21 dias de idade. Contudo, a administração de hipoxantina não alterou o imunoconteúdo das subunidades α1, α2 e α3 de Na^+,K^+-ATPase quando comparado ao grupo controle.

Visto que a Na^+,K^+-ATPase é uma enzima transmembrana e vulnerável ao estresse oxidativo, também avaliamos a peroxidação lipídica pela técnica de TBARS e o dano proteico pelo teor de tióis para melhor elucidar o efeito da hipoxantina sobre a diminuição da atividade de Na^+,K^+-ATPase. Devido ao alto

conteúdo lipídico e maior taxa de utilização de oxigênio o cérebro possui grande susceptibilidade à peroxidação lipídica e formação de radicais livres. A peroxidação lipídica pode causar alterações significativas na permeabilidade, fluidez e integridade das biomembranas, resultando em alteração na sinalização celular (Halliwell, 2011). Além disso, esse processo parece estar relacionado a vários distúrbios neuropsiquiátricos, por exemplo, transtorno bipolar e esquizofrenia (Romano et al., 2017). No entanto, observamos que a administração intraestriatal de hipoxantina não teve alteração sobre a peroxidação lipídica, o que pode sugerir que modificações na membrana lipídica não estão envolvidas na inibição da atividade Na^+,K^+-ATPase pela hipoxantina.

Considerando que algumas enzimas envolvidas no metabolismo energético apresentam grupos tióis em suas estruturas e que a perda desses grupos é um dos principais alvos do dano oxidativo (Rahal et al., 2014), também analisamos o conteúdo de grupos tióis em estrado de ratos de 21 dias de vida. Neste estudo, a injeção intraestriatal de hipoxantina diminuiu o conteúdo de grupamentos tióis de proteínas. Portanto, o estresse oxidativo provocado pela hipoxantina pode induzir diminuição desses grupos, possivelmente pela formação de pontes dissulfeto, modificando a estrutura secundária da Na^+,K^+-ATPase, responsável pela inibição enzimática.

Em resumo, os presentes resultados esclarecem alguns mecanismos de neurotoxicidade induzida pela hipoxantina em estriado de ratos de 21 e 60 dias de idade. A administração intraestriatal de hipoxantina aumentou os níveis de citocinas pró-inflamatórias, o imunoconteúdo de NF-κB nuclear e iNOS, diminuiu os níveis de nitrito, bem como ativou microglia e astrócitos. Além

disso, a administração intraestriatal de hipoxantina causou comprometimento neuroenergético resultando em depleção de ATP, disfunção mitocondrial e apoptose em estriado de ratos de 60 dias. Em ratos de 21 dias, a administração intraestriatal de hipoxantina também provocou desequilíbrio neuroenergético além de reduzir a atividade de Na^+,K^+-ATPase, via dano a proteínas por influência do estresse oxidativo causado pela hipoxantina. Estes estudos sugerem alguns mecanismos de toxicidade da hipoxantina sobre parâmetros inflamatórios, neuroenergéticos e oxidativos, sugerindo que esses processos podem estar envolvidos, pelo menos em parte, com distúrbios encontrados em pacientes com a doença de Lesch-Nyhan.

5. CONCLUSÕES

A administração intraestriatal de hipoxantina em ratos:

- Modificou o perfil inflamatório, visto pela alteração no imunoconteúdo de NF-κB, níveis de citocinas (IL-6 e TNF-α), bem como o imunoconteúdo de iNOS e níveis de nitritos;
- Provocou ativação microglial e astrocitária através do imunoconteúdo de Iba1 e GFAP, respectivamente;
- Alterou parâmetros de estresse oxidativo, como: níveis de espécies reativas e atividade de enzimas antioxidantes (SOD, CAT e GPx) causando dano ao DNA;
- Modificou a bioenergética estriatal visto pela alteração na atividade de enzimas da fosforilação oxidativa, níveis de ATP, bem como a regulação de creatina quinase citosólica e mitocondrial em estriado de ratos de 60 dias;
- Alterou a funcionalidade mitocondrial visto pela massa e o potencial mitocondrial, além de causar morte celular em estriado de ratos de 60 dias;
- Modificou a bioenergética estriatal visto pela alteração na atividade da piruvato quinase e enzimas da fosforilação oxidativa, bem como na regulação de creatina quinase citosólica e mitocondrial em estriado de ratos de 21 dias;
- Diminuiu a atividade da Na^+,K^+-ATPase e o conteúdo de grupos tióis em estriado de ratos de 21 dias;

6. PERSPECTIVAS

A fim de melhor compreender os mecanismos pelos quais a hipoxantina pode estar relacionada com alterações patológicas e melhor mimetizar com as condições apresentadas pela doença, temos como perspectivas:

- Desenvolver um modelo de administração crônica de hipoxantina em ratos Wistar;
- Investigar os efeitos do modelo sobre parâmetros comportamentais cognitivos e motores através das tarefas de campo aberto, reconhecimento de objetos, teste da trave, teste da escada e teste do cilindro;
- Verificar o efeito da administração crônica intraestriatal de hipoxantina sobre a histologia estriatal, analisando GFAP, NeuN e Tirosina hidroxilase, bem como o imunoconteúdo dos mesmos;
- Investigar parâmetros de viabilidade celular por citometria de fluxo através de marcação com Anexina-PI e cascatas de sinalização de sobrevivência e morte celular em estriado de ratos submetidos ao modelo;
- Avaliar o efeito da administração crônica intraestriatal de hipoxantina sobre a função mitocondrial, como: massa mitocondrial e potencial de membrana, MitoTracker Green e MitoTracker Red, respectivamente, bem como a atividade dos complexos da cadeia respiratória e os níveis de ATP intracelular em estriado de ratos.

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