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# O SULFITO E O TIOSSULFATO INDUZEM DISFUNÇÃO BIOENERGÉTICA EM CÓRTEX CEREBRAL DE RATOS

PORTO ALEGRE, JULHO DE 2013

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Bacharel em Biomedicina.

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**RESUMO**

A sulfito oxidase (SO) é uma enzima que catalisa a conversão de sulfito a sulfato, a qual consiste na reação final da via de degradação dos aminoácidos sulfurados cisteína e metionina. A deficiência da SO é um erro inato do metabolismo caracterizado bioquimicamente pelo acúmulo cerebral de sulfito, tiossulfato e S-sulfocisteína e clinicamente por disfunção neurológica e atrofia cortical. Embora evidências demonstrem uma ação tóxica do sulfito, os mecanismos envolvidos no dano neurológico ainda não estão totalmente elucidados. Portanto, o objetivo deste trabalho foi investigar os efeitos *in vitro* do sulfito e do tiossulfato sobre parâmetros de metabolismo energético em córtex cerebral de ratos jovens. Ratos Wistar machos de 30 dias de idade foram eutanasiados por decapitação e tiveram o córtex cerebral dissecado e homogeneizado em tampão específico para cada técnica. As amostras foram pré-incubadas com os metabólitos em concentrações que variaram de 1 a 500 μM durante 30 min a 37 ºC. Após a incubação, foram determinadas a produção de $CO_2$ a partir de glicose marcada radioativamente (U-$^{14}$C), as atividades dos complexos enzimáticos da cadeia transportadora de elétrons (complexos I – IV) e as atividades das enzimas creatina quinase (CK) e $Na^+,K^+$-ATPase. Verificamos que o sulfito inibiu a atividade do complexo IV da cadeia respiratória mitocondrial, indicando um dano na transferência de elétrons. Por outro lado, o tiossulfato não alterou as atividades dos complexos avaliados. Além disso, ambos os metabólitos inibiram a atividade da creatina quinase total (tCK) e das suas isoformas mitocondrial e citosólica, sugerindo um comprometimento do tamponamento e transferência intracelular de energia em córtex cerebral de ratos. O efeito inibitório do sulfito sobre a atividade da tCK foi atenuado por melatonina, enquanto que a inibição causada pelo tiossulfato sobre esta enzima foi prevenida ou atenuada por melatonina, glutationa e reduzida pelo inibidor da óxido nítrico sintase $N^{\omega}$-nitro-L-arginina metil éster, indicando envolvimento de espécies reativas de oxigênio e nitrogênio nestes efeitos. Por outro lado, a produção de $CO_2$ e a atividade da enzima $Na^+,K^+$-ATPase não foram alteradas pelos metabólitos. Nossos achados sugerem que a disfunção energética causada pelo sulfito e pelo tiossulfato pode estar envolvida no dano cortical encontrado nos pacientes afetados pela deficiência da SO.

# 1. INTRODUÇÃO

## *1.1 Deficiência da enzima sulfito oxidase (SO)*

A sulfito oxidase (SO) é a enzima terminal da via da degradação dos aminoácidos sulfurados cisteína e metionina, realizando a conversão de sulfito a sulfato na maioria dos tecidos em mamíferos, incluindo o cérebro (Hobson et al., 2005). A SO está localizada no espaço intermembranas da mitocôndria na forma de dímero com duas subunidades idênticas. Além dos aminoácidos sulfurados, o sulfito também pode ser derivado de $H_2S$, o qual é constantemente produzido por diversas células de mamíferos (Mitsuhashi et al., 1998, 2005). A SO também oxida o sulfito proveniente do meio exógeno (Basheer et al., 2007; Johnson e Duran, 2001), já que esse composto e seus derivados são amplamente utilizados em conservantes de alimentos e na indústria farmacêutica a fim de manter a estabilidade e a potência de alguns medicamentos (Chapman, 1993; Derin et al., 2006; Gunnison e Palmes, 1973; Taylor et al., 1986). A ação da SO mantém os níveis plasmáticos de sulfito em até 10 µM (Ji et al., 1995).

A deficiência da SO é uma doença neurometabólica de caráter autossômico recessivo que pode ocorrer devido a uma deficiência isolada da SO ou devido à deficiência do cofator molibdênio. O molibdênio na SO encontra-se complexado como molibdopterina, o qual também é essencial para as atividades das enzimas xantina oxidase e aldeído oxidase (Edwards et al., 1999; Tan et al., 2005). Portanto, os portadores da deficiência de molibdênio também são deficientes nas atividades dessas três enzimas.

### *1.1.1 Aspectos Clínicos e Laboratoriais*

O quadro clínico da deficiência isolada da SO é similar ao da deficiência do molibdênio (Chan et al., 2002). Entretanto, os sintomas na deficiência isolada da SO geralmente têm início tardio e são mais moderados, enquanto que a deficiência do molibdênio pode ser uma doença fatal. Os sintomas incluem disfunção neurológica grave e progressiva, convulsões tônico-clônicas logo após o nascimento, hipotonia axial, hipertonicidade periférica e atraso no desenvolvimento, além de subluxação do cristalino e morte prematura (Edwards et al., 1999; Johnson e Duran, 2001).

As alterações bioquímicas tipicamente encontradas na deficiência da SO (deficiência isolada da SO e deficiência do cofator molibdênio) incluem excreção urinária excessiva de sulfito inorgânico, tiossulfato e S-sulfocisteína (Basheer et al., 2007; Herken et al., 2009), além de eventual acúmulo de altas quantidades de ácido lático (Basheer et al., 2007),Eichler et al., 2006). Já os achados neuropatológicos característicos incluem perda massiva de neurônios, desmielinização e proliferação glial com anormalidades na substância branca. Também já foi evidenciado atrofia cortical, leucoencefalopatia e anormalidades nos gânglios basais, tálamo e cerebelo (Basheer et al., 2007; Bindu et al., 2011; Rosenblum, 1968).

### *1.1.2 Diagnóstico*

Para o diagnóstico das duas formas da deficiência da SO, o sulfito é facilmente detectado em urina fresca através de uma fita indicadora de sulfito (Johnson e Duran 2001). Entretanto, vários estudos indicam que esse teste não é confiável devido à rápida oxidação do sulfito a sulfato em temperatura ambiente (Hobson et al., 2005; Ngu et al., 2009; Sass et al., 2010). Um teste mais confiável é a identificação de S-sulfocisteína na urina através da espectrometria de massas (Hobson et al., 2005; Johnson e Rajagopalan, 1995).

A confirmação do diagnóstico pode ser feita através da medida da atividade enzimática em fibroblastos cultivados e análise mutacional (Sass et al., 2010). O diagnóstico pré-natal também é possível através do monitoramento da atividade da SO em biópsia das vilosidades coriônicas.

### *1.1.3 Tratamento*

O tratamento para a deficiência da SO ainda é bastante limitado. Na maioria dos casos, o tratamento sintomático é realizado com o objetivo de controlar as convulsões, apresentando geralmente pouco sucesso (Sass et al., 2010). Contudo, com a utilização de drogas antiepilépticas, tais como vigabatrina, os pacientes têm apresentado melhora nas convulsões (Johnson e Duran 2001). Em alguns casos, uma dieta com baixa quantidade de proteína e teor reduzido de aminoácidos contendo enxofre tem sido utilizada.

### *1.1.4 Fisiopatologia*

Os mecanismos responsáveis pelo aparecimento dos sintomas neurológicos ainda não estão totalmente estabelecidos, embora haja evidências apontando para uma ação tóxica do sulfito. Estudos prévios *in vivo* demonstraram que o sulfito induziu peroxidação lipídica e diminuiu as concentrações de glutationa (GSH), a capacidade antioxidante total e a atividade da catalase em cérebro e fígado de ratos (Chiarani et al., 2008; Derin et al., 2009). Estudos com ratos nocaute para a enzima SO também evidenciaram que concentrações aumentadas de sulfito induziram lipoperoxidação no hipocampo e diminuíram a capacidade antioxidante do plasma, além de causarem déficit cognitivo (Herken et al., 2009; Küçükatay et al., 2005).

Estudos em culturas de células Neuro-2a e PC12 evidenciaram que o sulfito aumentou a geração de espécies reativas de oxigênio e causou diminuição do ATP. Observou-se também que o sulfito alterou o potencial de membrana mitocondrial quando glutamato foi utilizado como substrato. Além disso, o acúmulo de ácido lático apresentado por alguns pacientes indica possível disfunção mitocondrial. O sulfito também diminuiu a produção de NADH a partir de glutamato e inibiu a enzima glutamato desidrogenase em mitocôndrias de cérebro de ratos (Zhang et al., 2004). Vincent e colaboradores (2004) ainda demonstraram que o sulfito aumentou a produção de espécies reativas de oxigênio em células renais, acompanhado por uma depleção de ATP, que pode ser explicada por uma inibição das enzimas mitocondriais glutamato desidrogenase e malato desidrogenase.

## ***1.2 Fosforilação Oxidativa***

A fosforilação oxidativa é o processo pelo qual o $O_2$ é reduzido a $H_2O$ por elétrons doados pelo NADH e $FADH_2$, os quais fluem através de vários pares redox (cadeia respiratória), gerando ATP a partir de ADP e Pi (Nelson e Cox, 2013). Em eucariotos, a fosforilação oxidativa ocorre nas mitocôndrias, mais especificamente na cadeia respiratória, e é responsável pela maior parte da energia produzida pela célula.

As mitocôndrias são organelas envoltas por uma membrana externa, facilmente permeável a pequenas moléculas e íons, e por uma membrana interna, impermeável à

maioria das moléculas e íons, incluindo prótons (Nelson e Cox, 2013). A membrana interna contém transportadores específicos para a passagem de substâncias como o piruvato, glicerol-fosfato, malato, ácidos graxos e outras moléculas essenciais às funções mitocondriais (Abeles, Frey e Jencks, 1992). O fluxo de elétrons do NADH e $FADH_2$ até o $O_2$ se dá através de complexos enzimáticos ancorados na membrana mitocondrial interna. Essa transferência de elétrons é impulsionada por um crescente potencial redox existente entre o NADH e o $FADH_2$, os outros complexos enzimáticos da cadeia respiratória e o $O_2$, que é o aceptor final dessa cadeia de reações de oxidação.

A cadeia respiratória é composta por vários complexos enzimáticos e uma coenzima lipossolúvel, a coenzima Q ou ubiquinona (Di Donato, 2000). O complexo I, também chamado de NADH desidrogenase ou NADH:ubiquinona oxidorredutase, transfere os elétrons do NADH para a ubiquinona. O complexo II (sucinato desidrogenase) reduz à ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do sucinato a fumarato no ciclo do ácido cítrico. O complexo III citocromo $bc_1$ ou ubiquinona-citocromo *c* oxidoredutase catalisa a redução do citocromo *c* a partir da ubiquinona reduzida. Na parte final da cadeia de transporte de elétrons, o complexo IV (citocromo *c* oxidase) catalisa a transferência de elétrons de moléculas reduzidas de citocromo *c* para $O_2$, formando $H_2O$. São necessárias quatro moléculas de citocromo *c* para reduzir completamente uma molécula de $O_2$. Todos esses complexos possuem grupamentos prostéticos específicos para desempenharem o papel de aceptores e doadores de elétrons (Abeles, Frey e Jencks, 1992).

A respiração mitocondrial, portanto, é sustentada por substratos ligados a NADH ou a $FADH_2$. No entanto, a membrana mitocondrial interna é impermeável a essas moléculas, necessitando sistemas de transferência desses equivalentes reduzidos do citosol para a matriz mitocondrial. Nesse contexto, a oxidação do NADH formado no citosol é possibilitada por sistemas chamados lançadeiras, que transferem elétrons do NADH do citosol para a matriz, através de moléculas capazes de serem transportadas através da membrana mitocondrial interna.

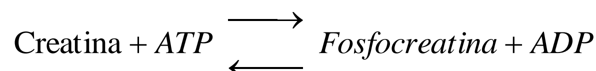
O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Assim, cria-se um gradiente eletroquímico transmembrana, utilizado por um quinto

complexo proteico, o complexo V ou ATP sintase, para a síntese de ATP. Dessa forma, a oxidação de substratos energéticos está acoplada ao processo de fosforilação do ADP, ou seja, quando o potencial de membrana é dissipado pelo fluxo de prótons a favor do gradiente eletroquímico, a energia liberada é utilizada pela ATP sintase, que atua como uma bomba de prótons ATP-dependente (Nelson e Cox, 2013).

### *1.3 Creatina quinase*

A enzima creatina quinase (CK) (E.C.2.7.3.2) catalisa a reação reversível de transferência do grupamento fosfato do ATP para o grupamento guanidino da creatina (Cr), formando fosfocreatina (PCr) e ADP.

A reação catalisada pela CK é a seguinte:



$$\text{Creatina} + ATP \rightleftarrows Fosfocreatina + ADP$$

A CK está presente em grandes quantidades em tecidos que requerem alta demanda de energia, como o músculo esquelético, o músculo cardíaco e o cérebro (Wyss e Kaddurah-Daouk, 2000). Foram identificadas cinco isoenzimas de CK (duas mitocondriais e três citosólicas), cujas subunidades são produzidas por genes distintos com expressão tecido-específica. As isoenzimas citosólicas (Cy-CK) são encontradas como moléculas diméricas, compostas por dois tipos de subunidades (CK-B e CK-M), originando três diferentes isoformas: CK-MM (predominante em músculo esquelético adulto), CK-BB (predominante em cérebro) e CK-MB (predominante em músculo cardíaco) (Manos et al., 1991; Molloy et al., 1992). As duas isoenzimas mitocondriais, Mi-CK ubíqua e Mi-CK sarcomérica, são encontradas no espaço intermembranas, formando moléculas homodiméricas ou homooctaméricas prontamente interconversíveis (Wyss e Kaddurah-Daouk, 2000). A Mi-CK octamérica é considerada a forma predominante e ativa *in vivo*, sendo muito importante para a função da enzima (Soboll et al., 1999).

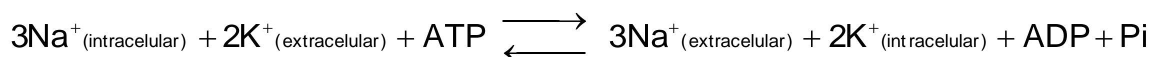
A Mi-CK interage simultaneamente com as membranas mitocondriais interna e externa, permanecendo acoplada à translocase de nucleotídeos de adenina, canal transportador do ATP da matriz mitocondrial para o espaço intermembranas. O grupamento γ-fosfato do ATP é transferido para a Mi-CK no espaço intermembranas e

dessa para a Cr, formando ADP e PCr. A PCr deixa a mitocôndria e se difunde através do citoplasma até os sítios de consumo de energia, onde, por ação das isoenzimas citosólicas (CK-MM, CK-MB ou CK-BB), irá regenerar o ATP e formar novamente Cr. A Cr liberada por retornar a mitocôndria fechando o ciclo (Wyss e Kaddurah-Daouk, 2000).

### *1.4 $Na^+$,$K^+$-ATPase*

A enzima $Na^+$,$K^+$-ATPase é uma proteína transmembrana constituída por dois tipos de subunidades: a subunidade α de 110 kD, que contém os sítios catalíticos e de ligação de íons, e a subunidade β, que é uma glicoproteína de 55 kD de função desconhecida, formando uma estrutura dimérica (αβ)2. A função dessa enzima é translocar os cátions $Na^+$ e $K^+$ através da membrana plasmática contra seus gradientes de concentração, utilizando a energia fornecida pela hidrólise ATP. A enzima transporta simultaneamente 3 íons $Na^+$ para fora e 2 íons $K^+$ para dentro da célula. A saída de $Na^+$ capacita as células animais a controlar osmoticamente seu conteúdo hídrico. Visto que três cargas positivas são transportadas para o meio extracelular e somente duas são transportadas para o meio intracelular, o fluxo de íons $Na^+$ e $K^+$ produz um gradiente eletroquímico através da membrana celular (Lingrel e Kuntzweiler, 1994). Esse gradiente é usado como fonte de energia para a despolarização e repolarização do potencial de membrana, para a manutenção e regulação do volume celular, para transporte ativo, transporte dependente de íon $Na^+$, de glicose, de aminoácidos, de neurotransmissores e para cotransporte/antiporte de outros íons (Geering, 1990). Todas as células eucarióticas superiores consomem ATP por elas produzido para a manutenção das concentrações citosólicas e extracelulares de $Na^+$ e $K^+$, sendo que esse consumo pode ser da ordem de 40 a 60 % nas células neuronais (Whittan, 1962).

A reação catalisada pela $Na^+$,$K^+$-ATPase é a seguinte:



$$3Na^+_{(intracelular)} + 2K^+_{(extracelular)} + ATP \rightleftarrows 3Na^+_{(extracelular)} + 2K^+_{(intracelular)} + ADP + Pi$$

## 2. ARTIGO CIENTÍFICO

# Disturbance of brain energy metabolism provoked by sulfite and thiosulfate: a potential pathomechanism involved in the neuropathology of sulfite oxidase deficiency

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*Abbreviations*: cCK, cytosolic creatine kinase; CK, creatine kinase; DCIP, 2,6-dichloroindophenol; GSH, reduced glutathione; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; mCK, mitochondrial creatine kinase; MEL, melatonin; Pi, inorganic phosphate; ROS, reactive oxygen species; SO, sulfite oxidase; tCK, total creatine kinase; TRO, trolox.

**Abstract**

Sulfite oxidase (SO) deficiency is biochemically characterized by tissue accumulation and high urinary excretion of sulfite, thiosulfate and S-sulfocysteine. Affected patients present severe neurological symptoms and cortical atrophy, whose pathophysiology is still poorly established. Therefore, in the present work we investigated the *in vitro* effects of sulfite and thiosulfate on important parameters of energy metabolism in cerebral cortex of young rats. We verified that sulfite inhibited the activity of complex IV, whereas thiosulfate did not alter any of the activities of the respiratory chain complexes. It was also found that sulfite and thiosulfate markedly reduced the activity of total creatine kinase (CK) and its mitochondrial and cytosolic isoforms, implying that these metabolites impair brain cellular energy buffering and transfer. In contrast, the $^{14}CO_2$ production from [U-$^{14}$C] glucose and the activity of synaptic $Na^+,K^+$-ATPase were not altered by sulfite or thiosulfate. We also observed that the inhibitory effect of sulfite and thiosulfate on CK activity was prevented or attenuated by melatonin, glutathione and the nitric oxide inhibitor $N^{\omega}$-nitro-L-arginine methyl ester, indicating the involvement of reactive oxygen and nitrogen species in these effects. Therefore, it may be presumed that disturbance of cellular energy provoked by sulfite and thiosulfate contribute to the neurological symptoms and abnormalities found in patients affected by SO deficiency.

*Keywords:* Sulfite oxidase deficiency; sulfite; thiosulfate; bioenergetic dysfunction; rat brain.

## 1. Introduction

Sulfite oxidase (SO; EC 1.8.3.1), encoded by *SUOX* gene, is an enzyme localized in the mitochondrial intermembrane space that catalyzes the oxidation of sulfite to sulfate, which is the terminal reaction of the metabolism of sulfur amino acids [1]. SO also detoxifies the sulfite derived from exogenous sources, such as sulfiting agents used in pharmaceuticals and as preservatives in food [2-5]. The importance of SO becomes obvious in cases of SO deficiency. The deficiency of this enzyme can be caused by mutations in the *SUOX* gene (isolated SO deficiency) or in any gene that affects the synthetic pathway of its molybdenum cofactor (molybdenum cofactor deficiency) [6].

Patients affected by SO deficiency (isolated SO deficiency and molybdenum cofactor deficiency) present severe neurological dysfunction characterized by encephalopathy, psychomotor retardation and generalized seizures and may have a fatal outcome at an early age. Dislocated lens and feeding difficulties are also commonly observed [2, 7-10]. Neuropathological findings include massive neuronal loss and gliosis in cerebral cortex, atrophy in the cerebral white matter and abnormalities in basal ganglia and cerebellum [2, 11-16]. Biochemically, the disorder is characterized by tissue accumulation and high urinary excretion of sulfite and its related metabolites thiosulfate and S-sulfocysteine [16].

The mechanisms underlying the pathogenesis of the severe brain damage in SO deficiency are still poorly elucidated. However, previous studies demonstrated that sulfite induces lipid peroxidation and decreases antioxidant defenses in brain and erythrocytes of normal and SO-deficient rats [17-21]. Moreover, chemical studies demonstrated that sulfite generates a sulfite radical that could mediate lipid peroxidation, and leads to the formation of superoxide anion in the presence of oxygen [22, 23]. It has been also showed that sulfite decreases mitochondrial membrane potential and ATP synthesis in mitochondria prepared from rat brain and kidney, as well as in Neuro-2a and PC12 cells [24, 25]. Finally, a recent study demonstrated that administration of sulfite causes loss of pyramidal neurons in hippocampus of normal and SO-deficient rats [26].

Considering that the potential neurotoxic effects of thiosulfate have not yet been investigated and that the exact mechanisms exerted by sulfite are not fully established, in the present study we evaluated the *in vitro* effects of these metabolites on bioenergetics,

by determining the activities of the respiratory chain complexes I to IV, creatine kinase (CK) and $Na^+,K^+$-ATPase activities in brain of young rats.

## 2. Material and Methods

### *2.1 Animals*

Thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS – Brazil, were used. The animals had free access to water and 20 % (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil) and were maintained on a 12:12 h light / dark cycle in air conditioned constant temperature (22°C ± 1°C) colony room. The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, and followed the "National Institutes of Health guide for the care and use of Laboratory animals" (NIH Publications No. 80-23, revised 2001). All efforts were made to minimize the number of animals used and their suffering.

### *2.2 Reagents*

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Sulfite and thiosulfate were dissolved on the day of the experiments in the buffer used for each assay and the pH was adjusted to 7.4. The biochemical parameters were determined in the presence of various concentrations of sulfite and thiosulfate (1-500μM), whereas control groups did not contain these metabolites in the incubation medium. Parallel experiments were carried out to detect any interference of sulfite and thiosulfate on the techniques utilized to measure the biochemical parameters.

### *2.3 Sample preparation*

Animals were killed by decapitation and the brain was immediately removed, and kept on an ice plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. For the determination of $CO_2$ production, the homogenates were prepared in Krebs–Ringer

bicarbonate buffer, pH 7.4. For the activities of the respiratory chain complexes I–III, II, II–III and IV, cerebral cortex was homogenized (1:20, w/v) in SETH buffer (250mM sucrose, 2.0mM EDTA, 10 mM Trizma base and 50IU. $mL^{-1}$ heparin), pH 7.4. For total creatine kinase (tCK) activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. The homogenate was centrifuged at 800*g* for 10min and the supernatant was kept at -70°C until being used for enzymatic activity determination. For preparation of mitochondrial and cytosolic fractions, the homogenates were centrifuged at 800*g* for 10 min at 4°C and the pellet discarded [27]. The supernatant was then centrifuged at 27,000*g* for 30 min at 4°C. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (mCK) enzymatic assay. The supernatants were further centrifuged at 125,000*g* for 60min at 4°C, the microsomal pellet discarded, and the cytosol (supernatant) was used for the determination of cytosolic creatine kinase (cCK) activity. The period between tissue preparation and measurement of the parameters was always less than 5 days, except for mCK and cCK assays, which were performed in the same day of the preparations. We used samples containing approximately 0.01–1mg protein in the assays.

*2.4 Preparation of synaptic plasma membrane*

For the measurement of $Na^+$,$K^+$-ATPase activity, cerebral cortex was homogenized (1:10, w/v) in 0.32M sucrose solution containing 10mM HEPES and 0.1mM EDTA. Synaptical plasma membranes were then prepared according to the method of Jones and Matus [29], modified by Wyse, et al. [30] using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0M. After centrifugation at 69,000*g* for 2h, the fraction at the 0.8-1.0M sucrose interface was taken as the membrane enzyme preparation.

*2.5 Determination of $CO_2$ production*

Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11$cm^3$) in a volume of 0.45mL. Flasks were pre-incubated at 35°C for 10min in the presence of sulfite or thiosulfate in a metabolic shaker (90 oscillations

min$^{-1}$) with 625μM n-dodecyl-β-D-maltoside in order to permeabilize the mitochondrial membranes. Controls did not contain the metabolites in the incubation medium. After pre-incubation, [U-$^{14}$C] glucose (0.055μCi) plus 5.0mM of unlabeled glucose was added to the incubation medium. The flasks were gassed with a mixture of $O_2/CO_2$ (95:5) and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 60 nm / 4 nm piece of Whatman 3 filter paper were hung from the stoppers. After 60min incubation at 35°C in a metabolic shaker (90 oscillations.min$^{-1}$), 0.2mL of 50% trichloroacetic acid was supplemented to the medium and 0.1mL of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30min to complete $CO_2$ trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Reis de Assis et al. 2004). Results were calculated as pmol $CO_2$.h$^{-1}$.g tissue$^{-1}$ and expressed as percentage of control.

*2.6 Respiratory chain complex I–IV activities*

The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate/cytochrome c oxidoreductase (complex II–III) were determined according to Fischer, et al. [31]. The activity of NADH/cytochrome c oxidoreductase (complex I–III) was assayed according to the method described by Schapira, et al. [32] and that of cytochrome c oxidase (complex IV) according to Rustin, et al. [33]. The methods used to measure these activities were slightly modified, as described in details in a previous report [34]. The samples were pre-incubated with sulfite or thiosulfate at 3 °C for 30min. Controls did not contain the metabolites in the incubation medium. The activities of the respiratory chain complexes were calculated as nmol.min$^{-1}$.mg protein$^{-1}$ and the results were expressed as percentage of control.

*2.7 Creatine kinase (CK) activity*

The activities of tCK, mCK and cCK were measured according to Hughes [35] with slight modifications [36]. In brief, the reaction mixture consisted of 50mM Tris buffer, pH 7.5, containing 7.0mM phosphocreatine, 7.5mM $MgSO_4$, and cortical preparations in a final volume of 0.1mL. Sulfite or thiosulfate were added to the medium

and submitted to a pre-incubation at 37 °C for 30min. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02mL of 50mM p-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes [35]. The color was developed by the addition of 0.1mL of 2% α-naphtol and 0.1mL of 0,05% diacetyl in a final volume of 1.0mL and read after 20min at $\lambda = 540$nm. Results were calculated as μmol of creatine.$min^{-1}$.mg $protein^{-1}$ and expressed as percentage of control. In some experiments, the antioxidants trolox (soluble α-tocopherol, TRO, 5 μM), melatonin (MEL, 1000 μM), the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 500 μM) or reduced glutathione (GSH, 10 μM) were co-incubated with 500μM sulfite or thiosulfate.

*2.8 Determination of $Na^+$,$K^+$-ATPase activity*

The reaction mixture for the $Na^+$,$K^+$-ATPase assay contained 5mM $MgCl_2$, 80mM NaCl, 20mM KCl, 40 mM Tris-HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3μg of protein) previously incubated with sulfite or thiosulfate at 37 ˚C for 30min. The enzymatic assay occurred at 37 ˚C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3mM. The reaction was stopped by the addition of 200μL of 10% trichloroacetic acid. $Mg^{2+}$-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. $Na^+$, $K^+$-ATPase activity was calculated by the difference between the two assays [30]. Released inorganic phosphate (Pi) was measured by the method of Chan, et al. [37]. Enzyme-specific activities were calculated as nmol Pi released.$min^{-1}$.mg $protein^{-1}$ and expressed as percentage of control.

*2.9 Protein determination*

Protein content was measured by the method of Lowry et al. [40], using bovine serum albumin as a standard.

*2.10 Statistical analysis*

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the median was used for statistical calculations. Data were analyzed using

one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Linear regression analysis was also used to test dose-dependent effects. Only significant F values are shown in the text. Differences between groups were considered significant at $P<0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

**3. Results**

First, we investigated the *in vitro* effect of sulfite and thiosulfate on $CO_2$ production from [U-$^{14}$C] glucose in cortical homogenates from 30-day-old rats in order to evaluate whether these compounds could affect the aerobic glycolytic pathway and the citric acid cycle activity. We found that none of the tested metabolites was able to alter this parameter (Figure 1).

Next we evaluated the effect of sulfite and thiosulfate on the activities of the respiratory chain complexes I to IV in rat cerebral cortex. Sulfite significantly inhibited complex IV activity [$F_{(4,15)}=4.438$, $P<0.05$] in a dose-dependent manner [$\beta=-0.650$, $P<0.01$], whereas no significant alterations were induced by this metabolite on the activities of the respiratory chain complexes I-III and II-III (Fig. 1). It should be stressed here that complex II activity could not be measured in the presence of sulfite because this compound interfered with the assay. Moreover, thiosulfate did not affect any of the complex activities (Fig. 2).

The effect of sulfite and thiosulfate on tCK activity in cortical supernatants was also examined. We found that tCK activity was significantly inhibited by sulfite [$F_{(4,15)}=36.717$, $P<0.001$] and thiosulfate [$F_{(4,20)}=250.306$, $P<0.001$] in a dose-dependent manner [Sulfite: $\beta=-0.721$, $P<0.001$; Thiosulfate: $\beta=-0.824$, $P<0.001$] (Fig. 3A). We then investigated the effect of these compounds on the activity of mCK and cCK isoforms. Fig. 3B and 3C show, respectively, that mCK and cCK activities were inhibited by sulfite [mCK: $F_{(4,15)}=55.250$, $P<0.001$; cCK: $F_{(4,18)}=15.368$, $P<0.001$] and thiosulfate [mCK: $F_{(4,20)}=58.537$, $P<0.001$; cCK: $F_{(4,15)}=33.312$, $P<0.001$]. The inhibition induced by these metabolites on mCK and cCK activities presented a dose-

dependent fashion [Sulfite: mCK: β=-0.687, $P<0.01$; cCK: β=-0.653, $P<0.01$; Thiosulfate: mCK: β=-0.844, $P<0.001$; cCK: β=-0.906, $P<0.001$].

In order to evaluate whether the significant reduction of tCK activity caused by sulfite and thiosulfate was mediated by the oxidation of critical groups of the enzyme that are susceptible to reactive species attack, cortical supernatants were pre-incubated with sulfite (500 μM) or thiosulfate (500 μM) in the presence of the antioxidants TRO (5 μM), MEL (1000 μM), L-NAME (500 μM) and GSH (10 μM). It was verified that MEL attenuated the inhibition of tCK activity caused by sulfite [$F_{(5,18)}=34.794$, $P<0.001$] (Fig. 4A). Furthermore, GSH fully prevented, whereas MEL and L-NAME attenuated the inhibitory effect induced by thiosulfate on tCK activity [$F_{(5,26)}=50.402$, $P<0.001$] (Fig. 4B).

Finally, we tested the effect of sulfite and thiosulfate on $Na^+,K^+$-ATPase activity from synaptic plasma membranes of cerebral cortex of rats. It can be seen in Table 1 that these metabolites did not change $Na^+,K^+$-ATPase activity.

## 4. Discussion

Isolated SO deficiency and molybdenum cofactor deficiency are biochemically characterized by tissue accumulation and high urinary excretion of sulfite, thiosulfate and S-sulfocysteine [16]. Although severe neurological symptoms are observed in affected patients, the exact underlying mechanisms involved in the neuropathology of these disorders are poorly established. However, accumulation of high amounts of lactic acid in some affected patients indicates mitochondrial dysfunction [2, 41]. Therefore, in the present study we evaluated the *in vitro* effects of sulfite and thiosulfate on important parameters of energy metabolism in brain of young rats.

We verified that sulfite selectively inhibited complex IV activity of the respiratory chain. Since complex IV represents the rate-limiting enzyme of the mitochondrial respiratory chain [42], it may be suggested that this inhibition caused by sulfite could contribute, at least in part, to the decrease of ATP synthesis and increase of ROS generation demonstrated in previous studies [17, 18, 24, 25]. Our present data indicate that oxidative phosphorylation is compromised by sulfite.

We also investigated the effect of sulfite and thiosulfate on the activities of CK and found that both compounds inhibited tCK, as well as the activities of the isoforms mCK and cCK in cerebral cortex. Considering that the CK system is essential for the transport of high-energy phosphate from the sites of energy production in the mitochondrial matrix to cytosolic sites of energy consumption, maintaining a stable ATP level in living cells [43, 44], the present data indicate that sulfite and thiosulfate impair brain cellular energy buffering and transfer.

It should be emphasized here that CK has vulnerable amino acid residues, including cysteine thiol groups, which are very reactive to oxidants [44-46]. Furthermore, it has been shown that brain CK activity decreases after exposure to agents that promote generation of free radicals, probably by oxidation of these amino acid residues of the enzyme that are critical for its functional activity [44, 47-52]. Therefore, we next evaluated the effect of antioxidants on the inhibitory effect caused by sulfite and thiosulfate on tCK activity and found that MEL attenuated the inhibitory effect of sulfite, whereas MEL and GSH attenuated or fully prevented the inhibition of tCK activity induced by thiosulfate. These findings suggest that hydroxyl radical, which is mainly scavenged by MEL, as well as by GSH [53-56], is probably involved in these effects. Regarding to the thiosulfate-induced inhibition of tCK activity, we verified that L-NAME also attenuated this effect, implying that thiosulfate mediates the generation of reactive nitrogen species. This is in line with previous studies showing that CK can be rapidly inactivated by peroxynitrite and nitric oxide-derived oxidants that mainly target the active cysteine sites of the enzyme [44, 50].

We also verified that sulfite and thiosulfate did not affect $CO_2$ formation from glucose implying that these metabolites do not disturb the aerobic glycolysis, citric acid cycle activity and neurotransmission. Similarly, sulfite and thiosulfate were also not able to alter $Na^+$,$K^+$-ATPase activity, which corroborates with previous findings [17] and indicate that both metabolites do not compromise the membrane potential generation and the cellular volume control.

At the present it is difficult to determine the pathophysiological relevance of our present data, since the concentrations of sulfite and thiosulfate in the brain of patients with SO deficiency are unknown. However, it should be stressed that significant

alterations of the biochemical parameters verified in our study were achieved with micromolar concentrations of the evaluated metabolites. Furthermore, it should be considered that the concentrations of sulfite and thiosulfate significantly increase in affected patients during catabolic stress situations, such as infections, which increase the availability of sulfur amino acids due to intense proteolysis [16]. It is also noteworthy that, among human brain structures, cerebral cortex presents the highest expression of SO, suggesting that this brain region needs higher enzyme levels, perhaps due to greater formation of sulfite during metabolism [65]. Therefore, the deficiency of this enzyme may predispose the cerebral cortex to damage by excess of sulfite and thiosulfate, which is in accordance with the clinical evidence showing severe cortical damage in several patients [65].

In conclusion, this is the first report showing that the metabolites accumulating in SO deficiency markedly inhibit CK activity and moderately impair the electron flow through the respiratory chain. In case these findings are confirmed *in vivo* and in tissues from patients affected by isolated SO deficiency and molybdenum cofactor deficiency, it is tempting to speculate that bioenergetic dysfunction may contribute, at least in part, to the neurological damage found in these disorders.

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**Legends to figures**

**Fig. 1.** Effect of sulfite and thiosulfate on $^{14}CO_2$ production from [U-$^{14}$C] glucose in rat cerebral cortex. Cerebral cortex homogenates were incubated in the presence of sulfite and thiosulfate (1-500 µM). Values are means ± standard deviation for six independent experiments (animals) and are expressed as percentage of controls (Controls: $^{14}CO_2$ production [$^{14}CO_2$ . $h^{-1}$ . g $tissue^{-1}$]: Sulfite: 922 ± 111; Thiosulfate: 798 ± 117). Controls did not contain the tested compounds in the incubation medium.

**Fig. 2.** Effect of sulfite and thiosulfate on the activities of the respiratory chain complexes I–IV in rat cerebral cortex. Cerebral cortex supernatants were incubated in the presence of sulfite or thiosulfate (1-500 µM). Values are means ± standard deviation for four to six independent experiments (animals) and are expressed as percentage of controls (Controls: (A) Complex I-III activity [nmol cytochrome *c* reduced . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 4.14 ± 1.40, Thiosulfate: 2.60 ± 0.27; (B) Complex II activity [nmol DCIP reduced . $min^{-1}$ . mg $protein^{-1}$]: Thiosulfate: 9.97 ± 0.98; (C) Complex II-III activity [nmol cytochrome *c* reduced . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 35.0 ± 4.32, Thiosulfate: 27.4 ± 3.70; (D) Complex IV activity [nmol cytochrome *c* oxidized . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 96.7 ± 12.3, Thiosulfate: 67.1 ± 10.1. Controls did not contain the tested compounds in the incubation medium. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to controls (Duncan multiple range test).

**Fig. 3.** Effect of sulfite and thiosulfate on total (A), mitochondrial (B) and cytosolic (C) creatine kinase (CK) activities in rat cerebral cortex. Cerebral cortex supernatants were incubated in the presence of sulfite or thiosulfate (1-500 µM). Values are means ± standard deviation for four to five independent experiments (animals) and are expressed as percentage of controls (Controls: (A) Total creatine kinase (tCK) activity [nmol creatine . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 5.20 ± 0.78, Thiosulfate: 6.55 ± 0.59; (B) Mitochondrial creatine kinase (mCK) activity [nmol creatine . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 1.73 ± 0.35, Thiosulfate: 6.46 ± 1.02; (C) Cytosolic creatine kinase (cCK) activity [nmol creatine . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 5.41 ± 0.60, Thiosulfate: 6.52 ± 1.04. Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

**Fig. 4.** Effect of antioxidants on the inhibition of total creatine kinase (tCK) activity induced by sulfite (A) and thiosulfate (B) in rat cerebral cortex. The antioxidants trolox (TRO, 5 μM), melatonin (MEL, 1000 μM), the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 500 μM) or reduced glutathione (GSH, 10 μM) was co-incubated with 500 μM sulfite or thiosulfate and the activity of the enzyme measured afterward. Values are means ± standard deviation for four to ten independent experiments (animals) and are expressed as percentage of controls (Controls: tCK [nmol creatine . $min^{-1}$ . mg $protein^{-1}$]: Sulfite: 3.78 ± 0.81; Thiosulfate: 6.72 ± 2.54. Controls did not contain the tested compounds in the incubation medium. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls; $^{\#\#\#}P < 0.001$, compared to 500 μM sulfite or thiosulfate (Duncan multiple range test).

**Abreviations:**

CK – Creatine kinase

- tCK – Total creatine kinase
- mCK – mitochondrial creatine kinase
- cCK – cytosolic creatine kinase

GSH – Glutathione

L-NAME - $N^{\omega}$-nitro-L-arginine methyl ester

MEL – Melatonine

ROS – Reactive oxygen species

SO – Sulfite oxidase

TRO – Trolox

**FIGURES**

Figure 1

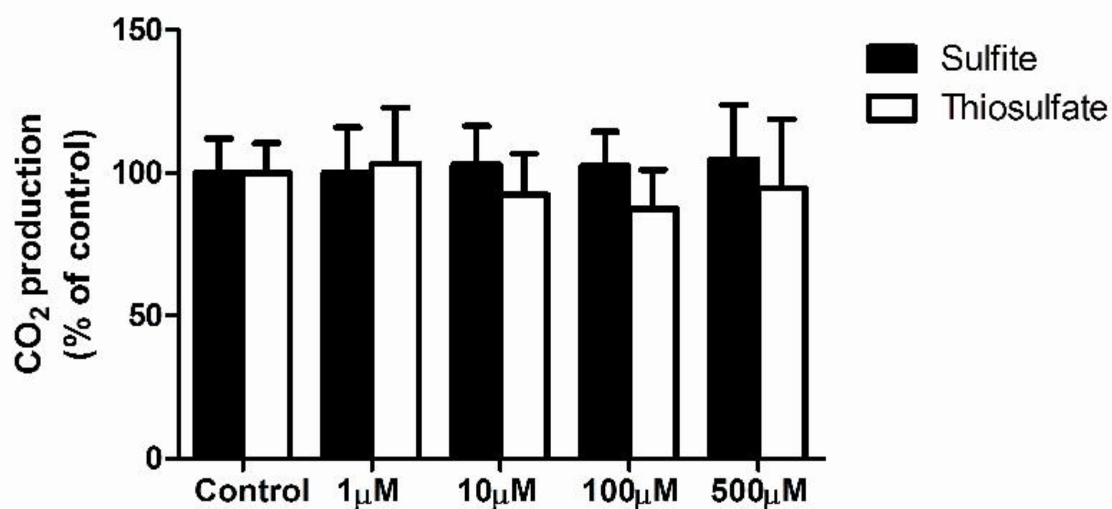


Figure 2

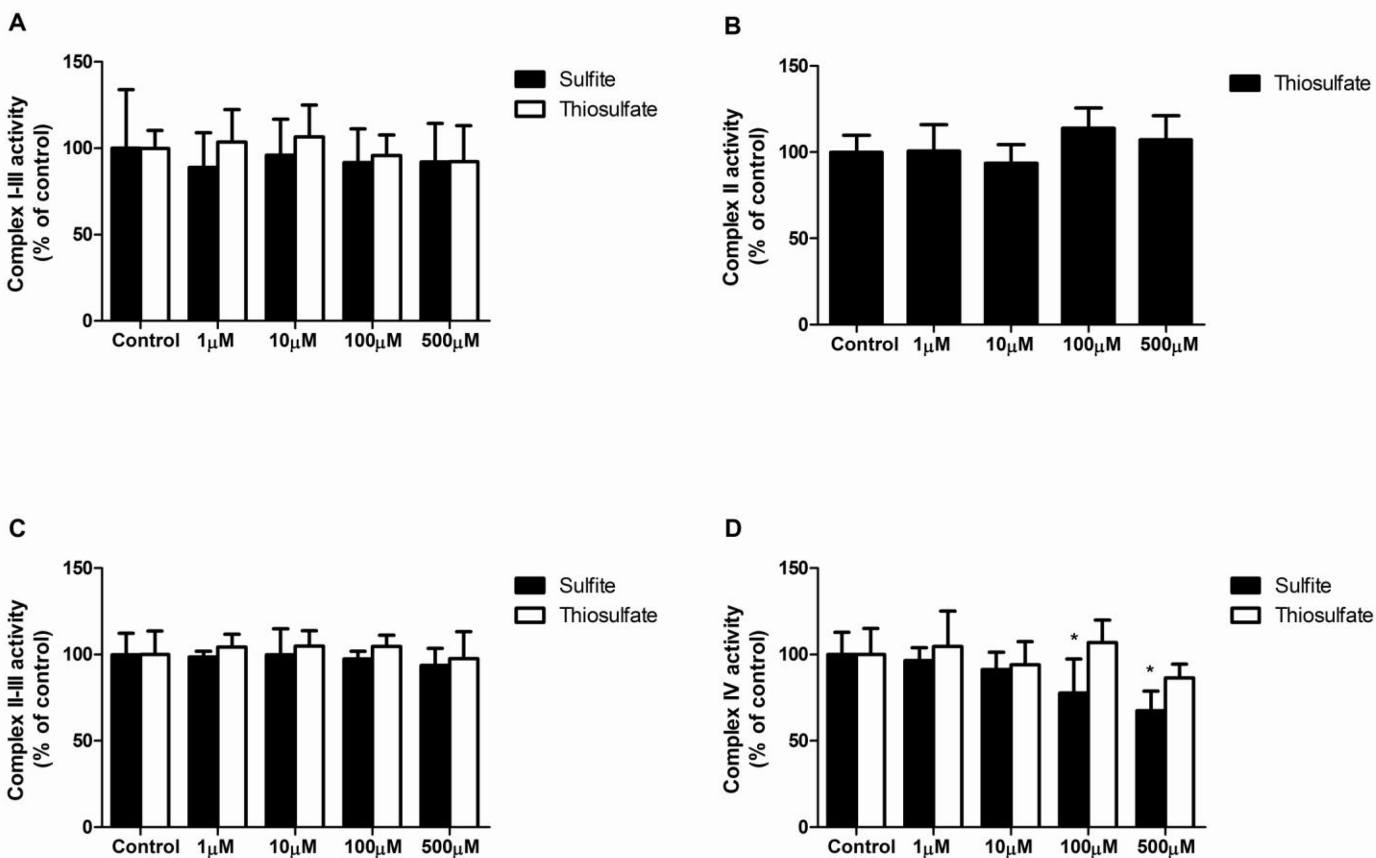

Figure 3

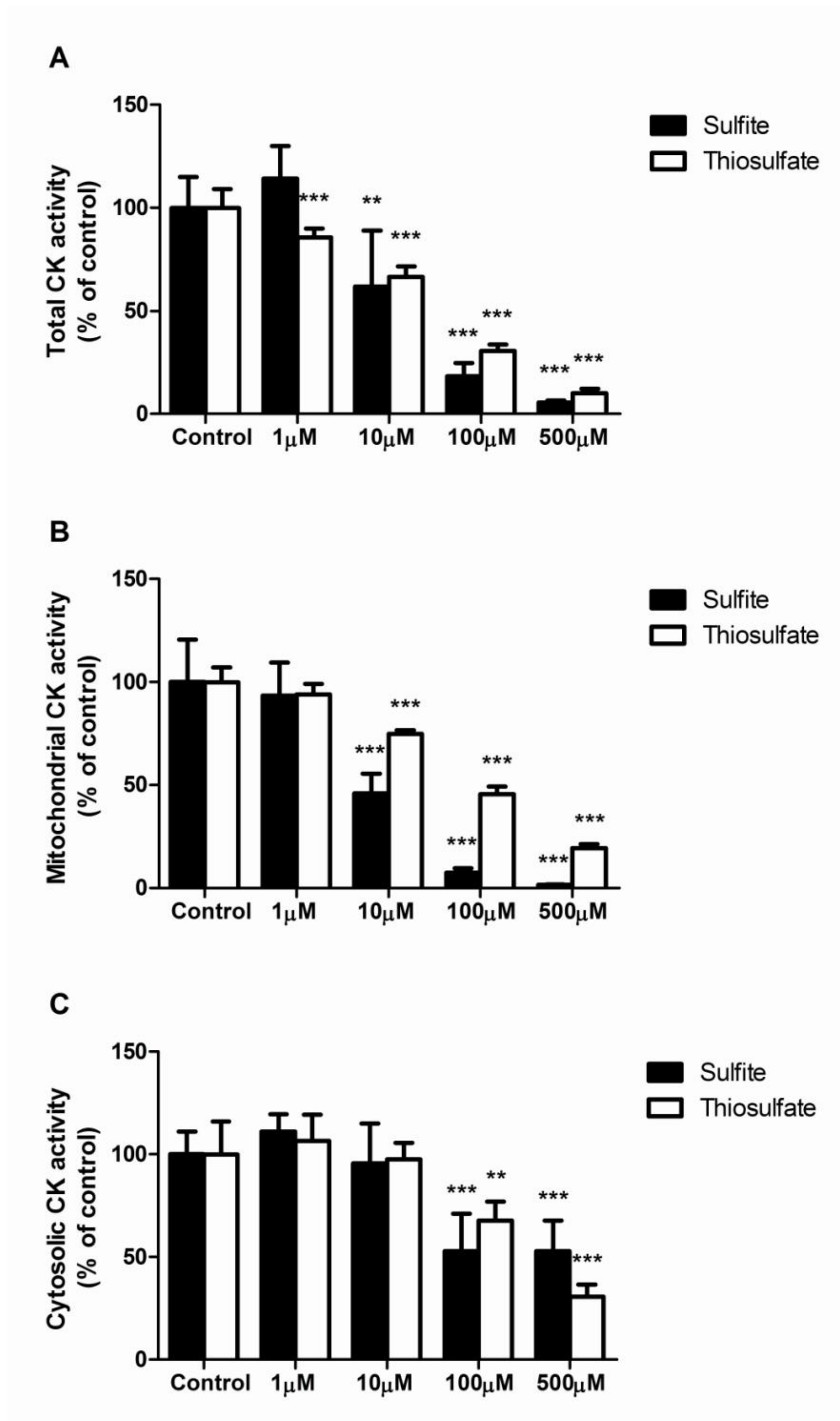

Figure 4

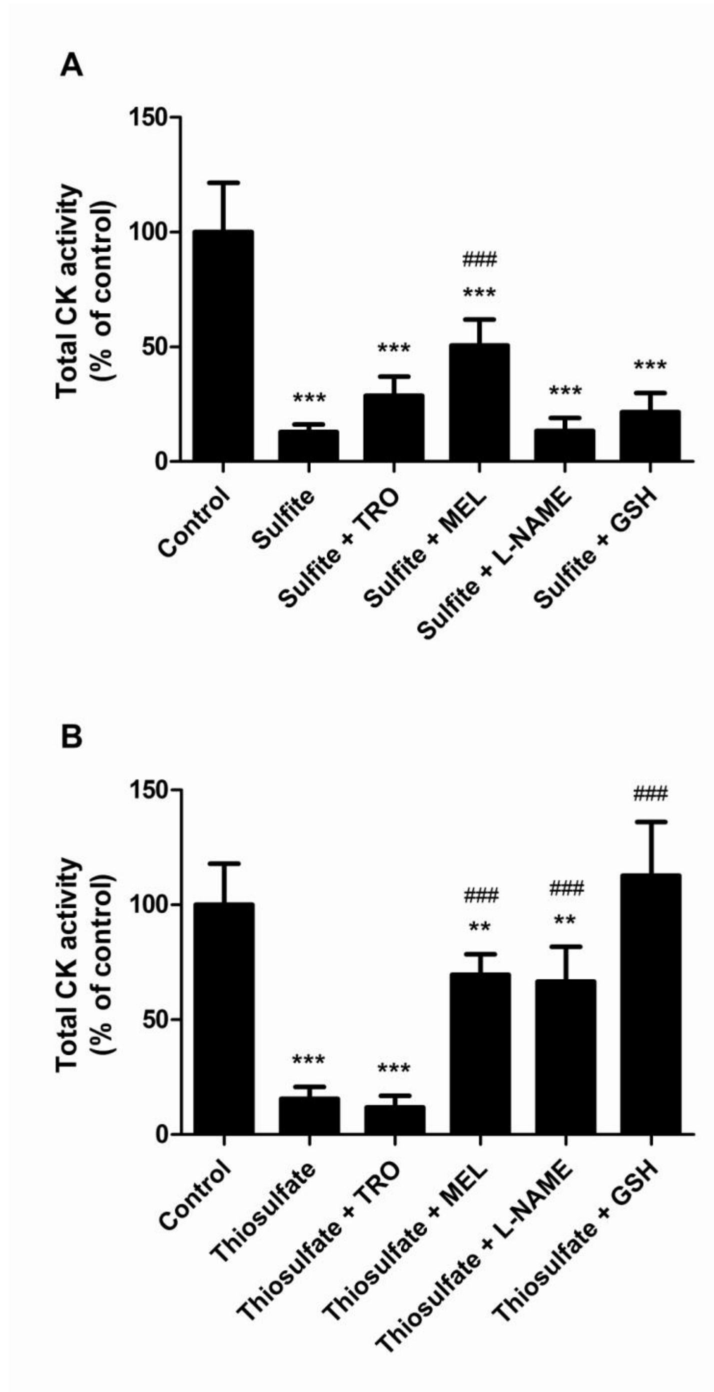

Table 1. Effect of sulfite and thiosulfate on $Na^+$,$K^+$-ATPase activity in rat cerebral cortex

| | $Na^+$,$K^+$-ATPase activity | | | | |
|---|---|---|---|---|---|
| | Control | 1 μM | 10 μM | 100 μM | 500 μM |
| Sulfite | 100 ± 27.6 | 116 ± 17.8 | 117 ± 16.0 | 108 ± 21.2 | 105 ± 13.7 |
| Thiosulfate | 100 ± 14.0 | 99.8 ± 10.8 | 98.0 ± 10.0 | 102 ± 16.4 | 101 ± 20.7 |

Values are means ± standard deviation for five to six independent experiments (animals) and are expressed as percentage of controls. (Controls: $Na^+$,$K^+$-ATPase activity [nmol Pi released . $min^{-1}$ . mg of $protein^{-1}$]: Sulfite: 715 ± 197; Thiosulfate: 1250 ± 175). No significant differences between groups were detected (one-way ANOVA).

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## 3. CONCLUSÃO

O presente trabalho evidenciou que o sulfito e o tiossulfato, metabólitos acumulados na deficiência da SO, inibem a atividade da CK, e que o sulfito prejudica o fluxo de elétrons na cadeia respiratória por inibir a atividade do complexo IV, em córtex cerebral de ratos jovens. Além disso, foi verificado o envolvimento de espécies reativas na inibição da atividade da CK causada pelo sulfito e tiossulfato, visto que os antioxidantes melatonina, glutationa e o inibidor da óxido nítrico sintase $N^{\omega}$-nitro-L-arginina metil éster preveniram esse efeito inibitório. Caso os resultados aqui apresentados sejam confirmados em tecidos de pacientes portadores da deficiência da SO, pode-se especular que alterações no metabolismo energético contribuem, ao menos em parte, para o dano neurológico encontrado nessa doença.

## 4. PERSPECTIVAS

Este trabalho tem como perspectivas o estudo dos efeitos *in vitro* dos mesmos metabólitos acumulados na deficiência da SO sobre parâmetros de estresse oxidativo, tais como a medida da atividade da enzima aconitase do ciclo de Krebs, a oxidação da 2',7'-diclorofluoresceína e a produção de nitratos e nitritos, além da avaliação de função mitocondrial. Também pretendemos estudar os efeitos *ex vivo* desses metabólitos em córtex cerebral de ratos através de injeções intracerebrais.

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## 6. ANEXOS

Normas da revista “Molecular Genetics and Metabolism”, para qual o trabalho será
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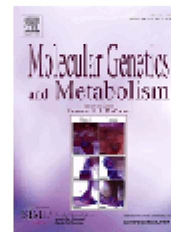

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*Molecular Genetics and Metabolism* is a contribution to the understanding of the **metabolic basis of disease.** The journal publishes articles describing investigations that use the tools of **biochemistry** and **molecular biology** for studies of normal and diseased states. In addition to original research articles, occasional minireviews reporting timely advances as well as brief communications and letters to the editor are considered.

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