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CREATINA PREVINE ALTERAÇÕES EM PARÂMETROS DE MEMÓRIA, ATIVIDADE DA ACETILCOLINESTERASE E ESTRESSE OXIDATIVO EM RATOS SUBMETIDOS À INJEÇÃO INTRAESTRIATAL DE GUANIDINOACETATO

PORTO ALEGRE

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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 Bacharela em Biomedicina.

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Orientadora: Profª. Drª. Angela T.S. Wyse

PORTO ALEGRE

2016

DEDICO ESTE TRABALHO

À minha família, que sempre possibilitou meus estudos.

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RESUMO

A deficiência de guanidinoacetato metiltransferase (GAMT) é um erro inato do metabolismo (EIM) detectado entre as síndromes da deficiência de creatina, sendo causada por uma mutação no gene que codifica a enzima GAMT, o que leva a uma diminuição na síntese de creatina e um consequente aumento na concentração de guanidinoacetato (GAA). Pessoas que sofrem dessa síndrome começam a apresentar sintomas neurológicos na infância como: hipotonia muscular, movimentos extrapiramidais involuntários e convulsões. O objetivo geral do presente projeto foi estudar a neurotoxicidade do GAA, através de estudo do comportamento (teste de reconhecimento de objetos), atividade da enzima acetilcolinesterase (AChE) e testes bioquímicos de estresse oxidativo (enzimas antioxidantes e oxidação da 2,7-diclorofluoresceína), bem como o papel da creatina como neuroprotetora utilizando um modelo de cirurgia estereotáxica em ratos adultos. Ratos Wistar de 60 dias de vida foram submetidos a cirurgia estereotáxica após um pré-tratamento com creatina ou salina que durou 7 dias. Após a cirurgia os animais receberam uma administração intraestriatal sendo divididos em quatro grupos: (1) controle (infusão intraestriatal de salina e pré-tratamento com salina), (2) Salina + GAA (infusão intraestriatal de GAA e pré-tratamento com salina), (3) Creatina + GAA (infusão intraestriatal de GAA e pré-tratamento com creatina), (4) Creatina + Salina (infusão intraestriatal de salina e pré-tratamento com creatina). Uma hora após a administração intraestriatal os ratos foram decapitados para análise bioquímica do estriado ou submetidos ao teste comportamental. Resultados mostraram que a administração intraestriatal de GAA foi capaz prejudicar a performance de ratos no teste de reconhecimento de objetos, além de aumentar a atividade da AChE, aumentar a produção de espécies reativas e diminuir a atividade de enzimas antioxidantes. Creatina se mostrou capaz de prevenir a maioria das alterações, com exceção da diminuição da atividade da enzima superóxido dismutase (SOD). É possível supor que as alterações bioquímicas aqui encontradas contribuem para as alterações neurológicas apresentadas por pacientes com deficiência de GAMT. É possível que a suplementação com creatina possa ser um tratamento adjuvante.

Palavras chave**:** Deficiência de guanidinoacetato metiltransferase; Creatina; Guanidinoacetato; Neurotoxicidade; Acetilcolinesterase; Estresse oxidativo

LISTA DE ABREVIAÇÕES

ACh - Acetilcolina

AChE - Acetilcolinesterase

AGAT - L-arginina: glicina amidinotransferase

ADP - Adenosina difosfato

ATP - Adenosina trifosfato

CAT - Catalase

DCF - 2,7-Dicloro Fluoresceína

EIM - Erros Inatos do Metabolismo

GAA - Guanidinoacetato

GAMT - Guanidinoacetato Metiltransferase

SAM - S-adenosilmetionina

SNC - Sistema Nervoso Central

SOD - Superoxide Dismutase

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1. INTRODUÇÃO

1.1 Erros inatos do metabolismo

Erros inatos do metabolismo (EIM) são doenças genéticas hereditárias, majoritariamente autossômicas recessivas que se caracterizam pela síntese de uma proteína anômala, geralmente uma enzima, que apresentará atividade parcial ou totalmente reduzida. Essas alterações podem levar ao bloqueio de rotas metabólicas com consequente acúmulo de substrato, diminuição na síntese do produto ou até formação de produtos tóxicos por rotas metabólicas alternativas (Scriver, 2008).

Os EIM são considerados raros quando analisados separadamente, porém, em conjunto, os cerca de 1000 EIM descritos atingem um a cada mil nascidos vivos (Mak et al., 2013). A classificação mais utilizada para os EIM é realizada de acordo com a área do metabolismo afetada (Scriver, 2001), podendo ser: EIM de ácidos orgânicos, aminoácidos, glicídios, lipídios, glicosaminoglicanos, glicoproteínas, purinas e pirimidinas, enzimas eritrocitárias, metais, lipoproteínas, hormônios e proteínas plasmáticas, dentre outros.

A deficiência da Guanidinoacetato Metiltransferase (GAMT) foi o primeiro EIM detectado entre as síndromes da deficiência de creatina, sendo o foco deste trabalho.

1.2 Deficiência de Guanidinoacetato Metiltransferase (Deficiência de GAMT)

O N-amino-imino-metil-glicina, conhecido como guanidinoacetato (GAA) é um aminoácido pertencente à classe dos compostos guanidínicos. Esses compostos são substâncias caracterizadas pela presença do grupo básico guanidino em sua estrutura (H_2N-C(=NH)-NH-), e exercem um importante papel biológico, incluindo a participação da arginina na síntese de uréia e da creatina na contração muscular (Wyss et al., 2007).

O GAA é sintetizado nos rins, onde é obtido principalmente da arginina e glicina (Takeda et al., 1992). Após a síntese renal, é transportado pelo fígado, onde recebe um grupamento metil doada pela S-adenosilmetionina (SAM), em uma reação catalisada pela

enzima Guanidinoacetato Metiltransferase (GAMT)., dando origem à creatina (Gordon, 2010).

A deficiência de GAMT foi o primeiro EIM detectado entre as síndromes da deficiência de creatina, sendo descrita como de natureza genética hereditária, causada por uma mutação no gene que codifica a enzima GAMT, o que leva a uma diminuição na síntese de creatina e um consequente aumento na concentração de GAA (Stöckler, *et al.*, 1994; Stöckler, *et al.*, 1996; Schulze, *et al.*, 2001; Gordon, 2010). As alterações bioquímicas decorrentes desse EIM incluem excreção urinária contendo muito GAA e pouca creatina, altas concentrações de GAA no cérebro e em outros tecidos, e redução drástica de creatina no cérebro e nos músculos esqueléticos dos pacientes afetados (Stöckler, *et al.*, 1996; Schulze, *et al.*, 2003). Pessoas que sofrem dessa síndrome começam a apresentar sintomas neurológicos na infância como: hipotonia muscular, movimentos extrapiramidais involuntários, convulsões, fala arrastada e até mesmo autismo (Stöckler, *et al.*, 1996; Schulze, *et al.*, 2001; Arias-Dimas, *et al.*, 2006; Gordon, 2010).

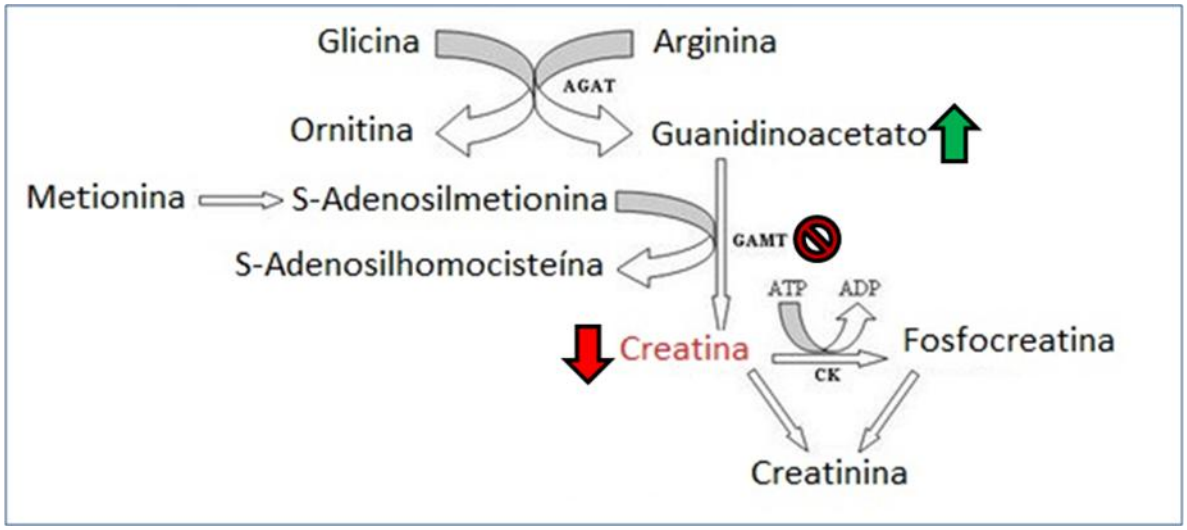

Figura 1. Bloqueio metabólico no metabolismo da creatina encontrado da deficiência de GAMT. Abreviações: AGAT, arginina: glicina-amidinotransferase; GAMT, guanidinoacetato metiltransferase; ATP, adenosina trifosfato; ADP, adenosina difosfato; CK, creatina quinase. Adaptado de Casey and Greenhaff, 2000.

Diversos estudos buscam opções de tratamentos para aliviar os sintomas apresentados pelos pacientes com deficiência de GAMT. A suplementação oral de

creatina em doses de 400-670 mg por kg de peso corporal por dia pode resultar em melhoras, principalmente no quadro de movimentos involuntários extrapiramidais e ataques epiléticos. Apesar de um recente estudo demonstrar que suplementação com creatina diminui os níveis plasmáticos de GAA em indivíduos normais (Peters et al, 2015), é importante ressaltar que o mesmo não parece ocorrer em pacientes com deficiência na enzima GAMT (Stöckler, *et al.*, 1996; Gordon, 2010). Para que haja uma melhora ainda maior no quadro clínico, estudos sugerem que uma diminuição na ingestão de um dos aminoácidos precursores do GAA, a arginina, combinada com suplementação de ornitina também podem prevenir as convulsões características da doença (Dhar *et al.*, 2009; Gordon, 2010; Stockler et al., 2014).

1.3 Creatina

A creatina é um ácido orgânico nitrogenado que exerce diversos papéis benéficos *in vivo* e *in vitro*, tais como efeitos protetores na hipóxia, isquemia cerebral, dano muscular, e oxidativo (Wyss, *et al.*, 2007; Andres, *et al.*, 2008). Sua suplementação pode inclusive ser utilizada como estratégia para o crescimento e a manutenção muscular e óssea (Schlattner, *et al.*, 2006). Muitos de seus efeitos benéficos provêm da ação da enzima creatina cinase (CK) sobre a creatina, o que gera a fosfocreatina, que é de extrema importância para diversos processos metabólicos, funcionando como reserva energética, transferindo reversivelmente seu grupo N-fosforil para o ADP quando este está em altas concentrações, regenerando assim ATP (Wallimann, *et al.*, 1992; Saks, *et al.*, 2004; Sauer e Schlattner, 2004). Aproximadamente dois terços da creatina são transformados em fosfocreatina pelas isoformas de CK (Wallimann, *et al.*, 2011). Somado a isso, a creatina possui propriedades antioxidantes *per se* (Sestili, *et al.*, 2006; Young, *et al.*, 2010) e sua suplementação possui ação neuroprotetora em doenças neurodegenerativas (Wyss e Schulze, 2002; Hersch, *et al.*, 2006; Bolaños, *et al.*, 2009; Pastula, *et al.*, 2012). A reserva energética fornecida pelo sistema creatina/creatina quinase também ajuda a prevenir a sobrecarga da cadeia transportadora de elétrons, reduzindo a geração de espécies reativas que tem o poder de causar estresse oxidativo e induzir a dissociação do citocromo C da membrana interna mitocondrial, evitando eventos de ativação da apoptose, o que sugere proteção cerebral ao acúmulo de GAA (Kolling e Wyse, 2010; Meyer et al, 2006).

1.4 Acetilcolinesterase

A acetilcolina (ACh) é um neurotransmissor clássico sintetizado pela enzima colina acetiltransferase a partir de acetato e colina, sendo armazenado em vesículas nos neurônios pré-sinápticos. A acetilcolinesterase (AChE) é uma enzima responsável pela degradação da ACh liberada na fenda sináptica, e que parece ajudar a co-regular a transmissão colinérgica (Geula e Darvesh, 2004). Diversos estudos apontam que esta enzima desenvolve papéis importantes em várias desordens neurológicas, inclusive na doença de Alzheimer (Giacobini, 2004; Lane, *et al.*, 2004; Lane, *et al.*, 2006; Sindi, et al., 2015). Inibidores da AChE permitem que o neurotransmissor ACh permaneça mais tempo na fenda sináptica, o que promove acentuada melhora no aprendizado e memória em ratos isquêmicos tratados com anticolinesterásicos (Borlongan, *et al.*, 2005).

Por desempenhar um papel fundamental no SNC e coordenar propriedades anti-inflamatórias que podem evitar um estado de estresse oxidativo, é importante que a atividade da AChE esteja regulada, já que poderia trazer consequências neurológicas indesejadas. Entretanto, estudos apontam que compostos guanidínicos, como o GAA, alteram a atividade dessa enzima (Delwing-De Lima, *et al.*, 2010), levando inclusive a alterações comportamentais (Zugno, *et al.*, 2008), o que pode estar associado com as alterações neurológicas presentes nos pacientes deficientes em GAMT.

Embora a fisiopatologia da deficiência de GAMT seja pouco conhecida, algumas alterações bioquímicas e comportamentais já foram observadas em alguns estudos. Neste contexto, ratos submetidos à cirurgia estereotáxica, que receberam uma injeção intraestriatal de GAA, decapitados 30 min após essa injeção, tiveram um aumento na atividade da AChE, além de apresentarem um prejuízo na aquisição, consolidação e evocação da memória aversiva, sugerindo que o GAA afeta todos os passos da formação da memória (Zugno, et al., 2008). Portanto, é de nosso interesse elucidar os mecanismos responsáveis por essas alterações**.**

1.4 Estresse Oxidativo

As espécies reativas são constantemente produzidas no organismo em níveis basais, principalmente durante o processo de respiração celular, e possuem diversas

funções fisiológicas (Halliwell, 2012). O organismo possui diversos mecanismos para evitar que os efeitos das espécies reativas se tornem muito prejudiciais ao nosso corpo, entre eles estão as defesas enzimáticas (Superóxido Dismutase [SOD], Catalase [CAT] e Glutationa Peroxidase [GPx]) e as não-enzimáticas (Glutationa reduzida [GSH], vitaminas, entre outras) (Halliwell e Gutteridge, 2007). Entretanto, em situações patológicas essas espécies reativas podem causar um desequilíbrio entre as espécies reativas e as defesas antioxidantes. A persistência desse desequilíbrio leva ao estresse oxidativo, que pode causar dano em diferentes tipos de biomoléculas, incluindo DNA, lipídeos e proteínas (Halliwell e Whiteman, 2004; Nelson e Cox, 2008). Além disso, o estresse oxidativo pode desencadear respostas inflamatórias locais e sistêmicas, que por sua vez intensificarão o próprio estresse oxidativo.

O cérebro é um órgão sensível ao estresse oxidativo, uma vez que o sistema nervoso central (SNC) apresenta um elevado consumo de oxigênio em consequência do alto consumo de ATP. Nesse sentido, existem diversas evidências que apontam a correlação entre o estresse oxidativo e a ocorrência de doenças neurodegenerativas, tais como Parkinson, Alzheimer, esclerose lateral amiotrófica, epilepsia, entre outras (Halliwel, 2001; Moreira, et al., 2005; Vidoni et al., 2016).

Estudos do nosso grupo já demonstraram que o excesso de GAA inibe a atividade da creatina cinase, o complexo II da cadeira respiratória e a captação de glutamato, provavelmente através de estresse oxidativo (Zugno, *et al.*, 2007; Zugno, *et al.*, 2007).

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral do presente projeto foi estudar os mecanismos de neurotoxicidade do GAA, incluindo estudos bioquímicos e comportamentais, bem como o papel da creatina como neuroprotetora.

2.2. Objetivos específicos

1) Avaliar a memória/aprendizado de ratos submetidos à administração intraestriatal de GAA, utilizando a tarefa comportamental de reconhecimento de objetos;

2) Investigar parâmetros bioquímicos que possam estar envolvidos nas possíveis alterações de memória e comportamento, tal como atividade da AChE em estriado de ratos submetidos à injeção instraestriatal de GAA;

3) Avaliar alguns parâmetros de estresse oxidativo, tais como: atividade das enzimas antioxidantes (SOD e CAT) e oxidação da 2,7-dicloro fluoresceína (DCF) em estriado de ratos submetidos à injeção instraestriatal de GAA;

4) Investigar o papel neuroprotetor da creatina sobre as alterações bioquímicas e comportamentais encontradas no presente estudo.

Os resultados, bem como a metodologia do trabalho serão mostrados na forma de artigo científico a ser submetido para uma revista científica;

Além do trabalho experimental também foi escrito uma revisão sobre o tema - ver anexo A.

3. ARTIGO CIENTÍFICO

O artigo intitulado “**Effect of guanidinoacetate on memory and oxidative status in striatum**” foi formatado conforme normas para publicação junto ao periódico Journal of Inborn Errors of Metabolism & Screening

Effect of guanidinoacetate on memory and oxidative status in striatum

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Abstract

Guanidinoacetate Methyltransferase deficiency is a disorder of the creatine metabolism, altering guanidinoacetate (GAA) and creatine levels. Patients present convulsions and mental retardation, whose mechanisms are unclear. In this study we investigated the effects of an intrastriatal administration of GAA on non-aversive behavioral test, acetycholinesterase (AChE) activity and parameters of oxidative stress such as 2′7′dichlorofluorescein (DCFH) oxidation and antioxidant enzyme activities. Adult rats received a single intrastriatal GAA administration or saline. Animals were subjected to behavioral or striatum biochemical tests 1 hour after the infusion. In the novel object recognition test, the time exploring the novel object increased in the control group ($P < 0.05$), while the same did not happened in treated animals. GAA significantly increased AChE ($P < 0.01$) and decreased the activity of Superoxide dismutase (SOD) ($P < 0.01$) and catalase (CAT) ($P < 0.01$) as well as increased DCF oxidation ($P < 0.01$) Creatine prevented all alterations, except SOD activity.

Key words: Guanidinoacetate Methyltransferase – GAMT Deficiency; Creatine; Guanidinoacetate; Neurotoxicity; Acetylcholinesterase; Oxidative stress

3.1 Introduction

Guanidinoacetate (GAA) is a metabolite of glycine in which the amino group can be converted into a guanidine. It is highly involved in the metabolism of creatine, being its direct precursor. [1]

Creatine has been known for its essential functions in muscle, contributing to a transient intracellular storage of metabolic energy mainly in the form of phosphocreatine that is nothing more than creatine with a phosphate group.[1] Mammals take about half of their creatine from diet (mostly from meat and fish), and synthesize the other half through a relative simple reaction that occurs mainly in the kidney and liver involving a two-step pathway with two enzymes and one membrane carrier.[2] The enzymes involved are L-arginine: glycine amidinotransferase (AGAT) and *N*-guanidinoacetate methyltransferase (GAMT), while the membrane carrier is named SLC6A8. The phosphorylation of creatine is made possible by the creatine kinases (CK).[3] This is an efficient way to store energy not only to skeletal muscle, but also to organs that require a great amount of energy in order to efficiently develop their activities in human body, like the brain.[4] The phosphocreatine has a slightly higher diffusion capacity than ATP, and it can reversibly transfers its N-phosphoryl group to ADP when this nucleotide is at high concentrations, regenerating ATP and preventing the tissue from running out of energy. [5,6,7].

In 1994, Stöckler et al, described a new inborn error of metabolism (IEM) that was addressed as the first inborn error of metabolism of creatine. The new disease was named after the affected enzyme: guanidinoacetate methyltransferase (GAMT) deficieny.[8] This metabolic disease is an autosomal

recessively inherited disorder caused by mutations in the *GAMT* gene that leads to depleted levels of creatine and excessive concentrations of GAA in skeletal muscle, blood, brain and other tissues.[9,10,11] It is a rare disease with approximately 110 cases reported so far, mostly from Europe and the Middle East.[12] The cerebral tissue relies upon a continuous energy supply in order to maintain its functions properly. Therefore, with creatine in low levels, the brain becomes the most affected organ in GAMT deficiency patients. Neurological symptoms are common and variable among individuals affected by GAMT deficiency, including intractable epilepsy, intellectual impairment, autism, auto mutilating behavior, extra pyramidal syndrome, slurred speech and hypotonia. [11,13,14,15]

Supplementation with creatine has been used as pharmacological treatment in GAMT deficiency for a long time.[11] The energetic reservoir provided by the system creatine/creatine kinase helps to prevent the overload of the mitochondrial respiratory chain, reducing generation of reactive species that have the power to cause oxidative stress and induce cytochrome C dissociation from the inner mitochondrial membrane, initiating early apoptotic triggering events.[16] A recent study revealed that creatine is able to prevent damage induced in vitro by oxidizing agents in both nucleated and non-nucleated cells, suggesting that creatinine may possess similar properties.[20] These antioxidant proprieties presented by creatine appear to be beneficial not only to GAMT,[21] but also to other innate errors of metabolism where creatine was able to prevent lipid peroxidation and imbalance of redox homeostasis.[22,23]

The cholinergic system plays a key role in the modulation of learning and memory in mammals[24] and is also related with modulation of inflammatory pathways[25]. Achetylcholinesterase (AChE) is the enzyme responsible for the degradation of acetylcholine (ACh) released in the synaptic cliff, and has a role in the co-regulation of cholinergic transmission[26]. Several studies have shown that this enzyme plays an important role in several neurological disorders[27,28]. For developing a fundamental role in the central nervous system (CNS) and regulating anti-inflammatory properties that can avoid oxidative stress, it is important that the activity of this enzyme remain regulated, since it could bring unwanted neurological consequences. However, studies claim that guanidine compounds, like GAA, alter the activity of AChE, leading to behavioral abnormalities [29, 30] that may be associated with the neurological alterations found in patients with GAMT deficiency.

3.2 Aim

In the present study, we investigated the effects of an intrastriatal administration of GAA on the process of memory acquisition (via novel object recognition tasks), as well as AChE activity in striatum adult rats. We also evaluated some parameters of oxidative stress such as 2′7′dichlorofluorescein (DCFH) oxidation and antioxidant enzyme activities. The neuroprotective role of creatine on the possible biochemical changes observed in this model was also investigated. Our hypothesis is that the alterations caused by GAA may be associated with oxidative insult and that creatine might prevent such damage. Striatum was used because patients with GAMT deficiency present basal ganglia abnormalities[31].

3.3 Materials and Methods

3.3.1 Animals and reagents

Sixty-day-old wistar rats weighing 180–200 g were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial 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 Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All efforts were made to minimize the number of animals used and their suffering. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

3.3.2 Pretreatment with creatine

The animals used for experiments in this study were subjected to a pretreatment for 7 days, receiving a daily intraperitoneal injection of creatine (50 mg/kg), or saline.[32] During this pretreatment, stereotaxic surgery was performed in the animals in order to facilitate the administration of GAA, as described below.

3.3.3 Surgery and intrastriatal administration

Surgery and intrastriatal infusion were performed, according to Folbergrova et al. (2001). Animals were anesthetized by intraperitoneal injection

of ketamine and xylazine (100 mg/kg and 14 mg/kg, respectively). The heads of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: −0.5 mm; L: −2.5 mm; DV: −2.5 mm). The cannula was fixed with acrylic cement. Experiments were performed at 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a 5 μL Hamilton micro syringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum.[33]

The animals were divided into four groups: Group 1 (control group), rats that suffered surgery and received intrastriatal infusion of saline and pretreatment with saline; group 2 (GAA-treated), rats that received 10 μM intrastriatal infusion of GAA solution (0.02 nmol/striatum) and pretreatment with saline; group 3 (GAA-treated plus creatine), rats that received 10 μM intrastriatal infusion of GAA solution (0.02 nmol/striatum) and pretreatment with creatine; group 4 (Saline-treated plus creatine), rats that received intrastriatal infusion of saline and pretreatment with creatine. The volume administered intrastriatally (saline or GAA solution) was 2 μL. One hour after intrastriatal infusion, the rats were decapitated without anesthesia (for biochemical studies) or subjected to the behavioral assessment.

3.3.4 Behavioral procedures

Behavioral procedures were performed between 10 a.m. to 3 p.m. in a controlled light and sound room, by a researcher blind to the animal's experimental condition. GAA was injected before the training session, in order to

evaluate the process of memory acquisition. The test session was performed 1h after the training to assess short-term memory.

One day before the training session, all animals were habituated to walk freely in the empty arena for 5 min. The arena used was a black wooden box (50 × 50 × 50 cm). In the training session, two identical objects were placed equidistant from the sidewalls. In this chamber, each animal performed a trial of 5 min. After each trial, the apparatus was cleaned to alleviate olfactory cues. In the second trial, the test session, one of the objects was substituted by a different. An experimenter registered the time of object exploration, i.e., touching it with paws or exploring it by olfaction with direct contact of the snout [34]. The object discrimination index was calculated in the test session, as follows: the difference in exploration time divided by the total time spent exploring the two objects {[(B − A)/(A + B)] where B is the new object and A is the familiar object}. Rats without memory impairment explore the new object for more time when compared with the old one [35].

3.3.5 AChE activity assay

AChE activity was determined by the method of Ellman et al.[40] For AChE assay, striatum was homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000 × g, being the supernatants used for the enzymatic AChE analyses. Hydrolysis rate was measured at acetylthiocholine (S) concentration of 0.8 mM in 1 ml assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of striatum homogenate was added to the reaction mixture and

preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25°C. All samples were run in duplicate. Protein was measured by the Coomassie Blue method according to Bradford.[41]

3.3.6 Oxidative stress parameters

3.3.6.1 Tissue Preparation

The striatum was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, to determine the oxidative stress parameters. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C; the pellet was discarded and the supernatant was immediately separated and used for the measurements.

3.3.6.2 2′,7′-dichlorofluorescein (H_2DCF) oxidation assay

Reactive species production were measured second to LeBel et al. (1992) method [36], based on the oxidation of 2′,7′-dichlorofluorescein (H_2DCF). Samples (60 µL) were incubated for 30 min at 37 °C in the dark with 240 µL of 100 µM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96 wells plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by reactive species presenting in samples. The last reaction produces the fluorescent compound dichlorofluorescein (DCF) which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/mg protein.

3.3.6.3 Superoxide dismutase assay (SOD)

SOD activity assay is based on the auto-oxidation ability of pyrogallol, a process highly dependent on superoxide, which is the substrate for SOD. The inhibition of this compound autoxidation occurs in the presence of SOD, whose activity is then indirectly assayed at 420 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA) [37]. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units per mg of protein.

3.3.6.4 Catalase assay (CAT)

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). The method is based on the disappearance 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 [38.] One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein.

3.3.6.5 Protein determination

Protein concentration was measured by the method of Lowry et al. using bovine serum albumin as standard. [39]

3.3.7 Statistical analysis

The parametric data for four groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test when F-test was significant. Values of $P < 0.05$ were considered statistically significant. All analyzes and graphics were performed using GraphPad Prism 5.1 software program in a compatible computer.

3.4 Results

In the object recognition test, control rats spent less time on the familiar object in the test session when compared to the training (Figure 1A and 1B), thus, the time exploring the novel object increased in the control group ($P \leq 0.05$), while the same did not happened in animals that received intrastriatal infusion of GAA. Creatine per se exerts no effect on this parameter and when associated with GAA was able to prevent the alterations ($P \leq 0.01$). The group treated with GAA also exhibited a reduced discrimination index ($p < 0.05$) (Figure 1C).

We evaluated the effect of GAA intrastriatal infusion and/or creatine administration on AChE activity in striatum of 60-day-old rats. Figure 2 shows that GAA infusion increased AChE in rat striatum ($P \leq 0.01$) after 1h from the infusion. Creatine treatment *per se* did not alter this parameter, but prevented the effect caused by GAA.

Since AChE can be altered by oxidative stress, we also investigated the effect of GAA intrastriatal infusion and/or creatine administration on antioxidant

enzymes, superoxide dismutase (SOD), catalase (CAT), and on reactive species production indicated by the 2′,7′-dichlorofluorescein (DCFH) oxidation. Figure 3 shows that GAA infusion significantly decreased the activity of SOD ($P \leq 0.01$) in rat striatum after 1h from the infusion, and that creatine was not able to reverse such alteration ($P \leq 0.001$). As we can see in Figure 4, GAA intrastriatal infusion also caused a decline in CAT activity ($P \leq 0.01$) under the same circumstances. In this case creatine was able to prevent the alteration and did not altered the activity of this enzyme per se. Furthermore, the reactive species production was increased in rats that suffered GAA intrastriatal infusion when compared with control group ($P \leq 0.01$) (Figure 5). Once again, creatine treatment per se did not alter this parameter, but prevented the effect caused by GAA.

3.5 Figures

Figure 1

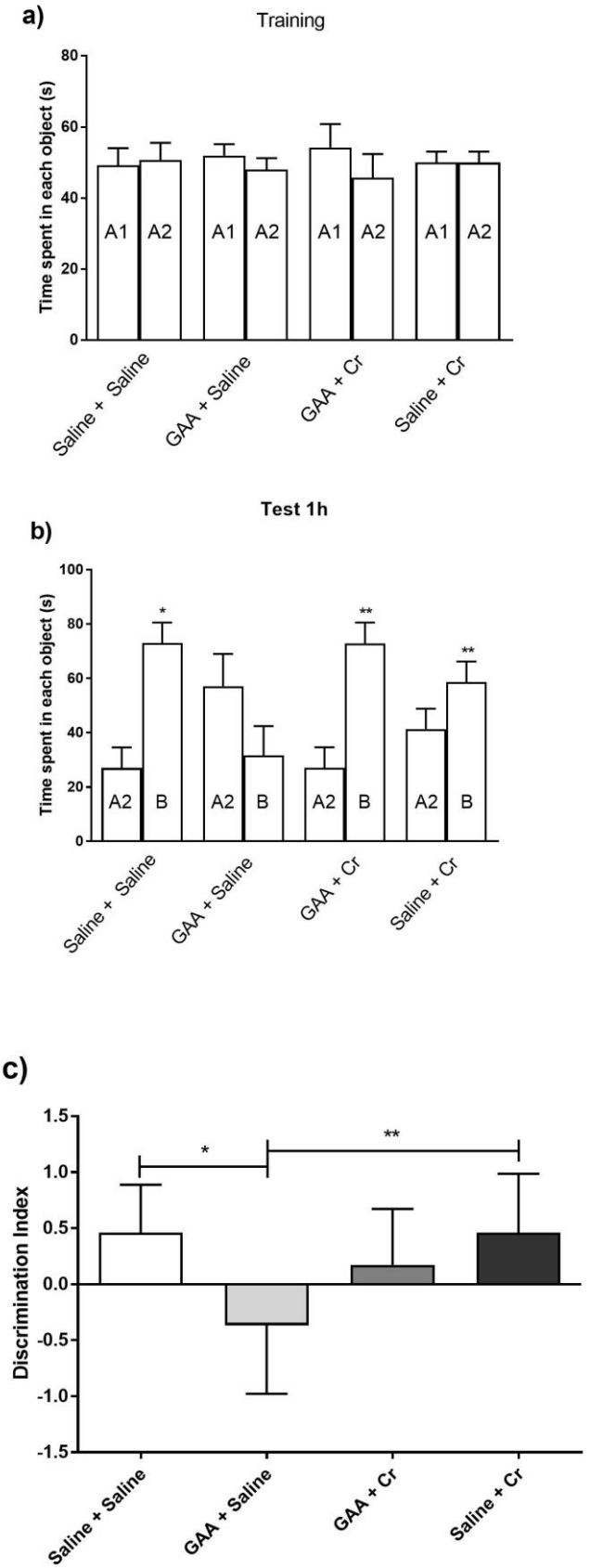


Figure 2

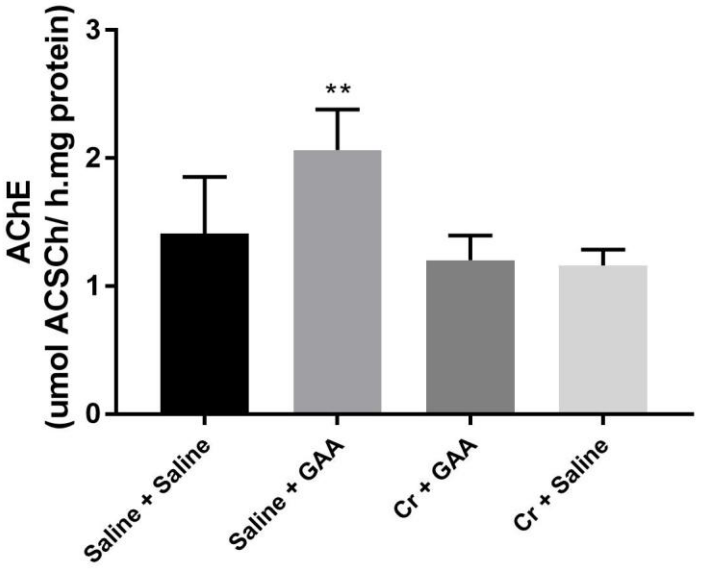


Figure 3

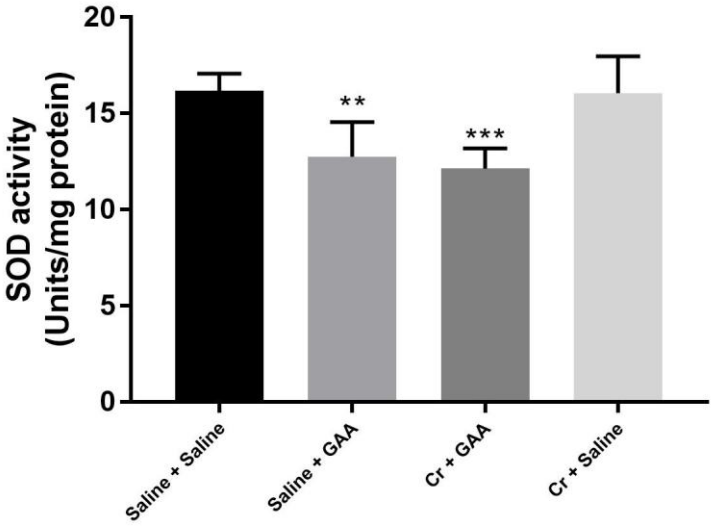


Figure 4

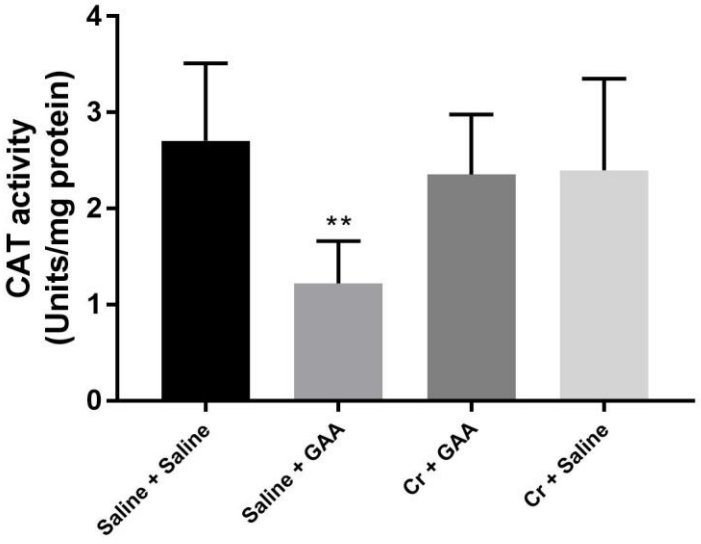
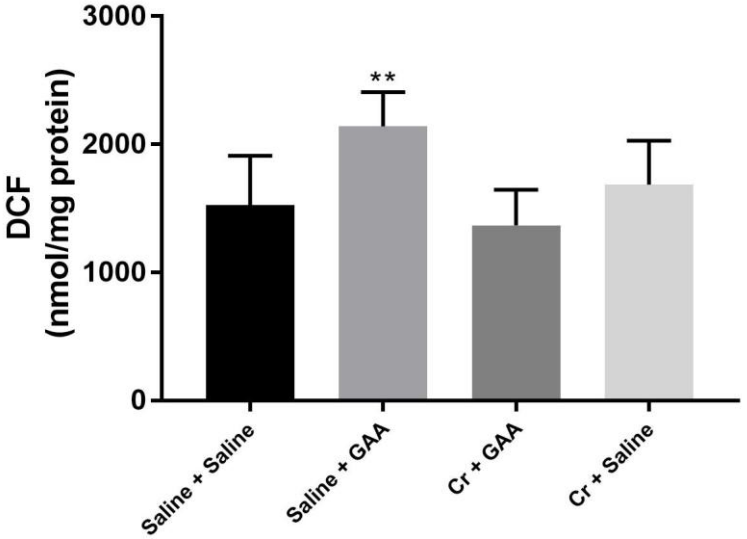


Figure 5



3.6 Discussion

Patients with (GAMT) deficiency present depleted levels of creatine and excessive concentrations of GAA in skeletal muscle, blood, brain and other tissues.[9,10,11] Neurological symptoms are common and variable among individuals affected by GAMT deficiency, including intractable epilepsy, intellectual impairment, autism, auto mutilating behavior, extra pyramidal syndrome, slurred speech and hypotonia. [11,13,14,15] In this sense, the brain becomes the most affected organ in GAMT deficiency patients, but the pathophysiology of this disease is still unclear.

In the present study, we investigated the effects of an intrastriatal administration of GAA on the process of memory acquisition (via novel object recognition tasks), AChE activity, an important enzyme related with learning and memory[29,30], as well as some parameters of oxidative stress such as 2′7′dichlorofluorescein (DCFH) oxidation and antioxidant enzyme activities (SOD, CAT) in striatum of Sixty-day-old wistar rats. Furthermore we investigated the role of creatine on the possible biochemical changes observed in this model, since supplementation with creatine has been used as pharmacological treatment in GAMT deficiency for a long time.[11]

Results show that rats that suffered an intrastriatal infusion of GAA had their performance in the novel object recognition test impaired. This is in agreement with previous data that also found learning and memory deficits in rats subjected to the same model.[45] Nonetheless, the cholinergic system plays a crucial role in cognitive function, including memory.[45] Previous studies have shown that cholinergic neurotransmission modulates aversive conditioning.[46]

Our results showed that GAA significantly increased AChE activity, implying that GAA accumulation affects memory processing, and that AChE probably plays a role in this alteration.

Free radicals formation seems to be involved in a large number of human diseases. Due to accumulation of toxic metabolites, inborn errors of metabolism consist in diseases where this excessive free radical formation is almost always present. Aerobes constantly make reactive species, but modulate their actions by synthesizing antioxidants. This balance allows some reactive species to perform useful functions while minimizing oxidative damage[43]. However, this is not the case on this model. Results showed that GAA intrastriatal infusion increased DCF oxidation, an index of reactive species production. In addition to this, the activities of the antioxidant enzymes SOD and CAT were impaired. Studies have shown that an intrastriatal injection of GAA in rats inhibits the activity of crucial enzymes for the generation of energy in the CNS. These enzymes are Na^+, K^+-ATPase, complex II in the mitochondrial respiratory chain and creatine kinase.[42,44] Alterations in such important enzymes may lead to depleted ATP availability, intensifying the overload of the mitochondrial respiratory chain, and increasing even more the occurrence of oxidative stress.

The cognitive decline observed in this study is probably not caused by a single factor like AChE, but by a range of mechanisms that must be considered.[47] GAA seems to act as a direct agonist of $GABA_A$ receptors with a less efficient GABA-mimetic potential than GABA itself. However, at high concentrations, like the ones found in GAMT patients, GAA is able to cause chronic stimulation in $GABA_A$ and, consequently, receptors undergo

desensitization. This effect causes an increase in neuronal excitability due to internalization of $GABA_A$ receptors and a decreased response to GABA.[48,49]

Creatine has been shown as a safe approach in the prevention and treatment of central nervous system disorders, such as neurodegenerative diseases and cognitive impairments. [50] Considering that more studies are necessary to establish the effects of creatine on experimental models, we also evaluated the effect of this compound on impairments elicited by GAA intrastriatal administration. Creatine concurrent administration prevented the effects caused by GAA in the activity of CAT and in the DCF oxidation, but had no effect on SOD decrease. Furthermore, creatine was able to prevent the increase in AChE activity, allowing ACh to remain for more time in the synaptic cliff, improving the outcome in the object recognition test. In this context, studies report that creatine may be a cytoprotector independent of the antioxidant status of enzymatic defenses, indicating that creatine can act as a direct scavenger of a range of radicals, including superoxide anion and peroxynitrite.[17,18] This effect happens regardless if creatine is in or out a mammalian cell.[19] This is in agreement with previous studies from our group that showed that creatine can act as an antioxidant, preventing the lipid peroxidation in rats submitted to intrastriatal injection of GAA [21].

In summary, our findings demonstrated that intrastriatal administration of GAA affected antioxidants enzymes and caused an increase in the production of reactive species. Furthermore, the activity of the enzyme AChE suffered an increase. Therefore, it is possible to hypothesize that the alteration of this important enzyme for memory and cognition associated with changes in

parameters of oxidative stress may contribute to brain damage causing behavioral and cognitive deficits like the one observed in this study. If this also occurs in GAMT deficiency patients, it is possible that creatine supplementation might be beneficial as a form of adjunct treatment.

3.7 Acknowledgements

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3.8 Conflict of interest

The authors declare that they have no conflict of interest.

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3.10 Figure Legends

Figure 1. Effect of GAA intrastriatal infusion and/or creatine administration on the novel object recognition test. (a) Results in the training session (b) Results in the test session (c) Discrimination index. Data are expressed as mean±SD for 10-12 animals in each group.*p<0.05; **$p < 0.01$; as evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. Cr: Creatine; GAA: Guanidinoacetate.

Figure 2. Effect of GAA intrastriatal infusion and/or creatine administration on AChE activity in striatum of 60-day-old rats. Data are expressed as mean±SD for 7-10 animals in each group. **$p < 0.01$; as evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. AChE: Acetycholinesterase; Cr: Creatine; GAA: Guanidinoacetate.

Figure 3. Effect of GAA intrastriatal infusion and/or creatine administration on SOD activity in striatum of 60-day-old rats. Data are expressed as mean±SD for 7-10 animals in each group. **$p < 0.01$, ***p<0,001; as evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. SOD: Superoxide dismutase; Cr: Creatine; GAA: Guanidinoacetate.

Figure 4. Effect of GAA intrastriatal infusion and/or creatine administration on CAT activity in striatum of 60-day-old rats. Data are expressed as mean±SD for 7-10 animals in each group. **$p < 0.01$; as evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. CAT: Catalase; Cr: Creatine; GAA: Guanidinoacetate.

Figure 5. Effect of GAA intrastriatal infusion and/or creatine administration on the oxidation of DCF in striatum of 60-day-old rats. Data are expressed as mean±SD for 7-10 animals in each group. $^{**}p < 0.01$; as evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. DCF: 2′,7′-dichlorofluorescein; Cr: Creatine; GAA: Guanidinoacetate.

4. CONCLUSÃO

Em resumo, nossos resultados demonstraram que a administração intraestriatal de GAA apresentou um efeito considerável nas atividades de enzimas antioxidantes (SOD e CAT) além de aumentar a produção de espécies reativas (medido através da oxidação do DCF). Além disso, houve um aumento na atividade da enzima AChE. Assim sendo, é possível levantar a hipótese que a alteração dessa importante enzima para a memória e cognição, associada as mudanças nos parâmetros de estresse oxidativo podem contribuir para o dano cerebral, causando déficits comportamentais e cognitivo como o observado neste estudo. Se isso de fato ocorre em pacientes com deficiência na enzima GAMT, é possível que a suplementação com creatina possa apresentar benefícios como tratamento adjuvante.

5. PERSPECTIVAS

- Avaliar conteúdo de BDNF em estriado de ratos submetidos à injeção instraestriatal de GAA;
- Avaliar função mitocondrial (massa e potencial) e níveis de ATP em ratos submetidos à injeção instraestriatal de GAA;
- Avaliar expressão gênica e imunoconteúdo de AChE em estriado de ratos submetidos ao modelo de injeção intraestriatal de GAA;
- Avaliar a captação de glutamato e o imunoconteúdo dos seus transportadores (GLAST e GLT-1) em ratos submetidos à injeção instraestriatal de GAA;
- Avaliar marcadores inflamatórios (IL-6 e TNF-α, IBA1) em ratos submetidos à injeção instraestriatal de GAA;

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ANEXO A - REVIEW INTITULADO "**Guanidinoacetate Methyltransferase Deficiency: A Review of Guanidinoacetate Neurotoxicity"**

Abstract

Guanidinoacetate Methyltransferase (GAMT) deficiency is an autosomal recessively inherited disorder of the metabolism of creatine that leads to depleted levels of creatine and excessive concentrations of guanidinoacetate. Patients affected develop neurological symptoms during childhood, such as muscular hypotonia, involuntary extrapiramidal movements, convulsions, slurred speech, and even autism. Although the pathophysiology of GAMT-deficiency is unclear, neurological dysfunction is commonly found in this disease and it has been mainly attributed to reduction of creatine or/and increase of guanidinoacetate levels. Reports from literature suggest that guanidinoacetate may interfere with neuronal $GABA_A$ receptors and cause epilepsy in man. Preclinical studies show that guanidinoacetate increases free radical formation and decreases brain antioxidant defenses, inducing alteration in oxidative status. Guanidinoacetate also impairs energy metabolism in brain. The discussion of this review focuses on various and latest studies addressing GAMT deficiency, creatine metabolism, as well as addresses the question of neurotoxicity guanidinoacetate.

Key words: Guanidinoacetate Methyltransferase – GAMT Deficiency; Creatine; Guanidinoacetate; Neurotoxicity.

Guanidiniacetate and creatine metabolism — an overview

Guanidinoacetate (GAA) is a metabolite of glycine in which the amino group can be converted into a guanidine. It is highly involved in the metabolism of creatine, being its direct precursor. [1]

Creatine has been known for its essential functions in muscle, contributing to a transient intracellular storage of metabolic energy mainly in the form of phosphocreatine that is nothing more than creatine with a phosphate group.[1] Mammals take about half of their creatine from diet (mostly from meat and fish), and synthesize the other half through a relative simple reaction that occurs mainly in the kidney and liver involving a two-step pathway with two enzymes and one membrane carrier.[2] The enzymes involved are L-arginine: glycine amidinotransferase (AGAT) and *N*-guanidinoacetate methyltransferase (GAMT), while the membrane carrier is named SLC6A8. As suggested by the names of the relevant enzymes, this endogenous synthesis of creatine requires three amino acids, glycine, arginine and methionine.[3] The first step of creatine biosynthesis is catalyzed by AGAT, and occurs mainly in kidney, more frequently in the mitochondria intermembrane space and in lower levels in the cytoplasm.[4] It condenses the amino acids arginine and glycine to produce GAA. GAMT transfers a methyl group from *S*-adenosylmethionine (SAM) to GAA to produce creatine. The majority of creatine that arise from this second step is produced in the liver.[4] The creatine produced in the liver is secreted in the bloodstream by an unknown mechanism and

distributed throughout the body, where it actively enters the cells using specific creatine transporter, SLC6A8, a Na^+- and Cl^- -dependent symporter.[5,6] The phosphorylation of creatine is made possible by the creatine kinases (CK).[7] This is an efficient way to store energy not only to skeletal muscle, but also to organs that require a great amount of energy in order to efficiently develop their activities in human body, like the brain.[8] The phosphocreatine has a slightly higher diffusion capacity than ATP, and it can reversibly transfers its N-phosphoryl group to ADP when this nucleotide is at high concentrations, regenerating ATP and preventing the tissue from running out of energy. [9, 10, 11]. Approximately two thirds of creatine are transformed in phosphocreatine by the isoforms of CK,[12] showing the importance of this molecule as intracellular storage of metabolic energy. Creatine and phosphocreatine may undergo a non enzymatic reaction of dehydration and cyclization to form creatinine that freely diffuses to the bloodstream and leaves the body in the urine. This molecule is frequently used as a marker of the renal function.[13] Creatine synthesis is regulated physiologically in kidney by down-regulation of AGAT, and in developing brain cells by retro regulation of AGAT by creatine levels.[14] Creatine transporter (SLC6A8) is regulated by creatine levels in the bloodstream, and is inhibited by high levels of GAA.[1] Furthermore, the ingestion of creatine supplement has been shown to decrease the rate of endogenous synthesis. [14]

GAMT deficiency

In 1994, Stöckler et al, described a new inborn error of metabolism (IEM) that was adressed as the first inborn error of metabolism of creatine. The new disease was named after the affected enzyme: guanidinoacetate methyltransferase (GAMT) deficieny.[15] This metabolic disease is an autosomal recessively inherited disorder caused by mutations in the *GAMT* gene that leads to depleted levels of creatine and excessive concentrations of GAA in skeletal muscle, blood, brain and other tissues.[16,17,18] It is a rare disease with approximately 110 cases reported so far, mostly from Europe and the Middle East.[19] The cerebral tissue relies upon a continuous energy supply in order to maintain its functions properly. Therefore, with creatine in low levels, the brain becomes the most affected organ in GAMT deficiency patients. Neurological symptoms are common and variable among individuals affected by GAMT deficiency, including intractable epilepsy, intellectual impairment, autism, auto mutilating behavior, extra pyramidal syndrome, slurred speech and hypotonia. [16,17,20,21]

Supplementation with creatine has been used as pharmacological treatment in GAMT deficiency for a long time.[17] Nonetheless, one of the major issues surrounding GAMT deficiency and the brain is the difficulty that creatine has to penetrate the blood brain barrier (BBB) due to the small amount of SLC6A8 transporter present in the microcapillaries, the absence of the same transporter in astrocytes lining them, and the limited diffusion of creatine through extracellular matriz surrounding BBB.[22] To provide sufficient levels of creatine in

healthy individuals, the brain has its own synthesis expressing AGAT and GAMT locally. In the cortex and other brain regions, AGAT and GAMT are dissociated and the intermediate GAA has to be transported between AGAT- and GAMT-expressing cells to complete the synthetic pathway.[23] The presence of this endogenous creatine synthesis is supported by studies that demonstrated that despite the inability to capture creatine from periphery, patients with SLC6A8 deficiency still have normal levels of this molecule in their central nervous system (CNS), while GAMT deficiency patients presented a dramatic reduction in comparison.[24] The phenotypic diversity in patients with GAMT deficiency may be partially explained by the variety of functions that creatine seems to play in the brain. Apart from its functions in energy regeneration, researchers proposed that creatine may act as a neurotransmitter in the CNS modulating GABAergic and/or glutamatergic neurons.[25] This theory is supported by studies that show that rat brain synaptosomes express creatine transporter SLC6A8,[26] implying the existence of a creatine reuptake mechanism in axon terminal membrane.[27]

The energetic reservoir provided by the system creatine/creatine kinase also helps to prevent overload of the mitochondrial respiratory chain, reducing generation of reactive species that have the power to cause oxidative stress and induce cytochrome C dissociation from the inner mitochondrial membrane, initiating early apoptotic triggering events.[28] In fact, several studies claim that creatine can not only prevent the generation of reactive species, but also act as a direct scavenger of a range of radicals, including

superoxide anion and peroxynitrite.[29,30] This effect happens regardless if creatine is in or out a mammalian cell.[31] A recent study revealed that creatine is able to prevent damage induced in vitro by oxidizing agents in both nucleated and non-nucleated cells, suggesting that creatinine may possess similar properties.[32] These antioxidant proprieties presented by creatine appear to be beneficial not only to GAMT,[33] but also to other innate errors of metabolism where creatine was able to prevent lipid peroxidation and imbalance of redox homeostasis.[34,35]

Neurotoxicity of GAA

Despite the fact that decreased levels of creatine play a key role in the physiopathology of GAMT deficiency, the evidence shows that elevated levels of GAA in the brain is the major responsible for triggering the neurological symptoms of patients affected by this disease, particularly the epileptogenic action.[21] Under physiological conditions, no transport of GAA occurs at the BBB, but there is a way out via taurine transporters in the Blood Cerebrospinal Fluid Barrier (BCSFB).[22] Under GAMT deficiency there is uptake of GAA from the periphery at BBB, and the exit of GAA from CSF to blood may be increased using both TauT and SLC6A8 which is also capable of transporting GAA.[22,29] The accumulation of GAA also happens via the brain endogenous AGAT activity, that is plainly functional. It is suggested that high concentrations of GAA can interfere with neuronal $GABA_A$ receptors but not $GABA_B$-receptors and cause the neurological dysfunction behind the symptoms presented by GAMT deficiency

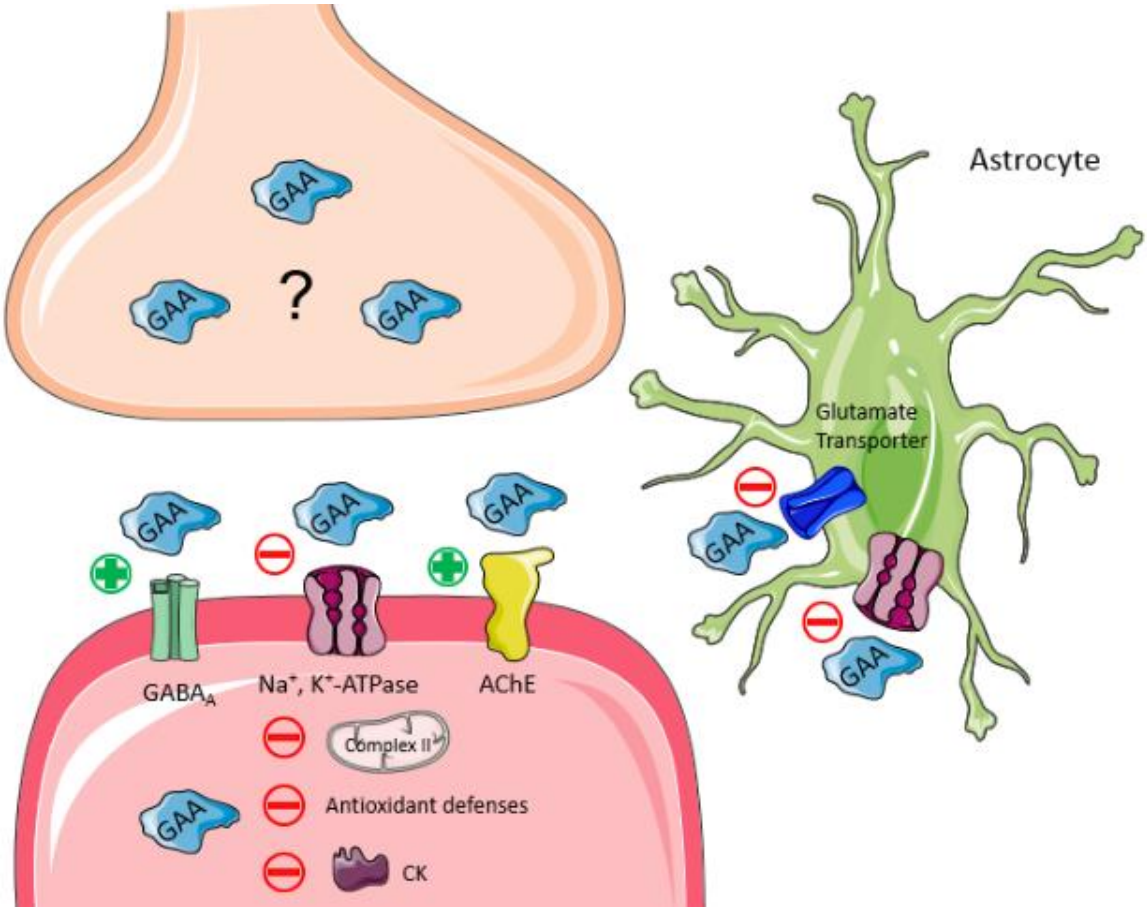
patients.[36] GAA seems to act as a direct agonist of $GABA_A$ receptors with a less efficient GABA-mimetic potential than GABA itself. However, at high concentrations, like the ones found in GAMT patients, GAA is able to cause chronic stimulation in $GABA_A$ and, consequently, receptors undergo desensitization. This effect causes an increase in neuronal excitability due to internalization of $GABA_A$ receptors and a decreased response to GABA.[37,38] The basal ganglia output nuclei globus pallidus and substantia nigra pars reticulata are involved in both movement and seizure control through GABAergic projections that appear altered in experiments conducted in vitro.[37] Therefore, the motor dysfunctions and particularly seizure activity in patients with GAMT deficiency may be explain by interference of GAA in these interconnections present in the basal ganglia.

In addition, studies have shown that an intrastriatal injection of GAA in rats leads to a series of biochemical alterations and abnormalities in behavioral tests. Firstly, the intraestriatal injection appeared to cause a decrease in antioxidant defenses at the same time that inhibit the activity of crucial enzymes for the generation of energy in the CNS. These enzymes are Na^+, K^+-ATPase, complex II in the mitochondrial respiratory chain and creatine kinase.[39,40] Nonetheless, the cholinergic system plays a crucial role in cognitive function, including memory, and results showed that GAA significantly increased acetylcholinesterase (AChE) activity and impaired retention of an inhibitory avoidance task, implying that GAA accumulation affects all steps of memory processing following distinct time-dependent mechanisms.[41] Furthermore, another study showed that GAA markedly inhibited glutamate uptake by striatum, what can be linked to the neurological dysfunction characteristic of GAMT-deficient patients, especially the

generalized convulsions.[42] These series of heterogenic alterations appear to be involved in the pathophysiology of the neurological features present in patients with GAMT-deficiency.

Suggested mechanisms of neurochemical effects of GAA accumulation in brain are summarized in figure 1.

Figure 1.



Clinical cases and possible treatments to GAMT deficiency

The diagnosis of GAMT deficiency can be made based upon a series of tests that include creatinine excretion in 24-hour urine, Sakaguchi staining reaction of guanidino compounds in urine samples, GAA excretion measured quantitatively, and magnetic resonance spectroscopy to detect accumulation of GAA and depletion of creatine in the brain.[43] Measurements of GAMT activity are now available for some cell types.[44] Once the pathology is confirmed, the treatment of GAMT deficiency aims for both repletion of cerebral creatine and reduction of GAA concentrations.

Oral supplementation with creatine (administered as creatine- monohydrate) at high doses can restore brain levels and result in improvements to the patients mostly in the involuntary extrapiramidal movements and convulsions.[16,17] The benefits of this supplementation can be reached only with long term treatment due to the slow cross of creatine through BBB. The positive effects may take months or even years to appear. The doses used are normally around 400 mg-2g/kg/ day. This dose appears to be safe, since excess of creatine may cause oxidative stress.[32] A pre-supplementation investigation of kidney function might be considered for reasons of safety, but in normal healthy subjects appears unnecessary.[45] A recent study performed by Peters et al. (2015) showed that the supplementation with creatine (3 g per day) decreases GAA concentrations in plasma of Bangladeshi adults without GAA deficiency.[46] However, It is important to highlight that creatine supplementation *per se* do not fully normalize GAA levels in patients with GAMT deficiency. In order to achieve an even better clinical outcome, studies suggest that lowering the ingestion of one of the precursor

substrates of GAA, arginine, as well as high-dose L-ornithine supplementation are necessary.[16,47,48] L- ornithine acts via competitive inhibition of AGAT. Furthermore, sodium benzoato administration may reduce the production of GAA via conjugation with glycine to form hippuric acid that is quickly excreted.[18] Nonetheless, a recent treatment applied in a 6 year old GAMT deficiency patient showed no further benefits of using sodium benzoato combined with creatine and arginine restriction, which questions the relevance of this additional supplementation.[49]

This evidence-based treatment using creatine, ornithine, and sodium benzoate supplements along with dietary protein restriction was able to improve seizures and development in five patients with GAMT deficiency.[50] The average age of diagnosis in this study was 25.8 ± 26.2 months, but one of the subjects was diagnosed earlier, at 8 days from birth. The remarkable fact is that this subject treated at birth remains developmentally normal at 12 months of age, while the others still face neurological dysfunctions. This is in agreement with adult and child cases of GAMT deficiency, where diagnosis and treatment later in life correlates with persistency of motor and neurological impairments, despite minor improvements.[49,51,52]

A larger study collected data on 48 patients with GAMT deficiency worldwide,[48] and showed that the clinical presentation of this innate error of metabolism is indeed highly variable, and best treated with supplementation with creatine and L-ornithine, and low intake of arginine. The median age at diagnosis in this study was 51 months, and then again, those who were diagnosed earlier in life

developed normally or showed only borderline developmental outcomes than those who were not. This may be due to the fact that BBB in developmental brain is not as tightly regulated as in adults, and this can facilitate the cross of exogenous creatine into the brain. Furthermore, 3D organotypic brain cell cultures were heavily affected by increased levels of GAA, that caused axonal hypersprouting and an increase in non-apoptotic cell death.[14] This strongly affects brain cell development in an irreversible way and may explain why later diagnosis fails to normalize completely the symptoms presented by GAMT deficiency patients.

Although early treatment seems to prevent permanent damage from occur during CNS development, it is important to note that treatment should continue through lifetime, as we can see in the case of one of the patients in the study conducted by Stockler et al., 2014. Treatment was started within the first3 weeks of life. At 32 months her development was normal while her late treated brother (who also have GAMT deficiency) faced more difficulties. However, at age four year the treatment was discontinued, and she was affected by episodes of febrile seizures. She recommenced the treatment, and even though her current status is not as good as in the beginning of treatment, it is still better than her late-treated brother. This provides evidence of the importance of searching for new Prenatal Diagnosis of GAMT, like measurements in the amniotic fluid and direct sequencing of the GAMT gene in newborns**.**[53,54]

Conclusion

In conclusion, GAMT deficiency is a rare innate error of metabolism of creatine that results in severe neurological effects. It is a treatable disorder, since supplementation with creatine, depletion of arginine in the diet, and supplementation with ornithine considerably ameliorates the symptoms. The seizures do not respond to conventional epileptic treatment and there still controversy about the effectiveness of using sodium benzoate supplementation. Moreover, early diagnosis and start of treatment improve the outcome, and may even lead, if begun pre-symptomatically, to normal development. Ideally, treatment should start before the creatine pool supplied from maternal body during gestation ends. This makes GAMT deficiency an important target to pre- natal diagnosis. The treatment must be uninterrupted and always guided by health professionals in order to avoid nutritional deficits and low compliance. Nevertheless, a lot of study is still needed to better understand the mechanism behind all the neurological dysfunctions presented by patients, and finding a better way to remove brain GAA is the main goal of researches in the field.

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Legends:

Figure 1. Suggested mechanisms of neurochemical effects of GAA accumulation in brain. Under GAMT deficiency there is uptake of GAA from the periphery and GAA synthesis in the neuron occurring mainly in the mitochondria intermembrane space via AGAT. GAA leaves the neuron through an unknown mechanism and due to the lack of functional GAMT it accumulates in the synaptic cliff and within the neuron itself. This accumulation leads to a series of imbalances in enzymes, receptors and redox state. GAA seems to act as a direct agonist of $GABA_A$ receptors, leading to their desensitization and internalization. It appears to cause a decrease in antioxidant defenses at the same time that inhibits the activity of crucial enzymes for energy generation: Na^+, K^+-ATPase, complex II in the mitochondrial respiratory chain and creatine kinase. GAA significantly increased AChE activity, leading to less ACh available in the synaptic cliff. Furthermore, GAA markedly inhibited glutamate uptake by astrocytes.