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AVALIAÇÃO DOS EFEITOS DE ANTIMICROBIANOS SOBRE PARÂMETROS GLUTAMATÉRGICOS, OXIDATIVOS E INFLAMATÓRIOS EM CULTURAS PRIMÁRIAS DE ASTRÓCITOS.

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 a obtenção do título de Bacharel(a) em Biomedicina.

Área de habilitação: Bioquímica

Orientador: Prof. Dr. André Quincozes dos Santos.
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RESUMO

Os astrócitos são células altamente dinâmicas que mantêm a homeostase do sistema nervoso central, bem como, regulam sistemas de neurotransmissores e atuam no processamento das informações sinápticas, do metabolismo energético, das defesas antioxidantes e da resposta inflamatória. Baseado nisso, o cultivo dessas células vêm sendo amplamente utilizadas para estudos metabólicos referentes à resposta inflamatória, ao estresse oxidativo, ao sistema glutamatérgico e à senescência. Manter o microambiente celular asséptico é fundamental para que possamos entender a funcionalidade astrocitária *in vitro* sem inferência de microrganismos, para isso comumente é realizada a adição de antimicrobianos aos meios de cultivos. A partir disso, percebemos que existe uma carência de protocolos padronizados na literatura acerca das classes e concentrações desses fármacos. Portanto, neste trabalho avaliamos alterações em parâmetros glutamatérgicos, oxidativos e inflamatórios em culturas de astrócitos frente aos antimicrobianos gentamicina e penicilina/estreptomicina adicionados ao meio de cultivo em diferentes concentrações. Nas células cultivadas com gentamicina foram observadas algumas alterações no metabolismo energético, como o aumento da captação de glutamato dose-dependente e a diminuição da captação de glicose e do transportador de glicose tipo 1 (GLUT1). Também foi observado aumento nos níveis de glutationa (GSH), porém nenhuma alteração na atividade da enzima superóxido dismutase foi encontrada. Ainda, observamos uma elevação nos níveis de interleucina (IL)-1β e uma redução nos níveis de IL-10. Já as células cultivadas com penicilina/estreptomicina também apresentaram variações no metabolismo energético como: aumento da captação de glutamato dose-dependente e aumento da captação de glicose. Além disso, foram notadas alterações no citoesqueleto de actina e elevação nos níveis de IL-6. Tais achados sugerem, portanto, que a adição desses fármacos ao meio de cultivo interfere nas funções metabólicas astrocitárias. Logo, o uso não padronizado desses antimicrobianos pode explicar as discrepâncias entre experimentos realizados por diferentes grupos de pesquisas.

Palavras-chave: astrócitos, antimicrobianos, gentamicina, penicilina, estreptomicina.

ABSTRACT

Astrocytes are dynamic glial cells that maintain central nervous system homeostasis, regulating neurotransmitter systems, and process synaptic information, energy metabolism, antioxidant defenses, and inflammatory response. In the sense, astrocyte cultures have been widely used as tool for metabolic studies concerning the inflammatory response, oxidative stress, glutamatergic system and senescence. In order to obtain reliable results, these cultures need to be aseptic and it is commonly obtained through the addition of antimicrobials to the culture medium. In line with this, we noticed that there is a lack of protocols in the literature about the class and concentration of these drugs added to the culture medium. Therefore, in this work, we evaluated changes in glutamatergic, oxidative and inflammatory parameters in primary astrocytes cultured with the antimicrobials gentamicin and penicillin/streptomycin in different doses. Cells cultivated with gentamicin presented changes in energy metabolism, such as a dose-dependent increase in glutamate uptake and a decrease in glucose uptake and in the expression of glucose transporters type 1 (GLUT1). We also observed an increase in glutathione (GSH) levels, but no change in superoxide dismutase activity. Additionally, an increase in interleukin (IL)-1β levels and a reduction in IL-10 levels were observed. Astrocytes cultivated with penicillin/streptomycin also presented variations in energy metabolism: a dose-dependent increase in glutamate uptake as well as in glucose uptake. Furthermore, actin cytoskeleton and IL-6 levels were also changed. Such findings suggest that the addition of antimicrobials to the culture medium alters the astrocyte metabolism. Therefore, the non-standard use of these drugs might explain changes between experiments performed by different research groups.

Keywords: astrocytes, antimicrobials, gentamicin, penicillin, streptomycin.

1. INTRODUÇÃO

1.1 SISTEMA NERVOSO CENTRAL

O parênquima do sistema nervoso central (SNC) é constituído por dois grandes tipos celulares: os neurônios, responsáveis pela transmissão e processamento de informações, e as células gliais, que possuem diversos papéis que garantem o adequado funcionamento cerebral (Brady *et al.*, 2012). As células gliais são divididas em oligodendrócitos, microglia e astrócitos. Os oligodendrócitos são responsáveis principalmente pela síntese de mielina, as células microgliais são as células fagocíticas envolvidas principalmente na resposta inflamatória e as células astrocitárias desempenham uma série de funções essenciais para a homeostase do SNC (Gomes *et al.*, 2013), destacam-se, estas, como componentes majoritários (Raine *et al.*, 2006; Kettenmann *et al.*, 2008).

1.2 ASTRÓCITOS

Em 1846, o patologista Rudolf Virchow descreveu pela primeira vez a neuroglia e a caracterizou como sendo células pequenas e de forma arredondada, que preenchiam o espaço extracelular e faziam parte do tecido conectivo (Somjen *et al.*, 1988). Os astrócitos constituem aproximadamente 50% do número total de células do SNC. Possuindo, ainda, a maior diversidade funcional do cérebro com uma capacidade dinâmica de modificar seu fenótipo no decorrer da vida (Shao *et al.*, 1994). Com o avanço das técnicas o estudo das propriedades do SNC, a compreensão sobre a funcionalidade das células astrocitárias apresenta-se muito mais complexa e importante do que se imaginava anteriormente.

Os astrócitos são células altamente variáveis em sua morfologia e função, sendo cruciais para a manutenção da homeostase do SNC. Estas células são responsáveis por captar glutamato – o principal neurotransmissor excitatório do SNC – através da atividade dos transportadores de glutamato-1 (GLT-1) e transportador glutamato-aspartato (GLAST) (Anderson *et al*., 2000; Danbolt *et al.*, 2001). Os astrócitos também são essenciais na conversão de glutamato em glutamina através da enzima glutamina sintetase (GS), sendo a glutamina gerada nos astrócitos por essa enzima e lançada de volta para os terminais pré-sinápticos para ser utilizada na síntese *de novo* do neurotransmissor glutamato nos neurônios (Schousboe *et al.*, 2013). Após essa conversão, o glutamato pode ser utilizado como uma importante fonte energética neuronal (Bak *et al.* 2006).

Entretanto, o substrato mais utilizado nas demandas energéticas cerebrais é a glicose e os astrócitos também expressam transportadores de glicose do tipo 1 (GLUT1) na membrana dos seus processos celulares adjacentes aos capilares sanguíneos (Mergenthaler *et al*., 2013; Benarroch *et al*., 2014). Desta maneira, eles captam glicose do sangue e a utilizam, em parte oxidativamente, para gerar ATP e suprir a energia necessária para suas rotas metabólicas, e em parte anaerobicamente, levando a um aumento do lactato intracelular (Pellerin *et al.*, 2002).

Além disso, os astrócitos são essenciais para a manutenção das defesas antioxidantes no SNC. Esse sistema de defesa antioxidante pode ser dividido em não enzimático e enzimático. Em relação à defesa antioxidante não enzimática, os astrócitos são responsáveis pela síntese de glutationa (GSH), um tripeptídeo formado pelos aminoácidos glutamato, cisteína e glicina, que exerce seu efeito antioxidante reagindo diretamente com as espécies reativas de oxigênio (EROs) (Pope *et al.*, 2008). Paralelamente, no que se refere à defesa antioxidante enzimática, devem ser destacadas as três principais enzimas antioxidantes: (1) superóxido dismutase (SOD), capaz de dismutar o radical superóxido a peróxido de hidrogênio; (2) catalase (CAT), responsável por detoxificar o peróxido de hidrogênio a oxigênio e água; e (3) glutationa peroxidase (GPx) agente inativador de peróxidos orgânicos e que possui importância vital na redução dos níveis de GSH (Bélanger *et al.*, 2009; Kimelberg *el al*., 2010).

Ainda na abrangência de suas funções, essas células também são capazes de reconhecer os eventos iniciais do processo inflamatório, bem como responder e amplificar a sinalização microglial (Wang *et al.*, 2008). Durante o processo inflamatório os astrócitos liberam uma gama de citocinas anti e pró-inflamatórias com intuito de atrair leucócitos e aumentar sua proliferação no local da inflamação (Jensen *et al.*, 2013). Em relação às citocinas anti-inflamatórias liberadas pelos astrócitos pode ser ressaltada a interleucina - 10 (IL-10), a qual pode manter a homeostase imune (Oliveira *et al*., 2016). Por outro lado, dentre as principais citocinas pró-inflamatórias produzidas e secretadas por astrócitos, estão o fator de necrose tumoral α (TNF-α), a interleucina 1β (IL-1β) e a interleucina-6 (IL-6) (Jensen *et al.*, 2013). Estes mediadores inflamatórios quando secretadas pelo astrócitos, são responsáveis por induzir o aumento do processo inflamatório (Oliveira *et al*., 2016).

Outro importante papel dos astrócitos é sua capacidade de responder a lesões no SNC, sejam elas resultantes de patologias, traumas, insultos químicos ou doenças genéticas, através da ativação do processo de astrogliose (ou gliose reativa) (Sofroniew *et al.*, 2009). Os astrócitos

possuem uma proteína marcadora de diferenciação celular, a proteína glial fibrilar ácida (GFAP), uma proteína que apresenta filamentos intermediários do tipo III e peso molecular aparente de 50kDa (Bramanti *et al.*, 2010; Yang *et al.*, 2015). Com o intuito de conter os danos causados por diferentes lesões, os astrócitos alteram sua morfologia e se proliferam, tornando-se reativos (processo caracterizado por rápida síntese de filamentos intermediários de GFAP) formando uma cicatriz glial, que impede a proliferação, principalmente, de mediadores inflamatórios (Sofroniew *et al.*, 2009).

1.3 CULTURA DE ASTRÓCITOS CORTICAIS

Como os astrócitos são responsáveis por inúmeras e distintas funções relacionadas à homeostase do SNC, a cultura dessas células pode ser utilizada para caracterizar propriedades bioquímicas, farmacológicas e morfológicas do SNC. Essa metodologia vem sendo amplamente utilizada como modelo para estudos metabólicos relacionados à resposta inflamatória, ao estresse oxidativo, ao sistema glutamatérgico e à senescência (Pertusa *et al.*, 2007; Xiang *et al.*, 2014; Bakshi *et al.*, 2015; Copetti-Santos *et al.*, 2015).

Com base nisso, nos últimos anos, nosso grupo - assim como outros - vem buscando elucidar o papel dos astrócitos em situações patológicas que acometem o SNC (Bellaver *et al.*, 2015b, 2016; Caito *et al.*, 2013; Xiang *et al.*, 2014), uma vez que essas células emergem como um promissor alvo para moléculas profilático-terapêuticas. A cultura de células consiste em processos de isolamento e manutenção da viabilidade e da proliferação celular de um determinado tipo de tecido. Isso ocorre em um sistema *in vitro* constituído por nutrientes e fatores essenciais para sua sobrevivência perante condições específicas de temperatura, pH e osmolaridade (Barbosa *et al*., 2015).

Em 1961, Hayflick&Moorhead foram pioneiros no uso de antibióticos para prevenir a contaminação bacteriana em cultivos celulares, onde a adição desses fármacos aos meios de cultivo permitiu que a técnica fosse continuadamente disseminada e utilizada arbosa *et al*., 2015). Para a manutenção destas culturas, observa-se na literatura a adição não padronizada de antimicrobianos de diferentes classes e em diferentes concentrações ao meio de cultivo (Meshitsuka *et al*., 2008; Zhu *et al.*, 2015). Apesar de se acreditar que os antibióticos possam influenciar no modo como as culturas celulares respondem aos estímulos a que são expostas, ainda não existem estudos robustos relacionados a tal tema.

1.4 ANTIMICROBIANOS

Antimicrobianos são compostos naturais, sintéticos ou semi-sintéticos, que são capazes de inibir o crescimento ou causar a morte de fungos ou bactérias; sendo eles classificados como bacteriostáticos ou bactericidas, respectivamente (Walsh *et al.*, 2003). Após a descoberta da penicilina por Alexander Fleming, em 1928, vários outros antimicrobianos foram descobertos e aprimorados, como a estreptomicina e a gentamicina, ambos da classe dos aminoglicosídeos (Guimarães *et al.*, 2010).

A penicilina é classicamente obtida a partir de um extrato de cultura fluida do fungo *Penicillium notatum* e pertence ao grupo dos betalactâmicos – antibióticos que possuem o anel azetidinona de quatro membros (ou anel betalactâmico). Seu núcleo fundamental – ácido 6-aminopenicilênico – é o requisito estrutural para sua atividade biológica, que faz com que este antimicrobiano aja de modo eficaz contra bactérias gram-positivas e apresente baixa toxicidade (Guimarães *et al*. 2010). Esse fármaco tem como alvo a enzima transpeptidase e atua inibindo a formação de ligação cruzada entre cadeias de peptideoglicano, impedindo a formação correta da parede celular bacteriana (Suárez *et al.*, 2009; Guimarães *et al*., 2010).

A estreptomicina, por sua vez, é isolada a partir de um microrganismo do solo, o *Streptomyces griseus*, e é efetiva contra bactérias gram-negativas. Esse fármaco pertence ao grupo dos aminoglicosídios e apresenta efeito bactericida por ligar-se especificamente à subunidade 30S dos ribossomos bacterianos. Isso impede o movimento do ribossomo ao longo do RNAm e, consequentemente, interrompe a síntese proteica (Doi *et al.*, 2016; Guimarães *et al*., 2010). A estreptomicina pode ser administrada pelas vias intramuscular profunda e intravenosa e, por possuir elevada toxicidade (Brunton *et al.*, 2012), geralmente é administrada em associação a outros fármacos, como ocorre com sua combinação à penicilina, a qual promove um efeito bactericida sinérgico (Guimarães *et al*., 2010; Wargo *et al*., 2014). Paralelamente, devido a sua alta toxicidade e ao fato de ser menos ativa do que outros membros de sua classe contra bastonetes gram-negativos aeróbicos (Brunton *et al.*, 2012), esse antibiótico não é mais utilizado em monoterapias, o que faz com que seja substituído por outros fármacos.

Para substituições monoterápicas, o antibiótico substituto costuma ser a gentamicina, a qual é utilizada no tratamento de infecções graves causadas por bactérias gram-negativas resistentes a outros fármacos, embora ela também apresente um certo grau de toxicidade (Brunton

et al., 2012; Destache *et al*., 2014). A gentamicina, que também faz parte da classe dos aminoglicosídeos, é produzida por um actinomiceto – *Micromonospora purpúrea* (Ghaffar *et al*., 2015). Seu mecanismo de ação, semelhante ao citado para a estreptomicina, consiste na interrupção da síntese proteica (Guimarães *et al*., 2010). Muitos tipos diferentes de infecções podem ser tratadas com esse antibiótico; todavia, devido a sua moderada toxicidade, o uso prolongado deve ser restrito ao combate às infecções potencialmente fatais e àquelas em que um agente menos tóxico é contraindicado ou é menos efetivo (Brunton *et al.*, 2012).

Por fim, todos os antibióticos citados anteriormente são utilizados tanto no tratamento de patologias *in vivo* quanto em técnicas laboratoriais *in vitro*. Diferentes concentrações destes antimicrobianos são extensivamente utilizadas em meios de cultivo celular, sendo a utilização deles considerada apenas como uma medida adicional de segurança, não como um método de esterilização dos meios de cultura. Além disso, estes antimicrobianos são utilizados, na maioria dos casos, apenas como viabilizadores do cultivo celular, não devendo influenciar nas funções celulares propriamente ditas. Considerando isso, na escolha do antibiótico, deve-se principalmente levar em conta dois fatores: o grau de citotoxicidade do antibiótico e a resposta celular do mesmo, uma vez que a interação entre ambos é muito variada (Freshney *et al.*, 2006; Belenky *et al*., 2015).

2. JUSTIFICATIVA

Considerando: i) a ampla utilização da cultura de astrócitos como ferramenta de estudos para elucidar o papel destas células em modelos experimentais, ii) a utilização não padronizada de antibióticos por diferentes grupos de pesquisa no preparo da cultura astrocitária, e iii) a possibilidade da obtenção de resultados não fidedignos devido ao uso variável destes antimicrobianos, torna-se relevante elucidar o efeito de diferentes classes/concentrações de antimicrobianos em culturas de astrócitos. Portanto, esse trabalho de conclusão de curso contribui para elucidar a influência que os antimicrobianos podem exercer sobre a funcionalidade astrocitária basal e ainda ajuda a esclarecer divergências observadas em estudos com culturas de astrócitos realizadas por diferentes grupos de pesquisa.

3. OBJETIVO GERAL

Avaliar alterações em parâmetros glutamatérgicos, oxidativos e inflamatórios relacionadas à utilização de diferentes classes e concentrações de antimicrobianos em culturas primárias de astrócitos corticais.

3.1 OBJETIVO ESPECÍFICO 1

Verificar alterações em proteínas gliais e em parâmetros glutamatérgicos a partir da cultura de astrócitos em um meio de cultivo contendo penicilina/estreptomicina ou gentamicina, ambos em diferentes concentrações. Para este fim, os seguintes parâmetros foram avaliados: (a) imunoconteúdo de GFAP, (b) captação de glutamato, (c) imunoconteúdo dos transportadores GLT-1 e GLAST e (d) atividade e imunoconteúdo da enzima GS.

3.2 OBJETIVO ESPECÍFICO 2

Avaliar se diferentes classes e concentrações de antimicrobianos promovem alterações na captação de glicose astrocitária. Para isso, após as culturas terem sido mantidas em meio contendo penicilina/estreptomicina ou gentamicina, os seguintes parâmetros foram avaliados: (a) captação de glicose e (b) imunoconteúdo de GLUT1.

3.3 OBJETIVO ESPECÍFICO 3

Verificar se alterações em classes e concentrações de antimicrobianos promovem alterações em parâmetros oxidativos. Visando alcançar isso, após as culturas terem sido mantidas em meio contendo penicilina/estreptomicina ou gentamicina, os seguintes parâmetros foram avaliados: (a) níveis de GSH e (b) atividade da enzima antioxidante SOD.

3.4 OBJETIVO ESPECÍFICO 4

Determinar alterações inflamatórias em culturas primárias de astrócitos corticais expostos a diferentes classes e concentrações de antimicrobianos. Para essa determinação, os seguintes parâmetros foram avaliados: (a) TNF-α, IL-1β, IL-6 e IL-10.

4. ARTIGO

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Antimicrobials changed glutamatergic, oxidative and inflammatory parameters in primary astrocyte cultures

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ABSTRACT

Astrocytes are dynamic glial cells that maintain central nervous system homeostasis, processing synaptic information and regulating neurotransmitter systems, energy metabolism, antioxidant defenses, and inflammatory response. In this sense, astrocyte cultures have been widely used as tool for studies concerning glial functionality. In order to obtain reliable results, these cultures need to be aseptic and it is commonly obtained through the addition of antimicrobials to the culture medium. In line with this, we noticed that there is a lack of protocols in literature about the class and concentration of these drugs added to the culture medium. Hence, in this work we evaluated the changes in glutamatergic, oxidative and inflammatory parameters of astrocytes primary cultured with the antimicrobials gentamicin and penicillin/streptomycin at different doses. Cells were cultivated with gentamicin presented changes in energy metabolism, such as a dose-dependent increase in glutamate uptake and a decrease in glucose uptake as well as in the expression of glucose transporters type 1 (GLUT1). We also observed an increase in glutathione (GSH) levels, as well as levels of interleukin (IL)-1β and a reduction of IL-10. Astrocytes cultivated with penicillin/streptomycin also presented variations in energy metabolism: a dose-dependent increase in glutamate uptake and glucose uptake. In addition, actin cytoskeleton and IL-6 levels were also changed. Such findings suggest that the addition of antimicrobials to the culture medium alters the astrocyte metabolism. Therefore, the non-standard use of these drugs might explain between experiments performed by different research groups.

Keywords: astrocytes, cultures, antimicrobials, gentamicin, penicillin, streptomycin

1. INTRODUCTION

Astrocytes are important cells in the central nervous system (CNS), they are involved in the maintenance of the extracellular environment and in the stabilization of cell-cell communications under physiological and pathological conditions [1–5]. These cells participate in a diverse CNS functions, including regulation of neurotransmitter systems, ionic homeostasis, metabolic support, energy metabolism, synaptic information processing (as part of the tripartite synapse), plasticity and neuronal excitability, antioxidant defenses, the maintenance of the blood-brain barrier, and inflammatory response [1–3, 6–10]. These cells release anti- and pro-inflammatory cytokines in regenerative or protective processes such as interleukin (IL)-10, IL-1β, IL-6, and tumor necrosis factor α (TNF- α), respectively [11]. Astrocytes also have a characteristic marking of cell differentiation, a glial fibrillary acidic protein (GFAP), an intermediate type III filament that can be altered when the CNS suffers injury [12, 13].

Astrocytes are also crucial cells in glutamatergic transmission homeostasis performing glutamate uptake through high-affinity glutamate transporters: glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST) [14–17]. This glutamate might be used as a substrate for oxidation in the tricarboxylic acid cycle, synthesize the tripeptide glutathione (GSH) – the major brain antioxidant – or glutamine through the enzyme glutamine synthetase (GS) [18–21]. Moreover, astrocyte cells take up glucose through type 1 glucose transporters (GLUT1) [1, 22, 23].

Cultured astrocytes have been extensively used to characterize the biochemical, pharmacological and morphological properties of the CNS. Based on that, several studies sought to elucidate the role of astrocytes in physiological and pathological conditions [24–27], since these cells emerge as a promising target for prophylactic therapeutic molecules. Cell cultures consist in the isolation and maintenance of a certain type of tissueto maintain viability and cell proliferation. This occurs in an *in vitro* system a favorable environment for cell survival [28].

In order to maintain the cellular environment without contamination, different protocols added antimicrobial agents to the culture medium. Generally, they are added non-standardized in relation to both classes and concentrations [29–31]. These drugs must have not influence in the cellular responses, although there are few studies elucidating the impact of antimicrobial drugs in astrocyte functionality. The most common antimicrobials added to the culture medium

are gentamicin (aminoglycosides), penicillin (beta lactam) and streptomycin (aminoglycosides); The latter two being administered together, because induced a synergistic bactericidal effect [32].

Moreover, standard protocols for antimicrobial use in cell culture medium are not reported in the literature as well the concentrations that are able to influence cellular responses. Therefore, the aim of the present study was evaluate the changes in glutamatergic, oxidative and inflammatory parameters related to the use of different classes and concentrations of antimicrobials in cortical astrocyte cultures.

2. MATERIAL AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and other materials for cell cultures were purchased from Gibco (Carlsbad, CA, USA) ; ELISA for TNF- α and IL-1β were purchased from PeproTech (Rocky Hill, NJ, USA); polyclonal anti-GFAP was purchased from Dako (Carpinteria, CA, USA); polyclonal anti-GLUT1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-GS and anti-GAPDH were from Sigma (St. Louis, MO, USA); polyclonal anti-GLAST and anti-GLT1 were from Alpha Diagnostic (San Antonio, TX, USA); L-[^{3}H]-glutamate, 2-Deoxy-D-[1,2-^{3}H]glucose ([^{3}H]2DG), nitrocellulose membrane and ECL kit were from Amersham (Buckinghamshire, UK). Lastly, both antimicrobials Penicillin/Streptomycin and Gentamicin were purchased from Gibco (Carlsbad, CA, USA).

Animals

Newborn male Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil) and maintained in a controlled environment (12 hours light/12 hours dark cycle; 22 ± 1ºC; ad libitum access to food and water). All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul's Animal Care and Use Committee (process number 29180).

Primary astrocyte cultures

The cortices were removed from the animals and dipped in Hank's Balanced Salt Solution (HBSS). These structures were undergo enzymatic, mechanical and centrifugal dissociation steps. The cells were seeded and cultured in DMEM/F12 culture medium containing 10% fetal bovine serum (SFB) in a 37 °C incubator in an atmosphere containing 5% CO_2 until they reach confluence, which occurs around 10 days [25, 33]. The culture environment was contained the different concentrations of the following antibiotics: penicillin [20 U/ml]/streptomycin [0.02 mg/ml], penicillin [100 U/ml]/streptomycin [0.1 mg/ml], penicillin [200 U/ml]/streptomycin [0.2 mg/ml] or gentamicin [0.1 mg/ml, 0.05 mg/ml, 0.025 mg/ml]. Once the cells reached confluence, they were used for neurochemical experiments.

Immunocytochemistry

Immunocytochemistry was performed as described previously by our group [34]. Briefly, cell cultures were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After blocking overnight with 4% albumin, the cells were incubated overnight with anti-GFAP (1:400) at 4°C; this was followed by PBS washes and incubation with a specific secondary antibody conjugated with Alexa Fluor® 488 (green staining) for 1 hour at room temperature. For all immunostaining-negative controls, the reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was excluded. For actin-labeling analyses, the cells werencubated with 10 mg/ml rhodamine-labeled phalloidin in PBS for 45 min and two washes with PBS. Cell nuclei were stained with 0.2 μg/ml of 4',6'-diamidino-2- phenylindole (DAPI). The cells were visualized with a Nikon inverted microscope and the images were transferred to a computer with a digital camera (Sound Vision Inc.).

Western Blot Analysis

Astrocytes were solubilized in lyses solution containing 4 % SDS, 2-mM EDTA and 50-mM Tris–HCl (pH 6.8). Samples were separated by SDS/PAGE (45 mg protein per sample), and transferred to nitrocellulose membranes, which were then incubated overnight (4 °C) with one of the following antibodies: anti-GFAP (1:1000), anti-GLT-1 (1:1000), anti-GLAST (1:1000), anti-GS (1:10000), anti-GLUT1 (1:1000) or anti-GAPDH (1:1500). GAPDH was used as a loading control. Then, the membranes were incubated with a peroxidase-conjugated

anti-rabbit immunoglobulin (IgG) or with peroxidase conjugated anti-mouse (IgG) at a dilution of 1:20000 for 2 hours. Chemiluminescence signals were detected in an Image Quant LAS4010 system (GE Healthcare) using an ECL kit [34]. The results are expressed as percentages relative to the lowest antimicrobial concentration.

Glutamate uptake

After the cells reached confluence, the glutamate uptake was performed as previously described [35] with some modifications. Briefly, astrocytes were incubated at 37°C in HBSS containing the following components (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 Na_2HPO_4, 0.44 KH_2PO_4, 4.17 $NaHCO_3$ and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.33 µCi/ml L-[2,3-^{3}H] glutamate. The incubation was stopped after 7 minutes by removal of the medium and rinsing the cells twice with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Incorporated radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake, considered "specific uptake", was obtained by subtracting the sodium-independent uptake from the total uptake.

Glutamine synthetase activity

After the cells reached confluence, the GS (EC 6.3.1.2) activity was performed as previously described [36]. Briefly, cell homogenate (0.1 ml – about 50 µg) was added to 0.1 ml of the reaction mixture containing (in mM): 10 $MgCl_2$, 50 L-glutamate, 100 imidazole-HCl buffer (pH 7.4), 10 2-mercaptoethanol, 50 hydroxylamine-HCl and 10 ATP, and incubated for 15 minutes (37°C). The reaction was stopped by the addition of 0.4 ml of a solution containing (in mM): 370 ferric chloride, 670 HCl, and 200 trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated using standard quantities of γ-glutamylhydroxamate treated with a ferric chloride reagent.

2-Deoxy-D-[1,2-^{3}H]glucose ([^{3}H]2DG) uptake

After cells reached confluence, basal glucose uptake and glutamate-stimulated glucose uptake were assessed as previously described [37]. The cells incubated with DMEM/F12 1%FBS for 2 hours at 37°C. The medium was then replaced by DMEM/F12 supplemented with

1%FBS containing 1 µCi/ml [3H]2DG for 20 minutes at 37°C. After incubations, the cells were rinsed with HBSS and lysed overnight with NaOH 0.3 M. Incorporated radioactivity was measured in a scintillation counter.

Glutathione content

After the cells reached confluence, GSH levels were assessed as previously described [34]. Astrocyte homogenates (by about 50 µg) were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% metaphosphoric acid. The supernatant was assayed with o-phthaldialdehyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 µM). GSH concentrations were calculated as nmol/mg protein.

Superoxide dismutase (SOD) activity

SOD (EC 1.15.1.1) activity was determined using the RANSOD kit from Randox (Autrim, UK). The SOD activity (based on the degree of the inhibition of formazan dye formation) in the lysed cells was spectrophotometrically measured at 505 nm. The inhibition of the produced chromogen was proportional to the activity of SOD present in the sample. The results are expressed as percentages relative to the control conditions.

Inflammatory Response Measurement

The TNF-α levels were measured in an extracellular medium using a rat TNF-α ELISA kit from Thermo. The levels of interleukins were measured in an extracellular medium using ELISA kits for IL-1β, IL-6 and IL-10 from Thermo. The results are expressed in nanogram per milliliter. The average minimum sensitivity of the ELISA kit detection is 0.4 ng/ml of cytokines.

3. RESULTS

Evaluation of astrocytic cytoskeleton proteins cultivated with different antimicrobial

The purity of the astrocyte cultures was assessed using cytoskeletal protein GFAP. Immunocytochemical analyses showed an intense cytoplasmic immunolabeling for GFAP and gentamicin or penicillin/streptomycin did not change the immunolabeling (Fig. 1a; Fig 2a).

Using immunoblotting analyses, we observed no significant changes in GFAP expression relative to the 0.025 mg/ml concentration (adopted as control) in cells cultivated with gentamicin (Fig. 1b). In the same way, the astrocytes cultivated with penicillin/streptomycin, did also not change GFAP expression relative to the 20 U/ml or 0.02 mg/ml concentration (adopted as control) (Fig. 2b).

Typically, cultured rat astrocytes show a polygonal to fusiform and flat morphology, and these characteristics were also observed here in cortical astrocytes. Furthermore, actin cytoskeleton is the major determinant of the cell morphology, and our astrocyte cultures cultivated with gentamicin showed significant staining for actin with parallel arrangement of stress fiber (Fig. 1a), whereas those cultivated with penicillin/streptomycin showed changes in parallel arrangement (Fig. 2a).

Increase in antimicrobial concentrations is accompanied by increase in glutamate uptake

A significant dose-dependent increase was observed in the glutamate uptake in astrocytes cultivated with both gentamicin (from 3.7 to 6.1 nmol/mg protein/min, $P = 0.0004$, compared to the lower concentration 0.025 mg/ml with 0.1 mg/ml; Fig. 3a) and penicillin/streptomycin (from 3.8 to 8 nmol/mg protein/min, $P < 0.001$, compared to the lower concentration 20 U/ml/0.02 mg/ml with 200 U/ml/0.2 mg/ml; Fig. 3d). These cells remove glutamate from the extracellular environment through the activity of GLT-1 and GLAST transporters [17]. In this sense, we did not observe changes in the expression of GTL-1 and GLAST in cells cultivated with both antibiotics (Fig. 3b, c, e, f).

Once taken up by astrocytes, glutamate is converted into glutamine by GS, which is constitutively expressed by astrocytes. The western blotting analyses indicate no alteration in the GS expression as well as in the GS activity in the cells cultivated with two antimicrobials (Fig. 4a, b, c, d).

Glucose uptake and GLUT1 transporter expression showed changes in astrocytes cultivated with antimicrobials

Astrocytes are capable of capturing blood glucose, providing energy for maintaining neuronal activity. This process is performed by the GLUT1 transporter, which is expressed by these cells [22]. Based on that, we observed that glucose uptake was significant reduced (from 27 to 22 nmol/mg protein/min, $P = 0.0447$; Fig. 5a) in astrocytes cultivated with 0.1 mg/ml of

gentamicin compared to the lowest concentration 0.025 mg/ml. We also observed a significant decrease (from 16% to 5%, P = 0,0529; Fig.5b) in the GLUT1 transporter expression in astrocytes cultivated in a medium containing the highest concentration of gentamicin. On the other hand, cells cultivated with 200 U/ml/0.2 mg/ml penicillin/streptomycin in the medium presented an increase in glucose uptake (from 83 to 127 nmol/mg protein/min, P = 0.0076; Fig. 5c). Penicillin/Streptomycin, in all evaluated concentrations, did not change GLUT1 expression levels (Fig 5d).

Alteration in the system of antioxidant defenses in the astrocytic cells cultivated with gentamicin and penicillin/streptomycin

We evaluated the levels of GSH - non-enzymatic antioxidant defense - and we observed a significant increase in GSH content only in the astrocytes cultivated with 0.1 mg/ml of gentamicin compared to 0.025 mg/ml (from 2,9 to 3,9 nmol/mg protein, P =0.0185; Fig 6a). However, in cells cultivated with penicillin/streptomycin, GSH levels did not change (Fig. 6c). Concerning to the enzymatic antioxidant defenses we evaluated the SOD activity – capable of dismuting the radical superoxide to hydrogen peroxide. Thus, we did not observe significant alterations in SOD activity of astrocytes cultivated with gentamicin nor penicillin/streptomycin (Fig. 6b, d).

Changes in the inflammatory response in astrocytes exposed to distinct antimicrobial

Astrocytes actively participate in the inflammatory response releasing anti- and proinflammatory cytokines [38]. As shown in Fig. 7b in cells cultivated with gentamicin increased the levels of IL-1β (from 39 to 46 ng/ml, $P < 0.0001$, compared the concentration 0.025 mg/ml and 0.05 mg/ml with 0.1 mg/ml). While the anti-inflammatory IL-10 levels were decreased (from 41 to 37 ng/ml, P = 0.0300, compared the concentration 0.025 mg/ml with 0.1 mg/ml; Fig. 7d) in the same cultures. However, TNF-α and IL-6 levels showed no significant alterations among different concentrations. On the other hand, in cells cultivated with penicillin/streptomycin, we observed an increase in the proinflammatory cytokine IL-6 levels (from 34 to 40 ng/ml, P = 0.0185 compared the concentration 20 U/ml/0.2 mg/ml with 200 U/ml/0.2 mg/ml; Fig. 7g). At the same time, the other pro inflammatory cytokines TNF-α and IL-1β (Fig. 7e, f) demonstrated no significant changes as well as we did not observe alterations in the anti-inflammatory cytokine IL-10 (Fig. 7h).

4. DISCUSSION

The astrocytes are majority in the CNS and represent approximately half of the cells of the human brain [39, 40]. These cells are highly variable in morphology and function, being essential in CNS homeostasis [39]. Cell culture is a widely used technique, since it is possible to recreate an ideal environment for the cells *in vitro*, mimicking pathophysiological conditions that occur *in vivo*. This environment consists of nutrients and essential factors for its survival, and also needs to be free from microbial contaminations, as the coexistence of cells with microorganisms might alter specific cellular responses. In this sense, antimicrobials are usually added to the cell culture medium in order to make the environment aseptic. Among the most used drugs to obtain this asepsis are gentamicin, penicillin and streptomycin; the latter two being administered together.

Gentamicin - produced by an actinomycete, *Micromonospora purpurea* - is an antimicrobial that has a half-life of 2.5 hours. It belongs to the class of aminoglycosides and is used against Gram-negative bacteria resistant to other drugs, at the same time that it presents a certain degree of toxicity [41]. On the other hand, the Penicillin is part of the beta-lactam group, has a half-life of 0.5 to 0.7 hours and is obtained from a fluid culture extract of the fungus *Penicillium notatum*; Being even effective against Gram-positive bacteria and presenting low toxicity [32]. Last, the Streptomycin - isolated from a soil microorganism, *Streptomyces griseus;* its half-life is 2 to 3 hours and belongs to the class of aminoglycosides - which is effective against Gram-negative bacteria and has high toxicity [32].

Taking back the context of the astrocytic cultures management and considering the above antimicrobials, in this work we evaluate the energetic metabolism of these cells,-which we focused on the glutamate uptake. This function has great importance for the proper CNS functioning. Sodium-dependent astrocytic uptake is the most important for the maintenance of physiological extracellular levels of glutamate, avoiding its excitotoxic potential. This process is accomplished through the activity of GLT-1 and GLAST transporters [42], which are expressed by astrocytes. Based on these concepts, we observed a dose-dependent increase in glutamate uptake in cells cultivated with both antimicrobials gentamicin and penicillin/streptomycin, which could indicate some cells responsive profile against these two drugs [43]. Furthermore, we verified the immunocontent of the two astrocytic glutamate transporters and no sig-

nificant changes were observed in cells cultivated with both antimicrobials. Therefore, the antimicrobials were able to increased glutamate uptake, but not have been sufficient to modulate the glutamate transporters expression [44].

After glutamate is taken up by astrocytic transporters, it might be converted to glutamine by the enzyme GS - an exclusively astrocytic enzyme in the CNS. This allows these cells an important communication route with the neurons through the glutamate-glutamine cycle [45]. We evaluated this enzyme - both immunocontent and enzymatic activity - and observed no significant changes in the cells cultivated with both antimicrobials. As increased glutamate uptake did not impact on GS activity, two possible metabolic targets for this neurotransmitter may be indicated: (I) its direct use as an energy source by conversion to α-ketoglutarate [46]; or (II) its use in GSH biosynthesis [47].

GSH is a non-enzymatic antioxidant defense that plays its antioxidant effect by reacting with reactive oxygen species (ROS), or by being oxidized by the glutathione peroxidase enzyme [47, 48]. From these concepts, we evaluated the levels of GSH and no significant changes were observed in the cells that had the antimicrobial penicillin/streptomycin added to their culture medium [46]. On the other hand, in cells cultivated with gentamicin, there was an increase of GSH levels at all concentrations evaluated. In particular, a increase in the highest concentration (0.1 mg/ml). This alteration might be justified by the increase in glutamate uptake. As it is used in the synthesis of GSH - the enzyme γ-glutamyl-cysteinyl synthetase catalyzes the bind between glutamate and cysteine to form the γ-glutamyl-cysteinyl dipeptide, which might follow the astrocytic GSH pathway or might be exported from astrocytes and be used as a substrate for neuronal GSH biosynthesis [47]. With regard to the enzymatic antioxidant defenses we can highlight the SOD, which is able to dismutate the superoxide radical to hydrogen peroxide [49]. After evaluating the activity of this enzyme, we observed no significant change in the cells cultivated with both antimicrobials. This tends to indicate that was not necessary a cellular defense - action of SOD - in response to these antimicrobials; since GSH, being an important antioxidant defense, might be reacting and containing any excessive production of ROS [20, 50].

Still on the energetic metabolism of these cells, we evaluated GLUT1 – the main astrocytic transporter of glucose - that captures and distributes glucose in the intracellular environ-

ment, having two important destinations in the astrocytic cells: the glycolytic-and the glycogenic pathways [22]. When evaluating the immunocontent of this transporter and the glucose uptake, in cells cultivated with gentamicin, we identified a significant reduction of both glucose uptake and expression. This occurred only in the medium with the highest antimicrobial concentration. In addition, the evaluation of glucose uptake in response to penicillin/streptomycin, the highest concentration of antimicrobial was also highlighted, but this time as a significant increase. The significant reduction - uptake and immunocontent - in the culture medium containing gentamicin might be explained by the cells needs a stimulus to capture glucose. For this, we can suggest as an explanation the increase of glutamate uptake, as it already serves as an important energetic source for the cell [51]. Based on that, since the cell apparently does not need a high glucose demand, because it has glutamate as energetic source, it is possible that it leads to a reduction of GLUT1 expression, which promotes a reduction in glucose uptake. Also, in cells cultivated with penicillin/streptomycin, these antimicrobials were able to increase the glucose uptake [46], but not sufficient to modulate the GLUT1 expression.

In addition to the functions already mentioned, these cells can also recognize the early inflammatory process events, as well as respond and amplify microglial signaling [3]. These cells release a range of anti and proinflammatory cytokines [3, 52]. Therefore, we evaluated the levels of these cytokines and observed significant alterations in both culture medium; indicating that these drugs are causing an inflammatory process. In addition, changes in the levels of these cytokines - pro or anti-inflammatory - may indicate damage to immune homeostasis (IL-10) [53, 54] and/or activate the inflammatory process (IL-6 and IL-1β) [55, 56].

The cytoskeletal intermediate protein, GFAP - immunocontent and immunocytochemistry were analyzed - no significant changes were observed in cells cultivated with gentamicin; once the presence of significant changes in protein expression may indicate cell damage [13]. This unaltered levels of GFAP may be due to the action of GSH - it refers to the physiological state of the cell - containing ROS overproduction [47, 57]. The actin cytoskeleton was also analyzed, which is a highly dynamic structure that is continuously reorganized whenever the cell responds to the environment, either by altering its shape or by suffering division [58]. In cells cultured with gentamicin, no changes were observed in actin structure. In cells cultivated with penicillin/streptomycin, we also observed no significant changes in both GFAP immunocontent and immunocytochemistry. In the actin cytoskeleton, we observed modifications in the

classical arrangement. The presence of modifications may indicate signal transmission, which leads to an adequate cellular response [58]. Thus, concomitant with the increase observed in proinflammatory cytokines might lead the actin cytoskeleton reorganization [59].

Finally, the findings here described suggest that antimicrobials used in culture medium to prevent contamination may alter astrocytic functionality. Thus, the non-standard use of such drugs in cell cultures may explain possible contrasts between experiments performed by different research groups. Thus, it is important to formulate standardized protocols on the addition of these antimicrobials to the culture medium.

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Conflict of interest

The authors declare there are no conflicts of interest.

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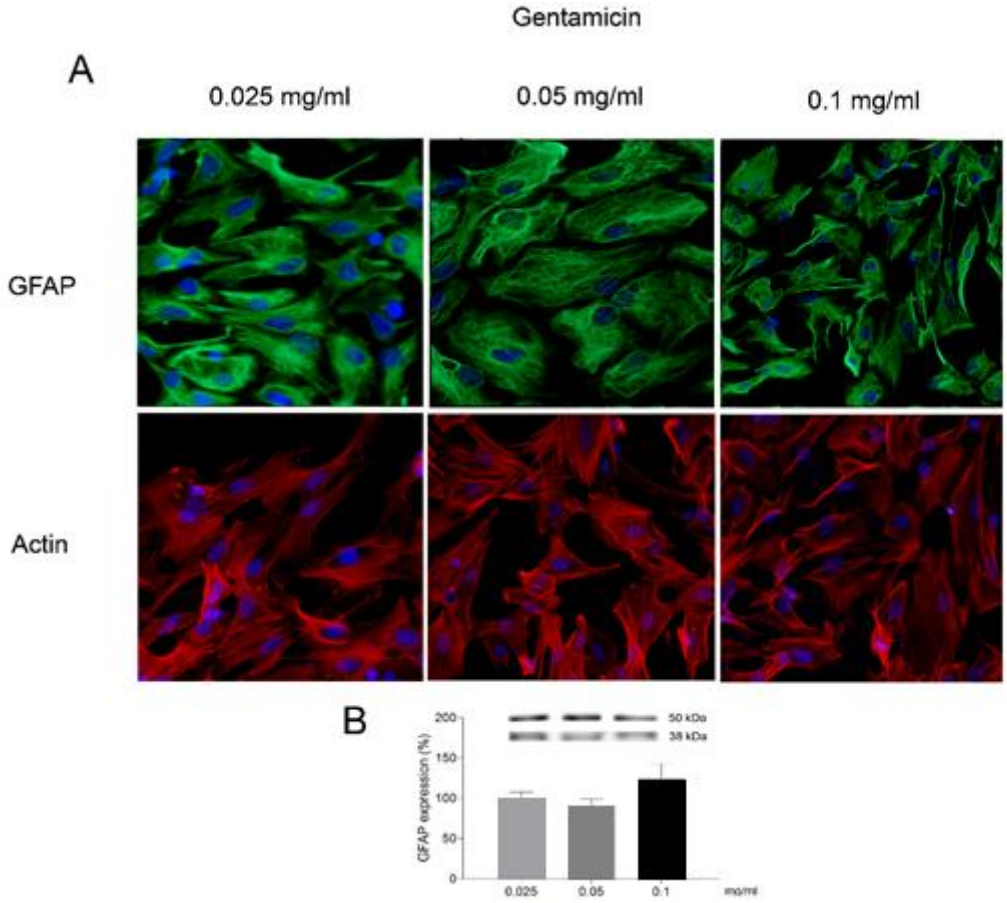

Fig 1. Evaluation of astrocytic cytoskeleton proteins cultivated with gentamicin. (A) Immunostaining for GFAP and staining for actin in different concentrations of gentamicin (B) Representative immunoblot of GFAP (*n*=6). The *bar graph* corresponds to the mean ± S.E.M. and represents the percentages relative to the 0.025 mg/ml concentration (adopted as control). The data were generated in two independent experiments and analyzed statistically using one-way ANOVA followed by Tukey's test.

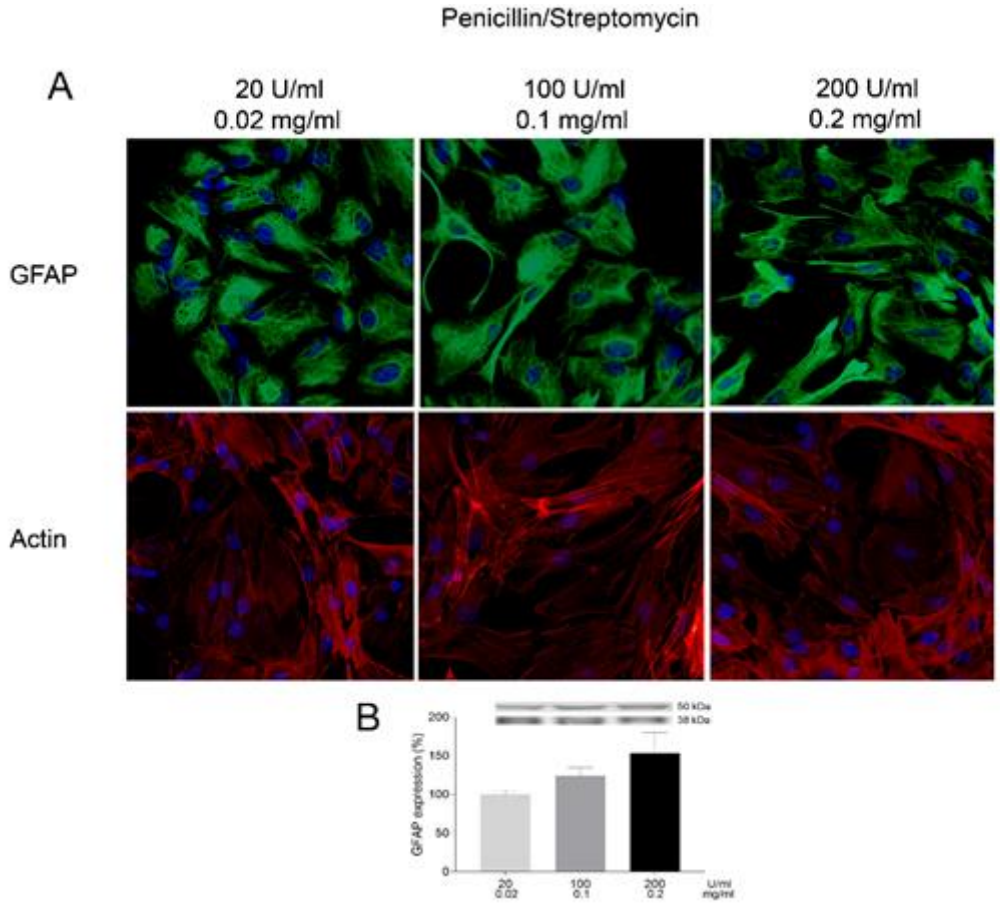

Fig 2. Evaluation of astrocytic cytoskeleton proteins cultivated with penicillin/streptomycin. (A) Immunostaining for GFAP and staining for actin in different concentrations of penicillin/streptomycin. (B) Representative immunoblot of GFAP (*n*=6). The *bar graph* corresponds to the mean ± S.E.M. and represents the percentages relative to the 20 U/ml/0.02 mg/ml concentration (adopted as control). The data were generated in two independent experiments and analyzed statistically using one-way ANOVA followed by Tukey's test.

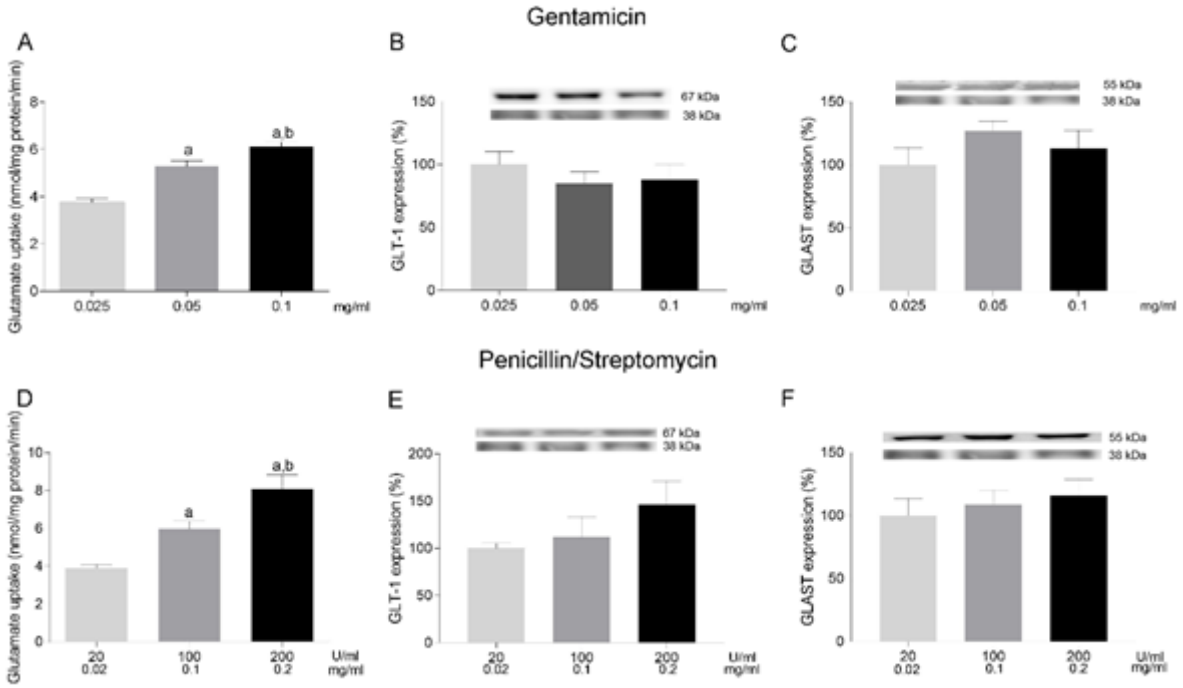

Fig 3. Increase in antimicrobial concentration induced by increase in glutamate uptake. (A) Glutamate uptake. Quantification of (B) GLT-1 and (C) GLAST in astrocytes cultivated with gentamicin. (D) Glutamate uptake, (F) Quantification of GLT-1 and (G) GLAST in astrocytes cultivated with penicillin/streptomycin. Data are expressed as the mean ± S.E.M. and represents the percentages relative to the lowest antimicrobial concentration (adopted as control). The data were generated in four independent experiments with n=6 and analyzed statistically using one-way ANOVA followed by Tukey's test. Values of $p < 0.05$ were consider significant. *a* Indicates differences from lowest antimicrobial concentration; *b* Indicates differences from intermediate antimicrobial concentration.

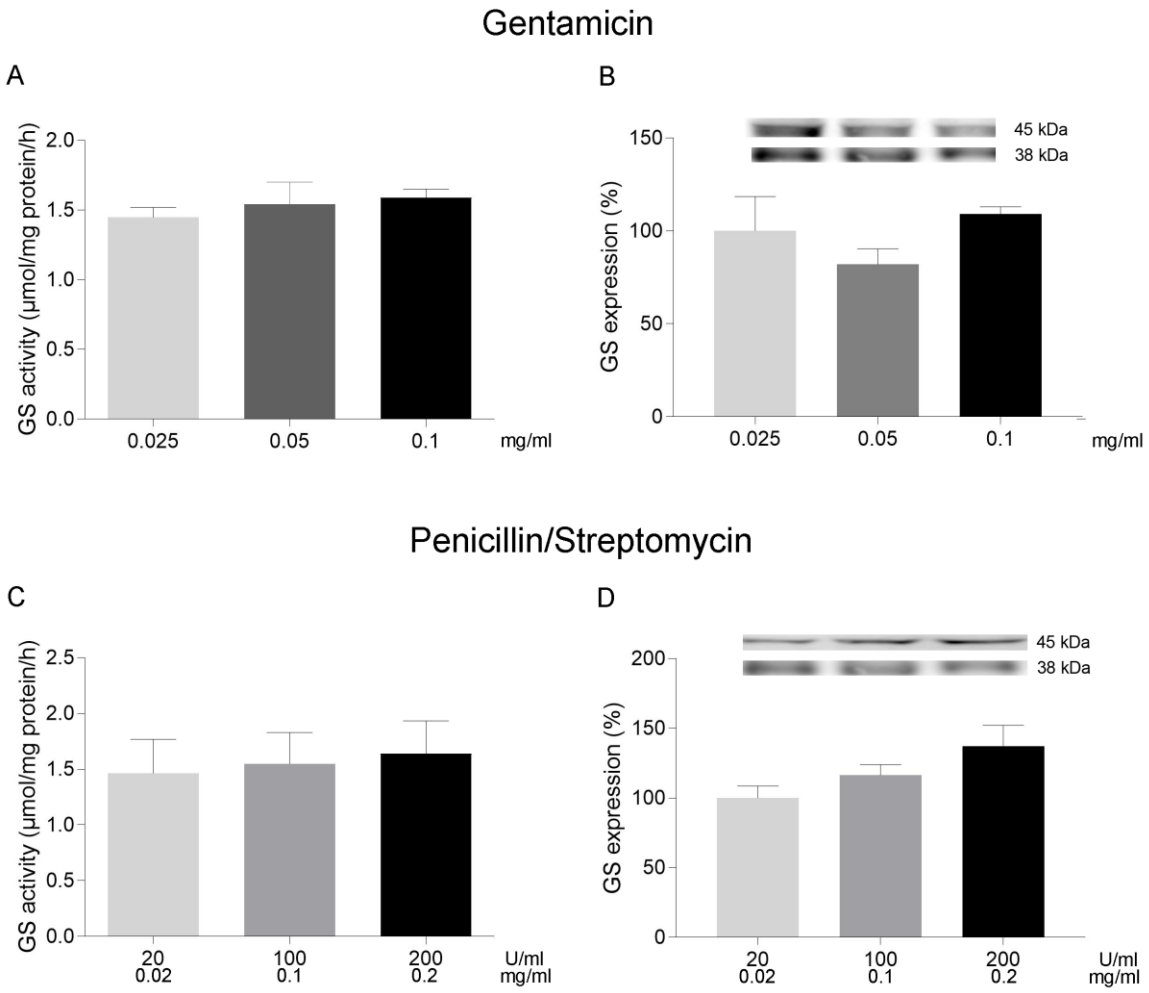

Fig 4. Effects of antimicrobials on GS activity and expression in astrocyte primary cultures. (A) GS activity and (B) GS immunocontent in astrocytes cultivated with gentamicin. (C) GS activity and (B) GS immunocontent in astrocytes cultivated with penicillin/streptomycin. Data are expressed as the mean ± S.E.M. and represents the percentages relative to the lowest antimicrobial concentration (adopted as control). The data were generated in four independent experiments with *n*=6 and analyzed statistically using one-way ANOVA followed by Tukey's test.

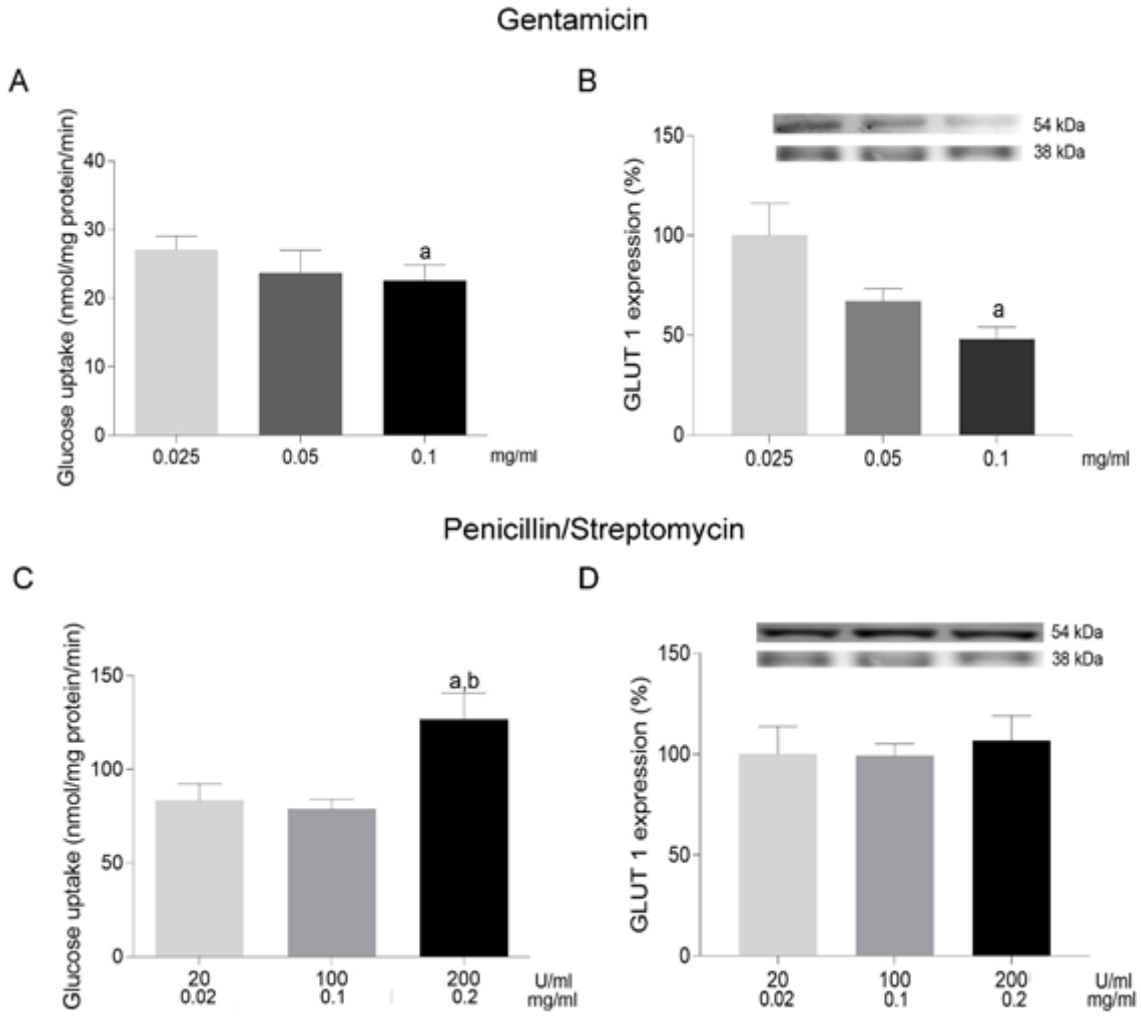

Fig 5. Glucose uptake and GLUT1 transporter expression showed changes in astrocytes cultivated with antimicrobials. (A) Glucose uptake and (B) GLUT1 expression in astrocyte cultivated with gentamicin (C) Glucose uptake and (D) GLUT1 expression in astrocytes cultivated with penicillin/streptomycin. Immunoblots (n=6) data are expressed as the mean ± S.E.M. and represents the percentages relative to the lowest antimicrobial concentration (adopted as control). Analyzed statistically using one-way ANOVA followed by Tukey's test. Values of $p < 0.05$ were consider significant. *a* Indicates differences from lowest antimicrobial concentration; *b* Indicates differences from intermediate antimicrobial concentration.

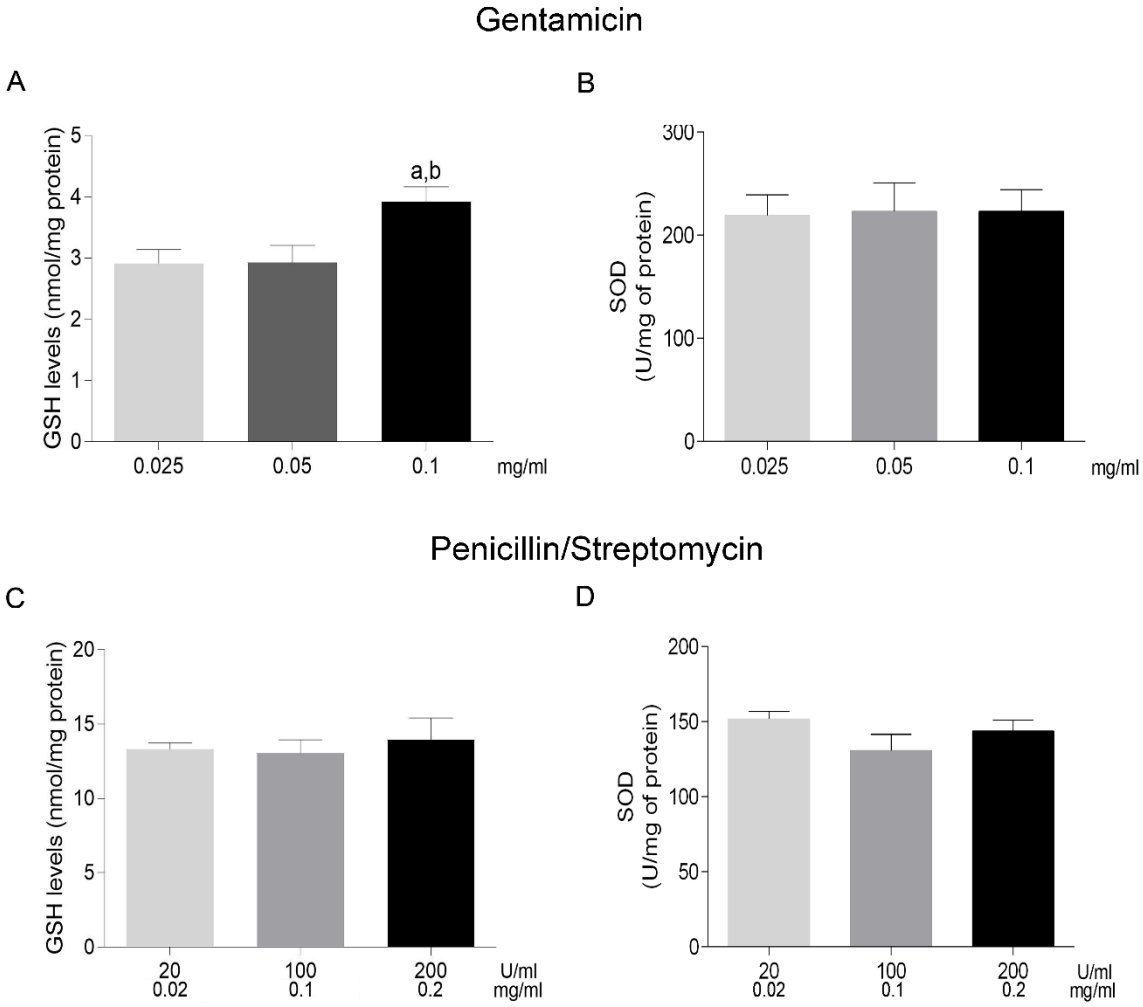

Fig 6. Alteration in the system of antioxidant defenses in the astrocytic cells cultivated with gentamicin and penicillin/streptomycin. (A) GSH levels and (B) SOD activity in astrocytes cultivated with gentamicin. (C) GSH levels and (D) SOD activity in astrocytes cultivated with penicillin/streptomycin. Analyzed statistically using one-way ANOVA followed by Tukey's test. Values of $p < 0.05$ were consider significant. *a* Indicates differences from lowest antimicrobial concentration; *b* Indicates differences from intermediate antimicrobial concentration.

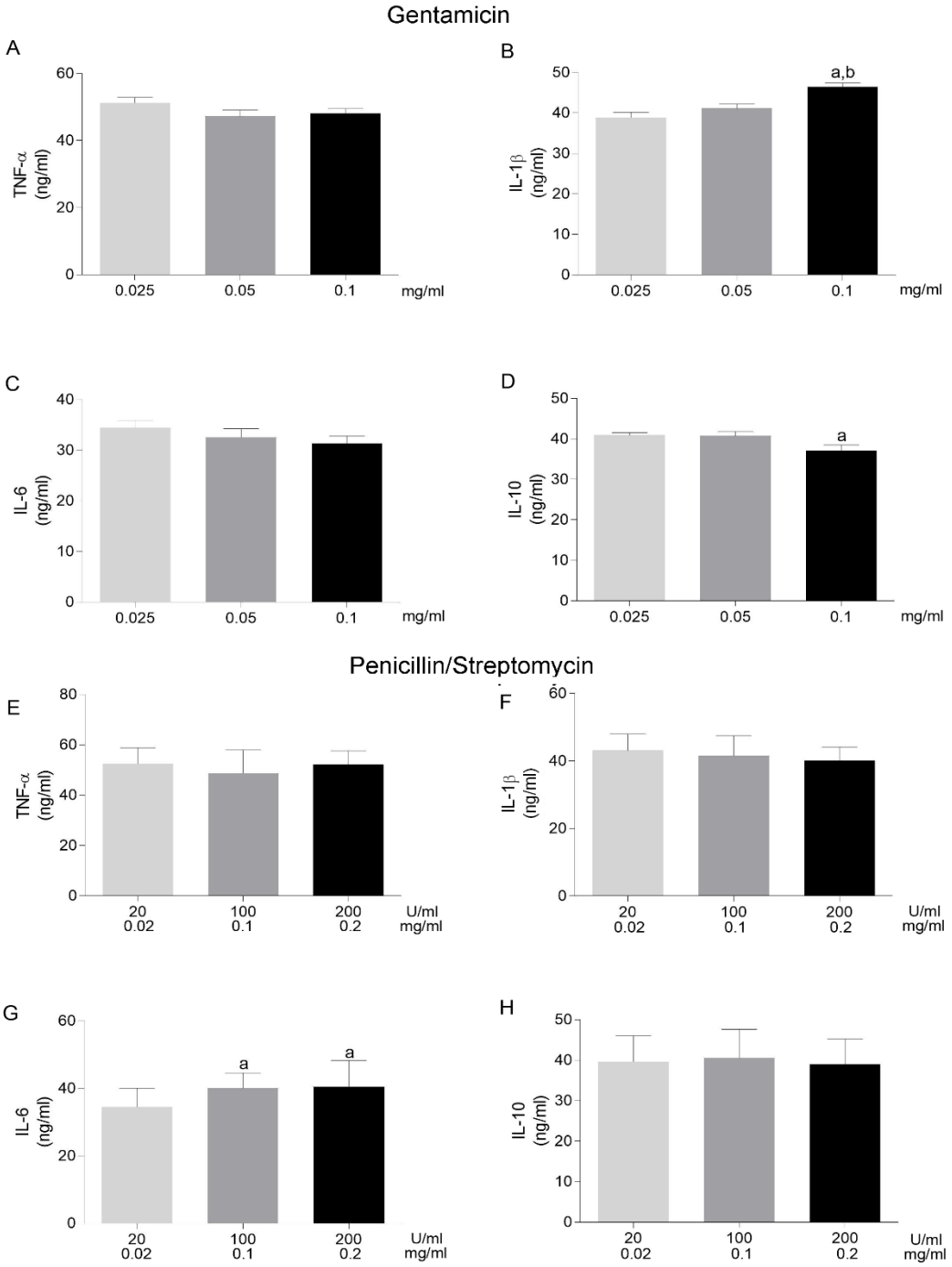

Fig 7. Changes in the inflammatory response of astrocytes exposed to distinct antimicrobial. (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-10 levels in astrocytes cultivated with gentamicin. (E) TNF-α, (F) IL-1β, (G) IL-6 and (H) IL-10 levels in astrocytes cultivated with penicillin/streptomycin. Analyzed statistically using one-way ANOVA followed by Tukey's test. Values of $p < 0.05$ were consider significant. *a* Indicates differences from lowest antimicrobial concentration; *b* Indicates differences from intermediate antimicrobial concentration.

5. CONCLUSÕES E PERSPECTIVAS

Os resultados obtidos neste trabalho sugerem que os antimicrobianos adicionados aos meios de cultivos interferem no metabolismo celular. Pois, observamos algumas alterações no metabolismo energético, nas defesas antioxidantes, no citoesqueleto de actina e, ainda, na respostas inflamatória. Sendo assim, o uso não padronizado desses antimicrobianos pode explicar as discrepâncias entre experimentos realizados por diferentes grupos de pesquisas. A partir disso, percebemos que existe uma necessidade de formular protocolos acerca dos tipos de fármacos e de suas concentrações que serão adicionadas aos meios.

Como perspectivas pretendemos avaliar a ceftriaxona, esse antimicrobiano também pode ser adicionado aos meios de cultivo como uma técnica de assepsia. Esse fármaco demonstrou ser capaz de atuar diretamente na modulação do sistema glutamatérgico, aumentando a captação de glutamato e a expressão de GLT-1 (Rothstein *et al.*, 2005). Além disso, pretendemos realizar mais experimentos neuroquímicos como: avaliação do imunoconteúdo e secreção dos fatores tróficos o fator neurotrófico derivado da glia (GDNF) e o fator neurotrófico derivado do encéfalo (BDNF), atividade das enzimas CAT e GPx e avaliar produção das espécies reativas de oxigênio (DCFH).

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA MOLECULAR AND CELLULAR BIOCHEMISTRY

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The title page should include: The name(s) of the author(s); A concise and informative title; The affiliation(s) and address(es) of the author(s); The e-mail address, and telephone number(s) of the corresponding author If available, the 16-digit ORCID of the author(s)

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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Please provide 4 to 6 keywords which can be used for indexing purposes.

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Manuscripts should be submitted in Word; Use a normal, plain font (e.g., 10-point Times Roman) for text; Use italics for emphasis; Use the automatic page numbering function to number the pages; Do not use field functions; Use tab stops or other commands for indents, not the space bar; Use the table function, not spreadsheets, to make tables; Use the equation editor or MathType for equations; Save your file in docx format (Word 2007 or higher) or doc format (older Word versions); Manuscripts with mathematical content can also be submitted in LaTeX. LaTeX macro package (zip, 182 kB)

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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols. Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

REFERENCES

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

Negotiation research spans many disciplines [3]. This result was later contradicted by Becker and Seligman [5]. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-0080955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J

Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb.

http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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Vector graphics containing fonts must have the fonts embedded in the files.

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If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

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Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.

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To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

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Do not include titles or captions within your illustrations.

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Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.

No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.

Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

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