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EFEITOS ANSIOLÍTICOS DE ACETIL-L-CARNITINA EM PEIXES-ZEBRA

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RESUMO

A acetil-L-carnitina (ALC) é a forma acetilada do aminoácido L-carnitina capaz de modular diversos alvos relacionados a fisiopatologia dos transtornos mentais. A ALC atravessa a barreira hematoencefálica de uma maneira mais eficaz do que a L-carnitina. Estudos demonstraram que a ALC possui atividade antioxidante, neuroprotetora e reguladora da neuroplasticidade, aumentando a expressão de BDNF e de receptores metabotrópicos de glutamato. Dessa forma, considerando o mecanismo multialvo da ALC, o objetivo deste estudo foi investigar os efeitos desse composto sobre parâmetros comportamentais e bioquímicos em peixes-zebra. Diferentes grupos de animais foram tratados com ALC (0,1, 1 e 10 mg/L) e posteriormente submetidos aos testes de tanque-novo, claro-escuro e estresse por perseguição. Animais controle não foram submetidos a nenhum tratamento, mas foram submetidos as mesmas condições experimentais. Posteriormente, os animais foram eutanasiados e os encéfalos coletados para a avaliação de parâmetros relacionados ao estresse oxidativo. ALC apresentou efeito ansiolítico no teste de tanque novo e de claro/escuro, além de prevenir o comportamento ansiogênico induzido por um estressor agudo (estresse por perseguição). Além disso, a ALC foi capaz de prevenir a lipoperoxidação induzida pelo estresse agudo no encéfalo dos peixes-zebra. Os dados apresentados aqui justificam a continuidade das investigações dos potenciais efeitos da ALC no tratamento de transtornos psiquiátricos relacionados ao estresse.

Palavras-chave: Acetil-L-carnitina. Ansiedade. Estresse oxidativo. Peixe-zebra.

ABSTRACT

Acetyl-L-carnitine (ALC) is the acetylated form of the amino acid L-carnitine capable of modulating various targets related to the pathophysiology of mental disorders. ALC crosses the blood-brain barrier more effectively than L-carnitine. Studies have shown that ALC has antioxidant, neuroprotective and neuroplasticity activity, increasing the BDNF and metabotropic glutamate receptors expression. Thus, considering the multi-target mechanism of ALC, the objective of this study was to investigate the effects of this compound on behavioral and biochemical parameters in adult zebrafish. Different groups of animals were treated with ALC (0.1, 1 and 10 mg/L) and subsequently submitted to the novel tank, light/dark and chasing stress tests. Control animals were not subjected to any treatment but were submitted to the same experimental conditions. After the behavioral tests, the animals were euthanized, and the brains were collected for the evaluation of parameters related to oxidative stress. ALC showed anxiolytic effect in the novel tank and light/dark tests, as well as was able to prevent anxiety-induced by chasing stress. In addition, ALC was able to prevent lipid peroxidation induced by acute stress in the zebrafish brain. The data presented here justify the continuation of investigations of the potential effects of ALC in the treatment of stress-related psychiatric disorders.

Keywords: Acetyl-L-carnitine. Anxiety. Oxidative stress. Zebrafish.

SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E SIGLAS

5-HT Serotonina

6-OHDA 6-hidroxidopamina

AKT Proteína Cinase

ALC Acetil-L-carnitina

ATP Adenosina Trifosfato

BAX Regulador Apoptótico

BCL-2 Proteínas Antiapoptóticas

CAT Catalase

COMT Catecol-O-Metil Transferase

DNA Ácido Desoxirribonucleico

DSM-V Manual Diagnóstico e Estatístico de Transtornos Mentais 5ª Edição

EROs Espécies Reativas de Oxigênio

EUA Estados Unidos da América

GABA Ácido Gama-Aminobutírico

GSH Glutationa Reduzida

GSK 3b Glicogênio Sintase Cinase 3 beta

ISRS Inibidores Seletivos da Receptação de Serotonina

ISRSN Inibidores Seletivos da Receptação de Serotonina e da Noradrenalina

MAO Monoaminaoxidase

NAC N-acetilcísteina

NMDA N-metil D-Aspartato

NPSH Grupos Tióis não-proteicos

SOD Superóxido Dismutase

TBARS Substâncias Reativas ao Ácido Tiobarbitúrico

UCS Estresse Crônico Imprevisível

1 INTRODUÇÃO

A acetil-L-carnitina (ALC) é a forma acetilada do aminoácido L-carnitina capaz de modular diversos alvos relacionados a fisiopatologia dos transtornos mentais. Recentemente, estudos pré-clínicos e clínicos demonstraram os efeitos da ALC em parâmetros relevantes para ansiedade, esquizofrenia e transtornos do humor. Dessa forma, considerando o mecanismo multialvo da ALC, o objetivo deste estudo foi investigar os efeitos desse composto sobre parâmetros comportamentais e bioquímicos em peixes-zebra, agregando informações sobre os resultados obtidos em diferentes modelos de ansiedade em peixes-zebra, bem como sobre o potencial mecanismo de neuromodulação da ALC.

1.1 Revisão de literatura

O estresse pode ser definido como uma reação complexa desencadeada pelo organismo frente a estressores de origens variadas. Tal reação é extremamente importante para que o organismo responda adequadamente as demandas do dia a dia (Galvão-Coelho et al., 2015; McEwen and Morrison, 2013). Entretanto, quando essas demandas ultrapassam a capacidade adaptativa do organismo, isso pode predispor o indivíduo a doenças como hipertensão, diabetes, disfunções sexuais e cognitivas, depressão e ansiedade (Portugal et al., 2016).

De acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-V), os transtornos de ansiedade incluem transtornos que compartilham características de medo e angústia excessiva e perturbações comportamentais. Os transtornos de ansiedade incluem transtorno de ansiedade generalizada, transtorno

de ansiedade social, fobias específicas e transtorno de ansiedade de separação. Enquanto o medo é a resposta emocional à ameaça iminente real ou percebida, a ansiedade é a antecipação de ameaça futura (Craske et al., 2009).

As bases neurobiológicas dos transtornos de ansiedade não estão completamente esclarecidas, mas diversos sistemas de neurotransmissores estão envolvidos (Griebel and Holmes, 2013; Holmes et al., 2003). Recentemente, pesquisas têm associado transtornos psiquiátricos com o estresse oxidativo, o qual pode ser definido como o desequilíbrio entre a formação e remoção de agentes oxidantes no organismo, decorrente da geração excessiva de espécies reativas de oxigênio (EROs) e/ou da diminuição de antioxidantes endógenos (Maes et al., 2011; Ng et al., 2008). Resultados recentes do nosso grupo mostraram que N-acetilcisteína, uma molécula que possui atividade antioxidante, apresenta atividade ansiolítica e anti-estresse em peixes-zebra (Marcon et al., 2016) e camundongos (Mocelin et al., 2015). Dessa forma, abordagens que envolvam diminuição e/ou prevenção de estresse oxidativo poderão contribuir com o tratamento de psicopatologias como ansiedade e depressão.

Os primeiros fármacos utilizados para o tratamento dos transtornos de ansiedade foram os benzodiazepínicos. Esses compostos ligam-se a um sítio específico no receptor de $GABA_A$, potencializando o efeito inibitório do neurotransmissor. Após a ligação do GABA ao seu receptor, ocorre abertura do canal de cloreto e influxo desse íon para o meio intracelular, inibindo diversos eventos pós-sinápticos de transdução de sinal. Os efeitos ansiolíticos são mediado pelos receptores $GABA_A$ que contêm a subunidade α_2, enquanto a sedação ocorre por meio da subunidade α_1 (Griebel and Holmes, 2013). Clinicamente os benzodiazepínicos apresentam a vantagem de ter um início de ação rápido, causando redução da

ansiedade, da agressividade ou até sedação, entretanto, podem apresentar efeitos colaterais. Um dos efeitos colaterais mais pronunciados desta classe de fármaco é a amnésia anterógrada. Tais efeitos têm sido demonstrados em animais e humanos (Andreatini et al., 2001; Levitan et al., 2013). Os benzodiazepínicos também possuem um potencial de causar dependência. A exposição crônica provoca modificação na neurotransmissão gabaérgica que contribui para o desenvolvimento de tolerância, dependência e síndrome de abstinência (Nordon et al., 2009; Schallemberger et al., 2016).

Atualmente, o tratamento farmacológico considerado de primeira linha para transtornos de ansiedade são os antidepressivos inibidores seletivos da receptação de serotonina (ISRS) e inibidores seletivos da receptação de serotonina e da noradrenalina (ISRSN) (Griebel and Holmes, 2013). Os ISRS atuam no neurônio pré-sináptico inibindo especificamente a recaptação de serotonina. O aumento da disponibilidade sináptica de serotonina estimula a função de receptores (5-HT) pós-sinápticos, e acredita-se que a estimulação desses receptores contribui para os efeitos adversos característicos dessa classe de fármacos, incluindo efeitos gastrointestinais (náuseas, vômitos) e sexuais (demora ou comprometimento do orgasmo). A fluoxetina, a paroxetina, a sertralina e o escitalopram são exemplos de fármacos dessa classe. Dos fármacos ISRSN, a venlafaxina foi mais amplamente estudada, mas a duloxetina também já apresenta eficácia comprovada. No entanto, os ensaios clínicos com ISRS e ISRSN mostram 60 a 70% de resposta terapêutica. Além disso, em pacientes que fazem uso prolongado de ISRS ou ISRSN, a disfunção sexual e o embotamento afetivo se apresentam como limitações importantes (Amaral, 2014; Braga et al., 2010; Castillo et al., 2000).

Apesar da ampla variedade de tratamentos disponíveis para os transtornos de ansiedade, os efeitos adversos relacionados ainda causam problemas relevantes e contribuem para a baixa adesão ao tratamento. Relevante para este projeto estima-se que em torno de 50% dos pacientes sejam refratários a esses tratamentos, justificando o grande interesse no desenvolvimento de novos fármacos mais efetivos. É provável que esse objetivo só seja alcançável com o desenvolvimento de fármacos com mecanismo de ação inovador.

1.2 Acetil-L-carnitina (ALC)

O uso de suplementos nutricionais tem crescido ao longo das últimas décadas. Entre as substâncias que têm recebido grande atenção, destaca-se a ALC que é popularmente utilizada como coadjuvante na redução de gordura corporal e como termogênico (Brass, 2000; Silva et al., 2012).

A carnitina, um nutriente essencial da dieta, atua como carreador de ácidos graxos para o interior da mitocôndria onde ocorre a β-oxidação. Altas concentrações de carnitina podem ser encontradas em tecidos e células nas formas livre ou como acilcarnitinas, como a acetil-L-carnitina (Steiber et al., 2004). ALC possui inúmeras funções biológicas como auxiliar a captação de acetil-CoA na mitocôndria durante a oxidação dos ácidos graxos, estimular a produção de acetilcolina e a síntese de proteínas e de fosfolipídios, prevenindo a morte neuronal (Di Cesare Mannelli et al., 2010; Jones et al., 2010).

Além disso, a ALC possui atividade neuroprotetora e antioxidante (Chapela et al., 2009; Jones et al., 2010). A administração de ALC aumenta os níveis celulares de GSH em astrócitos de rato, facilita o transporte de GSH através da barreira

hematoencefálica (Kido et al., 2001) e eleva os níveis de GSH na via nigroestriatal (Fariello et al., 1988). Esse aumento dos níveis de GSH poderia proteger tanto sinaptossomas quanto mitocôndrias do estresse oxidativo mediado por espécies reativas de oxigênio (Drake et al., 2002). A administração da ALC diminuiu a lipoperoxidação em encéfalos de ratos idosos, além de prevenir a formação de radicais livres e a oxidação de proteínas em córtex frontal dos cães submetidos a isquemia cerebral e reperfusão (Calvani and Arrigoni-Martelli, 1999). A ingestão crônica oral de 500 mg/kg de ALC por roedores por 25 dias alterou o metabolismo energético cerebral e provocou aumento significativo nas concentrações de serotonina no córtex e noradrenalina no hipocampo de ratos (Smeland et al., 2012). A suplementação crônica de ALC causou diminuição do metabolismo de glicose em lactato, indicando aumento nos níveis de energia em algumas regiões do encefálicas (Smeland et al., 2012).

Estudos mostraram que a ALC aumentou a expressão do receptor metabotrópicos mGlu2 no córtex pré-frontal e hipocampo por meio de mecanismos epigenéticos mediados por acetilação das histonas e do fator de transcrição NFkb, possuindo potencial para o tratamento de transtornos do humor e de ansiedade, com um bom perfil de tolerabilidade. Além disso, ALC aumentou a neurogênese em camundongos submetidos ao protocolo de estresse crônico imprevisível (Cuccurazzu et al., 2013; Nasca et al., 2013). ALC também mostrou eficácia terapêutica sobre sintomas depressivos em pacientes com fibromialgia (Leombruni et al., 2015) e em pacientes idosos (Pettegrew et al., 2002).

Em outro estudo realizado em ratos, a administração crônica de ALC nas doses de 50 e 75 mg/kg, mas não 10 e 100 mg/kg, mostrou efeito sobre a ansiedade no teste de labirinto em cruz elevado (Levine et al., 2005). Recentemente, Lau e

colaboradores (2016) demonstraram que o tratamento por via oral durante três dias com ALC preveniu o aumento de ansiedade induzido por um protocolo de estresse por contenção no teste de claro escuro em ratos (Lau et al., 2016).

O tratamento com L-carnitina associado a uma dieta hipoproteica é capaz de conferir proteção contra o dano oxidativo a biomoléculas (lipídios, proteínas, enzimas e DNA) (Jones et al., 2010). Levando em consideração que o sistema nervoso central é extremamente sucetível ao estresse oxidativo devido ao grande conteúdo lipídico, intervenções capazes de prevenir e/ou reverter tais insultos poderiam ser utilizados no tratamento de transtornos psiquiátricos (Wang et al., 2014).

O antagonista do receptor NMDA cetamina reduziu os níveis de ATP em embriões peixes-zebra. Além disso, diminuiu a proteína mitocondrial e o potencial de membrana, causando deficiência de ATP mediada por disfunção mitocondrial. A maior parte do ATP gerado pelas mitocôndrias cardíacas é utilizada para sua contração e relaxamento. Embriões tratados com cetamina mostraram estrutura cardíaca anormal, comprometendo o desenvolvimento do coração. Essa atividade foi abolida com o co-tratamento com ALC, prevenindo os efeitos deletérios induzidos pela cetamina (Robinson et al., 2018). Um estudo também mostrou os efeitos da ALC sobre o comportamento e estresse oxidativo em peixes-zebra submetidos a um protocolo de estresse crônico imprevisível (UCS). O UCS induziu comportamento ansiogênico e estresse oxidativo enquanto o tratamento com ALC preveniu tais efeitos (Marcon et al., 2019).

Recentemente, uma equipe que incluiu pesquisadores de diversas instituições como a Universidade Rockefeller (EUA), a Universidade de Duke (EUA) e o Instituto Karolinska (Suécia), conduziu um experimento com roedores que concluiu que a ALC tinha um efeito antidepressivo de ação rápida nos animais e acredita-se que seu

mecanismo de ação impeça o disparo excessivo de neurônios excitatórios, mas essa função precisará ser mais explorada. A equipe também recrutou 71 pacientes com diagnóstico de depressão, homens e mulheres com idades entre 20 e 70 anos. Também recrutaram 45 pessoas saudáveis como grupo de controle. Quando comparados ao grupo de controle pareado por idade e sexo, os pacientes com depressão tinham níveis sanguíneos substancialmente mais baixos de ALC. Esses resultados sugerem que a ALC pode ser um potencial marcador biológico no transtorno depressivo maior (Nasca et al., 2018).

A ALC é uma molécula multialvo capaz de modular diversas vias relacionadas à fisiopatologia dos transtornos psiquiátricos relacionados ao estresse. Entretanto, ainda são poucos os estudos que mostram os efeitos desse composto em modelos animais de ansiedade. Dessa forma, a proposta desse trabalho foi avaliar os efeitos de ALC sobre parâmetros comportamentais e bioquímicos em peixes-zebra a fim de contribuir para a elucidação dos efeitos desse composto sobre o sistema nervoso central bem como estabelecer os possíveis mecanismos relacionados.

2 OBJETIVOS

2.1 Objetivo geral

Investigar os efeitos da exposição aguda à acetil-L-carnitina (ALC) sobre parâmetros comportamentais e bioquímicos em peixes-zebra.

2.2 Objetivos específicos

São objetivos específicos desta pesquisa:

a) avaliar os efeitos da ALC nos testes de tanque novo e claro-escuro em peixes-zebra;
b) investigar os efeitos da ALC sobre o comportamento e parâmetros de estresse oxidativo em peixes-zebra submetidos a um protocolo de estresse agudo (perseguição por rede).

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Anxiolytic and anti-stress effects of acute administration of acetyl-L-carnitine in zebrafish.

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Anxiolytic and anti-stress effects of acute administration of acetyl-L-carnitine in zebrafish

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ABSTRACT

Studies have suggested that oxidative stress may contribute to the pathogenesis of mental disorders. In this context, molecules with antioxidant activity may be promising agents in the treatment of these deleterious conditions. Acetyl-L-carnitine (ALC) is a multi-target molecule that modulates the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, acetylcholine production, protein, and membrane phospholipid synthesis, capable of promoting neurogenesis in case of neuronal death. Moreover, neurochemical effects of ALC include modulation of brain energy and synaptic transmission of multiple neurotransmitters, including expression of type 2 metabotropic glutamate (mGlu2) receptors. The aim of this study was to investigate the effects of ALC in zebrafish by examining behavioral and biochemical parameters relevant to anxiety and mood disorders in zebrafish. ALC presented anxiolytic effects in both novel tank and light/dark tests and prevented the anxiety-like behavior induced by an acute stressor (net chasing). Furthermore, ALC was able to prevent the lipid peroxidation induced by acute stress in the zebrafish brain. The data presented here warrant further investigation of ALC as a potential agent in the treatment of neuropsychiatric disorders. Its good tolerability also subsidizes the additional studies necessary to assess its therapeutic potential in clinical settings.

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INTRODUCTION

Acetyl-L-carnitine (ALC) facilitates the movement of acetyl-CoA into the mitochondria during the oxidation of fatty acids in mammals (*Chapela et al., 2009*). Moreover, this molecule is widely consumed as a dietary supplement for physical exercise (*Ribas, Vargas & Wajner, 2014*; *Nicassio et al., 2017*). Recently, preclinical and clinical studies have demonstrated the effects of ALC on parameters relevant to anxiety, schizophrenia, and mood disorders; with onset of action faster than antidepressant drug and exert

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neuroprotective, neurotrophic, and analgesic effects (*Levine et al., 2005*; *Wang et al., 2015*; *Traina, 2016*; *Singh et al., 2017*; *Nasca et al., 2017*; *Chiechio, Canonico & Grilli, 2017*).

A growing body of evidence suggests that psychiatric disorders such as anxiety and depression are associated with oxidative damage (*Ortiz et al., 2017*; *Niedzielska et al., 2016*; *Schiavone, Colaianna & Curtis, 2015*; *Cobb & Cole, 2015*; *Ng et al., 2008*), since a decrease in antioxidant capacity can impair the organism's protection against reactive oxygen species and cause damage to fatty acids, proteins, and DNA (*Maes et al., 2011*). Superoxide and hydroxyl radical (free radicals) or hydrogen peroxide and their derivatives (non-radical molecules) called reactive oxygen species (ROS) are responsible for causing oxidative damage (*Smaga et al., 2015*). The antioxidant defense mechanism they are the non-enzymatic (i.g. glutathione) and enzymatic antioxidants (i.g. superoxide dismutase and catalase) which show a trend to decrease in neuropsychiatric diseases (*Ozcan et al., 2004*; *Hassan et al., 2016*). Preclinical and clinical research has evaluated antioxidant compounds (i.g. N-acetylcysteine, resveratrol and curcumin) in the treatment of psychiatric disorders, and it has been reported that these compounds are able to protect against oxidative stress-induced neuronal damage, preventing lipid peroxidation and behavioral changes (*Mecocci & Polidori, 2012*; *Berk et al., 2014*; *Wang et al., 2014*; *Mocelin et al., 2015*; *Patel, 2016*; *Santos et al., 2017*).

With simple, rapid and cheaper tests when compared with rodents, zebrafish have been used as a powerful complementary model for the study of a variety of neuropsychiatric diseases through behavioral and biochemical parameters (*Stewart et al., 2015*; *Mocelin et al., 2015*; *Marcon et al., 2016*; *Marcon et al., 2018*; *Khan et al., 2017*). There are several behavioral protocols extensively used and described for this species, such as the novel tank and light/dark tests. The novel tank diving test is based on an anti-predatory defense mechanism that induces fish to swim at the bottom of the tank, whereas the light/dark test evaluates anxiety based on the innate preference of adult zebrafish to dark over light areas (*Levin, Bencan & Cerutti, 2007*; *Gebauer et al., 2011*; *Khan et al., 2017*; *Pittman & Piato, 2017*).

In addition to its role in lipid metabolism, ALC also possesses free radical scavenging properties, and may thus protect the cells from oxidative damage by acting as an antioxidant (*Gülçin, 2006*; *Sepand et al., 2016*). Therefore, the aim of this study was to investigate the effects of ALC in zebrafish by examining behavioral and biochemical parameters relevant to anxiety and mood disorders in zebrafish.

MATERIALS AND METHODS

Animals

A total of 240 adult zebrafish (*Danio rerio,* F. Hamilton 1822) wild-type short fin strain (6-month-old, 3–4 cm long) 50:50 male/female ratio were purchased from Delphis aquariums (Porto Alegre, Brazil). The fish were kept for 15 days in a closed acclimation tank system of 16 L (40 × 20 × 24 cm) identical to the experimental tanks. Housing conditions consisted only of a tank with water, heater, filter and aeration system, and were maintained as previously described in *Marcon et al. (2016)*. The tanks contained non-chlorinated, aerated

tap water (pH 7.0 ± 0.3; temperature 26 ± 1 °C; total ammonia at <0.01 mg/L; nitrite <0.01 mg/L; dissolved oxygen at 7.0 ± 0.4 mg/L; alkalinity at 22 mg/L $CaCO_3$ and total hardness at 5.8 mg/L), with a light/dark cycle of 14/10 h (lights on at 06:00 am). The fish were fed twice a day with a commercial flake fish food (Alcon BASIC®; Alcon, São Paulo, Brazil). On the experimental days, all the fish were only fed early in the morning before behavioral testing began. The order of testing was counterbalanced so that fasting time was randomized across experimental groups. All experiments were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (#30992/2015).

Drug and experimental design

O-Acetyl-L-carnitine hydrochloride (ALC, CAS number 5080-50-2) was acquired from Sigma-Aldrich (St Louis, Missouri, USA). In all experimental protocols (novel tank, light/dark, and acute chasing stress tests), the animals were treated or not with ALC (0.1, 1.0 and 10.0 mg/L) in a beaker for 10 min. In the first protocol, immediately after the treatment, the animals were placed in the novel tank test (NTT) for 6 min. In the second protocol, after the treatments, the animals were placed in the light/dark test (LDT) for 5 min. Finally, in the third protocol, the animals were treated as previously and then chased with a net for 2 min. Then, the animals were placed in the NTT. The biochemical analyses were performed in animals submitted to this last protocol. A control group was submitted to the same experimental conditions (stressed or not) but without treatment. Different sets of animals were used in each experimental protocol. The experimental design is shown in Fig. 1 and was based on the previously published study by *Mocelin et al. (2015)*. All behavioral tests were performed between 09:00 am and 16:00 pm. The researchers who performed the behavioral tests and analyzed the data were unaware of the allocation of animals to the experimental groups. The concentrations were based on previous studies with another antioxidant compound (N-acetylcysteine) and pilot studies with a wider concentration range. The same concentrations were used in a chronic study with ALC (M Marcon, R Mocelin, A Araujo, A Herrmann & A Piato, 2018, unpublished data). We do not attempt to extrapolate the drug concentrations we used in a fish study to human dosage since there is not a straightforward calculation to be done. Since the half-life and other pharmacokinetic parameters of ALC in zebrafish are not known, it is difficult to precisely compare the concentration range that we observed here with the dose range for humans.

Novel tank test (NTT)

The novel tank test followed the protocol already described in *Mocelin et al. (2015)*. Briefly, the animals were separately moved to the apparatus (2.7-L tank, 24 × 8 × 20 cm, virtually divided into three equal horizontal zones and filled with standard tank water up to 15 cm) and video recorded for 6 min to be later analyzed by the ANY-maze™ software (Stoelting Co., Wood Dale, IL, USA). To evaluate exploratory behavior and locomotion we measured the parameters: total distance moved (m), number of transitions between zones, time spent in the upper and bottom zones of the tank, and number of transitions to the upper zone. Total distance and crossings were used as an indicator of overall locomotor activity. The

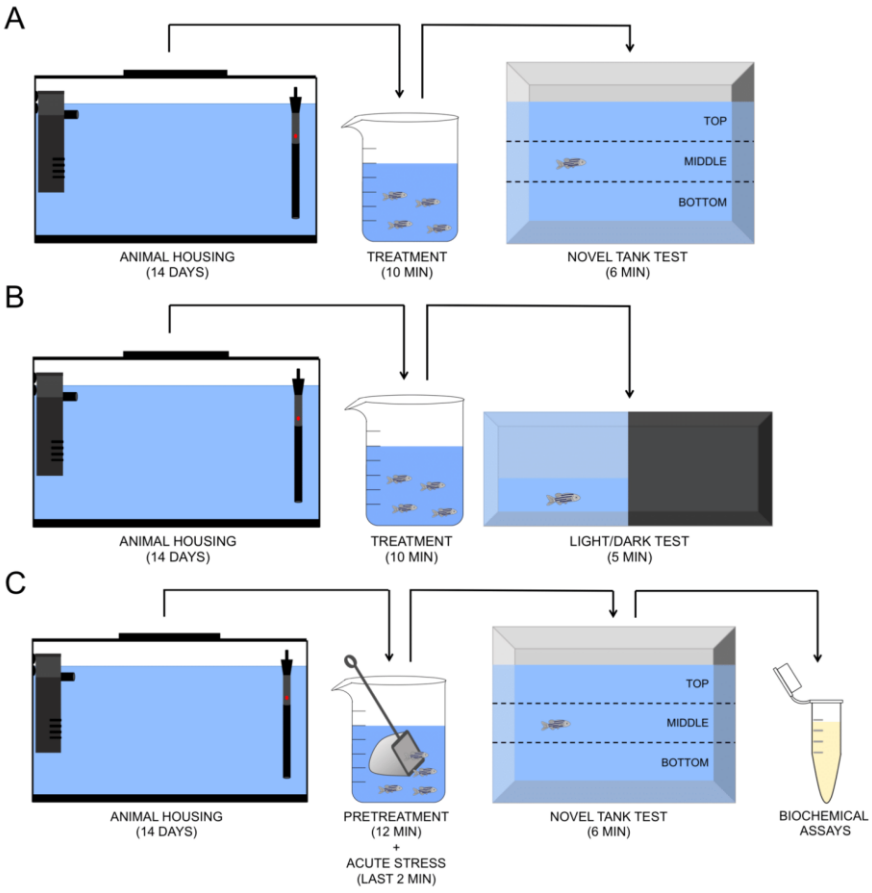

Figure 1 **Schematic representation of the experimental protocol. Novel tank test (A), light/dark test (B), and acute chasing stress and biochemical assays (C).**

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upper zone of the tank corresponds in rats and mice protocols to the periphery region of the open-field test. Alterations in time spent and number of crossings to this zone are frequently used as a parameter of anxiety in zebrafish (*Mocelin et al., 2015*; *Giacomini et al., 2016*; *Marcon et al., 2016*; *Mocelin et al., 2017*; *Marcon et al., 2018*).

Light/dark test (LDT)

The light/dark test followed the protocol already reported by *Gebauer et al. (2011)*. Specifically, the apparatus consisted of a glass tank (18 × 9 × 7 cm) divided by a raised glass into a dark and a white compartment of equal sizes, with the water level set at 3 cm and the partition raised 1 cm above the tank floor. One fish at a time was positioned in the white zone of the apparatus immediately after treatment. We recorded the number of

crossings and the time spent in the white compartment for 5 min. Zebrafish have a natural preference for dark environments and the white compartment is very anxiogenic for this species; anxiolytics increase the time spent in the white compartment (*Maximino et al., 2010*; *Mocelin et al., 2015*).

Acute chasing stress test (ACS)

The acute stress protocol was performed according to the previous study published by *Mocelin et al. (2015)*. Briefly, the animals were treated for 12 min and then chased for the last 2 min with a net before being moved to the novel tank, where they were recorded for 6 min. The behavioral parameters were quantified as described above for the NTT.

Tissue preparation

Samples were collected and prepared as previously reported by *Mocelin et al. (2018)*. Specifically, after the ACS fish were anesthetized by immersion in cold water and euthanized by decapitation. Each independent sample was then obtained by pooling four brains, which were homogenized on ice in 600 µL phosphate buffered saline (PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 10,000 g for 10 min at 4 °C in a cooling centrifuge, and the supernatant was packed in microtubes for further assays.

Protein determination

Protein was determined by the Coomassie blue method described in detail by Bradford (1976). Specifically, we used bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as standard and the absorbance of samples was measured at 595 nm.

Lipid peroxidation (TBARS)

Lipid peroxidation was measured by the quantification of thiobarbituric acid reactive species (TBARS) production according to the method reported by (*Draper & Hadley, 1990*). More specifically, we followed the protocol described by *Mocelin et al. (2018)*, in which 50 µL of the sample (80–100 µg protein) was mixed with 75 µL of trichloroacetic acid (TCA 10%; Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 6,000 rpm for 5 min at 4 °C in a cooling centrifuge. In the supernatants were added to 75 µL thiobarbituric acid (TBA 0.67%; Sigma-Aldrich, St. Louis, MO, USA), then homogenized in a vortex for 5s and heated at 100 °C for 30 min. TBARS levels were measured by absorbance (532 nm) in a microplate reader, using malondialdehyde (MDA; Sigma-Aldrich, St. Louis, MO, USA) as a standard, and results were expressed as nmol MDA/mg protein.

Reduced thiol (SH) and Non-protein thiols levels (NPSH)

SH and NPSH levels were determined and measured at 412 nm in a microplate reader according to the method described by *Ellman (1959)*. More specifically, we followed the steps described by *Mocelin et al. (2018)*. Briefly, for SH the samples (60–80 µg protein) were added to 10 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) dissolved in ethanol, developing yellow color after 1 h. The NPSH were similarly assessed, except that the sample was mixed with equal volumes of the 10% trichloroacetic acid (TCA) and centrifuged (6,000 rpm, 5 min). The supernatant was used for the biochemical assay. Results were expressed as µmol SH/mg protein.

Superoxide dismutase (SOD) and catalase (CAT) activities

SOD and CAT activities were determined according to the method reported by *Misra & Fridovich (1972)* and *Aebi (1984)*, respectively. The protocol followed the more specific details described by *Dal Santo et al. (2014)*. Specifically, SOD activity was quantified in a microplate reader (480 nm) by testing the inhibition of radical superoxide reaction of the sample (20–30 μg protein) in the presence of adrenalin, monitoring adrenochrome formation in a medium containing a glycine-NaOH buffer (pH 10) and adrenaline (1 mM). CAT activity was assessed by measuring the decrease in H_2O_2 absorbance in a microplate reader (240 nm). The assay mixture consisted of sample (20–30 μg protein), phosphate buffered saline (pH 7.4), and 5 μL H_2O_2(0.3 M). Results were expressed as units/mg protein.

Statistics

Normality and homogeneity of variance of the data were checked by D'Agostino-Pearson and Levene tests, respectively. Results were analyzed by one- or two-way ANOVA followed by Tukey's post hoc test. Two-way ANOVA was used to identify the main effects of stress and treatment, as well as their interactions. Data are expressed as a mean + standard error of the mean (S.E.M.). The level of significance was set at $p < 0.05$.

RESULTS

Behavioral parameters

Figure 2 shows the effects of ALC (0.1, 1.0 and 10.0 mg/L) on the novel tank test in zebrafish. ALC significantly increased the time spent in the top (0.1 and 1.0 mg/L, Fig. 2D) and decreased the time spent the bottom (0.1 mg/L, Fig. 2E) zone of the tank ($F_{3,77} = 8.0$, $p = 0.0001$ and $F_{3,77} = 5.6$, $p = 0.0016$, respectively). Locomotor parameters of groups treated with ALC (0.1, 1.0, and 10.0 mg/L) did not differ from control (Figs. 2A and 2B).

In the light/dark test, ALC (0.1 and 10.0 mg/L) significantly increased the time spent in the lit side of the tank when compared to control ($F_{3,92} = 3.6$, $p = 0.0161$, Fig. 3B). The number of crossings between the light and dark compartments was not altered by any of the concentrations ($F_{3,92} = 0.9$, $p = 0.4284$, Fig. 3A).

Figure 4 shows the effects of ALC in the acute chasing stress (ACS) in zebrafish and Table 1 summarizes the two-way ANOVA analysis. As expected, ACS decreased the distance total traveled, crossings, entries and time in the top area (Figs. 4A–4D, respectively) and increased the time in the bottom area (Fig. 4E). ALC (0.1, 1.0 and 10.0 mg/L) prevented the effects of ACS on the time in the top and bottom areas in the novel tank test (Figs. 4D and 4E). Also, ALC (0.1 mg/L) prevented the effects of ACS on the total distance traveled.

Biochemical parameters

Figure 5 shows the effects of ALC (0.1, 1.0 and 10.0 mg/L) on oxidative status. ACS significantly increased lipid peroxidation (TBARS), non-protein sulfhydryl (NPSH) and superoxide dismutase (SOD) activity (Figs. 5A, 5C and 5D, respectively), but did not alter sulfhydryl (SH) content and catalase (CAT) activity (Figs. 5B and 5E, respectively). Treatment with ALC (0.1, 1.0 and 10.0 mg/L) prevented oxidative damage as measured by

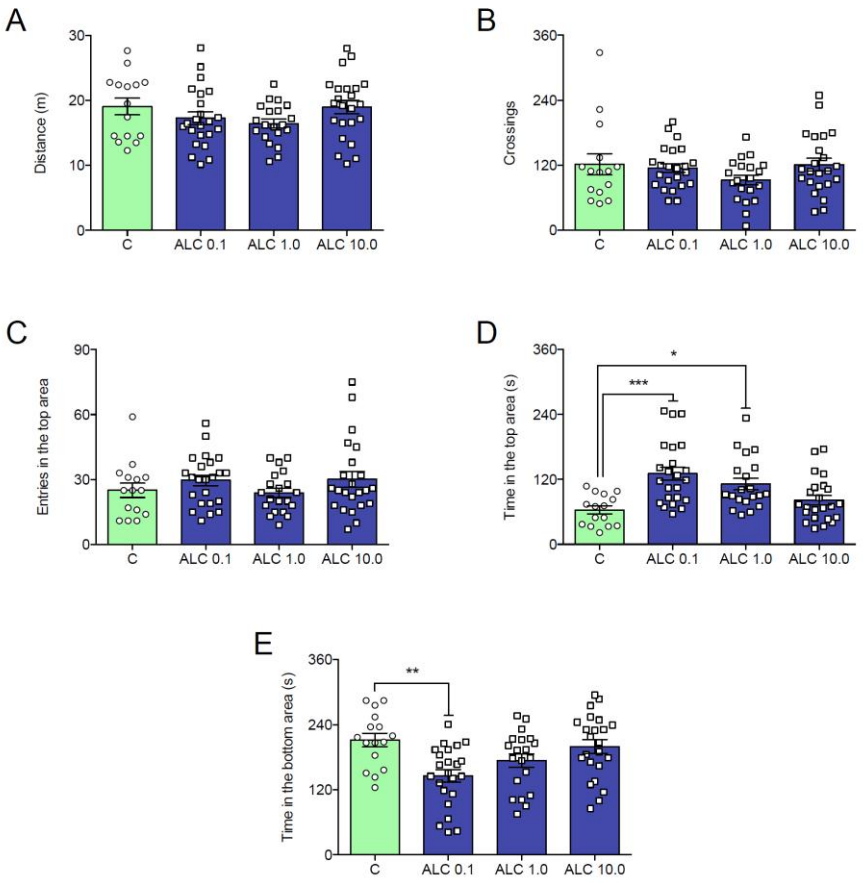

Figure 2 Effects of ALC (0.1, 1.0 and 10.0 mg/L) behavioral parameters in zebrafish submitted to the novel tank test. (A) distance traveled, (B) number of crossings, (C) entries and (D) time in the upper zone, and (E) time in the bottom zone. The data are presented as the mean + S.E.M. One-way ANOVA followed by Tukey post hoc test. $n = 15–23$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group.
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TBARS. ALC also prevented the increase of antioxidant defenses as measured by NPSH (0.1 mg/L) and SOD (0.1, 1.0 and 10.0 mg/L). Two-way ANOVA analyses were summarized in Table 2.

DISCUSSION

Here, we showed for the first time that ALC presents anxiolytic effects in both novel tank and light/dark tests in zebrafish. Moreover, ALC was able to prevent the anxiogenic effects and lipid peroxidation induced by an acute stress protocol. These results indicate a potential use of ALC in mental disorders related to stress.

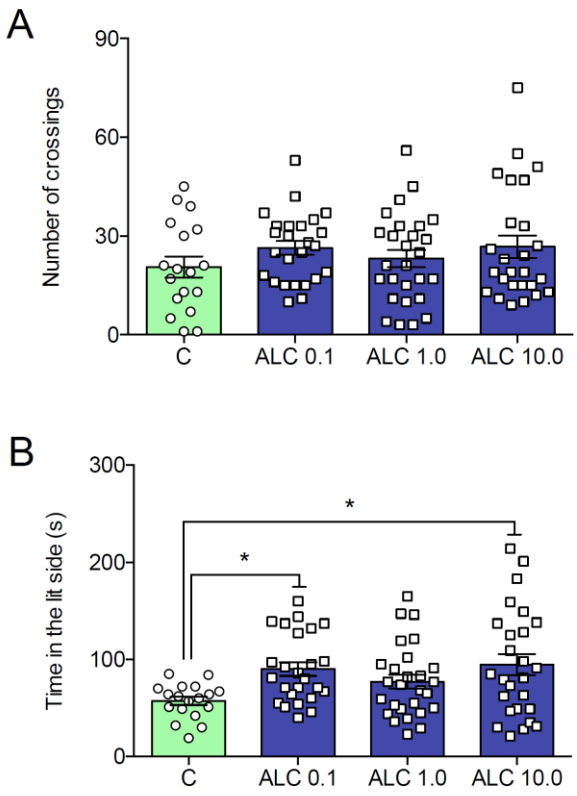

Figure 3 Effects of ALC (0.1, 1.0 and 10.0 mg/L) in the light/dark test in zebrafish. (A) number of crossings and (B) time in the lit side. The data are presented as the mean + S.E.M. One-way ANOVA followed by Tukey post hoc test. $n = 18–27$. $^*p < 0.05$ vs. control group.

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ALC increased the time spent in the upper as well as decreased the time spent in the bottom zones of the tank. Previous studies have shown that anxiolytic drugs such as buspirone, fluoxetine, diazepam, and ethanol increase the time spent in this zone (*Bencan, Sledge & Levin, 2009*; *Egan et al., 2009*; *Gebauer et al., 2011*). In the light/dark test, ALC increased the time spent in the lit side of the tank. This effect was observed with other drugs as clonazepam, bromazepam, diazepam, buspirone, and ethanol (*Gebauer et al., 2011*). Additionally, multi-target drugs other than ALC, for instance, N-acetylcysteine (NAC) and taurine, also increase the time in the lit side in the LTD in zebrafish (*Mocelin et al., 2015*; *Mezzomo et al., 2015*). In both NTT and LDT, ALC presented biphasic response. We can only speculate that different mechanisms of action may be involved in the effects of low versus high dose, but lower and higher concentrations would have to be tested for us to have a bigger picture of the dose–response relationship. ALC also prevented the locomotor impairment and anxiogenic behavior induced by the chasing stress protocol. Recently, our

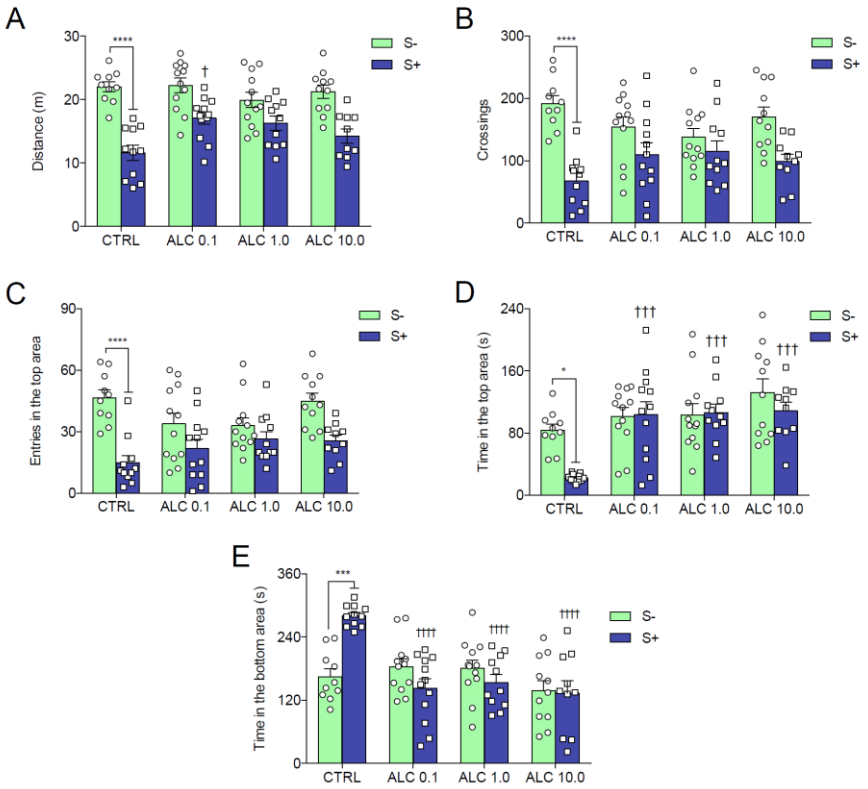

Figure 4 Effects of ALC pretreatment against stress-induced changes in behavioral parameters in zebrafish. (A) distance traveled, (B) number of crossings, (C) entries and (D) time in the upper zone, and (E) time in the bottom zone. The data are presented as the mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 10$–12. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. control group (S−); $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ vs. stressed control group (S+).

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group has shown that fluoxetine, diazepam, and NAC prevented the effects of a similar stress protocol in zebrafish (*Mocelin et al., 2015*; *Giacomini et al., 2016*).

The anxiolytic and antidepressant effects of ALC have been already reported in rodents (*Levine et al., 2005*; *Wang et al., 2015*; *Lau et al., 2017*). ALC modulates the cholinergic system by increasing acetyl-CoA content and choline acetyltransferase activity. Moreover, it modulates GABAergic, dopaminergic and glutamatergic neurotransmitter systems (*Chapela et al., 2009*; *Nasca et al., 2013*; *Wang et al., 2014*; *Singh et al., 2016*; *Chiechio, Canonico & Grilli, 2017*). In rats, ALC decreased the immobility time in the forced swim test and increased sucrose preference in 3 days of treatment, whereas 14 days were necessary to obtain the same effects with clomipramine (*Nasca et al., 2013*).

Table 1 Results of two-way analysis of variance (ANOVA) of behavioral analysis and the interaction between treatment with ALC and acute chasing stress.

Dependent variable	Effects	*F*-value	*DF*	*P*-value
Total distance	Interaction	3.46	3,81	**0.0201**
	ALC	2.39	3,81	0.0745
	Stress	71.34	3,81	**0.0001**
Crossings	Interaction	4.04	3,81	**0.0099**
	ALC	0.10	3,81	0.9583
	Stress	37.11	3,81	**0.0001**
Entries in the top	Interaction	3.47	3,81	**0.0198**
	ALC	1.18	3,81	0.3215
	Stress	36.10	3,81	**0.0001**
Time in the top	Interaction	2.72	3,81	**0.0499**
	ALC	9.81	3,81	**0.0001**
	Stress	4.86	3,81	**0.0303**
Time in the bottom	Interaction	9.02	3,81	**0.0001**
	ALC	9.03	3,81	**0.0001**
	Stress	0.84	3,81	0.3613

Notes.
DF, degrees of freedom.
Significant effects ($p < 0.05$) are given in bold.

Under normal conditions, damage by reactive oxygen species (ROS) is kept in control by efficient antioxidant systems, such as SOD and CAT enzymes, as well as non-enzymatic scavengers (*Schiavone et al., 2013*; *Schiavone, Colaianna & Curtis, 2015*; *Sandi & Haller, 2015*). Studies have demonstrated that ALC protects cells against lipid peroxidation and membrane breakdown through hydrogen peroxide scavenging (*Kumaran et al., 2003*; *Gülçin, 2006*), and can promote the expression of antioxidant enzymes such as SOD and CAT (*Augustyniak & Skrzydlewska, 2010*; *Li et al., 2012*).

Even though the ACS protocol increased antioxidant defenses (NPSH and SOD), it also caused lipid peroxidation (TBARS), which may indicate a possible adaptive response to ROS production during stressful conditions. Similar results were observed in zebrafish and reported in a previous study from our group using acute restraint stress (*Dal Santo et al., 2014*). Even though detection of MDA levels by HPLC would be a more specific indicator of lipid peroxidation, the TBARS assay we used in this study has been reported by many previous articles using samples from zebrafish and other animals (*Mihara & Uchiyama, 1978*; *Sunderman et al., 1985*; *Armstrong & Browne, 1994*; *Yagi, 1998*; *Kim et al., 2011*; *Basu et al., 2014*; *Yavuzer et al., 2016*). The association of these factors could be related to the prevented effects of ALC, and that our results indicate a deficit in antioxidant defenses against lipid peroxidation in zebrafish submitted to the ACS protocol, providing

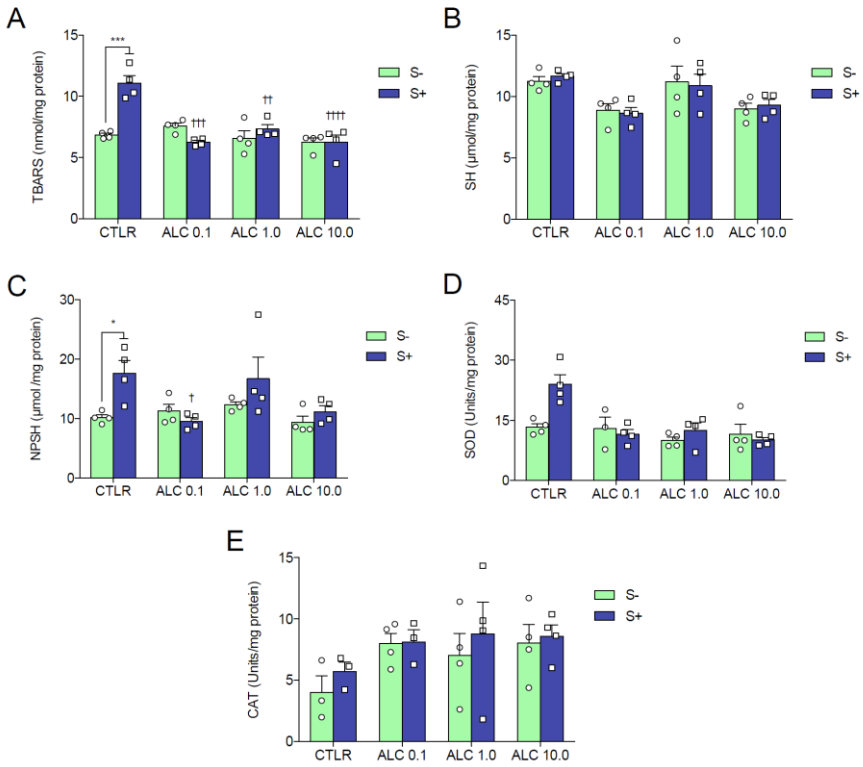

Figure 5 Effects of ALC pretreatment against stress-induced changes in biochemical parameters in zebrafish. (A) thiobarbituric acid reactive substances, (B) sulfhydryl, (C) non-protein sulphydryl, (D) superoxide dismutase, and (E) catalase. The data are presented as the mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 3$–4. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control group (S−); $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger\dagger}p < 0.0001$ vs. stressed control group (S+).

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further evidence for the hypothesis of an association between behavior and ROS with the pathophysiology of mental disorders stress-related and their prevention by ALC.

CONCLUSION

ALC is already widely used as supplementation for people who want to lose weight/fat burner, but only a few studies assessed its effects on stress-related outcomes. In addition to its antioxidant actions, ALC is also able to restore mitochondrial function, which is relevant to combat the dysregulation of fatty acid metabolism in the mitochondria-associated with psychiatric disorders. Furthermore, there is evidence that ALC increases expression of metabotropic glutamate receptors via epigenetic mechanisms (*Nasca et al., 2013*), which is also relevant for the pathophysiology of depression and other stress-related disorders.

Table 2 Results of two-way analysis of variance (ANOVA) of biochemical analysis and the interaction between treatment with ALC and acute chasing stress.

Dependent variable	Effects	*F*-value	*DF*	*P*-value
Lipid peroxidation	Interaction	14.70	3,24	**0.0001**
(TBARS)	ALC	14.39	3,24	**0.0001**
	Stress	8.80	1,24	**0.0067**
Sulfhydryl	Interaction	0.14	3,24	0.9339
(SH)	ALC	7.80	3,24	**0.0008**
	Stress	0.01	1,24	0.9289
Non-protein thiol	Interaction	2.73	3,24	0.0665
(NPSH)	ALC	3.63	3,24	**0.0273**
	Stress	6.35	1,24	**0.0188**
Superoxide dismutase	Interaction	5.46	3,23	**0.0055**
(SOD)	ALC	9.93	3,23	**0.0004**
	Stress	4.26	1,23	0.0504
Catalase	Interaction	0.13	3,21	0.9393
(CAT)	ALC	1.89	3,21	0.1626
	Stress	0.87	1,21	0.3606

Notes.
DF, degrees of freedom.
Significant effects ($p < 0.05$) are given in bold font.

Our study adds to a growing body of literature demonstrating the role of antioxidants in modulating behavior and oxidative homeostasis. The data presented here thus warrants further investigation of ALC as a potential agent in the treatment of neuropsychiatric illness. Its novel mechanism of action and good tolerability also subsidize the additional studies necessary to assess its therapeutic potential in clinical settings.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Angelo Piato is an Academic Editor for PeerJ.

Author Contributions

- Lais Pancotto performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Ricieri Mocelin conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Matheus Marcon conceived and designed the experiments, performed the experiments, approved the final draft.
- Ana P. Herrmann conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Angelo Piato conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All experiments were approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (#30992/2015).

Data Availability

The following information was supplied regarding data availability:

The raw data is provided as Data S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5309#supplemental-information.

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4 DISCUSSÃO E CONCLUSÃO

Nesse estudo mostramos pela primeira vez os efeitos ansiolíticos e antiestresse da acetil-L-carnitina (ALC) em peixes-zebra. A ALC aumentou o tempo de permanência na zona superior do aquário no teste de tanque novo e o tempo de permanência no lado claro no teste de claro/escuro. Nas concentrações utilizadas não foram observados efeitos sobre a atividade exploratória e locomoção. Além disso, animais submetidos ao estresse agudo por perseguição por rede apresentaram diminuição na distância percorrida, o número de cruzamentos e entradas e tempo de permanência na zona superior do aquário enquanto a ALC preveniu esses efeitos. Em relação aos parâmetros de status oxidativo, o estresse agudo induziu lipoperoxidação e aumentou os níveis de tióis não-proteicos e a atividade da enzima superóxido dismutase (SOD), sem afetar os níveis de sulfidrilas (SH) e da catalase (CAT). O tratamento com ALC preveniu a lipoperoxidação e o aumento da superóxido dismutase.

No teste de tanque novo, o tempo de permanência e o número de entradas na zona superior do aquário é frequentemente utilizado como uma medida de ansiedade. Ou seja, intervenções ansiogênicas como o estresse agudo por perseguição (de Abreu et al., 2014; Giacomini et al., 2016) ou contenção (Piato et al., 2011) diminuem tanto o tempo como o número de entradas na zona superior, enquanto ansiolíticos como a fluoxetina, a buspirona, o diazepam e o etanol aumentam o tempo de permanência na zona superior e o número de entradas por reduzir a ansiedade (Bencan et al., 2009; Gebauer et al., 2011; Maximino et al., 2014).

No teste de claro/escuro, a ALC aumentou o tempo de permanência no lado claro do aparato. Esse efeito foi observado com outros ansiolíticos como clonazepam, bromazepam, diazepam, buspirona e etanol (Gebauer et al., 2011; Maximino et al.,

2014). Do mesmo modo, compostos com ação multialvo como a taurina e N-acetilcisteína também aumentam o tempo de permanência no lado claro do aparato (Mezzomo et al., 2016; Mocelin et al., 2015).

Em condições normais, o dano por espécies reativas de oxigênio (EROs) é prevenido por eficientes sistemas antioxidantes enzimáticos como SOD e a CAT e não-enzimáticos (Sandi and Haller, 2015; Schiavone et al., 2012). Estudos demonstraram que o ALC protege as células contra a peroxidação lipídica e a degradação da membrana por meio da depuração de peróxido de hidrogênio (Gülçin, 2006) e aumentou a expressão de enzimas antioxidantes como a SOD e a CAT (Augustyniak and Skrzydlewska, 2010). Embora o protocolo de estresse agudo tenha aumentado as defesas antioxidantes (NPSH e SOD), também causou a peroxidação lipídica (TBARS), o que pode indicar uma possível resposta adaptativa à produção de ROS durante condições estressantes. Resultados semelhantes foram observados em peixe-zebra e relatados em um estudo anterior do nosso grupo usando estresse por contenção (Dal Santo et al., 2014).

Os dados obtidos nessa dissertação corroboram com os disponíveis na literatura e agregam informações sobre os efeitos desse composto em diferentes modelos de ansiedade em peixes-zebra bem como sobre o potencial mecanismo de neuromodulalção da ALC. Entretanto, mais estudos são necessários para ampliar a caracterização dos efeitos da ALC sobre transtornos de ansiedade bem como melhorar a caracterização do mecanismo de ação.

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