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N-acetil-L-Cisteína e o extrato hidroalcoólico de *Psidium guajava* exercem efeito protetor frente à toxicidade glutamatérgica em larvas de *Danio rerio* (peixe-zebra)

Dissertação de mestrado

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Dissertação apresentada ao programa de Pós-Graduação Stricto sensu em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Jeferson Franco

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"*A menos que modifiquemos nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo*".

Albert Einstein

RESUMO

O glutamato (Glu) atua como o principal transmissor excitatório no sistema nervoso central dos vertebrados, no entanto em altas concentrações, pode provocar a estimulação excessiva dos neurônios, em um processo denominado excitotoxicidade. Essa patologia está envolvida em inúmeras doenças neurodegenerativas, bem como lesões cerebrais. No entanto, os agentes farmacoterapêuticos clinicamente disponíveis contra a excitotoxicidade glutamatérgica não são completamente eficazes, portanto pesquisas sobre novos compostos são necessárias. Nesse estudo esperou-se elucidar o efeito protetor da N-Acetil-L-Cisteína (NAC) e do extrato hidroalcoólico de *Psidium guajava* (PG) contra a excitotoxicidade em um modelo de efeitos de exposição ao glutamato de larvas de *Danio rerio* (peixe-zebra). Para este propósito utilizou-se larvas com 3 dias pós-fertilização (dpf), expostas 10 υM de Glu, em placas de 96 poços, durante 24 horas (h). Após a finalização dos tratamentos foram feitas análises comportamentais e de viabilidade celular. A exposição ao glutamato resultou em aumento significativo na produção de EROs, diminuição na viabilidade mitocondrial, aumento no dano à cromatina e apoptose. Concomitantemente, a exposição ao glutamato resultou em mudanças comportamentais, como a distância percorrida e a resposta ao toque. Por outro lado, o tratamento com NAC demonstrou um efeito protetor evitando o dano celular e alterações comportamentais, enquanto o tratamento com PG preveniu contra o dano celular. Nossos dados mostram pela primeira vez um efeito protetor do NAC e PG contra o dano induzido pelo glutamato em larvas de peixe-zebra, sugerindo que ambos os compostos têm potencial terapêutico contra processos excitotóxicos.

Palavras-Chave: Estresse Oxidativo; apoptose; antioxidantes; zebrafish; excitotoxicidade glutamatérgica.

ABSTRACT

Glutamate (Glu) acts as the main excitatory transmitter in the central nervous system of vertebrates. However, in high concentrations, glutamate can cause excessive stimulation of neurons in a process called excitotoxicity. This pathology is involved in numerous neurodegenerative diseases as well as brain injuries. However, clinically available pharmacotherapeutic agents against glutamatergic excitotoxicity are not completely effective, so research on new compounds is necessary. In this study we hope to elucidate the protective effect of N-Acetyl-L-Cysteine (NAC) and the hydroalcoholic extract of *Psidium guajava* (PG) against excitotoxicity in a model of glutamate exposure effects of *Danio rerio* (zebrafish) larvae. For this purpose, 3-day post-fertilization (dpf) larvae exposed 10 υM Glu were used in 96-well plates for 24 hours (h). After the completion of the treatments, behavioral and cellular viability analyzes were performed. Exposure to glutamate resulted in a significant increase in ROS production, decrease in mitochondrial viability, increase in chromatin damage, and apoptosis. Concomitantly, exposure to glutamate resulted in behavioral changes, such as distance traveled and touch response. On the other hand, NAC treatment demonstrated a protective effect avoiding cell damage and behavioral changes, while PG treatment prevented cell damage. Our data show for the first time a protective effect of NAC and PG against glutamate-induced damage in zebrafish larvae, suggesting that both compounds have therapeutic potential against excitotoxic processes.

Key words: Oxidative stress; apoptosis; antioxidant; glutamatergic excitotoxicity; natural compounds.

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE ABREVIATURAS

AC	Ácido Cítrico
AMPA	α-Amino-3-Hydroxi-Metil-5-4-Isoxazolpropioico
ATP	Adenosina Trifosfato
Ca^{2+}	Cálcio
CNS	Central Nervous System
DA	Doença De Alzheimer
DH	Doença De Huntington
DMSO	Dimethyl Sulfoxide
DP	Doença De Parkinson
dpf	Dias Pós-Fertilização
EM	Esclerose Múltipla
EO	Estresse Oxidativo
EROs	Espécies Reativas De Oxigênio
g	Gramas
GLAST	Transportador de glutamato aspartato
GLT-1	Transportador de glutamato 1
GSH	Glutationa
H_2O_2	Peróxido De Hidrogênio
hpf	Horas Pós-Fertilização
LCTs	Lesões Cerebrais Traumáticas
mGluRs	Segundos Mensageiros Através De Proteína G
NAC	N-Acetil-L-Cisteína
NMDA	N-Metil-D-Asparto
O_2	Oxigênio
O_{2^-}	Ânion Superóxido
OH^-	Radical Hidroxila
OMS	Organização Mundial Da Saúde
PG	*Psidium Guajava*
poliQ	Poliglutaminas
PTAE	Proteínas Transportadores De Aminoácidos Excitatórios
ROS	Reactive Oxygen Species
SNC	Sistema Nervoso Central
βa	β-Amilóide

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta dissertação. A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, que se encontra no item **MANUSCRITO**. No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referências. O item **CONCLUSÕES**, encontrado no final desta dissertação, apresenta interpretações e comentários gerais sobre os resultados dos manuscritos presentes neste trabalho. As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO.**

SUMÁRIO

1. INTRODUÇÃO

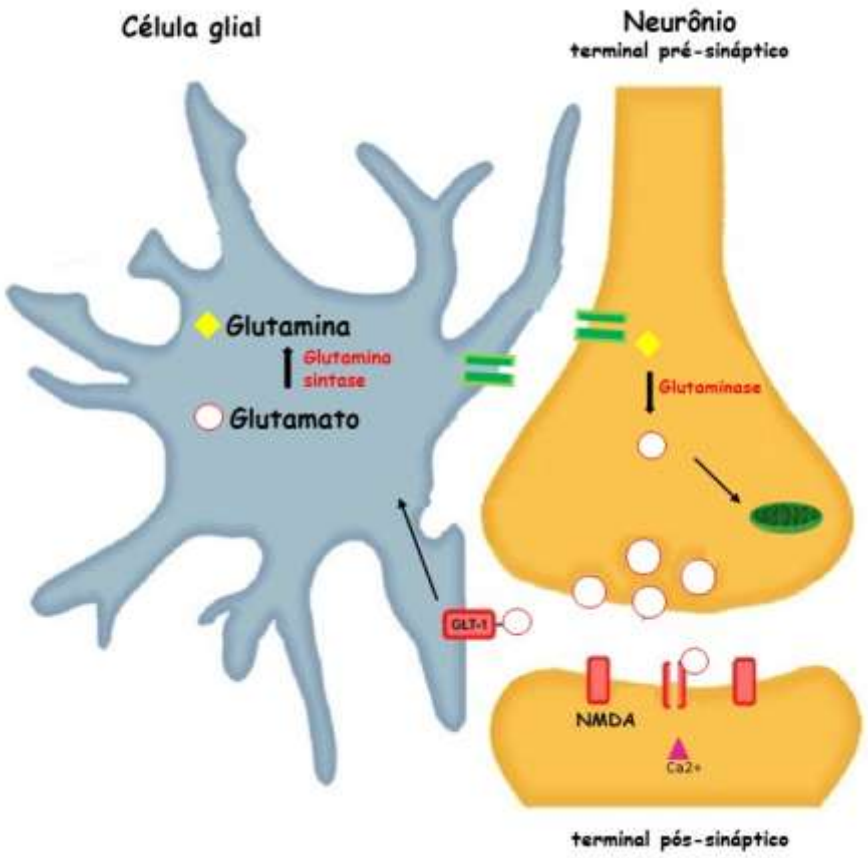
1.1. Excitotoxicidade glutamatérgica

O glutamato é o aminoácido livre mais abundante no sistema nervoso central (SNC) e atua como o principal neurotransmissor excitatório (MELDRUM, 2000), estando envolvido em diversas funções fisiológicas do cérebro, tais como: aprendizado e memória (RIEDEL; PLATT; MICHEAU, 2003) desenvolvimento e envelhecimento (SEGOVIA et al., 2001), proliferação, migração celular, diferenciação e morte (JANSSON; ÅKERMAN, 2014). Esse neurotransmissor não tem a capacidade de atravessar a barreira hematoencefálica, portanto é sintetizado por neurônios precursores localizados no cérebro, como a glutamina, ou através do α-cetoglutarato, por transaminassão, que consiste na transferência de um grupo amino para um α-cetoácido (KVAMME, 1998). Quando a glutamina é liberada pelas células gliais, e absorvida pelos neurônios, ela entra nos terminais pré-sinápticos, onde será metabolizada em glutamato pela enzima glutaminase.

O destino do glutamato nas células depende da condição metabólica, podendo seguir para o metabolismo oxidativo através do ciclo do ácido cítrico (AC) e gerar adenosina trifosfato (ATP), ou ser transformado em glutamina, pela enzima glutamina sintase (figura 1). Desta forma, os terminais sinápticos cooperam com as células da glia para manter um suprimento adequado do glutamato. Esta sequência de eventos é referida como o ciclo glutamato-glutamina. Além disso, quando as concentrações extracelulares de glutamato forem menores que 0,2 mM, grande parte do glutamato absorvido pelos astrócitos será convertido à glutamina, enquanto que em concentrações acima de 0,2 mM, pode seguir pela via do AC (SONNEWALD et al., 1993).

A função de sinalização não é dependente da natureza química do glutamato, mas de como as células são programadas para responder quando expostas a ele, uma vez que, os receptores de glutamato estão situados na superfície das células, e medeiam a maioria das neurotransmissões excitatórias no SNC de mamíferos (OZAWA; KAMIYA; TSUZUKI, 1998). Esses receptores podem ser ionotrópicos ou metabotrópicos. Os receptores ionotrópicos são canais iônicos que quando ativos se tornam permeáveis a cátions como sódio, cálcio e potássio e são classificados como N-metIl-D-asparto (NMDA), α-amino-3-hydroxi-5 metilisoxazol-4-propionato (AMPA) e cainato (KARAKAS; REGAN; FURUKAWA, 2015). Os receptores metabotrópicos são moléculas ligadas a sistemas de segundos mensageiros através de proteína G (mGluRs), modulando a atividade de diversas enzimas como, adenilato ciclase, guanilato

ciclase e fosfolipase C (GOLUBEVA et al., 2016). Estes receptores estão localizados nas células gliais, nos terminais pré-sinápticos, onde atuam na regulação da transmissão sináptica, e nos terminais pós-sinápticos, estão ligados a modulação da atividade dos canais iônicos, portanto estão relacionados tanto a efeitos excitotóxicos quanto inibitórios (SIMEONE; SANCHEZ; RHO, 2004).



Fonte: Adaptado de NEWINGTON; HARRIS; CUMMING, 2013.

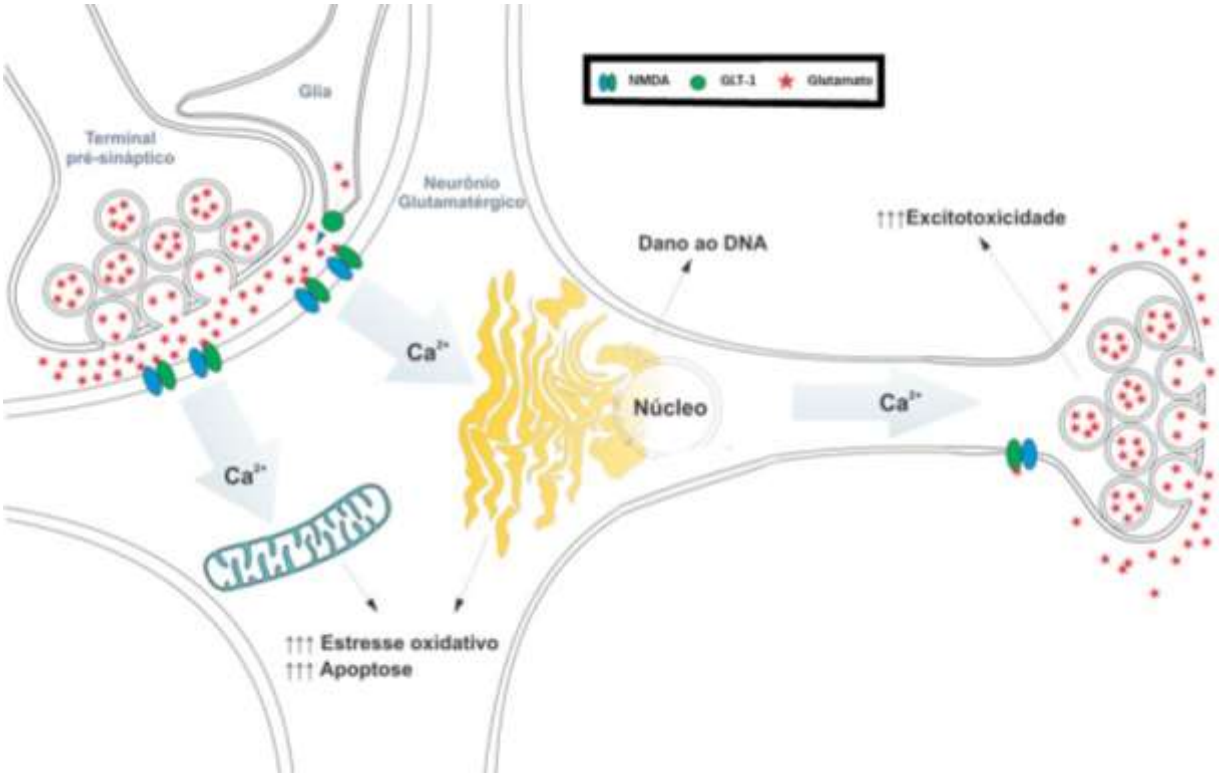
Figura 1: Síntese de glutamato **[**A glutamina é convertida em glutamato pela enzima glutaminase. O glutamato sintetizado é direcionado pra a síntese de ATP, ou então liberado na fenda pré-sináptica, onde irá se ligar a seus receptores NMDA e desempenhar sua função de neurotransmissor, e quando em excesso, é recolhido por suas proteínas transportadoras GLT-1].

O glutamato exerce sua função neurotransmissora a partir do líquido extracelular, onde ocorre a dinâmica de liberação e/ou remoção do glutamato, já que não há enzimas que possam degradá-lo, por isso, as concentrações de glutamato a níveis fisiológicos, são moduladas, exclusivamente, por uma família de proteínas transportadoras, chamada "Proteínas Transportadores de Aminoácidos Excitatórios" (PTAE) (KIM et al., 2011). Essas proteínas estão localizadas tanto em células neuronais quanto gliais e mantém a homeostase do glutamato extracelular (BEART; O'SHEA, 2007). A retirada de forma eficiente de glutamato da sinapse por esses transportadores é necessária para a neurotransmissão excitatória normal e prevenção contra toxicidade, pois, mesmo sendo um importante neurotransmissor, em altas concentrações, pode provocar a estimulação excessiva dos neurônios, causando inúmeras consequências, que vão desde danos severos à morte neuronal (LAU; TYMIANSKI, 2010a).

Esse processo, de estimulação excessiva, é denominado excitotoxicidade glutamatérgica. A excitotoxicidade ocorre quando os níveis de glutamato, no meio extracelular, se tornam muito mais elevados do que o normal (DANBOLT, 2001), causando a ativação prolongada dos seus receptores ionotrópicos e metabotrópicos, possibilitando a entrada de cálcio (Ca^{2+}) em grandes quantidades, na célula (SCHINDER et al., 1996), podendo resultar alterações bioenergéticas, que aumentam a carga oxidativa e ativam a apoptose (SATTLER; TYMIANSKI, 2000). Os receptores NMDA são altamente permeáveis ao Ca^{2+}, e tendem a permanecer abertos por mais tempo, em relação aos demais receptores de glutamato, portanto, sua ativação tem sido considerada como a principal consequência da excitotoxicidade (SATTLER; TYMIANSKI, 2000). As concentrações excessivas de Ca^{2+} intracelular causam a ativação de vias intracelulares que irão conduzir processos fisiológicos, para tentar balancear o excesso e garantir a homeostase celular. Porém, quando os sistemas de manutenção celular são comprometidos, e não consegue controlar os níveis de Ca^{2+}, poderá ocorrer a formação de processos patológicos (DONG et al., 2006; GIORGI et al., 2012). Nesse sentido, os astrócitos são muito importantes, já que representam a principal defesa contra a excitotoxicidade, sendo capazes de absorver o glutamato que está em excesso na fenda sináptica, graças à presença do transportador de glutamato 1 (GLT-1) e do transportador glutamato-aspartato (GLAST), que são, quantitativamente, os principais sequestradores de glutamato (BENARROCH, 2010; SHIGERI; SEAL; SHIMAMOTO, 2004).

As PTAE são moduladas pelo estado redox das células, ou seja, pelo balanço entre espécies reativas de oxigênio (EROs) e o sistema de defesas antioxidantes. Assim, se essas

proteínas não conseguir absorver o acumulo de glutamato na fenda pós-sináptica, o aumento do influxo de Ca^{2+}, poderá causar uma sobrecarga nas mitocôndrias, levando a produção aumentada de EROs, o que resultará no comprometimento da absorção de glutamato, e subsequentemente, morte celular (DOBRACHINSKI et al., 2017).



Fonte: Adaptado de PINTO; RESENDE, 2014

Figura 2: Excitotoxicidade glutamatérgica [o glutamato, quando em excesso no terminal sináptico, superativa os receptores NMDA, permitindo a entrada de grandes quantidades de Ca^{2+} nas células, acarretando inúmeras patologias].

1.2. Excitotoxicidade *versus* Lesões cerebrais e doenças neurodegenerativas

O glutamato pode funcionar como uma toxina poderosa capaz de induzir e/ou estar intimamente relacionado na patologia de inúmeras doenças neurodegenerativas, e atuar como mecanismo secundário em lesões cerebrais, tais como traumatismo craniano e acidente vascular cerebral (AVC).

1.2.1. Lesões cerebrais

Estudos mostram que a exposição a lesão cerebral traumática (LCT), isquemia ou AVC induz um aumento maciço na liberação de glutamato, seguido pelo influxo de íons Ca^{2+} e produção de radicais livres, culminando em morte neuronal (LAI; ZHANG; WANG, 2014; SZYDLOWSKA; TYMIANSKI, 2010). Além disso, essas patologias podem ocorrer por vários anos, sem a manifestação de sinais ou sintomas, dependendo do local da lesão. Portanto, intervenções terapêuticas são necessárias para evitar a cascata da excitotoxicidade do glutamato (QUILLINAN; HERSON; TRAYSTMAN, 2016).

As LCT geralmente envolvem danos mecânicos aos tecidos cerebrais (lesão primária), bem como reações que se propagam e acarretam lesões no tecido circundante, um fenômeno conhecido como lesão secundária (XIONG; MAHMOOD; CHOPP, 2013). Esses mecanismos secundários podem ser altamente complexos, envolvendo vias de sinalização celular inter-relacionadas, que incluem disfunção mitocondrial (ARUNDINE; TYMIANSKI, 2004), estresse oxidativo (RAGHUPATHI, 2004) e lesão excitotóxicas, que são umas das principais contribuintes para a cascata de lesões secundárias a jusante após as LCTs (PARK; BELL; BAKER, 2008).

1.2.2. Doenças neurodegenerativas

As doenças neurodegenerativas compõe uma importantes questões de saúde pública, sendo caracterizadas por disfunções progressivas e perda de neurônios, induzida por déficits neurológicos específicos (HAGUE; KLAFFKE; BANDMANN, 2005). Estudos sugerem que a excitotoxicidade desempenha um papel crucial em grande parte dos distúrbios neurodegenerativos, porém, de forma distinta de uma doença para outra, de acordo com o local onde a neurodegeneração ocorre (DONG; WANG; QIN, 2009a), pois mesmo o glutamato, sendo um aminoácido essencial para a neurotransmissão excitatória, sua hiperatividade pode, causa morte neuronal (BERLIOCCHI LAURA; BANO DANIELE; NICOTERA PIERLUIGI, 2005).

A perda de neurônios ocasionada pelos fenômenos excitotóxicos estão envolvidos em doenças como: doença de Alzheimer, doença de Parkinson e esclerose múltipla e doença de Huntington (OBESO et al., 2008; VALLEJO-ILLARRAMENDI et al., 2006; VELLIQUETTE; O'CONNOR; VASSAR, 2005). No entanto os processos excitotóxicos são, provavelmente,

fatores patogênicos secundários nessas doenças, e o grau de contribuição para a fisiopatologia de cada uma, não é claro (DOBLE, 1999).

A doença de Alzheimer (DA) é um distúrbio neurodegenerativo do sistema nervoso central associado à perda cognitiva e de memória progressiva. Uma das causas dessa doença é o acumulo do peptídeo β-amilóide (βA) em placas senis. Esse acumulo serve como fator de iniciação para múltiplas vias neurotóxicas, incluindo a excitotoxicidade (PARAMESHWARAN; DHANASEKARAN; SUPPIRAMANIAM, 2008). Acredita-se que o acumulo de glutamato e a ativação excessiva dos receptores NMDA, aumente a produção das formas patológicas de βA, da Tau, outra proteína relacionada com a DA (VELLIQUETTE; O'CONNOR; VASSAR, 2005). Além disso, estudos relatam evidência de alterações no metabolismo de transporte do glutamato, principalmente do GLT-1. Essas evidências excitotóxicas também são investigadas na Esclerose múltipla (EM).

A EM é um distúrbio neurodegenerativo humano de etiologia desconhecida, no entanto nos pacientes com essa doença, as concentrações de glutamato estão aumentadas no líquido cefalorraquidiano (SARCHIELLI et al., 2003) além disso, a excitotoxicidade contribui para a perda de oligodendrócitos e axônios (GROOM; SMITH; TURSKI, 2003; MATUTE; DOMERCQ; SÁNCHEZ-GÓMEZ, 2006). A morte das células neuronais, também tem sido relacionada à Doença de Parkinson (DP), um distúrbio neurológico causado pela perda de neurônios dopaminérgicos da substância negra e a consequente queda maciça do conteúdo de dopamina no corpo estriado (MEREDITH et al., 2009; OBESO et al., 2008).

A combinação do aumento da ativação do receptor de NMDA com a diminuição da captação de glutamato, também tem sido relacionado com a Doença de Huntington (DH). Uma doença neurodegenerativa hereditária fatal, causada pela expansão do códon CAG (citosina-adenina-guanina) para glutamina, resultando em um poliglutaminas (poliQ), sendo caracterizada por discinesias com comprometimento cognitivo e psiquiátrico associado (VELDMAN et al., 2015)

Contudo, considerando a indispensável presença de glutamato no SNC, não é surpreendente que problemas na transmissão glutamatérgica se relacionem com as lesões e doenças mencionada a cima. Tendo isso em vista, é de grande importância mais estudos para melhor compreensão dos processos excitotóxicos.

1.3. Estresse oxidativo *versus* Antioxidantes

O oxigênio (O_2) é uma molécula essencial para a vida aeróbica, mas também é um precursor para a formação de Espécies Reativas de Oxigênio (EROs). As EROs são radicais que possuem elétrons não pareados, como ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e radical hidroxila (OH^-) (PHANIENDRA; JESTADI; PERIYASAMY, 2015). Na homeostase redox, as EROs desempenham importantes funções fisiológicas, como na proliferação e diferenciação celular, participação no processo fagocítico de defesa contra infecções e atuando como fatores de transcrição na sinalização intracelular, induzindo a apoptose (HALLIWELL, 2006; SCHIEBER; CHANDEL, 2014). Entretanto, quando ocorre um aumento das EROs, que ultrapassam os níveis usados para sinalização, pode haver danos diretos à célula, pois passa a desempenhar efeitos citotóxicos sobre os fosfolipídios de membranas resultando em peroxidação lipídica e oxidação proteica (DRÖGE, 2002; KOZLOV et al., 2011), facilitando o desenvolvimento de uma variedade de patologias, incluindo a excitotoxicidade glutamatérgica (CHOUDHURY et al., 2017; RUEDA et al., 2016).

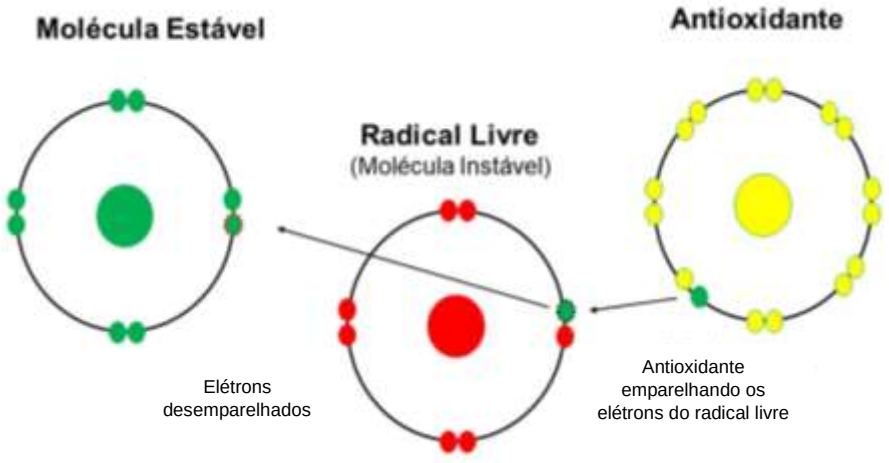
Durante os processos excitotóxicos, as mitocôndrias são muito importantes, pois armazenam o Ca^{2+} que entra nas células, quando as altas concentrações de glutamato superativa seus receptores (GUO et al., 2013; REGO; OLIVEIRA, 2003). No entanto, o acumulo excessivo de Ca^{2+} na matriz mitocondrial tem sido associado com a perda de potencial de membrana mitocondrial, desacoplamento de fosforilação oxidativa, abertura dos poros de permeabilidade mitocondrial e aumento da produção de EROs (DEPP et al., 2018). Todas essas consequências caracterizam a disfunção mitocondrial, que poderá causar inúmeros processos patológicos.

A disfunção mitocondrial associada à sobrecarga de Ca^{2+}, conduz um aumento do estresse oxidativo, desempenhando danos celulares associados à excitotoxicidade (FRANDSEN; SCHOUSBOE, 1993; REGO; OLIVEIRA, 2003). Uma das principais consequências desses processos é a produção de EROs, as quais induzem danos importantes ao SNC, o qual é suscetível a essas moléculas, por demandar alto consumo de oxigênio e por conter altos níveis de ácidos graxos poli-insaturados (SALIM, 2017).

A interação das EROs com biomoléculas, pode causar danos a lipídios e proteínas, culminando na fragmentação e condensação do DNA, fator que está relacionado com várias doenças neurodegenerativas. Visto isso, pesquisas indicam que a sobrecarga de Ca^{2+} intracelular associada à excitotoxicidade induz apoptose (ANKARCRONA et al., 1995; GIORGI et al., 2012)..

No entanto, as células possuem alguns mecanismos de defesa contra os danos induzidos pelos radicais livres. Os problemas ocorrem quando a produção de EROs excede sua eliminação pelos sistemas de proteção antioxidante ou quando estes são danificados, levando ao estresse oxidativo (EO). O EO decorre de um desequilíbrio entre a geração de compostos oxidantes e o desempenho de sistemas de defesa de enzimas antioxidantes (BIRBEN et al., 2012).

Os antioxidantes, tem a capacidade de eliminar EROs, por serem moléculas estáveis que doam um elétron a um radical livre, reduzindo sua capacidade de danificar outras biomoléculas (HALLIWELL, 2006). Essas moléculas podem ser antioxidantes endógenos enzimáticos e não-enzimáticos. Dentre os principais antioxidantes endógenos enzimáticos, encontram-se a superóxido dismutase, glutationa peroxidase, glutationa S-transferase, catalase. E não enzimático, o tripeptídeo glutationa (GSH) (BIRBEN et al., 2012; HALLIWELL, 2006). No entanto, o efeito tóxico de muitos agentes, pode causar o comprometimento desses sistemas de defesas endógenos. Por isso, estudos têm avaliado a capacidade de antioxidantes sintéticos ou naturais de combaterem os efeitos nocivos das EROs e atuar como potenciais tratamentos farmacoterapêuticos (BALASAHEB NIMSE; PAL, 2015; LIU et al., 2018b; ZHANG; LAU; MONKS, 2011).

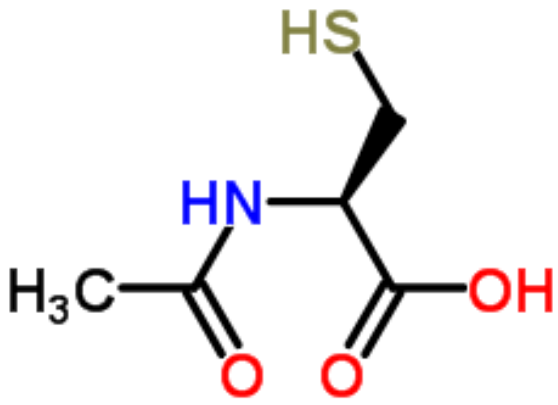


Fonte: Adaptado de encurtador.com.br/ceKSW.

Figura 3: Função de um antioxidante [os radicais livres danificam interagem com biomoléculas para tentar emparelhar a camada de elétrons, no entanto os antioxidantes doam seus elétrons para os radicais livres os instabilizando].

1.4. N-acetil-L-Cisteína

Dentre os antioxidantes sintéticos encontra-se a N-acetil-L-cisteína (NAC), um medicamento aprovado pela "Food and Drug Administration" dos Estados Unidos, com um histórico de segurança de 40 anos (EAKIN et al., 2014). A NAC, é um derivado da cisteína com um grupo acetil ligado ao seu átomo de nitrogênio, e como a maioria dos tióis, pode ser oxidado por uma grande variedade de radicais (Fig. 4) (SAMUNI et al., 2013). As altas propriedades antioxidantes da NAC resultam do composto sulfidrila, que permite a eliminação direta das EROs, bem como L-cisteína, que garante capacidade antioxidante indireta. A L-cisteína é conhecida como um precursor da GSH reduzida, um importante antioxidante endógeno (ELBINI DHOUIB et al., 2016). A atividade da NAC aumenta a concentração de GSH nas células e, portanto, é capaz de restaurar a homeostase redox e reduzir o EO. Além disso, é um potente sequestrador de radicais livres como resultado de suas reações nucleofílicas com as EROs (ARUOMA et al., 1989; RADOMSKA-LEŚNIEWSKA et al., 2016). Nesse sentido, estudos já demonstraram que a NAC protege as células neuronais contra oxidação, reduzindo as EROs (CHOI et al., 2011; FUKUI et al., 2009a).



Fonte: https://goo.gl/mBm3a4

Figura 4: Fórmula estrutural da NAC

Além disso, a NAC também pode regular o fluxo de glutamato através dos seus transportadores GLT-1 e do Sistema X_c^- (que troca glutamato por cistina na proporção de 1:1) (REISSNER; KALIVAS, 2010). O GLT-1 depende diretamente da cisteína, enquanto que o Sistema X_c^- depende da cistina, resultante da oxidação da cisteína (BRIDGES; NATALE; PATEL, 2012; SANACORA; BANASR, 2013). Portanto, tendo em vista a capacidade antioxidante através da interação com a GSH, e a capacidade de regular os transportadores de

glutamato, é possível que a NAC possa minimizar o estresse oxidativo, bem como evitar processos excitotóxicos (KRZYZANOWSKA et al., 2016; WHILLIER et al., 2009).

1.5. *Psidium guajava*

Acredita-se que dois terços das espécies de plantas do mundo tenham importância medicinal, e quase todas elas têm excelente potencial antioxidante (KRISHNAIAH; ROSALAM SARBATLY; NITHYANANDAM, 2010). Desde a descoberta desse potencial, estudos com extratos de plantas vem aumentando, visto que suas propriedades podem combater o estresse oxidativo induzido por uma variedade de doenças (KASOTE et al., 2015). Além disso a Organização Mundial da Saúde (OMS) também afirma que as plantas seriam a melhor fonte para obter diferentes tipos de tratamentos e medicamentos (PORWAL; SINGH; GURJAR, 2012).

A *Psidium guajava*, Linnaeus 1753 (PG), conhecida como "goiabeira" (Fig. 5), é uma espécie arbustiva e arbórea de pequeno porte, da família Myrtaceae, considerada nativa do México, no entanto, de uso popular em diversos países, pois cresce em todas as áreas tropicais e subtropicais do mundo e adapta-se a diferentes condições climáticas. Essa planta é conhecida por conter propriedades medicinais (GUTIÉRREZ; MITCHELL; SOLIS, 2008). Além do consumo do fruto, as folhas são tradicionalmente utilizadas, para o tratamento de diarreia, disenteria, flatulência e cólica abdominal, através de chás. O efeito antidiarreico está relacionado com o conteúdo em flavonoides, que atuam como antagonistas do Ca^{2+} nas fibras musculares lisas (LOZOYA et al., 2002). Também há relatos de uso para gastroenterite, germes patogênicos e antibacterianos intestinais, anti-inflamatório, para diabetes, hipertensão, cáries, feridas, alívio da dor e redução da febre (BASHA; KUMARI, 2012; GONÇALVES et al., 2008; JANG et al., 2014a; OJEWOLE, 2006; SOMAN et al., 2013; SRIWILAIJAROEN et al., 2012).

As folhas dessa planta, apresenta compostos, tais como, lipídios, hidratos de carbono, proteínas, vitaminas, óleos essenciais, taninos, saponinas, flavonoides, esteróis e triterpenos (THENMOZHI; RAJAN, 2015). Esses compostos fenólicos, especialmente os flavonoides, apresentam benefícios à saúde e fornecem à planta, elevadas propriedades antioxidantes, o que a torna uma excelente alternativa para combater o estresse oxidativo (CARRASCO-POZO; GOTTELAND; SPEISKY, 2010) e com isso, auxiliar no tratamento e prevenção de uma série de doenças neurodegenerativas.

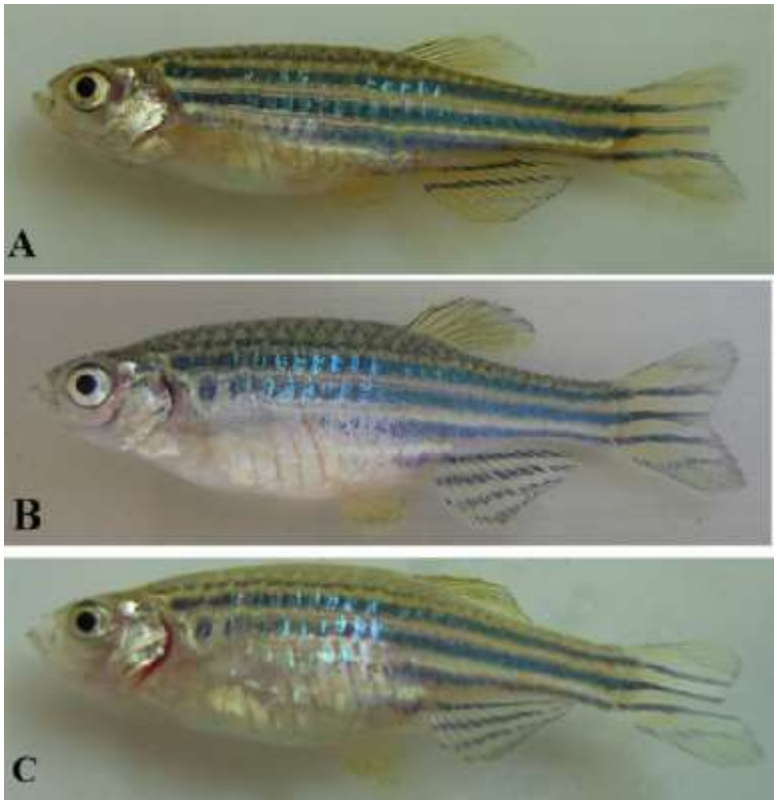
Fonte: https://goo.gl/7pPh7X

Figura 5: *Psidium guajava* Linnaeus. (1753)

1.6. *Danio rerio* (peixe-zebra)

A utilização de modelos animais tem sido muito eficaz nos campos de pesquisas de tratamentos de doenças devido a seus aspectos biológicos similares a humanos. O *Danio rerio* Hamilton-Buchanan (1822) (Fig. 6) foi introduzido nas pesquisas científicas como modelo animal por George Streisinger em 1981 (DAMMSKI et al., 2011; GRUNWALD, 2002), e vem

sendo amplamente utilizado como modelo, no ponto de vista científico em todas as suas fases de desenvolvimento, tanto para pesquisas sobre o potencial farmacológico de novas drogas, como na toxicologia de contaminantes ambientais, embriologia, biologia do comportamento, genética, bioquímica entre várias outras (BAMBINO; CHU, 2017; COLWILL; CRETON, 2011; COSTA-SILVA et al., 2018b; FETCHO, 2007; LI et al., 2018).

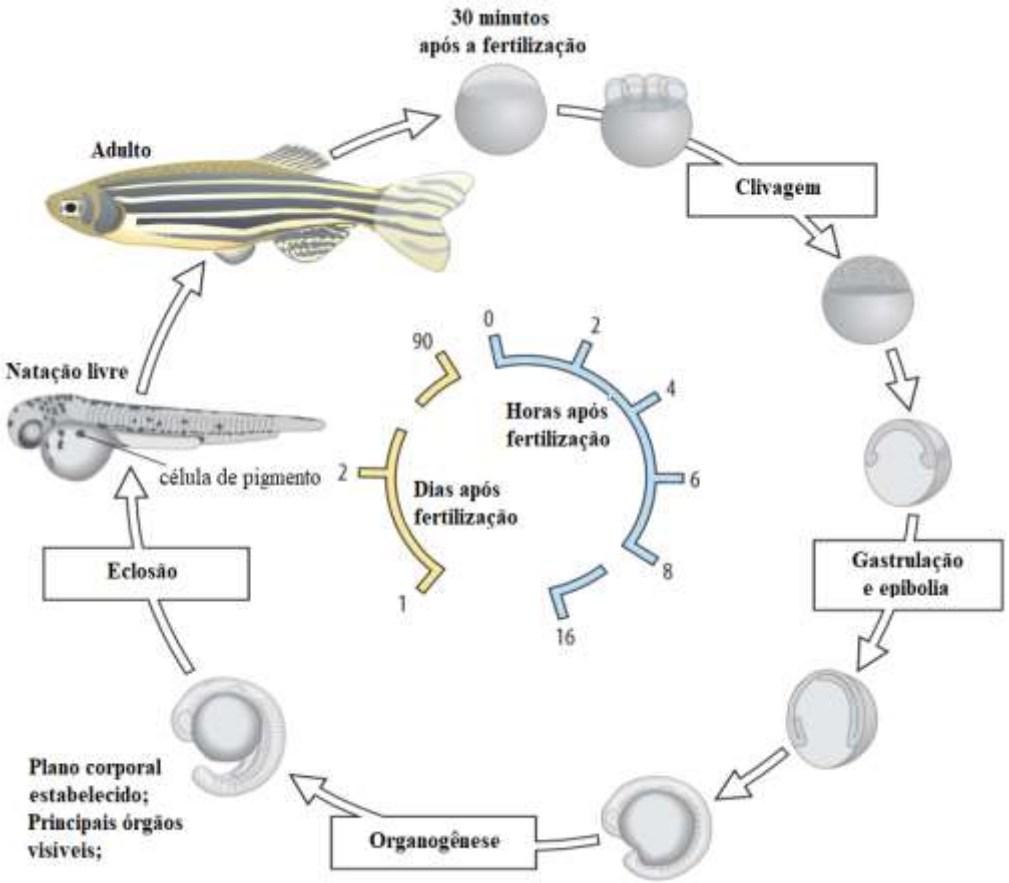


Fonte: AVDESH et al., 2012.

Figura 6: Dimorfismo sexual do peixe-zebra –[(A) macho com o corpo esguio e coloração dourada entre as listras, e as (B-C) fêmea com a barriga estendia, e coloração prateada entre as listras].

Essa espécie, conhecida popularmente no Brasil como peixe-zebra ou paulistinha, é um peixe teleósteo de água doce, da família Cyprinidae, nativo do sul e sudeste da Ásia. Seu desenvolvimento inclui estágio embrionário, larval, juvenil e adultos (Fig. 7), sendo um processo rápido, no qual a formação da gástrula começa aproximadamente 6 horas após a fertilização. Em 48 horas os embriões já estão completamente formados, pronto para eclodir dos ovos. Após 3 meses, atingem a maturidade sexual e possuem alta taxa reprodutiva, cuja fêmeas são capazes desovar cerca de 200-300 ovos por dia, embora o intervalo entre posturas dependa da nutrição, qualidade da água e intensidade de produção, (DAMMSKI et al., 2011;

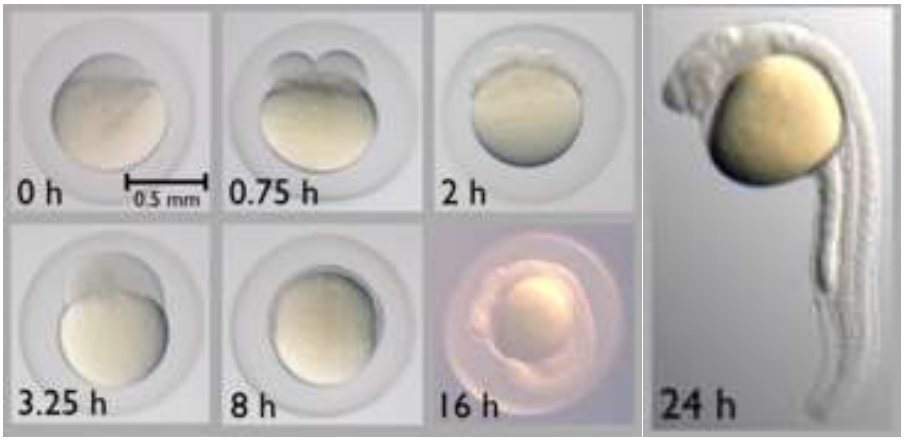
GRUNWALD, 2002). As fêmeas são facilmente diferenciadas dos machos, pois possuem a barriga estendida, e a colocação mais prateada, enquanto que os machos apresentam o corpo mais esguio e com tonalidades douradas.



Fonte: Adaptado e traduzido D'COSTA; SHEPHERD, 2009.

Figura 7: Ciclo de desenvolvimento do peixe-zebra

Os embriões são transparentes, sendo possível acompanhar todo desenvolvimento até minutos antes da eclosão (Fig. 8). Isso permite analisar diversos parâmetros, tais como: mortalidade, frequência cardíaca, formação de somitos, taxa de eclosão, e após 24 horas já é possível observar desfechos morfológicos. Aos 4 dias pós-fertilização (dpf), as larvas têm aproximadamente 4 mm de comprimento, entre 4 e 5 dpf as larvas começam a nadar e a bexiga natatória infla gradualmente (COSTA-SILVA et al., 2018a; KIMMEL C., BALLARD W., KIMMEL S., ULLMAN B., 1995).



Fonte: https://goo.gl/sn1PCz

Figura 8: Desenvolvimento embrionário-larval – [a transparência dos embriões possibilita acompanhar o desenvolvimento da primeira divisão celular até o momento da eclosão].

De acordo com estudos, 70% das proteínas codificantes em genes humanos são relatadas no zebrafish, e 84% dos genes conhecidos estão associados com doenças humanas. Estes dados destacam a importância do modelo na pesquisa de doenças humanas (HOWE et al., 2013) e a oportunidade de acelerar o processo de descoberta de novas drogas que possam servir como estratégias terapêuticas (RICO et al., 2011; STERN; ZON, 2003). Portanto, essas características, juntamente com o rápido desenvolvimento, alta taxa de reprodução, fecundação externa e transparência dos embriões e larvas (WESTERFIELD, M., 2000), fazem deste um dos mais importantes modelos de organismos vertebrados para pesquisas biológicas. Além disso, esse modelo apresenta um repertório comportamental bastante complexo, desde o estágio larval até adulto, pois parece ser conservado evolutivamente e se assemelha com espécies de mamíferos (EGAN et al., 2009; SPENCE et al., 2008). Por essa razão, tem sido utilizado com sucesso em pesquisas que abordam as mais diversas respostas comportamentais, o tornando uma potencial ferramenta para rastreio de alto rendimento (GERLAI, 2010; MATHUR; GUO, 2010; STEWART et al., 2011).

2. OBJETIVOS

2.1. Objetivo geral

Avaliar o efeito protetor da N-Acetil-L-cisteína e do extrato hidroalcoólico de *Psidium guajava* em um modelo de excitotoxicidade induzida pela exposição de larvas de *Danio rerio* ao glutamato.

2.2. Objetivo específico

- Determinar a concentração de Glutamato e do extrato de *Psidium guajava*, a ser utilizado no estudo;
- Avaliar alterações comportamentais e sensório-motoras relacionadas ao potencial neurotóxico, em larvas de peixe-zebra expostas ao Glutamato;
- Avaliar alterações no processo apoptótico, assim como marcadores de viabilidade mitocondrial e celular nas larvas expostas ao Glutamato;
- Analisar o efeito protetor de N-acetil-L-cisteína e do extrato hidroalcoólico de *Psidium guajava* nas larvas de peixe-zebra expostas ao Glutamato.

-

3. RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito, o qual foi submetido à revista *Neurochemical Research*, ISSN: 1573-6903. Além disso, os itens Materiais e Métodos, Discussão e Referências Bibliográficas, encontram-se no manuscrito, os quais estão dispostos na forma em que foram submetidos para publicação.

3.1. Manuscript Details

Manuscript number	BBR_2019_774
Title	N-acetyl-L-Cysteine and the hydroalcoholic extract of *Psidium guajava* protects against glutamatergic excitotoxicity in zebrafish larvae
Article type	Research Paper

Abstract

Glutamate is the main excitatory neurotransmitter in the vertebrate's central nervous system, however, at high concentrations, it may induce a process called excitotoxicity due to excessive neuronal stimulation. This phenomenon is involved in numerous neurodegenerative diseases and brain injury. The clinically available pharmacotherapeutic agents against glutamatergic excitotoxicity are not completely effective; therefore, research on novel compounds is necessary. In this study, the main objective was to evaluate the potential protective effects of N-acetyl-L-cysteine (NAC) and the hydroalcoholic extract of *Psidium guajava* (PG) against excitotoxicity in a model of glutamate exposure effects of *Danio rerio* larvae (zebrafish). For this purpose, zebrafish larvae, at three days after fertilization (dpf), were exposed for 24h to 10 υM glutamate, and hallmarks of glutamate toxicity were evaluated. Exposure to glutamate resulted in a significant increase in ROS steady-stead production, a decrease in mitochondrial viability, increase in chromatin damage, and apoptosis. Concomitantly, glutamate exposure resulted in behavioral changes, such as distance traveled and touch response. On the other hand, the treatment with NAC demonstrated a protective effect avoiding the cellular damage and behavioral alterations, whereas the treatment with PG prevented against the cellular damage induced by glutamate. Our data show for the first time a protective effect of NAC and PG against glutamate- induced damage in zebrafish larvae, suggesting that both compounds have therapeutic potential against excitotoxic processes.

Keywords: Oxidative stress; apoptosis; antioxidant; glutamate; natural compounds.

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N-acetyl-L-Cysteine and the hydroalcoholic extract of *Psidium guajava* protects against glutamatergic excitotoxicity in zebrafish larvae

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Abstract

Glutamate is the main excitatory neurotransmitter in the vertebrate's central nervous system, however at high concentrations; it may induce a process called excitotoxicity due to excessive neuronal stimulation. This phenomenon is involved in numerous neurodegenerative diseases and during brain injury. The clinically available pharmacotherapeutic agents against glutamatergic excitotoxicity are not completely effective; therefore, research on novel compounds is necessary. In this study, it was expected to elucidate the potential protective effects of N-acetyl-L-cysteine (NAC) and the hydroalcoholic extract of *Psidium guajava* (PG) against excitotoxicity in a model of glutamate exposure effects of *Danio rerio* larvae (zebrafish). For this purpose, zebrafish larvae at three days after fertilization (dpf) were exposed for 24h to 10 □□ glutamate, and hallmarks of glutamate toxicity were evaluated. Exposure to

glutamate resulted in a significant increase in ROS steady-stead production, a decrease in mitochondrial viability, increase in chromatin damage, and apoptosis. Concomitantly, glutamate exposure resulted in behavioral changes, such as distance traveled and touch response. On the other hand, the treatment with NAC demonstrated a protective effect avoiding the cellular damage and behavioral alterations, whereas the treatment with PG prevented against the cellular damage induced by glutamate. Our data show for the first time a protective effect of NAC and PG against glutamate-induced damage in zebrafish larvae, suggesting that both compounds have therapeutic potential against excitotoxic processes.

Keywords: Oxidative stress; apoptosis; antioxidant; glutamate; natural compounds;

Introduction

Glutamate is the most abundant free amino acid and acts as the main excitatory transmitter in the vertebrate central nervous system (CNS) with up to 40% of all synapses being glutamatergic [1]. It is found in more than 80% of all neurons [2], being involved in several physiological functions of the brain, such as cognition, memory, behavior, movement, sensation and in the formation of neural network during development [3–5]. However, the excessive activation of glutamate receptors may lead nerve cells to death in a process referred to as excitotoxicity [6]. Excitotoxic phenomena are involved in numerous neurodegenerative diseases such as Alzheimer's disease [7], Parkinson's disease [8], multiple sclerosis [9] and Huntington's disease [10]. As well as a secondary mechanism after Traumatic Brain Injury [11]. Considering the importance of this neurotransmitter in the CNS, it is not new that glutamate is involved in the pathophysiology of numerous brain diseases, in which, glutamate acts as a potent neurotoxin capable of inducing damage and cell death [12].

Excitotoxicity occurs when prolonged activation of glutamate ionotropic and metabotropic receptors takes place [13]. The primary ionotropic receptors activated by glutamate are the N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5 methylisoxazole-4-propionate (AMPA) and kainic acid (KA) receptors. The NMDA type is highly permeable to Ca^{2+}, and calcium overload has been considered as an essential mechanism during

excitotoxicity, resulting in mitochondrial membrane depolarization, bioenergetic failure, increase in the oxidative load and activation of apoptosis pathways [14] [15, 16].

In this sense, mitochondria play an essential role in cell survival during excitotoxicity, as ATP producers through oxidative phosphorylation as well as regulators of intracellular calcium (Ca^{2+}) homeostasis. On the other hand, these organelles are a primary endogenous source of reactive oxygen species (ROS) [17]. Therefore, its homeostatic imbalance is associated with the generation of superoxide, leading to the alteration of the membrane potential and increasing ROS generation [18]. Even though ROS are essential for cell signaling, its excessive production can disrupt normal cell function and promote damage to cellular lipids, nucleic acids, and proteins, facilitating the development of a variety of pathologies, including glutamate neurotoxicity [19, 20].

Glutamate excitotoxicity is associated with higher cellular levels of ROS [21]. However, cells usually have a few mechanisms by which they defend against damage induced by free radicals. Problems occur when the production of ROS exceeds their elimination by the antioxidant protection systems or when the latter is damaged, leading to oxidative stress [22]. Therefore, exogenous antioxidants acquired through a proper diet, as well as, synthetic antioxidants used as pharmacological drugs tend to inhibit oxidative stress induced by free radicals and have a propensity to block or delay apoptosis [23, 24]. Among the synthetic antioxidants, there is the N-acetyl -L-cysteine (NAC). The high antioxidant properties of the NAC result from the sulfhydryl compound, which provides cells with an extra antioxidant capacity [25].

In addition to synthetic antioxidants, plant-derived compounds can also neutralize neurotoxicity *in vivo* [26–28], thus, being widely studied in various models of disorders of the CNS, including neurodegenerative diseases, stroke and trauma [29–31]. Psidium guajava Linnaeus 1752 (PG), popularly known as guava, belonging to the family Myrtaceae. This family which comprises 140 genera and 3.500 species of trees and shrubs, distributed in tropical and subtropical regions of Australia, Asia, and America. PG flourish in a variety of soils, in which it rapidly propagates and bears fruits [32].

The leaves of this species are widely used in folk medicine, through the infuse of water for the alleviation of diarrhea, dysentery, flatulence, and abdominal colic is a common practice initially inherited from traditional Aztec medicine [39]. Also, antimicrobial, antitussive,

sedative, antidiabetic, and antioxidant properties have also been demonstrated for this plant [33–37]. The wide range of beneficial effects of PG has been attributed to its high content of antioxidants such as ascorbic acid, flavonoids and phenolic compounds including quercetin, gallic acid and caffeic acid [38] [39]. Moreover, other secondary metabolites such as tannins, saponins, steroids [40, 41], carbohydrates, and glycosides [42]. Some studies report that plant extracts rich in polyphenols reduce oxidative stress, mitochondrial dysfunction, and cell death [43].

The *Danio rerio* Hamilton-Buchanan 1822 (Zebrafish) has a high degree of genetic similarity with humans, as well as, brain structures and signaling pathways, being increasingly used in scientific research [44, 45]. Additionally, this model offers advantages such as low maintenance cost, fast development, high reproduction rate, external fertilization, and embryo transparency [46, 47]. Also, the larval stage of zebrafish has been used as a platform in neurobehavioral assays, due to its unique characteristics such as the small size of the larvae and the ability to live in 96-well plates allowing the screening of new drugs and compounds, without generating large amounts of residues [46]. For these and other reasons, zebrafish larvae have been proposed as an alternative model which could bridge the gap between simple assays based on cell or tissue culture, and biological validation in whole organisms such as rodents [46, 47].

Thus, the present study was undertaken to evaluate the potential protective effect of N-Acetyl-L-cysteine and hydroalcoholic extract of *Psidium guajava* in a model of excitotoxicity induced by exposure of *Danio rerio* larva to glutamate.

Material and Methods

Chemicals

L-Glutamic acid monosodium salt; N-acetylcysteine; 2,7-dichlorofluorescein diacetate (DCFH2-DA); Sybr Green; N,N′,N′-Tetramethylacridine-3,6-diamine (Acridine Orange); Resazurin sodium salt (R7017); HEPES Minimum 99.5%; Albumin from bovine serum (BSA, A6003); Dimethyl sulfoxide (DMSO); Sodium chloride (NaCl); Sodium hydroxide (NaOH); Potassium Chloride (KCl); Potassium dihydrogen phosphate (KH_2PO_4); Disodium hydrogen phosphate (Na_2HPO_4); Ethylenediamine tetra-acetic acid (EDTA); Triton X-100; Agarose; Agarose low-melting, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Zebrafish maintenance and reproduction

The larvae used were obtained through the reproduction of the adult *Danio rerio* fish, kept in a Zebtec® system under appropriate water conditions (pH 7.2, 400 □S conductivity, 28 °C temperature), with a 14 h/10 h photoperiod and fed on commercial flocked fish food supplemented with *Artemia sp*., as established elsewhere [47]. The experimental protocols used in this work were approved by the local Ethics committee (CEUA –Unipampa: protocol 003-2016)[48]. The zebrafish larvae were maintained in system water in a B.O.D. (Biochemical Oxygen Demand) incubator at 28 °C until the beginning of the experiments.

Preparation of the hydroalcoholic extract of *Psidium guajava* (PG)

The stem barks of *Psidium guajava* were collected in São Gabriel, Rio Grande do Sul (30° 19’ 27 1” S 54° 20 ’43 7 W), Brazil, in August 2017. The plant material was identified by Ms. Nathane Rodrigues and stored in herbarium Bruno Edgar Irgang (HBEI), of de Federal University of Pampa (UNIPAMPA), and a voucher specimen was deposited (number 1578).

The dried leaves were macerated and weighed, resulting in 250.5 grams (g) of fresh leaves. Afterward, they were distributed in two glass containers, and 1.5 liters of ethyl alcohol PA + 1.5 liters of distilled water were added to each vessel. The vials were kept sealed and protected from light for 15 days. After the content has been subjected to the lyophilization process and using a rotary evaporator, and the end was obtained 30.8 g of hydro-alcoholic PG.

Exposure of zebrafish embryo/larvae to Glu, NAC, and PG

To determine the concentration of glutamate to be used, a number of 50 Zebrafish embryos per group, at 24 h post-fertilization (hpf) were exposed to different concentrations of Glu (5, 10, 20, 50 □□) in Petri dishes in a final volume of 10 mL, these concentrations were based on a previous study [49]. Embryos were kept in B.O.D. throughout the treatment period. After setting a sublethal concentration, larvae with three dpf were exposed to 10□M of glutamate for 24 hours. Behavioral and biochemical tests were performed.

The pre-treatment with 750 □□ NAC was performed, based on a recent study in our laboratory, where no morphological and behavioral changes were detected [50]. Therefore, 72 hpf larvae were pre-exposed for one hour to the NAC, after this period, Glu was added, in the NAC solution, at a final concentration of 10 □□ (Fig.1b). The dilutions of the reagents were done in a solution of 0.5% DMSO and the larvae were kept in B.O.D. during the 24-hour treatment period. Similarly, a dose-response curve of the PG was also performed using embryos with up to ±4 hpf.

Mortality

Following the treatments, embryos mortality was evaluated at 168 hpf time point, every 24 hours. Embryos viability was analyzed by observation of eggs coagulation and absence of the heartbeat in a stereomicroscope according to previously published protocols [47].

Behavior

The behavioral tests were performed after the exposure periods. For this, a 24-well plate was used, in which water of the Zebtec system was placed in two wells, then one larva per well was placed to carry out the filming. The experimental procedures were performed on a stable surface and in a controlled environment to avoid variations in light, sound, and temperature. The behavioral activities of the zebrafish larvae were recorded through the novel tank test in a single 360-sec session. The behavioral repertoire of the larvae was recorded using a webcam connected to a laptop at a rate of 30 frames / s using appropriate video tracking software (ANY-mazeTM, Stoelting CO, USA).

Determination of ROS generation and mitochondrial viability *in vitro*

For the evaluation of reactive oxygen species (ROS), previously established procedures were used [50], with small modifications. Briefly, 15 larvae were used for n, with 96hpf, homogenized in 20 mM HEPES buffer, in the powerlyzer by 30 to 2000 vibrations. Then, for

detection of ROS, the supernatant was incubated in the in a black 96-well plate, with HEPES 20mM buffer and DCF-DA 10 mM. The readings were done in 4 times: 0.20, 40, and 60 min, at 525 nm.

Mitochondrial viability was measured by resazurin assay (fluorescence) with homogenates of 20 larvae *per* group. The assay is based on the ability of viable mitochondria to convert resazurin into a fluorescent compound resorufin [51]. The fluorescence was acquired in a fluorescence plate reader (Perkin Elmer Enspire 2300) at 544 nm of excitation and 590 nm emission after 1h of incubation at room temperature.

Genotoxicity

Comet assay

Genotoxicity will be assessed by breaks in the double helix of deoxyribonucleic acid through the comet test according to [52] with some modifications. To this end, slides containing 1% agarose (diluted in PBS) were prepared and placed at 40 °C for drying. Then, 20 larvae per group were homogenized manually with a pestle for 5 min with of Dulbecco's Modified Eagle Medium (DMEN) plug, centrifuged for 10 min at 3000 rpm, for further removal of the supernatant and resuspension of the pellet in 1% PBS buffer. To complete the slide preparation, the sample was placed in 0.75% low melting agarose, and added to the coverslip, to spread the contents. The slides were placed at 4 °C for 15 min and then added in a lysis solution containing 100mM EDTA, 2.5M NaCl, 1% Triton X-100 and 10% DMSO (pH 13.0) in the dark at 4 °C overnight. After this time, the slides were immersed in a neutralizing solution containing 400mM Tris for 30 min. For the unwinding of the DNA, the slides were immersed for 30 min in a horizontal electrophoresis tank containing an alkaline buffer (12 g/L NaOH and 0.37 g/L EDTA) at 25 V and 300 mA. After the electrophoresis run, the slides were washed in distilled water, fixed with 70% alcohol for 5 min, and placed in the refrigerator for 1h. Then the slides were stained with Sybr Green for 5 min. All slides were analyzed by fluorescence microscopy (Olympus 1x71) at 100× magnification, with an exposure of 1044.7 ms, in the green light (Olympus U-RFL-T UV light) and an image analysis system (Q- Capture). The obtained images were analyzed by ImageJ/Open Comet image analysis software.

Determination of cell death

Acridine Orange in vivo

For this assay were used larvae with 96 hpf. Acridine Orange fluorescent dye was used for the detection of cells in the process of apoptosis. Ten larvae were placed in a 2 mL microtube containing system water and the reagent at the final concentration of 5 □g/mL and then incubated in the dark for 20 min [53]. After, which the embryos were washed one time in system water, then fixed in slides for microscopy with 1.5% methylcellulose and images obtained by fluorescence microscopy (Olympus 1x71) at 40× magnification, with an exposure of 500 ms, in the green light (Olympus U-RFL-T UV light) and an image analysis system (Q-Capture). The images obtained were analyzed by ImageJ software;

Total protein quantification

Protein content was determined using bovine serum albumin (BSA) as standard, according to [54].

Statistical analysis

Normality tests (Kolmogorov Smirnov) and homogeneity (Bartlett's test) was applied. The results were expressed as mean ± standard error (S.E.M.), for the nonparametric data, the analysis was done through the Kruskal-Wallis test and Dunn's post-test. The parametric data were analyzed by the One-Way (ANOVA) and Tukey post-test, considering the significant results when $p \leq 0.05$.

Results

Determination of concentrations of Glu and PG

To determine the concentration of Glu to be used, a dose curve (10, 20, and 50 □□ were performed. At the end of the 120hrs treatment, the exposure resulted in decreased larval survival in a dose-dependent manner (Fig. 2).

For the remaining tests, the concentration of 10 □M was used, since it showed a significantly lower survival rate than the control.

After defining a security concentration of PG to be used, the embryos were exposed to the 0.005 ppm concentration of the extract until the third day after fertilization. The Glu was then added in a concentration of 10 □M for 24 hours (Fig. 1a).

The increase in ROS and changes in mitochondrial viability, induced by glutamate, is attenuated by NAC and PG

The production of ROS, measured as oxidation of the fluorescent dye DCF-DA, was significantly increased in the larvae exposed to Glu ($p < 0.01$) when compared to controls. On the other hand, NAC co-treatment and PG completely blocked this condition, by reducing Glu induced ROS to control levels (Fig. 3a – 3c).

Exposure to glutamate causes a significant decrease in mitochondrial viability ($p <0.05$) compared to control. However, pre-treatment with NAC and PG resulted in a significant increase in viability at the control level, as well as in NAC-only treatment and PG (Fig. 3b – 3d).

NAC and PG prevents genotoxic damage and glutamate-induced cell death

By comet test, a significant increase ($p <0.001$) in comet length and Comet area, compared to control, observed in larvae exposed to Glu 10 □M, could be observed. Corroborating this, there was a significant increase ($p <0.001$) in apoptotic cells stained with acridine orange. However, the NAC and PG pre-treatment completely blocked DNA fragmentation as parameters of comet length and comet area (Fig. 4) and the number of apoptotic cells (Fig. 5).

NAC prevents changes in the behavioral profile of the larvae, induced by glutamate

Larvae exposed to Glu 10 □M presented a significant ($p < 0.001$) decrease of distance traveled ($p <0.001$), max speed ($p <0.001$) and mean speed ($p < 0.05$), after the treatment. However, NAC pre-treatment kept the parameters at the control level (Fig. 6).

Glu exposed larvae did not respond to a tail touch and were incapable of swimming ($p < 0.01$). However, this impairment on motor behavior was significantly diminished in pre-treated with NAC, and the swimming capacity was re-establish to the control levels in larvae (Fig. 6f). However, pre-treatment with the PG did not demonstrate significant changes in the behavior (Fig. 7).

Discussion

In this study, we investigated the potential protective, and antioxidant effect of N-acetylcysteine and *Psidium guajava* extract against oxidative stress and behavioral changes induced by glutamate toxicity in zebrafish larvae, in order to collaborate with research for pharmacotherapeutic agents, since potential treatments against excitotoxic process are still scarce.

The larval model was used as a tool to track changes in the pathophysiological processes of excitotoxicity in order to identify compounds with therapeutic potential to avoid or minimize oxidative damage. This model has the potential to be used in the research of many treatments since in addition to the genetic similarity with humans and the low maintenance cost, they have the undeveloped blood-brain barrier of up to 10 dpf, [55] allowing the direct uptake of the compounds to which they were exposed. In addition, the ability to live in 96-well plates allows the reduction of the amounts of residues used in the research, since a larva can live in a medium of 200 □L. Substantial evidence points to excitotoxicity as a fundamental mechanism involved in human neurodegenerative conditions, including both acute disorders such as stroke and traumatic brain injury, and chronic disorders such as Huntington's, Parkinson's, and Alzheimer's diseases [7, 8, 56–58]. Excitotoxicity is a pathological process capable of causing structural damage and functional deficits due to the accumulation of glutamate, which causes excessive depolarization of the postsynaptic neuron and, consequently, ionic and energetic homeostasis disorders, generating reactive oxygen species (ROS) [59]. The increase of ROS production and the calcium overload in mitochondria may result in glutamate uptake impairment and cellular death [60].

In this work it was demonstrated that there was a significant reduction of the survival of the Glu-exposed larvae in relation to the control, in a dose-dependent manner. The same result was observed in a treatment with transgenic Zebrafish larvae [49]. Therefore, from the survival curve of different concentrations of Glu, we chose the concentration of 10 □M, because it presented a significantly lower survival rate than the control, but it allowed evaluating endpoints of cellular viability and behavior.

Our results demonstrated that exposure to Glu caused an increase in ROS steady-stead formation in exposed larvae and decreased mitochondrial viability. Mitochondria are important during the excitotoxicity process, as they can store Ca^{2+}, which enters the cells when the

glutamate receptors are activated [13]. Therefore a homeostatic imbalance may lead to mitochondrial dysfunction, which is often associated with increased ROS production [18]. Besides that, the reactivity of ROS with DNA, proteins, and lipids facilitates the action of a variety of pathologies, including cell death by apoptosis, affecting the survival of exposed organisms [61]. ROS derived from mitochondria are involved in the initiation phase of apoptosis, contributing to cell death signaling [62]. The set of proteases responsible for the apoptotic processes are called caspases. Studies already demonstrate that these proteases are involved in the excitotoxicity of cerebrocortical [63] and cerebellar cultures [64], also, studies have suggested that caspase activation occurs downstream of Ca^{2+} influx and mitochondrial dysfunction [12]. In this sense, our results are in line with literature demonstrated that treatment with Gu induced genotoxicity and cell death by apoptosis.

Studies have shown that high levels of neuronal cytosolic Ca^{2+} exceed the capacity of intracellular regulatory mechanisms, leading to metabolic imbalances, such as free radical formation and cell death [14, 65]. Therefore, Ca^{2+} overload may have caused damage to the mitochondria, increasing ROS production. Growing evidence suggests that glutamate excitotoxicity induced oxidative stress mediates by mitochondrial dysfunction [66, 67]. As well as, several types of research suggest that mitochondria are at the center of oxidative stress and neuronal death, induced by oxidation [66, 68]. However, the mechanisms of oxidative stress induced by glutamatergic excitotoxicity are not fully understood. Therefore, the understanding of such a process is a key factor for the development of pharmacological interventions that minimize the pathologies of this process.

The use of synthetic antioxidants and natural compounds with antioxidant and protective activity has intensified in recent years as a treatment for a wide variety of toxicological conditions [69, 70]. The synthetic antioxidant NAC can modulate glutamate-induced toxicity. Studies have shown that NAC decreased oxidative stress and improved mitochondrial function in the striatum of Huntington disease models [71]. Our results demonstrated the ability of the NAC to prevent the effects of glutamatergic toxicity, which was able to minimize the increase in ROS production. This compound has this scavenger capacity thanks to the sulfhydryl group, which allows the direct elimination of ROS, such as superoxide radical, hydrogen peroxide and hydroxyl radical, as well as, L-cysteine, which guarantees indirect antioxidant capacity, known as a precursor of GSH [72, 73]. Therefore, possibly this intervention in the exacerbated

production of ROS, has collaborated in the prevention of DNA damage and cell death by apoptosis, indicated by the comet assay and Acridine Orange, respectively.

On the other hand, astrocytes and microglial cells express glutamate receptors and play an important role in the induction of cell death [74, 75]. Although, the oxidative stress induced by excitotoxicity may be compromising the functioning of these cells, causing the process of cell death to accelerate, leading to physiological and, consequently, behavioral damages. Also, motor neurons are particularly susceptible to excitotoxicity [76]. However, pre-treatment with NAC prevented locomotor and sensory-motor damage, which we can observe through behavioral tests. Studies have shown that NAC can cross the blood-brain barrier and directly donate cysteine to the central nervous system [77], also, NAC can regulate the amount of Ca^{2+} by donating cysteine to glutamate transporter 1 (GLT-1) [78], because of both transport cystine into astrocytes [79]. GLT-1 is directly dependent on cysteine , which mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the plasma membrane and depends on cystine derived from cysteine oxidation [80, 81]. Given the ability of NAC to increase GSH production and decrease glutamate in the synaptic cleft by GLT-1 [82, 83], we suggested in our study that NAC can the ability to modulate glutamate-induced toxicity, inhibiting oxidative stress rescuing motor functions, reinforcing the hypothesis of the involvement of oxidative stress in the larvae, induced by glutamate.

Natural antioxidants have also received attention due to their pharmacological and biological activities [84]. *Psidium guajava pomífera L*. (Myrtaceae), popularly known as guava. It is a plant of popular use in different countries, adapts to different climatic conditions and has been described as containing several medicinal properties [85]. Studies have already demonstrated the ability to eliminate free radicals [86], possibly due to the presence of compounds in their leaves, such as lipids, carbohydrates, proteins, vitamins, essential oils, tannins, saponins, flavonoids, sterols and triterpenes [85]. These phenolic compounds, present several health benefits and provide the plant with high antioxidant properties, which makes it an excellent alternative to combat oxidative stress, and studies on the potential protective effect of plants suggest that polyphenolic compounds can reduce the mitochondrial calcium load, resulting from excitotoxicity [43, 87]. Through the results, it was possible to observe its protective and antioxidant potential, as it prevented the production of ROS, as well as protected larvae from mitochondrial damage and DNA, as well as interfered with cell death. However, it did not demonstrate significant results related to behavioral changes.

Based on the results, we suggest that the mechanisms of oxidative stress observed after glutamatergic toxicity in zebrafish larvae occurred due to mitochondrial homeostatic dysregulation, favoring the exacerbated production of ROS. The high concentration of these molecules possibly caused damage to the DNA, leading to cell death. On the other hand, the pre-treatment with the antioxidants NAC and PG, demonstrated a great potential of protection, since they were able to prevent the damages caused by the glutamatergic toxicity. Also, the polyphenolic compounds present in the extract PG can be acting to reduce the calcium load. Thus, we suggest that NAC and PG are excellent candidates for the treatment of excitotoxic processes.

In conclusion, our study indicates that the synthetic antioxidant NAC and the extract of *Psidium guajava* have potential protective effects against glutamate-induced excitotoxicity in Zebrafish larvae, suggesting that both have potential therapeutic accounts for the excitotoxic processes.

Future Direction

Our results demonstrated the protective effect of two antioxidants to treat excitotoxic processes. Given the lack of treatments for such lesions in numerous neurodegenerative diseases and brain lesions, further studies should be performed to more accurately elucidate the mechanisms of action of these antioxidants and thereby to develop novel pharmacotherapeutic compounds to minimize and/or prevent glutamatergic excitotoxicity.

Acknowledgments

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Figures

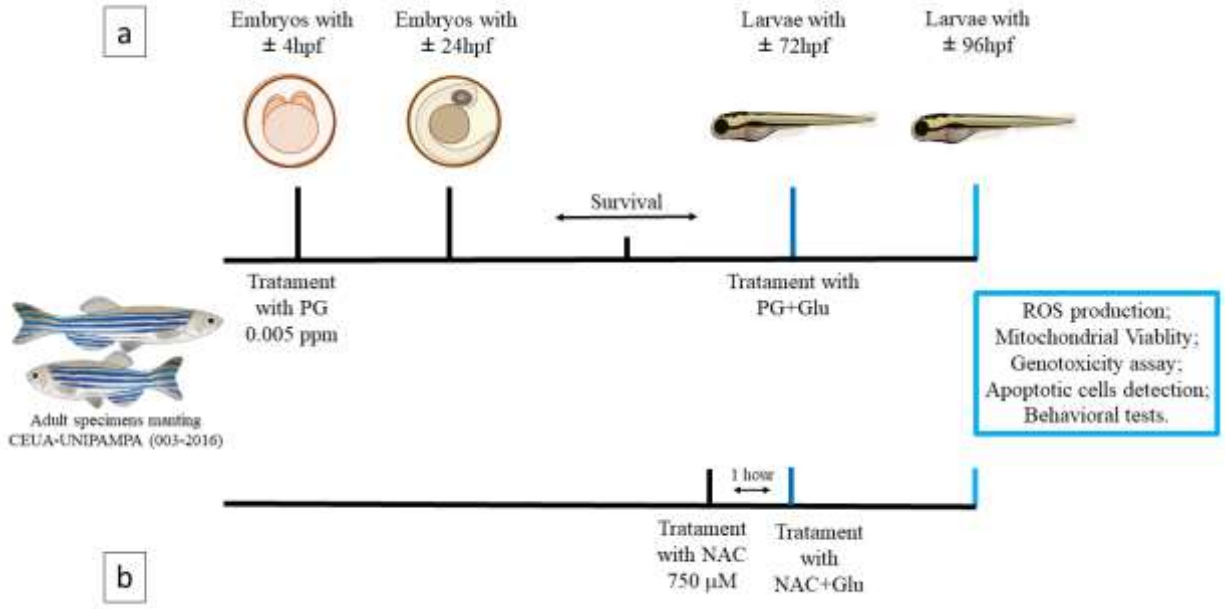


Figure 1: Experimental design of exposure to PG (a) and to NAC (b) and subsequent experiments.

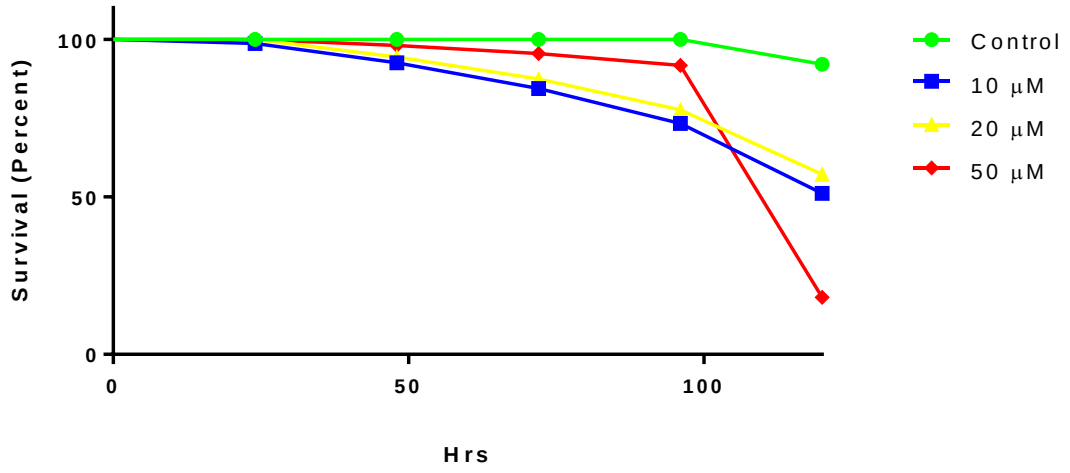


Figure 2: Evaluation of survival of larvae treated with different concentrations of glutamate. (a) The number of live larvae exposed to different concentrations of glutamate after 24 hpf. Data are expressed as mean ± SEM from 100 embryos by group. Statistical analysis was performed by comparing the survival curves with a log-rank (Mantel–Cox) test.

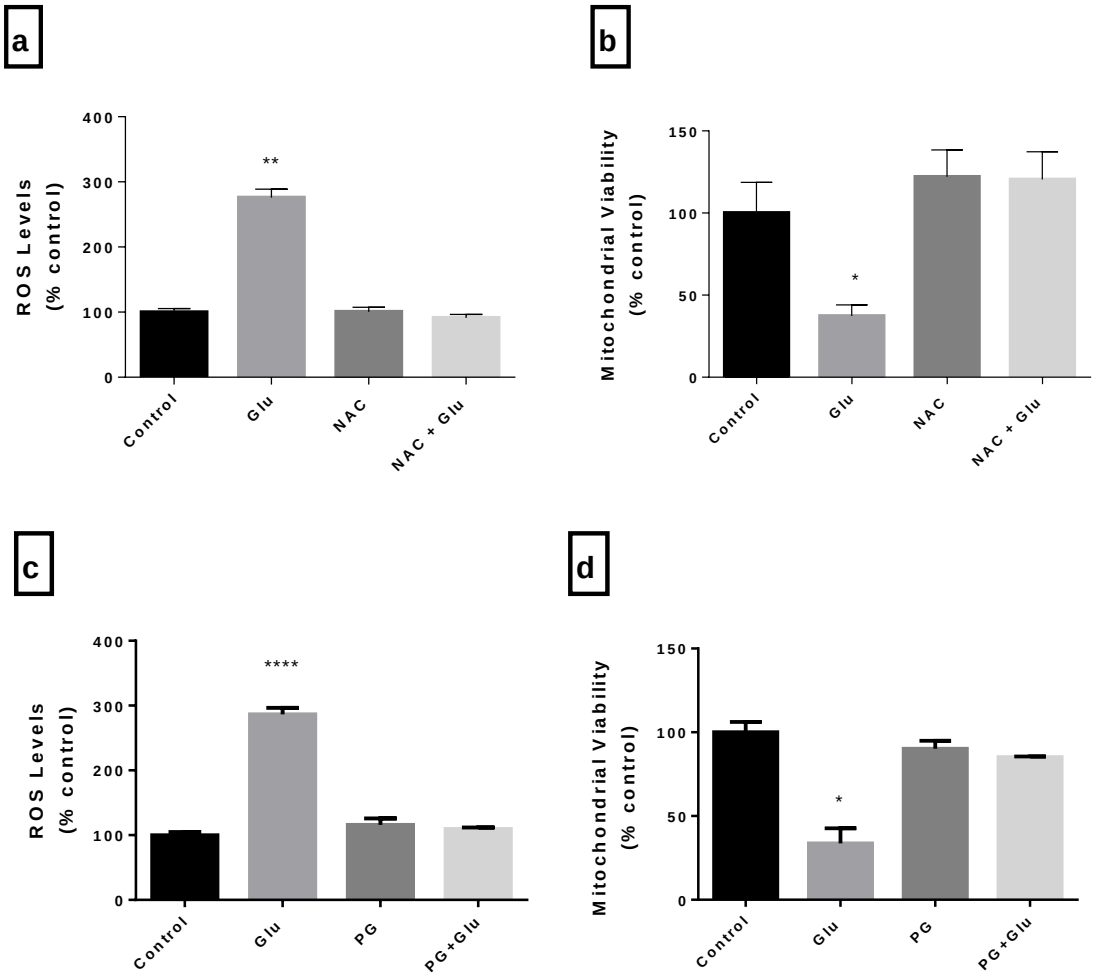
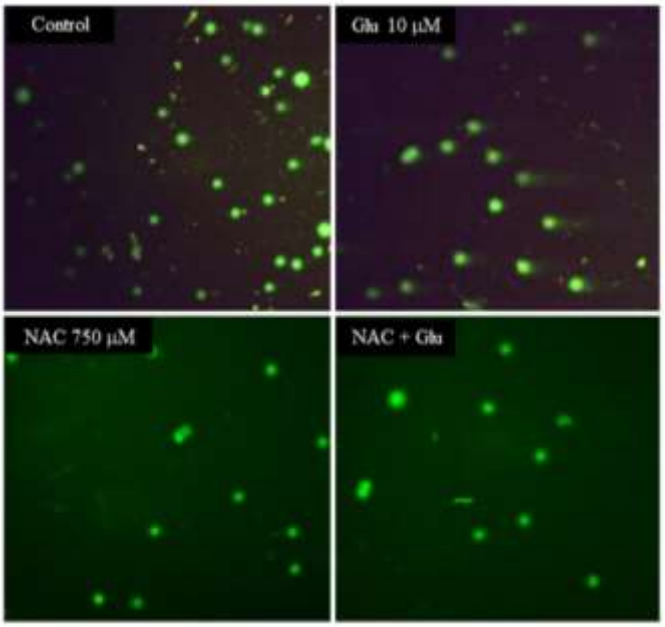
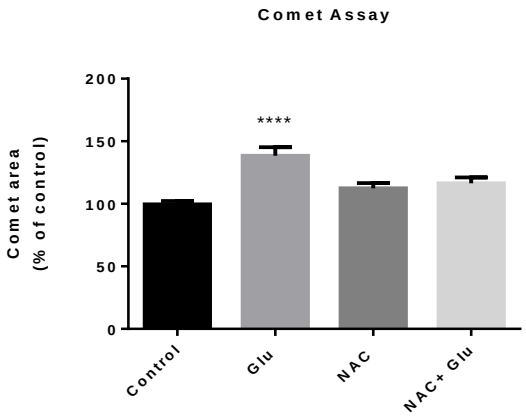


Figure 3: Changes in ROS steady-stead formation and mitochondrial viability. (a) quantification of DCF-DA relative fluorescence from Glu and NAC (b) Resazurin fluorescence assay from Glu and NAC (c) quantification of DCF-DA relative fluorescence from Glu and PG (d) Resazurin fluorescence assay from Glu and PG. Data are expressed as mean ± SEM (% of control) each group and analyzed by one-way ANOVA followed by Kruskal- Wallis and Dunn's Multiple Comparison Test ****$p < 0.0001$, **$p < 0.01$ and *$p < 0.05$.

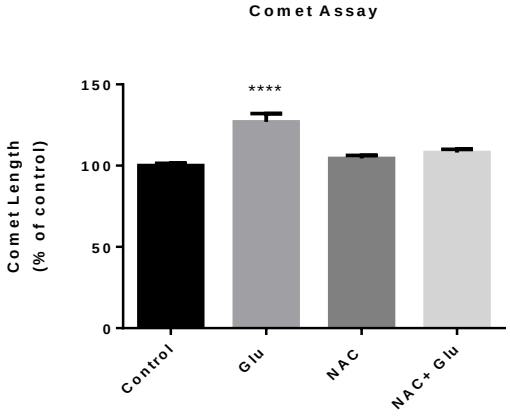
a


Control
Glu 10 µM
NAC 750 µM
NAC + Glu

b


Comet Assay
Comet area (% of control)
200
150
100
50
0
****
Control
Glu
NAC
NAC+ Glu

c


Comet Assay
Comet Length (% of control)
150
100
50
0
****
Control
Glu
NAC
NAC+ Glu

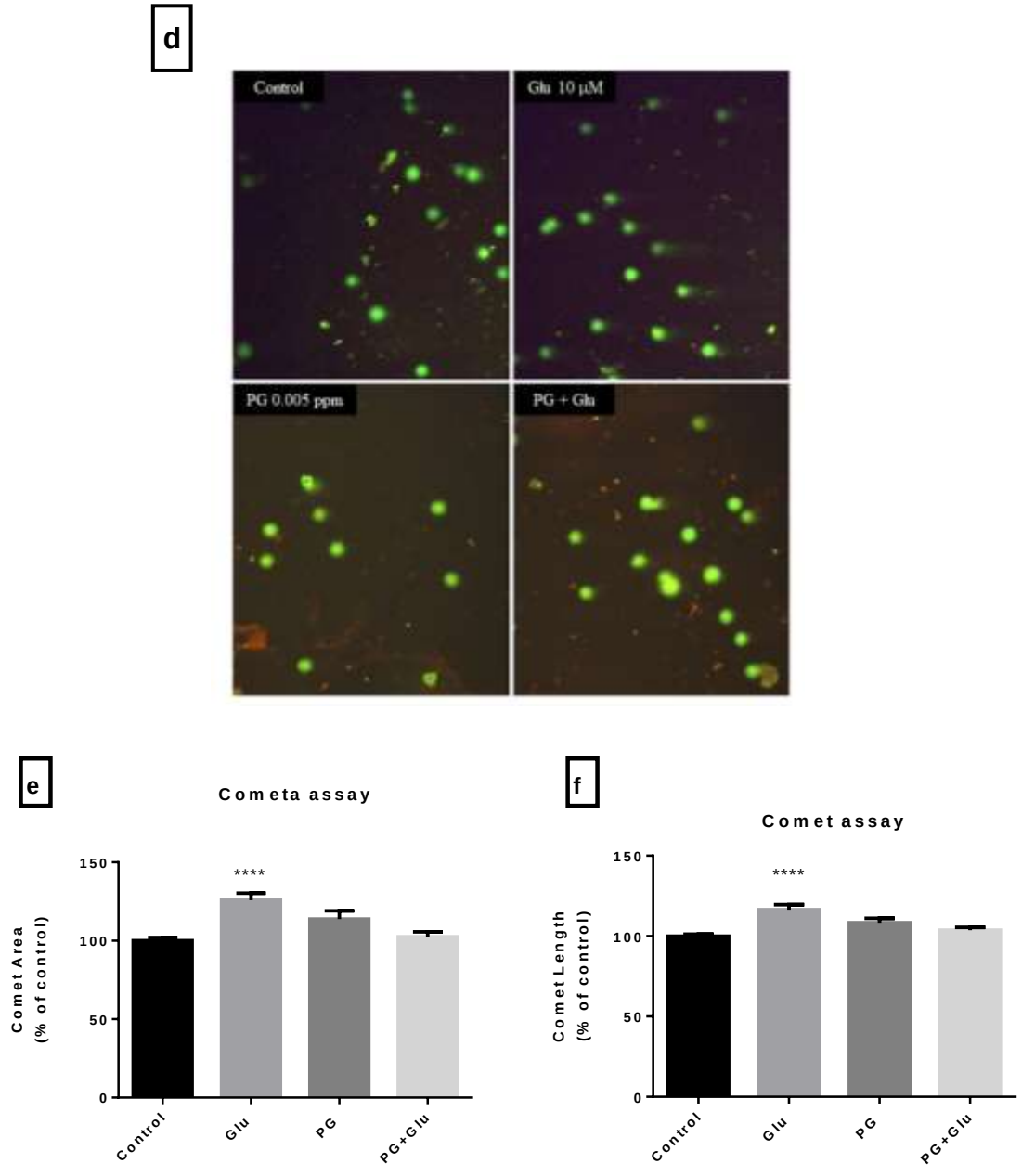


Figure 4: Determination of genotoxicity. (a) Representative images of single cell gel electrophoresis (comet assay) of larvae at 4 dpf (control, Glu, NAC, NAC+Glu); (b) Comet area Glu and NAC; (c) comet length Glu and NAC; (d) Representative images of single cell gel electrophoresis (comet assay) of larvae at 4 dpf (control, Glu, PG, PG+Glu) (e) Comet area Glu and PG; (f) comet length Glu and PG. Data were expressed in microns as the average (% of control) of ±100 different cells and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test****$p < 0.0001$ and ***$p < 0.001$.

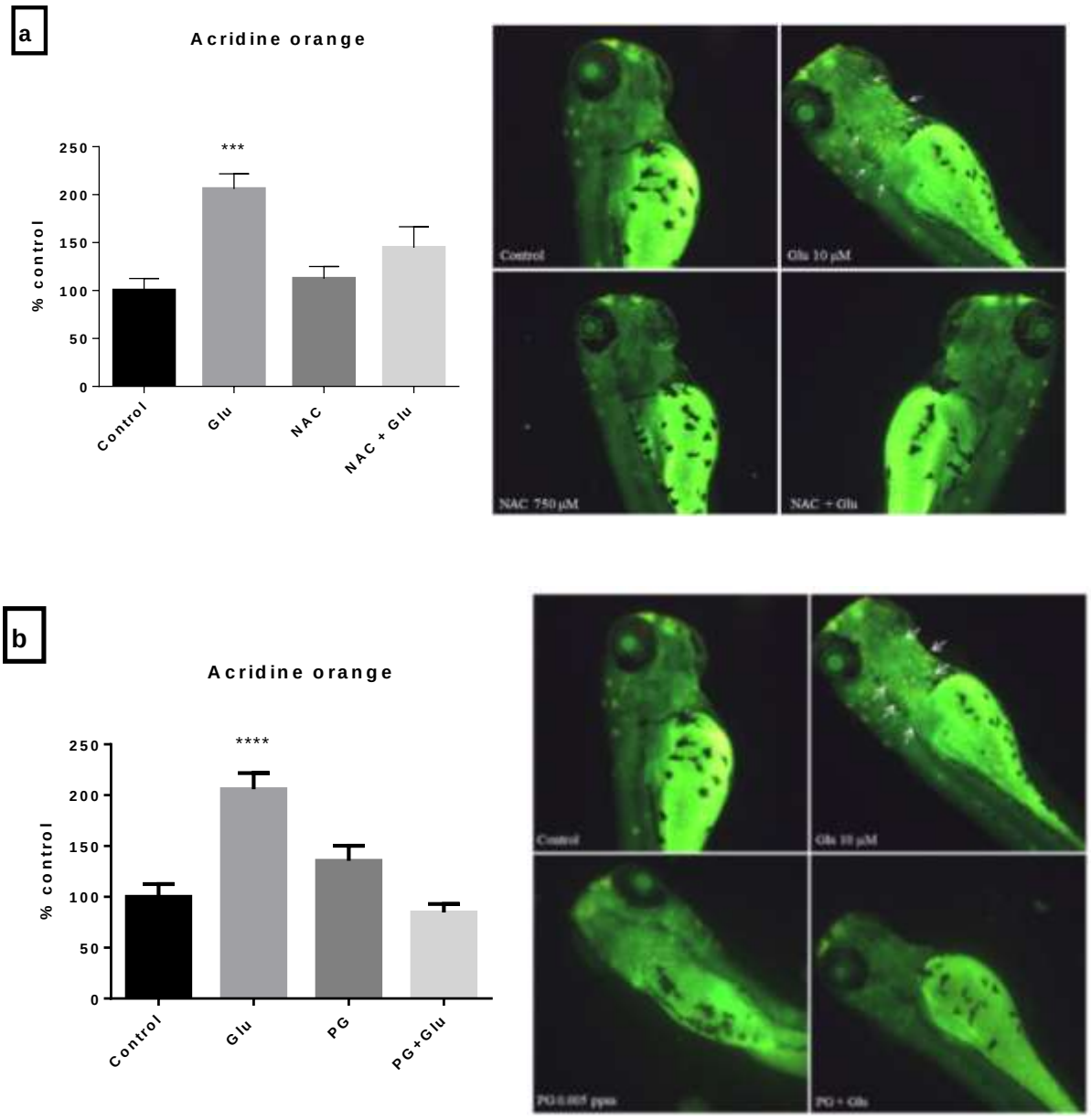


Figure 5: Determination of cell death. Quantification of Acridine Orange relative fluorescence from (a) Glu and NAC (b) Glu and PG. Apoptotic cells were determined of larvae at 4 dpf indicated by the white arrow. Data are expressed as mean ± SEM (fold change) from 10 embryos analyzed individually for each group and analyzed by Kruskal- Wallis and Dunn's Multiple Comparison Test ****$p < 0.001$ and ***$p < 0.001$.

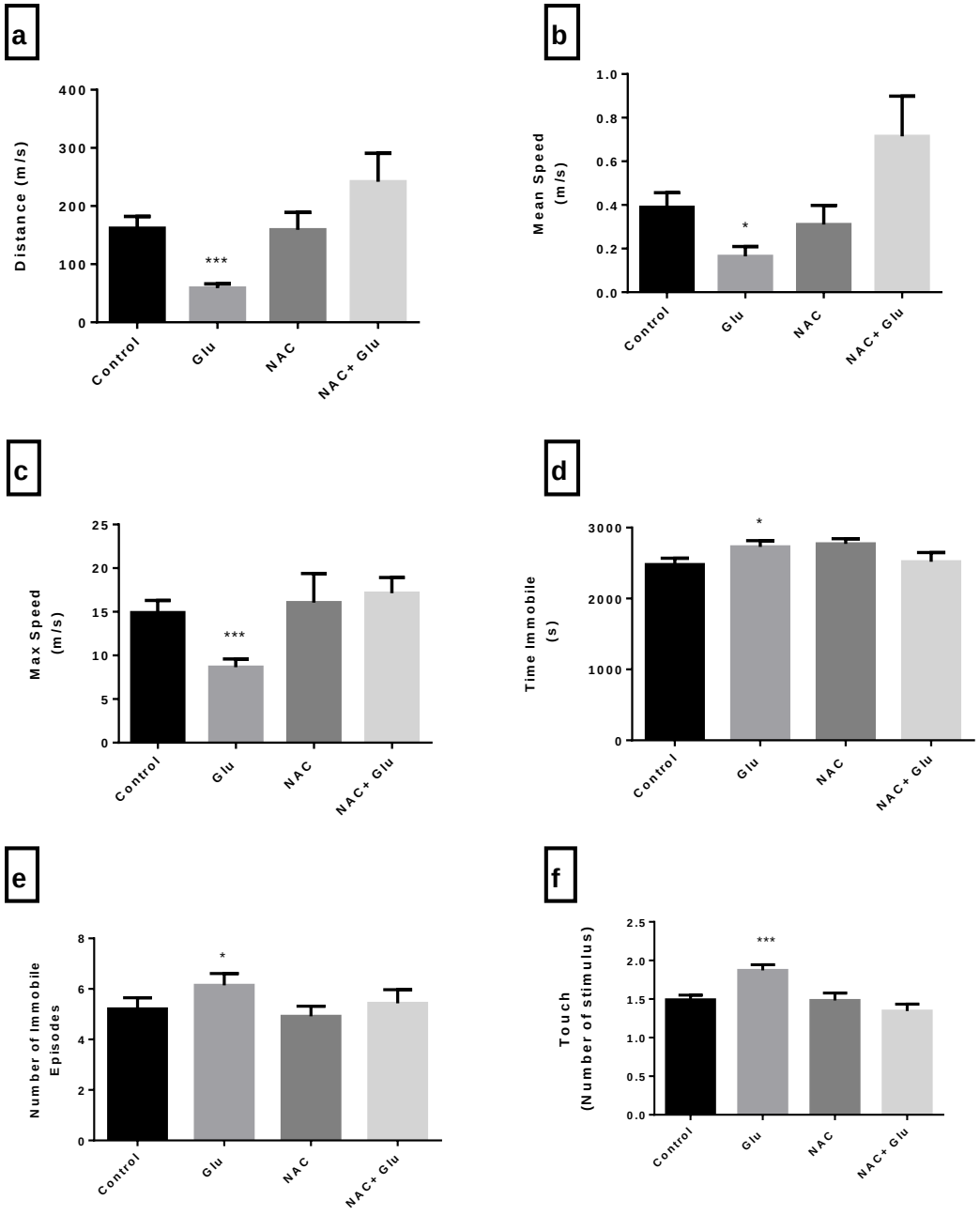


Figure 6: Analyses of swimming behavior and sensory-motor capacity from tratament with Glu and NAC. (a) Distance traveled of larvae 4 dpf , expressed by meters/secunds; (b) Mean speed expressed by mean of meters/secunds; (c) Max speed expressed by speed of meters/secunds; (d) Time immobile expressed by time in seconds; (e) Immobile episodes expressed by number of episodes; (f) touch stimulus, expressed as the number of stimuli necessary to first swim. For all these data, 30 embryos were used individually for each group and results are expressed as mean ± SEM, statistically analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$; **$p < 0.01$; *$p< 0.05$.

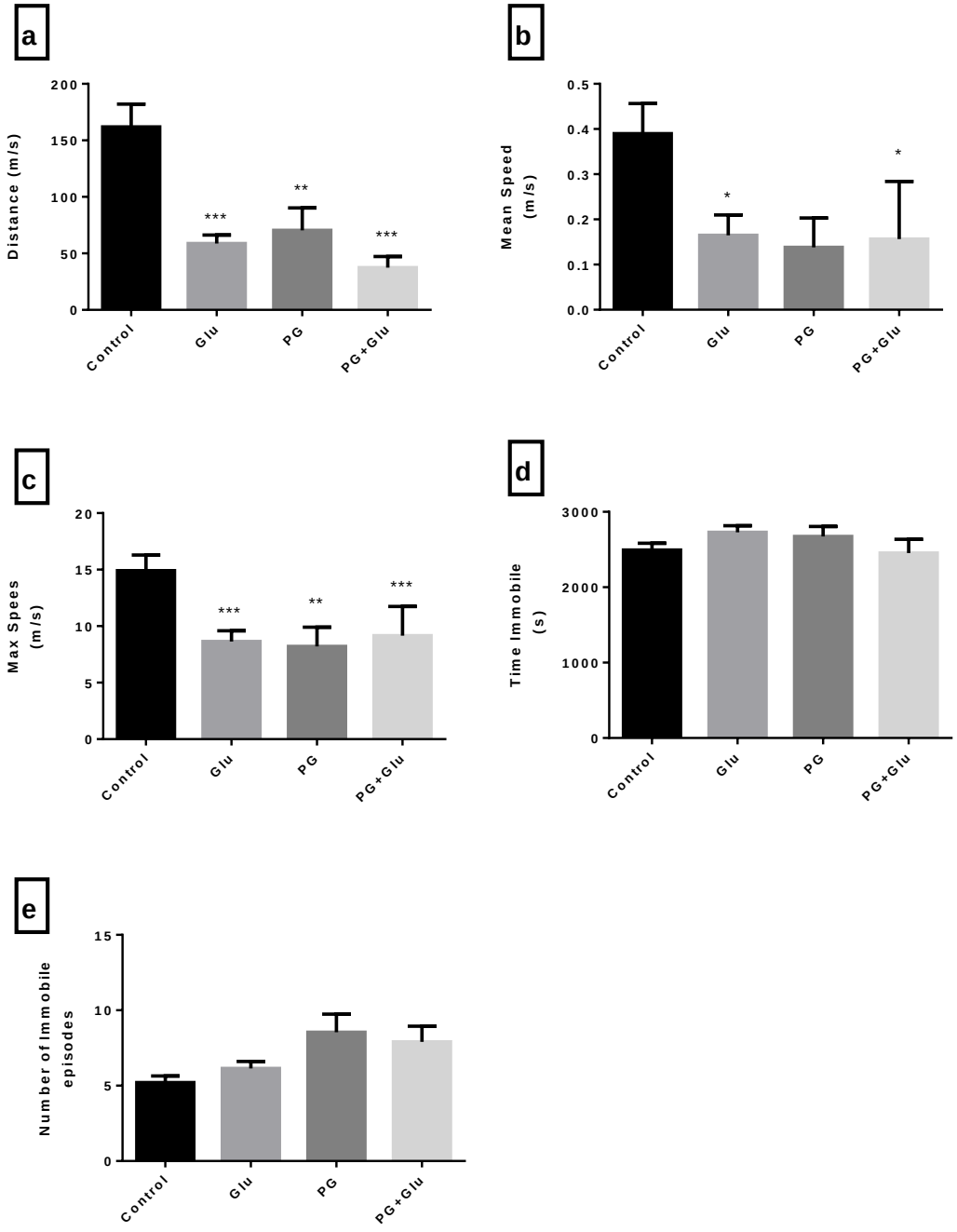


Figure 7: Analyses of swimming behavior from tratament with Glu and PG. (a) Distance traveled of larvae 4 dpf, expressed by meters/secunds; (b) Mean speed expressed by mean of meters/secunds; (c) Max speed expressed by speed of meters/secunds; (d) Time immobile expressed by time in seconds; (e) Immobile episodes expressed by number of episodes. For all these data, 30 embryos were used individually for each group and results are expressed as mean ± SEM, statistically analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

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4. CONCLUSÕES

Com base nos resultados obtidos neste trabalho, foi possível observar que a exposição das larvas de peixe-zebra ao glutamato resultaram em uma série de alterações nos parâmetros, fisiológicos e comportamentais, portanto, apesar desse neurotransmissor ser muito importante para o SNC, em altas concentrações, pode acarretar na produção elevada de EROs, danos genotóxicos e morte celular por apoptose. Essas consequências estão envolvidas em inúmeras doenças neurodegenerativas, bem como em lesões cerebrais traumáticas, causando déficits motores, como visto nos resultados obtidos neste trabalho.

Além disso, os resultados também indicam que o peixe-zebra é um modelo relevante para o estudo dos processos excitotóxicos, visto que resultados semelhantes foram observados em outros trabalhos (MCCUTCHEON et al., 2016; VAN DEN BOSCH et al., 2006), com isso, a sua utilização auxilia no avanço de novos tratamentos para uma série de patologias que tem a excitotoxicidade glutamatérgica como consequência secundária.

Nesse sentido, esse trabalho demonstrou o efeito de dois antioxidantes com potencial protetor contra a excitotoxicidade, sendo um antioxidante sintético (N-Acetil-L-Citeína- NAC) e um composto de origem vegetal (extrato hidroalcoólico de *Psidium guajava* - EPG) os quais evitaram a produção de EROs, danos genotóxicos e morte celular, evitando o estresse oxidativo envolvido na toxicidade glutamatérgica, e a NAC, em especial, foi capaz de prevenir alterações comportamentais. Por fim, conclui-se que ambos antioxidantes estudados são bons candidatos de tratamentos para prevenir e/ou evitar os processos excitotóxicos, envolvidos em inúmeras doenças neurodegenerativas, bem como, lesões cerebrais.

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