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# AÇÃO PROTETORA DA ASTAXANTINA SOBRE O EFEITO DO ETANOL NO CÉREBRO: ANÁLISE ELETROFISIOLÓGICA EM RATOS ALBINOS ADULTOS

RICARDO ABADIE GUEDES

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*Aos meus Pais e meus irmãos, por sempre terem sido minhas fontes de coragem e inspiração.*

# ÍNDICE

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# RESUMO

O consumo de bebidas alcoólicas é um caso de freqüente abuso de drogas, que está associada com uma ampla variedade de distúrbios patológicos, afetando diversos órgãos inclusive o cérebro. Em estudo anterior a este, ficou demonstrado que, no cérebro em desenvolvimento o aporte de etanol facilita a propagação da depressão alastrante cortical (DAC), um fenômeno neural relacionado com a excitabilidade, presente em diversas espécies animais. Este efeito eletrofisiológico foi atenuado por um extrato etanólico de carotenóides de camarão (*Litopenaeus vannamei*). Foram investigados aqui os efeitos da astaxantina pura, o principal carotenóide encontrado no camarão, sobre a DAC. Ratos Wistar machos adultos foram tratados por gavagem, durante 18 dias, com 2,5, 10 e 90µg/kg/dia de astaxantina dissolvida em etanol. A DAC foi registrada na superfície cortical, entre um e três dias, após o final do tratamento. Quatro grupos tratados respectivamente apenas com etanol (3g/kg/d), água destilada e óleo de soja (com e sem astaxantina) foram também estudados para a comparação com os grupos etanol + astaxantina. Os ratos tratados com etanol demonstraram maiores velocidades de DAC (valores médios em mm/min, por hora de registro variando entre 4,08 ± 0.09 e 4.12 ± 0.16), comparados ao grupo água destilada (3,19 ± 0,13 e 3,27 ± 0,06). A adição de astaxantina ao etanol resultou em velocidades menores de DAC de maneira dose dependente, variando entre 3,68 ± 0,09 e 3,97 ± 0,22 para a dose de 2,5 µg⁄kg⁄d, entre 3,29 ± 0,09 e 3,32 ± 0,07 para a dose de 10 µg⁄kg⁄d e entre 2,89 ± 0,13 e 2,92 ± 0,11 para a dose de 90 µg⁄kg⁄d. As velocidades dos grupos óleo de soja (com e sem astaxantina) não foram estatisticamente diferentes do grupo 10µg/kg/d + etanol e grupo água destilada. Os resultados demonstraram o efeito antagônico da astaxantina, contrapondo-se à facilitação da propagação da DAC induzida por etanol. Provavelmente, as propriedades antioxidantes dos carotenóides estão envolvidas em tais efeitos.

Palavras-chave: Etanol, Depressão Alastrante Cortical, Antioxidantes, Carotenóides, Astaxantina.

# ABSTRACT

The consumption of alcoholic drinks is a frequent drug-abuse situation, which is associated to a wide variety of pathological disturbances affecting several organs, including the brain. We have previously shown in the developing rat brain that ethanol intake facilitates the propagation of cortical spreading depression (CSD), an excitability-related neural phenomenon present in several animal species. This electrophysiological effect was attenuated by a shrimp (Litopenaeus vannamei) carotenoids extract. Here we investigated the effects of pure astaxanthin, the main carotenoid found in shrimp, on CSD. Adult Wistar male rats were treated per gavage, during 18 days, with 2.5, 10 or 90 µg⁄kg⁄d astaxanthin dissolved in ethanol and CSD was recorded on the cortical surface 1 to 3 days thereafter. Four groups, treated respectively with ethanol (3 g⁄kg/d), distilled water and soybean oil with- and without astaxanthin were also studied for comparison with the ethanol + astaxanthin groups. Ethanol-treated rats displayed higher CSD-velocities (mean values, in mm⁄min, per hour of recording ranging from 4.08 ± 0.09 to 4.12 ± 0.16), compared to the distilled watergroup (from 3.19 ± 0.13 to 3.27 ± 0.06). Addition of astaxanthin to ethanol lead to lower CSD velocities in a dose-dependent manner, ranging from 3.68 ± 0.09 to 3.97 ± 0.22 for the 2.5 µg⁄kg⁄d-dose, from 3.29 ± 0.09 to 3.32 ± 0.07 for the 10 µg⁄kg⁄d-dose, and from 2.89 ± 0.13 to 2.92 ± 0.11 for the 90 µg⁄kg⁄d-dose. The velocities of the soybean oil groups (with and without astaxanthin) were not statistically different from the 10 µg⁄kg⁄d astaxanthin + ethanol and distilled water groups. The results demonstrate the antagonistic effect of astaxanthin against the ethanol-induced facilitation of CSD propagation. Probably carotenoid antioxidant properties are involved in such effects.

Key Words: Ethanol, Cortical Spreading Depression, Antioxidants, Carotenoids, Astaxanthin.

## 1. INTRODUÇÃO

Os carotenóides constituem o grupo de pigmentos orgânicos mais disseminados na natureza, sendo encontrados nos organismos fotossintéticos como as algas, nos fungos e bactérias, bem como em todos os grupos de animais, dos protozoários até o homem (VILELA, 1976).

Os carotenóides são também denominados isoprenóides ou terpenóides, por sua derivação estrutural de moléculas de isopreno (C5) (Figura 1). Do ponto de vista químico, os carotenóides, em sua maioria, são tetraterpenóides (C40) formados por oito unidades isoprenóides unidas, numa orientação cabeça-cauda, exceto no centro da molécula em que a seqüência se inverte (cabeça-cabeça). Duas unidades de geranilgeranil difosfato (C20), por exemplo, unem-se, numa orientação "cauda-cauda", formando o esqueleto comum de 40 unidades carbônicas, que funciona como o molde, padrão do qual a maioria das variantes individuais é derivada (BRITON, 1995).

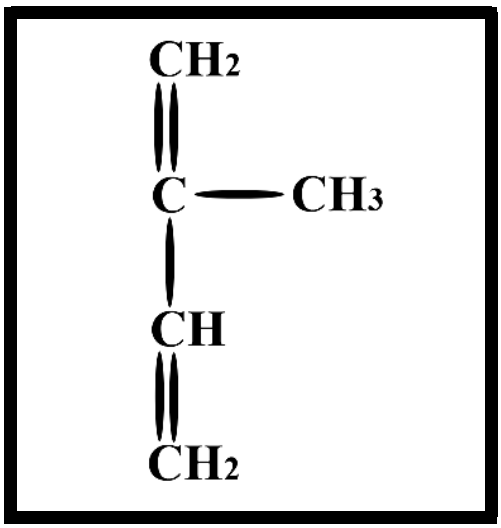


**Figura 1** - Estrutura molecular do isopreno

Apesar das mais de 600 diferentes conformações de carotenóides conhecidas, as quais já foram isoladas e caracterizadas a partir de fontes naturais, todas essas estruturas podem ser consideradas variações de um mesmo tema estrutural. A maioria dos carotenóides é constituída por oito unidades isoprenóides. Alguns sofrem excisão de um ou mais átomos de carbono, enquanto outros recebem adição de unidades isopreno, nos grupamentos terminais. Os que possuem menos de 40 carbonos são denominados apocarotenóides e os que possuem mais de 40 são os norcarotenóides (MELÉNDEZ-MARTINEZ, et al., 2007). Segundo Britton (1995) o esqueleto carbônico, arcabouço comum dessas moléculas, pode ser modificado: pela ciclização de uma ou de ambas as extremidades da cadeia, pelo nível de hidrogenação da molécula ou pela adição de grupos funcionais que contenham ou não moléculas de oxigênio.

Os carotenóides que não possuem oxigênio, em sua estrutura, são classificados como carotenos (β caroteno e licopeno) e, os que o contém, xantofilas (zeaxantina, astaxantina) (Figura 2) (MCNULTY et al., 2008). A característica comum dos carotenóides é possuir uma cadeia poliênica hidrofóbica central, ou seja, com várias ligações duplas e simples alternadas entre os átomos de carbono; o que lhes confere, como grupo, sua configuração molecular distinta, sua reatividade química e suas propriedades de absorbância (BRITON, 1995).

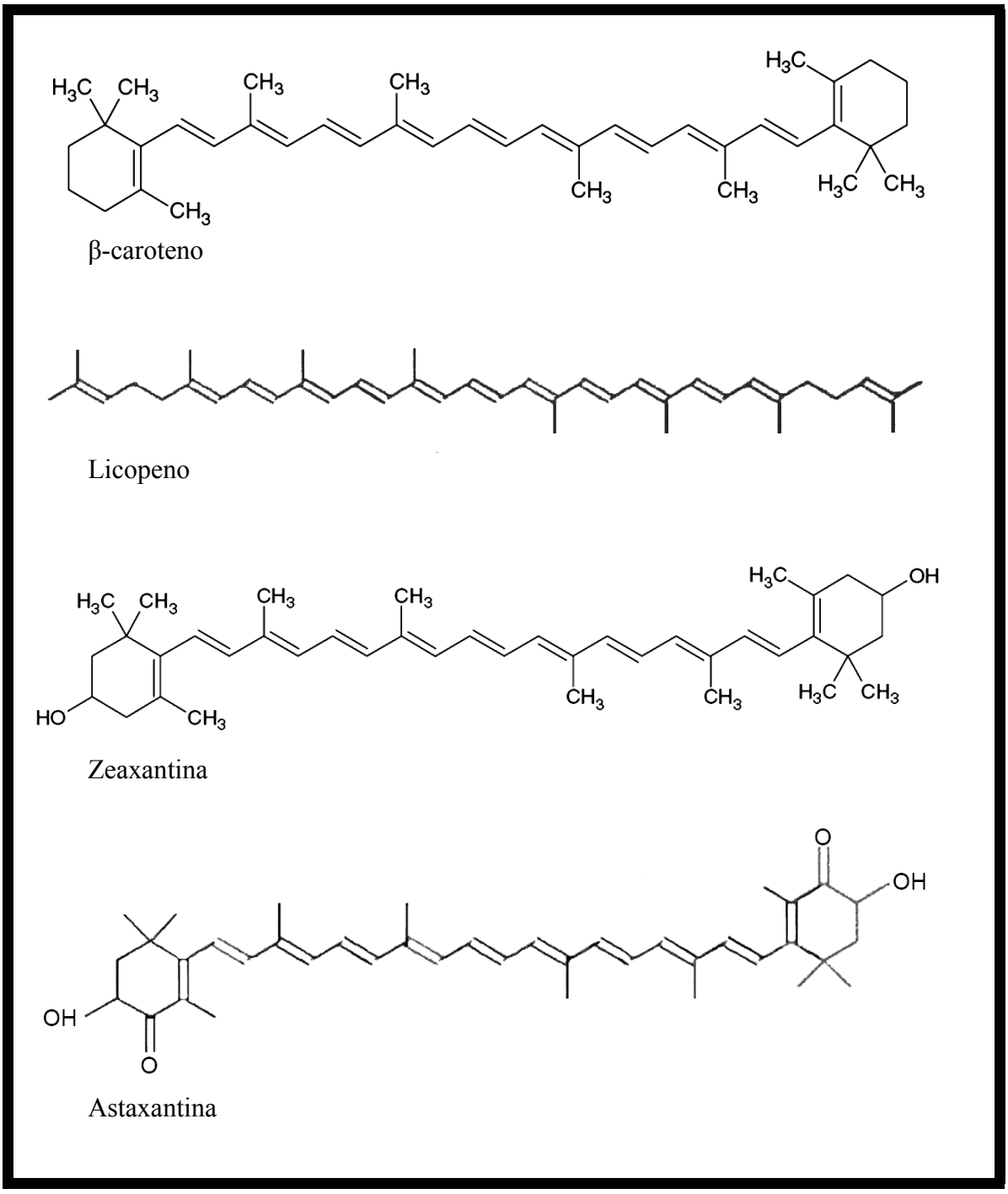


**Figura 2 -** Estrutura molecular dos carotenóides

Os carotenóides podem ser sintetizados por muitas bactérias, todas as algas fotossintetizantes e plantas superiores, bem como por alguns fungos (WEBER e

DAVOLI, 2003). Nas plantas, os carotenóides presentes nas flores ajudam a atrair os insetos, que ajudam na polinização (WEBER e DAVOLI, 2003).

Nas plantas (onde são primariamente produzidos), os pigmentos carotenóides exercem muitas funções como, por exemplo, de captação de luz, fotoproteção, dissipação de excessos de energia e desativação de oxigênio singleto. Carotenóides e xantofilas atuam principalmente associados a proteínas e à clorofila, nos complexos coletores de luz inseridos nos complexos-antena, nas membranas tilacóides, em que funcionam como reguladores do fluxo de energia e estabilizadores da estrutura protéica. A capacidade, que os carotenóides possuem, de deslocar os elétrons $\boldsymbol{\pi}$ (pi) ao longo de todo o sistema de ligações conjugadas, na região central do esqueleto carbônico, é o que lhes confere sua atividade distinta. Esta capacidade de condução permite que eles atuem como "fios moleculares", transportando elétrons para as clorofilas ***a*** e ***b*** e, subseqüentemente, para os centros de reação de fotossíntese nas plantas. Podem atuar também na direção inversa de transmissão, e dissipar para as proximidades, em forma de calor, a energia excedente absorvida de estados mais excitados da clorofila, ou de radicais reativos formados durante a quebra da água, no processo para a fixação de carbono (BRITON, 1995; FORMAGGIO et al., 2001).

A falta destes pigmentos (carotenóides) capazes de coletar luz nos comprimentos de onda, nos quais a clorofila não age de forma eficiente e de debelar o eventual excesso de energia de ativação absorvido pela clorofila, pode gerar intensa formação de radicais livres nos complexos-antena (FORMAGGIO et al., 2001). Experimentos em que herbicidas inibem a enzima fitoeno sintase levam à produção de eventos de foto-oxidação que destroem moléculas de clorofila (MELÉNDEZ-MARTINEZ et al., 2007; PENDON et al., 2006). Todas essas informações revelam parte da função biológica

dessas moléculas, na compatibilização entre a existência da vida, e a alta reatividade da molécula-chave para a geração de energia nos organismos aeróbicos, o oxigênio.

Os animais, entretanto, não são capazes de produzir carotenóides, tendo que adquiri-los a partir da dieta. A natureza hidrofóbica desses pigmentos determina a sua localização celular nos animais, normalmente associada aos compartimentos lipídicos. No entanto, podem formar complexos hidrossolúveis estáveis com proteínas, lipoproteínas ou glicoproteínas. Tais complexos são encontrados, principalmente, em invertebrados aquáticos como camarões, lagostas e caranguejos (MELÉNDEZ-MARTINEZ et al., 2007; BRITTON, 1995).

Em muitos animais os carotenóides são responsáveis pelos padrões de coloração que provêem camuflagem ou apelo visual para atração sexual. Alguns podem ser precursores utilizáveis na formação de β-caroteno e vitamina A, e também apresentam importante atividade antioxidante, ou seja, são capazes de eliminar radicais livres (WEBER e DAVOLI, 2003).

À medida que o interesse pela atividade fisiológica antioxidante foi aumentando, com o conhecimento crescente dos danos biológicos causados pelos radicais livres, mais estudos começaram a investigar os impactos dessas substâncias antioxidantes em modelos de estresse oxidativo. Estudos anteriores demonstraram que o tratamento suplementar com β-caroteno reduziu a incidência de certos tipos de câncer. No entanto, em alguns grupos específicos, como de fumantes por exemplo, outras suplementações (vitamina A e C) podem aumentar a probabilidade de desenvolvimento de câncer de pulmão (YOUNG e LOWE, 2001; HUGHES, 2001).

Com o intuito de obterem um suposto aumento na longevidade, ou na qualidade de vida, muitas pessoas passaram a fazer uso indiscriminado e continuado de vitaminas,

principalmente a A, C e E. Alguns estudos apresentaram resultados sugerindo que tal prática de auto-medicação pode levar a significante ação oxidativa de tecidos e oncogênese, em relação a grupos que não tomaram suplementação vitamínica (HALLIWELL, 1996; HUGHES, 2001 ). A partir da averiguação de tais dados, sugere-se a necessidade de um maior conhecimento dos potenciais efeitos que diferentes dosagens de vitaminas ou outras substâncias, rotuladas como antioxidantes, podem ter sobre o organismo, em diferentes condições.

As recentes descobertas sobre os efeitos dos radicais livres nos organismos vivos, em geral, e no organismo humano em particular, geraram o crescente interesse no estudo de substâncias antioxidantes. As espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) são produzidas naturalmente como conseqüência do metabolismo aeróbico e são integrantes da homeostase do oxigênio nos tecidos. A homeostase do oxigênio consiste na manutenção do balanço entre agentes oxidantes e antioxidantes, podendo ter seu equilíbrio modificado por disfunções biológicas, como, por exemplo, nos processos inflamatórios. Quando a homeostase não é mantida, o ambiente pode tornar-se propício ao estresse oxidativo (SEIFRIED et al., 2007). Aproximadamente de 1 a 3% do oxigênio, que o corpo consome, é convertido em espécies reativas de oxigênio. Dentre as quais temos: o radical superóxido, o peróxido de hidrogênio e o radical hidroxila. Todos subprodutos metabólicos normais, formados constantemente pelas mitocôndrias nas células (SEIFRIED et al., 2007).

A produção controlada de espécies reativas de oxigênio é, na verdade, de grande importância para o metabolismo, células de defesa as produzem para que ocorra a eliminação de microorganismos patógenos e de células com crescimento acelerado. A versatilidade funcional das espécies reativas de oxigênio se traduz pelo seu papel na

prevenção de doenças, no auxílio ao sistema imune para a eliminação de microorganismos, na mediação da sinalização celular, bem como no exercício de um papel principal, em processos apoptóticos. Tal versatilidade indica quais são as possíveis conseqüências de flutuações significativas no equilíbrio entre radicais livres e antioxidantes no organismo. O efeito de um desequilíbrio vai depender da concentração das espécies reativas, da duração da exposição celular às mesmas, bem como do tipo celular atingido (SEIFRIED et al., 2007; HUGHES, 2001).

Uma grande classe de moléculas responsáveis pela transdução de sinais, as proteína-kinases mitose-ativadas (MAPKs), também são influenciadas por estas espécies reativas, que funcionam neste caso como mediadores das vias de transdução (.MARTINDALE e HOLBROOK, 2002). As MAPKs compõem-se de três subfamílias (ERKs, JNKs e p38 kinases) e, embora suas atividades possam se sobrepor, uma regula principalmente a proliferação celular (ERKs), sendo mais sensível a sinais extracelulares. As outras duas (JNKs e p38 kinases) são mais fortemente ligadas às respostas ao estresse, levando, em último caso, à apoptose ou necrose (HOLBROOK e IKEYAMA, 2002).

As ERKs atuam coordenando a sinalização através de fatores de crescimento como o fator de crescimento plaqueta-derivado, fator de crescimento de fibroblastos e fator de crescimento epidermal (GEF). Já foi verificada a ativação de receptores de fator de crescimento epidermal (GEFR) por espécies reativas de oxigênio, de maneira similar ao que ocorre quando da sua ligação com o próprio fator de crescimento (HOLBROOK e IKEYAMA, 2002), sugerindo a interferência dos radicais livres na ativação de receptores celulares.

A ativação do receptor induz a uma autofosforilação que, finalmente, resulta no

recrutamento das ERKs e da NADPH oxidase, com conseqüente aumento da produção de ânions superóxido e peróxido de hidrogênio (FINKEL, 1998). O aumento temporário das espécies reativas pode então elevar ou mimetizar a associação receptor-ligando, facilitando a autofosforilação desses receptores e induzindo cascatas de sinalização. Estudos de Drodge (2002) verificaram que a eliminação do peróxido de hidrogênio pela catalase inibe a autofosforilação dos receptores EGF, e do fator de crescimento de nervos (NGF). O conhecimento do papel das espécies reativas, como mensageiros em vias de sinalização, tem gerado interesse a respeito de como esse fato poderia ajudar a esclarecer os mecanismos de resposta para diferentes estímulos extracelulares (DRODGE, 2002; JOHNSON e LAPADAT, 2002; MARTINDALE e HOLBROOK, 2002).

Por outro lado, os radicais livres estão envolvidos na deterioração das células através do estresse oxidativo das biomoléculas, ao que parece, favorecendo o aparecimento e o desenvolvimento de diversas doenças degenerativas crônicas, como o câncer, além de doenças cardiovasculares (TAPIERO et al., 2004). Uma enzima, por exemplo, que tenha algum de seus aminoácidos alterado, pode perder total ou parcialmente sua atividade; ou ainda manter uma atividade modificada. Os danos mais sérios são aqueles ocorridos no material genético. O acúmulo de mutações no DNA (troca de bases, por exemplo) pode levar à oncogênese. Essas evidências ilustram como as espécies reativas, interagindo com substratos biológicos, podem gerar danos às biomoléculas, afetando a saúde humana (BARREIROS et al., 2006).

O estresse oxidativo é uma condição definida como desequilíbrio fisiológico entre elementos oxidantes e antioxidantes no corpo humano, com maior atuação dos agentes oxidantes. É uma situação que pode gerar danos em proteínas, peroxidação de

lipídios de membranas celulares, assim como em outras biomoléculas como DNA e RNA. Nos organismos vivos a peroxidação pode ocorrer naturalmente, em certas condições, sob controle enzimático, para geração de mediadores inflamatórios lipídio-derivados. Além disso, durante o estresse oxidativo, pode acontecer também de forma não enzimática (ROMERO et al., 1998). As bicamadas lipídicas que envolvem as células são compostas de diferentes lipídios anfipáticos, principalmente fosfolipídios ricos em ácidos graxos poliinsaturados permeados por proteínas associadas. Estas, ao interagir com espécies reativas como peróxido de hidrogênio, ânion superóxido, peroxinitritos e radical hidroperoxil, entre outros, pode levar à peroxidação dos lipídeos de membrana em sistemas biológicos. Quando estes envoltórios lipoprotéicos delimitantes do conteúdo celular são danificados, o resultado é a perda de integridade da membrana, com alteração da sua fluidez e da sinalização intracelular e, conseqüentemente, da função da célula (HUGHES, 2001).

Foi descrito um efeito do hidroxinonenal, um subproduto da peroxidação lipídica, que consiste na redução da concentração de glutationa e da atividade da enzima glutaniona-peroxidase, e de indução da apoptose neuronal pelo favorecimento do acúmulo de peptídeos β-amilóides associados à doença de Alzheimer (ROMERO et al., 1998).

A astaxantina é uma molécula, que é considerada um potente antioxidante biológico, por exibir forte poder de eliminação de radicais livres. Em animais, essa molécula protege membranas celulares e tecidos contra peroxidação lipídica e dano oxidativo do LDL-colesterol (MIKI, 1991). Devido a sua ação, essa molécula está sendo amplamente investigada. Essa atividade antioxidante, porém, depende de uma série de fatores, tais como estrutura química, local ou sítio de ação, potencial para interação com

outros antioxidantes e vitaminas, concentração do carotenóide e pressão parcial de oxigênio (YOUNG e LOWE, 2001). Lordan e colaboradores (2008) demonstraram a capacidade da astaxantina, em baixas concentrações, de proteger uma linhagem de células monocíticas humanas, contra apoptose induzida por oxidação do colesterol das membranas. Por outro lado, concentrações maiores de astaxantina não demonstraram a mesma proteção, revertendo o seu efeito. O trabalho de Santocono e colaboradores (2007), por sua vez, sugere ação protetora deste e de outros carotenóides, ao dano no DNA, causado por duas diferentes moléculas oxidantes: óxido nítrico (não reativa, mas que produz $N_2O_3$) e peroxinitrito. No mesmo trabalho, a astaxantina não apresentou proteção efetiva contra o ânion nitroxil, sugerindo uma interação diferente entre o carotenóide em questão e diferentes radicais oxidativos.

Das considerações anteriormente expostas, deduz-se que moléculas com ação antioxidante, como a astaxantina, podem ter alguma ação benéfica, quando o organismo é submetido a condições de elevada produção de radicais livres, como o consumo prolongado de etanol (TAPIERO et al., 2004). Nessa situação, certa variedade de compostos, como o malondialdeído (MDA) e o hidroxinonenal (HNE), é formada quando hidroperóxidos de lipídeos decompõem-se nos sistemas biológicos, tornando esses compostos em indicadores da peroxidação de lipídeos (JORDÃO JUNIOR et al., 1998; ROMERO et al., 1998). Estudos demonstraram que, sob tais condições, os carotenóides são capazes de proteger tecidos como a mucosa gástrica e o cérebro de injúrias provocadas pelo etanol (KIM et al., 2005; MITCHELL et al., 1999). Propriedades antioxidantes têm se mostrado importantes na proteção do cérebro dos efeitos deletérios dos radicais livres, particularmente em doenças neurodegenerativas (OCHIAI et al., 2007).

O etanol é uma das drogas de consumo mais difundidas no mundo, sendo amplamente utilizado em todas as culturas e grupos sociais humanos (MODY, 2008). Embora estudos tenham sugerido que a sua ingestão, em pequenas doses, possa eventualmente trazer certos benefícios à saúde, a sua ingestão abusiva é prejudicial (POIKOLAINEN, 1995). Em levantamento da organização mundial de saúde, OMS, verificou-se que 4,0% da mortalidade mundial são conseqüências diretas do consumo de etanol (WHO, 2007).

Ao considerar economias de mercado de pobreza intermediária, como o Brasil, o álcool é o principal fator causal de doenças e morte, sendo seu impacto percentual estimado entre 8% e 14,9%. No Brasil, portanto, estima-se que mais de 10% dos problemas de saúde são causados pelo consumo de etanol (MELONI e LARANJEIRA, 2004).

A ingestão excessiva de etanol, principalmente entre adultos jovens, constitui uma situação de freqüente abuso da droga, que está associada a uma larga variedade de distúrbios patológicos capazes de afetar vários órgãos, inclusive fígado, rins, coração, pâncreas e cérebro (LIEBER, 1995; FENG e FAINGOLD, 2008). Os efeitos deletérios provocados pelo álcool podem ser a conseqüência da sua toxicidade, através de seus metabólitos secundários e/ou a formação de espécies reativas de oxigênio (BARDEN et al., 2007).

A oxidação do etanol está associada a uma mudança na homeostase da oxi-redução nos hepatócitos, gerando desordens metabólicas. O etanol por si, a hiperlactacidemia e níveis elevados de NADH aumentam a atividade da enzima xantina-oxidase que, por sua vez, resulta na produção de superóxido (DAS e VASUDEVAN, 2007). Outra rota de produção de radicais livres, proporcionada pelo etanol, é a geração

de hidroxietil realizada pela Citocromo P450 2E1 nos microssomos, uma vez que a quantidade de citocromo correlaciona-se com a peroxidação lipídica e a formação de superóxido (MANTLE e PREEDY, 1999). Kamimura e colaboradores (1992) demonstraram que os níveis plasmáticos de hidroxinonenal e malondialdeído aumentam, significativamente, em doença alcoólica hepática experimental. A formação de hidroxinonenal reduz a atividade enzimática da glutationa peroxidase, prejudicando a eliminação de superóxido de hidrogênio nos tecidos (KAMIMURA et al., 1992).

O álcool, ainda, pode induzir a formação de espécies reativas, pela reação da enzima aldeído-oxidase, que gera oxi-radicais e pelo aumento da disponibilização de NADH para as mitocôndrias que, com o metabolismo oxidativo alterado, respondem com o aumento do efluxo de radicais livres (DAS e VASUDEVAN, 2007).

Níveis elevados de danos oxidativos no DNA, lipídios e proteínas têm sido reportados por uma série de ensaios em estudos *post-mortem* em tecidos de pacientes com síndrome de Parkinson, Alzheimer e esclerose lateral amiotrófica e, ao menos algumas dessas mudanças podem ter ocorrido no início do desenvolvimento da doença (HALLIWELL, 2001). O acúmulo de danos oxidativos, em neurônios, pode ser primária ou secundariamente a causa para o aumento da incidência das doenças neurodegenerativas acima referidas, em populações idosas (RAO e BALACHANDRAN, 2002).

A isquemia cerebral, por exemplo, leva a danos no cérebro causados por diversos mecanismos patogênicos que também são deflagrados por neurotrauma. Estes mecanismos incluem, entre outros, excitotoxicidade, produção excessiva de radicais livres, inflamação e apoptose (TAPIERO et al., 2004). A observação, de que um defeito genético na esclerose lateral amiotrófica afeta a enzima antioxidante superóxido

dismutase, direcionou o foco de pesquisadores para o papel que as espécies reativas de oxigênio exercem na excitotoxicidade e morte neuronal (DYKENS, 1994). Além do mais, isquemia cerebral ou trauma, ambos deflagram mecanismos autoprotetores similares, incluindo a produção de proteínas de choque térmico, citocinas antiinflamatórias e antioxidantes endógenos. Algumas terapias neuroprotetoras procuram agir na minimização da ativação de vias tóxicas, aumentando a atividade dos mecanismos neuroprotetores endógenos (LEKER e SHOHAMI, 2002).

A ação nociva de radicais livres sobre o sistema nervoso pode ser combatida, sob condições fisiológicas, por substâncias antioxidantes, como os carotenóides (HALLIWELL, 2001; RAO e BALACHANDRAN, 2002; MITCHELL et al., 1999).

O modelo experimental utilizado neste trabalho, caracterizado pelo fenômeno da "depressão alastrante da atividade elétrica cortical cerebral", ou depressão alastrante cortical (DAC), tem suas propriedades influenciadas por espécies reativas de oxigênio combatidas pelos carotenóides (DO CARMO e MARTINS-FERREIRA, 1992; NETTO e MARTINS-FERREIRA, 1989).

A DAC é um interessante fenômeno neural, cujos mecanismos, embora não totalmente esclarecidos, parecem ter pontos em comum com os mecanismos de certas doenças neurológicas humanas, como a epilepsia (LEÃO, 1944), a enxaqueca (LEHMENKÜHLER et al., 1993; ROGAWSKI, 2008; VAN DEN MAAGDENBERG et al., 2004), e a isquemia cerebral (DREIER et al., 2006; FABRICIUS et al., 2006; TAKANO et al., 1996). A DAC foi descrita inicialmente por Leão (1944), como uma onda propagável de supressão da atividade elétrica espontânea e provocada em resposta à estimulação de um ponto da superfície do córtex cerebral. Na Figura 3 pode ser visto o

desenho esquemático das posições de colocação dos dois eletrodos (1 e 2) para registro da DAC.

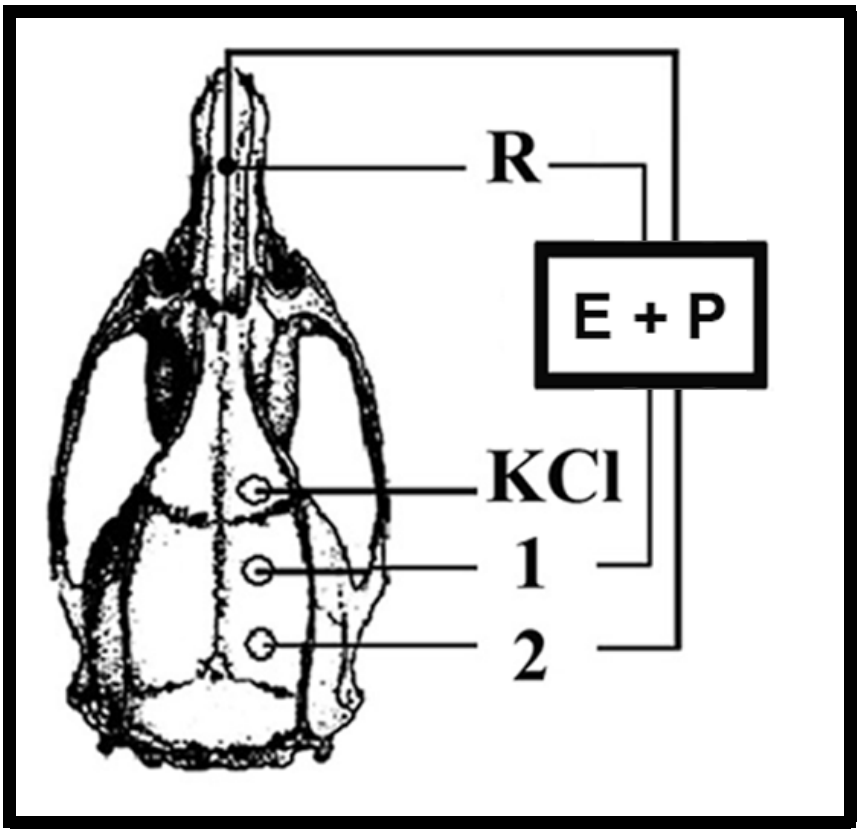


**Figura 3 -** Localização dos orifícios para deflagração e registro da DAC. O registro da atividade elétrica cortical espontânea (E) e da variação lenta de potencial (P) durante a DAC foi feito nos pontos 1 e 2, (osso parietal), contra um eletrodo de Referência (R, no osso nasal). O orifício para aplicação do estímulo (KCl), necessário para deflagrar a DAC, está indicado no osso frontal.

Concomitantemente à depressão da atividade eletrocorticográfica, demonstrou-se também a ocorrência de outras características típicas do fenômeno, como uma variação lenta de voltagem (LEÃO, 1947), e um fluxo transmembrana, reversível, de água e íons (KRAIG e NICHOLSON, 1978). A variação lenta de voltagem é demonstrada na Figura 4, e um esquema de propagação da DAC na Figura 5.

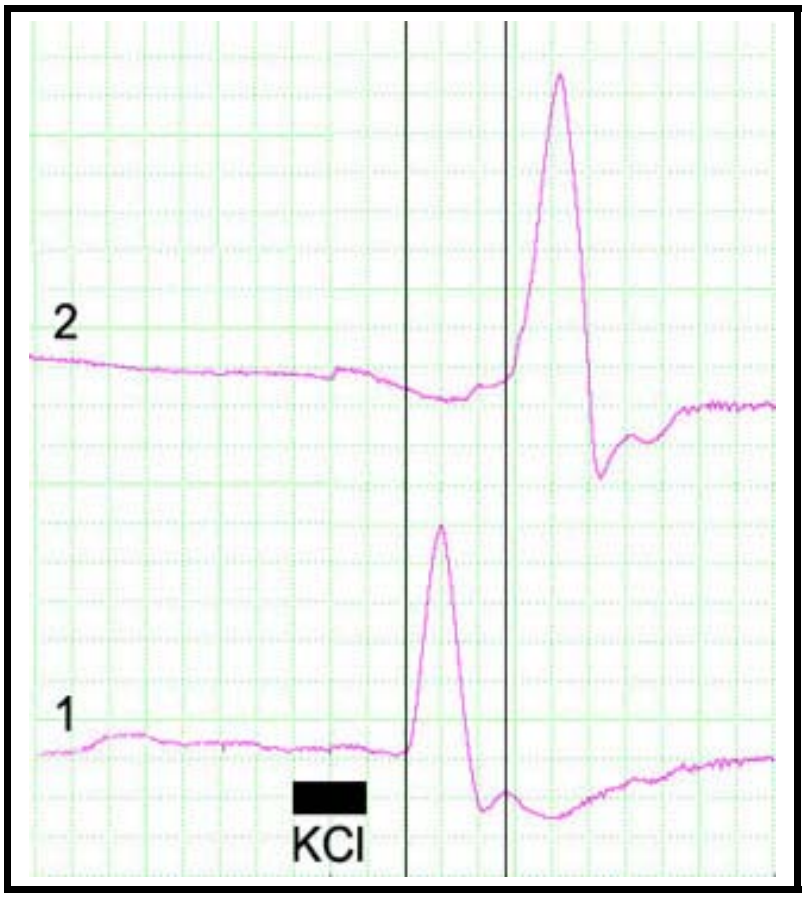


**Figura 4 -** Variação lenta de voltagem característica da Depressão alastrante cortical (DAC). As linhas verticais delimitam o **tempo** (**T)** decorrido entre a passagem da DAC pelos dois pontos corticais de registro (indicados pelos números 1 e 2 no desenho da figura 3). O cálculo da velocidade de propagação da DAC é feito segundo a fórmula **V= D/T**, onde **V** é a velocidade e **D** é a distância inter-eletrodos. As variações de voltagem têm em média 10 mV (negativo para cima) e a barra escura horizontal indica o tempo de 1 minuto em que o estímulo (KCl) usado para deflagrar a DAC foi aplicado à frente do ponto 1.

A exata natureza dos processos subjacentes à DAC continua a ser objeto de muita investigação. Assim, além da influência das espécies reativas de oxigênio, anteriormente referida, tem sido sugerida a participação, na DAC, de alguns íons, como o potássio (GRAFSTEIN,1956), o cloreto (GUEDES e DO CARMO, 1980), e o cálcio (SIESJO e BENGTSSON, 1989), como também de aminoácidos excitatórios (GUEDES et al.,1988; VAN HARREVELD e FIFKOVÀ, 1970) e outros neurotransmissores (GUEDES et al., 1987; 1992; 2002; RODRIGUES et al., 1988).

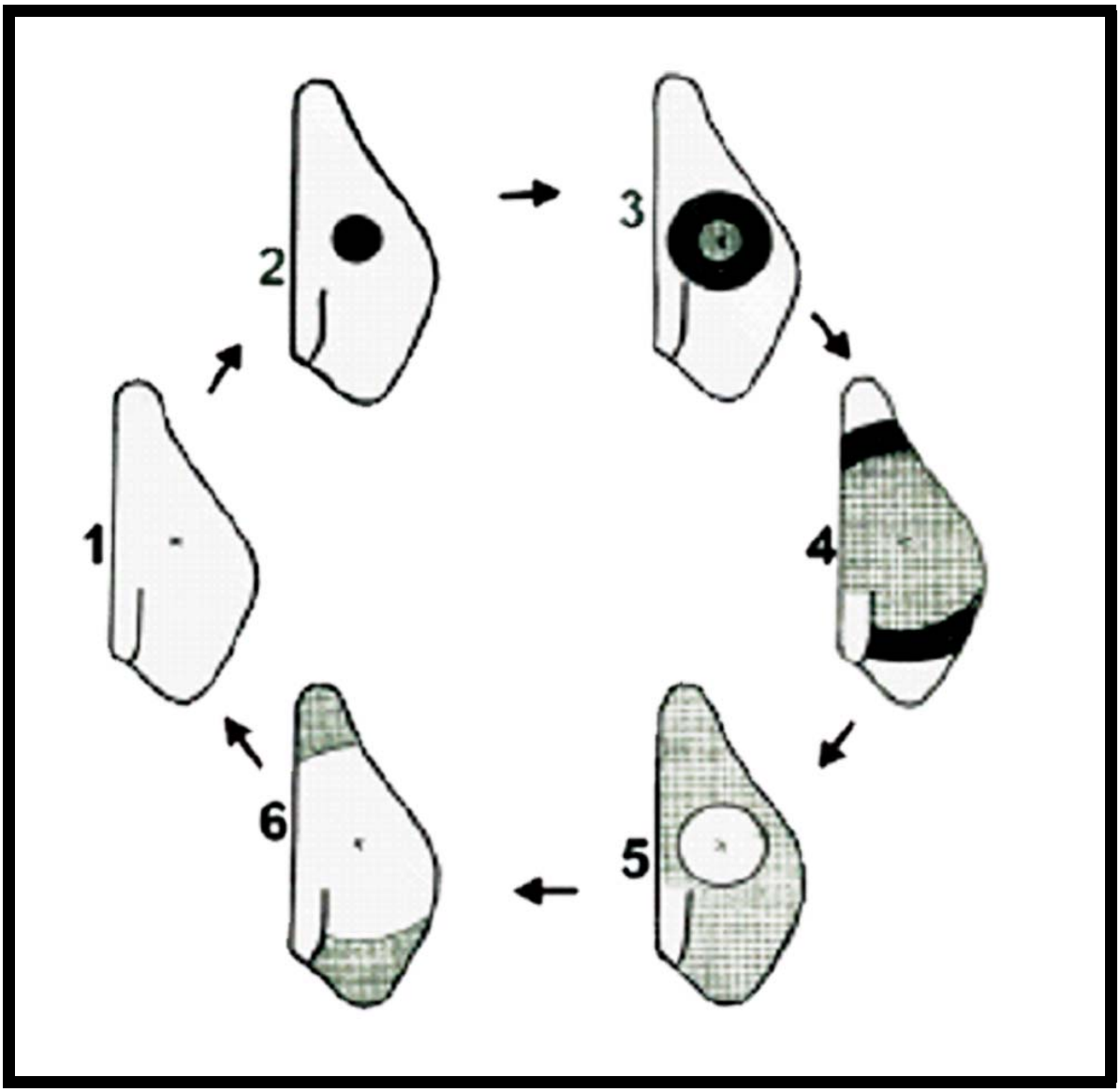


**Figura 5 -** Esquema de propagação da DAC. Ciclo da DAC deflagrada a partir de um ponto no córtex cerebral. As regiões claras representam áreas com atividade elétrica normal, as negras indicam atividade elétrica suprimida; as regiões hachuradas indicam o tecido em estado refratário (obtido de GUEDES, 2005).

A investigação da DAC pode fornecer informação sobre mudanças fisiológicas na atividade elétrica de células nervosas corticais. Conforme demonstrado em animais de laboratório, o tecido nervoso torna-se mais susceptível à DAC, sob o efeito de tratamento com etanol, em comparação a um grupo controle tratado com água destilada (GUEDES e FRADE, 1993; BEZERRA et al., 2005). Essa maior susceptibilidade do tecido cortical à DAC é expressa por um aumento significativo da sua velocidade de

propagação; variações nessa velocidade de propagação constituem o principal indicador da sensibilidade cerebral à DAC.

A deficiência dietética de vitaminas antioxidantes como vitaminas C e E resulta em uma susceptibilidade aumentada para a DAC, tanto em ratos jovens (ÉL-BACHÁ et al., 1998), quanto em ratos senis (GUEDES et al., 1996). Além do mais, nesse sentido, foi recentemente demonstrado que a vitamina E, associada com etanol, teve um efeito protetor maior sobre a atividade elétrica epileptiforme, induzida por penicilina, no córtex de ratos, em comparação com essas duas substâncias usadas separadamente (KOZAN et al., 2007). Estudos feitos por Bezerra e colaboradores (2005) mostraram também que um extrato etanólico de carotenóides do camarão (*Litopenaeus vannamei*) exerce uma ação protetora frente aos efeitos do etanol, na depressão alastrante cortical (DAC).

Assim, o principal propósito do presente estudo foi analisar os efeitos de diferentes doses de astaxantina, sobre mudanças na propagação da depressão alastrante cortical, em ratos adultos tratados com etanol. Tomou-se por hipótese que o tratamento com astaxantina pura poderia mimetizar os efeitos, sobre a DAC, do extrato etanólico de camarão, no cérebro de ratos adultos.

Portanto, neste trabalho, foi analisada a ocorrência de alterações na propagação da DAC, em ratos adultos (78 a 81 dias de idade), previamente tratados por gavagem (ilustrado na Figura 6), durante 18 dias, com diferentes doses (2,5 – 10 e 90 μg/kg/d) do carotenóide comercial puro, astaxantina (diluído em etanol), em comparação com outros dois grupos, tratados respectivamente com o veículo em que o carotenóide foi dissolvido (etanol) e com água destilada. Definiu-se assim uma "curva dose-resposta" para as 3 diferentes doses utilizadas. Foram analisadas, então, as variações de

propagação da DAC ocorridas em função do tratamento com diferentes concentrações de astaxantina. Essas variações foram documentadas, por meio do registro eletrofisiológico cerebral.

**Figura 6 -** Processo de gavagem. Imagem ilustrativa obtida em MAIA, 2004.

A idéia deste estudo segue a linha de pesquisa iniciada com a colaboração entre os Laboratórios de Enzimologia do Departamento de Bioquímica/CCB e o de Fisiologia da Nutrição, do Departamento de Nutrição/CCS, ambos da Universidade Federal de Pernambuco. Um estudo inicial das variações da propagação da DAC sob o efeito do etanol utilizou, como parâmetro, a administração em ratos albinos de um extrato etanólico de carotenóides do camarão *Litopenaeus vannamei* (BEZERRA et al., 2005). Nesse extrato, a astaxantina aparece, dentre os outros carotenóides, como o mais abundante (STAINISLAW e BRITTON, 2001). A utilização da astaxantina pura deveu-

se, primeiro, ao fato de estar o carotenóide presente em maior concentração no extrato de camarão do experimento que precedeu este estudo e, segundo, porque parecia possuir capacidade antioxidante, até dez (10) vezes mais efetiva do que outro antioxidante, o α-tocoferol (MIKI, 1991).

A justificativa do presente estudo foi testar, em ratos albinos, a hipótese de que a astaxantina teria ação semelhante àquela do extrato de camarão sobre o efeito do etanol na DAC. Este estudo representa uma continuidade à pesquisa realizada com o extrato etanólico de carotenóides do camarão e permitiu estabelecer a diferenciação entre o efeito da influência de um único carotenóide, em comparação com um extrato contendo mais de um carotenóide, em concentrações distintas.

Os dados obtidos neste trabalho são consistentes com os descritos sobre outros efeitos protetores dos carotenóides no cérebro. Um possível mecanismo pelo qual esse carotenóide pode antagonizar os efeitos do etanol, na depressão alastrante cortical, poderia ser a ação removedora de espécies reativas de oxigênio da astaxantina. Contudo, para que se possa confirmar essa hipótese, níveis sangüíneos e cerebrais de espécies reativas de oxigênio deverão ser medidos, em futuros experimentos.

Por outro lado, acredita-se que a ação do etanol no cérebro ocorra por meio do sistema do ácido γ-amino-butírico (GABA) (KOZAN et al., 2007; KUMAR et al., 2003; SONN e MAYEVSKY, 2001). Nesse contexto, é interessante notar que os mecanismos mediados pelo GABA participam no fenômeno da depressão alastrante da atividade elétrica do córtex cerebral. Foi encontrado um aumento na velocidade de propagação da DAC no córtex de ratos tratados com Diazepam, um agonista do GABA (GUEDES et al., 1992). Além do mais, foi verificada uma intensificação do fenômeno da DAC em ratos tratados, de forma crônica, com etanol (BONTHIUS et al., 2001; GUEDES e

FRADE, 1993). Em ratos recém desmamados (30-40 dias de vida), tratados cronicamente com álcool, durante o desenvolvimento do SNC (gestação ou lactação), verificou-se um efeito protetor de um extrato de carotenóides do camarão, contra os efeitos do etanol sobre a DAC, no cérebro em desenvolvimento. Foram encontrados resultados que demonstraram que, ratos nascidos de mães previamente tratadas, durante a gestação ou lactação, com o extrato etanólico de carotenóides de camarão, apresentaram velocidades de DAC menores, em comparação com grupos tratados apenas com etanol (BEZERRA et al., 2005).

Neste trabalho, o tratamento por gavagem com astaxantina, por 18 dias, exerceu um efeito protetor de maneira dose dependente, com a variação das velocidades da depressão alastrante, de acordo com um modelo matemático, expresso pela fórmula $y = 2.9048 + 1.1988e^{(-x/9.0217)}$, que apresentou decaimento exponencial (com um $r^2 = 0.9998$).

Por causa da natureza lipofílica da astaxantina, foi necessário utilizar óleo de soja, como veículo, no grupo que recebeu apenas astaxantina (sem álcool). Na única dosagem testada, de 10μg/kg/d, não foram verificadas diferenças estatisticamente significativas, quando comparada com os grupos que receberam água destilada ou óleo de soja. Isso sugere que, se a astaxantina tem algum efeito sobre a DAC que seja independente da ação do etanol, ele será possivelmente percebido se forem utilizadas doses mais elevadas.

Em conclusão, este estudo demonstrou, pela primeira vez, que a astaxantina antagoniza os efeitos do etanol sobre a depressão alastrante cortical, no cérebro de ratos adultos jovens. Os presentes resultados estão em concordância com resultados anteriores (BEZERRA et al., 2005), sugerindo que a astaxantina seria, pelo menos em

parte, responsável pelos efeitos do extrato de carotenóides do camarão, anteriormente observados, sobre a depressão alastrante.

Estudos futuros, da atuação de diferentes carotenóides sobre a fisiologia do sistema nervoso central, podem ser concentrados em aspectos como a determinação de doses possivelmente benéficas, para proteção das células nervosas contra o estresse oxidativo, causado por diferentes condições patológicas. O nível de potencial oxidativo apresentado por diferentes doses destas moléculas certamente poderá ser também objeto de futuras investigações. Igualmente, pode-se procurar levantar que impacto estas moléculas antioxidantes apresentariam sobre diferentes aspectos do desenvolvimento, migração, proliferação e diferenciação dos diversos tipos celulares do SNC.

# OBJETIVOS

## Objetivo Geral

Investigar se o antioxidante astaxantina, influencia a ação do etanol no cérebro de ratos albinos adultos.

## Objetivos Específicos

Tratar ratos adultos cronicamente, por gavagem, com três diferentes doses de astaxantina dissolvida em etanol.

Nessas condições, caracterizar a propagação da depressão alastrante no córtex cerebral desses animais, comparando-as com aquelas de um grupo controle tratado apenas com etanol.

Adicionalmente comparar também com outros dois grupos controles tratados com óleo de soja, com e sem astaxantina (veículo não alcoólico para astaxantina), e com água destilada (em lugar do etanol).

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## ANEXO I

Artigo publicado na revista Neuroscience Letters. Este artigo contém os resultados iniciais do projeto que originou esta dissertação.

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# Shrimp carotenoids protect the developing rat cerebral cortex against the effects of ethanol on cortical spreading depression

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**Abstract**

Cortical spreading depression is a neural phenomenon present in several animal species. Spreading depression features, like velocity of propagation, depends on several chemical and metabolic factors, as for example, anti-oxidants. Here we studied spreading depression-velocity changes in weaned rat-pups born from dams treated on a daily basis, either during gestation or lactation, with a carotenoid ethanolic extract (30 μg/kg/day) prepared from shrimp waste (heads). These pups were compared with age-mated ones, whose mothers were treated either with the vehicle (ethanol) or with distilled water. Compared to the distilled water-group (mean values, in mm/min, per hour of recording ranging from $3.02 \pm 0.26$ to $3.15 \pm 0.27$ [treatment during gestation; $n = 7$], and from $3.03 \pm 0.25$ to $3.22 \pm 0.30$ [lactation; $n = 11$]), ethanol-treated rats displayed higher spreading depression-velocities (from $3.74 \pm 0.06$ to $3.82 \pm 0.08$ [gestation; $n = 7$], and from $4.26 \pm 0.32$ to $4.33 \pm 0.34$ [lactation; $n = 11$]; $p < 0.05$). Compared to the ethanol-group, carotenoid-treatment lead to lower spreading depression-velocities ($p < 0.05$), ranging from $3.38 \pm 0.09$ to $3.42 \pm 0.12$, $n = 7$ (gestation) and $3.58 \pm 0.13$ to $3.62 \pm 0.17$, $n = 12$ (lactation). Carotenoid-treatment during lactation was shown to be significantly more effective than that during gestation ($p < 0.05$), in lowering spreading depression-velocity. The results suggest a protective action of shrimp carotenoids against the ethanol effects on spreading depression. This protective effect could be related to the carotenoid antioxidant properties, as previously indicated by evidence showing spreading depression-effects of other antioxidants.
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*Keywords:* Spreading depression; Shrimp carotenoids; Ethanol; Brain development; Antioxidants; Free radical injury

Spreading depression of brain electrical activity is a phenomenon first described in the rabbit cerebral cortex by Leão [21] as a propagating "wave" of suppression of the spontaneous electrical activity in response to stimulation of one point on the cortical surface. Concomitantly with the electrocorticographic depression, a slow negative potential change [22], as well as other spreading depression typical features, like transmembrane water- and ion flow [18], have been shown to occur.

The exact nature of the processes underlying spreading depression continues to be an object of much investigation. Roles have been suggested for some ions, like potassium [9], chloride [15], and calcium [34], as well as for excitatory amino acids [12,38] and other neurotransmitters [3,10,13,14,33]. Evidence is also available suggesting a possible role for reactive oxygen species in spreading depression [6,7,26]. The deleterious reactive oxygen species action on the nervous tissue can be counteracted, under physiological conditions, by antioxidant substances like carotenoids [17,30]. These molecules are lipophilic and tend to accumulate in lipophilic compartments, like membranes or lipoproteins. In those places, they exert their scavenging effects, protecting against reactive oxygen species produced in certain pathological states, like alcoholism [37]. Animals are not biochemically capable of biosynthesizing carotenoids, but they can accumulate and/or convert precursors, which they obtain from the diet. In crustaceans, they are present as a carotenoprotein in some associations in the carapace, legs parts, blood, eyes, eggs, hepatopancreas and ovary [20].

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The main purpose of the present study is to analyze the occurrence of spreading depression-changes in weaned rat-pups born from dams treated either during gestation or lactation, with a shrimp-carotenoid ethanolic extract. An abstract containing part of the present results has appeared [1].

The shrimp (*Litopenaeus vannamei*) processing waste (heads) was provided by EMPAF Ltd. The waste was packed in plastic bags and stored at −20 °C before use. The protease used was Alcalase (2.4 l FG) provided by Novozyme Brazil S.A. All others reagents used were of analytical grade, provided by Sigma or Merck.

In a reaction vessel (100 mL), shrimp heads (30 g) were minced and mixed with 30 mL of 0.5% Alcalase solution. The proteolytic hydrolysis was carried out for 2 h at 40 °C. Afterwards, the protease was inactivated by heating (100 °C for 10 min), and the supernatant and carotenoprotein (sediment) were separated by centrifugation at 10,000 × *g* for 10 min [8]. Then, the carotenoids were extracted using 90% (v/v) ethanol. Carotenoid concentration and pigment amounts were calculated according to Davies [4] following the formula: $Q = (\mathrm{OD} \times V)/E$, where $Q$ denotes carotenoids amounts (μg); OD stands for optical density (470 nm); $V$ is the volume (mL); and $E = 2500$ (astaxanthin specific extinction coefficient). Ethanolic extraction was carried out in absence of light and in a $N_2$ atmosphere.

The rats utilized in this study were handled in accordance with the "Principles of Laboratory Animal Care" (National Institutes of Health, USA) and with the norms of the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco. They were reared in polyethylene cages (51 cm × 35.5 cm × 18.5 cm) in a room maintained at 21 ± 1 °C with a 12 h light/12 h dark cycle (lights on at 7:00 a.m.). Female Wistar rats received, either during gestation days 7–21 or during lactation days 7–25, per gavage, 30 μg/kg/day of carotenoids extracted from shrimps and diluted in ethanol (3.8 mL/kg). Their pups were compared to pups, born from dams which had been treated in the same way either with the vehicle (ethanol-group) or with distilled water. After weaning (25th postnatal day), pups were fed the same lab chow diet, previously given to their mothers (Purina do Brasil Ltd. containing 23 g protein/100 g diet), until the day of the electrophysiological recording (36–40 days). For that purpose, the animal was intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose. A tracheal cannula was inserted and three trephine holes were made on the right side of the skull. These holes were aligned in the anteroposterior direction and parallel to the midline. One hole was positioned on the frontal bone (2 mm in diameter) and was used to apply the stimulus to elicit spreading depression. The other two holes were drilled on the parietal bone (3–4 mm in diameter) and were used to record the propagating spreading depression wave. Rectal temperature was continuously monitored and maintained at 37 ± 1 °C by means of a heating blanket.

Cortical spreading depression was elicited at 20 min intervals by 1 min application of a cotton ball (1–2 mm in diameter) soaked in 2% KCl solution, applied to the anterior hole, drilled at the frontal region. Both the slow potential change and the spontaneous cortical electrical activity accompanying spreading depression were continuously recorded for 4 h, by using a pair of Ag–AgCl agar-Ringer electrodes (one in each hole). These electrodes consisted of 5 cm-long plastic conic pipettes (0.5 mm tip inner diameter), filled with Ringer solution, solidified with the addition of 0.5% agar, into which a chlorided silver wire was inserted. The pipettes were fixed together with cyanoacrylate glue, so that the interelectrode distance was kept constant. Each pair of electrodes was connected to a lever, which could be vertically moved by turning around a screw, so that the electrode tips could be gently placed on the intact dura-mater, under low-power microscope control, without any excessive pressure on the cortical surface. A common reference electrode, of the same type, was placed on the nasal bones (see inset of Fig. 1).

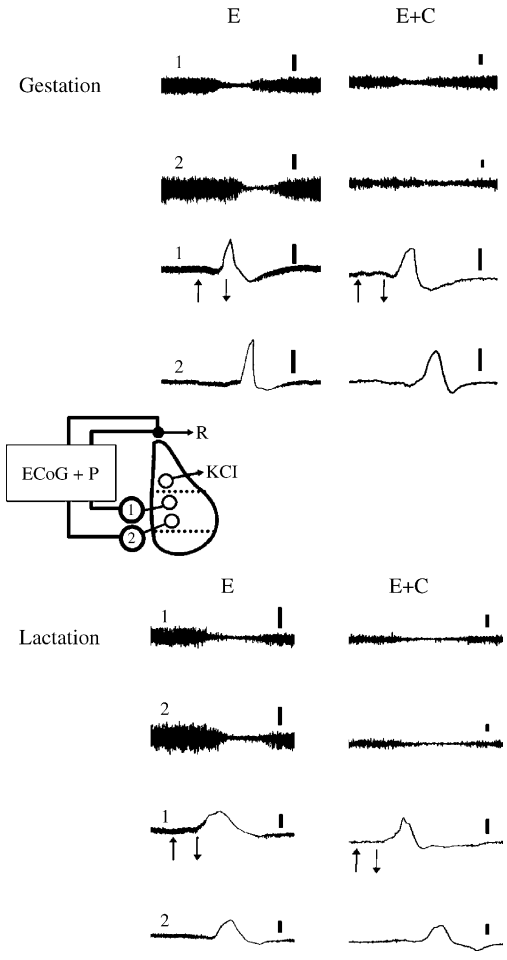


Fig. 1. Recordings of spontaneous cortical electrical activity (ECoG; two upper traces in each panel) and slow potential change (P; two lower traces) during spreading depression in rats whose mothers had been treated, either during gestation or during the lactation period, with ethanolic extract of shrimp carotenoids (30 μg/kg/day; groups E + C), or equivalent volumes (3.8 mL/kg/day) of vehicle (ethanol; group E). Spreading depression was elicited by applying a cotton ball (1–2 mm diameter) soaked in 2% KCl solution for 1 min, on the frontal cortex. Upwards and downwards arrows indicate respectively the beginning and the end of KCl stimulation. The right hemisphere inset shows the recording positions 1 and 2 (on the parietal cortex) and the position of the reference electrode (R), on the nasal bones, as well as the place of KCl stimulus (on the frontal cortex). The distance between the cortical electrodes was 4.0 mm in all examples in this figure. Note the slower spreading rates in the carotenoid-treated animals, as compared to the respective vehicle-treated controls. Vertical calibration bars equal 1 mV for the ECoG- and 10 mV for the P-recordings.

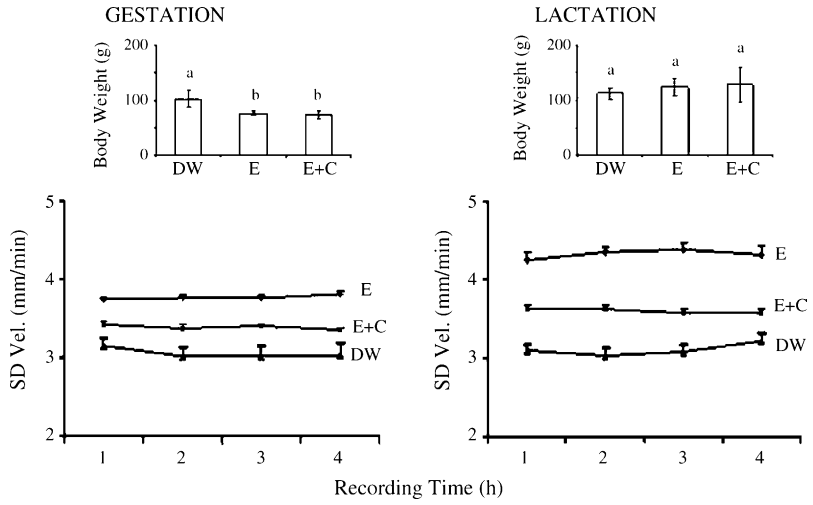


Fig. 2. Reduction of spreading depression-velocity in just-weaned rat pups (36–40 days of life) whose mothers had been treated with an ethanolic extract of shrimp carotenoids (30 μg/kg/day), either during the gestation or during the lactation period, as compared to corresponding vehicle- (ethanol; 3.8 mL/kg) and distilled water-treated controls. Data are expressed as mean ± standard deviation, per hour of recording. All groups were shown to be statistically different from each other (ANOVA plus Tukey test; $p < 0.05$). Insets show body weights. Values marked with distinct letters are significantly different (ANOVA plus Tukey-test; $p < 0.05$). E, E + C and DW refers to ethanol-, ethanol plus carotenoid- and distilled water-treatment, respectively.

The spreading depression velocity of propagation was calculated from the time required for a spreading depression wave to pass the distance between the two cortical electrodes (time between the two slow potential initial rising phases).

Body weights and spreading depression propagation rates were compared between groups by ANOVA, followed by a post-hoc (Tukey–Kramer) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$). All values are presented as means ± standard deviations.

Application of KCl for 1 min on a cortical point in the frontal region was very effective in eliciting a single spreading depression-wave, which propagated without interruption and was recorded on the parietal region of the same hemisphere, as documented by the electrophysiological recordings (electrocorticogram and slow potential changes) shown in Fig. 1. Since KCl-elicited spreading depression episodes were obtained at 20 min intervals, three measurements of spreading depression-velocity could be obtained in each hour of recording, and the mean velocity per hour could be calculated. The two distilled water-groups, born from mothers treated with distilled water during gestation ($n = 7$) and during lactation ($n = 11$) displayed mean spreading depression velocities (in mm/min) ranging respectively from 3.02 ± 0.26 to 3.15 ± 0.27 (distilled water during gestation), and from 3.03 ± 0.25 to 3.22 ± 0.30 (lactation). Compared to the distilled water-controls, ethanol treatment, both during gestation ($n = 7$) and lactation ($n = 11$), increased significantly ($p < 0.01$) the spreading depression-velocities. The mean values ranged respectively from 3.74 ± 0.06 to 3.82 ± 0.08 (ethanol-treatment during gestation), and from 4.26 ± 0.32 to 4.33 ± 0.34 (lactation). The carotenoid-treated groups displayed intermediate spreading depression velocities, significantly lower than those of the respective ethanol-groups, but higher than those of the distilled water-animals ($p < 0.05$). The carotenoid-rat velocities ranged from 3.38 ± 0.09 to 3.42 ± 0.12, (maternal carotenoid ethanolic extract-treatment during gestation; $n = 7$), and from 3.58 ± 0.13 to 3.62 ± 0.17, (lactation; $n = 12$). These data are presented at Fig. 2.

On the day of spreading depression-recording, both ethanol- and carotenoid-groups treated during gestation presented body weights lower ($p < 0.05$) than the distilled water controls (respectively 75.7 ± 2.3, 73.4 ± 6.6, and 102.2 ± 15.1 g). In contrast, ethanol- and carotenoid-treatment during lactation lead to mean weights higher that the corresponding distilled water-control, but the differences did not attain statistical significance (respectively 124.2 ± 16.3, 128.7 ± 30.7 and 112.1 ± 10.5 g). Body weight data are presented as inset in Fig. 2.

Ethanol is the most frequent abused drug, mostly among young adults and adolescents. Its ingestion produces a wide variety of pathological disturbance affecting a number of organs. Due to the fact of being a small molecule, soluble in both lipids and water, it permeates all tissues of the body and affects most vital functions of virtually all organs, including liver, kidney, heart, pancreas and brain, [23]. In line with this view, we found a decrease in pup body weights, in the groups treated with ethanol during gestation, as compared to the distilled water-control group. Treatment during lactation resulted in a tendency to higher body weights in comparison to the distilled water-group, but the difference was not significant. It is interesting to note that an improvement of lactation outcome, associated with ethanol treatment, has been recently described in malnourished rats [36].

Concerning the spreading depression effects, it has previously been demonstrated that adult rats treated with ethanol for 7 days became more susceptible to spreading depression, in comparison with distilled water-treated controls, as judged by the higher spreading depression velocities [16]. The present data showed similar spreading depression-effects (increase in velocity) in the weaned rat pups, whose mothers had been treated with ethanol both during gestation and suckling, as compared to the distilled water-controls. The ethanol-treatment during

the suckling period was more effective in facilitating spreading depression propagation than the treatment during gestation. Data also indicated a higher effectiveness, in the lactation group, of the shrimp carotenoid protective action against the ethanol effect on spreading depression. These results are in agreement with the well-established fact that the suckling period is more relevant to spreading depression-effects, as well as to a number of other biochemical and physiological alterations, than the gestation phase [5,25,32].

Excessive alcohol intake characterizes a situation, which can lead to a toxic degeneration in several organs. Under this condition, lipid peroxidation increases, as a consequence of increased oxidative stress produced by ethanol intake and its subsequent oxidation [31]. Increases in the rate of lipid peroxidation can enhance reactive oxygen species formation, which is potentially injurious to the brain tissue [2]. The reactive oxygen species effects are usually counteracted by homeostatic mechanisms, under physiological conditions. These homeostatic mechanisms involve the participation of antioxidant substances, like ascorbic acid, α-tocopherol and carotenoids [19]. Carotenoids include an array of substances that address different biological properties, such as enhancement of immune functions, cell protection from DNA damage, stimulation of gap junction intercellular communication, induction of detoxifying enzymes and cell proliferation inhibition [19,28,35].

Reactive oxygen species scavenging would be a possible mechanism whereby shrimp carotenoids could antagonize the ethanol effects on spreading depression. The proof of this mechanism would require blood- and brain reactive oxygen species levels to be measured, which has not been done presently. Despite that, it is very tempting to think first about this hypothesis, for the two following reasons: first, reactive oxygen species production in the nervous tissue has been shown to elicit spreading depression in the isolated chicken retina [26] and in the rat cortex [7]; second, treating rats with diets deficient in the antioxidant vitamins C an E increased cortical spreading depression-susceptibility, both in young adult [7] and old rats [11]. Interestingly, brain structural effects of ethanol in laboratory animals have been recently reported to be counteracted by α-tocopherol ([24], in rats) and ascorbic acid ([27], in frogs). Antioxidant properties have also been shown to be important in protecting the brain against the deleterious effects of free radicals, particularly in neurodegenerative diseases. However, little is known about the impact of dietary antioxidants upon the development and progression of these diseases [17].

Other antioxidant molecules, like taurine, have been shown to attenuate the effects of ethanol concerning the oxidative stress [29]. These authors found an increased lipid peroxidation, as well as a decrease of superoxide dismutase, catalase and glutathione-peroxidase activities in the hemolysate and tissues of ethanol-fed rats, as compared to the controls. They also reported a diminution of the contents of reduced glutathione, α-tocopherol and ascorbic acid. All these changes were shown to be attenuated by the administration of taurine simultaneously with ethanol.

In conclusion, the present study describes, for the first time, the antagonizing action of shrimp carotenoids on the brain spreading depression-susceptibility changes promoted by ethanol in the rat cerebral cortex. The data support previous evidence [7,11], on the spreading depression phenomenon, in favor of the hypothesis of a reactive oxygen species neutralizing action of antioxidant substances. The definitive proof of this hypothesis will require reactive oxygen species measurements in future experiments.

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## ANEXO II

Artigo publicado na revista Alcoholism: Clinical and Experimental Research. Este artigo contém os dados obtidos na parte final do projeto que originou esta dissertação.

# Dose-Dependent Effects of Astaxanthin on Cortical Spreading Depression in Chronically Ethanol-Treated Adult Rats

Ricardo Abadie-Guedes, Suzan Diniz Santos, Thiago Barbosa Cahú, Rubem Carlos Araújo Guedes, and Ranilson de Souza Bezerra

**Background:** The consumption of alcoholic drinks is a frequent drug-abuse situation, which is associated to a wide variety of pathological disturbances affecting several organs, including the brain. We have previously shown in the developing rat brain that ethanol intake facilitates the propagation of cortical spreading depression (CSD), an excitability-related neural phenomenon present in several animal species. This electrophysiological effect was attenuated by a shrimp (*Litopenaeus vannamei)* carotenoids extract. Here we investigated the effects of pure astaxanthin, the main carotenoid found in shrimp, on CSD.

**Methods:** Adult Wistar rats were treated per gavage, during 18 days, with 2.5, 10 or 90 μg/kg/d astaxanthin dissolved in ethanol (3 g/kg) and CSD was recorded on the cortical surface 1 to 3 days thereafter. Four groups, treated respectively with ethanol, distilled water and soybean oil with- and without astaxanthin were also studied for comparison with the ethanol + astaxanthin groups.

**Results:** Ethanol-treated rats displayed higher CSD-velocities (mean values, in mm/min, per hour of recording ranging from 4.08 ± 0.09 to 4.12 ± 0.16), compared to the distilled water-group (from 3.19 ± 0.13 to 3.27 ± 0.06). Addition of astaxanthin to ethanol lead to lower CSD-velocities in a dose-dependent manner, ranging from 3.68 ± 0.09 to 3.97 ± 0.22 for the 2.5 μg/kg/d-dose, from 3.29 ± 0.09 to 3.32 ± 0.07 for the 10 μg/kg/d-dose, and from 2.89 ± 0.13 to 2.92 ± 0.11 for the 90 μg/kg/d-dose. The velocities of the soybean oil groups (with and without astaxanthin) were not statistically different from the 10 μg/kg/d astaxanthin + ethanol and distilled water groups.

**Conclusion:** The results demonstrate the antagonistic effect of astaxanthin against the ethanol-induced facilitation of CSD propagation. Probably carotenoid antioxidant properties are involved in such effects.

**Key Words:** Ethanol, Cortical Spreading Depression, Antioxidants, Carotenoids, Astaxanthin.

CAROTENOIDS ARE MOLECULES that, in the mammal organism, tend to accumulate in lipophilic compartments, like membranes or lipoproteins. In those compartments, they exert their scavenging effects, protecting against reactive oxygen species produced in certain pathological states, like alcoholism (Kim et al., 2005; Mitchell et al., 1999; Tapiero et al., 2004). Although not biochemically capable of biosynthesizing carotenoids, mammals can accumulate and/or convert precursors obtained from the diet.

Astaxanthin, one of the dominant carotenoid in marine animals, is a powerful biological antioxidant. This molecule exhibits strong free radical scavenging activity and protects against lipid peroxidation and oxidative damage of LDL-cholesterol, cell membranes, cells, and tissues (Miki, 1991). In fact, it was previously observed (Bezerra et al., 2005) that a shrimp carotenoid ethanolic extract exert a protective action against the ethanol effects on the cortical spreading depression (CSD), an interesting excitability-related neural phenomenon. CSD has been first described as a cortical response consequent to electrical, mechanical or chemical stimulation of the tissue surface (Leão, 1944). This response consists of a reversible and slowly propagating "wave" of reduction of the spontaneous and evoked cortical electrical activity, with a simultaneous DC slow potential change of the tissue (Leão, 1947). CSD has been studied in vivo in several animal species (Gorgi, 2001), having also been recorded in the human brain (Mayevsky et al., 1996).

During CSD, while the spontaneous activity is depressed, "epileptiform waves", similar to those found in the epileptic EEG, usually appear and propagate (Leão, 1944). This led to

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the idea that perhaps CSD- and epilepsy mechanisms would share some common features (Leão, 1944, 1972). The same logic led some authors to postulate an important role for CSD in the physiopathology of migraine (Hadjikhani et al., 2001; Lehmenkühler et al., 1993) and brain ischemia (Takano et al., 1996). In all cases, current discussions often mention the possible involvement of either certain ions (Guedes and Do Carmo, 1980; Siesjö and Bengtsson, 1989), neurotransmitter activity (Gorelova et al., 1987; Guedes et al., 1987, 1992), or free radicals produced in the nervous tissue (El-Bachá et al., 1998; Guedes et al., 1996).

Experimental evidence indicates that the neural tissue naturally offers a certain degree of resistance to CSD propagation (Guedes and Do Carmo, 1980). When this resistance decreases (by some kind of experimental treatment), CSD propagates at higher velocities, as compared to normal conditions, and vice-versa. So, experimental procedures that either weaken or strengthen the brain ability to counteract CSD may provide valuable clues to the understanding of the phenomenon and of the pathologies related to them. The brain CSD susceptibility has been further characterized by several authors in rats submitted to environmental, pharmacological, and nutritional manipulations (Amâncio-dos-Santos et al., 2006; Costa-Cruz et al., 2006; De Luca et al., 1977; Fregni et al., 2007). One of the conditions studied was ethanol ingestion (Guedes and Frade, 1993; Sonn and Mayevsky, 2001).

Chronic ethanol ingestion, mostly in young adults, constitutes a frequent drug-abuse situation, which is associated to a wide variety of pathological disturbance affecting a number of organs, including liver, kidney, heart, pancreas, and brain (Lieber, 1995). The main purpose of the present study was to analyze the effects of different astaxanthin doses on changes in CSD propagation in the cerebral cortex of adult rat chronically treated with ethanol. It was hypothesized that treatment with pure astaxanthin would mimic the CSD-effects of the shrimp carotenoids ethanolic extract, in the adult rat cortex, as previously demonstrated (Bezerra et al., 2005).

## MATERIALS AND METHODS

Astaxanthin (purchased from Sigma, St. Louis, MO) was dissolved in pure ethanol, resulting in final concentrations of 2.5, 10, and 90 $\mu$g/ml. All others reagents used were of analytical grade, provided by Merck.

Astaxanthin concentration was calculated according to Davies (1976) following the formula: $Q = (OD \times V)/E$, where $Q$ denotes carotenoids amounts ($\mu$g); OD stands for optical density (470 nm); $V$ is the volume (ml); and $E = 2500$ (astaxanthin specific extinction coefficient). After prepared, the astaxanthin solutions were maintained in absence of light and in a $N_2$ atmosphere. The rats utilized in this study were handled in accordance with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, MD) and with the norms of the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco, Brazil. They were reared in polyethylene cages (51 × 35.5 × 18.5 cm) in a room maintained at 21 ± 1°C with a 12 h light/12 h dark cycle (lights on at 7:00 AM).

Male Wistar young adult rats (60 to 70 days of life) received for 18 days, per gavage, astaxanthin in doses of 2.5, 10 or 90 $\mu$g/kg/d, diluted in 95% ethanol (corresponding to a dose of 3 g/kg/d). This ethanol daily dose (3 g/kg/d) is within the range usually employed in rodents (Bonthius et al., 2001; Nash et al., 2007), and has been shown to produce blood alcohol levels effective in experimentally altering the nervous system (Nash et al., 2007).

These animals were compared to control rats that had been treated in the same way either with the vehicle (ethanol-group), or with distilled water, or with soybean oil (used as alternative vehicle to dissolve astaxanthin, which is not hydrosoluble). The influence of astaxanthin without ethanol was evaluated in another group treated with 10 $\mu$g/kg/d astaxanthin dissolved in soybean oil instead of ethanol. All groups had free access to water and a commercial lab chow diet (Purina do Brasil Ltd. containing 23 g protein/100 g diet), until the day of the electrophysiological recording (79 to 89 days), which corresponded to 1 to 3 days after terminating the ethanol treatment. During this 1 to 3 days post ethanol period, daily examination of the animals revealed a certain degree of increased locomotor activity and aggressivity in response to manipulation.

The surgical and CSD-recording procedures were performed as previously described (Bezerra et al., 2005). Briefly, the animal was intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose. A tracheal cannula was inserted and three trephine holes were made on the right side of the skull. These holes were aligned in the anteroposterior direction and parallel to the midline. One hole was positioned on the frontal bone (2 mm in diameter) and was used to apply the stimulus to elicit CSD. The other two holes were drilled on the parietal bone (3 to 4 mm in diameter) and were used to record the propagating CSD wave. The distance between the centers of contiguous holes was about 3 to 5 mm. Rectal temperature was continuously monitored and maintained at 37 ± 1°C by means of a heating blanket. CSD was elicited at 20-minute intervals by 1 minute application of a cotton ball (1 to 2 mm in diameter) soaked in 2% KCl solution, applied to the anterior hole, drilled at the frontal region. Both the slow DC-potential change and the spontaneous cortical electrical activity accompanying CSD were continuously recorded for 4 hours, by using a pair of Ag–AgCl agar-Ringer electrodes (one in each hole). These electrodes consisted of 5 cm-long plastic conic pipettes (0.5 mm tip inner diameter), filled with Ringer solution, solidified with the addition of 0.5% agar, into which a chlorided silver wire was inserted. The pipettes were fixed together with cyanoacrylate glue, so that the interelectrode distance was kept constant. Each pair of electrodes was connected to a lever, which could be vertically moved by turning around a screw, so that the electrode tips could be gently placed on the intact dura-mater, under low-power microscope control, without any excessive pressure on the cortical surface. A common reference electrode, of the same type, was placed on the nasal bones (see inset of Fig. 1). The DC-potential changes were recorded by connecting the electrodes to GRASS DC-amplifiers, and the ECoG was recorded with AC-amplification (band pass filters set at 1 to 35 Hz range). Both DC-recording and ECoG were performed in a model 7-D GRASS chart recorder.

The CSD velocity of propagation was calculated from the time required for a CSD-wave to pass the distance between the two cortical electrodes. In the measurement of CSD velocities, the initial point of each DC negative rising phase was used. Body weights and CSD propagation rates were compared between groups by a repeated measures ANOVA, followed by a post-hoc (Tukey–Kramer) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$). All values are presented as means ± standard deviations.

## RESULTS

### *Body Weights*

On the day of CSD recording, rats treated with distilled water, ethanol and astaxanthin ethanolic solutions during

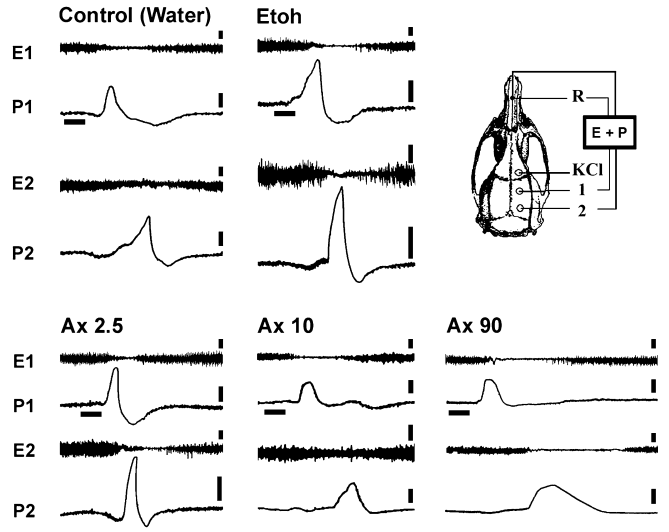


**Fig. 1.** Recordings of spontaneous cortical electrical activity (E; two upper traces in each panel) and slow potential change (P; two lower traces) during cortical spreading depression in adult rats (90 days of life) treated per gavage with astaxanthin at doses of 2.5, 10, and 90 $\mu$g/kg/d (respectively A × 2.5, A × 10, and A × 90), as well as in rats treated with vehicle- (ethanol; 3 g/kg; group Etoh) and distilled water-treated (DW) controls. Spreading depression was elicited by applying a cotton ball (1 to 2 mm diameter) soaked with 2% KCl solution for 1 minute on the frontal cortex. Horizontal bars under P1 indicate the time (1 min) of KCl stimulation. The right hemisphere inset shows the recording positions 1 and 2 (on the parietal cortex) and the position of the reference electrode (R), on the nasal bones, as well as the place of KCl stimulus (on the frontal cortex). The distance between the cortical electrodes was 4.0 mm in all examples in this figure. Note the slower spreading rates in the carotenoid-treated animals compared to the respective vehicle-treated controls. Vertical calibration bars equal 1 mV for the electrocorticogram and 10 mV for the P-recordings.

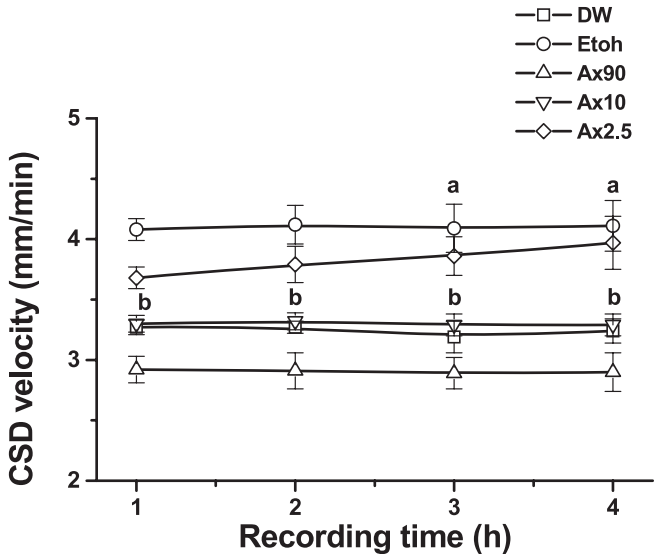


**Fig. 2.** Reduction of cortical spreading depression-velocity in young adult rats (90 days of life) treated per gavage with astaxanthin at doses of 2.5, 10, and 90 $\mu$g/kg/d (respectively A × 2.5, A × 10, and A × 90) compared to rats treated with vehicle- (ethanol; 3 g/kg; group Etoh) and controls treated with distilled water (DW). Data are expressed as means ± standard deviation, calculated in each hour of recording. (A) All groups significantly differed from the Etoh-group, except for the third and fourth hour values of group A × 2.5. (B) Furthermore, with the exception of group A × 10, all groups were shown to be statistically different from the DW controls (ANOVA plus Tukey–Kramer test; $p < 0.05$).

18 days were weighed. The mean body weights were: 355.7 ± 9.9 g ($n = 8$); 300.4 ± 14.3 g ($n = 7$); 274.9 ± 7.8 g ($n = 5$); 316.7 ± 11.3 g ($n = 6$); 293.6 ± 11.5 g ($n = 8$) for the rats treated with distilled water, ethanol, and 2.5 $\mu$g/kg, 10 $\mu$g/kg or 90 $\mu$g/kg astaxanthin ethanolic solutions, respectively. When compared with the initial mean weight (282.4 ± 5.2 g; $n = 36$), it was observed that the distilled water group showed a positive weight gain. In contrast, all ethanol-treated groups failed in increasing body weight. Moreover, it was also found that astaxanthin treatment did not change the negative ethanol effect on the weight gain. The body weights of the soybean oil groups treated with and without astaxanthin (respectively 332.7 ± 14.2 g; $n = 4$ and 327.8 ± 46.3 g; $n = 7$) were not statistically different from the DW-treated group.

### *Cortical Spreading Depression Propagation*

The electrophysiological recordings (electrocorticogram and slow DC-potential changes) during CSD are documented in Fig. 1. As a rule, application of KCl for 1 minute on a cortical point in the frontal region very effectively elicited a single CSD-wave, which propagated without interruption between the two recording electrodes and was recorded on the parietal region of the same hemisphere. Three measurements of CSD-velocity could be obtained in each hour of recording, since KCl-elicited CSD episodes were obtained at 20-minute intervals. The CSD-velocities are presented as mean ± SD velocity per hour, in all groups (Fig. 2).

### *Dose–Response Effect of Astaxanthin on Ethanol-Induced CSD Facilitation*

As can be seen in Fig. 2, the distilled water-group displayed mean CSD propagation velocities, (in mm/min, measured at each hour of recording) ranging from 3.19 ± 0.13 to 3.27 ± 0.06 ($n = 7$). The ethanol-treated rats presented higher CSD-velocities (from 4.08 ± 0.09 to 4.12 ± 0.16; $n = 7$; $p < 0.05$). Astaxanthin-treatment dose dependently leads to lower CSD-velocities, in comparison to the ethanol group. The mean CSD-velocities, per hour of recording, in the astaxanthin groups ranged as follows, respectively in the groups treated with 2.5, 10, and 90 $\mu$g/kg/d: from 3.68 ± 0.09 to 3.97 ± 0.22 ($n = 5$), from 3.29 ± 0.09 to 3.32 ± 0.07 ($n = 5$) and from 2.89 ± 0.13 to 2.92 ± 0.11 ($n = 5$). ANOVA showed that all astaxanthin-treated groups presented significantly lower CSD velocities, except for the third and fourth hour in the 2.5 $\mu$g/kg/d treatment. No significant differences were seen between the treatments with the 10 $\mu$g/kg/d astaxanthin dose and distilled water groups. The group treated with the highest astaxanthin dose (90 $\mu$g/kg/d) displayed the lowest CSD velocities (Fig. 2).

Figure 3 represents the average CSD velocities calculated for the whole 4-hour period. These velocities changed as a

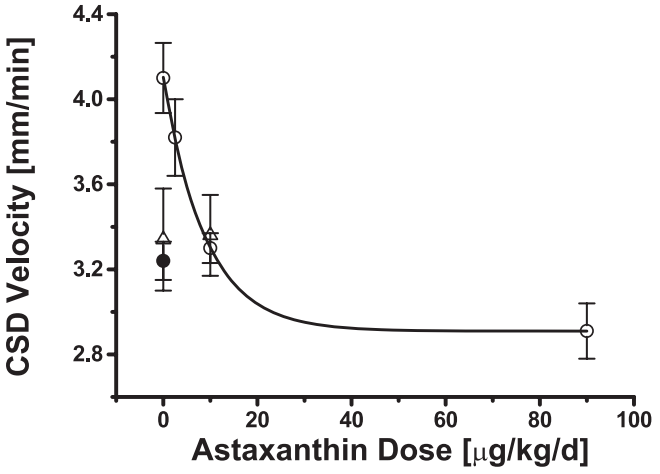


**Fig. 3.** Dose–response effects of astaxanthin dissolved in ethanol on cortical spreading depression velocity in the rat cortex (open circles). The curve resulted from plotting the mean CSD velocities (during the entire 4-h recording period) as a function of the astaxanthin doses. Each point represents the average CSD velocity for the respective group. Note that the values for the soybean oil groups (with and without astaxanthin; open triangles) are not different from that of the distilled water control group (black dot).

function of astaxanthin dose, following an exponential decay model ($r^2 = 0.9998$; $y = 2.9048 + 1.1988e^{(-x/9.0217)}$, where the $x$-variable is astaxanthin dose in $\mu$g/kg/d). The average CSD velocities for the DW-, as well as for the soybean oil- and 10 $\mu$g/kg/d astaxanthin + soybean oil groups are also presented in this figure for comparisons. These velocities were not statistically different from the 10 $\mu$g/kg/d astaxanthin + ethanol group.

## DISCUSSION

It is well established that alcoholism represents a condition in which reactive oxygen species (ROS) are produced (Tapiero et al., 2004). Under such a condition, antioxidant molecules like carotenoids have been shown to protect tissues, as for example gastric mucosa (Kim et al., 2005) and brain (Mitchell et al., 1999), from ethanol-induced injuries. Antioxidant properties have also been shown to be important in protecting the brain against the deleterious effects of free radicals, particularly in neurodegenerative diseases (Tapiero et al., 2004). Our data are consistent with the reported protective effects of carotenoids on brain tissue.

One possible mechanism whereby astaxanthin could antagonize the ethanol effects on CSD would be based on the reactive oxygen species scavenging action of astaxanthin. It is very tempting to think first about this hypothesis for the two following reasons: first, it has been already shown that reactive oxygen species production in the nervous tissue can elicit CSD in the isolated chicken retina (Netto and Martins-Ferreira, 1989) and in the rat cortex (El-Bachá et al., 1998); second, dietary deficiency of the antioxidant vitamins C an E resulted in increased CSD-susceptibility, both in young adult (El-Bachá et al., 1998) and old rats (Guedes et al., 1996). Furthermore, in this respect it has recently been demonstrated that $\alpha$-tocopherol associated to ethanol has a higher protecting effect against penicillin-induced epileptic activity, as compared with the actions of either drug alone (Kozan et al., 2007). However, to confirm this hypothesis, blood- and brain reactive oxygen species levels have to be measured in future experiments.

On the other hand, the ethanol action on the brain has generally been believed to occur through the $\gamma$-aminobutyric acid (GABA) system (Kozan et al., 2007; Kumar et al., 2003; Sonn and Mayevsky, 2001). In this context, it is interesting to note that GABA-mediated mechanisms participate in the phenomenon of cortical spreading depression. Guedes et al. (1992) found an increase in cortical spreading depression propagation velocity in the cerebral cortex of rats treated with the GABA-agonist diazepam. Moreover, studies have reported that rats chronically treated with ethanol present enhancement of the cortical spreading depression-phenomenon (Bonthius et al., 2001; Guedes and Frade, 1993). By treating developing rats with ethanol, we previously described a protective action of a shrimp carotenoids extract against the ethanol-effects on cortical spreading depression in the developing brain. It was also found that weaned rat pups born from dams treated during gestation or lactation with an ethanolic extract of shrimp carotenoids presented lower cortical spreading depression velocities, as compared with a group treated with ethanol (Bezerra et al., 2005).

The daily ethanol treatment per gavage during 18 days has presently been considered as chronic treatment. Sonn and Mayevsky (2001) used acute treatment, by intravenously infusing ethanol during the CSD recording session and found and impairing effect on CSD propagation, which is opposite to the results of Guedes and Frade (1993), Bezerra et al. (2005) and this work. Methodological differences, namely ethanol administration methods and durations, are likely to have contributed for the here commented different results. Furthermore, it is difficult to determine whether the present facilitating ethanol effect on CSD propagation has been due to the chronic ethanol treatment or correspond to an acute withdrawal effect. The first possibility seemed more plausible to us, in view of the findings of Guedes and Frade (1993), who observed the CSD propagation enhancement 1 hour after the last ethanol dose. However, we agree that additional experiments are necessary to further clarify this point.

Besides the problem of maternal drug consumption affecting their offspring, ethanol effects on the adult brain also deserves considerable attention, since chronic alcohol intake by young adults represents a worldwide public health problem nowadays. Such concern has been addressed in the present work, regarding the carotenoids protective role. Furthermore, as astaxanthin is the main carotenoid present in shrimp, it would be judged interesting to test astaxanthin action against the ethanol effects on CSD in young adult rats. The present results showed that astaxanthin administration per gavage during 18 days exerted a protective effect in a dose-dependent manner, with the CSD velocity varying according to a mathematic model presenting an exponential decay (with an $r^2 = 0.9998$).

Due to the lipophilic nature of astaxanthin, we had to use soybean oil as vehicle in the group receiving only astaxanthin (without ethanol). At the dose of 10 μg/kg/d, no statistical differences were seen, when compared with the DW-, or with the soybean oil groups, suggesting that if astaxanthin has effects on CSD that are independent of ethanol effects, it might occur at higher doses.

In conclusion, our data have demonstrated, for the first time, that astaxanthin antagonizes the ethanol effect on cortical spreading depression in the young adult rat brain. The present results are consistent with our previous data (Bezerra et al., 2005), suggesting that astaxanthin, the main shrimp carotenoid, could be, at least in part, the responsible for the previously observed cortical spreading depression-effects. Despite being clear that the mechanisms of ethanol action on the brain are mostly based on the GABAergic activity and on the ROS-production, and that both mechanisms influence cortical spreading depression propagation, the astaxanthin protective action on cortical spreading depression remains to be further clarified. Probably carotenoid antioxidant properties are involved in such effects.

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