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RICARDO ABADIE GUEDES

Efeitos do consumo de etanol e da sua abstinência sobre o cérebro: ação protetora de antioxidantes

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco, sob orientação do Prof.Dr Ranilson de Souza Bezerra e co-orientação do Prof. Dr Rubem Carlos Araújo Guedes

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À minha família

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RESUMO

O abuso do consumo do etanol e a ingestão insuficiente de antioxidantes são fatores externos que podem alterar a eletrofisiologia do cérebro. Estudos prévios demonstraram que a excitabilidade cerebral, relacionada com o fenômeno conhecido como depressão alastrante cortical (DAC), foi facilitada pelo consumo crônico de etanol, e o tratamento crônico com carotenóides atenuou esse efeito. Numa primeira etapa, investigou-se o efeito agudo da administração única de etanol (EtOH) sobre a DAC em ratos adultos e jovens previamente tratados (1 hora antes) com 10 μg/kg de astaxantina. Ratos Wistar machos (cinco grupos jovens e cinco adultos, 60 a 80 dias e 150 a 180 dias de idade respectivamente) receberam em duas gavagens com uma hora de intervalo, os seguintes tratamentos: grupos 1 e 2 receberam astaxantina na gavagem I combinada com EtOH (grupo 1) ou água (grupo 2) na gavagem II; os grupos 3 e 4 receberam óleo de oliva (o veículo no qual astaxantina foi dissolvido) na gavagem I combinado com EtOH (grupo 3) ou água (grupo 4) na gavagem II; grupo 5 recebeu água na gavagem I combinada com EtOH na gavagem II. A DAC foi registrada na superfície cortical por 4 horas. Comparado com os respectivos grupos controle água e óleo (grupos 2 e 4), o etanol em dose única (grupos 3 e 5) reduziu as velocidades de propagação da DAC e a astaxantina antagonizou esse efeito. Na segunda etapa, foi avaliado se outro antioxidante, não carotenoide (tocoferol), promoveria efeito similar à astaxantina. Os resultados comprovaram essa hipótese, indicando que a ação antagônica sobre o efeito do etanol na DAC não seria uma propriedade particular da astaxantina, mas provavelmente uma característica geral de moléculas antioxidantes. Em uma etapa final, o tema, no contexto da DAC, foi discutido em um artigo de revisão, que também faz parte do corpo desta tese.

Palavras-chave: Etanol, Astaxantina, Tocoferol, Antioxidantes, Depressão alastrante.

ABSTRACT

Ethanol consumption abuse and the insufficient intake of antioxidants are external factors that may alter the brain electrophysiology. Previous studies demonstrate that brain excitability, related with the phenomenon known as cortical spreading depression (CSD), was facilitated by chronic consumption of ethanol, and that chronic treatment with carotenoids attenuated these effect. On a first stage, it was investigated the acute effect of a single ethanol (Etoh) dose on the CSD in young and adults rats previously treated (1 hour before) with 10 µg/kg of astaxanthin. Male Wistar rats (five young and five adult groups, 60 to 80 and 150 to 180 days of age respectively) received in two gavages with one hour interval, the following treatments: groups 1 and 2 received astaxanthin on gavage I combined with Etoh (group 1) or water (group 2) on gavage II; groups 3 and 4 received olive oil (the vehicle in wich astaxanthin was dissolved) on gavage I combined with with Etoh (group 3) or water (group 4) on gavage II; group 5 received water on gavage I combined with Etoh on gavage II. CSD was recorded on the cortical surface for 4 hours. Compared with the respective control groups water and oil (groups 2 and 4), ethanol in a single dosis (groups 3 and 5) reduced the CSD propagation velocities and astaxanthin antagonized this effect. On a second stage, it was evaluated if a different non-carotenoid antioxidant, (tocopherol), would promote an effect similar to astaxanthin. The results confirmed this hypothesis, indicating that the antagonic action of ethanol on CSD would not be a particular property of astaxanthin, but probably a general feature of antioxidants molecules. In a final stage, the theme, on the CSD context, was discussed in a review article, wich is also part of this thesis.

Key words: Ethanol, Astaxanthin, Tocopherol, Antioxidants, Spreading Depression.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

AVAI – Anos de vida ajustados por incapacidade

CAT - Catalase

CSD – Cortical Spreading Depression

DAC – Depressão Alastrante Cortical

ERO – Espécies reativas de oxigênio

ERN – Espécies reativas de nitrogênio

EtOH – Etanol

GPx – Glutationa peroxidase

GSH - Glutationa

OMS – Organização Mundial de Saúde

OPS - Organización Panamericana de la Salud

PAHO – Pan American Health Organization

SOD – Superóxido dismutase

U.S. DOT – United States Department of Transportation

WHO – World Health Organization

SUMÁRIO

1 – INTRODUÇÃO

O alcoolismo, considerado um grave problema social, está associado a diferentes doenças, como o câncer (BOFFETTA et al., 2006; PARKIN, 2011), acidente vascular cerebral (PATRA et al., 2010), e lesão hepática (LU et al., 2010). Os danos causados à saúde pelo consumo do etanol podem estar ligados à produção de radicais livres e seus efeitos deletérios em diversos órgãos. Entre os órgãos afetados, o cérebro é um dos principais alvos do etanol. Nos estágios iniciais, o uso do álcool provoca sensações de prazer e bem estar e seu uso continuado pode criar uma relação de dependência, e estabelecer um padrão crônico de ingestão. Os efeitos danosos no organismo podem ser exacerbados, pois a adaptação do corpo à substância provoca, frequentemente, um aumento das dosagens requeridas para se atingir as sensações desejadas e, assim, ser satisfeita a demanda fisiológica.

O abuso do consumo do etanol e a ingestão insuficiente de antioxidantes são fatores externos que podem alterar a eletrofisiologia cerebral. Sobre esse tema, nossos estudos prévios durante o mestrado demonstraram que a excitabilidade cerebral, relacionada com o fenômeno conhecido como depressão alastrante cortical (DAC), foi facilitada pelo consumo crônico de etanol, e o tratamento crônico com carotenóides atenuou esses efeitos (ABADIE-GUEDES et al., 2008), indicando papel protetor desse antioxidante contra os efeitos do etanol no sistema nervoso.

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 consequência 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, balanço este que pode ser 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.

Este trabalho propôs estudar o tema com uma abordagem baseada no modelo eletrofisiológico da DAC, que é influenciável pelo álcool e pelos efeitos da sua abstinência. A DAC parece também estar associada a doenças neurológicas como a epilepsia, a isquemia cerebral e a enxaqueca clássica. Os dados deste trabalho pretenderam fornecer informações de relevância para o melhor entendimento do papel fisiológico de substâncias antioxidantes, sobre a atividade elétrica cerebral, e as conseqüências da modificação de seus padrões. Numa visão de médio prazo, pode-se prever que programas de saúde pública poderão ser beneficiados, direta ou indiretamente, com as informações obtidas neste estudo.

2 – REVISÃO DA LITERATURA

2.1 Uma breve história do consumo do álcool

O álcool é uma das substâncias com ação neural cujo consumo é mais difundido no mundo, sendo amplamente usada na maioria das culturas e grupos sociais humanos. Existem evidências da produção de bebidas intencionalmente fermentadas ainda no final da idade da pedra lascada, em

torno de dez mil anos antes de Cristo. O vinho aparece em pictogramas egípcios de 4000 anos a.C, e é possível que o consumo de cerveja tenha precedido o de pão (MODY, 2008). Por milhares de anos, na história da sociedade ocidental, a cerveja e o vinho foram utilizados como principais líquidos para debelar a sede, devido aos sérios problemas de saúde oriundos do consumo de água, uma vez que esta, naquela época, não passava por qualquer processo de purificação. Fontes de água pura, como nascentes de montanhas, não eram disponíveis para a maioria das populações. A cerveja e o vinho de então continham também, além do álcool, quantidades consideráveis de ácido acético e outros ácidos orgânicos. Esta acidez certamente contribuía, junto com o álcool, para o efeito de esterilização da água utilizada para diluir a cerveja e o vinho, normalmente numa proporção de 2:1. Por conta da diluição, a concentração de álcool nessas bebidas, utilizadas para matar a sede e como fonte de calorias, era geralmente baixa e assim causava poucas consequências adversas. Apesar do conhecimento dos efeitos do consumo de grandes quantidades das preparações alcóolicas, cuidados com os efeitos do álcool contido nas bebidas tornou-se centro de preocupação apenas muito tempo depois (VALLE, 1994).

O desenvolvimento da destilação foi responsável pela primeira grande mudança no modo e na extensão do consumo humano de álcool, desde o advento da fermentação da cerveja e da vinicultura. Mais importante, este desenvolvimento talvez tenha marcado a transição do consumo do vinho e da cerveja como fonte de hidratação e nutrientes, para o consumo de álcool em quantidades suficientes para serem perigosas, chamando atenção para o lado negativo do uso e do abuso do consumo do etanol. A destilação passou a

permitir a concentração e o isolamento do álcool em menores volumes, proporcionando bebidas com conteúdo e “potência” padronizados. O álcool foi comemorado como o melhor meio para o preparo de destilados farmacêuticos e considerado, durante parte da Idade Média, uma panacéia por si só (VALLE, 1994). Durante o período da ocorrência da peste negra na Europa, quando a população naquele continente foi reduzida em dois terços em uma geração, e medicações eficazes não eram conhecidas, bebidas destiladas eram prescritas pelos médicos aos enfermos por causa dos efeitos temporários de sensação de aquecimento e bem estar. O álcool destilado, nesse curto período, foi conhecido inclusive como “aqua vitae” (água da vida) e acreditava-se até que possuísse poderes mágicos (CLAUDIAN, 1970; FORBES, 1970; WILSON, 1973).

Apenas a partir do século 17, com o aumento do consumo do chá e do café, a realização de fervura da água foi amplamente difundida e o consumo de bebidas alcoólicas, como forma de manter o balanço hídrico, diminuiu drasticamente. O processo de fervura da água já era realizado, em grande parte da cultura oriental, há pelo menos 5000 anos, enquanto na sociedade ocidental não era apreciado, nem associado com a eliminação dos patógenos causadores de doenças. A grande diferença no consumo de líquidos entre orientais e ocidentais no passado, tendo os primeiros o costume de consumir chá feito com água fervida, e os últimos a cerveja e o vinho, levantam algumas questões a respeito do metabolismo humano do álcool. Em aproximadamente metade da população chinesa e japonesa, o metabolismo do álcool difere significativamente dos ocidentais devido a diferenças genéticas. Essas diferenças resultam de uma enzima, relacionada ao metabolismo do etanol, a

aldeído desidrogenase, que se apresenta pouco ou não funcional em parte da população oriental. A falta de função dessa enzima, por sua vez, provoca uma síndrome de sensibilidade ao álcool que torna os indivíduos portadores dessa condição pouco tolerantes a essa substância (VALLE, 1994).

Ao longo da história, de forma geral, o consumo de álcool tem um conteúdo ritualístico no seu uso em diversas culturas. Entretanto, com a modernização e mudança na organização econômica e social em muitas dessas culturas, seu uso passou a ser associado principalmente ao contexto recreativo ou à busca imediata do prazer, originando padrões de consumo de grandes quantidades de uma vez ou ao longo do tempo (RONZANI e FURTADO, 2010).

2.2 Álcool: consumo, comportamento e consequências

Os efeitos de relaxamento e desinibição de traços de personalidade proporcionados pela ingestão do etanol passam a ter um papel estimulante e desejado sobre o comportamento social. Entre tais efeitos desejados estão incluídos a “sensação de busca”, descrita como a evitação da monotonia, e definida como o desvio da rotina e a necessidade de mudança e ação, bem como a impulsividade, definida como agir no estímulo do momento, de modo não planejado (GRAU e ORTET, 1999). É criada assim uma expectativa, por meio da qual, se o álcool é consumido, então certas consequências comportamentais e afetivas, importantes no contexto social, se seguem. Trabalhos de investigação psicológica sugerem que essa expectativa sobre o resultado do consumo do etanol pode ser um importante mediador das

variáveis biológicas e ambientais prévias que influenciam o risco do abuso do álcool (como o alcoolismo). Seria uma associação causal, de substancial contribuição, para o álcool posteriormente consumido (YOUNG e OEI, 1993; JONES et al., 2001; GOLDMAN, 2002; CONNOR et al., 2011).

Alguns pesquisadores consideram hoje em dia o álcool etílico como uma "entidade" multifacetada que, dependendo de sua dosagem, pode ser utilizado como "lubrificante social", uma companhia sofisticada para um jantar, um benfeitor da saúde cardiovascular ou um agente de destruição (VALLE, 1998). O consumo abusivo de álcool é um problema mundial de saúde, que resulta em, aproximadamente, 2,5 milhões de mortes por ano (OMS, 2010), inclusive na perda da vida de centenas de milhares de jovens. Em 1990, 5% das mortes de pessoas entre 5 e 29 anos foram atribuídas às consequências desse consumo (MURRAY e LOPEZ 1997). A idade de início do consumo é um fator importante, pois quanto mais cedo o indivíduo começa a beber, parece ser mais provável tornar-se dependente posteriormente. GRANT e DAWSON (1997) verificaram o aparecimento de dependência de álcool em algum estágio da vida para mais de 40% de grupos de indivíduos, nos quais o início do consumo de bebidas alcoólicas antecedeu os 15 anos de idade, com uma média de redução entre 8 e 10% desse risco para cada ano extra de adiamento do início do consumo. Comparado com o contato mais tardio com a bebida, indivíduos acometidos de alcoolismo, que iniciam um consumo mais precoce parecem apresentar maior gravidade de dependência e problemas relacionados ao álcool (DOM et al., 2006). É sabido que o acesso ao álcool é um forte determinante do consumo (BABOR e CAETANO, 2005). Um estudo recente no Brasil, envolvendo 1900 estudantes com idades entre 11 e 21 anos,

indicou ser muito fácil adquirir álcool em lojas, e beber em um contexto social com amigos e parentes. Apenas 1% dos adolescentes relatou ter tentado comprar bebidas alcoólicas e não ter conseguido (VIEIRA et al., 2007).

Os jovens que começam a beber cedo, durante os anos da adolescência, aparentemente também experimentam maior risco de sofrer acidentes relacionados ao álcool, como aqueles com veículos automotores, quedas, queimaduras e afogamentos, do que usuários de iniciação em idades mais avançadas (HINGSON et al., 2000). O consumo abusivo é, dessa maneira, não apenas o fator causal de diversas doenças, mas também precursor de injúria e violência. Estudos verificaram relação próxima entre o consumo de álcool e aumento da agressividade (DUKE et al., 2011; LEJUEZ et al., 2010; DOM 2006). Nos Estados Unidos, 39% das fatalidades em acidentes automotivos envolveram o consumo de álcool em 2005 (U.S. DOT, 2005). Nas Américas, entre 20% e 50% das fatalidades em acidentes de tráfego estão relacionadas com o álcool (PAHO, 2007).

A maioria dos países reconhece os sérios problemas de saúde pública causados pelo consumo prejudicial de etanol e tem procurado adotar programas e políticas preventivas eficazes para mitigar as consequências negativas do exagero na ingestão dessa substância. Estima-se que em torno de dois bilhões de pessoas consumam bebidas alcóolicas no mundo e 76,3 milhões sejam diagnosticadas com algum distúrbio de uso de álcool (WHO, 2004). Os riscos do etanol para a saúde são muitos, e variam em tipo e intensidade. Alguns, mas não todos, são relacionados à dose e podem resultar diretamente ou dos efeitos do álcool, ou por sua interação com outros fatores. A intoxicação é frequentemente um importante mediador de injúria e a

dependência pode exacerbar significativamente seus riscos e causar exposição prolongada a seus perigos (BABOR e CAETANO, 2005).

O custo total causado pelo consumo excessivo de álcool nos Estados Unidos em 2006 foi estimado em 223,5 bilhões de dólares. Deste total, 161,3 bilhões (72,2%) devidos à perda de produtividade; 24,6 bilhões (11,0%) relativos ao aumento dos custos com saúde; 21,0 bilhões (9,4%) de custos com justiça criminal e 16,7 bilhões (7,5%) por outras causas (BOUCHERY et al., 2011).

Nas Américas, por exemplo, foi estimado que, em 2002, o álcool levou a 5,4% do total de mortes, uma proporção 68% maior, se comparado aos 3,7% da média global. Dessa totalidade 50,5% foram devidos a ferimentos, intencionais ou acidentais (REHM et al., 2006). O consumo *per capita* nas Américas foi aproximadamente 40% maior do que a média global em 2002, 8,7 litros comparados a 6,2 da média mundial (REHM et al., 2006). Quando analisado entre 26 fatores de risco e seu impacto sobre a carga de doenças que geram alguma incapacidade, o álcool é o principal fator nas Américas, enquanto é o quarto no mundo (REHM e MONTEIRO, 2005).

No caso de economias de mercado de pobreza intermediárias, como o Brasil, seu impacto percentual está estimado entre 8% a 14,9%. No Brasil, portanto, mais de 10% dos problemas de saúde são causados pelo consumo de etanol (MELONI e LARANJEIRA, 2004). A alta mortalidade entre os consumidores crônicos de bebidas alcoólicas, que fazem uso de doses consideradas elevadas, deve-se principalmente a suicídio, homicídio, ferimentos acidentais e doenças como cirrose hepática, derrame hemorrágico,

cânceres gástricos e da região superior do sistema respiratório (KLATSKY e ARMSTRONG, 1993; FARCHI et al., 1992).

O abuso do consumo é uma ameaça particularmente grave para o homem, principalmente por ferimentos, violência e doenças cardiovasculares. Globalmente uma porcentagem de 6,2% de mortes masculinas é atribuída ao álcool, comparado com 1,1% de morte de mulheres. Os homens superam em 4 vezes as mulheres em episódios de consumo excessivo, muito provavelmente, a razão pelas maiores taxas de mortalidade e invalidez. Posição socioeconômica e nível educacional mais baixo estão associados a risco aumentado de morte, doença ou ferimento relacionado ao álcool, determinantes que são maiores para o homem do que para a mulher (WHO, 2011). Ao redor do mundo, ocorre uma ampla variação no padrão de consumo, com os maiores níveis encontrados nos países desenvolvidos, principalmente no hemisfério norte; mas também na Argentina e Austrália. Baixas taxas de consumo podem ser encontradas, por exemplo, em países do norte da África e leste do Mediterrâneo, cujas regiões possuem uma grande representação de populações de fé islâmica, as quais apresentam altos níveis de abstenção (WHO, 2011).

A **Figura 1** mostra a distribuição *per capita* do consumo mundial de bebidas alcoólicas, em litros de etanol puro, segundo o último relatório da OMS (WHO, 2011). A **Figura 2** apresenta o padrão de distribuição de renda mundial e as respectivas expectativas de vida. As **Figuras 3** e **4** apresentam respectivamente os dados sobre morte e anos de vida ajustados por invalidez (AVAI), em relação a 19 fatores de risco e nível de renda. O índice conhecido pela sigla AVAI (anos de vida ajustados por invalidez) é um indicador calculado

utilizando-se os dados de morte prematura, anos vividos com incapacidade e expectativa de vida; e definido como uma medida "do tempo vivido com incapacidade e do tempo perdido devido à mortalidade prematura" (MURRAY, 1994). A **Figura 5** apresenta um resumo sobre os dados de consumo para o Brasil. As **Figuras 6** e **7** apresentam uma classificação de dez fatores de risco para, respectivamente, *causa mortis* e AVAI parametrizados por nível de renda.

O consumo individual de álcool pode ser descrito em termos de gramas de álcool consumido, ou por conteúdo alcóolico distinto de cada bebida. Uma dose padronizada, por exemplo, contém 10 gramas na Europa e entre 12 e 14 nos Estados Unidos e Canadá. O consumo de risco é um padrão que aumenta os perigos para a saúde, se duradouro. A OMS define esse nível de risco como uso de 20 a 40 gramas diários para mulheres e 40 a 60 gramas para os homens. O consumo prejudicial se refere ao que leva a consequências tanto para a saúde física quanto mental, e está definida pela OMS como mais de 40 gramas de álcool ao dia para mulheres e mais de 60 gramas por dia para os homens (OPS, 2008).

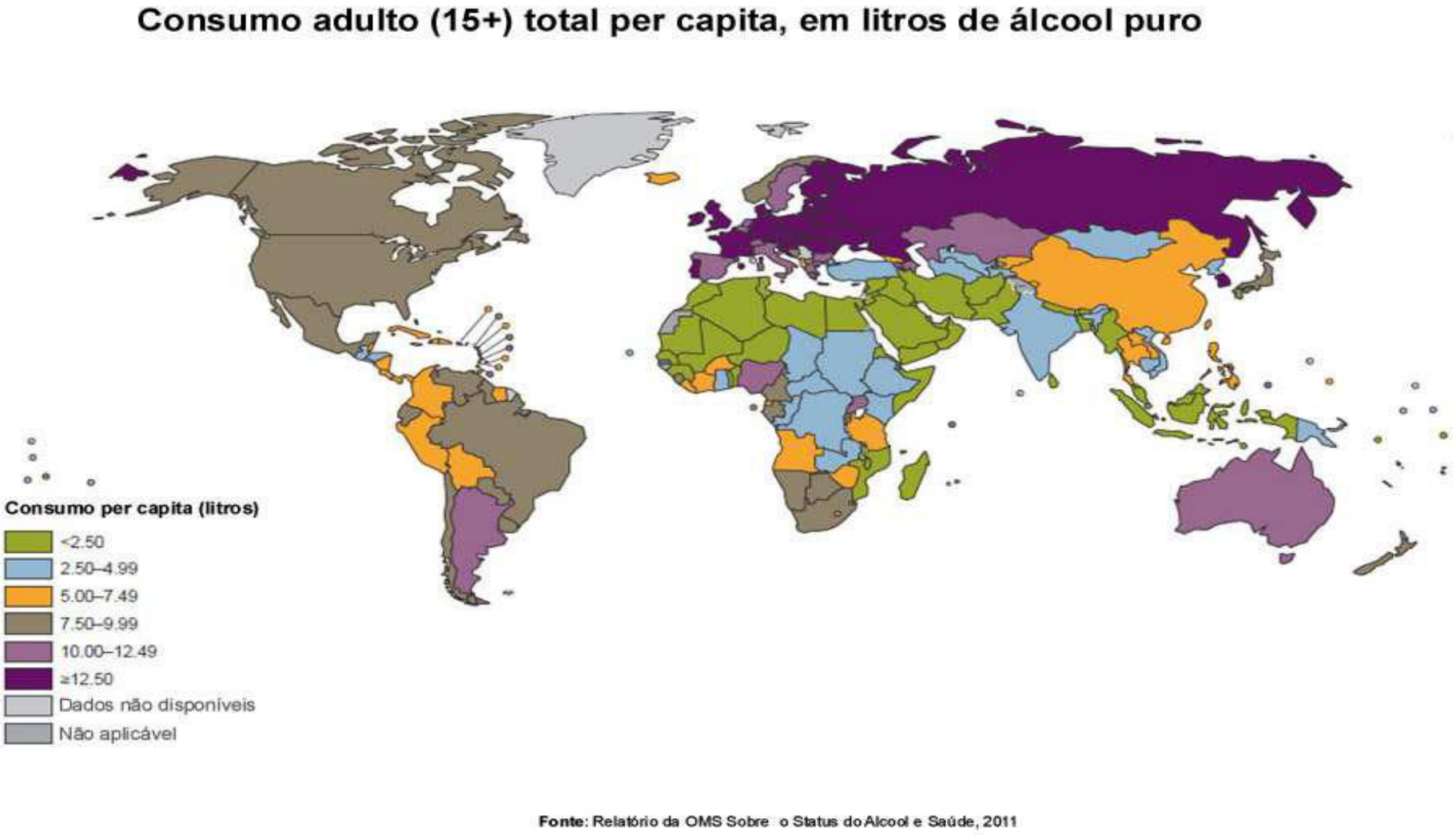


Figura 1: fonte WHO 2011

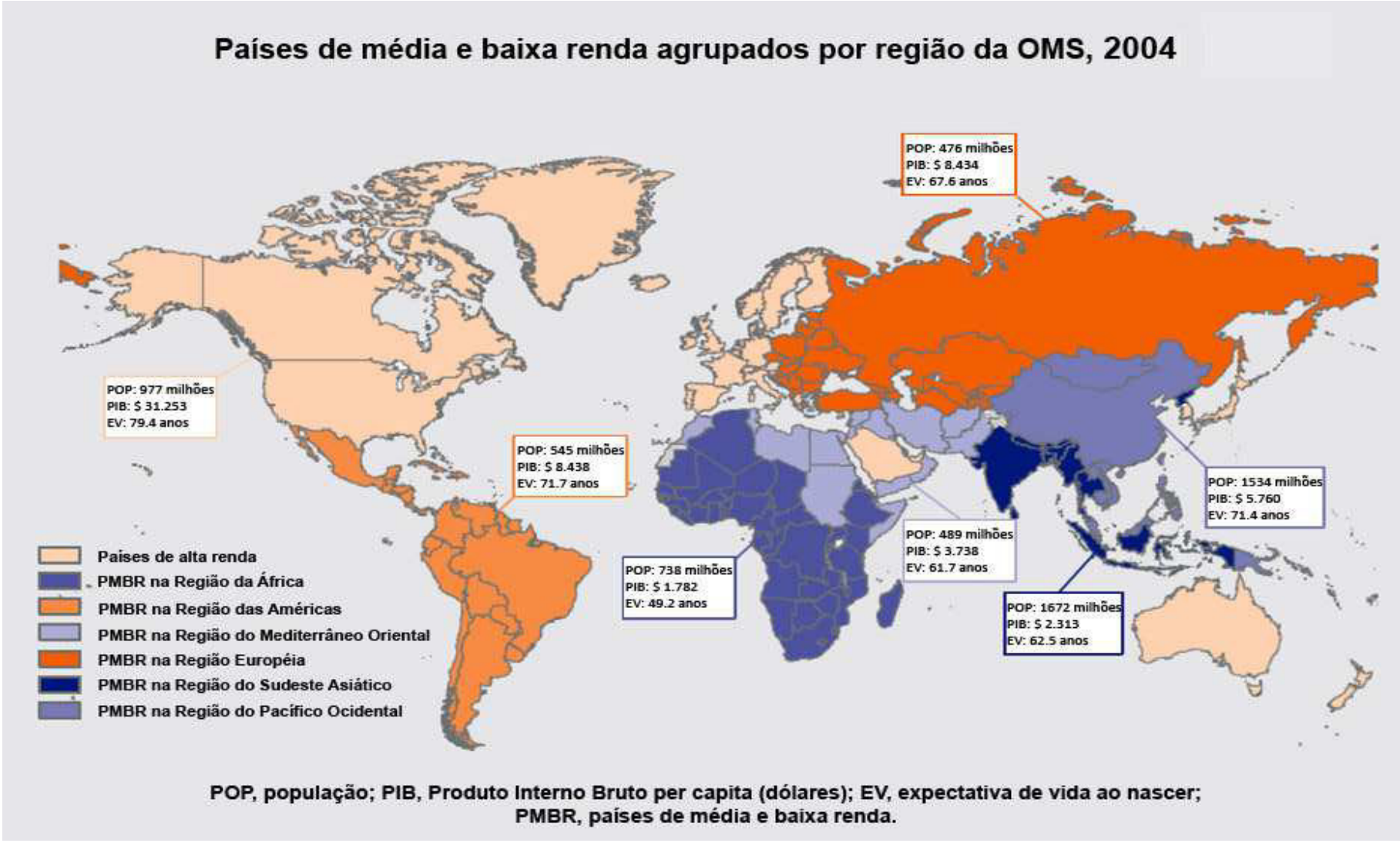


Figura 2: fonte WHO 2009

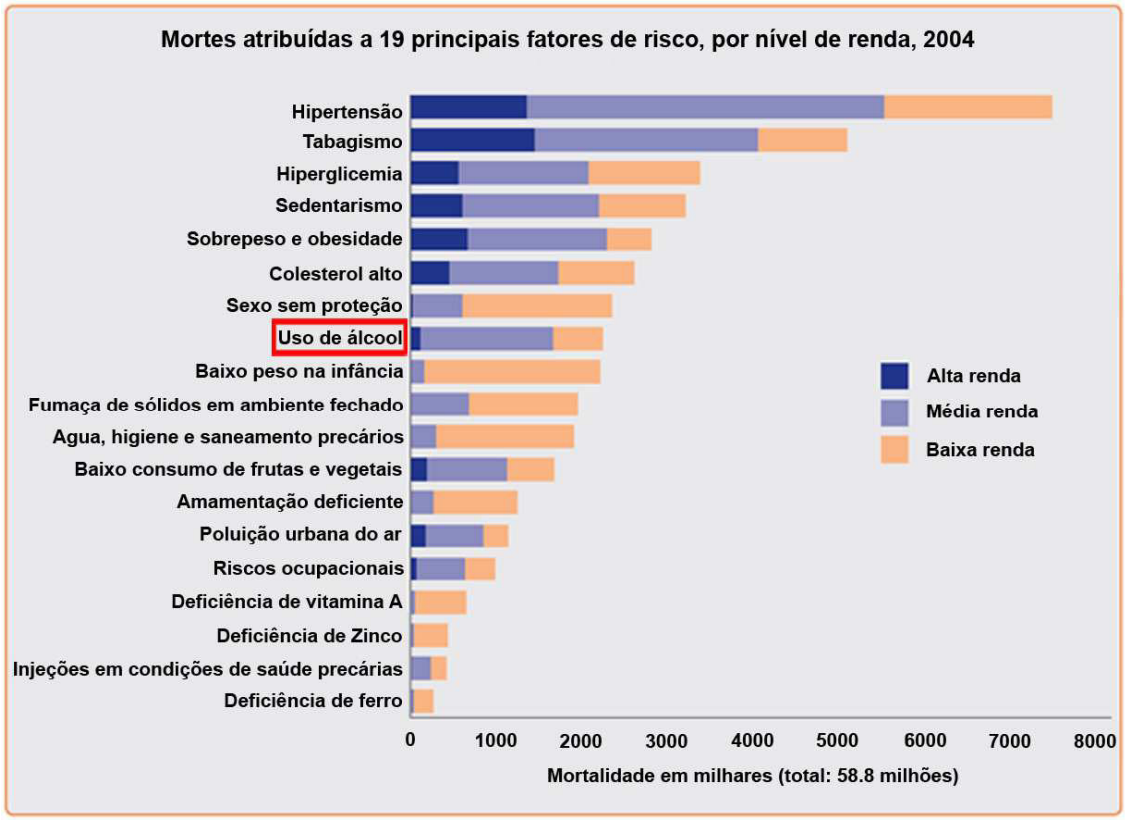


Figura 3: fonte WHO 2009

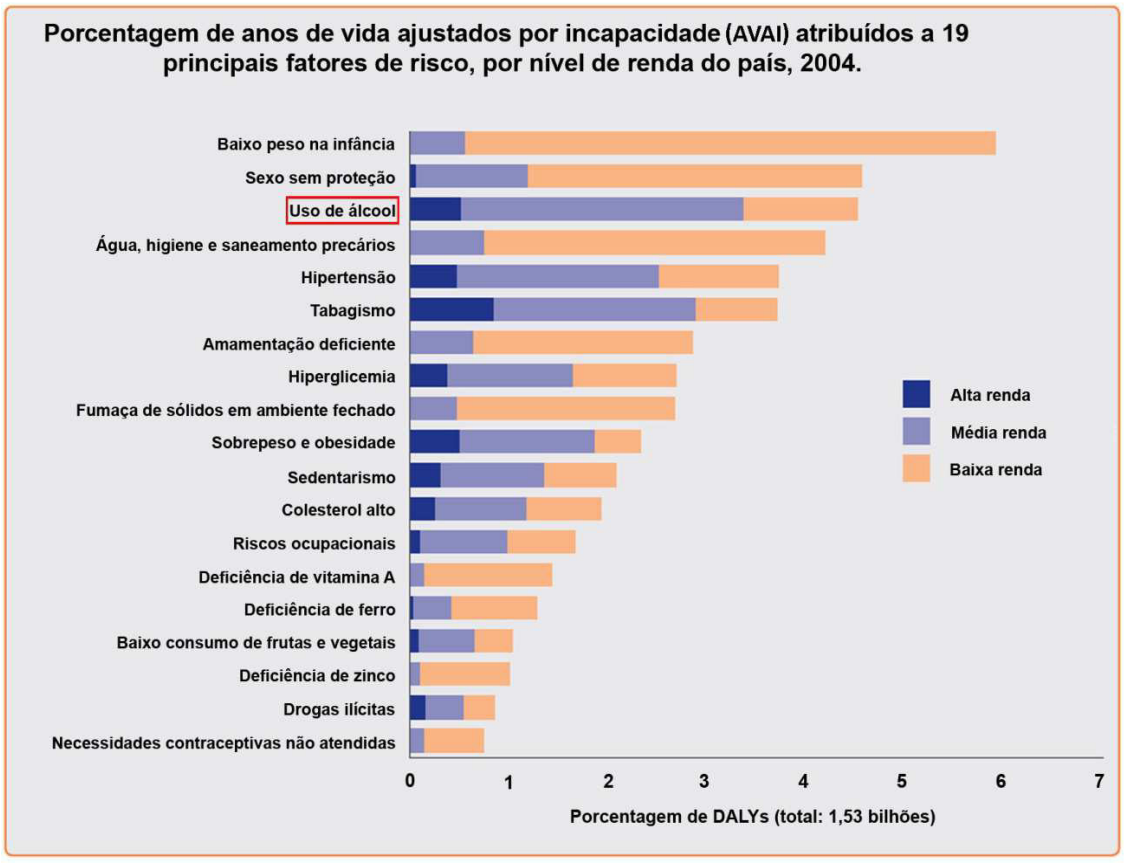


Figura 4: fonte WHO 2009

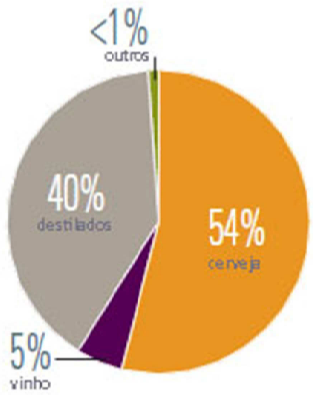
Brasil

Fonte: Nações Unidas, dados de 1990-2006

Contexto socioeconômico

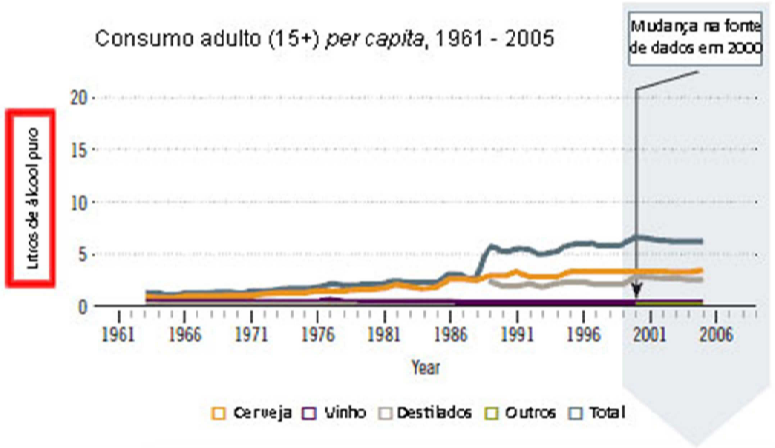
População:189 323 000 ➤ População 15+ anos: 72% ➤ População em área urbana: 85% ➤ Grupo de renda (Banco Mundial): Extrato superior da renda média

Registro do consumo adulto (15+) de álcool por tipo de bebida alcóolica (em litros de álcool puro), 2005



Consumo de álcool

Dados populacionais (referentes à população de 15 anos ou mais em litros de álcool puro).



Consumo adulto (15+) *per capita*, média de 2003 - 2005 (em litros de álcool puro)

Registrado	6.2
Não-registrado	3.0
Total	9.2
Região Americana-OMS	8.7

Estimativa de mudança em cinco anos no consumo *per capita* (15+) 2001-2005

Aumento

➤ Estável

Redução

Inconclusivo

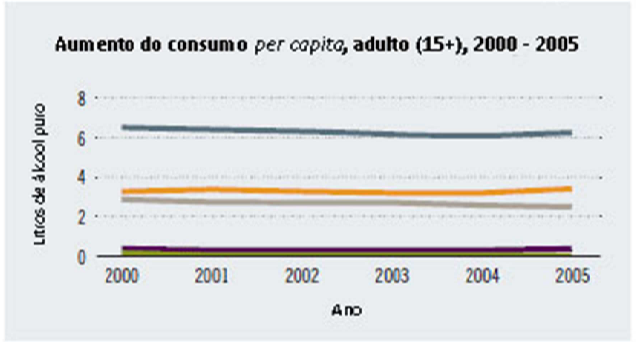


Figura 5: fonte WHO 2011

Classificação dos 10 principais fatores de risco causadores de morte por grupo de renda, 2004

	Fatores de risco	Mortes (milhões)	Porcentagem do total		Fatores de risco	Mortes (milhões)	Porcentagem do total
	Mundo				**Países de baixa renda**		
1	Hipertensão	7.5	12.8	1	Baixo peso na infância	2.0	7.8
2	Tabagismo	5.1	8.7	2	Hipertensão	2.0	7.5
3	Hiperglicemia	3.4	5.8	3	Sexo sem proteção	1.7	6.6
4	Sedentarismo	3.2	5.5	4	Água, higiene e saneamento precários	1.6	6.1
5	Sobrepeso e obesidade	2.8	4.8	5	Hiperglicemia	1.3	4.9
6	Colesterol alto	2.6	4.5	6	Fumaça de sólidos em ambiente fechado	1.3	4.8
7	Sexo sem proteção	2.4	4.0	7	Tabagismo	1.0	3.9
8	Uso de álcool	2.3	3.8	8	Sedentarismo	1.0	3.8
9	Baixo peso na infância	2.2	3.8	9	Amamentação deficiente	1.0	3.7
10	Fumaça de sólidos em ambiente fechado	2.0	3.3	10	Colesterol alto	0.9	3.4
	Países de média renda				**Países de alta renda**		
1	Hipertensão	4.2	17.2	1	Tabagismo	1.5	17.9
2	Tabagismo	2.6	10.8	2	Hipertensão	1.4	16.8
3	Sobrepeso e obesidade	1.6	6.7	3	Sobrepeso e obesidade	0.7	8.4
4	Sedentarismo	1.6	6.6	4	Sedentarismo	0.6	7.7
5	Uso de álcool	1.6	6.4	5	Hiperglicemia	0.6	7.0
6	Hiperglicemia	1.5	6.3	6	Colesterol alto	0.5	5.8
7	Colesterol alto	1.3	5.2	7	Baixo consumo de frutas e vegetais	0.2	2.5
8	Baixo consumo de frutas e vegetais	0.9	3.9	8	Poluição atmosférica urbana	0.2	2.5
9	Fumaça de sólidos em ambiente fechado	0.7	2.8	9	Uso de álcool	0.1	1.6
10	Poluição atmosférica urbana	0.7	2.8	10	Riscos ocupacionais	0.1	1.1

Países agrupados por renda bruta per capita - baixa renda (US$ 825 ou menos), alta renda (US$ 10 066 ou mais)

Figura 6: fonte WHO 2009

Classificação dos fatores de risco selecionados: 10 principais fatores de risco causais de AVAI por grupo de renda, 2004

	Fatores de risco	AVAI (milhões)	Porcentagem do total		Fatores de risco	AVAI (milhões)	Porcentagem do total
	Mundo				**Países de baixa renda**		
1	Baixo peso na infância	91	5.9	1	Baixo peso na infância	82	9.9
2	Sexo sem proteção	70	4.6	2	Água, higiene e saneamento precários	53	6.3
3	Uso de álcool	69	4.5	3	Sexo sem proteção	52	6.2
4	Água, higiene e saneamento precários	64	4.2	4	Amamentação deficiente	34	4.1
5	Hipertensão	57	3.7	5	Fumaça de sólidos em ambiente fechado	33	4.0
6	Tabagismo	57	3.7	6	Deficiência de vitamina A	20	2.4
7	Amamentação deficiente	44	2.9	7	Hipertensão	18	2.2
8	Hiperglicemia	41	2.7	8	Uso de álcool	18	2.1
9	Fumaça de sólidos em ambiente fechado	41	2.7	9	Hiperglicemia	16	1.9
10	Sobrepeso e obesidade	36	2.3	10	Deficiência de zinco	14	1.7
	Países de média renda				**Países de alta renda**		
1	Uso de álcool	44	7.6	1	Tabagismo	13	10.7
2	Hipertensão	31	5.4	2	Uso de álcool	8	6.7
3	Tabagismo	31	5.4	3	Sobrepeso e obesidade	8	6.5
4	Sobrepeso e obesidade	21	3.6	4	Hipertensão	7	6.1
5	Hiperglicemia	20	3.4	5	Hiperglicemia	6	4.9
6	Sexo sem proteção	17	3.0	6	Sedentarismo	5	4.1
7	Sedentarismo	16	2.7	7	Colesterol alto	4	3.4
8	Colesterol alto	14	2.5	8	Drogas ilícitas	3	2.1
9	Riscos ocupacionais	14	2.3	9	Riscos ocupacionais	2	1.5
10	Água, higiene e saneamento precários	11	2.0	10	Baixo consumo de frutas e vegetais	2	1.3

Países agrupados por renda bruta per capita - baixa renda (US$ 825 ou menos), alta renda (US$ 10 066 ou mais)

Figura 7: fonte WHO 2009

2.3 Álcool e neuropatogênese

A ingestão de álcool pode produzir, no organismo, uma grande variedade de distúrbios patológicos. O consumo excessivo e prolongado dessa substância caracteriza uma situação que pode levar à degeneração tóxica e disfunção em diversos órgãos. Por ser uma molécula pequena e solúvel tanto em água como em lipídeos, o etanol difunde-se, com facilidade, através das membranas mucosas esofágicas e estomacais. Após a absorção, termina por ser completamente oxidado, nunca ficando armazenado no corpo (DAS e VASUDEVAN, 2007). A relação entre o consumo de etanol e as consequências para a saúde é complexa e multidimensional. As condições patológicas decorrentes do consumo podem ser consideradas completamente atribuíveis ao etanol, o que identifica o consumo como causa necessária para o desenvolvimento da condição patológica, e na falta do qual tal condição não ocorreria. Além disso, têm-se as doenças infecciosas ou crônicas, nas quais o álcool é uma causa componente associada, ou seja, facilita a instalação do quadro e pode agravar suas consequências (REHM et al., 2010). Entre as patologias derivadas 100% do consumo de álcool podem ser citadas: intoxicação aguda, síndrome de dependência, estado de abstinência com ou sem delírio, desordem psicótica, degeneração do sistema nervoso devido ao álcool, polineuropatia alcoólica, cardiomiopatia alcoólica, fibrose e esclerose hepática, falência hepática, cirrose hepática, pancreatite aguda e crônica, dentre outras (EHLERS e CRIADO, 2010; REHM et al., 2010). Entre as patologias não completamente atribuíveis ao etanol, mas nas quais ele tem participação provável, podemos citar a epilepsia, Doença de Alzheimer, câncer de boca, de faringe, de esôfago, de estômago, de fígado, de reto, diabetes

mellitus, pneumonia, desordem depressiva unipolar e tuberculose, (PARKIN, 2011; REHM et al., 2010).

Estudos atuais discutem resultados que apontam para um aumento da expectativa de vida de consumidores moderados, em comparação a consumidores “pesados” (mais de três doses/dia) e a não-consumidores de bebida alcoólica, aqueles que nunca consomem mais de uma dose/mês (RIMM et al., 1996, RUITENBERG et al., 2002). Embora a toxicidade do etanol possa estar associada ao aparecimento de demência em bebedores abusivos, a ingestão moderada parece reduzir, em longo prazo, o risco de demência. Essa relação, entre o consumo e benefícios ou malefícios sofridos, foi verificada em estudos com indivíduos idosos, em que os hábitos de consumo de etanol foram determinados e acompanhados, desde anos antes de começarem a aparecer os casos de demência associados à idade (RUITENBERG et al., 2002).

2.4 Álcool e espécies químicas reativas

Os efeitos deletérios provocados pelo álcool podem ser consequentes à sua toxicidade direta, ou indireta, através de seus metabólitos secundários e/ou a formação de espécies reativas de oxigênio. A oxidação do etanol está associada com uma mudança na homeostase da oxi-redução nos hepatócitos, que levam a certas 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 do ânion superóxido. O álcool pode induzir a formação de espécies reativas ainda 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). É possível que, desta maneira, dano oxidativo possa acumular-se como uma consequência secundária do consumo e metabolização do etanol.

De fato, uma das maiores ameaças à homeostasia celular dos organismos aeróbicos é a formação dos intermediários reativos e os subprodutos do metabolismo do oxigênio (FRIDOVICH, 1978; HALLIWELL e CROSS, 1994). Essas espécies reativas são, ironicamente, derivadas de processos metabólicos e fisiológicos normais que são essenciais para a célula (YU, 1994). O processo evolutivo dotou os organismos aeróbicos com mecanismos bem balanceados para neutralizar os efeitos oxidativos maléficos do oxigênio e seus metabólitos reativos. Esses componentes protetores são denominados sistemas de defesa antioxidantes (DAVIES, 1986; HARRIS, 1992). Sua função é proteger a homeostase celular da ruptura por radicais livres e outras moléculas reativas criadas durante a redução do oxigênio (BENDICH et al., 1984; LUNEC et al., 1985). O desbalanceamento em favor dos radicais oxidativos gerados no metabolismo, sobre o seu efetivo controle pelos mecanismos antioxidantes, caracteriza a situação de estresse oxidativo. Nessa condição, a intrincada rede de defesa de enzimas e moléculas antioxidantes, integrada com outros componentes celulares dedicados ao reparo das moléculas danificadas ou alteradas, tornam-se insuficientes para a integridade da homeostase celular (RIVETT, 1985; SALO et al., 1998). Os dados experimentais disponíveis mostram que a remoção de peróxido de hidrogênio e oxigênio singleto, pelo sistema de defesa antioxidante, é essencial para a possibilidade de vida aeróbica saudável. A exposição ao oxigênio puro a

1 atm, por exemplo, por um período curto como 6 horas, causa inflamação do peito, rouquidão e tosse. Exposição por período mais prolongado leva a danos pulmonares (HALLIWELL e CROSS, 1994).

Mecanismos de defesa contra o estresse oxidativo induzido por radicais livres envolvem: (i) mecanismos preventivos, (ii) mecanismos de reparo, (iii) defesas físicas e (iv) defesas antioxidantes. As defesas antioxidantes enzimáticas incluem a superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT). Antioxidantes não-enzimáticos estão representados pelo ácido ascórbico (vitamina C), α-tocoferol (vitamina E), glutationa (GSH), carotenóides, flavonóides e outros antioxidantes. Sob condições normais, existe um balanço entre a produção de espécies reativas, as atividades e os níveis intracelulares desses antioxidantes. Esse balanço é essencial para a saúde e a sobrevivência dos organismos (VALKO et al., 2007).

Embora as espécies reativas sejam formadas normalmente no metabolismo para cumprir uma série de ações fisiológicas, ao reagir com uma grande variedade de componentes celulares, sua produção excessiva pode causar alterações morfológicas e funcionais nas células, inclusive o desbalanceamento da homeostase do cálcio intracelular, considerada a base dos mecanismos de injúrias por excitotoxicidade (TURRENS, 2003).

Outros estudos têm relatado que o dano oxidativo de biomoléculas parece estar envolvido com o aparecimento e o desenvolvimento de diversas doenças sistêmicas crônicas, como o câncer, além de doenças cardiovasculares, sendo essas duas as maiores causas de morbidade e mortalidade no mundo ocidental (TAPIERO et al., 2004). Humanos adultos jovens que consomem etanol de forma crônica têm uma maior probabilidade de

serem patologicamente afetados em diversos órgãos, como fígado, rins, coração pâncreas e cérebro (LIEBER, 1995). O estresse oxidativo, induzido pelo álcool, está relacionado com os sistemas mitocondriais e microssomais, envolvendo o metabolismo do etanol e a formação de espécies reativas de oxigênio e nitrogênio. Estas, quando produzidas em grande quantidade, podem provocar a depleção dos mecanismos antioxidantes endógenos e, assim, estabelecer um ambiente propício ao estresse oxidativo, pela atuação dos radicais livres (RASHBA-STEP et al., 1993; DAS e VASUDEVAN, 2007).

2.5 Sistema nervoso, espécies reativas de oxigênio e antioxidantes

Com relação à ação das espécies reativas de oxigênio **no sistema nervoso central**, é importante mencionar que essa ação, juntamente com outros fatores, tem sido associada, em países desenvolvidos, com a incidência aumentada de doenças neurodegenerativas, incluindo danos relacionados a estresse oxidativo (CHEN et al., 2009). Isso pode ser intensamente produzido em condições de sobrecarga de oxidantes no organismo, como em alimentação parenteral (CHEN et al., 2007), em casos de hipo-funcionamento dos mecanismos antioxidantes neurais, ou pelo consumo excessivo de etanol. Nessas condições, a suplementação dietética de antioxidantes pode ser benéfica (CODOÑER-FRANCH et al., 2010). Taxas aumentadas de peroxidação no cérebro, por exemplo, pode levar à produção excessiva de espécies reativas de oxigênio, as quais, se não forem neutralizadas por mecanismos antioxidantes, são altamente deletérias ao tecido cerebral. Essa neutralização fisiológica das espécies reativas pode falhar na presença de um

número de fatores, como deficiência nutricional (RAMESH et al., 2010), processo de envelhecimento (GEMMA et al., 2007) e o consumo de etanol (LOUREIRO et al., 2011). Esses fatores podem romper o cenário óxido-redutor normal do cérebro, levando a uma situação de aumento do estresse oxidativo no qual o cérebro torna-se susceptível ao desenvolvimento de certas disfunções neurológicas, como as que ocorrem na Doença de Alzheimer (AXELSEN et al., 2011), diabetes (SEIFRIED et al., 2007) e epilepsia (GROSSO et al., 2011). Propriedades antioxidantes têm se mostrado importantes na proteção do cérebro contra os efeitos deletérios dos radicais livres, particularmente em doenças neurodegenerativas (OCHIAI et al., 2007).

O etanol pode permear praticamente todo o tecido cerebral, exercendo a maior parte dos seus efeitos tóxicos através da produção de espécies reativas de oxigênio (TAPIERO et al., 2004). O tecido cerebral é particularmente vulnerável ao dano oxidativo, possivelmente por causa do seu alto consumo de oxigênio, baixo nível de enzimas antioxidantes, altos níveis de ferro livre e consequente produção de grandes quantidades de espécies reativas (LAMARCHE et al., 2004). 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, a excitotoxicidade, a produção excessiva de radicais livres, a inflamação e a apoptose. Além do mais, tanto a isquemia quanto o trauma cerebral deflagram mecanismos autoprotetores similares, nos quais se incluem a produção de proteínas de choque térmico, citocinas anti-inflamatórias e antioxidantes endógenos. A terapia neuroprotetora, utilizando antioxidantes, procura minimizar a ativação de vias tóxicas, aumentando a atividade dos mecanismos

neuroprotetores endógenos (LEKER e SHOHAMI, 2002). O acúmulo de danos oxidativos em neurônios pode ser, primária ou secundariamente, a causa para o aumento da incidência de doenças neurodegenerativas como neoplasias, doença de Parkinson, de Alzheimer e esclerose lateral amiotrófica, em populações idosas (HALLIWELL 2001; RAO e BALACHANDRAN; 2002).

O etanol perturba processos relevantes na formação do cérebro, podendo resultar em migração celular tardia e anômala, depressão da neurogênese e desenvolvimento estrutural e funcional aberrante (GONZÁLEZ et al., 2007). Na “síndrome do alcoolismo fetal”, uma série de disfunções comportamentais, intelectuais e neurológicas pode se desenvolver, como conseqüência da exposição do embrião, em formação, ao etanol, comprometendo o desenvolvimento normal do sistema nervoso central (COSTA et al., 2000; LIPINSKI et al., 2012; MATTSON et al., 2012). O hipocampo é um dos principais alvos da injúria nessa síndrome, podendo originar déficits em aprendizado e memória, os quais são encontrados em crianças acometidas de tal quadro. Moléculas com ação antioxidante como a vitamina E (α-tocoferol) e o β-caroteno protegem células nervosas, cultivadas a partir do hipocampo de ratos, contra os efeitos do etanol, em um modelo *in vitro* de síndrome do alcoolismo fetal associado com isquemia (MITCHELL et al., 1999). Uma das desordens neurológicas mais comumente identificada como sendo associada ao alcoolismo é a síndrome de Wernicke-Korsakoff. Esta síndrome é causada por deficiência de tiamina, que pode ser consequência de sua insuficiência na dieta ou por redução da absorção, provocada pelo consumo de álcool, que leva à lesão bilateral no diencéfalo (notadamente no tálamo e corpos mamilares). A sua característica mais marcante é a

deterioração da memória (FADDA e ROSSETTI, 1998; KESSELS e KOPELMAN, 2012; SUBRAMANYA et al., 2010). Nessa condição patológica pode ocorrer tanto amnésia anterógrada quanto retrógrada. Além da perda de memórias formadas anteriormente ao estabelecimento da síndrome, nos estágios avançados dessa neuropatologia, a dificuldade persistente na formação de novas memórias compromete a capacidade de aprendizado de novas informações, um estado frequentemente persistente e irreversível (FADDA e ROSSETTI, 1998; KESSELS e KOPELMAN, 2012; LOUGH, 2012).

Estudos recentes demonstraram que, na Doença de Alzheimer, a deposição de peptídeos β-amilóides e a morte neuronal estão relacionadas a uma crescente disfunção neural cognitiva. O estresse oxidativo observado na doença, provocado provavelmente por níveis aumentados de ferro e cobre na neurópila (regiões do sistema nervoso central com predominância de terminações axonais e dendríticas, mas com poucos corpos celulares), causa alteração no metabolismo de esfingolipídios e do colesterol, e proporciona o acúmulo celular de ceramidas de cadeia longa e colesterol (SMITH et al. 2000; CUTLER et al., 2004). Esse acúmulo de colesterol livre é tóxico para a célula, o que por sua vez pode levar a uma cascata neurodegenerativa que é evidenciada pelo aparecimento dos sintomas clínicos da doença. Foi verificado que o tratamento com α-tocoferol mostrou-se eficaz em reduzir a formação de ceramidas, e proteger neurônios contra a morte induzida pelo acúmulo de peptídeo β-amilóide (CUTLER et al., 2004).

O consumo de etanol provoca o acúmulo de lipídios no fígado (esteatose hepática), que em princípio não caracteriza uma condição patológica. A persistência de consumo abusivo, entretanto, pode favorecer o

desenvolvimento de esteatohepatite, com produção e acúmulo de lipídios tóxicos (como as ceramidas), ativação de citocinas pró-inflamatórias e desenvolvimento de resistência à insulina (DE LA MONTE et al., 2009; SETSHEDI et al., 2011). O aumento intracelular de ceramidas, bem como de diacilglicerol e acil-coenzima A de cadeia longa, tem sido demonstrado como sendo uma condição promotora da resistência à insulina. Isso ocorre por interferência na fosforilação de proteínas da via da insulina, incluindo o substrato do receptor de insulina (IRS 1 e 2), fosfatidil-inositol-3-quinase (PI3 quinase) e Proteína Quinase C (SILVEIRA et al. 2008). A Insulina, através de vias intracelulares complexas, que começam com a sua ligação ao seu receptor na superfície das células, transmite sinais pró-crescimento, pró-sobrevivência e de ativação do metabolismo energético (DE LA MONTE E WANDS, 2005).

No sistema nervoso central, as cascatas de sinalização da insulina e do fator de crescimento semelhante à insulina tipo 1 (IGF-1) têm funções críticas na regulação e manutenção de funções cognitivas e motoras. Essas moléculas sinalizadoras possuem seus receptores abundantemente expressos em vários tipos celulares por todo o cérebro, incluindo neurônios e oligodendroglia (BROUGHTON et al., 2007; D'ERCOLE e YE, 2008; FREUDE et al., 2008). Uma vez que a insulina e os fatores de crescimento semelhantes à insulina (IGF 1 e 2) mediam a sobrevivência, plasticidade, metabolismo energético e funções neurogliais, a debilitação prolongada nas respectivas cascatas de sinalização pode ter sérias consequências sobre a cognição e o comportamento (CHESIK et al., 2008; SCHUBERT et al., 2003). O etanol causa resistência à insulina por perturbar a composição de lipídeos de membrana causando assim alterações

significativas que prejudicam a ligação da insulina com o seu receptor, e subsequentemente toda a sequência de sinalização (COHEN et al., 2007; DE LA MONTE et al., 2008; HALLAK et al., 2001). A lipólise induzida pela resistência à insulina leva à geração de lipídios como as ceramidas, que podem aumentar ainda mais a resistência à insulina, a função mitocondrial e assim, a sobrevivência da célula (HOLLAND e SUMMERS, 2009; LANGEVELD e AERTS, 2009). Ceramidas são moléculas lipídicas sinalizadoras que modulam respostas celulares positivas ou negativas tais como proliferação, motilidade, plasticidade, inflamação e apoptose (SUMMERS, 2006). Em estados de lipólise associada à resistência à insulina, as ceramidas acentuam o desenvolvimento dessa resistência através da ativação de citocinas inflamatórias e inibição da sinalização estimulada pela insulina (BOURBON et al., 2002; HAJDUCH et al., 2001). Em humanos alcoólatras, a atrofia e degeneração da substância branca com expressão reduzida de gens relacionados à mielina, e grau aumentado de estresse oxidativo, foram associados com o aumento da expressão de gens para ceramidas (DE LA MONTE et al., 2008). As ceramidas produzidas em regiões externas ao sistema nervoso, como por exemplo, no fígado sob influência do álcool, ultrapassam a barreira hematoencefálica e podem disparar mecanismos apoptóticos no cérebro, numa condição de proteção reduzida das vias pró-sobrevivência e crescimento, proporcionadas pela insulina, formando um eixo hepato-cerebral de neurodegeneração (DE LA MONTE et al., 2009; SETSHEDI et al., 2011).

Em diversas células, o álcool prejudica a diferenciação e o crescimento celulares, interfere nos efeitos estimulantes de fatores tróficos e é capaz de alterar a expressão de proteínas do citoesqueleto (GUERRI et al., 2001).

Quando tais alterações causadas pelo etanol afetam as células gliais, podem levar à perturbação nas interações glia-neurônio e produzir defeitos no funcionamento do cérebro (GONZÁLES et al., 2007; GUERRI et al., 2001). Os astrócitos, que formam o maior grupo de células não excitáveis no sistema nervoso central de mamíferos, são células capazes de regular a atividade neuronal e a neurotransmissão sináptica. Apropriadamente estimulados por uma série de neuromoduladores, hormônios e neurotransmissores, os astrócitos respondem com um aumento na concentração intracelular de cálcio livre. Este aumento, por sua vez, regula eventos intracelulares como expressão gênica, diferenciação, respostas metabólicas, sistemas de transportes de íons e secreção de substâncias neuroativas (GONZÁLEZ et al., 2007). Entretanto, o aumento nos níveis de cálcio no citoplasma, como ocorre nos astrócitos durante a metabolização do etanol ingerido, pode levar a uma maior captação deste íon pelas mitocôndrias. O aumento da captação de cálcio pela mitocôndria leva essa organela à produção excessiva de espécies reativas de oxigênio; o aumento da produção das espécies reativas, por sua vez, leva à uma sobrecarga da concentração de cálcio citoplasmático, num jogo de puxa-empurra “de auto-alimentação” (GONZÁLEZ et al., 2007). Dessa maneira, pode-se visualizar que a manutenção de níveis elevados de cálcio intracelular é uma das formas pelas quais o álcool pode provocar, por estresse oxidativo, a morte dos astrócitos, tão importantes na manutenção da homeostase no sistema nervoso central. As espécies reativas produzidas pelas mitocôndrias são mediadores da sinalização molecular relacionada à via apoptótica mitocôndria-dependente, que envolve a ligação com proteínas anti e pró-

apoptóticas, liberação de citocromo c e a sinalização via p53 que levam à morte celular (NIIZUMA et al., 2009).

Estudos *in vitro* demonstraram que o aumento dos níveis de peróxido de hidrogênio e a redução da glutationa mitocondrial são os agentes causadores mais prováveis da apoptose induzida pelo etanol (TAPIERO et al., 2004). Estudos já demonstraram o efeito benéfico do α-tocoferol, em aumentar a sobrevivência de células cerebelares de rato frente à neurotoxicidade induzida pelo etanol, tanto *in vitro* como *in vivo*, sendo essa resistência associada ainda a uma elevação nos níveis de proteínas anti-apoptóticas (HEATON et al, 2004).

FISCHER e KITTNER (1998) descreveram uma redução dose-dependente da severidade de ataques epiléticos no modelo de abrasamento por pentilenotetrazol em ratos, para doses moderadas de etanol (0,5 – 1,5 g/kg, por 28 dias). Por outro lado, o tratamento subcrônico, com altas doses, seguido por longos períodos de abstinência, levaram a efeitos pró-convulsivos. SCORZA e colaboradores (2003) verificaram que a administração de etanol, seguida pela sua abstinência, provoca alterações neuropatológicas e aumento de crises epilépticas no modelo de epilepsia produzida pela pilocarpina. Eles concluíram que a síndrome da abstinência do álcool é um evento crucial no desenvolvimento de alterações funcionais e neuropatológicas associadas à epilepsia. Interações medicamentosas entre o etanol e drogas antiepilépticas têm sido também descritas (FISCHER, 2005). KOZAN e colaboradores (2007) verificaram que administração de α-tocoferol reduziu a freqüência média da atividade epileptiforme, principalmente associada com etanol, no modelo de epilepsia induzida por penicilina. Esta, quando injetada intracorticalmente, age bloqueando especificamente a transmissão sináptica de neurotransmissores

inibitórios (McDONALD e BARKER, 1977), facilitando assim a atividade epileptiforme. Por outro lado, o etanol parece estimular a inibição mediada pelos receptores inibitórios do GABA (gama-aminobutiric acid; em português, ácido gama-aminobutírico). Os receptores de glutamato do tipo NMDA, por sua vez, são inibidos pela exposição ao etanol, elevando o nível do limiar para a atividade epileptiforme, o que, associado à estimulação dos mecanismos GABAérgicos, aparentemente contribui para a redução encontrada na freqüência de espículas epileptiformes no eletroencefalograma. Durante os períodos de abstinência ao etanol, sugere-se que a falta da inibição elevada do sistema GABA mais a quantidade excessiva dos receptores excitatórios NMDA participem do efeito pró-convulsivante da abstinência do álcool (KOZAN et al., 2007). Uma possibilidade é que isto represente a base de um mecanismo de plasticidade.

As distintas populações celulares do cérebro normal geram atividade elétrica continuamente, durante todo o período de vida do organismo. Todas as funções regulatórias dependentes do cérebro no organismo dependem direta ou indiretamente dessa atividade elétrica. Em humanos, bem como em animais de laboratório, uma interessante maneira de adquirir informação importante sobre o funcionamento cerebral consiste em registrar a atividade elétrica produzida espontaneamente pelo cérebro. Esta técnica, denominada eletroencefalograma (EEG), pode ser definida como a medida das oscilações temporais do potencial elétrico entre dois pontos do cérebro. Por ser uma técnica não invasiva (um certo número de eletrodos é fixado sobre a pele do crânio e as oscilações de potencial elétrico são registradas de maneira pareada), é amplamente utilizada em humanos, auxiliando no diagnóstico de

diversas desordens neurológicas (GUEDES, 2005). Em animais experimentais, os registros podem ser realizados de maneira invasiva, sob anestesia, na qual o crânio é aberto cirurgicamente e os eletrodos de registro são posicionados sobre a superfície ou mesmo dentro do córtex; neste caso, é denominado eletrocorticograma (ECoG). Sob estresse oxidativo, como por exemplo, na injúria por isquemia/reperfusão, a atividade elétrica cerebral pode ser afetada em diferentes intensidades (KARA et al., 2011). Em animais sob estresse oxidativo, diversos relatos descrevem susceptibilidades alteradas para desordens relacionadas à excitabilidade do cérebro, tais como epilepsia e doenças degenerativas (GROSSO et al., 2011; AYYLDIZ et al., 2007; WALDBAUM e PATEL, 2010). A implicação fisiopatológica de tal conjunto de evidências, para a saúde humana, levou-nos a querer investigar em que extensão o desarranjo no estado redox poderia alterar a atividade elétrica do córtex cerebral. Para este propósito, temos usado como modelo experimental o fenômeno da depressão alastrante da atividade elétrica cortical (DAC).

2.6 Depressão alastrante cortical e espécies químicas reativas

A DAC é um fenômeno eletrofisiológico que foi primeiramente descrito por Aristides Leão (LEÃO, 1944; 1947). É definida como uma redução da atividade elétrica do córtex cerebral, em resposta à estimulação de um ponto cortical. Essa estimulação pode ser de natureza química, elétrica ou mecânica. A redução observadada no registro eletrocorticográfico propaga-se concentricamente (com velocidade na ordem de 2 a 5 mm/min) por toda a superfície do córtex, a partir do ponto inicialmente estimulado. Após alguns

minutos, a atividade elétrica retorna completamente ao normal, o que caracteriza o fenômeno como completamente reversível (LEÃO, 1944). Esse fenômeno já foi registrado em inúmeros vertebrados, como aves, mamíferos e mesmo em anfíbios (LEÃO, 1972; BURES et al., 1974; DO CARMO e MARTINS FERREIRA, 1984; GORJI, 2001), já tendo sido registrado em humanos (MAYEVSKY et al., 1996; DOHMEN et al, 2008; FABRICIUS et al, 2008).

A modulação imposta por diferentes tratamentos experimentais, sobre a velocidade com que a DAC se propaga, proporciona um indício sobre o estado geral do tecido nervoso quanto à facilidade ou dificuldade de transmissão dos sinais elétricos cerebrais. Esta informação, por sua vez, nos permite comparar as modificações do perfil de atividade elétrica no cérebro, resultantes de tratamentos ou condições impostas, para buscar um melhor entendimento dos eventos envolvidos na modificação da excitabilidade cerebral. Mudanças nas características da DAC relacionam o fenômeno a doenças neurológicas de interesse clínico, como a epilepsia, enxaqueca com aura, isquemia cerebral (LEHMENKÜHLER et al., 1993; LEÃO, 1944; GUEDES e CAVALHEIRO, 1997).

Durante a DAC, enquanto a atividade elétrica espontânea é reduzida, ondas “epileptiformes”, semelhantes àquelas descritas em pacientes epilépticos, usualmente aparecem e se propagam (LEÃO, 1944; GUEDES e DO CARMO, 1980). Essa e outras evidências experimentais sugerem que a DAC pode ter seus mecanismos relacionados aos de doenças humanas, como a epilepsia (LEÃO, 1944; 1972; GUEDES et al., 2009), a migrânea com aura, outrora denominada “enxaqueca clássica” (HADJIKHANI et al., 2001), e

também à isquemia cerebral (DOHMEN et al., 2008). Dentre outros possíveis fatores, os radicais livres produzidos no tecido nervoso são freqüentemente apontados como envolvidos na DAC (NETTO e MARTINS-FERREIRA, 1989; El-BACHÁ et al., 1998). Pelo exposto, a DAC foi utilizada neste estudo como modelo experimental, em prosseguimento à linha de pesquisa desenvolvida na UFPE/Departamento de Nutrição, onde a parte experimental deste trabalho foi realizada.

Evidências experimentais indicam que o tecido neural, em condições fisiológicas, oferece certo grau de resistência à propagação da DAC (GUEDES e DO CARMO, 1980). Quando essa resistência diminui, em função de algum tratamento experimental, a DAC passa a se propagar com velocidades mais altas do que as observadas em condições controle; o contrário ocorre, se a resistência cortical à DAC for experimentalmente aumentada. Assim, procedimentos experimentais, que enfraqueçam ou reforcem a capacidade cerebral de se contrapor à DAC, podem fornecer informações valiosas para a compreensão desse fenômeno e para as doenças a ele relacionadas. Na UFPE (no Departamento de Nutrição), a DAC vem sendo estudada em ratos submetidos a manipulações ambientais (SANTOS-MONTEIRO et al., 2000), farmacológicas (AMÂNCIO-DOS-SANTOS et al., 2006), nutricionais (ROCHA-DE-MELO et al., 2006) e hormonais (ACCIOLY et al, 2012). Dentre as diversas condições, que influenciam a DAC, está o consumo de etanol: o tecido nervoso torna-se mais susceptível à DAC, sob o efeito prolongado do etanol, em comparação com grupos tratados com água destilada (GUEDES e FRADE, 1993; SONN e MAYEVSKY, 2001).

Para testar a hipótese de que a ação facilitadora do etanol sobre a DAC está associada ao estresse oxidativo induzido pelo álcool, BEZERRA e colaboradores (2005) registraram a DAC em ratos tratados cronicamente (por 18 dias) com um extrato alcoólico de carotenóides do camarão (*Litopenaeus vannamei*). Eles verificaram uma redução da velocidade de propagação da DAC, em comparação com ratos tratados apenas com o etanol. Esse estudo, do qual resultou a publicação do artigo (BEZERRA et al, 2005), sugere a aceitação da hipótese acima e fez parte do trabalho de conclusão do curso de graduação do doutorando que ora apresenta esta tese.

O extrato de carotenóides do camarão, acima descrito, possui na sua composição vários carotenóides, sendo a astaxantina o mais abundante. Assim, em prosseguimento ao trabalho acima, no seu mestrado, o presente doutorando investigou a possibilidade de que os efeitos desse extrato de carotenóides fossem devidos, senão exclusivamente, ao menos principalmente à astaxantina. Os resultados confirmaram essa hipótese: o tratamento com astaxantina pura (comercial) neutralizou os efeitos do tratamento crônico com etanol sobre a DAC, de forma semelhante ao observado com o extrato de carotenóides do camarão; o trabalho resultou na publicação de mais um artigo (ABADIE-GUEDES et al., 2008).

Duas perguntas se seguiram a esse trabalho e foram sequencialmente investigadas durante o doutorado: a primeira pergunta objetivou verificar se, em comparação com o tratamento crônico, o tratamento agudo com etanol (apenas um dia) teria efeito sobre a DAC e se a astaxantina, também ministrada por um dia, neutralizaria tal efeito.

A segunda pergunta procurou investigar se a ação protetora dos carotenóides sobre o efeito do etanol na DAC seria restrita apenas a essa classe de antioxidantes (carotenóides) ou se outras moléculas com ação antioxidante, mas que não sejam carotenóides, também a exerceriam. Para responder a essa pergunta, decidiu-se investigar uma molécula que tivesse, ao lado da ação antioxidante, um importante papel metabólico e fisiológico. A vitamina E (α-tocoferol) atende a esses critérios, sendo que alguns dos seus efeitos sobre o sistema nervoso já têm sido estudados (CHERUBINI et al., 2008; CUDDIHY et al., 2008; MARINO et al., 2004; SHICHIRI et al., 2007). Assim, analisou-se a ação da vitamina E sobre a DAC, na presença do etanol, e essa ação foi comparada com aquela previamente descrita para a astaxantina. Os resultados desses experimentos revelaram que o tocoferol antagoniza os efeitos do etanol sobre a DAC, de forma semelhante ao carotenóide astaxantina.

3.0 OBJETIVOS

3.1 Objetivo geral

Estudar os efeitos de diferentes regimes de consumo de álcool sobre a atividade elétrica no córtex de ratos Wistar, e comparar a ação de dois diferentes tipos de moléculas antioxidantes em modificar o efeito do etanol sobre a eletrofisiologia nesses animais.

3.2 Objetivos específicos

1) Caracterizar o efeito agudo da administração de etanol sobre a DAC, em animais adultos jovens de duas faixas distintas de idade; e avaliar se o tratamento agudo com astaxantina se oporia a tal efeito;

2) investigar se o α-tocoferol modificaria as alterações na propagação da DAC causadas pelo etanol, bem como pela sua abstinência;

(3) comparar eventuais modificações com aquelas previamente observadas com a astaxantina.

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CAPÍTULO I

The Impairing Effect of Acute Ethanol on Spreading Depression is Antagonized by Astaxanthin in Rats of 2 Young-Adult Ages

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Background: Ethanol (EtOH) abuse and insufficient ingestion of antioxidants are external factors that can alter brain electrophysiology. Our previous studies have demonstrated that the excitability-related brain electrophysiological phenomenon known as cortical spreading depression (CSD) was facilitated by chronic EtOH intake, and chronic treatment with carotenoids attenuated this effect. Here, we investigated the acute effect of a single EtOH administration on CSD in young and adult rats previously (1 hour) treated with 10 μg/kg of astaxanthin.

Methods: Male Wistar rats (5 young- and 5 adult groups, 60 to 80 and 150 to 180 days of age, respectively) were treated by 2 gavage procedures at 1-hour interval as follows: groups 1 and 2 received astaxanthin in gavage I combined with EtOH (group 1) or water (group 2) in gavage II; groups 3 and 4 received olive oil (the vehicle in which astaxanthin was dissolved) in gavage I combined with EtOH (group 3) or water (group 4) in gavage II; group 5 received water in gavage I combined with EtOH in gavage II. CSD was recorded on the cortical surface for 4 hours.

Results: Compared to the respective water and oil controls (groups 2 and 4; CSD velocities: 3.73 ± 0.09 and 3.78 ± 0.07 mm/min in the young groups; 2.99 ± 0.10 and 3.05 ± 0.19 mm/min in the adult groups), a single dose of EtOH (groups 3 and 5) decreased CSD propagation velocities (3.29 ± 0.23 and 3.16 ± 0.10 mm/min in the young groups; 2.71 ± 0.27 and 2.75 ± 0.31 mm/min in the adult groups). Astaxanthin antagonized the impairing effect of acute EtOH on CSD (group 1; mean velocity: 3.70 ± 0.19 and 3.13 ± 0.16 mm/min for the young and adult groups, respectively).

Conclusions: The results showed an antagonistic effect of acute EtOH treatment on CSD propagation that was reverted by astaxanthin. The EtOH–astaxanthin interaction was not influenced by the age, as it was found in both young and adult animals.

Key Words: Acute Ethanol, Astaxanthin, Antioxidants, Spreading depression.

HUMAN YOUNG ADULTS who consume ethanol (EtOH) chronically have a high probability of being pathologically affected in several organs, like liver, kidney, heart, pancreas, and brain (Lieber, 1995). EtOH can permeate almost all the brain tissue, exerting most of its toxic effects through the production of reactive oxygen species (ROS; Tapiero et al., 2004). Antioxidant molecules like carotenoids have also been shown to be important in protecting the brain against the deleterious effects of free radicals, particularly in neurodegenerative diseases (Mitchell et al., 1999; Tapiero et al., 2004). Among carotenoids, astaxanthin is predominant in marine organisms, like algae, crustaceans, and fish. It is a powerful biological antioxidant, strongly scavenging free radicals and protecting the living tissue against lipid peroxidation and oxidative damage (Miki, 1991). Recently, it has been pharmacokinetically demonstrated that several rat tissues, including the brain, present high affinity for astaxanthin (Choi et al., 2011). Moreover, besides being a potent antioxidant, astaxanthin has also anticancer and anti-inflammatory activities (Higuera-Ciapara et al., 2006).

Contrasting with the extensive investigation of the brain effects of chronic EtOH, the impact of a single dose of EtOH has not been investigated in detail, although some recent studies suggested important, gene-related (Guo et al., 2011; de Licona et al., 2009), as well as cortical excitability (Conte et al., 2008), effects of this acute condition. Also, no systematic investigation has addressed the role of age in the effects of EtOH on the brain.

The aim of this study was to investigate, in rats of 2 young-adult ages, the effects of a single dose of EtOH on the propagation of cortical spreading depression (CSD), a phenomenon related to brain excitability. CSD has been first described as a cerebral cortex response elicited by electrical, mechanical, or chemical stimulation of 1 point of the tissue. This response is expressed as a reversible and slowly

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propagating "wave" of depolarization causing a depression in spontaneous and evoked cortical electrical activity, with a simultaneous direct current (DC) slow potential change in the tissue (Leão, 1944, 1947). Besides being much studied in several animal species (Gorji, 2001; Guedes, 2005), CSD has also been recorded in the human brain, both in vitro (Gorji and Speckmann, 2004) and in vivo (Fabricius et al., 2008). During CSD, the neurons and probably glial cells suffer depolarization, causing brain electrical silence, which led some researchers to recently start using the term "spreading depolarization" as the causal event of spreading depression (Dreier, 2011). It is largely assumed that CSD is involved in the production of the symptoms of migraine aura (Lauritzen et al., 2011) and would also causally related to other neurological diseases like epilepsy and stroke (Lauritzen et al., 2011; Leão, 1944).

Nutritional, environmental, and pharmacological factors have been shown to influence brain CSD propagation in rats (Amâncio-dos-Santos et al., 2006; Amaral et al., 2009; Guedes, 2005; Tenório et al., 2009). Current discussions on the possible CSD mechanisms often mention the involvement of neurotransmitter activity (Gorelova et al., 1987; Guedes et al., 1987, 1992), and free radicals produced in the brain (El-Bachá et al., 1998; Guedes et al., 1996). Experimental evidence indicates that conditions that either weaken or strengthen the ability of the brain to counteract CSD may shed new light on the phenomenon and the diseases related to it. One of such conditions is EtOH ingestion: acute EtOH ingestion has impaired CSD propagation (Sonn and Mayevsky, 2001), whereas daily ingestion for 7 or more days facilitated it (Abadie-Guedes et al., 2008; Guedes and Frade, 1993). We have previously demonstrated that the antioxidant molecules known as carotenoids exert an antagonistic action against the effect of chronic EtOH on CSD in rats (Abadie-Guedes et al., 2008; Bezerra et al., 2005).

In this study, we hypothesized that CSD propagation would be influenced (i) by the age of animals; (ii) by a single dose of EtOH; and that (iii) the EtOH effect would be counteracted by a single astaxanthin administration.

MATERIALS AND METHODS

Astaxanthin (purchased from Sigma, St. Louis, MO) was dissolved in olive oil, to a final concentration of 10 μg/ml. Then, the astaxanthin solution was kept in the dark and in an N_2 atmosphere.

The animals (male Wistar rats) were handled in accordance with the norms of the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco, Brazil (where the experiments had been conducted), which complies with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, MD). They were reared in polypropylene cages (51 × 35.5 × 18.5 cm) in a room maintained at 21 ± 1°C with a 12 hour light/12 hour dark cycle (lights on at 7:00 AM). Five groups of Wistar young (60 to 80 days of age) and 5 groups of adult rats (150 to 180 days old) were given 2 gavages (a method consisting of administering substances directly into the stomach through a cannula inserted in the mouth) at a 1-hour interval, as follows: groups 1 and 2 received astaxanthin in gavage I combined with 3 g/kg EtOH (group 1) or water (group 2) in gavage II; groups 3 and 4 received olive oil (the vehicle in which astaxanthin was dissolved) in gavage I, combined with 3 g/kg EtOH (group 3) or water (group 4) in gavage II; finally, group 5 received water in gavage I combined with 3 g/kg EtOH in gavage II. Astaxanthin-treated rats received 10 μg/kg of this carotenoid per gavage, diluted in 95% EtOH, as previously described (Abadie-Guedes et al., 2008). This EtOH dose (3 g/kg) is within the range usually employed in experiments with 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).

All groups had free access to water and a commercial laboratory chow diet (containing 23 g protein per 100 g diet; Purina do Brazil Ltd, Paulínia, SP, Brazil).

Immediately after gavage II, the CSD recording session was carried out, as previously described (Bezerra et al., 2005). Briefly, under anesthesia with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose (Sigma; injected intraperitoneally), a tracheal cannula was inserted and 3 trephine holes were made on the right side of the skull, aligned along 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 2 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. At 20-minute intervals, CSD was elicited by application, for 1 minute, of a cotton ball (1 to 2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) placed on the frontal cortical surface through the hole drilled on that region. On the 2 parietal holes, both the slow DC-potential change and the reduction in the spontaneous cortical electrical activity accompanying CSD were continuously recorded for 4 hours using a pair of Ag–AgCl agar-Ringer electrodes (1 in each hole), as previously described (Abadie-Guedes et al., 2008). A common reference electrode, of the same type, was placed on the nasal bones (see insert of Fig. 1). The DC-potential changes were recorded by connecting the electrodes to GRASS DC-amplifiers (Astro-Med Industrial Park, West Warwick, RI), 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 (Astro-Med Industrial Park). In some experiments, DC-recording was also computer-digitalized and recorded. The CSD velocity of propagation was calculated from the time required for a CSD wave to cover the distance between the 2 cortical electrodes. In the measurement of CSD velocities, the initial point of each DC negative rising phase was used. A repeated measure analysis of variance (ANOVA) followed by a post hoc (Tukey–Kramer) test was used to compare body weights and CSD propagation rates between groups. Differences between groups were accepted as significant when $p \leq 0.05$. All values are presented as means ± standard deviations.

RESULTS

As previously observed (Bezerra et al., 2005), CSD was consistently elicited by 2% KCl applied for 1 minute on a cortical point in the frontal region. Once elicited, CSD propagated and was recorded by the 2 electrodes placed on the parietal cortex of the stimulated hemisphere. Examples of the electrophysiological recordings (electrocorticogram and slow potential changes) are shown in Fig. 1.

As described in Materials and Methods, in both young and adult ages, 2 groups of animals were treated with EtOH,

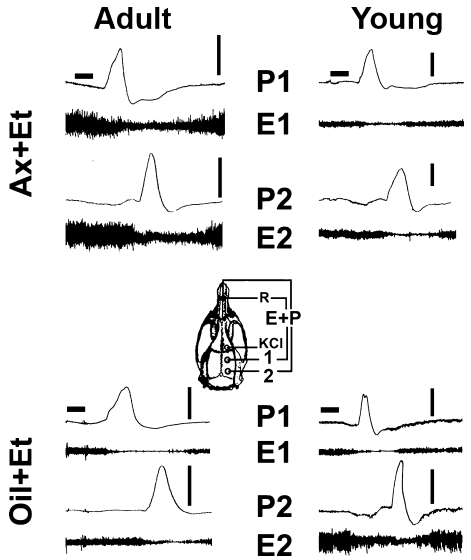

Fig. 1. Recordings of spontaneous cortical electrical activity (E; second and fourth traces in each panel) and slow potential change (P; first and third traces) during cortical spreading depression in two 150 to 180 day old and two 60 to 80 day old rats treated with 2 gavages at a 1-hour interval. The 2 animals of the upper row (marked with Ax + Et) were treated with 10 μg/kg astaxanthin dissolved in olive oil in gavage I and with 3 g/kg ethanol (EtOH) in gavage II. The 2 animals in the lower row (marked with oil + Et) were treated with olive oil in gavage I and with EtOH in gavage II. 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 over P1 indicate the time (1 minute) 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). Vertical calibration bars equal 1 mV for the electrocorticogram and 10 mV for the P-recordings (negative upward).

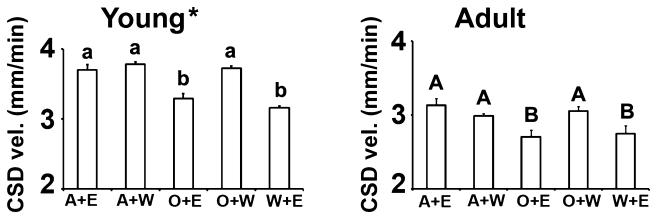

Fig. 2. Reduction in cortical spreading depression velocity in the cortical surface of young and adult rats treated with 10 μg/kg astaxanthin in gavage I combined with 3 g/kg ethanol (EtOH) (group A + E) or water (group A + W) in gavage II. These groups were compared with groups treated with olive oil (the vehicle in which astaxanthin was dissolved) in gavage I combined with 3 g/kg EtOH (group O + E) or water (group O + W) in gavage II; finally, group W + E received water in gavage I combined with 3 g/kg EtOH in gavage II. Data are expressed as mean ± SEM. Asterisk indicates that all young-values are higher than the corresponding adult-values. Different capital and lowercase letters represent significant difference in the means of the different treatments administered per gavage (ANOVA plus Tukey test; $p < 0.05$). CSD, cortical spreading depression.

one of which in combination with oil (group 3) and the other with water (group 5). EtOH acute treatment was associated with lower CSD propagation velocities (mean values in mm/min: 3.29 ± 0.23 and 3.16 ± 0.10 in the young groups; 2.71 ± 0.27 and 2.75 ± 0.31 in the adult groups), as compared to the respective water and oil controls (groups 2 and 4; CSD velocities: 3.73 ± 0.09 and 3.78 ± 0.07 in the young groups; 2.99 ± 0.10 and 3.05 ± 0.19 in the adult groups). This impairing effect of EtOH on CSD was counteracted by astaxanthin (group 1 mean velocity: 3.70 ± 0.19 and 3.13 ± 0.16 for the young and adult groups, respectively). Treatment with astaxanthin alone (10 μg/kg without EtOH; group 2) did not modify the CSD propagation velocity. All data are presented in Fig. 2.

DISCUSSION

Three main findings were demonstrated in this work. First, a single dose of EtOH impairs the propagation of CSD in the rat cortex. Second, a previous single administration of astaxanthin reverses this neurophysiologic effect of acute EtOH. Third, within the age-range of this study (60 to 180 days), the interaction between the EtOH and astaxanthin effects on CSD did not depend on the animal's age. Furthermore, CSD propagation has previously described as being age-dependent (Guedes et al., 1996); this finding has been presently confirmed. Although a different methodology was employed in this study, our data on CSD deceleration associated to acute EtOH treatment are generally in agreement with those of Sonn and Mayevsky (2001). The major methodological discrepancy between these studies lies in the fact that those authors administered EtOH by intravenous continuous infusion, whereas in our experiments, EtOH was administered by gavage (see Materials and Methods).

It is important to mention the contrasting effects produced by acute as compared to chronic EtOH administration. Guedes and Frade (1993) treated adult rats with EtOH for 7 days and found an increase in CSD propagation velocity. In line with that Bezerra and colleagues (2005) and Abadie-Guedes and colleagues (2008) also found a facilitation of CSD propagation after treating rats with EtOH for 18 days. Taken together, these data suggest an important difference between acute and chronic EtOH actions on CSD.

CSD can be used as a physiological index that is valuable for understanding the relationship between oxidative stress and neural function. The resistance offered by the brain tissue to CSD propagation can be augmented or diminished by experimental conditions, resulting in lower or higher CSD velocities of propagation, respectively (Merkler et al., 2009; Rocha-de-Melo et al., 2006). Experimental increasing or decreasing the brain ability to counteract CSD may help in understanding the phenomenon and the diseases related to it.

Several clinically relevant conditions have been investigated, concerning EtOH effects on CSD. These conditions include nutritional, metabolic, environmental, hormonal, and pharmacological factors. Some of these conditions facilitate CSD, as for example malnutrition (Rocha-de-Melo et al., 2006), hypoglycemia (Costa-Cruz et al., 2006), and γ-aminobutyric acid (GABA)-enhancing drugs (Guedes et al., 1992), while others like serotoninergic drugs (Amâncio-dos-Santos et al., 2006), hypernutrition (Rocha-de-Melo

et al., 2006), aging (Guedes et al., 1996), and peripheral afferent stimulation (Monte-Silva et al., 2007) impair CSD.

In humans, alcohol consumption is characterized by single episodes of EtOH intake, in the beginning. Later on, alcohol consumption becomes a habit, with drinking events repeated at short intervals. Most of the clinical and experimental investigations focus on this late chronic phase, and little attention has been given to the acute intake period. In a recent clinical study, Conte and colleagues (2008) described distinct electrophysiological alterations in patients diagnosed as chronic EtOH addicts, as compared with healthy volunteers who drank a single EtOH dose. In line with these findings, our results in rats demonstrate CSD propagation alterations even after a single dose intake, stressing the importance of further investigating this point.

It is known that ROS can be formed as a consequence of EtOH metabolism (Tapiero et al., 2004), and the presence of antioxidants may help in protecting tissues such as gastric mucosa (Kim et al., 2005) and brain (Mitchell et al., 1999) from the deleterious effects of EtOH. The use of the CSD phenomenon as a neurophysiologic model allowed us to demonstrate a consistency between our data and those previously described in the literature, regarding the protective effect of astaxanthin against EtOH action on the brain tissue (Abadie-Guedes et al., 2008; Bezerra et al., 2005). In addition, the data obtained herein raise the issue of the effective impact of a single EtOH dose on brain electrophysiological phenomena. It was also here observed that 1 astaxanthin dose alone was able to counteract EtOH action on CSD.

Although our findings can be explained by different mechanisms, we hypothesize that the most likely mechanism is based on the chemical properties of astaxanthin, which make this molecule a very effective antioxidant. This molecule is composed by 2 carbonyl and 2 hydroxyl groups, and 11 conjugated ethylenic double bonds, forming a polyene system that is probably responsible for its potent antioxidant power, acting by donating the electrons and reacting against free radicals. Generally, the final products will have a more stable structure (Briton, 1995; Tapiero et al., 2004). We suggest that ROS scavenging would be a possible mechanism whereby astaxanthin could antagonize the EtOH effects on CSD. However, the measurement of blood and brain ROS levels, which has not been carried out in the present work, would be required to prove our hypothesis. Notwithstanding that, we think that this hypothesis is very plausible, mainly because ROS production in the nervous tissue has been shown to elicit CSD in the isolated chicken retina (Netto and Martins-Ferreira, 1989) and in the rat cortex (El-Bachá et al., 1998). In addition, CSD susceptibility has been proven to be increased in rats with diets deficient in the antioxidant vitamins C and E (El-Bachá et al., 1998; Guedes et al., 1996). Furthermore, brain structural effects of EtOH in laboratory animals are counteracted by antioxidant molecules like α-tocopherol in rats (Marino et al., 2004) and ascorbic acid in frogs (Peng et al., 2005). Recently, a neuroprotective effect of astaxanthin against oxidative stress by cerebral ischemia has been reported in rats. The authors have suggested that such effect is also related to the antioxidant activities of astaxanthin (Lee et al., 2010). These pieces of evidence reinforce our hypothesis in favor of an antioxidant-dependent mechanism for the brain protective effect of astaxanthin.

Another possible mechanism of EtOH action on CSD would involve the GABA system. Several actions of EtOH have been attributed to occur via the GABA system (Kozan et al., 2007; Kumar et al., 2003; Sonn and Mayevsky, 2001). Interestingly, administration of GABAergic compounds modulates CSD (Guedes et al., 1992).

In conclusion, the demonstration of EtOH effects on the CSD model, and the interaction of EtOH with antioxidants like astaxanthin resemble findings observed in a number of clinical disorders (Lauritzen et al., 2011). The appropriate extrapolation of our experimental model to the humans certainly requires caution and presents some limitations. However, our previous pieces of evidence (Abadie-Guedes et al., 2008; Bezerra et al., 2005) and the present study highlight the possibility of testing antioxidant molecules like astaxanthin in clinical trials in the development of more effective strategies for treating such human diseases, like alcoholism.

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CAPÍTULO II

Special issue article

The use of cortical spreading depression for studying the brain actions of antioxidants

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Objectives: We review the main adverse effects of reactive oxygen species (ROS) in the mammalian organism, introducing the reader on the worldwide problem of the ROS neurophysiological impact on the developing and the adult brain, and discussing the neuroprotective action of antioxidant molecules.
Methods: We briefly present the electrophysiological phenomenon designated as 'cortical spreading depression' (CSD), as a parameter of normal brain functioning. We highlight recent electrophysiological advances obtained in experimental studies from our laboratory and from others, showing how to investigate the ROS effects on the brain by using the CSD phenomenon.
Results: Under conditions such as aging, ROS production by photo-activation of dye molecules and ethanol consumption, we describe the effects, on CSD, of treating animals with (1) antioxidants and (2) with antioxidant-deficient diets.
Discussion: The current understanding of how ROS affect brain electrophysiological activity and the possible interaction between these ROS effects and those effects of altered nutritional status of the organism are discussed.

Keywords: EEG, Cortical spreading depression, Carotenoids, Reactive oxygen species, Brain development, Malnutrition

Introduction: the redox scenario in the central nervous system

In the mammalian organism, the production of free radicals such as the reactive oxygen species (ROS) is considered an important event occurring with variable intensity at distinct phases of life, from the intrauterine period until late aging. The excessive production of ROS is certainly deleterious for the organism, but under physiological conditions this event can efficiently be maintained under control by mechanisms involving antioxidant molecules. However, several external factors can modify this physiological control, either increasing or decreasing the efficiency of the mechanisms that protects the organism against the harmful effects of ROS.[1]

Concerning the ROS action on the central nervous system, the increased incidence of neurodegenerative diseases in developing countries has been associated to several factors, including neural damage due to oxidative stress.[2] This can be intensely produced in conditions of oxidant load of the organism, as in parenteral nutrition,[3] or in cases of hypo-functioning of neural antioxidant mechanisms, when dietary antioxidant supplementation can be beneficial.[4] For example, increased rates of cerebral lipid peroxidation can lead to the excessive production of ROS which, if not neutralized by antioxidant mechanisms, are highly deleterious to the brain tissue. This physiological ROS neutralization can fail in the presence of a number of factors, like nutritional imbalance,[5] aging process,[6] and the consumption of ethanol.[7] These factors can disrupt the normal brain redox scenario, leading to a situation of increased oxidative stress in which the brain becomes susceptible to the development of certain neurological dysfunctions, like those occurring in Alzheimer's disease,[8] diabetes,[1] and epilepsy.[9]

In the last two decades, a considerable scientific effort has been produced to clarify whether antioxidant supplementation can help in preventing these various dysfunctions. The implication is that understanding the specific molecular effects of ROS on brain functioning is of great importance in determining the role of antioxidants on these diseases and may certainly help in shedding light on the epidemiological findings, which sometimes point to opposite neuropsychological effects of antioxidants.

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The action of ROS in the developing brain

As in other organs, the biochemical and morphological organization of the brain can be disrupted by the oxidative stress subsequent to the imbalance between the increase in the ROS production and the decreased effectiveness of antioxidant systems. When this occurs early in life, during the so-called 'brain growth spurt' period,[10] the deleterious consequences for the brain functioning can be particularly severe,[1] as compared with the adult brain. The brain growth spurt period corresponds to the highest speed of developmental processes like neurogenesis, gliogenesis, and cell migration, in the neural tissue.[11] Under such conditions, dietary antioxidants can avoid the increase of intracellular ROS concentration, thus preventing cell damage.[12] Basic processes such as neuronal excitability and learning- and memory-dependent functions may be affected to some extent by oxidative stress, and this can contribute to the appearance of important disabling neuropathological disorders. These in many cases are permanent, and can affect an impressive number of children in several parts of the world.[1,8,9] This certainly has prompted several research groups to investigate the neural protective effects of antioxidant molecules, mainly those from nutraceutical- and dietary sources, both in laboratory animals and in humans, using *in vivo* and *in vitro* models.

We will next present data from our laboratory documenting the effects of ROS on the brain electrophysiological activity in both developing and adult animals.

Antioxidants and brain electrophysiology

The distinct cell populations of the normal brain continuously generate electrical activity, as long as the organism is alive. All brain-dependent regulatory functions in the organism depend directly or indirectly on this electrical activity. In humans, as well as in laboratory animals, an interesting way of getting important information on brain functioning consists in recording the electrical activity produced spontaneously by the brain. This technique is denominated electroencephalogram (EEG), and can be defined as the measurement of the temporal oscillations of electrical potential between two points of the brain. Because EEG is a non-invasive technique (a number of electrodes are fixed on the scalp and the potential oscillations are recorded pair wise), it is widely used in humans, aiding in the diagnosis of several neurological disorders.[13] In experimental animals, the recording can be performed in an invasive manner, under anesthesia, in which the skull is surgically opened and the recording electrodes are placed on the cortical surface and even inside the cortex; in this case, it is denominated 'electrocorticogram' (ECoG).

Under oxidative stress, as for example in ischemia/reperfusion injury, the brain electrical activity may be more or less affected.[14] In animals under oxidative stress, several reports document altered susceptibility to disorders related to brain excitability, such as epilepsy,[9,15,16] and neurodegenerative diseases.[12,17] Collectively, the physiopathological implications of such pieces of evidence for the human health led us to investigate to what extent the redox disorders could alter the electrical activity of the brain. For that purpose we have used, as an experimental model, the cortical spreading depression (CSD) phenomenon (see[13,18] for a review).

CSD as a tool for studying brain antioxidant effects

CSD was originally described in the 1940s by the Brazilian scientist Aristides Leão, at that time a young PhD student working at Harvard University, USA. The phenomenon was then described as being a reversible and propagated 'wave-like' electrophysiological response of the cerebral cortex subsequent to its punctual stimulation. The response consists of a reduction (depression) of the spontaneous and evoked electrical activity of the cerebral cortex.[19] During CSD there is a depolarization of neurons and probably also of glial cells, and this led some researchers recently to start using the term 'spreading depolarization' as a synonym for spreading depression.[20] Simultaneously to the neuronal depolarization, a reversible dilation of the pial blood vessels,[21] and a slow potential change (also called DC potential change) of the tissue have been also described by Leão.[22] In contrast to what some may think, the term 'depression', in this context, is not directly related with the psychiatric disease, named also by that term. Rather, it has an electrophysiological meaning, signifying that the amplitude of the ECoG activity at the depressed region tends to zero. Once elicited, CSD concentrically propagates to remote cortical regions, while the initially depressed point starts to recover. The complete recovery of the tissue usually requires about 5–10 minutes, as evaluated by the restoration of the pre-depression ECoG pattern and DC level of the slow potential change. Because this DC variation has an 'all-or-none' feature, it is very suitable for estimating the CSD velocity of propagation. This velocity usually ranges from 2 to 5 mm/minute in all vertebrate species so far studied, from fishes to mammals including the human species, and is considered very low when compared with the much higher conduction velocity of neuronal action potentials, which in mammals is in the order of m/second. The very low CSD velocity is compatible with a mechanism of propagation of humoral nature, in which the release of one or more chemical compounds from

the depolarized neural cells would diffuse through the extra-cellular space, and would 'contaminate' the neighbor cells, which then become electrophysiologically depressed.[23] Once depressed, these neighbor cells would release the CSD-eliciting chemical factors; the neurotransmitter glutamate and the ion potassium, among others, are the most frequently postulated candidates.[20] The released chemical factors would then contaminate other cells, and so on, giving rise to a positive feedback loop that is very effective in maintaining the auto-regenerative propagation of CSD.

We believe that the CSD phenomenon is an interesting electrophysiological model that is valuable for understanding the relationship between oxidative stress, nutrition, and neural development and function. Experimental evidence indicates that the resistance offered by the brain tissue to CSD propagation can be increased or decreased by experimental manipulations, resulting in lower or higher CSD velocities of propagation, respectively.[24,25] Experimental procedures that either augment or diminish the brain ability to counteract CSD may help in understanding the phenomenon and the diseases related to it. Therefore, CSD can be considered as a real electrophysiological tool, which can aid in understanding the normal and pathologic brain.[26]

In previous studies, we have characterized alterations in brain CSD susceptibility in animals submitted to environmental, pharmacological, and nutritional manipulations.[27–32] One of the conditions studied was the manipulation of antioxidants, which will be reviewed and discussed in the following sections.

CSD, aging, and dietary antioxidants

During the mammalian aging process, the production of ROS is an important event, and the oxidation of DNA, proteins, and lipids by ROS, when accumulated in the aged brain, can functionally affect it.[33] This includes functions that are dependent on the brain excitability.[34] With that in mind, we investigated in rodents (albino rats and gerbils) the influence of aging on brain susceptibility to CSD (for details, see[35]).

In albino rats and Mongolian gerbils (*Meriones unguiculatus*) with ages ranging from 2.5 to 24 months (rats) and from 1.5 to 58 months (gerbils), under anesthesia with urethane + cloralose mixture (1000 + 40 mg/kg, respectively, intraperitoneally), CSD was recorded during 2 hours on two points of the parietal cortex. For each animal, the mean CSD velocity of propagation was calculated based on the time spent for the CSD-wave to cross the inter-electrode distance. We plotted the CSD velocities as a function of ages and the correlation coefficients between the two variables, as well as the equations for the computed regression lines were calculated. In both rodent species, we found a significant ($P < 0.05$) negative correlation between CSD velocities and ages (Fig. 1). The lower CSD velocity found in aged animals is apparently contrasting with the tendency of the aged brain in presenting higher levels of lipid peroxidation, which lead to the formation of ROS[36] and would accelerate CSD. However, in the rat brain the levels of the antioxidant molecule ascorbic acid (AA) are preserved in the aged cerebral cortex,[37] and this could help explaining the lower CSD velocity in the aged rat. Other brain changes related to aging such as alteration of the intra- and extracellular brain volumes[38] may also help in explaining this effect.

In view of the above-commented evidence of increased ROS production during aging, and also because ROS can elicit retinal spreading depression,[39] we decided to investigate the CSD propagation in

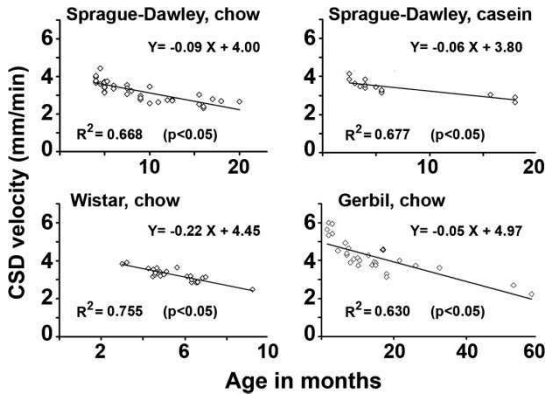

Figure 1 Mean CSD velocities of propagation as a function of the age of rats (Wistar and Sprague-Dawley strains) and gerbils (*Meriones unguiculatus*). Each point in the graphics corresponds to the mean velocity for one animal. The equations for the computed regression lines, as well as the corresponding correlation coefficients are given in each graph. In all cases there is a significant negative correlation between CSD velocities and age. Adapted from published data of our lab.[35]

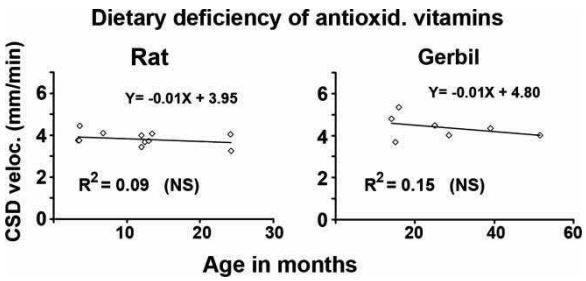

Figure 2 CSD velocities of propagation in rats and gerbils of different ages, as described in Fig. 1, showing absence of significant correlation between the two variables, after the animals had been submitted to dietary deficiency of the antioxidant vitamins C and E for 4–6 weeks. Adapted from published data of our lab.[35]

animals deprived of antioxidant vitamins in their diet. Under this condition, the significant negative correlation between CSD velocity and animal's age disappeared (Fig. 2), suggesting the involvement of brain ROS in the aging effect on CSD.

CSD, photo-activation-induced ROS, and dietary antioxidants

It has been demonstrated in the isolated retina that CSD can be elicited when a *bolus* of the photo-sensible dye Rose Bengal, which had previously been applied to the preparation in darkness, is illuminated.[39] The photo-activation of this dye is known to produce ROS (singlet oxygen[40]). Thus, we decided to test if illumination of another light-sensible molecule, the yellow-colored neutral dye riboflavin, which has biological importance as vitamin B2, could elicit CSD in the rat cortex *in vivo*. Riboflavin is capable of reducing oxygen to the ROS superoxide when activated by photons.[41]

We studied two groups of adult Wistar rats: one control ($n = 9$), fed a normal chow diet, and one deficient group ($n = 15$), which received during 4–6 weeks previously to the experiment a diet free from the antioxidant vitamins C and E.[42] In urethane + cloralose anesthetized rats, a 1–2 hours 'baseline' CSD recording was performed, when CSD was elicited at 20-minute intervals by conventional stimulation with 2% KCl applied for 1 minute to a point of the frontal cortical surface. After this baseline period, tests were performed with 1.0 mM riboflavin applied to the same frontal region and illuminated for 1–3 minutes by a 40 W white light bulb, placed 10–15 cm over the cortical surface. As can be seen in Table 1, in the control group, this procedure elicited CSD in 29.7% of the cases (11 out of 37 applications, with CSD appearing in 7 out of the 9 rats of this group). In the antioxidant-deficient animals, the effectiveness of photo-activated riboflavin to elicit SD increased significantly to 62.8% (44 out of 70 riboflavin applications; 15 out of 15 rats; $P < 0.05$). Neither illumination of an equivalent volume of Ringer solution applied to the same region nor the application of riboflavin without illumination did elicit CSD (Fig. 3).

The results demonstrate[42] that photo-activated riboflavin is capable of eliciting CSD in the rat cerebral cortex and that dietary deficiency of the antioxidant vitamins C and E can enhance brain susceptibility to this process, suggesting that the increased levels of free radicals in the brain, which probably occur under dietary antioxidant deficiency, might be involved in this effect.

CSD, ethanol-induced ROS, and shrimp antioxidants

The enhanced formation of ROS in the brain can occur because of excessive alcohol ingestion, which produces lipid peroxidation. This increased ROS production is potentially injurious to the brain tissue[36] and the ROS effects are usually counteracted by mechanisms involving antioxidant substances, like carotenoids, which can be found in several foods from vegetal and animal origin. An interesting animal source of carotenoids is represented by the crustaceans like shrimps, where carotenoids are present as a carotenoprotein in some associations in legs parts, blood, eyes, eggs, hepatopancreas, ovary, and the carapace.[43]

Table 1 Number of applications of riboflavin and percentage of SD elicitation in control and vitamin-deficient rats

	Control group			Deficient group		
Rat no.	A = applications	B = elicitations	B = (as % of A)	A = applications	B = elicitations	B = (as % of A)
1	3	0	0.0	6	1	16.7
2	5	0	0.0	3	1	33.3
3	6	1	16.7	5	2	40.0
4	7	2	28.6	6	3	50.0
5	3	1	33.3	4	2	50.0
6	3	1	33.3	5	3	60.0
7	4	2	50.0	5	3	60.0
8	3	2	66.7	6	4	66.7
9	3	2	66.7	3	2	66.7
10				3	2	66.7
11				6	4	66.7
12				6	5	83.3
13				3	3	100.0
14				4	4	100.0
15				5	5	100.0
Total	37	11		70	44	
Average	4.1	1.2		4.7	2.9	
Mean			32.8			64.0
±SD			±25.1			±24.6

The groups are significantly different, either by using the Rank sum test ($P < 0.05$) or Student's *t*-test ($P < 0.01$). Data from our previous paper.[42]

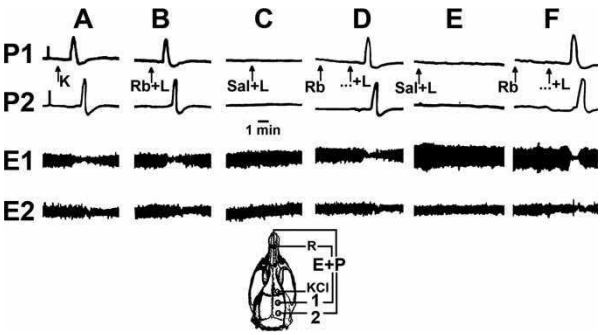

Figure 3 ECoG and DC recordings in the parietal cortex of a rat, showing CSD elicitation by the classical KCl stimulation (A), and also by photo-activation of riboflavin (Rb), seen in (B). Illumination of an equivalent volume of Ringer solution did not elicit CSD (C and E). The same negative result was observed when riboflavin was applied without illumination (first arrow in D and F). CSD appeared 3–4 minutes later when riboflavin was illuminated (second arrow in D and F). Vertical bars at P1 and P2 in A correspond to -°©–10 mV. Modified from a figure of our previous publication.[42]

We had initially treated adult rats, with one daily ethanol application per gavage during 7 days,[44] and demonstrated a facilitating effect on CSD propagation, as compared with water-treated controls (Fig. 4). More recently, we tested whether a shrimp carotenoid extract (obtained from shrimp heads) could have a protecting effect on the CSD actions of ethanol. A carotenoid ethanolic extract (30 μg/kg/day) prepared from shrimp heads was given per gavage to pregnant or lactating rat dams, and we analyzed CSD in their pups, which were compared with age-mated ones from mothers treated either with the vehicle (ethanol) or with distilled water. In line with the initial experiments,[44] ethanol treatment was associated with higher ($P < 0.05$) CSD velocities as compared with the distilled water group. Carotenoid

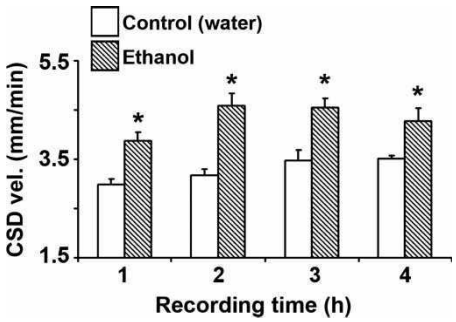

Figure 4 Effect of ethanol treatment on CSD propagation velocities in 80–100-day-old adult rats ($n = 10$). Ethanol was administered daily per gavage during 7 days. For comparison, a control group ($n = 8$) received distilled water by the same gavage procedure. One hour after the last gavage, a 4-hour-CSD recording session was initiated. Data are expressed as mean ± standard deviation. Asterisks indicate that the ethanol-treated group presented significantly higher CSD propagation velocities, as compared with the control group ($P < 0.05$; *t*-test). (Data from our previous publication.[44])

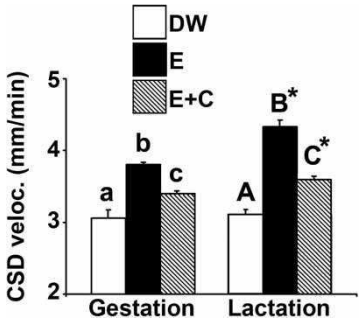

Figure 5 Mean CSD velocities in the cerebral cortex of just-weaned rat pups (36–40 days of life) whose mothers had been treated with 30 μg/kg/day of an ethanolic extract of shrimp carotenoids (E + C), either during the gestation or during the lactation period, as compared to corresponding controls treated with vehicle – ethanol (E; 3 g/kg) or distilled water (DW). Data are expressed as mean ± standard deviation. Asterisks represents higher values ($P < 0.05$) comparing groups treated during gestation and lactation. Different capital and lowercase letters represent significant difference ($P < 0.05$) in the means of the different treatments administered per gavage (analysis of variance (ANOVA) plus Tukey's test; $P < 0.05$). Modified from a figure of our previous publication.[45]

treatment resulted in lower CSD velocities ($P < 0.05$), when compared to the vehicle (ethanol) group, and this effect was more intense in the groups treated during lactation, as compared with the groups treated in the gestation period.[45] The results are shown in Fig. 5, and suggest a protective action of shrimp carotenoids against the ethanol effects on spreading depression.

Because astaxanthin is the most abundant carotenoid present in the shrimp, we proposed that the CSD effects of the shrimp extract were caused by astaxanthin. To test this proposition, we analyzed CSD in adult rats (60–70 days of life) treated per gavage for 18 days with pure astaxanthin (from Sigma Co., St Louis, MO, USA) in doses of 2.5, 10, or 90 μg/kg/day, diluted in 95% ethanol (corresponding to a dose of 3 g/kg/day of ethanol), which is within the range of ethanol dose usually employed in rodents.[46,47] Three control groups were treated, respectively, with the vehicle (ethanol group), or with distilled water, or with soybean oil (used as alternative vehicle to dissolve astaxanthin, which is not hydrosoluble). In an additional group, the influence of astaxanthin without ethanol was evaluated by treating the animals with 10 μg/kg/day astaxanthin dissolved in soybean oil instead of ethanol. From the weaning until the day of the electrophysiological recording (79–89 days), all animals had free access to water and a commercial lab chow diet (Purina do Brazil Ltd, São Lourenço da Mata, PE, Brazil containing 23 g protein/100 g diet). Fig. 6 represents the average CSD velocities calculated for the whole 4-hour period. Compared with the ethanol group, the CSD

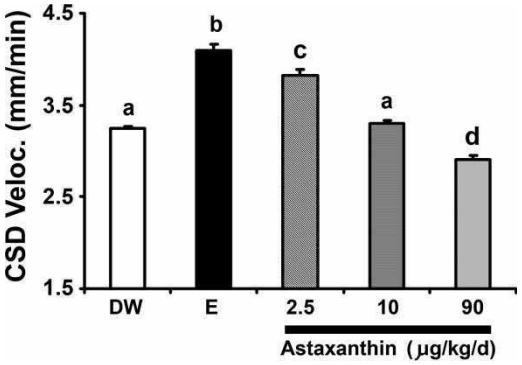

Figure 6 Dose–response relationship of the antagonizing astaxanthin effect on CSD propagation in adult rats (90 days of life) treated per gavage with astaxanthin at doses of 2.5, 10, and 90 μg/kg/day compared to rats treated with vehicle- (ethanol, E; 3 g/kg) and controls treated with distilled water (DW). Data are expressed as means ± standard deviation, calculated in each hour of recording. Different lowercase letters represent significant difference ($P < 0.05$) in the means of the different treatments administered per gavage (ANOVA plus Tukey's test; $P < 0.05$). Modified from a figure of our previous publication.[48]

velocities in the astaxanthin groups decreased as a function of astaxanthin dose, following an exponential decay model, which is represented by the equation $y = 2.9048 + 1.1988e^{(-x/9.0217)}$; ($r^2 = 0.9998$), in which the y-variable is the CSD velocity of propagation, and the x-variable is astaxanthin dose in μg/kg/day.[48] The average CSD velocities for the soybean oil and 10 μg/kg/day astaxanthin + soybean oil groups (not shown in the figure) were not statistically different from the water-treated control group. We conclude that the effectiveness of astaxanthin in antagonizing the CSD effects of ethanol supports our suggestion that the similar effect in the rats previously treated with the carotenoid extract is probably due to the high proportion of astaxanthin present in that extract.[48]

Interaction antioxidant/nutrition on CSD features

When occurring in the initial phase of mammal's life, a deficient nutrition can alter negatively, and sometimes permanently, the anatomical, biochemical, and physiological organization of the brain. This initial period is the phase in which proliferation, growth, migration, and myelination of the brain cells are occurring with the highest rate.[11,18] In the rat, this period corresponds to the first 3 weeks of post-natal life, i.e. the suckling period.[49]

It had been demonstrated that early malnutrition enhances CSD propagation,[25,50] but no information was available regarding a possible nutrition/antioxidants interaction on CSD. Recently,[51] we addressed this issue by studying the CSD features in the cerebral cortex of well-nourished and malnourished developing rats treated chronically (21 days) with another antioxidant, AA. This molecule (also denominated vitamin C) is present in the brain and can exert biphasic modulating effects (i.e. it can exert antioxidant or prooxidant action) in distinct models of excitability-related brain disorders.[52,53] We addressed the following two questions in the brain of weaned young rats, subjected to malnutrition during lactation followed by nutritional recovery: (i) How does daily enteral administration of AA during the brain development affect CSD propagation, and (ii) if so, how this effect would be influenced by the previous brain nutritional condition. Wistar suckling rats were malnourished during the entire lactation period by feeding their lactating dams a deficient diet (with only 8% of protein), and were compared to well-nourished controls (maternal diet with 23% protein). From post-natal days 7–28, part of each nutritional group was treated with 60 mg/kg/day of L-AA. CSD propagation was analyzed when the pups were 30–40 days old. In line with previous findings (see[18] for an overview), malnutrition was associated with higher ($P < 0.05$) CSD velocities of propagation and DC-amplitudes, as compared to the well-nourished controls. AA treatment resulted in an increased CSD amplitudes and propagation velocities ($P < 0.05$), as compared to non-treated ('naïve'; Nv) and saline-treated (Sal) controls, in both well-nourished and malnourished conditions. Data are presented in Fig. 7. We conclude that the chronic treatment of developing rats with AA at the dose of 60 mg/kg/day facilitated CSD

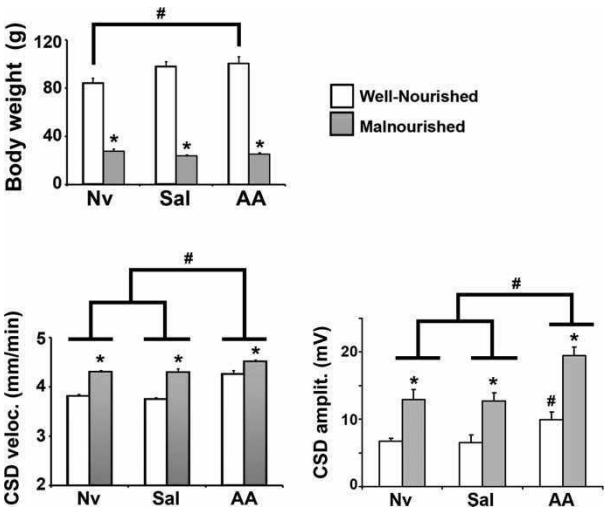

Figure 7 Body weight, and CSD velocity and amplitude of well-nourished and malnourished 30–40-day-old rats treated from the 7th to the 28th post-natal days with 60 mg/kg/day of ascorbic acid (AA group) or saline (Sal group) or no treatment (naïve – NV group). Asterisks indicate significant difference ($P < 0.05$) between well-nourished and the corresponding malnourished groups. The symbol # shows significant difference between the AA group and the indicated controls (ANOVA plus Tukey's test). Modified from a figure of our previous publication.[51]

propagation, and this effect is not significantly modulated by malnutrition. Considering that other antioxidants have antagonized CSD,[42,48] and considering also that AA can eventually have prooxidant action, and thus can cause neurotoxicity,[3,52,54] we suggest that AA at the dose of 60 mg/kg/day acts as prooxidant on the CSD model, as indicated by the larger amplitudes of the CSD DC-shifts and the faster CSD propagation, observed in the AA-treated rats.[51] In fact, this AA dose of 60 mg/kg/day has been previously reported by others as being prooxidant in the rat.[52] All the evidence notwithstanding, we think that future experiments for measuring blood and brain ROS levels, as well as studying developing rats treated with lower AA doses, or studying developed animals treated at adulthood, shall confirm this hypothesis.

Relations to neurological disorders, hypotheses, and concluding remarks

The involvement of neuronal and glial oxidative damage in several neurological disorders has been postulated by various authors based on a number of clinical and experimental pieces of evidence. For example, oxidative stress in the mitochondria has been implicated in the neuronal injury induced by seizures, possibly contributing to the pathogenesis of temporal lobe epilepsy,[16] and hippocampal oxidative stress has been postulated as causally involved in age-related impairment in learning and memory.[55] In addition, deficient redox regulation during development has been speculated to play a role in schizophrenia.[12] The same is true concerning the role of ROS in Alzheimer's disease.[56] On the other hand, dietary treatment with antioxidants, and environmental enrichment have been shown to additively protect and even enhance cognitive function in aged humans,[57] and this has been also demonstrated in animal models.[58] A variety of foods usually present in the human diet, like nuts, vegetables, fruits, cereals, tea, wine, as well as some fishes and crustaceans, are natural sources of antioxidant compounds. As previously stressed in the Introduction, a diet that is rich in such foods may aid in protecting the organism against neurodegenerative diseases that depends on increased oxidative damage; also, it may decrease pro-inflammatory cytokines in the brain by reducing oxidative damage.[59] This implies that the systematic and long-lasting consumption of adequate amounts of the antioxidant-rich foods should be recommended as an effective way to prevent, or at least minimize, those neurological diseases related to increased oxidative stress during the aging process. At an epidemiological level however, it is doubtful whether many human beings would long lastingly adhere to this nutritional intervention that sometimes would represent a radical change in their alimentary style.[6]

The analysis of CSD features under conditions of dietary antioxidant deficiency as well as under increased redox imbalance constitutes a promising experimental approach for the electrophysiological investigation of the brain effects of nutritional and non-nutritional conditions of great neuropathological relevance that can increase the energy demands in the brain, and are associated to important human diseases, such as brain ischemia, epilepsy, and migraine, which are also influenced by disorders of the brain redox homeostasis. Regarding the involvement of CSD in the migraine, it is important mentioning data from others[60] that suggest a beneficial effect of antioxidants on the frequency or severity of migraine. Recently, an increase in the activity of the enzyme superoxide dismutase has been reported in the rat cortex 1 hour after CSD.[61] Such pieces of evidence highlight the clinical importance of the present data, as CSD has been postulated as being involved in the pathophysiology of brain stroke, epilepsy, and migraine. A number of possible mechanisms for the reported effects has been proposed, including the induction of neuroprotective genes,[61] and the involvement of nitric oxide.[62]

Certainly, the appropriate data extrapolation between species (e.g. from rat to humans) is not a simple task, requiring great caution and presenting some limitations. Notwithstanding such limitations, from the converging body of evidence here presented it turns out that the development of experimental models devoted to investigating this theme is highly desirable, and this is particularly true in the case of the electrophysiological models. The comprehension of the mechanisms by which such conditions affect CSD propagation may be helpful in the development of more effective strategies for treating those diseases.

Acknowledgements

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CAPÍTULO III

Title: Antagonizing effect of α-tocopherol on the ethanol action in spreading depression in the rat cortex

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Abstract

Background: We have previously demonstrated in rats that acute and chronic treatment with ethanol respectively decelerated and accelerated the propagation of cortical spreading depression (CSD), and the antioxidant carotenoid astaxanthin antagonized such effects (Abadie-Guedes et al, 2008; 2012). However, it remained to clarify whether other non-carotenoid antioxidants would have the same effect. Here we investigated this possibility by testing the alpha-tocopherol.

Methods: Male Wistar adult rats of two age-groups (juvenile [J]; 60-80 days, and adult [A]; 150-170 days; n=8-10 for each group) received per gavage acute (1 day) or chronic treatment (21 days) with 3g/kg/d ethanol combined with 300 ug/kg (acute treatment), or 85ug/kg/d (chronic treatment) of tocopherol, or only vehicle (olive oil for tocopherol and water for ethanol). Under urethane+chloralose anesthesia, the CSD was recorded in two points of the cortical surface during 4h and the CSD velocity of propagation was calculated, based on the time spent for CSD to pass the interelectrode distance.

Results: In both age-groups, acute and chronic ethanol respectively decelerated and accelerated CSD, as compared to the corresponding control (vehicle) groups. Addition of tocopherol to the ethanol treatments antagonized the CSD effects of ethanol, bringing back the CSD velocities to the levels of the vehicle groups ($P<0.05$; ANOVA plus Tukey test).

Conclusion: Data reinforce the antagonistic role of antioxidants on brain processes involved in CSD, and also suggest that the action of antioxidants on CSD is not a particular property of carotenoids, but rather a general feature of antioxidant molecules.

Introduction

In young and adult humans, the consumption of ethanol (EtOH) is a current worldwide health problem with serious social and physiological implications. This drug is generally of easy access to a large contingent of persons. Also, its amphipathic features enables a prompt action on several organs, like liver, kidney, heart, pancreas, and brain (Lieber, 1995). The production of reactive oxygen species (ROS) by EtOH metabolism results in toxic effects on the brain, mainly because the fact that this drug can easily permeate the brain tissue (Tapiero et al., 2004). Therefore, the investigation of such effects in neurophysiological models can shed light on the mechanisms underlying the brain EtOH actions.

In previous studies, the phenomenon of cortical spreading depression (CSD) has been used in order to investigate the neural protective action of the carotenoids, a particular class of antioxidant molecules, against chronic and acute EtOH effects (Bezerra et al, 2005; Abadie-Guedes et al, 2008; 2012). Initially, this protective effect was observed in the developing brain of rats treated with a shrimp carotenoid extract (Bezerra et al, 2005). As the most abundant carotenoid in shrimps is astaxanthin, we tested whether the protective effect of the shrimp carotenoid extract would be due to astaxanthin. The results confirmed that astaxanthin similarly antagonized the facilitation of CSD induced by *chronic* treatment with ethanol, and this astaxanthin effect was dose-dependent (Abadie-Guedes et al, 2008). We have further investigated the effects of astaxanthin on the *acute* action of ethanol on CSD. In contrast to the chronic treatment, acute ethanol (single dose) decelerated CSD propagation, and this effect was also antagonized by astaxanthin (Abadie-Guedes et al, 2012).

In fact, several studies have reported the antagonizing ethanol effect of different antioxidants (Hancock and Miller, 2006; Tiwari and Chopra, 2011; Ibraim et al, 2012),

and this includes tocopherols (Heaton et al, 2011; Tiwari et al, 2009; Kozan et al, 2007). This molecule plays an important role in modifying the ethanol-induced oxidative stress, acting as proton donator to scavenging reactive oxygen species.

In this study, we investigated the hypothesis that non-carotenoid antioxidant molecules could also antagonize the ethanol effect on CSD, by testing the α-tocopherol, reinforcing the role of antioxidants on brain processes involved in CSD.

Methods

The animals (male Wistar rats) were handled in accordance with the norms of the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco, Brazil, which complies with the "Principles of Laboratory Animal Care"(National Institutes of Health, Bethesda, MD). They were reared in polypropylene cages (51 x 35.5 x 18.5 cm) in a room maintained at 21± 1°C with a 12 hour light/12 hour dark cycle (lights on at 7:00 AM).

Five groups of Wistar young (60 to 80 days of age) and 5 groups of adult rats (150 to 170 days old) received the treatment by gavage (a method consisting of administering substances directly into the stomach through a cannula inserted in the mouth). Two of the five groups (here named groups 1 and 2) received acute treatments (one-day gavage) and the other three groups (named groups 3, 4 and 5) were submitted to chronic treatment (one daily gavage during 21 days). In the moment of the treatment, tocopherol (purchased from Sigma, St. Louis, MO) was dissolved in olive oil, or in ethanol, respectively for the experiments with acute and chronic ethanol treatment. All manipulation of tocopherol was done in absence of light. The young and adult rats of the acute treatment groups received two gavages separated by a 1-hour interval. In gavage I, they received tocopherol (300 mg/kg). One hour later, they received in gavage

II 3 g/kg EtOH (group 1) or water (group 2). In the acute groups, we performed the CSD recordings immediately after the treatment.

The animals of the three groups on chronic treatment (groups 3, 4 and 5) received, during 21 days, a single daily gavage containing ethanol plus tocopherol (groups 3 and 4), or only ethanol (group 5). The reason of treating the chronic groups with only one gavage is to avoid excessive, long-lasting stress represented by two gavages per day during 21 days. In this case, tocopherol was dissolved directly in ethanol (groups 3 and 4). The difference between groups 3 and 4 consisted in the time point when CSD was recorded: group 3 was recorded immediately after the treatment, while group 4 was recorded 5-10 days thereafter (to simulate an "ethanol abstinence period"). In order to avoid excessive accumulation of tocopherol in the tissues, in the chronic groups the dose of tocopherol was reduced from 300mg/kg to 85 mg/kg/day. Finally, in group 5, only ethanol was administered, and CSD was recorded after a 5-10 days abstinence period. The dose of ethanol used in these experiments (3 g⁄kg) is within the range usually employed in experiments with 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).

All groups had free access to water and a commercial laboratory chow diet (containing 23 g protein per 100 g diet; Purina do Brazil Ltd, Paulínia, SP, Brazil).

The CSD recording session was carried out, as previously described (Bezerra et al., 2005). Briefly, under anesthesia with a mixture of 1 g⁄kg urethane plus 40 mg⁄kg chloralose (Sigma; injected intraperitoneally), a tracheal cannula was inserted and 3 trephine holes were made on the right side of the skull, aligned along 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 2 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. At 20-minute intervals, CSD was elicited by application, for 1 minute, of a cotton ball (1 to 2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) placed on the frontal cortical surface through the hole drilled on that region. On the 2 parietal holes, both the slow DC-potential change and the reduction in the spontaneous cortical electrical activity accompanying CSD were continuously recorded for 4 hours using a pair of Ag–AgCl agar-Ringer electrodes (1 in each hole), as previously described (Abadie-Guedes et al., 2008). A common reference electrode, of the same type, was placed on the nasal bones (see insert of Fig. 1). The DC-potential changes were recorded by connecting the electrodes to GRASS DC-amplifiers (Astro-Med Industrial Park, West Warwick, RI), 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 (Astro-Med Industrial Park). In some experiments, DC-recording was also computer-digitalized and recorded. The CSD velocity of propagation was calculated from the time required for a CSD wave to cover the distance between the 2 cortical electrodes. In the measurement of CSD velocities, we used as a reference the initial point of each DC negative rising phase. Body weight intragroup differences between before and after chronic treatment was analyzed by paired t-test. A repeated measure analysis of variance (ANOVA) followed by a post hoc (Tukey–Kramer) test was used to compare CSD propagation rates between groups. Differences between groups were accepted as significant when $p<0.05$. All values are presented as means±S.D.

Results

Body weights

After the 21 days of gavage, the young rats treated chronically displayed a significant weight gain of 12.7%, 22.0% and 21.3% body weights in groups 3, 4 and 5, respectively, as compared to the pre-gavage weight (247.6±21.5g; $P<0.05$; paired t-test). In the chronic adult rats, no significant weight gain was observed. Rather, the group 3 (without the "abstinence period") displayed a 3.8% weight reduction after the 21 days gavage treatment ($P<0.05$), as compared with the pre-gavage weight (394.6±30.6g). The groups 4 and 5 presented no weight change.

Cortical Spreading Depression Propagation

Fig. 1 shows the electrophysiological recordings of the slow DC-potential changes during CSD. The CSD eliciting stimulus (application of 2% m/v KCl solution for 1 min on a cortical point in the frontal region) was effective in triggering a single CSD-wave, which propagated and was recorded by the two recording electrodes on the parietal region of the same hemisphere.

Confirming our previous results, the CSD propagation velocities were altered in the groups treated only with ethanol, with acute treatment decelerating, whereas chronic treatment accelerating CSD. In both cases, addition of tocopherol antagonized the ethanol CSD effects, and these findings were observed both in young and adult animals (Figure 2).

In the young groups, the mean±s.d. CSD velocities of propagation (in mm/min) were 3.05±0.06 and 4.37±0.31 for the acute and chronic ethanol treated groups; in the

acute tocopherol treatment, with or without ethanol, the CSD velocities were 3.35±0.09 and 3.42±0.16, respectively; in the chronic tocopherol treatment, the CSD velocity was 3.46±0.12 and 3.25±0.08, respectively for the groups in which the CSD recordings were taken with or without 5-10 days abstinence from ethanol.

In the adult groups, the CSD velocities were 2.66±0.24 and 3.88±0.31 for the acute and chronic ethanol treatment, respectively; in the acute condition, tocopherol, restored the CSD velocity to 3.21±0.08 and 3.13±0.09 for the groups with or without ethanol, respectively, comparable to the value previously reported for control rats ($P<0.05$); this effect of tocopherol was also observed in the chronic condition, where the mean velocities were 3.23±0.08 and 3.26±0.08 for the groups in which the CSD recordings were taken with or without 5-10 days abstinence from ethanol.

Discussion

It has been largely accepted that the mechanisms of ethanol action on the brain involve the increase of reactive oxygen species in the tissue [Tapiero et al, 2004]. In this context, the scavenging of reactive oxygen species would be a useful mechanism whereby antioxidant molecules would help in antagonizing the ethanol effects on the brain. In line with this possibility, the present study strongly suggested that α-tocopherol antagonizes the CSD effects of ethanol, in the acute as well as in the chronic condition, in both young and adult rats.

The brain protective effects of antioxidant molecules have been largely discussed in the literature. The clinical and experimental results collectively demonstrate a positive action of antioxidants in counteracting reactive oxygen species occurring in several neurological diseases. Among them, the disturbances provoked by the abusive

consumption of ethanol are of great impact on the human health (Rehm et al, 2010). Several models on laboratory animals have been used to study this problem. Concerning the neurophysiological approach, several authors have demonstrated that the phenomenon of cortical spreading depression can provide useful information on the brain effects of ethanol (Sonn and Mayevsky, 2001; Guedes and Frade, 1993), and these effects are antagonized by the antioxidant carotenoid astaxanthin (Bezerra et al, 2005; Abadie-Guedes et al, 2008; 2012), probably by its scavenging action against reactive oxygen species. In this context, it is interesting to comment that reactive oxygen species produced in the nervous tissue can elicit CSD [Netto and Martins-Ferreira, 1989; El-Bachá et al, 1998).

In the present study, we found that the antagonizing action of α-tocopherol against the ethanol effects on CSD occurred in a way similar to that observed for astaxanthin. These findings support the hypothesis that the previously demonstrated effect of astaxanthin on the ethanol action on CSD is rather a general effect of antioxidant molecules and not a particular property of carotenoids. In fact, other antioxidant molecules, like taurine, have been shown to attenuate the effects of ethanol concerning the oxidative stress (Pushpakiran et al, 2004). Moreover, the comparison of young and adult groups demonstrated that tocopherol effect did not depend on the animal's age. The comparison between CSD velocities of young and adult groups has also confirmed previous evidence in favor of an inverse relationship between CSD velocity and the ages of the animals (Guedes et al, 1996; Batista-de-Oliveira et al, 2012).

The usual ethanol consumption by humans begins with single episodes of intake of alcoholic drinks. As time passes, alcohol ingestion events may become frequent, characterizing the passage from the acute to a chronic consumption. The sudden interruption of long-term frequent ethanol use generates a syndrome known as

abstinence, or withdrawal. The clinical features of abstinence include psychological symptoms like depression and anxiety, and also signs like hyperactivity and tremors. In some cases, rigidity and convulsion may be present and in extreme situations, coma and death may occur. Ethanol abstinence may also increase the production of reactive oxygen species (Jung and Metzger, 2010). When we have compared the group with 5-10 days of abstinence from ethanol (present results) with the group without abstinence (our previous study; Abadie-Guedes et al, 2008), we concluded that, under our experimental conditions, the abstinence did not influence the ethanol effect on CSD propagation.

The contrasting CSD effects produced by acute (Abadie-Guedes et al, 2012) as compared to chronic EtOH treatment (present study) deserve comment. While chronic ethanol accelerates CSD (Guedes and Frade, 1993; Bezerra et al, 2005; Abadie-Guedes et al, 2008), acute ethanol decelerates it (Sonn and Mayevsky, 2001; Abadie-Guedes et al, 2012), suggesting the participation of different mechanisms acting in the two conditions.

In conclusion, our findings confirms the hypothesis of an antagonistic role of antioxidants on brain processes involved in CSD. Since another class of antioxidant (carotenoids; Bezerra et al, 2005; Abadie-Guedes et al, 2008; 2012) had also antagonizing the ethanol effects on CSD, we would postulate that the action of antioxidants on CSD is a general feature of antioxidant molecules.

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Figure legends

Fig. 1. Examples of recordings of the slow potential changes (P) occurring during cortical spreading depression (CSD) in two young rats (60-80 days of life; upper panels) and two adult animals (150-170 days; lower panels). The 2 animals of the right panels were chronically treated with ethanol (3 g/kg/day, per gavage, for 21 days). The two rats in the left panels received ethanol plus tocopherol (85 μg/kg/day, for the same period). In each panel, the black horizontal bar indicates the time (1 minute) of application of a cotton ball (1 to 2 mm diameter) of 2% KCl, which usually elicited a single episode of CSD. The central 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). Traces marked with P1 and P2 are CSD recordings obtained in the cortical points 1 and 2, respectively. Vertical calibration bars equal 10 mV for the P-recordings (negative upward). The vertical interrupted lines delimit the latency for a CSD wave to propagate from cortical point 1 to point 2. Note that in the tocopherol-treated rats the latencies are longer as compared to the corresponding animals treated only with ethanol.

Fig. 2. Effect of α-tocopherol on the acute and chronic ethanol actions in the propagation velocity of cortical spreading depression (CSD) of young and adult rats. In each age group, treatments with ethanol and tocopherol, administered isolated or combined, were compared with controls treated with the vehicles (see convention in the upper part of the figure). T = tocopherol; E = ethanol; W = water. Data are expressed as

mean ± S.D. Asterisks indicate that the groups chronically treated with ethanol have higher CSD velocities as compared with the corresponding tocopherol controls. The symbol # indicates that the groups acutely treated with ethanol (data from our previous study; Abadie-Guedes et al, 2012) display lower CSD velocities than the tocopherol controls ($P<0.05$; ANOVA plus Tukey test).

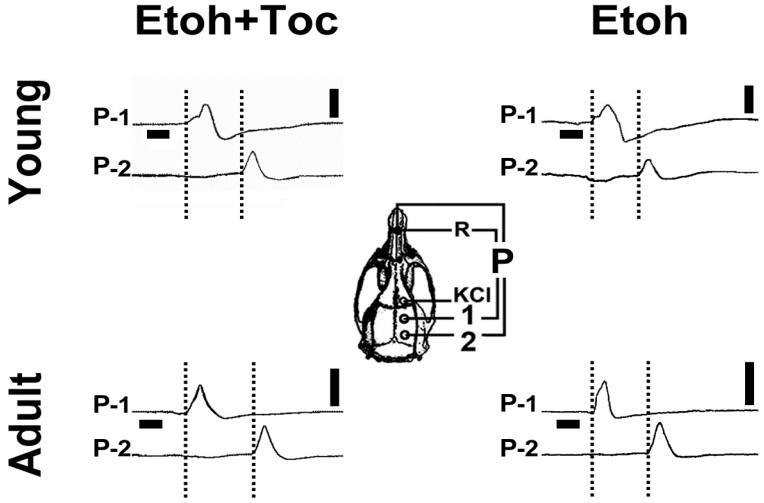

Figura 1

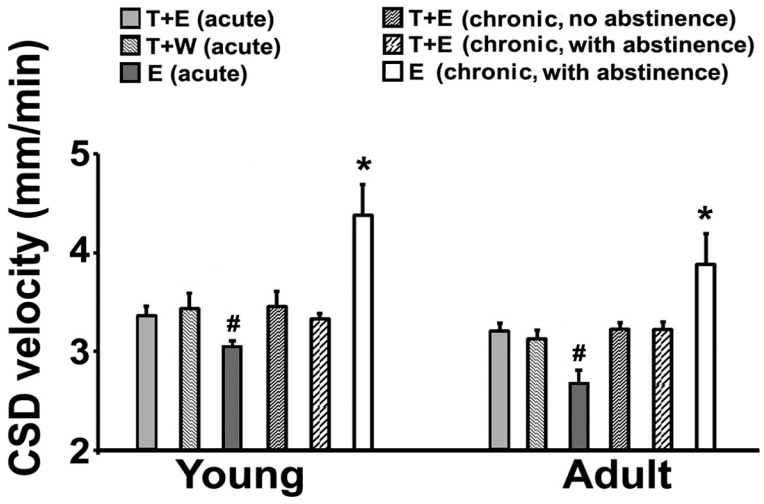

Figura 2

5. CONCLUSÕES

Os resultados deste trabalho demonstraram efeitos opostos do álcool sobre a atividade elétrica do córtex cerebral, a depender da duração do consumo (agudo ou crônico). Foi verificado também que tanto os antioxidantes astaxantina quanto o α-tocoferol antagonizaram a ação do álcool sobre o sistema nervoso, o que sugere um efeito protetor contra espécies químicas reativas formadas, no modelo de estresse oxidativo (consumo de etanol) utilizado neste trabalho. A idade foi um fator que não influenciou na interação do álcool e das molécuas antioxidantes utilizadas no experimento. Na etapa em que foi avaliado se outro antioxidante, não carotenoide (tocoferol), promoveria efeito similar à astaxantina (verificado em estudos anteriores, BEZERRA et al., 2005). Os resultados comprovaram essa hipótese, indicando que a ação antagônica sobre o efeito do etanol na DAC não seria uma propriedade particular da astaxantina, mas provavelmente uma característica geral de moléculas antioxidantes.

Os resultados referentes ao primeiro objetivo acima originaram um artigo que foi publicado na revista ***Alcoholism: Clinical and Experimental Research*** (ABADIE-GUEDES et al, 2012). Os resultados referentes aos outros objetivos originaram outro artigo (anexo a esta tese), a ser submetido para publicação, também na revista acima.

Adicionalmente, o doutorando participou como co-autor de um trabalho de revisão sobre o uso da depressão alastrante cortical para o estudo da ação de antioxidantes no cérebro. Este trabalho foi recentemente publicado na revista Nutritional Neuroscience (GUEDES et al, 2012).

ANEXO I

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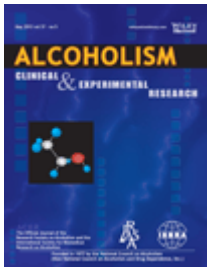

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ANEXO II

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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 ± 0.26 to 3.15 ± 0.27 [treatment during gestation; $n = 7$], and from 3.03 ± 0.25 to 3.22 ± 0.30 [lactation; $n = 11$]), ethanol-treated rats displayed higher spreading depression-velocities (from 3.74 ± 0.06 to 3.82 ± 0.08 [gestation; $n = 7$], and from 4.26 ± 0.32 to 4.33 ± 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 ± 0.09 to 3.42 ± 0.12, $n = 7$ (gestation) and 3.58 ± 0.13 to 3.62 ± 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.

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).

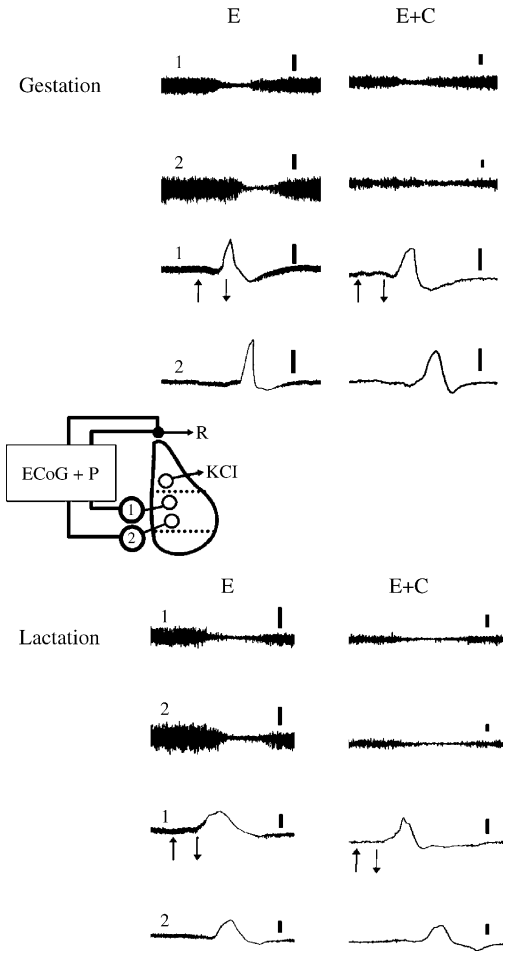

Fig. 1. Recordings of spontaneous cortical electrical activity (ECoG; two upper traces in each panel) and slow potential change (P; two lower traces) during spreading depression in rats whose mothers had been treated, either during gestation or during the lactation period, with ethanolic extract of shrimp 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.

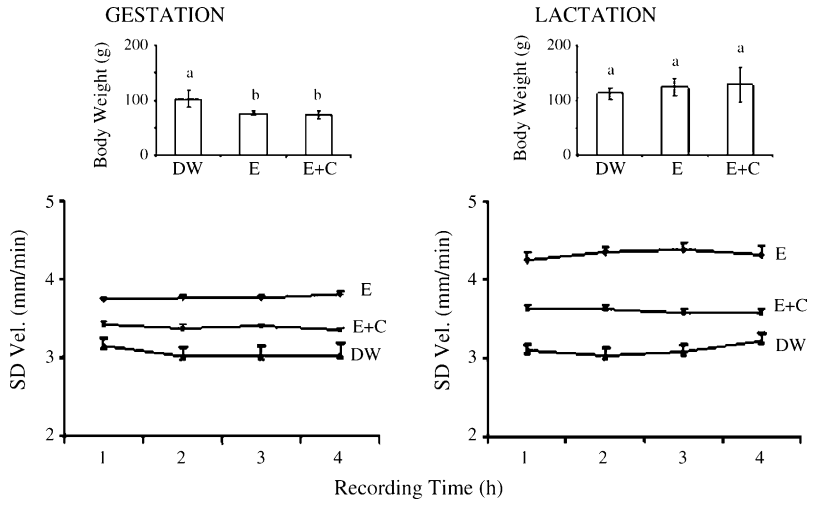

Fig. 2. Reduction of spreading depression-velocity in just-weaned rat pups (36–40 days of life) whose mothers had been treated with an ethanolic extract of shrimp carotenoids (30 μg/kg/day), either during the gestation or during the lactation period, as compared to corresponding vehicle- (ethanol; 3.8 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 III

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

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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 μg/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 (μg); 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 μg/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 μg/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

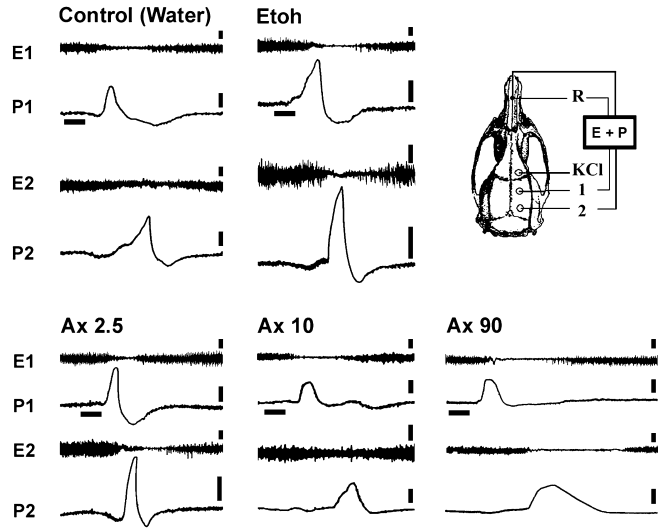

Fig. 1. Recordings of spontaneous cortical electrical activity (E; two upper traces in each panel) and slow potential change (P; two lower traces) during cortical spreading depression in adult rats (90 days of life) treated per gavage with astaxanthin at doses of 2.5, 10, and 90 μg/kg/d (respectively 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.

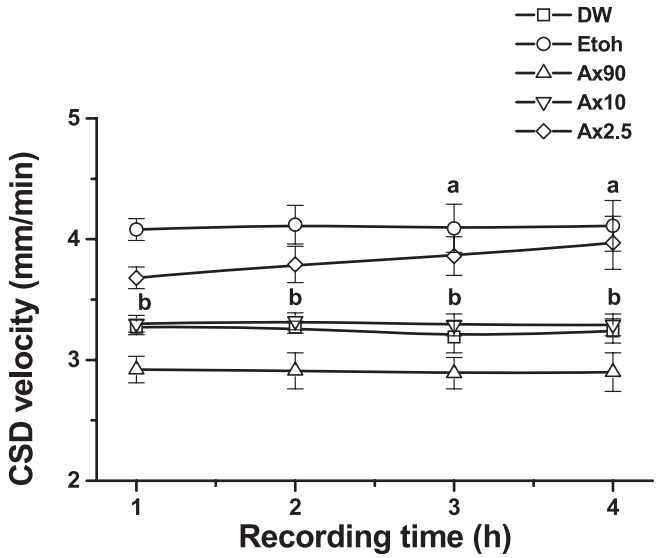

Fig. 2. Reduction of cortical spreading depression-velocity in young adult rats (90 days of life) treated per gavage with astaxanthin at doses of 2.5, 10, and 90 μg/kg/d (respectively A × 2.5, A × 10, and A × 90) compared to rats treated with vehicle- (ethanol; 3 g/kg; group Etoh) and controls treated 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 μg/kg, 10 μg/kg or 90 μg/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 μg/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 μg/kg/d treatment. No significant differences were seen between the treatments with the 10 μg/kg/d astaxanthin dose and distilled water groups. The group treated with the highest astaxanthin dose (90 μg/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

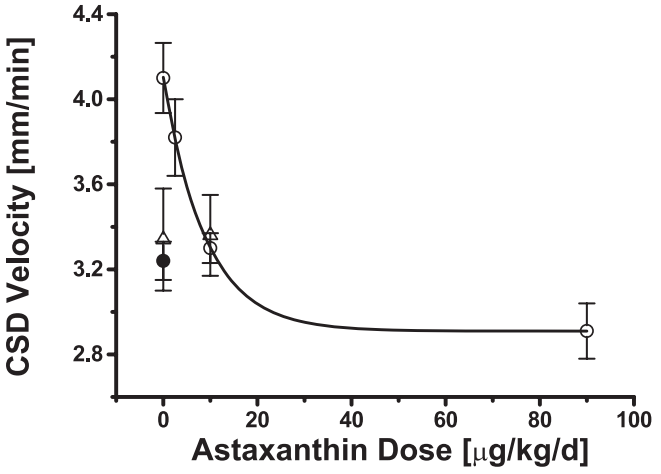

Fig. 3. Dose–response effects of astaxanthin dissolved in ethanol on cortical spreading depression velocity in the rat cortex (open circles). The curve resulted from plotting the mean CSD velocities (during the entire 4-h recording period) as a function of the astaxanthin doses. Each point represents 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 μg/kg/d). The average CSD velocities for the DW-, as well as for the soybean oil- and 10 μg/kg/d astaxanthin + soybean oil groups are also presented in this figure for comparisons. These velocities were not statistically different from the 10 μg/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 α-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 γ-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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