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AÇÃO DO LICOPENO NA PROTEÇÃO CONTRA DANOS INDUZIDOS NO DNA *IN VIVO* E *IN VITRO*

Doutoranda: Clarissa Scolastici
Orientadora: Dra. Daisy Maria Fávero Salvadori
Co-orientador: Dr. Luís Fernando Barbisan

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SUMÁRIO

I - RESUMO

O licopeno é um pigmento natural sintetizado por plantas e microorganismos, e encontrado principalmente no tomate. É um isômero acíclico do β-caroteno sem atividade pró-vitamínica-A, sendo um dos mais potentes agentes antioxidantes. Diversos pesquisadores têm demonstrado o efeito protetor do licopeno contra danos no DNA e sobre a carcinogênese quimicamente induzida, embora os mecanismos envolvidos nesses processos não estejam, ainda, totalmente esclarecidos. Assim, o presente estudo objetivou avaliar o potencial quimioprotetor do licopeno contra danos no DNA e sobre a hepaticarcinogênese em roedores. O efeito anticarcinogênico do licopeno em fígado de ratos foi avaliado utilizando-se os focos GST-P positivos (modelo de hepatocarcinogênese de média duração proposto por Ito et al., 1988) como biomarcadores de lesões pré-neoplásicas. Para avaliação do efeito antigenotóxico e antimutagênico do carotenóide, bem como de seu possível mecanismo de ação, foram utilizados, respectivamente, o teste do cometa e o teste do micronúcleo em duas linhagens celulares *in vitro:* células de ovário de hamster chinês (CHO) e células de hepatoma humano (HepG2). Mutágenos de ação direta (peróxido de hidrogênio, oxido de 4-nitroquinolina - 4NQO e metil metanosulfonato - MMS) e indireta (dietilnitrosamina -DEN), foram utilizados como indutores de danos no DNA in *vitro.* Os resultados mostraram que o licopeno, na concentração 300 ppm, reduziu significativamente os danos no DNA induzidos pela DEN na etapa de iniciação da hepatocarcinogênese de rato, embora não tenha sido observada redução do número e área dos focos GST-P-positivos. Os resultados dos experimentos *in vitro* mostraram que o tratamento prévio e simultâneo com licopeno (10, 25, e 50 μM) foi eficaz em reduzir os níveis de danos no DNA induzidos pelo H_2O_2 e pela DEN em células HepG2, quando avaliados tanto teste do cometa como do micronúcleo. Redução do nível de danos no DNA foi também detectada em células CHO quando o carotenóide foi administrado antes ou simultaneamente ao H_2O_2, 4-NQO e MMS. Concluindo, nossos resultados confirmam o

efeito protetor do licopeno contra danos quimicamente induzidos no DNA, muito embora tal atividade seja dependente do protocolo de tratamento utilizado. Por outro lado, não foi detectado efeito quimioprotetor do carotenóide sobre o desenvolvimento de lesões pré-neoplásicas induzidas pela DEN em fígado de ratos.

II . ABSTRACT

Lycopene is a natural pigment synthesized by plants and microorganisms, and mainly found in tomatoes. It is an acyclic isomer of β-carotene with no vitamin A activity, and one of the most potent antioxidants. Several studies have showed the chemopreventive effect of lycopene on chemically-induced DNA damage and on chemical hepatocarcinogenesis, however its mechanism of action needs to be clarified. The present study aimed to evaluate the protective activity of lycopene on chemically-induced DNA damage and on rodent hepatocarcinogenesis. Anticarcinogenic potential of lycopene was evaluated using GST-P positive foci (medium-term hepatocarcinogenesis model described by Ito et al., 1988) as a biomarker of preneoplasic lesion. Lycopene antigenotoxicity and antimutagenicity, as well as its mechanism of action were investigated in two cell lines (CHO and human hepatoma cell – HepG2) using the comet assay and micronucleus test, respectively. Direct-acting (hydrogen peroxide; 4-nitroquinoline 1-oxide - 4NQO; methyl methanesulphonate - MMS) and indirect-acting (n-nitrosodiethylamine – DEN) mutagens were used to induce *in vitro* DNA damage. The results showed that lycopene (300 ppm) reduced DEN-induced DNA damage at the initiation step of *in vivo* hepatocarcinogenesis, although no effect was observed on the number and area of GST-P positive foci in liver of rats. Data from *in vitro* experiments showed that lycopene (10, 25 and 50 μM) was effective in reducing DNA damage induced by H_2O_2 and DEN in HepG2 cells, both in comet assay and in micronucleus test. In CHO cells the chemopreventive acitivity of lycopene was visualized only for primary DNA damage in the comet assay. In conclusion our data confirmed the chemopreventive effect of lycopene on chemically-induced DNA damage, although such activity depends on the treatment schedule used. On the other hand, no protective action of this carotenoid on DEN-induced preneoplasic lesion in liver of rats was detected.

III – REVISÃO DE LITERATURA

Considerações Iniciais

Avaliações recentes indicam, que se medidas rigorosas de controle não forem adotadas, o câncer se converterá na principal causa de morte em muitos países no início desse século. Sabe-se, hoje, que fatores de risco como estilo de vida, dieta, agentes infecciosos e predisposição genética podem aumentar a incidência de doenças degenerativas como o câncer. No entanto, considerando que nem sempre é possível prevenir doenças relacionadas a mutações apenas reduzindo a exposição humana a agentes mutagênicos e carcinogênicos, vários estudos têm objetivado a identificação de compostos que atuem na proteção contra danos no DNA, diminuindo, assim, a taxa de mutação e alterações celulares (Ribeiro et al., 2005).

Os primeiros estudos realizados para identificação de compostos com atividade protetora contra danos no DNA, data da metade do século 20. A idéia de que é possível desenvolver estratégias nutricionais que atuem protegendo o homem contra danos genéticos e doenças relacionadas, é suportada por estudos epidemiológicos que mostram que 20% a 60% de todos os cânceres estão relacionados à dieta (Doll & Peto, 1981). Além disso, sabe-se que milhões de novos casos por ano poderiam ser prevenidos pela ação de compostos da dieta (Arggawal & Shishodia, 2006).

Estudos epidemiológicos demonstram que as incidências regionais do câncer estão diretamente relacionadas aos hábitos de vida, condições sócio-econômicas e, principalmente, à alimentação (Doll & Peto, 1981; Arggawal & Shishodia, 2006). Por esse motivo, a ligação entre hábitos alimentares e risco de câncer tem sido alvo de diversos estudos epidemiológicos, pré-clinicos e experimentais (Milner, 2002). Esses estudos demonstram que a identificação da ocorrência natural de carcinógenos e anticarcinógenos alimentares é útil não apenas para o

entendimento da carcinogênese como, também, para novas estratégias de prevenção (Arggawal & Shishodia, 2006).

A quimioprevenção do câncer pode ser entendida como uma forma de prevenir a doença por meio da administração de um ou mais compostos químicos durante as etapas iniciais da carcinogênese (Sporn et al., 1976; Sporn & Suh, 2002). Os agentes quimioprotetores podem ser classificados em três categorias, de acordo com seus efeitos nas diferentes fases da carcinogênese: 1) inibidor da formação do carcinógeno ativo; 2) bloqueador de metabólito ativo e, 3) supressor do desenvolvimento neoplásico (Wattenberg, 1985). Entretanto, é difícil a classificação precisa dos agentes quimiopreventivos, visto que os mecanismos de ação da maioria dos compostos são desconhecidos, e muitos deles atuam no processo de carcinogênese por diferentes mecanismos (Stoner et al., 1997).

Vários dos inibidores da carcinogênese já identificados são constituintes naturais dos alimentos (Ames et al., 1995; Arggawal & Shishodia, 2006). Assim, a investigação dos mecanismos pelos quais a dieta poderia influenciar o desenvolvimento do câncer pode contribuir para a elucidação de aspectos fundamentais da carcinogênese e do comportamento biológico das neoplasias malignas, e ter impacto sobre estratégias de prevenção e tratamento dessa patologia (Arggawal & Shishodia, 2006).

Danos oxidativos e agentes antioxidantes

Nos últimos anos, os agentes antioxidantes têm merecido grande atenção devido ao seu potencial terapêutico sobre doenças relacionadas à produção de radicais livres (Ratnam et al., 2006). Estes são moléculas ou espécies químicas altamente reativas que contêm um par de elétrons não pareado que podem induzir estresse oxidativo (Sies, 1997). O estresse oxidativo pode lesar lipídeos, proteínas, enzimas, carboidratos e a molécula de DNA de células e tecidos resultando em danos de membrana, fragmentação do DNA, alterações estruturais de

proteínas e enzimas ou mesmo a morte celular (Beckman & Ames, 1998). Entre as conseqüências do estresse oxidativo estão o desenvolvimento do câncer, as desordens neurodegenerativas, as doenças cardiovasculares e o diabetes (Ratnam et al., 2006).

Os compostos antioxidantes são definidos como substâncias que “neutralizam” os radicais livres e previnem os danos causados pelos mesmos. Sua ação protetora se dá pela desintegração dos radicais oxidantes, antes mesmo que estes reajam com alvos biológicos, prevenindo, portanto, reações em cadeia ou a ativação de espécies de oxigênio altamente reativas (Azzi et al., 2004).

Os agentes antioxidantes podem ser classificados em dois grandes grupos: os enzimáticos e os não enzimáticos. Alguns desses antioxidantes são produzidos endogenamente, e incluem enzimas, moléculas com baixo peso molecular e co-fatores enzimáticos. A maioria dos agentes antioxidantes não enzimáticos é obtida da dieta e podem ser polifenóis, vitaminas, minerais, compostos organo-sulforados e carotenóides (Ratman et al., 2006).

Os carotenóides representam um importante grupo de pigmentos naturais que são sintetizados por plantas e por alguns microrganismos (Goodwin, 1986). Amplamente distribuídos na natureza, os carotenóides apresentam ações protetoras contra determinados tipos de cânceres (Mares-Perlman et al., 2002). Estudos epidemiológicos têm contribuído para elucidar o papel dos carotenóides da dieta na prevenção e terapia de diversas doenças. Duas hipóteses são consideradas sobre o papel anticarcinogênico dos carotenóides: a primeira, está relacionada à sua atividade pró-vitamina A, como é o caso do β-caroteno; a segunda, à sua propriedade antioxidante intrínseca, como é o caso do licopeno (Rao & Agarwal 1999).

Licopeno

O licopeno, um dos mais de 600 carotenóides encontrados na natureza, vem sendo bastante estudado devido sua atividade antioxidante (Bramley, 2000). Trata-se de um carotenóide acíclico, composto por 40 átomos de carbono e 56 de hidrogênio, unidos por 11 duplas ligações conjugadas (Figura 1) (Holloway, 2000). É uma molécula acíclica, simétrica e sem atividade pró-vitamina A. O licopeno apresenta isomerização *cis-trans*, mas com raras exceções a configuração mais encontrada em plantas é a *all-trans*, que é termicamente mais estável (Emenhiser et al., 1995). Embora a sua principal fonte seja o tomate, o licopeno também pode ser encontrado em uma grande variedade de frutas e vegetais como melancia, goiaba e mamão papaia (Bramley, 2000).

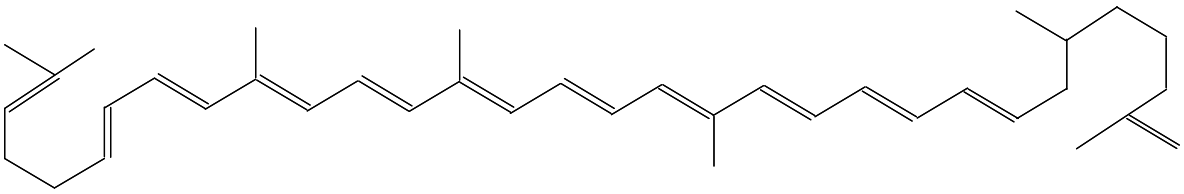

Figura 1. Fórmula estrutural do licopeno *all-trans*

Recentes estudos experimentais e epidemiológicos têm demonstrado que a dieta rica em licopeno pode ser associada à diminuição do risco de patologias crônicas como o câncer e as doenças cardiovasculares (Rao et al., 2006). Além disso, níveis séricos ou teciduais elevados de licopeno são inversamente relacionados com doenças crônicas (Rao et al., 2006).

Embora a atividade antioxidante do licopeno pareça ser a principal responsável pelos seus efeitos benéficos, evidências como o aumento das comunicações intercelulares via *gap junctions* (Zhang et al., 1991), a inibição da proliferação celular, o estímulo ao metabolismo

de xenobióticos e a modulação do processo inflamatório (Blum et al., 2005) são outros mecanismos propostos.

Diversos estudos *in vitro* e *in vivo* têm demonstrado o papel do licopeno na prevenção do câncer. Estudos com culturas de células têm sido realizados a fim de evidenciar os efeitos moleculares e bioquímicos do licopeno em linhagens normais e transformadas (Rao & Agarwal, 1999; Rao et al., 2006). Segundo Coutryman et al. (1991), o licopeno (10 mmol/L) inibe em cerca de 40% o desenvolvimento de linhagens leucêmicas humanas e, em associação com a vitamina D, inibe a proliferação celular e induz a apoptose (Amir et al., 1999). Além disso, o carotenóide induz aumento da expressão do gene da conexina 43, que codifica uma proteína presente nas *gap junction*, levando a um efeito anticarcinogênico (Zhang et al., 1992). Culturas de células normais de fígado de rato expostas ao tetracloreto de carbono apresentaram redução no número de lesões oxidativas e aumento da sobrevida após o acréscimo de licopeno ao meio, sendo esses resultados decorrentes da ação supressora da lipoperoxidação (Kim, 1994). Quando comparado ao alfa e beta-caroteno, o licopeno inibiu, de modo mais expressivo, a proliferação *in vitro* de células tumorais de endométrio, de mama (MCF-7) e de pulmão (NCI-H226) (Levy et al., 1995). Em estudos com células AH109A1 de hepatoma de ratos foi sugerido que a propriedade antioxidante do licopeno pode inibir a característica de invasividade destas células (Kozubi et al., 2000). Em Jurkat E6.1 (células malignas de linfócitos T), o tratamento de licopeno por 24 h induziu a apoptose, embora de maneira menos eficaz que o beta-caroteno (Muller et al., 2002); em células epiteliais normais de próstata humana (PrEC) o licopeno diminuiu significativamente o crescimento celular (Obermuller-Jevic et al., 2003). Tange et al. (2005) mostraram que o tratamento de três linhagens celulares de próstata (células andrógeno independente, DU145 e PC-3, e células andrógenos dependente, LNCaP) com o licopeno inibiu a proliferação celular, principalmente

nas linhagens andrógeno independente. Diminuição da proliferação celular foi também observada em células SiHa (células de cânceres cervical) (Brewer et al., 2006).

Em células de hepatoma humano (HepG2) pré-tratadas com o licopeno foi observada uma proteção contra os efeitos toxicos da aflatoxina (Reddy et al., 2006). Ainda em hepatócitos, em células Hep-1, o licopeno reduziu significativamente a capacidade invasiva dessas células (Hwang e Lee et al., 2006).

Estudos realizados em roedores confirmam a atividade protetora do licopeno observada em cultura de células. Em camundongos com neoplasia de mama houve retardo do crescimento tumoral após ingestão crônica de licopeno. Este resultado foi atribuído à redução na liberação de prolactina pela ingestão do carotenóide (Nagasawa et al., 1995). Em ratas com tumores de mama, foi observado que após o tratamento com licopeno por 16 semanas houve redução do número e tamanho dos tumores; no mesmo modelo, o beta-caroteno não apresentou tal atividade (Sharoni et al., 1997). Com relação ao sistema nervoso central, o licopeno inibiu o crescimento e desenvolvimento de células de glioma em ratos (Wang et al., 1989). Foi também detectada em ratos, a diminuição do número de tumores de bexiga, após 12 semanas de administração de suco de tomate (Okajima et al., 1998). O efeito do licopeno em cânceres gástricos induzidos pela N-metil-N-nitro-N-nitrosoguanidina e pelo cloreto de sódio saturado também foi investigado em ratos da linhagem Wistar. Os resultados mostraram que o carotenóide inibiu significativamente o processo carcinogênico pela modulação da peroxidação lipídica (Velmurugan & Nagini, 2005). Também por este mecanismo, foi observado o efeito quimioprotetor do licopeno na carcinogênese oral induzida pela 12-dimethilbenz[a]anthraceno (DMBA) em Hamster (Bhuvaneswari et al., 2004). Em modelo de carcinogênese de múltiplos órgãos em camundongos B6C3F1 foi demonstrado o efeito quimioprotetor do licopeno sobre a carcinogênese de pulmão, diminuindo a incidência e multiplicidade dos tumores (Kim et al., 1997)..

Em cólon, Narisawa et al. (1998) observaram redução na incidência de tumores induzidos pela N-metil-N-nitrosuréia em ratos tratados por 35 semanas com suco de tomate (1,7 mg de licopeno/kg/animal). Em estudo anterior, os mesmos autores haviam relatado a inibição da formação de focos de criptas aberrantes (FCA), após administração de pequenas doses de licopeno (0,12 e 0,24 mg/dia) (Narisawa et al., 1996). Wargovich (1996) avaliou o papel do carotenóide administrado via ração (0,038 e 0,075 g/kg) nas fases de formação e progressão de criptas aberrantes induzidas por azoximetano. Os resultados mostraram efeito protetor do licopeno na fase de formação, mas não durante a progressão das lesões. A avaliação da biodisponibilidade, distribuição, atividade antioxidante e sobre neoplasias de colón mostrou que o licopeno tem importante ação sobre o estresse oxidativo e apresenta tendência de proteção na etapa de promoção de FCA induzidas pelo azoximetano (Charu et al., 1999). No entanto, Kim (1998) não observou efeito do licopeno em focos de criptas aberrantes induzidas pela dimetilhidrazina, quando administrado via água de beber (0,0025% e 0,005 %).

São poucos os estudos da ação do licopeno sobre a hepatocarcinogênese induzida quimicamente. Em ratos, a administração do carotenóide reduziu a área de focos de hepatócitos alterados induzidos pela dietilnitrosamina e pelo 2-acetilaminofluoreno (Astrog et al., 1997). Toledo et al. (2003), também demonstraram o efeito do licopeno na prevenção de danos no DNA e no desenvolvimento de focos GST-P positivos em ratos submetidos ao modelo do hepatócito resistente. Por outro lado, Gradelet et al. (1999) não observaram efeito significativo sobre leões pré-neoplásicas induzidas pela aflatoxina. Do mesmo modo, não foi observado efeito do carotenóide no modelo de carcinogênese de tumores hepáticos espontâneos utilizando ratos Long-Evans Cinnamon (LEC) (Watanabe et al., 2001).

Os dados apresentados mostram que ainda não é claro o papel licopeno na prevenção da hepatocarcinogênese (Astorg et al., 1997; Watanabe et al., 2001). Além disso, segundo a Agência Internacional de Pesquisa do Câncer (IARC), não há evidências acerca da capacidade quimioprotetora do licopeno em seres humanos e são ainda inadequadas e limitadas as evidências em outras espécies animais (Vainio & Bianchini, 2002).

Hepatocarcinogênese química

Carcinogênese é um termo geral utilizado para denotar o processo complexo de desenvolvimento do câncer. Esse processo é considerado um fenômeno ativo podendo ser induzido por vários agentes químicos, físicos ou biológicos (Pitot & Dragan, 1991).

A carcinogênese química pode ser induzida por cancerígenos diretos, que parecem não requerer qualquer conversão prévia, ou indiretos, que agem por meio de metabólito reativo originado após a sua metabolização (Venitt, 1994). O desenvolvimento do câncer induzido quimicamente é considerado um processo em múltiplas etapas (Pitot & Dragan, 1991). Há evidências que sugerem que uma neoplasia pode se desenvolver em três etapas básicas denominadas iniciação, promoção e progressão (Pitot et al., 1996), sendo duas delas já bem caracterizadas operacionalmente: a iniciação e a promoção.

Na etapa de iniciação o agente químico provoca alterações irreversíveis em genes relacionados aos processos de proliferação e diferenciação celular (Bos & Kreijl, 1992). A ocorrência eventual de proliferação celular reparativa ou compensatória, “fixa” essas lesões de DNA, impedindo o reparo eficiente e a eliminação (morte celular) dessas células, originando, então, as células-filhas iniciadas (Kaufmann et al., 1991). A morte celular por apoptose de células com DNA mutado, pode representar um importante mecanismo protetor contra o desenvolvimento da neoplasia (Grasl-Kraupp et al., 2000).

A etapa de promoção envolve a seleção e proliferação clonal das células iniciadas, com formação de lesões pré-neoplásicas (Pitot, 1993). Os agentes promotores, que atuam provocando essa expansão, o fazem por meio do aumento da proliferação celular (Cohen & Ellwein, 1990; 1991) e da inibição da apoptose (Schulte-Hermann et al., 1990).

O último estágio do desenvolvimento neoplásico, a progressão, caracteriza-se por instabilidade genômica e contínua evolução das características independentes, como mudanças bioquímicas das células malignas, aumento da proliferação celular, invasão, anaplasia e metástase (Pitot & Dragan, 1991). A irreversibilidade desse estágio é assumida pelas alterações no genoma da célula (Pitot & Dragan, 1991).

São grandes os progressos no estudo da hepatocarcinogênese desde o primeiro modelo de câncer de fígado descrito por Sasaki & Yoshida em 1935. Várias modificações foram realizadas com a finalidade de se obter um modelo que permitisse o estudo das várias etapas do processo carcinogênico. O sistema de dois estágios na formação do tumor hepático foi introduzido por Peraino et al. (1971). Desde então, diversos modelos têm sido descritos para o estudo da hepatocarcinogênese química induzida em ratos, sendo parte deles baseado nos conceito de iniciação e promoção da carcinogênese química (Ito et al., 1988; 1989; 1996; 2000).

O câncer hepático induzido quimicamente em roedores também ocorre através do desenvolvimento de alterações seqüenciais. A iniciação é causada por carcinógenos genotóxicos que causam danos no DNA. A falha na correção desses danos seguida da proliferação celular leva à formação inicial de focos de hepatócitos alterados fenotipicamente (FHA). Os FHA progridem para nódulos, adenomas e, por fim, para o câncer. A demonstração de que essas alterações estão seqüencialmente associadas, indica que os focos representam etapas pré-neoplásicas, podendo ser considerados marcadores histológicos da futura neoplasia hepática (Farber & Sarma, 1987; Bannasch et al., 2003).

Diferentes tipos de FHA, induzidos por carcinógenos químicos, têm sido identificados no fígado de ratos, na dependência das alterações de seus componentes citoplasmáticos, como glicogênio, retículo endoplasmático, ribossomos e peroxissomos (Bannasch et al., 1989; Bannasch & Zerban, 1992; Su & Bannasch, 2003). Assim, sob coloração de rotina com hematoxilina e eosina (H&E), as alterações permitem classificar os focos de hepatócitos alterados de acordo com a sua morfologia. O conhecimento de que as células dos focos exibem atividade enzimática diferente das células normais, permite marcá-las imuno e histoquimicamente, por meio de marcadores enzimáticos, tais como, glutamiltranspeptidase (GGT), adenosiltrifosfatase (ATPase), glicose-6 fosfatase (G-6-Pase) e glutationa S-transferase, forma placentária (GST-P) (Bannasch et al., 1989; Ittrich et al., 2003). Essa última, a forma placentária da glutationa S-transferase (GST-P), foi estabelecida como um dos melhores marcadores dos focos pré-neoplásicos no fígado de rato (Ito et al., 1989; 2000).

Dentre os modelos de hepatocarcinogênese de média duração destaca-se o protocolo proposto por Ito et al. (1988) desenvolvido a partir do modelo experimental desenvolvido por Solt e Faber (1976). Nesse modelo, a iniciação da hepatocarcinogênese é realizada pela administração intraperitoneal de uma dose única de dietilnitrosamina (DEN) (200 mg/kg de peso corpóreo) na segunda semana de experimento. Na quinta semana, os animais são submetidos à hepatectomia parcial de dois terços e sacrificados na oitava. Atualmente, este modelo vem sendo também utilizado para a identificação de compostos quimioprotetores da hepatocarcinogênese, em especial substâncias antioxidantes naturais e sintéticas (Ito et al., 1996).

Sistema teste in vitro – células CHO e HepG2

Até o momento, a maioria dos testes de mutagenicidade e genotoxidade tem sido realizada em sistemas *in vitro*, particularmente em bactérias, devido ao menor custo e rapidez dos

experimentos (Fergunson, 1994). No entanto, uma vez que em procariotos os mecanismos envolvidos nos processos de reparo do DNA e/ou metabolismo das substâncias não são os mesmos de células de mamíferos, tornou-se necessário o uso de linhagens que melhor reproduzissem as condições *in vivo*. Outra possibilidade na utilização de células de mamíferos é a capacidade de certas linhagens de metabolizar/detoxificar xenobióticos (promutágenos) sem a utilização de sistemas exógenos, pois possuem complexos enzimáticos semelhantes aos encontrados no fígado humano (Valentin-Severin et al., 2003).

Linhagens celulares de ovário de hamster chinês são freqüentemente utilizadas em estudos de mutagênese devido sua facilidade de crescimento e manipulação, além do pequeno número de cromossomos e menor variabilidade genética (Fiore et al., 2002; Aardema et al., 2006). Células de ovário de hamster chinês, ou células CHO, derivam de uma linhagem de células epiteliais obtida por biopsia de ovário de uma hamster chinesa adulta. O seu cultivo teve início em 1957 por TT Puck e colaboradores, na Faculdade de Medicina do Colorado, em Denver -EUA (Puck et al., 1958).

Por outro lado, a linhagem celular derivada de hepatoma humano - HepG2 - é bastante utilizada por preservar os sistemas enzimáticos de metabolização (Uhl et al., 2000). Estas células mantêm as enzimas de fase I e II que participam da ativação e detoxificação de carcinógenos reativos com o DNA (Uhl et al., 2000). Estas enzimas são geralmente perdidas durante o cultivo de células *in vitro*. Assim, o uso das células HepG2 pode reproduzir com maior fidelidade os efeitos de substâncias que necessitam de metabolização para exercer seus efeitos genotóxicos e mutagênicos. As células HepG2, isoladas em 1979 de um hepatoblastoma de um garoto argentino de 11 anos de idade (Aden et al., 1979), têm sido utilizadas com bastante sucesso em diferentes tipos de estudos (Majer et al., 2005) e com diferentes parâmetros de análise (micronúcleo, troca de cromátides irmãs e aberrações

cromossômicas) (Natarajan et al., 1991; Knasmuller et al., 2004). Mais recentemente o teste do cometa foi também realizado nesse tipo celular (Uhl et al., 1999; 2000; Knasmuller et al., 2004).

Teste do cometa e do micronúcleo

O teste do micronúcleo é um dos métodos mais utilizados para avaliar danos cromossômicos. Trata-se de uma metodologia simples, rápida e capaz de detectar, além de quebras, perdas cromossômicas (aneuploidia). Os micronúcleos constituem-se em pequena massa nuclear delimitada por membrana, separada do núcleo principal e formada durante a telófase da mitose ou meiose, quando o envelope nuclear é reconstituído ao redor dos cromossomos das células filhas; são resultantes de fragmentos acêntricos ou de cromossomos inteiros não incluídos no núcleo principal. Como o micronúcleo (MN) se manifesta apenas em células que passam por um ciclo de divisão, foi desenvolvido um método que detecta tais células por apresentarem dois núcleos quando têm sua citocinese bloqueada. O bloqueio da citocinese é obtido pela adição de citocalasina B (isolada do fungo *Helminthosporum dermatoideum*) ao meio de cultura, a qual impede a polimerização dos filamentos de actina que formam o anel de microfilamentos ao final da divisão celular (Falck et al., 1997). Essa técnica permite maior precisão na avaliação dos danos, pois os resultados não sofrem interferências de alterações na cinética celular causadas por citotoxicidade. Além disso, o teste do MN pode ser aplicado a vários tipos celulares podendo ser útil para avaliar quebras e perdas cromossômicas, rearranjo (pontes nucleoplasmáticas), inibição da divisão celular, amplificação de DNA e células necrótica e apoptótica (Fenech et al., 2005).

Por outro lado, o teste do cometa vem também se tornando uma ferramenta importante para estudos dos mecanismos de genotoxicidade e carcinogênese. O ensaio tem se mostrado

bastante sensível para detectar danos primários no DNA de células individualizadas (Tice et al., 1991; Moler et al., 2006).

Rydberg e Johanson (1978) foram os primeiros pesquisadores a utilizarem um teste para quantificar diretamente danos no DNA em células individualizadas. Com a finalidade de aumentar a sensibilidade do teste para a detecção desses danos, Ostling e Johanson (1984) desenvolveram uma técnica utilizando eletroforese em microgel que, após a introdução de novas variáveis, passou a ser conhecida como o teste do cometa. A técnica em microgel de agarose envolve eletroforese em condições alcalinas (pH > 13), a qual permite detectar quebras de fita simples, dupla e sitios álcali-lábeis (Moller et al., 2006). O método baseia-se na lise, eletroforese e marcação fluorescente do DNA de uma pequena quantidade de células suspendidas numa fina camada de agarose e colocadas sobre uma lâmina de microscópio. A corrente elétrica faz com que fragmentos de DNA migrem mais rapidamente para um dos pólos resultando em imagens com aparência de cometas (Tice et al, 1991)

Agentes genotóxicos e carcinogênicos

No presente estudo, o potencial protetor do licopeno foi avaliado sobre o efeito de compostos reconhecidamente genotóxicos, mutagênicos e carcinogênicos, que atuam por diferentes mecanismos de ação. Foram eles: o peróxido de hidrogênio (H_2O_2), o metil metano sulfanado (MMS), a 4-nitroquinolina (4-NQO) e a dietilnitrosamina (DEN).

Peróxido de hidrogênio (H_2O_2)

O peróxido de hidrogênio é um composto que interage diretamente com o DNA e causa grandes danos à molécula pela formação de espécies de oxigênio altamente reativas. Esse estresse oxidativo pode induzir quebras de fita simples ou dupla (SSBs e DSBs) e ainda causar danos às bases do DNA. Mais de 20 tipos de danos de base já foram identificados após

o estresse oxidativo. O dano mais comum que ocorre em purinas é o 7,8-dihidro-8-oxoguanina (8-oxo-G), enquanto o mais freqüente nas pirimidinas é a formação da timina glicol (Tg) (Slupphaug et al., 2003).

Metil Metano Sulfonado (MMS)

O metil metano sulfonado é um agente alquilante do DNA, bastante utilizado nos últimos anos em experimentos de mutagênese e de recombinação gênica. O MMS transforma a base guanina em 7-metilguanina e a adenina em 3-metilguanina, causando, respectivamente, pareamento errôneo de bases e bloqueio da replicação do DNA. Os danos causados por agentes alquilantes são predominantemente reparados pelo sistema de reparo por excisão de bases (Lundim et al., 2005).

4-nitroquinolina óxido (4-NQO)

O óxido de nitroquinolina é um composto eletrofílico com grande ação carcinogênica e mutagênica, e que mimetiza a ação da radiação UV. A 4-NQO forma aductos de DNA que causam vários tipos de lesões, como quebra de fita simples, formação de dímeros de pirimidina, formação de sítios abásicos e oxidação de bases. Essas lesões podem ser rapidamente reparadas pelo sistema de excisão de bases ou nucleotídeos (Mambo et al., 2003). Embora a 4-NQO necessite ser metabolizada a 8-hidroxideoxiguanosina para exercer o seu potencial carcinogênico por danos oxidativos (Arima et al., 2006), a mesma é considerada um mutágeno de ação direta (Gomes-Carneiro et al., 2006).

Dietilnitrosamina (DEN)

A dietilnitrosamina é um potente carcinógeno genotóxico utilizado como agente iniciador em protocolos de hepatocarcinogênese. Após metabolização, é altamente reativa

com o DNA, formando, de maneira proporcional à dose, aductos pró-mutagênicos como O^6-etil-dioxi-guanosina e a O^4- e O^6-etil-dioxi-timidina. A bioativação da DEN é realizada por isoenzimas (CYP2E1) do citocromo P450 (Verna et al., 1996).

IV - OBJETIVOS DO ESTUDO

Objetivos Gerais

Com base nas informações apresentadas, o presente estudo objetivou avaliar o efeito protetor do licopeno contra danos quimicamente induzidos no DNA *in vivo* e *in vitro* sobre a hepatocarcinogênese química experimental.

Objetivos Específicos

- avaliar o efeito antigenotóxico (teste do cometa) e antimutagênico (teste do micronúcleo) do licopeno em células de ovários de hamster chinês (CHO) e células de hepatoma humano (HepG2) expostas ao peróxido de hidrogênio, metilmetanosulfonato, óxido de nitroquinolina e dietilnitrosamina;

- avaliar o efeito protetor do licopeno sobre danos no DNA e sobre lesões pré-neoplasicas GST-P positivas induzidos pela dietilnitrosamina em modelo de hepatocarcinogênese, em ratos Wistar machos.

V- ARTIGO 1:

De acordo com as normas da revista: Cancer Epidemiology, Biomarkers & Prevention

Lycopene inhibits DNA damage in liver of male Wistar rats submitted to a medium-term hepatocarcinogenesis assay

Abstract

Lycopene, an acyclic isomer of beta-carotene, with no vitamin A activity, has been shown to exert antioxidant, antimutagenic and anticarcinogenic activities both in *in vitro* and *in vivo* systems. However, the results concerning its chemopreventive potential on chemical hepatocarcinogenesis are ambiguous. The aim of the present study was to investigate the antigenotoxic and anticarcinogenic effects of dietary lycopene in liver of Wistar rats (medium term hepatocarcinogenesis assay) treated with the hepatocarcinogen diethylnitrosamine (DEN). Three treatment protocols were used: lycopene before, after, or simultaneously to DEN. Number and area of glutathione S-transferase positive (GST-P+) liver foci (preneoplastic lesion) and level of DNA damage in liver cells were used as end-points. The results showed that treatments with lycopene, at concentrations of 30, 100, and 300 ppm, did not reduce the number and area of GST-P+ liver foci. However, a significant reduction of DEN-induced DNA damage was detected when the highest dose of the carotenoid was administered before the carcinogen. The data suggest a chemopreventive effect of lycopene

against DNA damage, but no clear effectiveness on the hepatocarcinogenesis induced by DEN.

Key words: chemoprevention; diethylnitrosamine; DNA damage; GST-P liver foci; hepatocarcinogenesis; lycopene.

1. Introduction

In the past decades a number of DNA-protective and anticarcinogenic effects have been detected in plant-derived foods (1 .Knasmuller et al., 2002). Each of the classes of plant-derived foods may have unique phytochemical that interact with the host to confer preventive benefit by regulating enzymes important in metabolizing xenobiotics and carcinogens, by modulating nuclear receptors and cellular signaling of proliferation and apoptosis, and by acting indirectly through antioxidant actions that reduce proliferation and protect DNA from damage (2 .Aggarwal and Shishodia, 2006). Epidemiological and experimental studies have confirmed that natural constituents containing in a large percentage of fruit and vegetables reduce the risk of many types of cancer and other degenerative human diseases (2. Aggarwal and Shishodia, 2006).

Lycopene is a natural pigment synthesized by plants and microorganism, and mainly found in tomatoes (3. Gruenwald et al., 2003). It is a carotenoid, an acyclic isomer of β-carotene, with no vitamin A activity (4. Rao & Argawal 2000). Although the antioxidant properties of lycopene are thought to be primarily responsible for its beneficial properties, some studies have suggested that mechanisms such as modulation of intercellular gap junction communication (5. Zhang et al., 2002), hormonal and immune system (6. Levy et al., 1995), and metabolic pathways may also be involved (7. Astorg et al., 1997).

Lycopene consumption seems to be associated with lower risk for several cancers, particularly those of the gastrointestinal tract, lung, urinary bladder and prostate (8 Bhuvaneswari & Nagini, 2005). However, with regard the hepatocarcinogenesis, the results have proven ambiguous. Gradelet et al. (9) have reported no significant effect of this carotenoid on aflatoxin B1-induced liver pre-neoplastic foci in rats; no protective effect in the spontaneous hepatocarcinogenesis model has been also described (10. Watanabe et al., 2001). Contrarily, other authors have showed that dietary lycopene significantly reduced the incidence of diethylnitrosamine (DEN)-induced liver preneoplastic foci (7. Astorg et al., 1997; 11. Toledo et al., 2003). Nevertheless, similar effect was not detected when the carotenoids was administrated via drink water (12. Kim et al., 1997).

The DEN-partial hepatectomy (PH) model has proven to be a consistent bioassay for the detection of chemical hepatocarcinogens and for the assessment of the beneficial potential of chemopreventive agents (13. 14 Ito et al., 1988, 1996; 15. Moore et al., 1999). This 8-week-long medium-term rat liver assay uses as endpoint putative preneoplastic foci of altered hepatocytes that express the placental form of the enzyme glutathione S-transferase (GST-P) (13. Ito et al., 1988). It has been indicated as a practical approach for the assessment of the potential hazard or benefit of chemicals, when associated with other surrogate end-points (15. Moore et al., 1999). On the other hand comet assay is a technically simple and fast methodology that detects primary DNA damage in many cell types, both *in vitro* and *in vivo* (16. Tice et al., 1991; 17. Moler et al., 2006). The simple version of the alkaline comet assay may detect DNA strand breaks, alkaline labile sites, transient repair sites (17. Moler 2006).

The present investigation was designed to evaluate antigenotoxic and anticarcinogenic potential of lycopene in rat liver by using DNA damage (comet assay) and GST-P positive foci as end-points.

2. Material and Methods

2.1. Animals

Seven weeks old male Wistar rats, weighting approximately 80 g, were obtained from Centro Multidisclipinar de Investigação Biológica (CEMIB-UNICAMP, Campinas - SP, Brazil) and acclimated for a two weeks period before the beginning of the experiments. Animals were maintained in an experimental room under controlled conditions of temperature (22 ± 2^{o} C), humidity (50 ± 10%), and 12 h light/dark cycle, with *ad libitum* access to water and commercial diet (NUVILAB – Nuvital, Curitiba - PR, Brazil). Body weight, and water and food consumption were measured three times a week during the experimental period. The University Ethical Committee for Animal Research approved the protocols used in this study.

2.2 Chemicals

Diethylnitrosamine (DEN; CAS 7756; Sigma – USA) was used to induce liver preneoplastic lesion. The carcinogen, diluted in physiological saline solution, was i.p. injected at a single dose of 20 (for the comet assay) or 100 mg/kg body weight (GST-P foci and cellular kinetic assays).

Immunohistochemical staining for detecting the GST-P positive foci hepatocytes, was performed using the anti-rat GST-P primary antibody purchased from Medical Biological

Laboratories Co. (Tokyo – Japan); biotinilated anti-rabbit and anti-mouse IgG secondary antibody, and avidin-biotin peroxidase complex kit from Vector Laboratories Inc. (CA, USA) were also used.

Lyc-o-mato®, from Lycored Natural Products Industries (Israel), containing 6% lycopene, was mixed and homogenized into a powdered commercial diet (NUVITAL) at concentrations of 30, 100, and 300 ppm. After pelletization, diet was given to the animals from different groups.

2.3. Experimental designs

A - Hepatocarcinogenesis assay (DEN-parcial hepatectomy model; based on Ito et al., 13)

Animals were randomly distributed into nine groups (10 rats in each) (Figure 1): Group 1, negative control, received an i.p. injection of NaCl 0.9% at the 2nd week, and regular commercial diet during the entire experimental period (10 weeks); Group 2, positive control, was treated with a single dose of DEN (100 mg/kg b.wt.) at the 2nd week, and it was fed with commercial diet during the 10 weeks; Groups 3, 4, and 5 (pre-initiation protocol) received lycopene at concentration of 30, 100, and 300 ppm, respectively, during the first 2 weeks before the single dose of DEN (100 mg/kg b.wt.) and, then, regular diet until the end of the experimental period; Groups 6, 7, and 8, initially fed with regular diet (during the first 2 weeks), received a single dose of DEN at the 2nd week and, then, it was fed with diet containing lycopene at concentrations of 30, 100, and 300 ppm, respectively, for 8 weeks (post-initiation protocol); Group 9, was fed with a diet containing 300 ppm lycopene during 10 weeks, and received an i.p. injection of NaCl 0.9% at the 2nd week. Animals from all groups were submitted to 70% partial hepatectomy at the 5th week, and

were sacrificed at the end of the experimental period (10 weeks) by an i.p injection of pentobarbital (40mg/kg, body wt).

B - Genotoxicity Assay

Rats were distributed into seven groups of 5 animals (Figure 2) groups 1 (negative control) and 2 (positive control) received regular diet during 2 weeks and, 4 h before the sacrifice, an i.p. injection of 0.9%NaCl and DEN (20 mg/kg), respectively; group 3, 4, and 5 were fed with diets containing 30, 100, and 300 ppm lycopene, respectively, during 2 weeks and, 4h before the sacrifice, they were i.p. injected with DEN; Group 6 was fed with 300 ppm lycopene during the entire experimental period.

2.4. Immunohistochemical staining (GST-P and morphometry)

At necropsy, liver from animals of the experimental designs *A* and *B* was immediately excised and weighted. Sagital section of each lobe was collected and fixed in 10% phosphate-buffered formalin (24h). Paraffin-embedded liver samples were cut into 5 µm thick sections for subsequent immunohistochemical staining for GST-P positive foci using the avidin-biotin complex (ABC) (18. Hsu et al., 1981). Number and area of GST-P positive foci greater than 0.15 mm in diameter, were measured using a color video image processor (KS-300, Carl Zeiss, Germany). Data were expressed as number (N/cm^2) and area (mm^2/cm^2) of GST-P positive foci *per* liver section (experimental design *A*), and GST-P positive hepatocytes *per* liver section (experimental design *B*).

2.5. Liver and peripheral blood cells collection for the comet assay

The liver was excised, washed in saline solution, and a small fragment of the left lobule was transferred to a Petri dish kept on ice. The fragment was washed, minced, and suspended into 1 ml of Hank´s balanced salt solution (HBSS), supplemented with 20 mM EDTA and 10% DMSO. Liver tissue was minced again and the suspension containing isolated cells was transferred to a tube maintained onto ice until the preparation of the slides. Blood samples from the negative control and the group treated only with 300 ppm lycopene were collected via periorbital vein plexus immediately before sacrifice.

2.6. Comet assay

DNA damage was measured using the comet assay under alkaline conditions (19. Singh et al., 1988). Volumes of 5 and 10 µl of peripheral blood and liver cell suspensions, respectively, were mixed with 120 µl of 0.5% low melting point agarose (37^{o}C), layered onto pre-coated slides with normal melting point agarose, covered with coverslip, and placed at 4 ^{o}C, for 5 min, for agarose solidification. Coverslip was gently removed, and slides submersed into a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris 10% DMSO, 1 % Triton X-100) for 24h. After lysis, slides were briefly washed in PBS and placed onto a horizontal electrophoresis unit filled with fresh electrophoresis alkaline buffer (300 mM NaOH and 1mM EDTA, pH >13), for 20 min, at 4ºC

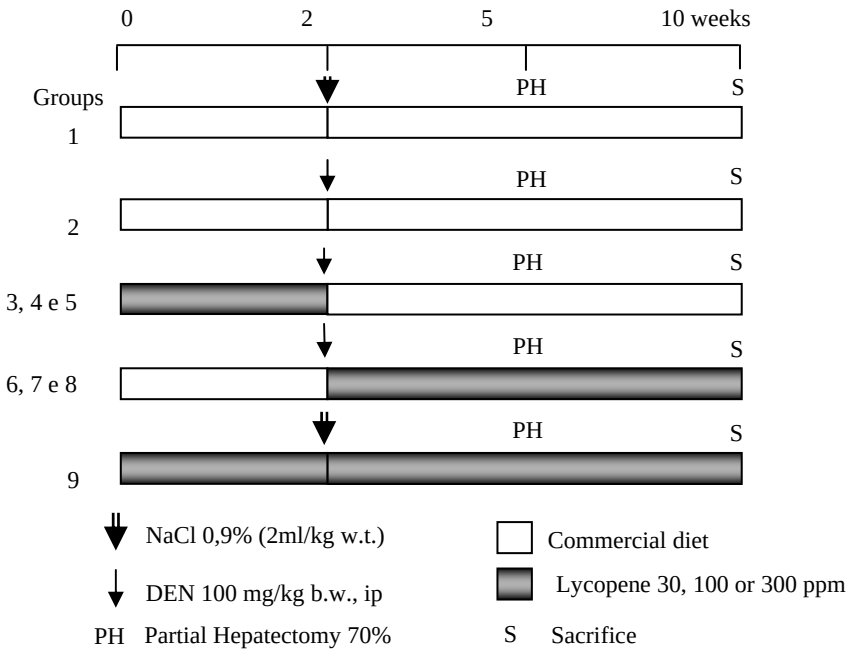

Fig. 1. Experimental design: hepatocarcinogenesis

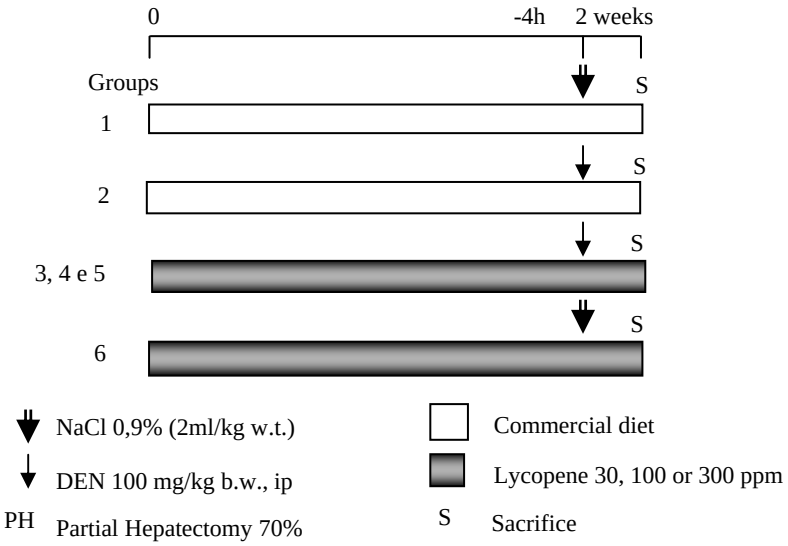

Fig. 2. Experimental design: genotoxicity assay

Electrophoresis was conducted at 4ºC, for 20 min, at 25 V and 300 mA. Slides were neutralized into a buffer (0.4 M Tris at pH7.5), dehydrated in absolute ethanol, and dried at room temperature. Before analysis, the slides were stained with 50µl ethidium bromide (20 µl/ml). Fifty randomly selected cells (nucleoids) per animal were examined at 400x magnification in a fluorescence microscope, using an automated image analysis system (Comet Assay II, Perceptive Instrument, UK). Two parameters were selected as indicators of DNA damage: tail intensity (% tail DNA, in % pixels), and tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units). Since none of the groups shows significant difference between these parameters, we chose tail moment for the presentation of the results.

2.7. Statistical analysis

The statistical analysis was done using the Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA, USA). Data were compared using one-way ANOVA (body and liver weights, and food consumption), and Kruskal-Wallis test (GST-P foci, comet assay, and mitosis and apoptosis data). The contrasts between groups were analyzed by Student-Newman-Keuls method. Significant differences was assumed when $P < 0.05$.

3. Results

Table 1 presents the final body weights, body weight gains, relative liver weights, food consumption and ingestion of lycopene from animals sacrificed 5 weeks after partial hepatectomy (PH). The estimated daily lycopene ingestions were 2.5, 8.7, and 25.17 mg/Kg b.wt, for diets containing 30, 100, and 300 ppm, respectively. None difference was detected between positive and negative control for the end-points. Significant reductions of relative liver weights were observed in the group treated only with the higher concentration of lycopene, when compared to the negative control and in those three groups treated lycopene after DEN injection (post-initiation protocol), when compared to the positive control.

Table 2 presents number and area of liver GST-P positive foci, and level of DNA damage (tail moment) in liver and blood cells of rats treated with lycopene and DEN. For GST-P+ foci, increased values were only observed in the positive control, when compared to the negative control and to the group treated only with the highest concentration of lycopene (300 ppm). For DNA damage, besides the statistically significant difference between positive and negative controls, data showed a significant reduction in liver cells when the animals were treated with lycopene (300 ppm) before DEN (pre-initiation phase). In blood cells, no difference was detected between the negative control and the group treated only with lycopene.

Table 1. Means (± SD) of body weight, body weight gain, relative liver weight, food consumption, and lycopene intake.

Treatment	Final body weight (g)	Body weight gain (g)	Relative liver weight (g)	Food consumption (g/rat/day)	Lycopene intake (mg/kg/day)
NaCl 0.9%	433.0 ± 22.6[a]	148.3 ± 17.3[a]	3.06 ± 0.19[a]	26.5 ± 26.1[a]	-
LYC3[1]+NaCl 0.9%	401.9 ± 28.6[a]	130.8 ± 24.6[a]	2.95 ± 0.15[b]	26.6 ± 26.0[a]	23.6 ± 3.7
DEN[2]	410.8 ± 66.2[a]	133.1± 22.1[a]	3.21 ± 0.30[a]	25.9 ± 25.0[a]	-
Pre-initiation					
LYC1+DEN	393.2 ± 41.1[a]	122.2 ± 18.2[a]	3.03 ± 0.18[a]	26.6 ± 25.1[a]	3.05 ± 0.35
LYC2+DEN	397.3 ± 26.4[a]	131.2 ± 22.2[a]	3.12 ± 0.17[a]	27.8 ± 27.1[a]	10.85 ± 1,34
LYC3+DEN	385.8 ± 33.0[a]	118.2 ± 20.9[a]	3.00 ± 0.21[a]	25.2 ± 25.0[a]	30.22 ± 3,57
Post-initiation					
DEN+LYC1	387.3 ± 27.1[a]	119.2 ± 21.6[a]	2.94 ± 0.26[b]	26.2 ± 25.3[a]	2.25 ± 0,31
DEN+LYC2	396.8 ± 30.4[a]	127.7 ± 23.5[a]	2.93 ± 0.15[b]	26.4 ± 25.7[a]	7.68 ± 0.96
DEN+LYC3	396.1 ± 34.8[a]	126.5 ± 26.1[a]	2.90 ± 0.13[b]	26.5 ± 25.9[a]	22.63 ± 3.30

[1] LYC1, LYC2, LYC3: lycopene (LYC1: 30 ppm; LYC2: 100 ppm; LYC3: 300 ppm).

[2] DEN: diethylnitrosamine (100 mg/kg, b. wt., i.p.) - positive control. Columns with values followed by different letters indicate significant difference ($p < 0.01$). One-Way ANOVA, Kruskall Wallis and Student-Newman-Keuls Method.

Table 2. Number and area of hepatic GST-P positive foci, and level of DNA damage (tail moment) in liver and blood cells of rats treated with lycopene and diethylnitrosamine.

Treatment	Number of animals	GST-P positive foci		DNA damage	
		Number (N/cm^2)	Area (mm^2/cm^2)	Liver cells	Blood cells
NaCl 0.9%	8	0.41 ± 0.53^a	0.007 ± 0.01^a	0.39 ± 0.13^a	0.36 ± 0.07^a
LYC3[1]	10	0.23 ± 0.29^a	0.001 ± 0.001^a	0.31 ± 0.06^a	0.37 ± 0.03^a
DEN[2]	10	8.93 ± 2.27^b	0.07 ± 0.02^b	2.30 ± 0.53^b	-
Pre-initiation					
LYC1+DEN	10	10.16 ± 4.98^b	0.09 ± 0.06^b	1.96 ± 0.26^b	-
LYC2+DEN	10	10.71 ± 3.72^b	0.10 ± 0.04^b	2.05 ± 0.44^b	-
LYC3+DEN	10	8.78 ± 2.63^b	0.07 ± 0.03^b	1.58 ± 0.32^c	-
Post-initiation					
DEN+LYC1	10	8.23 ± 4.23^b	0.09 ± 0.04^b	-	-
DEN+LYC2	10	10.27 ± 4.14^b	0.10 ± 0.06^b	-	-
DEN+LYC3	10	9.18 ± 3.25^b	0.09 ± 0.04^b	-	-

[1]LYC1, LYC2, LYC3: lycopene (LYC1: 30 ppm; LYC2: 100 ppm; LYC3: 300 ppm). [2]DEN: diethylnitrosamine (GST-P assay: 100 mg/kg, b.w.; comet assay: 20 mg/kg, b.w.). Values are mean ± SD. Columns with values followed by different letters indicate significant difference ($p < 0.01$).Kruskal-wallis test, One-Way ANOVA and Student-Newman-Keuls methods.

Discussion

Lycopene is a carotenoid mainly found in tomatoes, and is one of the most efficient antioxidants (8. Bhuvaneswari and Nagini, 2005). Epidemiological and experimental studies have suggested a strong association between lycopene and protection against a variety of cancers (8. Bhuvaneswari & Nagini, 2005). However, for liver cancer the results have proven ambiguous. Thus, this study evaluated the chemopreventive effect of lycopene in rats submitted to a hepatocarcinogenesis model.

Initially, our data showed difference in the relative liver weights among the positive control and those groups treated with lycopene after DEN (post-initiation protocol). This finding should not be considered as an important biomarker of toxicity, since the results of the body gain weight, final body gain and food consumption did not show any significant difference. Moreover, no indications of toxicity was detected in the histopathological analysis. According to epidemiological studies, neither adverse effect upon lycopene supplementation nor lycopene toxicity has been reported (20 Banhegyi, 2005). Mellert et al. (2002. 21) have described that male and female Wistar rats treated with a synthetic crystalline lycopene, at dose levels of 500, 1500, and 3000 mg/kg body weight/day, during a 13-week, do not present significant toxicological findings.

Lycopene did not present protective effect on GST-P positive hepatocytes foci development. Similar results were observed in the hepatocarcinogenesis assay, in which no difference in the number and area of the GST-P positive foci were also detected in pre- and post-initiation protocols. Same findings have been reported by Gradelet et al. (9. 1998), showing that lycopene (300 mg/Kg b.w.) is not able to reduce the number and size of aflatoxin B1-induced liver preneoplastic foci. Furthermore, (10. Watanabe et al. (2001) using a spontaneous hepatocarcinogenesis model have also showed that long-term administration of lycopene (0.005% per 6 weeks) does not reduce the risk of cancer in

Long-Evans Cinnamon rats. Contrarily, Astorg et al., (7) have demonstrated a significant decrease in the size of DEN-induced gamma-glutamyl transpeptidase- and glutathione S-transferase-positive foci, and a reduction of liver volume occupied by these foci, in rats under diets containing 300 ppm lycopene. According to these authors, lycopene does not appear to act through its antioxidant properties, but rather through its modulating effect on the liver enzyme-activating DEN (cytochrome P-450 2E1). Inhibitory effect of lycopene on hepatic preneoplastic lesions (GST-P + foci) induced in Wistar rats has been also described (11. Toledo et al., 2003).

The apparent discrepancy concerning the chemopreventive effect of lycopene on liver carcinogenesis could be explained by differences on its chemical reactivity. Herein, it must be taken into account that different chemicals have been used to initiate hepatocarcinogenesis. Perhaps, lycopene, dependent of its antioxidant ability, has reacted differently with the carcinogens when administered before or simultaneously. On the other hand, the doses used during initiation and promotion phases could not be sufficient to inhibit development of preneoplastic lesions (10. Watanabe et al., 2001).

Oxidative stress is described to play an important role in the process of hepatocarcinogenesis (22. Shiota et al., 2002). In addition, in DEN-induced rat hepatomas a general collapse of the enzymatic antioxidant systems has been also observed (23. Boitier, et al., 1995). Furthermore, it is known that DEN biotransformation produces promutagenic metabolites that can react to DNA inducing damage and initiation of liver carcinogenesis (24. Verna et al., 1996). Therefore, the investigation of DNA lesions during hepatocarcinogenesis should be emphasized (25. Uemura et al., 1996). When we evaluated the effect of lycopene on DEN-induced primary DNA damage in liver cells, our results showed a protective activity of the highest concentration of lycopene (300 ppm) administered before the carcinogen. Other authors had also investigated the effect of

lycopene preventing DNA damage. 26. Park et al. (2005) have demonstrated a reduction of DNA damage in Hep3B human hepatoma cell line treated with the carotenoid (0.1-50 μM); animals treated with lycopene (70 mg/Kg b.w.) presented lower level of DNA strand breakage in liver cells, as depicted by the comet assay (11. Toledo et al., 2003); five days pretreatment with lycopene (10 mg/kg body weight, ip) had almost completely prevented liver biomolecule oxidative damage induced by ferric nitrilotriacetate (Fe-NTA) (27. Matos et al., 2001).

The chemoprotective activity of lycopene currently observed could be related to its antioxidant potential. However, several studies have demonstrated that the inhibitory effects of lycopene on carcinogenesis could also involve upregulation of detoxification systems (8. Bhuvaneswari & Nagini, 2005). Thus, besides the antioxidant properties, carotenoids can prevent initiation of liver carcinogenesis by modulating the metabolism of the carcinogen through activation of the cytochrome P-450 2E1 enzymatic complex (7. Astorg et al., 1997).

In conclusion, our results demonstrated a chemopreventive effect of lycopene on the initiation, but not on the promotion step of DEN-induced hepatocarcinogenesis. Other studies are being carried out, trying to elucidate the mechanisms of action of lycopene on carcinogenesis.

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VI – ARTIGO 2:

De acordo com as normas da revista: Mutation Research

Antigenotoxicity and antimutagenicity of lycopene in HepG2 cell line evaluated by the comet assay and micronucleus test

Abstract

Epidemiological studies have provided evidence that high consumption of tomatoes effectively reduces the risk of reactive oxygen species (ROS)-mediated diseases such as cancer. Tomatoes are rich sources of lycopene, a stable and potent singlet oxygen- quenching carotenoid. In addition to its antioxidant properties, lycopene shows an array of biological effects including antimutagenic and anticarcinogenic activities. In the present study, the chemopreventive action of lycopene was examined on DNA damage and clastogenic or aneugenic effects of hydrogen peroxide (direct mutagen) and n-nitrosodiethylamine (DEN; indirect mutagen) in the metabolically competent human hepatoma cell line (HepG2 cells). Lycopene (97%), at concentrations of 10, 25, and 50 μM, was tested under three protocols: before, simultaneously, and after treatment with the mutagen, using the comet and micronucleus assays. The results showed that lycopene significantly reduced the genotoxicity and mutagenicity of H_2O_2 in all of the conditions tested. For DEN, significant reductions of primary DNA damage (comet assay) were detected when the carotenoid (all of the doses) was added in the cell culture medium before or simultaneously with the mutagen. In the micronucleus test, the protective effect of lycopene was observed only when added prior to DEN treatment. Since DEN is a procarcinogen that requires biotransformation to form DNA

adducts, we believe that lycopene, besides its antioxidant ability, might also act by modulating the enzymes responsible for the mutagen metabolism. In conclusion, our results suggest that lycopene is a suitable agent for preventing chemically nduced DNA and chromosome damage although further studies should be performed to better understand the mechanisms and conditions underlying its chemopreventive activity.

Key words: lycopene, antigenotoxicity, antimutagenicity, HepG2 cells.

1. Introduction

Since the hypothesis by Peto et al. (1) that ß-carotene might reduce the incidence of cancer, several epidemiological studies have shown that cancer risk is inversely related to the consumption of foods rich in carotenoids. Lycopene, a carotenoid mainly found in tomatoes, is an acyclic isomer of β-carotene, but with no vitamin A activity (2). It is a natural pigment synthesized by plants and microorganism (3), and one of the most potent antioxidants (4).

In addition to its antioxidant properties, lycopene has an array of biological effects including cardioprotective, anti-inflammatory, antimutagenic and anticarcinogenic activities (5). The mechanisms underlying the inhibitory effects of lycopene on carcinogenesis and mutagenesis could involve radical oxygen species (ROS) scavenging, up-regulation of detoxification (6), interference with cell proliferation (7), induction of gap-junctional communication (8), inhibition of cell cycle progression, and modulation of signal transduction pathways (5).

Different cell types have been used to determine genotoxicity and antigenotoxicity of some natural compounds (9). Since many carcinogens require metabolic activation to react with DNA, the use of cells that possess endogenous biotransforming activity can reduce some of the problems associated with the use of exogenous activation mixtures such as S9 mix (10).

Human hepatoma cell lines, the most promising being the HepG2 cell line, appear to be a practical alternative for assessing genotoxicity or antigenotoxicity (11). HepG2 cells are easy to handle and contain several enzymes responsible for the activation of various xenobiotics (12; 13).

Among short-term mutagenicity/genotoxicity assays, the micronucleus (MN) and the comet assays have been widely used for identifying chemopreventive agents. These two tests are sensitive, easy to perform, and can be carried out with various cell lines, including HepG2 (11; 14). The difference is basically due to variations in the type of DNA alterations detected by these two assays: the MN test detects irreparable lesions that manifest as chromosome aberrations and/or aneugenic effects while the comet assay detects primary DNA lesions. Thus, these assays were used to investigate the chemopreventive activity of lycopene on DNA damage induced in HepG2 cells by a direct (hydrogen peroxide) and an indirect (n-nitrosodiethylamine) mutagen.

2. Materials and Methods

2.1. Cells and culture conditions

HepG2 cells were grown as monolayer cultures in DMEM medium (Cultilab, Brazil) supplemented with 15% fetal calf serum (Cultilab - Brazil) and antibiotics, penicillin (100 U/ml) and streptomycin (0.1 mg/mL). Cells were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. Confluent cells were trypsinized for 10 minutes. Next, 10 mL complete medium was added and cells were centrifuged at 180 *g* for 5 minutes. Cell suspensions at densities of 0.25 x 10^6 cells per well or 1 x 10^6 cells per plate were seeded in 24-well plates and in 94-mm plastic dishes for the comet assay and micronuclei assay, respectively. Subcultures were performed on the day before the experiments.

2.2 Chemicals

Lycopene (> 96%), obtained from Lycored Natural Products (Israel), was dissolved into the culture medium plus Tween 80 (980 µl culture medium + 20 µl Tween) at concentrations of 10, 25, and 50 µM, just before use. The mutagens, n-nitrosodiethylamine (DEN) (Sigma - USA) and hydrogen peroxide (Merck - USA), were dissolved into DMEM medium just before treatment. The doses of mutagens and lycopene were determined by following preliminary studies.

2.3. Cell treatment for the comet assay

The different treatment protocols are summarized in Figure 1. Each protocol was performed in triplicate to ensure reproducibility.

- *Pre-treatment:* twenty-four hours after seeding, the medium was removed and cells were treated for 1 hour with lycopene at one of the three concentrations (10, 25, or 50 µM). Cells were then washed with PBS and treated with DEN (5 µg/mL) or H_2O_2 (0.1 mM) for 1 hour or 10 minutes, respectively, since DEN needs a longer exposure time to exert genotoxicity. After treatments, the cells were washed twice with PBS, tripsinized, centrifuged at 180 *g* for 3 min and resuspended into 100 µL of fresh medium.

- *Simultaneous treatment:* twenty-four hours after seeding, the medium was removed, and cells were simultaneously treated with lycopene (10, 25, or 50 µM) and with DEN (5 µg/mL) or H_2O_2 (0.1 mM) for 1 hour or 10 minutes, respectively. Then, cells were washed twice with PBS, tripsinized, centrifuged at 180 *g* for 3 min and resuspended into 100 µL of fresh medium.

- *Post-treatment:* twenty-four hours after seeding, the medium was removed and cells treated with DEN (5 µg/mL) or H_2O_2 (0.1 mM) for 1 hour or 10 minutes, respectively. Cells were then washed with PBS, and lycopene (10, 25, or 50 µM, in 10 mL medium) was added for 1

hour at 37°C. After this period, cells were washed twice with PBS, tripsinized, centrifuged at 180 *g* for 3 min, and resuspended into 100 µL of fresh medium.

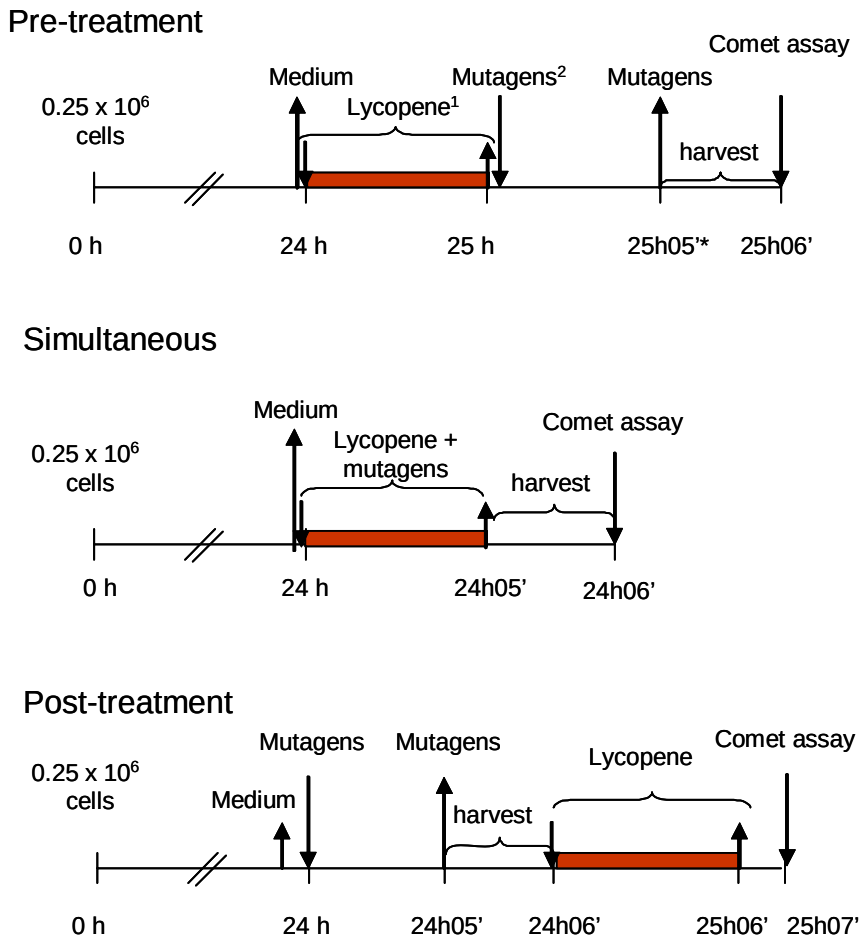

Figure 1: *Single cell gel electrophoresis assay - Experimental design*
[1]Lycopene: 10, 25 and 50 µM; [2]hidrogen peroxide (0.1mM); n-nitrosodiethylamine (5 µg/mL)
*one hour for DEN

The comet assay was performed by following the protocol of Tice et al. (15). Briefly, a volume of 10 µl of cells was mixed with 120 µl of 0.5% low-melting point agarose at 37°C, layered onto a precoated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification at 4°C, the coverslip was removed and the slides immersed into a lysis solution (2.5M NaCl, 100mM EDTA, 10 mM Tris-HCL buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for at least 1 hour. After lysis, slides were exposed to alkaline buffer (pH > 13) for 20 minutes and subjected to electrophoresis to

20 minutes (25V, 300mA). Then, the slides were neutralized with 0.4 M Tris-HCl buffer (pH 7.5), fixed in absolute ethanol and stored at 4^{o}C until analysis. Fifty randomly selected cells per treatment (well) were examined at 400x magnification, on a fluorescence microscope by using an automated image analysis system (Comet Assay II, Perceptive Instruments, UK). The parameter selected as indicator of DNA damage was tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units)

2.4 *Cytokinesis-block micronucleus (CBMN) assay*

The different treatment schedules are summarized in Figure 2.

- *Pre-treatment:* twenty-four hours after seeding, the medium was removed and cells were treated with lycopene at one of the three concentrations (10, 25, or 50 µM). Then, the cells were washed with PBS and treated with DEN (5 µL/mL) or H_2O_2 (0.27 mM) for 1 hour or 10 minutes, respectively. After these treatments, cells were washed twice with PBS, and incubated with medium containing cytochalasin B (final concentration 6 µg/mL) for 48 hours.

- *Simultaneous treatment:* twenty-four hours after seeding, the medium was removed and cells were simultaneously treated with lycopene (10, 25, or 50 µM) and with the mutagen (DEN: 5 µL/mL or H_2O_2: 0.27mM) for 1 hour or 10 minutes. Then, cells were washed twice with PBS and incubated with medium containing cytochalasin B for 48 hours.

- *Post-treatment:* twenty-four hours after seeding, the medium was removed and cells were treated with DEN (5 µL/mL) for 1 hour or H_2O_2 (0.27mM) for 10 minutes. Then, cells were washed with PBS, and lycopene (10, 25, or 50 µM, in 10 mL medium) was added for 1 hour at 37^{o}C. After this period, cells were washed twice with PBS, and incubated with medium containing cytochalasin B for 48 hours.

After harvesting, cells were treated with cold hypotonic solution (KCL 0.075 M) and fixed with methanol:acetic acid (3:1). Coded slides were stained with 3% aqueous Giemsa solution

for 15 minutes, and 1,000 binucleated cells per culture with intact cytoplasm were scored for the incidence of micronucleus formation.

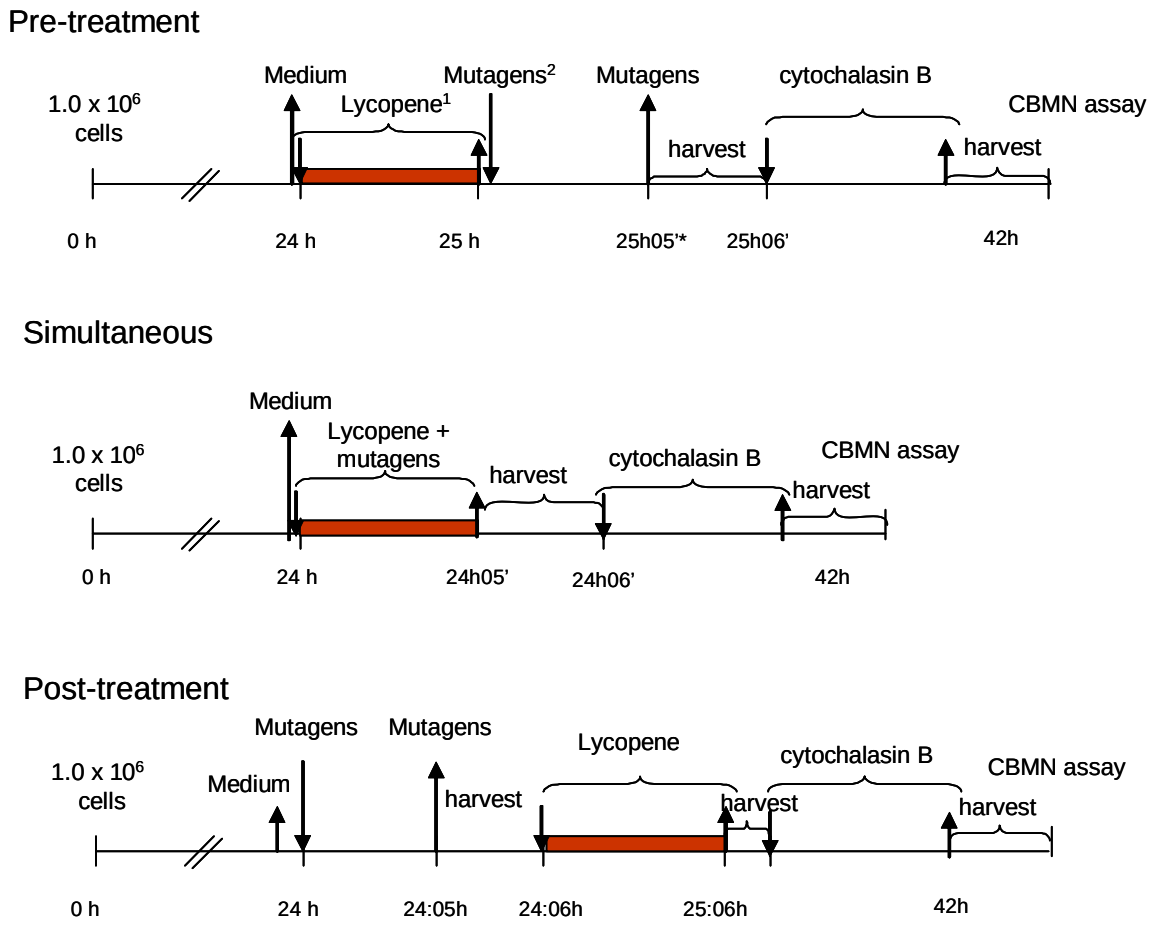

Figure 2: The cytokinesis-block micronucleus (CBMN) assay - *Experimental design*

[1]Lycopene: 10, 25 and 50 µM; [2]hidrogen peroxide (0.27mM); n-nitrosodiethylamine (5 µg/mL)
*one hour for DEN

2.5. Statistical analysis

DNA damage levels (tail moment) were compared between the treatments by using the Kruskal-Wallis test; frequencies of micronucleated cells were analyzed by using the X^2 test. *p* value < 0.05 was considered for statistical significance.

Results

Table I shows the level of DNA damage (tail moment) in HepG2 cells exposed to H_2O_2 or DEN and lycopene (10, 25, and 50 μM) at three different treatment schedules. The results showed that, for all the conditions, lycopene significantly reduced the genotoxicity of H_2O_2 ($P < 0.01$). For DEN, significant reductions of DNA damage were detected only when lycopene was used before or simultaneously with the mutagen ($P < 0.01$).

The frequencies of micronucleated binucleated HepG2 cells after exposure to lycopene (10, 25, and 50 μM) and H_2O_2, or DEN are presented in Table II. Pre and simultaneous treatments with lycopene (all the doses) significantly decreased the frequency of micronucleated cells induced by the direct-acting mutagen H_2O_2. The data also showed a protective effect of lycopene on DEN-induced micronucleated cells, but only when it was used before the indirect-acting mutagen treatment ($P < 0.05$).

Table I. Mean (±SD) DNA damage (tail moment) in HepG2 cells exposed to lycopene and H_2O_2 or DEN

Lycopene treatments	Tail moment (X ± SD)				
	C-	C+	LYC10[1]	LYC25	LYC50
LYC / H_2O_2					
Prior to H_2O_2	0.93±01.03[a]	3.62±2.95[b]	2.58±2.25[c]**	2.35±2.39[c]**	2.01±2.07[c]**
Simultaneously	0.75±1.29[a]	2.89±2.30[b]	1.32±1.55[c]**	1.57±1.49[dc]**	1.97±1.91[dc]**
After H_2O_2	0.36±0.67[a]	2.32±2.32[b]	1.06±1.30[c]**	1.55±1.71[c]**	1.35±1.66[c]**
LYC / DEN					
Prior to DEN	0.56±1.25[a]	2.55±2.68[b]	0.92±1.32 [a]**	0.95±1.34[a]**	1.08±1.6 [a]**
Simultaneously	0.95±1.26[ac]	3.56±2.92[b]	0.83±1.4[a]**	1.46±1.93[ac]**	1.52±1.87 [ac]**
After DEN	0.74±0.94[a]	1.50±2.13 [b]	1.12±1.73[b]	1.33±1.95[b]	2.06±2.63[b]

H_2O_2 (0.1 mM); DEN: n-nitrosodiethylamine (5 µg/mL); C- and C+: negative and positive control, respectively; [1]LYC: lycopene (LYC10: 10µM; LYC25: 25 µM; LYC50: 50 µM); Lines with values followed by different letters indicate statistically significant difference. ***P < 0.001* compared to C+.

Table II. Frequencies of micronuclei in HepG2 cells exposed to lycopene and H_2O_2 or DEN

Lycopene treatments	Micronucleated cells (number per 1,000 cells)				
	C-	C+	LYC10[1]	LYC25	LYC50
LYC / H_2O_2					
Prior to H_2O_2	19.0 ± 2.83[a]	129.3 ± 11.23[b]	111.7 ± 45.94[c]*	80.7 ± 18.34[c]**	68.7 ± 14.57[c]**
Simultaneously	19.0 ± 2.83[a]	129.3±11.23[b]	110.7 ± 1.55 [c]*	99.7 ± 3.51 [c]**	48.0 ± 9.54 [c]**
LYC / DEN					
Prior to DEN	33.5 ± 16.26[a]	107.0 ± 11.13[b]	85,7 ± 4.51 [c]*	84.0 ± 10.53[c]*	72.7 ± 28.15[c]**
Simultaneously	33.5 ± 16.26[a]	107.0 ± 11.13[b]	91.0 ± 11.53[b]	108.0 ± 3.60[b]	101.0 ± 7.21[b]

H_2O_2 (0.27 mM); DEN: n-nitrosodiethylamine (5 µg/mL); C- and C+: negative and positive control, respectively; [1]LYC: lycopene (LYC10: 10µM; LYC25: 25 µM; LYC50: 50 µM); Lines with values followed by different letters indicate significant difference. *$P < 0.05$; **$P < 0.001$ compared to C+.

Discussion

Lycopene is a more stable and potent singlet oxygen-quenching agent when compared to other carotenoids (5). It has been shown to exert a wide range of effects in biological systems, including anticarcinogenic activity (5). Among the mechanisms underlying the inhibitory effect of lycopene on carcinogenesis are ROS scavenging, up-regulation of detoxification systems, interference with cell proliferation, induction of gap-junctional communication, inhibition of cell cycle progression, and modulation of signal transduction pathways (5). In the present study, we investigated the chemopreventive activity of lycopene on DNA damage induced by H_2O_2 (a direct-acting mutagen) and DEN (an indirect-acting mutagen) in HepG2 cells by using two end-points: primary DNA lesions (single- and double-strand breaks and alkali-labile sites) and chromosome breakage or chromosome loss as depicted by the comet assay and micronucleus test, respectively.

HepG2 cells, with endogenous bioactivation capacity, retain many of the morphological characteristics of liver parenchymal cells (16) and contain several enzymes responsible for the activation of various xenobiotics (13; 14). Thus, this cell line has been widely and successfully used for direct or indirect mutagens screening (14; 10) and for identifying antimutagens (11). Some studies have demonstrated the lycopene protective effect on different types of injuries in HepG2 and other hepatic cells. It has been shown that lycopene opposes ethanol-induced oxidative stress and apoptosis in HepG2 cells overexpressing CYP2E1 (17). This carotenoid has also inhibited AFB1-induced toxicity and increased the cellular mitochondrial activity and survival of HepG2 cells (18). In SK-Hep1 human hepatoma cells lycopene has inhibited cell adhesion, invasion and migration (19). Protective effect on DNA damage and cell growth has also been observed in Hep3B cell line (20).

Our data showed that lycopene inhibited both primary DNA damage and the micronucleus when added to the cell culture medium before DEN, and only primary damage in the simultaneous treatment. Herein, the comet assay appeared to be more sensitive than the MN test to detect lycopene protective ability. The time between exposure and cell harvesting might have interfered in this result. DEN, a potent procarcinogen, needs metabolic biotransformation to produce the promutagenic adducts O6-ethyldeoxyguanosine and O4- and O6-ethyldeoxythymidine, which may initiate liver carcinogenesis (21; 22). In a previous study, we have found an antigenotoxic effect of lycopene on DEN-induced primary DNA damage in the liver of Wistar rats, *in vivo* (data still not published). Astorg et al. (6) also demonstrated a protective effect of this carotenoid against DEN-induced neoplasias. However, according to these authors, lycopene does not appear to act through its antioxidant properties, but rather through its modulating effect on the liver enzyme-activating DEN, cytochrome P-450 2E1. These results are in accordance with those of Toledo et al. (23), who

have shown a protective effect of lycopene in rats submitted to the “hepatocyte-resistant” model.

In the comet assay, differently from pre and simultaneous protocols, lycopene did not exert a protective effect when added after DEN, which reinforces its antioxidant activity and its action modulating DEN metabolism. Since DEN needs to be activated to react with DNA (22) and lycopene can modulate the metabolic pathway of indirect mutagens (6), we should suppose that it must be present per occasion of DEN exposure. However, a possible disturbance in the DNA-repair system when the carotenoid was added after DEN treatment cannot be discarded.

When the chemopreventive activity of lycopene was evaluated on the direct-acting mutagen H_2O_2, our data showed a clear protective action. This effect was detected both in the comet and micronucleus assays and at the three protocols used. Since H_2O_2 acts via reactive oxygen species (ROS), our results confirmed the antioxidant ability of lycopene. Previous studies have demonstrated that lycopene can inactivate hydrogen peroxide and nitrogen dioxide (24). Similar results were reported by Mortensen et al. (25), who showed the lycopene ability for scavenging nitrogen dioxide radicals in a pulse radiolysis technique. A protective effect of lycopene on chemically-induced DNA damage was also detected when HT29 cells were pre-incubated with an extract of mixed cruciferous and legume sprouts (rich in lycopene) and then challenged with H_2O_2 (26). In another study, lycopene was found to be at least twice as active as beta-carotene in protecting lymphocytes against NO_2^+ radical (27).

In conclusion, our results showed that lycopene could be a suitable agent for preventing chemically induced DNA and chromosome damage. However, further studies should be performed to better understand the mechanisms and conditions underlying its chemopreventive activity.

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VII – ARTIGO 3:

De acordo com as normas da revista: Food And Chemical Toxicology

Lycopene prevents chemically-induced DNA damage in Chinese hamster ovary cells

Abstract

Lycopene is a natural pigment synthesized by plants and microorganisms, and it is mainly found in tomatoes. It is an acyclic isomer of β-carotene and one of the most potent antioxidants. Several studies have demonstrated the ability of lycopene to prevent chemically induced DNA damage; however, the mechanisms involved are still not clear. In the present study, we investigated the antigenotoxic/antimutagenic effects of lycopene in Chinese Hamster Ovary Cells (CHO) treated with hydrogen peroxide, methylmethanesulphonate (MMS), and 4-nitroquinoline 1-oxide (4-NQO). Lycopene (97%), at concentrations of 10, 25, and 50 μM, was tested under three different protocols: before, simultaneously, and after the treatment with the mutagens. Comet and cytokinesis-block micronucleus assays were used to evaluate the level of DNA damage. Data showed that lycopene reduced the frequency of micronucleated cells induced by the three mutagens. However, this chemopreventive activity was dependent on the concentrations and treatment schedules used. Similar results were observed in the comet assay, although some enhancements of primary DNA damage were detected when the carotenoid was administered after the mutagens. In conclusion, our findings confirmed the chemopreventive activity of lycopene, and showed that this effect occurs under different mechanisms. Nevertheless, the data also show that further studies

should be performed before lycopene can be used as a strategy for preventing chemically induced DNA damage.

Key words: Lycopene; DNA damage; Antigenotoxicity, Antimutagenicity; CHO cells

I. Introduction

Dietary chemoprevention has emerged as a cost-effective approach to control most prevalent chronic diseases, including cancer. In particular, tomato and tomato products are recognized to confer a wide range of health benefits (Bhuvaneswari e Nagini, 2005). Epidemiological studies have provided evidence that high consumption of tomatoes effectively decrease the risk for cardiovascular disease and cancer by improving the antioxidant capacity (Bhuvaneswari e Nagini, 2005). Tomatoes are rich sources of lycopene, a natural pigment synthesized by plants and microorganisms (Gruenwald et al., 2003). It is an acyclic isomer of β-carotene with no vitamin A activity (Rao e Argawal 2000), but one of the most potent antioxidants.

It has been suggested that lycopene can prevent carcinogenesis by protecting critical biomolecules including DNA (Di Mascio et al., 1989). The anticancer activity of this carotenoid has been demonstrated both *in vitro* and *in vivo* (Bhuvaneswari e Nagini, 2005). It is believed that the mechanisms underlying the inhibitory effects of lycopene on carcinogenesis and mutagenesis could involve reactive oxygen species (ROS) scavenging, up-regulation of detoxification systems (Astorg et al., 1998), interference with cell proliferation (Pastori et al., 1997), induction of gap-junctional communication (Zhang et al., 1991), inhibition of cell cycle progression, and modulation of signal transduction pathways (Bhuvaneswari e Nagini, 2005).

In the present study we evaluated the ability of lycopene to prevent chemically induced primary DNA damage (as detected by the comet assay) and chromosome breakage or

loss (as detected by the micronucleus assay) in CHO cells. Besides its protective capacity, we tried to better understand the mechanisms involved in its chemopreventive activity.

2. Materials and Methods

2.1. Cell and culture conditions

CHO K-1 cells were grown to confluence in 94 mm plastic Petri dishes using Ham´s F10 medium supplemented with 10% fetal calf serum (Cultilab - Brazil) and antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/mL) at 37^{o}C in a humidified atmosphere of 5% CO_2. Confluent cells were detached with 0.15% trypsin (Cultilab - Brazil) for 5 minutes, 10 mL complete medium was added, and the suspension was centrifuged at 180 *g* for 5 minutes. Cells were seeded in 24-well plates at a density of 0.25 x 10^6 cells/well for the single cell gel electrophoresis assay, or in plastic Petri dishes (94mm) at a density of 1 x 10^6 per plate for the micronucleus assay.

2.2 Chemicals

Lycopene (> 96%), purchased from Lycored Natural Products (Israel), was dissolved into culture medium plus Tween 80 (980 µl culture medium + 20 µl Tween), at concentrations of 10, 25, and 50 µM, just before use. The mutagens, methyl methanesulphonate (MMS) (Sigma - USA), 4-nitroquinoline 1-oxide (4-NQO) (Sigma - USA) and hydrogen peroxide (Merck - USA), were dissolved into Ham´s F10 medium just before treatment. The doses of mutagens and lycopene were determined by following preliminary studies.

2.3. Single cell gel electrophoresis (comet) assay - Experimental design

The treatment protocols are summarized in Figure 1. Each protocol was performed in triplicate to ensure reproducibility.

- *Pre-treatment:* twenty-four hours after seeding, the medium was removed, and the cells were treated with lycopene (10, 25, or 50 µM) for 1 hour. Then, cells were washed with PBS and treated with MMS (80 µg/mL), 4-NQO (0.01 µM), or H_2O_2 (0.6mM) for 5 minutes. Further, cells were washed twice with PBS, detached with trypsin, centrifuged at 180 *g* for 3 min, and resuspended into 100 µL fresh medium.

- *Simultaneous treatment:* twenty-four hours after seeding, the medium was removed, and cells were simultaneously treated with lycopene and with the mutagen (MMS: 80 µg/mL; 4NQO: 0.01 µM; or H2O2: 0.6mM) for 5 minutes. Then, cells were washed twice with PBS, trypsinized, centrifuged at 180 *g* for 3 min and resuspended into 100 µL fresh medium.

- *Post-treatment:* twenty-four hours after seeding, the medium was removed, cells were treated with the mutagen (MMS: 80 µg/mL; 4NQO: 0.01 µM; or H_2O_2: 0.6mM) for 5 minutes, washed with PBS, and treated again with lycopene (10, 25, or 50 µM) for 1 hour at 37^{o}C. After this period, cells were washed twice with PBS, trypsinized, centrifuged at 180 *g* for 3 min, and resuspended into 100 µL fresh medium.

The single cell gel “comet” assay was performed according to Tice et al. (2000). Briefly, a volume of 10 µl of the cell suspension was added to 120 µl of 0.5% low-melting point agarose at 37^{o}C, layered onto a precoated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and the slides immersed into a lysis solution (2.5M NaCl, 100mM EDTA, 10 mM Tris-HCL buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about one hour. The slides were left into alkaline buffer (pH > 13) for 20 minutes, and electrophoresis was then conducted for 20 minutes, at 25V and 300mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed with absolute ethanol and stored under refrigeration until analysis. Fifty randomly selected cells per culture (plate) were examined at 400x magnification on a fluorescence microscope using an automated image analysis system

(Comet Assay II, Perceptive Instruments, UK). The metric parameter selected as an indicator of DNA damage was the tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units)

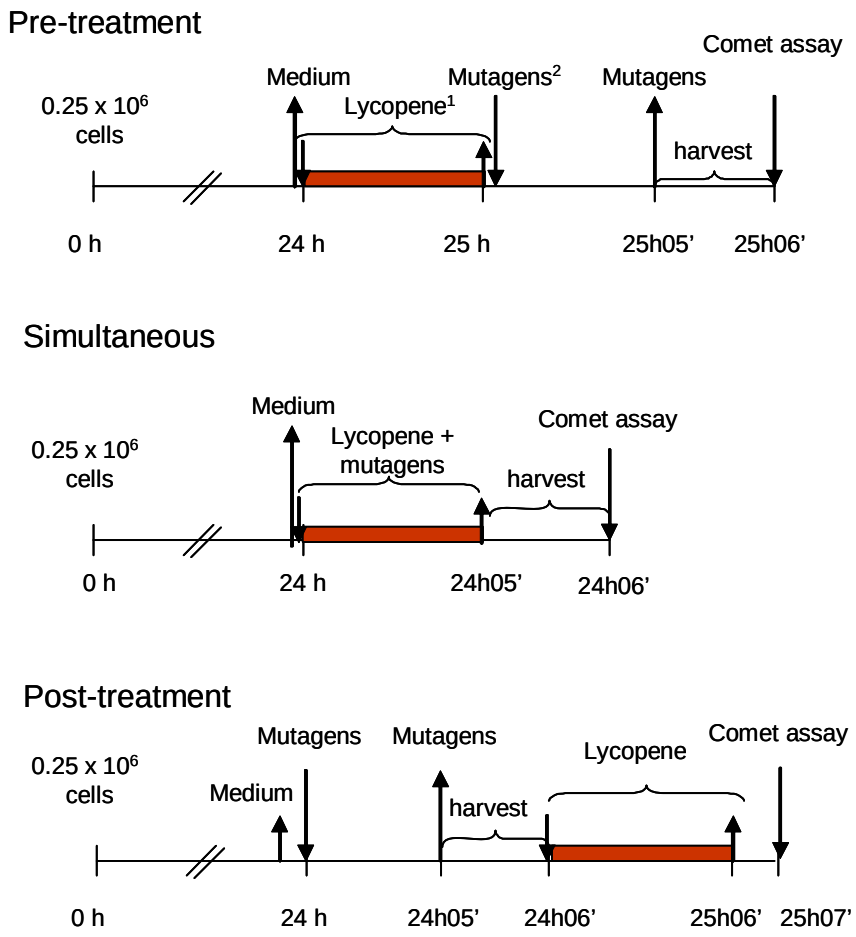

Figure 1: *Single cell gel electrophoresis assay - Experimental design*
1Lycopene: 10, 25 and 50 µM; 2methyl methanesulphonate (80 µg/mL); 4-nitroquinoline 1-oxide: (0.01 µM); hidrogen peroxide H2O2 (0.6mM)

2.4. *Cytokinesis-block micronucleus (CBMN) assay - Experimental designs*

Lycopene concentrations and protocols used for the micronucleus test were based on the results obtained in the comet assay (Figure 2). 4-NQO was not used in simultaneous and post-treatment protocols.

- *Pre-treatment:* twenty-four hours after seeding, the medium was removed, and cells were treated with different concentrations of lycopene (10, 25, or 50 µM) for 1 hour, washed with

PBS and then incubated with the mutagen (MMS 90 µg/mL; 4-NQO: 0.015 µM; or H_2O_2: 1mM) in fresh medium for 5 minutes. After this period, the medium was removed, the cells rinsed twice with PBS and incubated into a fresh medium containing cytochalasin B (final concentration 3 µg/mL) for a period of 24 hours, which was when the culture was harvested.

- *Simultaneous treatment:* twenty-four hours after seeding, the medium was removed, and cells were treated with one of the mutagens (MMS 90 µg/mL; 4NQO: 0.015 µM; or H_2O_2: 1mM) and with lycopene simultaneously, for 5 minutes, at room temperature. Further, cells were washed twice with PBS and then cytochalasin B was added into fresh medium.

- *Post-treatment:* twenty-four hours after seeding, the medium was removed, cells were treated with one mutagen (MMS 90 µg/mL; 4NQO: 0.015 µM, or H_2O_2: 1mM) for 5 minutes, then washed with PBS and treated with lycopene for one hour, at 37^{o}C. Thereafter, cells were washed twice with PBS, and cytochalasin B was added into fresh medium for another 24 hours.

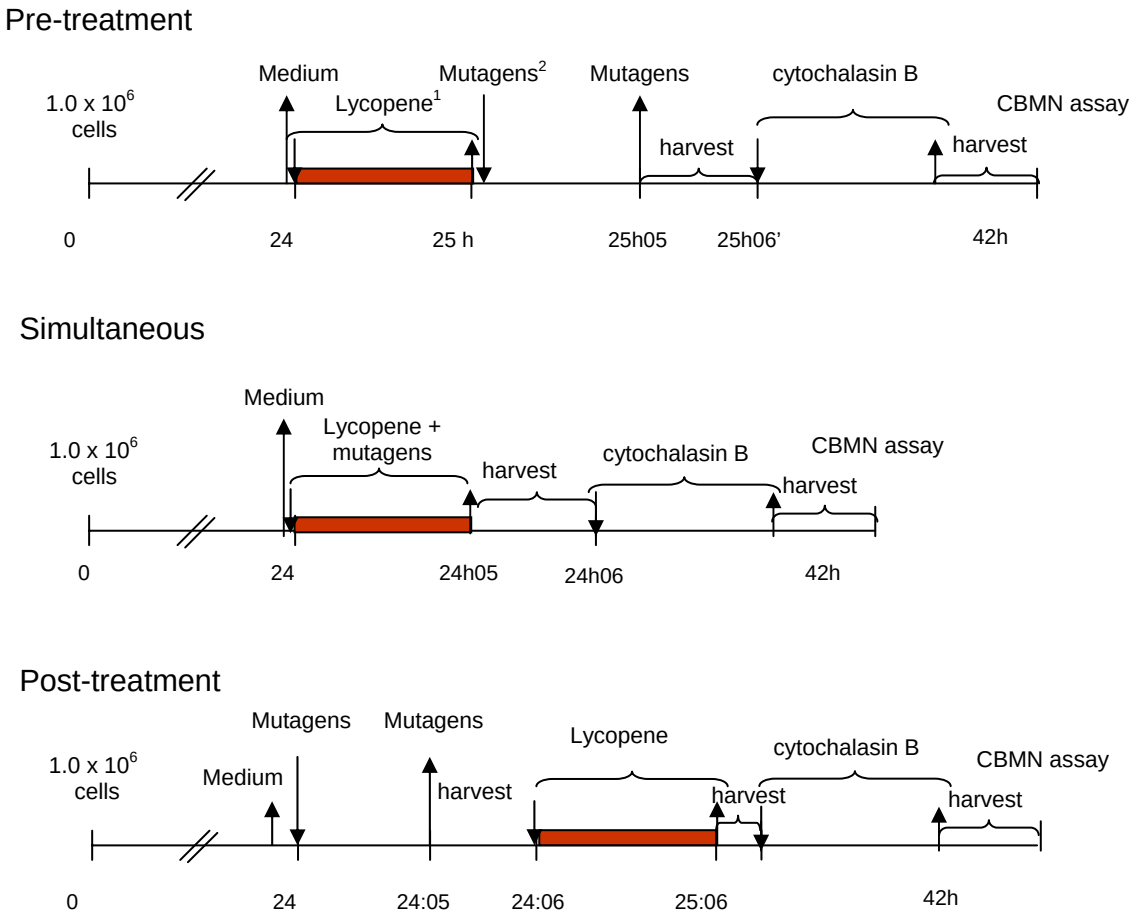

Figure 2: The cytokinesis-block micronucleus (CBMN) assay - *Experimental design*

[1]Lycopene: 10, 25 and 50 µM; [2]methyl methanesulphonate (80 µg/mL); 4-nitroquinoline 1-oxide: (0.01 µM); hidrogen peroxide H2O2 (0.6mM)

After the harvest time (42 hours), the cells were trypsinized, exposed to a cold hypotonic solution (KCL 0.075 M) and fixed with methanol:acetic acid (3:1). Coded slides were stained with 3% aqueous Giemsa solution for 15 minutes, and 1,000 binucleated cells per culture, with intact cytoplasm, were scored for the incidence of micronucleus formation.

2.5. Statistical analysis

The level of DNA damage (tail moment) was assessed using the Kruskal-Wallis test; the frequencies of MN were analyzed using the X^2 test. *p* value < 0.05 was considered for statistical significance.

3. Results

Table I shows the level of DNA damage (tail moment) in CHO cells exposed to mutagens and lycopene at three different treatment schedules. Statistically significant differences between negative and positive controls were observed for all of the mutagens at pre and simultaneous protocols. Nevertheless, for post-treatment protocol, only 4-NQO and MMS induced slight, but significant increase of DNA damage ($P < 0.05$). H_2O_2 did not present significance when compared to the negative control. On the other hand, data mainly showed that the three concentrations of lycopene significantly reduced the genotoxicity of H_2O_2 and MMS at pre and simultaneous treatments; for 4-NQO, only 10 μM lycopene had this inhibitory effect at these two treatment protocols. Conversely, 50 μM lycopene increased the level of DNA lesions when used after 4-NQO treatment. Similar results were observed when lycopene was administered after mutagens, i.e., the carotenoid increased the level of DNA damage induced by MMS, 4-NQO, and H_2O_2 (exception for 10 and 50 μM lycopene/ H_2O_2).

The frequencies of micronucleated CHO cells after exposure to the three treatment protocols are presented in Table II. The results showed statistically significant differences between negative and positive controls, for all of the mutagens in all of the protocols. Lycopene, at the three concentrations (10, 25, and 50 μM), significantly reduced the number of micronucleated cells (MNC) induced by H_2O_2 and MMS, when used simultaneously with the mutagens. Similar effect was observed when the carotenoid was administered prior to

MMS (for all the concentrations); prior to (25 μM) and after (50 μM) H_2O_2; and prior to 4-NQO (10 μM).

Table I. DNA damage (tail moment) in CHO cells treated with lycopene and three different mutagens.

Lycopene treatment protocols	Tail moment (X ± SD)				
	C -	C +	LYC10[1]	LYC25	LYC50
LYC / H_2O_2					
Prior to H_2O_2	0.13 ± 0.18^{a}	3.82 ± 2.25^{b}	2.54 ± 2.59^{c}*	2.83 ± 2.54^{c}*	2.18 ± 2.15^{c}**
Simultaneous	0.48 ± 0.76^{a}	3.89 ± 2.88^{b}	1.37 ± 2.64^{c}**	0.87 ± 1.15^{a}**	1.18 ± 1.35^{c}**
After H_2O_2	0.24 ± 0.32^{a}	0.65 ± 0.85^{ab}	1.03 ± 1.16^{b}	1.14 ± 1.63^{c}*	0.68 ± 1.00^{ab}
LYC / MMS					
Prior to MMS	0.33 ± 0.59^{a}	7.17 ± 3.71^{b}	4.33 ± 3.27^{cd}**	5.13 ± 3.38^{d}*	4.20 ± 3.27^{c}**
Simultaneous	0.26 ± 0.53^{a}	8.46 ± 2.82^{b}	3.28 ± 2.86^{c}**	3.79 ± 2.26^{c}**	4.52 ± 4.45^{c}**
After MMS	0.30 ± 0.63^{a}	1.87 ± 1.45^{b}	2.64 ± 1.81^{c}*	3.27 ± 2.16^{c}**	3.13 ± 2.41^{c}**
LYC / 4-NQO					
Prior 4-NQO	0.41 ± 0.70^{a}	3.54 ± 3.36^{b}	2.33 ± 2.32^{c}**	3.99 ± 2.49^{b}	2.97 ± 2.30^{b}
Simultaneous	0.42 ± 0.56^{a}	4.15 ± 3.46^{b}	2.99 ± 3.14^{c}*	4.02 ± 3.44^{b}	5.43 ± 3.60^{d}*
After 4-NQO	0.26 ± 0.44^{a}	2.27 ± 3.17^{b}	3.78 ± 3.73^{c}**	4.33 ± 3.79^{cd}**	5.17 ± 4.06^{d}**

H_2O_2 (0.6 mM); MMS: methylmethanesulphonate (80 μg/mL); 4-NQO: 4-nitroquinoline1-oxide (0.01 μM); C- and C+: negative and positive control, respectively; [1]LYC: lycopene (LYC10: 10μM; LYC25: 25 μM; LYC50: 50 μM); Lines with values followed by different letters indicate significant difference. *$P < 0.05$, **$P < 0.001$ (compared with C+).

Table II. Frequencies of micronucleated CHO cells after exposure to lycopene and three different mutagens.

Treatment	Micronucleated cells (number per 1,000 cells)				
	C-	**C+**	**LYC10[a]**	**LYC25**	**LYC50**
LYC / H_2O_2					
Prior to H_2O_2	6.5±0.71[a]	84.0±64.01[b]	74.0±32.07[b]	37.0±11.26[c]**	71.0±14.17[b]
Simultaneous	6.5±0.71[a]	84.0±64.01[b]	44.33±8.39[c]**	49.66±29.83[c]**	59.00±33.60[c]**
After H_2O_2	6.5±0.71[a]	84.0±64.01[b]	-	-	65.00±15.13[c]*
LYC / MMS					
Prior to MMS	29.5±2.12[a]	123.33±6.65[b]	58.67±9.07[c]**	61.0±12.12[c]**	67.67±7.03[c]**
Simultaneous	29.5±2.12[a]	123.33±6.65[b]	82.0±17.0[c]**	92.67±27.06[c]**	60.0±13.31[c]**
After MMS	29.5±2.12[a]	123.33±6.65[b]	-	-	115.0±27.87[b]
LYC / 4-NQO					
Prior 4-NQO	16.0±5.65[a]	65.0±11.31[b]	37.0±17.06[c]**	58.67±7.37[b]	70.0±11.79[b]

H_2O_2 (0.6 mM); MMS: methylmethanesulphonate (80 µg/mL); 4-NQO: 4-nitroquinoline1-oxide (0.01 µM); C- and C+, negative and positive control, respectively; [a]LYC: lycopene (LYC10: 10µM; LYC25: 25 µM; LYC50: 50 µM). Columns with values followed by different letters indicate significant difference. ***P < 0.001*,; **P < 0.05* (compared with C+). Statistical analysis: X^2 *test.*

Discussion

It is known that the mechanisms underlying the inhibitory effects of lycopene on carcinogenesis may involve ROS scavenging, up-regulation of detoxification systems, interference with cell proliferation, induction of gap-junctional communication, inhibition of cell cycle progression, and modulation of signal transduction pathways (Bhuvaneswari and

Nagini 2005). A number of *in-vitro* studies have been carried out to investigate this chemopreventive activity (Rao e Argawal, 2000). Among the effects of lycopene, reduction of DNA damage (Reddy et al., 2006; Muzandu et al., 2005; Astley et al., 2004; Yeh and Hu, 2000), induction of apoptosis (Muller et al., 2002; Reddy et al., 2006) and inhibition of cell proliferation (Tang et al., 2005; Nahum et al., 2006) have been described.

In the present study, we investigated the antigenotoxicity and antimutagenicity of lycopene in CHO cells treated with direct mutagens: MMS, 4-NQO or H_2O_2. Hydrogen peroxide is able to interact with DNA through highly reactive oxygen and radical species causing extensive oxidative damage (Ratnam et al., 2006). The DNA alkylating agent methyl methanesulfonate (MMS) modifies guanine (to 7-methylguanine) and adenine (to 3-methlyladenine) causing base mispairing and replication blocks, respectively (Beranek, 1990). 4-NQO is a powerful chemical mutagen and carcinogen that induces DNA oxidative lesions (Arima et al., 2006) and DNA adducts (Kim et al., 2006). The single cell gel electrophoresis (comet) assay and the cytokinesis-block micronucleus assays were used to evaluate primary DNA damage and chromosome breakage and/or loss, respectively. The comet assay is a technically simple and fast methodology that detects genotoxicity in any cell type, both *in vitro* and *in vivo* (Tice et al., 1991; Moler et al., 2006). The simple version of the alkaline comet assay allows to detect DNA strand breaks, alkaline labile sites, and transient repair sites (Moler 2006). On the other hand, the micronucleus assay has emerged as one of the preferred methods for assessing chromosome aberration.

When the chemopreventive activity of lycopene was evaluated by the comet assay, data showed that this carotenoid was able to reduce the level of DNA damage induced by H_2O_2, MMS, and 4-NQO under different treatment protocols. For H_2O_2–induced DNA lesions, the protective effect of lycopene was observed at pre and simultaneous protocols. Conversely, in the post-treatment protocol, lycopene increased the level of DNA damage

induced by H_2O_2. Herein, two aspects must be considered: the apparent absence of H_2O_2 genotoxicity in this protocol and the lycopene ability to increase DNA damage induced by this mutagen. The H_2O_2 failure in inducing genotoxicity could be due to the dose used and/or because the time between the treatment and cell harvesting might be enough for DNA lesions to be repaired. Lower level of DNA damage was also observed for MMS and 4-NQO under the same protocol.

Lowe et al. (1999), using HT29 cells, showed that lycopene only afforded protection against DNA damage (induced by xanthine/xanthine oxidase) at relatively low concentrations (1-3 μM). At higher concentrations (4-10 μM), the ability to protect the cell against such oxidative damage was rapidly lost and, indeed, the presence of the carotenoid increased the extent of DNA damage. Recently, an increased level of oxidative DNA damage product in LNCaP human prostate cancer cell treated with lycopene (5 μM) was also described (Hwang e Bowen, 2005). Young e Lowe (2001) demonstrated that lycopene, at high concentrations, may lose its effectiveness as an antioxidant. Moreover, Yeh et al. (2005) showed that this carotenoid enhances UVA-induced oxidative stress in C3H cells. The authors suggest that under UVA irradiation, lycopene may produce oxidative products that are responsible for the prooxidant effects. Additionally, a study using Hs68 cells has demonstrated that lycopene can be either an antioxidant or a prooxidant depending on the oxidants used. Although it is unclear whether lycopene may have prooxidant activity, the authors caution that it may be premature to undertake clinical trials with lycopene (Yeh e Hu, 2000). Based on these findings, we believe that the increased DNA damage caused by lycopene in the current study could be attributed to the concomitant prooxidative activities under certain *in-vitro* conditions. However, it should be emphasized that no cytotoxicity was detected (data not presented). Differently, in the micronucleus assay, lycopene presented protective activity

against H_2O_2-induced DNA damage in all of the treatment protocols, but with no dose-response relationship.

Because of its antioxidant properties, lycopene has gained attention and become one of the most studied chemopreventive agents. The high number of conjugated dienes makes lycopene one of the most potent singlet oxygen quencher among natural carotenoids (Di Mascio et al., 1989, Matos et al., 2006). To be an effective antioxidant, a molecule such as a carotenoid would have to remove free radicals, such as OH-, from the system either by reacting with them to yield harmless products or by disrupting free-radical chain reactions. It has been shown that carotenoids can be effective antioxidants in organic solutions under defined conditions (Lieber, 1993). Several competing reactions are possible. Some of these may be antioxidant reactions, but others may lead to a prooxindant action of the carotenoid (Britton, 1995).

Lycopene was found to be at least twice as active as beta-carotene in protecting lymphocytes against NO_2^+ radical (Bohm et al., 1995). It has also been demonstrated that lycopene can inactivate hydrogen peroxide and nitrogen dioxide, two well-documented genotoxins (Rao and Argawal, 1999). Mortensen et al. (1997), using the pulse radiolysis technique, demonstrated lycopene ability to scavenge nitrogen dioxide radicals. The antigenotoxicity of an extract of mixed cruciferous and legume sprouts (rich in lycopene) on H_2O_2-induced DNA damage was evaluated in HT29 cells by using the comet assay (Gill et al., 2004). The authors observed a significant reduction in DNA damage when the cells were pre-incubated with the extract for 24 hours and then challenged with H_2O_2.

Our data clearly showed the protective effect of lycopene, when administered before or simultaneously with MMS, both in the comet and micronucleus assays. Ambiguous results were observed for 4-NQO. It is known that 4-nitroquinoline 1-oxide (4NQO) is a powerful chemical mutagen and carcinogen (Arima et al., 2006). Among the damage induced by this

compound are oxidative DNA lesions (Arima et al., 2006) and DNA adducts (Kim et al., 2006). As mentioned before, concomitant antioxidative and prooxidative activities of lycopene could take place at the experimental conditions and doses used.

In summary, our results demonstrated that lycopene can prevent both primary DNA lesions and chromosome aberrations (breakage and loss). However, its chemopreventive activity depends on the dose and the treatment schedule used. -Moreover, lycopene may act differently depending on the mutagen to which the cells are exposed. Further studies must be performed in order to clarify the mechanisms and the conditions by which lycopene favorably modulate chemical mutagenesis.

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VIII - DISCUSSÃO GERAL

Diversos estudos epidemiológicos e experimentais *in vivo* e *in vitro* têm demonstrado o efeito do licopeno na prevenção de danos ao DNA (Reddy et al., 2006) e no desenvolvimento de alguns tipos de cânceres (Rao & Argawal, 2000, Bhuvaneswari & Nagini, 2005; Rao 2006). Considerando que a carcinogênese é um processo dinâmico que envolve uma gama de alterações em nível molecular, celular e histológico, o presente estudo objetivou avaliar o potencial quimioprotetor do licopeno, bem como seus possíveis mecanismos de anticarcinogenicidade.

Nossos resultados mostraram que o licopeno, na maior dose testada (300 ppm) e administrada antes da DEN, foi eficaz na prevenção de danos primários no DNA detectados pelo teste do cometa no modelo de hepatocarcinogênese *in vivo*. Entretanto, nesse experimento, não foi observado efeito protetor do carotenóide no desenvolvimento de focos GST-P positivos, indicando ausência de ação efetiva sobre a etapa de promoção da carcinogênese hepática em rato. Assim, nossa atenção foi direcionada para a atividade antigenotóxica do licopeno, uma vez que esta pode ser um mecanismo importante para a prevenção da iniciação do processo carcinogênico e de outras doenças relacionadas a alterações induzidas no material genético.

Portanto, visando contribuir para o esclarecimento dos mecanismos de ação do licopeno na prevenção de danos quimicamente induzidos no DNA foram realizados estudos *in vitro* utilizando-se duas linhagens celulares – HepG2 e CHO - sendo a primeira com atividade metabolizadora endógena. Essa estratégia foi adotada a fim de avaliar a atividade do carotenóide sobre um mutágeno de ação indireta, isto é, que necessite ser metabolizado à sua forma ativa para manifestar o caráter mutagênico. Assim, foi investigada a ação do licopeno sobre a DEN, cancerígeno previamente utilizado no modelo de hepatocarcinogênese *in vivo*.

Os resultados do experimento conduzido em células HepG2 indicaram que o carotenóide pode exercer importante atividade moduladora do metabolismo da DEN, uma vez que quando adicionado à cultura de células antes, ou simultaneamente ao mutágeno, reduziu sua atividade toxicogenética. Deste modo, esses achados corroboram aqueles obtidos *in vivo*, ocasião em que também foi observada a ação protetora do licopeno contra danos induzidos pela DEN em células de fígado de ratos. Apesar do efeito quimioprotetor do licopeno ser freqüentemente associado ao seu potencial antioxidante, no caso da prevenção de danos induzidos pela DEN sugere-se, também, uma ação do carotenóide sobre as vias de ativação e detoxificação metabólica (Bhuvaneswari & Nagini, 2005). Assim, o licopeno poderia prevenir danos no DNA pela modulação do metabolismo do carcinógeno, por meio da ativação do complexo enzimático P-450 (Astorg et al., 1997; Bhuvaneswari & Nagini, 2005).

Os sistemas testes *in vitro* também foram utilizados para avaliar diferentes protocolos de tratamento com o licopeno e o seu efeito do licopeno sobre outros tipos de danos quimicamente induzidos no DNA. Devido ao reconhecido papel antioxidante do licopeno, foi estudado seu efeito sobre danos oxidativos induzidos pelo peróxido de hidrogênio (H_2O_2) nas duas linhagens celulares (HepG2 e CHO). Em ambas, foi observada a redução do efeito toxicogenético do H_2O_2, com todas as concentrações de licopeno testadas, e quando adicionadas às culturas celulares antes ou simultaneamente ao mutágeno. As pequenas diferenças observadas entre as duas linhagens celulares poderiam ser explicadas pela presença de enzimas metabolizadoras endógenas nas células HepG2, já que os agentes antioxidantes naturais podem favorecer mudanças nos sistemas enzimáticos de defesa prevenindo o estresse oxidativo (Allia et al., 2006).

Os experimentos conduzidos em células CHO permitiram avaliar, também, o efeito do licopeno sobre outros dois mutágenos de ação direta, mas com diferentes mecanismos de

ação: o metilmetanosulfonato agente alquilante do DNA que modifica a guanina e a adenina (Beranek, 1990), e a 4 - óxido de nitroquinolina (4-NQO) potente mutágeno e carcinógeno que induz lesões oxidativas (Arima et al., 2006) e aductos no DNA (Kim et al., 2006). Os resultados nesse tipo celular mostraram clara ação protetora do licopeno quando administrado antes ou simultaneamente ao MMS no teste do cometa e do micronúcleo, muito embora o efeito sobre a 4-NQO tenha sido ambíguo (houve situações de aumento da atividade mutagênica do composto; fato já discutido no capítulo VI).

Concluindo, o presente estudo contribui para o avanço na identificação de agentes quimioprotetores naturais, em particular o licopeno, uma vez que os dados evidenciaram o efetivo potencial antigenotóxico e antimutagênico do carotenóide em células expostas a agentes mutagênicos e carcinogênicos. Os resultados mostraram, ainda, que o licopeno, além de sua reconhecida atividade antioxidante e capacidade de capturar radicais livres deletérios à célula e seus componentes, pode ter importante função sobre vias enzimáticas de metabolismo de agentes xenobióticos. No entanto, como foi observado que o efeito do licopeno pode ser dependente da condição de utilização, novos estudos devem ser conduzidos antes que este carotenóide possa ser adotado como um agente quimioprotetor contra danos no DNA e doenças geneticamente relacionadas.

IV - CONCLUSÕES

Com base nos nossos resultados podemos concluir que:

- o licopeno apresenta efeito protetor contra danos genéticos (danos primários no DNA e alterações cromossômicas) induzidos pela DEN em células hepáticas de ratos Wistar machos, *in vivo*;
- o licopeno, nas condições testadas, não apresenta efeito protetor sobre o desenvolvimento de focos GST-P positivos induzidos pela DEN em fígado de ratos Wistar macho;
- o licopeno apresenta atividade protetora do DNA contra danos oxidativos e alquilação induzidos, respectivamente, pelo H_2O_2 e MMS em células HepG2 e CHO *in vitro*;
- o licopeno apresenta efeito antigenotóxico e antimutagênico contra danos induzidos pela 4-NQO, mas na dependência da concentração e protocolo de tratamento utilizado;
- o licopeno pode atuar como potencializador de danos induzidos no DNA pela 4-NQO;
- outros estudos devem ser conduzidos antes que o licopeno seja rotineiramente utilizado como agente anticarcinogênico.

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