

PROGRAMA DE PÓS-GRADUAÇÃO EM ALIMENTOS E NUTRIÇÃO - PPGAN
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE – CCBS
UNIVERSIDADE FEDERAL DO ESTADO DO RIO DE JANEIRO –
UNIRIO

MONIQUE DE BARROS ELIAS CAMPOS

***Efeito da suplementação de licopeno e
betacaroteno sobre o metabolismo hepático em
diferentes modelos experimentais***

***Effect of lycopene and beta-carotene
supplementation on liver metabolism in different
experimental models***

Rio de Janeiro

2020

MONIQUE DE BARROS ELIAS CAMPOS

***Efeito da suplementação de licopeno e
betacaroteno sobre o metabolismo hepático em
diferentes modelos experimentais***

***Effect of lycopene and beta-carotene
supplementation on liver metabolism in different
experimental models***

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição, da Universidade Federal do Estado do Rio de Janeiro como requisito final para obtenção do título de Doutorado em Alimentos e Nutrição.

Orientador: Dr. Anderson Junger Teodoro

Co-orientadora: Dra. Vilma Blondet de Azeredo

Rio de Janeiro

2020

d198

de Barros Elias Campos, Monique
EFEITO DA SUPLEMENTAÇÃO DE LICOPENO E
BETACAROTENO SOBRE O METABOLISMO HEPÁTICO IN VIVO E
IN VITRO. / Monique de Barros Elias Campos. -- Rio
de Janeiro, 2020.
147f

Orientador: Anderson Junger Teodoro.
Coorientador: Vilma Blondet de Azeredo .
Tese (Doutorado) - Universidade Federal do
Estado do Rio de Janeiro, Programa de Pós-Graduação
em Alimentos e Nutrição, 2020.

1. Carotenoides. 2. Licopeno. 3. Betacaroteno.
4. Fígado. I. Junger Teodoro, Anderson, orient. II.
Blondet de Azeredo , Vilma, coorient. III. Título.

MONIQUE DE BARROS ELIAS CAMPOS

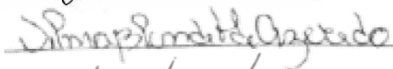
Efeito da suplementação de licopeno e betacaroteno sobre o metabolismo hepático em diferentes modelos experimentais

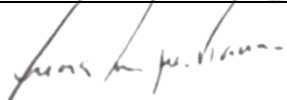
Effect of lycopene and beta-carotene supplementation on liver metabolism in different experimental models

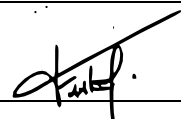
Tese de Doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição (PPGAN), da Universidade Federal do Estado do Rio de Janeiro.

Aprovada em 24 de março de 2020

Presidente: 

Avaliador 1:  Prof. Dr. Vilma Blondet de Azeredo
Pesquisadora UFF
SIAPE 0310645

Avaliador 2: 

Avaliador 3 (doutorado): 

Avaliador 4 (doutorado):  Jeandus Miranda Alves

Dedico este trabalho à minha querida mãe.

AGRADECIMENTOS

Agradeço a minha filha amada, pela companhia, pelos sorrisos, pela compreensão (ou não rs) nos momentos que precisei me dedicar ao doutorado. Ao meu marido Luiz Felipe, por todo amor, carinho, apoio e incentivo.

À minha família por estarem sempre por perto, pela dedicação e amor dados a mim, principalmente minha mãe, que apesar da ausência física, sinto sua companhia constantemente me dando forças e ânimo para continuar a caminhada.

Ao meu orientador, Anderson Teodoro, por toda dedicação, paciência, compreensão, e ajuda mesmo nas circunstâncias mais difíceis. Obrigada por acreditar em mim e por me acompanhar desde a graduação, sempre me orientando e apoiando meu crescimento.

À minha co-orientadora Vilma Blondet, que tanto me ajudou, orientou e incentivou as minhas escolhas nesse processo.

A todos os professores do Programa de Pós-Graduação em Alimentos e Nutrição pela oportunidade de realização do doutorado e aos professores da Escola de Nutrição da UNIRIO que fizeram parte da minha formação.

À todos que fizeram parte dessa banca. À todos os colegas do doutorado.

Enfim, a todos que de alguma forma contribuíram para o desenvolvimento desta Tese.

RESUMO

Os carotenóides são amplamente distribuídos na natureza, encontrados principalmente em frutas e legumes, dando origem a coloração amarela, laranja ou vermelha. Os carotenóides atuam como antioxidantes biológicos e parecem desempenhar um papel importante na saúde humana, protegendo células e tecidos dos efeitos nocivos dos radicais livres e do oxigênio singlete. Entre todos os carotenóides da dieta, o licopeno e o betacaroteno são um dos antioxidantes mais potentes de ocorrência natural e abundante. Os carotenóides têm características estruturais e químicas únicas que contribuem para propriedades biológicas específicas e atividades farmacológicas. O potencial antioxidante dos carotenóides pode ser responsável por seus benefícios à saúde. Relata-se que o consumo alimentar de produtos ricos em licopeno e betacaroteno está associado a uma diminuição do risco de câncer e doenças crônicas. Investigações adicionais serão necessárias para entender e avaliar o mecanismo pelo qual se provou que o licopeno, o betacaroteno e seus metabólitos possuem importantes atividades biológicas e benefícios à saúde. Este trabalho relata o entendimento atual do licopeno e betacaroteno em relação ao seu papel na saúde humana e na prevenção de vários tipos de câncer, principalmente na hepatocarcinogênese.

Palavras-chave: carotenóides; licopeno, β -caroteno; câncer de fígado; compostos bioativos

ABSTRACT

Carotenoids are widely distributed in nature, found in mainly vegetables and fruits, due to which yellow, orange or red color coloration is observed. Carotenoids act as biological antioxidants and seem to play an important role in human health by protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Among all the dietary carotenoids, lycopene and betacarotene is one of the most potent naturally and abundantly occurring antioxidant. Carotenoids has unique structural features and chemical features which contribute to specific biological properties and harmacological activities. The antioxidant potential of carotenoids may be esponsible for its associated health benefits. Dietary consumption of products rich in lycopene and betacarotene are reported to be associated with a decreased risk of cancer and chronic diseases. Further investigations will be required to understand and evaluate mechanism whereby lycopene, betacarotene and its metabolites are proven to possess important biological activities and health benefits. This review summarizes the current understanding of lycopene and betacarotene with respect to its role in human health and prevention of various cancer, mainly in hepatocarcinogenesis.

Keywords: carotenoids; lycopene; β -carotene; liver cancer; bioactive compounds;

SUMMARY

INTRODUÇÃO GERAL	15
-------------------------	-----------

CHAPTER 1

Effect of lycopene and betacarotene on the prevention of liver cancer – A mini review

ABSTRACT	21
1 INTRODUCTION	22
2 CAROTENOIDES	25
3 HEPATOCARCINOGENESE	26
4 CAROTENOIDES X ESTRESSE OXIDATIVO	26
5 LICOPENO	27
6 BETACAROTENO	29
CONCLUSION	
REFERENCES	30

CHAPTER 2

Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

ABSTRACT	57
1 INTRODUCTION	58
2 MATERIALS AND METHODS	59
2.1 <i>Materials</i>	59
2.2 <i>Cell Culture and Treatment Protocol</i>	60
2.3 <i>Cell Viability Assay</i>	60
2.4 <i>Cell Cycle Analysis</i>	61
2.5 <i>Incorporation of [14C]-acetate and lipids analysis</i>	61
2.6 <i>Microarray analyses: cRNA amplification, labeling and hybridization, and cDNA microarray</i>	62
2.7 <i>Analysis of gene expression</i>	62
2.8 <i>Preparation of total RNA and real-time polymerase chain reaction (PCR).</i>	62
2.9 <i>Statistical Analysis</i>	63
3 RESULTS	63
3.1 <i>Phenotype conversion of GRX</i>	
3.2 <i>Acetate incorporation into GRX cell lipids</i>	63
3.3 <i>Cell Proliferation and Cell Cycle of GRX Cells</i>	65
3.4 <i>Gene expression of lipocyte phenotype</i>	
3.5 <i>Microarray analysis</i>	
4 DISCUSSION	
CONCLUSION	66
REFERENCES	67

CHAPTER 3
Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell
Biomarkers in Rats

ABSTRACT	77
1 INTRODUCTION	78
2 MATERIAL & METHODS	79
<i>2.1 Samples</i>	79
<i>2.2 Experimental model</i>	81
<i>2.3 Analytical methods</i>	
<i>2.4 Cell cycle</i>	82
<i>2.5 Apoptosis assay</i>	83
<i>2.6 Data analysis</i>	84
3 RESULTS	85
4 DISCUSSION	
REFERENCES	86

CHAPTER 4

Effect of betacarotene and buriti juice on hepatic cells biomarkers in rats fed with high-fat diet

ABSTRACT	90
1 INTRODUCTION	91
2 MATERIAL & METHODS	92
2.1 <i>Samples</i>	92
2.2 <i>Experimental model</i>	93
2.3 <i>Analytical methods</i>	94
2.4 <i>Cell cycle</i>	94
2.5 <i>Apoptosis assay</i>	94
2.6 <i>Data analysis</i>	94
3 RESULTS	95
4 DISCUSSION	
REFERENCES	
CONCLUSÕES GERAIS E DESENVOLVIMENTO ADICIONAIS	116
GENERAL CONCLUSIONS AND FURTHER DEVELOPMETS	118
GENERAL REFERENCES	
ANEXOS	

O presente trabalho segue as normas da tese no formato de artigo definidos pelo Programa de Pós Graduação em Alimentos e Nutrição em 14 de maio de 2019.

Assim esta tese, está dividida em 4 capítulos:

I) Artigo de revisão bibliográfica: “Effect of lycopene and betacarotene on the prevention of liver cancer – A mini review”.

II) Artigo original que contempla resultados e discussão dos experimentos: “Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling”.

III) Artigo original que contempla resultados e discussão dos experimentos: “Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell Biomarkers in Rats”.

IV) Artigo Short Communication que contempla resultados e discussão dos experimentos: “Effect of betacarotene and buriti juice on hepatic cells biomarkers in rats fed with high-fat diet”.

INTRODUÇÃO GERAL

Nas últimas décadas têm se percebido alterações no estilo de vida e nos padrões e comportamentos alimentares dos indivíduos. Há um aumento no interesse em alimentos ultra processados com alta densidade energética, ricos em sódio, açúcar, conservantes e gorduras, principalmente gordura saturada e trans e pobres em micronutrientes e fibras (Suhett et al, 2019). Tais mudanças estão diretamente relacionadas com a elevada e crescente prevalência de excesso de peso no país, e com o perfil de morbi-mortalidade da população por doenças crônicas não transmissíveis (DCNT) (Neto et al, 2019). Esse comportamento está associado ao aumento do peso corporal, alterações metabólicas, como inflamação e estresse oxidativo, além de danos celulares e doenças crônicas não transmissíveis que envolvem diferentes tecidos, como o fígado (Wang et al, 2020; Bedê et al. 2020; Chooi et al, 2019).

Além da mudança do padrão alimentar atual da população, podemos destacar também a influência das “dietas em destaques”, como as cetogênicas, Low Carb ou Hight Fat, onde as pessoas estão consumindo uma dieta rica em gorduras (Rohit et al, 2019). A alta ingestão dietética de gordura, principalmente de ácidos graxos saturados, afeta diretamente a integridade e função de diversos tecidos, principalmente o fígado que pode ser agravado com o consumo de álcool (Charbonneau et al, 2007). Mais gordura é armazenada e o indivíduo avança para a obesidade. A alta ingestão de gordura combinada com altos níveis de estresse oxidativo resulta em aumento da carga de gordura hepática no ambiente e níveis reduzidos de antioxidantes, resultando em lipotoxicidade associado a um elevado nível de estresse oxidativo e redução da defesa antioxidante do organismo (Sies et al., 2005; Jaeschke et al., 2000). Dietas ricas em gordura saturada estimulam a síntese de citocinas pró-inflamatórias e o aumento das espécies reativas de oxigênio, estresse oxidativo e lesão nas células do tecido (Alegría-Ezquerro, 2008).

Nos hepatócitos, o estresse oxidativo e a peroxidação lipídica promovem danos à membrana plasmática, tornando-a vulnerável à apoptose e favorecendo a resposta inflamatória no tecido e no organismo de maneira geral (Geraldo et al., 2008; Oliveira &

Schoffen 2010; Bucchieri et al, 2002).

Existem evidências demonstrando que a hepatocarcinogênese, em ratos e em humanos, é um processo de múltiplos estágios precedido pelo aparecimento de focos de hepatócitos alterados e nódulos hepáticos hiperplásicos, sendo estes considerados lesões pré-neoplásicas decorrentes de expansão clonal a partir de hepatócitos iniciados após o processo inflamatório (Sherman et al., 1983; Teebor & Becker, 1981; Peres et al, 2003). O excedente metabólico do consumo excessivo de calorias também pode elevar a síntese de enzimas hepáticas, o que cria uma demanda excessiva no retículo endoplasmático (RE). Esse excesso de demanda no RE leva à indução de respostas metabólicas mediadas por estresse do ER, que foi associada ao desenvolvimento de câncer de fígado (Hetz et al, 2012; Fu et al, 2012; Malhi and Kaufman, 2011). Nesse contexto, podemos relacionar a incidência de câncer a fatores ambientais, sobretudo alimentares, predisposição genética, obesidade, entre outros fatores (Olthof et al, 2000)

Questões relacionadas com o papel da dieta na prevenção do câncer e tratamento são destaques a cada ano, incluindo resultados de estudos envolvendo vários alimentos, fitoquímicos e nutrientes; uso de abordagens complementares e alternativas para a prevenção e para o tratamento; e dietas ideais para aqueles que desejam prevenir o câncer ou a sua repetição. Atualmente, tem sido dada grande atenção a estratégias preventivas e, neste contexto, o uso de compostos bioativos presentes nos alimentos parece contribuir para este processo por diferentes mecanismos de ação, os quais são anticancerígeno, antioxidantes e anti-inflamatória (Upadhyaya et al, 2007).

Uma intervenção no estilo de vida que inclui mudança no comportamento alimentar aliado a perda de peso, dieta saudável e prática regular de exercícios é um dos tratamentos mais eficazes e seguros para diversas comorbidades e disfunções metabólicas associadas. Evidências recentes destacaram os efeitos preventivos e terapêuticos de certos compostos bioativos, especialmente aqueles ricos em carotenóides. Segundo alguns autores, a atuação dos carotenoides parece inibir o desenvolvimento de certas etapas da carcinogênese, através

do mecanismo antioxidante, que previne o estresse oxidativo, desativando as moléculas reativas de oxigênio singlete (Edge et al, 2018) ou pela ação direta antimutagênica, prevenindo a mutação celular. Já foi evidenciado a ação dos carotenoides, principalmente o licopeno e betacaroteno, na regulação das junções comunicantes, modificando a comunicação célula-célula, potencializando a resposta imune, na modulação da expressão do gene supressor tumoral ou inibindo o crescimento das linhas celulares tumorais (Zhang et al, 2002). A ação dos carotenoides sobre células cancerígenas têm sido intensamente estudada nas duas últimas décadas, principalmente seu metabolismo e biotransformação (Teodoro et al., 2009). A capacidade de um composto de inibir a proliferação das células cancerígenas é muito desejável. Em modelos experimentais distintos e na prática clínica, esse dois carotenoides têm sido amplamente estudados, no entanto, é pouco compreendido como o licopeno, betacaroteno ou a sinergia desses dois carotenoides regulariam a biologia dessas células. Modelos experimentais de hepatotoxicidade induzida pela dieta contribuíram para a elucidação da fisiopatologia de várias doenças hepáticas e também visam identificar e avaliar possíveis agentes dietéticos hepatoprotetores (Elias et al, 2019; Jesuz et al, 2019).

Nesse contexto, é importante estudar o possível efeito da suplementação de licopeno e/ou betacaroteno isolados e na matriz alimentar sobre o fígado atacadas por uma dieta rica em gorduras, avaliando seu potencial de proteção das células hepáticas ou de efeitos anti-proliferativo de células cancerígenas.

Assim esta tese dividida em 4 capítulos, apresentando 1 Artigo de revisão bibliográfica sobre licopeno e betacaroteno na prevenção de câncer de fígado (“Effect of lycopene and betacarotene on the prevention of liver cancer – A mini review”) e 3 Artigos originais que contempla resultados e discussão do tratamento de carotenoides sobre metabolismo hepático in vivo e in vitro (“Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling”, “Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell Biomarkers in Rats”, “Effect of betacarotene and buriti juice on hepatic cells biomarkers in rats fed with high-fat diet”);

Chapter 1

Hepatoprotective effects of lycopene and betacarotene in liver cancer – A mini review

Monique de Barros Elias¹, Anderson Junger Teodoro^{1*}

¹ Nutritional Biochemistry Core, Laboratory of Functional Foods,
Universidade Federal do Estado do Rio de Janeiro (UNIRIO),
Avenida Pasteur 296—Urca, Rio de Janeiro 22290-240,
Brazil; moniquebarros.nutri@gmail.com (M.d.B.E.)

* Correspondence: atteodoro@gmail.com; Tel.: +55-21-2542-7785

This manuscript was submitted to ... on February, 2020.

Chapter 2

Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

Monique de Barros Elias(a)*, Anderson Junger Teodoro(a)*, Felipe Leite Oliveira(b), Fatima Costa Rodrigues Guma(c), Renata Brum Martucci(b), Radovan Borojevic(d).

^a *Laboratory of Functional Foods, Universidade Federal do Estado do Rio de Janeiro, Av. Pastuer 296, Brazil, E-mail: atteodoro@gmail.com, Tel: +55 21 25427236*

^b *Federal University of Rio de Janeiro, Institute of Biomedical Sciences, Brazil*

^c *Federal University of Rio Grande do Sul, Biochemistry Department, Brazil*

^d *Faculty Arthur Sa Earp Neto Faculty of Medicine of Petropolis, RJ, Brazil*

This chapter was published on **Food Funct.**, (2019), vol 10, pg.1974-1984.
DOI: 10.1039/C8FO02369G

Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

Monique de Barros Elias^{1*}, Anderson Junger Teodoro^{1*}, Felipe Leite Oliveira², Fatima Costa Rodrigues Guma³, Renata Brum Martucci², Radovan Borojevic⁴

¹Nutritional Biochemistry Core, Laboratory of Functional Foods, Unirio, Rio de Janeiro, Brazil

²Laboratory of Cell Proliferation and Differentiation, Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Brazil

³Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

⁴Center of Regenerative Medicine, Faculty of Medicine - FASE, Petrópolis, Rio de Janeiro, Brazil

* These authors contributed equally to this work.

*Corresponding author:

Anderson Junger Teodoro, Universidade Federal do Estado do Rio de Janeiro, Escola de Nutrição,

Departamento de Ciência de Alimentos CEP 22290-240, Rio de Janeiro, RJ, Brazil,

Tel: +55 21 25427276; Fax: +55 21 25427752

e-mail: atteodoro@gmail.com

ABSTRACT

Hepatic stellate cells are liver-specific perivascular cells, identified as the major source of collagen in liver fibrosis, following their activation and conversion to myofibroblast-like cells. Lycopene is a carotenoid with biological activities and protective effect described in different pathologies, but little is known about its role in liver protection. We evaluated the influence of lycopene on cell cycle and lipid metabolism and monitored the possible pathways involved in lycopene inhibition of stellate cells activation. Lycopene induced expression of the lipocyte phenotype, with an accumulation of fat droplets in cytoplasm, with high synthesis and turnover of phospholipids and triglycerides. Cell proliferation analysis showed that lycopene reduced the growth of GRX cells. Lycopene induced an arrest in the G₀/G₁ phase, followed by a decrease of cells in G₂/M phase, regardless of the concentration of lycopene used. Lycopene modulated relevant signaling pathways related to cholesterol metabolism, cellular proliferation, and lipid metabolism. Also, lycopene treatment increased the expression of RXR- α , RXR- β , and PPAR γ , important biomarkers of liver regeneration. These results show that lycopene was able to negatively modulate events related to activation of hepatic stellate cells through mechanisms that involve changes in expression of cellular lipid metabolism factors, and suggest that this compound might provide a novel pharmacological approach for prevention and treatment of fibrotic liver diseases.

Keywords: lycopene, hepatic stellate cells, bioactive compounds, liver

Total number of text figures: 5

Total number of tables: 3

Conflict of interest: The authors have no competing interests.

INTRODUCTION

The liver has long been described as the major site of metabolism and accumulation of carotenoids and retinoids in the body. They are mostly found in lipid droplets of the hepatic stellate cells (HSCs), a population of liver nonparenchymal cells, resident quiescent in the perisinusoidal Disse's space. They are specialized in storage, metabolism, and release of vitamins, hormones and endogenous and exogenous lipid mediators. The HSCs represents the intracellular content of retinoids and carotenoids be very high. After retinol is converted to retinoic acid, it can be released into the blood circulation by the HSCs [1]. The HSCs may be directly or indirectly responsible for the maintenance of the circulating carotenoids homeostasis in periods of a low dietary uptake through the production of the intracellular retinoic acid [2]. HSCs can also uptake, store, isomerize and release into the blood circulation lycopene obtained from the alimentary sources [3].

Besides the storage and redistribution of lipid compounds in the body, HSCs are also the significant players of development and maintenance of hepatic fibrosis. The two activities required induction of HSCs into different and opposed metabolic functions and behaviors. Hepatic fibrosis is a liver tissue response to acute or chronic aggression. This is a response to products released by the injured tissues, mediated by the locally produced or circulating compounds, inflammatory mediators and cytokines [4,5]. Upon tissue injury, HSCs are activated and undergo transdifferentiation to myofibroblasts responsible for overproduction of collagen-rich extracellular matrix [6]. This activation of quiescent HSCs is the major component of hepatic fibrogenesis, including enhancement of proliferation and cell-specific downmodulation of hepatic lipid metabolism. Moreover, activated HSCs also produce pro-inflammatory mediators, which can enhance and further modulate the environment leading to hepatic fibrosis [7,8].

Controlling the degree of HSCs activation may have an antifibrotic protection effect for liver fibrosis. Peroxisome proliferator-activated receptor γ (PPAR γ), one of the PPAR isoforms, is highly

expressed in quiescent HSCs of the normal liver, but its expression and activity are dramatically decreased during the HSC activation *in vitro* and *in vivo* [9]. Upon binding to cognate ligands, PPAR undergoes a conformational change that releases the co-repressor, allowing its association to its mandatory pair, the X receptor for retinoic acid (RXR). This heterodimer (PPAR:RXR) binds to peroxisome proliferator response elements (PPRE), the specific DNA sequences in target gene promoters, leading to transcriptional activation [10,11]. Being one of the major controllers of the retinoic acid availability, HSCs are one of the major players in these events. However, the RXR can also bind the alternative ligands, including the acyclic carotenoid-derived ones, such as lycopene. This raises the question of the potential consequences of such alternative RXR ligands in HSCs, such as occurs upon lycopene supplementation. This question has been addressed in the present study.

Oxidative stress-related injuries are the potential causes of liver tissue injuries that can lead to hepatic fibrosis. Exogenous antioxidants, both synthetic and natural, have been used in alternative therapeutic approaches for this pathology of liver [12,13]. Epidemiological studies have indicated that food products that contain lycopene, the most potent antioxidant compound among carotenoids, have chemopreventive effects against cancers and other diseases [14]. Lycopene is a bright red carotene, a non-pro-vitamin A carotenoid is a highly unsaturated hydrocarbon containing eleven conjugated and two unconjugated double bonds. Its biological effects in humans have been attributed to mechanisms different from those mediated by the vitamin A, since lycopene lacks the β -ionone ring structure, and it cannot produce the vitamin A in the body [15]. In recent studies, lycopene has shown a protective effect against damage caused by oxidative stress causing liver toxicity, using a model induced by an overdose of acetaminophen. It was observed that lycopene was able to reduce both lipid peroxidation and protein carbonylation [13]. Moreover, it has been suggested that the normalization of liver function markers and lowering of liver damage may be related to the protective effect of lycopene against non-alcoholic liver

disease [8]. Lycopene can thus have a double and complementary effect on the activation of HSCs, underlying the evolution of liver fibrosis: lowering their activation level through modulation of the PPAR signaling pathway and protecting the cells of the oxidative stress.

The present study has addressed the question of lycopene effect on metabolism and activation, using an *in vitro* model of the murine GRX cell line representative of the murine HSCs. These cells are a useful model to study lycopene metabolism since they represent the murine hepatic stellate cells, both in their structural and metabolic properties [16,17,18]. Thus, the present study aimed to investigate the lycopene and their influence on cell cycle, lipid metabolism and the possible pathways involved in lycopene inhibition of activated stellate cells.

MATERIALS AND METHODS

Materials

All-trans lycopene was purchased from Sigma Chemical Company (St. Louis, MO, USA). Water-soluble (WS) lycopene (10%) was provided by Roche (Rio de Janeiro, RJ, Brazil). Dulbecco's cell culture medium and bovine serum albumin were obtained from Sigma, and fetal bovine serum (FBS) from Laborclin (Campinas, SP, Brazil). Tissue culture flasks and cell scrapers were obtained from Nunc (Roskilde, Denmark). All the chemicals were of analytical grade.

Cell Culture and Treatment Protocol

GRX cells were obtained from the Rio de Janeiro Cell Bank (UFRJ, Rio de Janeiro), which certified the cell identity and the absence of microbiological contaminants. GRX cells were plated in 25 cm² tissue culture flasks, 5x10⁶ cells/flask, and maintained routinely in the Dulbecco's medium

supplemented with 5% FBS and 2 g/L HEPES buffer, pH 7.4, under 5% CO₂ atmosphere. Under these conditions, they expressed the myofibroblast phenotype. In order to induce the lipocyte phenotype, cells (10⁴ cells/cm²) were incubated for 10 days in the standard culture medium. The culture medium was changed every 5 days and the incubated at different concentrations lycopene WS (1.0–5.0 μM lycopene water soluble, Roche) dissolved in water at 50°C. All the solutions were prepared every day and studied in the dark to maintain the lycopene stability.

Cell Viability Assay

GRX cells (5 x 10³ cells/seed) were seeded in 96-well plates, and after 24 hours they were incubated with different concentrations of lycopene ranging from 0.625–5.0 μM lycopene. The supernatant was removed every 2 to 3 days, cells were incubated it in the standard culture medium, and 10 μL of MTT (5 μg/mL) were added to each well, leaving the cells in the dark for two hours at 37°C. After incubation, the supernatant was discarded, and the precipitate dissolved in 150 μL dimethyl-sulfoxide. The plates were read in an enzyme-linked immunosorbent micro-plate reader (BioRad 2550, USA) at 570 nm. Cell viability was determined by MTT assay (Amresco, USA). The lycopene concentrations used in this work are by previous reports.

Cell Cycle Analysis

Cells were rinsed briefly with Ca-Mg free phosphate-buffered saline (PBS) and detached with trypsin at room temperature. After centrifugation, the cells were washed twice with PBS, 1x10⁶ cells were resuspended in 1.0 mL ice-cold VindeLov solution, containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/ml RNase and 50 μg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 minutes of incubation, the cell suspension was analyzed for DNA content by flow cytometry using a FACSCalibur

flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells containing the diploid DNA in G0–G1 (2n), S phase (>2n but <4n), and G2/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9, respectively. The percentage of cell population at a particular phase was estimated with EXPO32 V1.2 Analysis software. The cell dissociation procedure does not affect fluorescence under the used experimental conditions.

Incorporation of [^{14}C]-acetate and lipids analysis

All the cells were incubated with 0.25 $\mu\text{Ci/mL}$ [^{14}C]-acetate in 2.5 mL DMEM without serum for 24 hours, on the last days of incubation period with lycopene (5 and 10 days). The radioactive medium was removed, the monolayer washed with BSS-CM. The cells were collected, and centrifugation separated fractioned in fat droplets. The lipids were extracted from the fat droplets and from other cell fractions. Chloroform was evaporated under a nitrogen atmosphere, and the radioactive lipids were analyzed by Thin Layer Chromatography (TLC). Lipids were separated and identified by migration patterns using hexane:ethyl ether:acetic acid (80:20:1, v/v/v) as the mobile phase. The TLC plate was imaged and the incorporation of acetate (%) was quantified by densitometry.

Microarray analyses: cRNA amplification, labeling and hybridization, and cDNA microarray

Cells were incubated with 3,0 μM lycopene and all RNA was extracted from cells using Trizol followed by the RNA extraction kit for its purification (RNeasy Quiagen). The cells were centrifuged to remove the culture medium and subsequently lysed with guanidine isothiocyanate for inactivation of RNAses. After adding 70% ethanol, the sample was applied to the extraction column or silica membrane mini spin RNeasy and subjected to successive centrifugations. The RNA was eluted with 30 or 50 μL of ultra-pure water free of RNAses and stored at -70°C .

The concentration and purity of the extracted RNA were monitored by spectrophotometry of 2 μ L sample in the Nanodrop ND-1000. The quality and integrity of the extracted RNA were assessed by electrophoresis in agarose gel marked with ethidium bromide, and by electrophoresis in 2% agarose gel with Tris-Borate (TBE). The quantity of RNA was related to the number of cells. The degree of purity, determined by the ratio of the lengths 260nm/280nm, remained within the threshold between 1.8 and 2.0, indicating the absence of DNA or protein contaminants. The total RNA was characterized by electrophoresis, by the presence of the two bands for ribosomal RNA fractions 28s and 18s.

The cDNA synthesis, sample markup, and hybridization in DNA Microarrays blades (GeneChip[®] 1.0 HumanGene ST) were done according to the standard Affymetrix Protocol WT Analysis. The procedure consists of RNA marking where the total RNA samples were submitted to the reverse transcription reaction for the synthesis of cDNA, adding oligo dT and T7-primers and random primers, followed by an *in vitro* transcription reaction (IVT), a new cDNA synthesis with random primers, with fragmentation and UDG APE and "labeling" or marking with biotin terminal via TdT (transferase).

The marked cRNA, the hybridization cocktail, probes and controls containing fluorescent marker phycoerythrin were introduced in the DNA chips and, after incubation and washing steps, were read by a suitable scanner, providing the first experimental data defined by the intensity of the detected signals.

Analysis of gene expression.

Primary analysis of expression data was performed in the laboratory computers microarrays using GeneChip operating software AFLP[®] Operating Software (GCOS 1.4) and Expression Console [®] 1.1 for correction of noise and quality analysis. The algorithm used in this analysis is known as RMA (Statistical Algorithm) Detection Call (a qualitative measure that indicates whether the transcript is detected, undetected or marginal) for each set. The secondary analyses of the difference of expression (Fold Change)

were made with the use of standardization and statistics software Bioconductor Lawson.

For each gene, the ratio of signal intensities was calculated and transformed using differentially expressed genes (SDS) common to two different statistical methods (Limma and RankProd, both with $p < 0.05$). The biological pathways identified by the Ingenuity program show the SDRs with red and green colors, with red genes with increased expression and green being reduced. In addition, the colors are presented with different gradients, that is, the redder more expressive, the less red less expressive, the same way for the green color.

This software also provides normalization methods and the data of an array were normalized by a global lowess method, based on the local estimation of intensities and a regression calculation weighted toward similar spots. All these normalization steps were represented in Box plots which show averaged ratio of each slide before and after lowess within-array normalization. The t-test clustered significant genes.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were harvested after 5 and 10 days of treatment with 1, 3 or 5 μM lycopene. GRX cells were washed with BSS CMF, detached with trypsin and counted. To the volume equivalent of 1×10^6 cells, 1 mL Trizol (Gibco) was added and, after homogenization, 200 μL chloroform was added. The RNA was separated following the manufacturer's protocol, and total RNAs was quantified using spectrophotometry. cDNA was synthesized from 3 μg of the total RNA, added 0.5 mg of oligo-(dT) 12-18 and 200 units MLV (reverse transcriptase) (both from Gibco). After 60 min of incubation at 37°C , we raised the temperature to 95°C for 5 minutes and added water (4°C) until the volume of 100 μL .

The PCR reactions were carried with 2.5 μL cDNA, 0.5 U Taq polymerase, 1.5 mM MgCl_2 , 0.5 μL dNTP (10mM) and 0.1 μg of the sense and antisense of each primer (Table 1). The annealing temperature

and the number of cycles of amplification reactions varied according to the primer pair were made on the following the manufacturer's specifications. After electrophoresis, the gel was incubated in ethidium bromide solution and analyzed by transillumination (UV), with quantification of the bands by densitometry using the program Image Master VDS, with correction of the bands by the expression of α -actin in the same sample as described by Sun et al.[19].

Table 1. Primer sequences for the reverse transcription-quantitative polymerase chain reaction.

Primer	Sense	Annealing temperature (°C)	Size of the PCR product (pb)
Cx 43	5'ATCCAGTGGTACATCTATGG 3' 5'CTGCTGGCTCTGCTGGAAGG 3'	58	593
PPARγ	5'TTTTCAAGGGTGCCAGTTTC 3' 5'TCTGTGACGATCTGCCTGAG 3'	58	501
RARα	5'GCTTAACCAAAGGACGGATTCTTGG 3' 5'CGGTGTGCTGTAACCACTGACTGG 3'	52	425
RXRα	5'ACCCAGGTGAACTCTTCGTCCC 3' 5'CGGCTTCCAGAATCTTCTCTACAGG 3'	52	694
RXRβ	5'TTCTCCTCCTGGCCCACCTCTTACC 3' 5'CCGCAGCAGTATGACCTGATCGTCC 3'	52	796
PPARγ	5'CCTCATGAAGAGCCTTCCAA 3' 5'TTTTGTGGATCCGACAGTTT 3'	55	177
α-actin	5'GTGGGCCGCTCTAGGCACCA 3' 5'CTCTTTCATGTCACGCACGATTTC 3'	58	560

Statistical Analysis

The presented data are mean values $\pm$ SD of two independent experiments done in triplicate (n=6). Statistical comparisons were carried out by ANOVA and post hoc Tukey's test using Graph Pad Prism 4.0 and Statistical 6.0 program. All $p < 0.05$ values were considered statistically significant.

The data were normalized with the RMA method, available in program R. We searched for the higher stringency of the analyzes using differentially expressed genes (SDS) common to two different statistical methods (Limma and RankProd), both with p-value < 0.05 ; In this case there are

no significant values of p due to the number of samples. We used the 67 SDRs common to the Limma and RankProd methods.

RESULTS

Phenotype conversion of GRX

As described previously [3], under standard culture conditions, GRX cells formed monolayers and had a fibroblastoid morphology corresponding to the activated stellate cells in hepatic tissue (Figure 1A and 1B). Lycopene induced expression of the lipocyte phenotype accumulating fat droplets in their cytoplasm. Similar to other models, induction of lipid storage was not synchronized, and groups of cells with the typical lipocyte phenotype were present with cells that had just begun accumulation of lipid droplets. After 10 days, most of the cells reached the typical lipocyte phenotype (Figure 1C). The lipid nature of cell inclusions could be demonstrated by their affinity for the dye Oil Red O (Figure 1D).

Fig 1. GRX cells in the standard culture medium (A) and incubated with Lycopene 10% WS (C) for 10 days and after staining with oil-red (B and D respectively).

Acetate incorporation into GRX cell lipids

GRX cells were supplemented with lycopene for 5 and 10 days and were incubated with labeled acetate in the last days of culture. The cells were then fractionated; lipid droplets and other fractions were separated, and the incorporation of acetate was analyzed by TLC. GRX cells incorporated the labeled acetate into lipids indicating their synthesis and turnover (Table 2). The high total labeling of phospholipids indicated their high synthesis and turnover, particularly in the membranes, where it nearly doubled with the induction of the fat storing phenotype in cells at 10 days. The increased acetate incorporation into

triglycerides located in the cytoplasm, followed by the expected segregation into the droplets, indicated that the net syntheses of triglycerides were induced by lycopene to attend this new demand. The presence of labeled free fatty acids in droplets probably indicates that lipid transfer from the cytoplasm to droplets was similar to the formation of fatty droplets in adipocytes, mediated by acyl-transferases.

Table 2. Cell fractionation and composition of lipid droplets of GRX cells incubated with 3 μ M lycopene for 5 and 10 days.

Incubation time (days)	Total		Membrane		Cytoplasm		Nucleus		Lipid droplets
	Incorporation (%)								
	5	10	5	10	5	10	5	10	10
Fosfolipids	62,99	66,84	47,01	85,03	31,30	31,74	64,24	45,77	64,94
Monoacylglycerol	0,74	ND	4,01	ND	4,2	ND	2,22	ND	ND
Diacylglycerol	2,54	1,34	2,42	1,16	11,74	ND	1,28	2,51	ND
Triacylglycerol	8,11	17,58	3,62	1,28	22,28	43,62	14,08	17,43	26,65
Cholesterol	7,81	2,74	25,80	6,18	6,61	2,60	4,81	4,66	ND
Free fatty acid	6,83	3,62	10,81	3,67	19,44	2,19	5,15	10,23	5,25

Cell Proliferation and Cell Cycle of GRX Cells

Cell proliferation analysis showed that lycopene, from 48 hours on, was able to reduce the growth of GRX cells (Figure 2). Within two days, all cells incubated with different concentrations of lycopene grew 50 to 75% when compared to cells not incubated with lycopene, except the cells incubated with 0.5 μ M of lycopene (Figure 2A). After five days of treatment, all cells incubated with different concentrations of lycopene obtained a reduction of growth by 25%. After seven days of lycopene incubation, the cell growth profile showed no statistically significant differences (Figure 2C), being constant after 10 days of incubation (data not shown).

Fig 2. Growth profile of GRX cells incubated with different concentrations of lycopene (0.625-20 μ M) for 2 days (A), 5 days (B) and 7 days (C). Columns without common letters are statistically different ($p < 0.05$).

GRX cells were incubated with lycopene (1.0-5.0 μ M) for 2 to 7 days after the period of cell cycle analysis was performed. According to the Figure 3A, after 2 days incubation with lycopene, the fraction of GRX cells in G0/G1 phase increased, followed by a decrease of cells in the G2/M phase, regardless of the concentration of lycopene used ($p > 0.05$). This fact showed that there was an increase in intracellular lycopene content at the beginning of the formation of lipocyte phenotype. After 10 days of incubation, the cell cycle was normalized again, with no significant difference between the GRX cells incubated with lycopene and the control ones ($p > 0.05$) (Figure 3B).

Fig 3. Cell Cycle of untreated GRX cells (CT) and treated with lycopene (1-5 μ M) for 2 (A) and 7 days (B). The results are expressed as mean $\pm$ standard deviation and compared by Tukey test (* $p < 0.05$).

Gene expression of lipocyte phenotype

GRX cells were incubated in the presence and absence of lycopene for 10 days and then analyzed for possible expression of genes involved in the formation of lipid droplets.

Cells incubated with different concentrations of lycopene (1.0-5.0 μ M) showed an increased expression of RXR- α , RXR- β , and PPAR γ as compared to controls (Figure 4A-C). No change was detected in the expression of RAR as compared to the untreated groups of cells incubated with lycopene (Figure 4D-E).

Fig 4. Analysis by RT-PCR of untreated GRX cells (CT) and incubated with lycopene (1-5 μ M) and nuclear receptor expression RXR- α (A), RXR- β (B), PPAR- γ (C), connexin 43 (D) and RAR (E). The results are expressed as mean $\pm$ standard deviation (* $p < 0.05$).

Microarray analysis

The differentially expressed genes from the above analyses of Limma e Rank Prod discriminations categorized 67 genes and select 4 genes with a higher statistical difference (Table 3 and Figure 5). All these genes are involved in the metabolism of retinoic acid, but little is known about their activation through lycopene that has not the pro-vitamin A activity. We observed up-regulation of cellular growth and proliferation genes such as HLA-DPB1. Cell cycle-related genes were also modulated by lycopene treatment, with up-regulation of RGL2, C2 and CCHCR1 (Table 3).

Table 3. Top Bio Functions modified by lycopene in GRX cells by microarray analysis

Gene — DEG	Bio Function	Fold increase
C2	Cholesterol metabolism	8.215
HLA-DPB1	Cellular growth and proliferation	3.907
CCHCR1	Lipid metabolism Small Molecule Biochemistry Endocrine System Development and Function	3.244
RGL2	Cellular growth and proliferation Cell Cycle Genetic Disorder	2.530

Fig 5. Pathways and mechanisms of activation of lycopene in hepatic stellate cells. Red genes are activated by lycopene.

DISCUSSION

This study has described the several prominent sets of information on lycopene in the context of protection and modification of the hepatic stellate cell (HSC) phenotype. Studies suggest that HSCs are the primary source of ECM in liver injury [20,21]. During the progression of liver fibrosis, their proliferation plays a major role in ECM production extremely rich in collagen leads to scar deposition and liver fibrosis. HSCs are activated and transdifferentiated to myofibroblasts that typically express α -smooth muscle actin (α -SMA) and results in overproduction of the extracellular matrix (ECM) [22,23].

HSCs proliferation is a key step during the fibrogenic process [24,25]. Studies suggest having shown that lycopene can reduce the growth of different cancer cells [26–29]. In the present study, lycopene was able to reduce the growth of GRX cells and phenotype modification. Depending upon the way and the dose of lycopene administration, it may alter proliferation and production of cell cycle-regulatory proteins. [30,31]. Our results showed no influence of the concentration of lycopene used on cell cycle of GRX cells. The lycopene deposited in plasma, liver, and other tissues had no adverse effects, and no teratogenic effects were observed in rat studies [32,33].

We observed that lycopene induced an increase of cells in the G0/G1 phase after 2 days of incubation, followed by a decrease of cells in the G2/M phase. Palozza et al. [26] suggested that lycopene blocked the G1/S phase of the cell cycle, and the possible targets of this block could be the cyclin D1 and inhibitors of the cell cycle. It has been demonstrated that lycopene induced a G1/S cell cycle reduction, expressed by the upregulation of the cyclin A and p27 and downregulation of cyclins E and D1 [30,34,35,36]. Lycopene can induce cell cycle arrest at different stages in different human cancer cells [26,27,30,35–37].

During HSC conversion to the lipocyte phenotype, a reduction of GRX growth and induction of cell cycle arrest was observed, modifying the lipid composition and metabolism. A resting phenotype induction

mediated by lycopene can have a protective role in liver pathologies that cause fibrosis. At the same time, induction of the myofibroblasts phenotype with loss of lipid droplets can reduce the storage capacity of the lycopene in hepatic stellate cells.

The overall pattern of lipid accumulation was comparable to that observed by Martucci et al. [2], where triacylglycerols and free fatty acids were the major components of lipid droplets in GRX cells. This accumulation was accompanied by a significant change in lipid content of cell membranes, with an increase of phospholipids and a decrease in membrane cholesterol. These changes turn the membrane more fluid, ensuring greater mobility for rotations, and promoting greater protection against oxidation [38]. In addition, there was a decrease in the percentage of incorporation of mono and diglycerides and free fatty acid, both in total content and in cell fractions, but this was not observed in the nucleus.

Microarray technology provides a genomic approach to explore the markers and molecular mechanisms leading to hepatic fibrosis [39]. Accordingly, we used cDNA microarray analysis to detect the up-regulated genes associated with modification of HSC activation. Such analyses have already revealed roles in selected gene products in cell developmental and physiological functions. The activated pathways often involve retinoic acid, and we decided to test here its functional analog, the lycopene.

Microarray analysis identified many differentially expressed genes that are important in the cell cycle, cellular growth and proliferation and lipid metabolism. To confirm these results and determine the effects of lycopene on pathways other than those specifically involved in HSC, we performed a genome analysis using lycopene on GRX cells. Lycopene treatment modified the expression of 67 DEGS, but only 3 genes with statistical difference. Modification of some of these genes has been reported recently. We observed up-regulation of cellular growth and proliferation genes such as HLA-DPB1 and RGL2. Recent genome-wide studies have demonstrated that HLA-DPB1 gene may play an essential role on RNA expression and progression of HCV and HBV virus-related liver diseases [40,41]. The C2 protein plays a relevant role in

the regulation of intracellular cholesterol homeostasis by binding with free cholesterol, and this protein affects free cholesterol metabolism and regulates the HSCs activation [42].

The mechanisms of HSC activation are crucial for the understanding of liver fibrogenesis. Cytokines promote activation from Kupffer cells and platelets, among which TGF-beta is of great importance. It stimulates the HSC gene expression of ECM molecules, but inhibits proliferation and induces phenotypic transdifferentiation [43]. Previous studies demonstrated that the acyclic retinoic acid equivalent might be a possible metabolite of lycopene that induces apoptosis and regression of cell cycle. Activation of the retinoic acid signaling pathway could be involved in the activation of HSC. Lycopene has not the provitamin A activity, but it can form an acyclic retinoic acid equivalent, able to interact with the nuclear receptor of retinoic acid (RAR) [44].

Depletion of PPAR γ accompanies liver fibrosis and cirrhosis, in which activation of hepatic stellate cells occurs with α -actin expression, loss of the fat droplets, reduction of retinol content, increase in cell proliferation and increase in extracellular matrix production. These processes can be reversed with the restoration of PPAR γ expression [45].

PPARs are transcription factors that belong to the superfamily of nuclear receptors, which regulate expression of a large number of genes involved in biological processes, including energy and lipid metabolism, in response to environmental and dietary changes. PPARs belong to the type II family of nuclear receptors (NR), a group that encompasses non-steroid NRs that form obligate heterodimers with RXR. These heterodimeric receptors have functional domains for DNA and ligand binding. PPARs bind to recognition sequences in the promoter regions of their target genes and act directly to regulate gene transcription. [46,47].

The RXR also has an antifibrogenic protective effect on the liver. PPAR γ /RXR heterodimers regulate transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism,

neurodegenerative disorders, cancer and inflammation [48]. PPAR γ activators include prostanoids, fatty acids, thiazolidinediones and N-(2-benzoylphenyl) tyrosine analogs. RXR ligands include naturally occurring retinoic acid and synthetic retinoids. Specific ligands for RXR and PPAR receptors increase metabolic abnormalities associated with type 2 diabetes, obesity, and other cardiovascular risk factors. Although adipose tissue mediated some of the effects of PPAR γ /RXR ligands, other tissues also regulate the effects of these receptors. The activity of the PPAR γ /RXR heterodimer is influenced by posttranslational modifications, receptor turnover, polymorphisms, splice variants, coactivators and corepressors [49].

In human breast cancer cell lines on established in vitro, the RXR-selective ligand potentiates the antiproliferative and apoptotic responses of PPAR ligands. The molecular mechanisms regulating these effects are currently unclear [50]. PPAR and RXR ligands have been shown to differentially recruit subsets of transcriptional coactivator proteins to the receptor complex [51]. Combinations of these ligands may result in additional coactivator recruitment, leading to enhanced transcriptional activation and cellular effects.

In our study, GRX cells incubated with lycopene augmented their expression of RXR- α , RXR- β , and PPAR γ . PPAR signaling pathway appears to have been activated with the increased expression of PPAR and RXR, forming dimers in cells incubated with lycopene. Yang et al. [28] reported that lycopene increases expression of PPAR γ and RXR α , a direct target gene for the PPAR γ :RXR heterodimer.

Jun Wei [52] examined the regulation of HSC biology through a mechanism involved in the antifibrotic effect of PPAR, including the effect of endogenous PPAR on hepatic fibrosis. An increase in PPAR expression in quiescent primary HSC and PPAR depletion in culture-activated HSC was reported. Quiescent HSCs express adipocyte-specific genes, under the control of adipogenic transcription factor PPAR. Adipogenic transcriptional regulation by PPAR is thus required for the maintenance of the

quiescent HSC phenotype [53,54].

CONCLUSION

Our results indicate that lycopene induced a lipocyte phenotype in hepatic stellate cells, promoted cell cycle arrest in G₀/G₁ phase and decreased cell viability. During this process, there is an accumulation of lycopene in the cell membrane, with modification of membrane lipids. Also, we demonstrated that lycopene supplementation modulates molecular pathways by affecting the expression of cell cycle-related genes, cellular growth, proliferation and lipid metabolism probably by transcription activation of PPAR γ and RXR. Taken together, the present data suggest possible protective mechanism mediated by lycopene hepatic stellate cells.

ACKNOWLEDGMENTS

This research was supported by CNPq and CAPES grants of the Brazilian Federal Government, and FAPERJ grant of the Rio de Janeiro State Government.

REFERENCES

1. Sato M, Suzuki S, Senoo H. Hepatic stellate cells: unique characteristics in cell biology and phenotype. *Cell Struct Funct.* 2003;28: 105–12. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12808230>
2. Martucci RB, Ziulkoski AL, Fortuna VA, Guaragna RM, Guma FCR, Trugo LC, et al. β -Carotene storage, conversion to retinoic acid, and induction of the lipocyte phenotype in hepatic stellate cells. *J Cell Biochem.* 2004;92: 414–423. doi:10.1002/jcb.20073
3. Teodoro AJ, Perrone D, Martucci RB, Borojevic R. Lycopene isomerisation and storage in an in vitro model of murine hepatic stellate cells. *Eur J Nutr.* 2009;48. doi:10.1007/s00394-009-0001-6
4. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, et al. Senescence of Activated Stellate Cells Limits Liver Fibrosis. *Cell.* 2008;134: 657–667. doi:10.1016/j.cell.2008.06.049
5. Kong D, Zhang F, Zhang Z, Lu Y, Zheng S. Clearance of activated stellate cells for hepatic fibrosis regression: Molecular basis and translational potential. *Biomed Pharmacother.* 2013;67: 246–250. doi:10.1016/j.biopha.2012.10.002
6. Lu L, Wang J, Lu H, Zhang G, Liu Y, Wang J, et al. MicroRNA-130a and -130b enhance activation of hepatic stellate cells by suppressing PPAR γ expression: A rat fibrosis model study. *Biochem Biophys Res Commun.* 2015;465: 387–393. doi:10.1016/j.bbrc.2015.08.012
7. Paiva LA, Brand C, Bandeira-Melo C, Bozza PT, El-Cheikh MC, Silva PM, et al. Hepatic myofibroblasts derived from *Schistosoma mansoni*-infected mice are a source of IL-5 and eotaxin: controls of eosinophil populations in vitro. *Parasit Vectors.* 2015;8: 577. doi:10.1186/s13071-015-1197-3
8. Jiang W, Guo M-H, Hai X. Hepatoprotective and antioxidant effects of lycopene on non-alcoholic fatty liver disease in rat. *World J Gastroenterol.* Baishideng Publishing Group Inc; 2016;22: 10180–

10188. doi:10.3748/wjg.v22.i46.10180

9. Jin H, Lian N, Zhang F, Chen L, Chen Q, Lu C, et al. Activation of PPAR γ /P53 signaling is required for curcumin to induce hepatic stellate cell senescence. *Cell Death Dis.* 2016;7: e2189–e2189. doi:10.1038/cddis.2016.92
10. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, et al. PPAR α and PPAR γ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J. European Molecular Biology Organization*; 1996;15: 5336–48. Available: <http://www.ncbi.nlm.nih.gov/pubmed/8895578>
11. Zhang F, Lu Y, Zheng S. Peroxisome proliferator-activated receptor- γ cross-regulation of signaling events implicated in liver fibrogenesis. *Cell Signal.* 2012;24: 596–605. doi:10.1016/j.cellsig.2011.11.008
12. Kilic Suloglu, Ph.D. A, Girgin, Ph.D. APG, Selmanoglu, Ph.D. PG, Balci, MD S, Baydar, Ph.D. PT. Possible Effects of Lycopene and Silymarin on Rat Liver Functions and Oxidative Stress Markers. *Turkish J Biochem.* 2014;39: 344–350. doi:10.5505/tjb.2014.17136
13. Bandeira ACB, da Silva TP, de Araujo GR, Araujo CM, da Silva RC, Lima WG, et al. Lycopene inhibits reactive oxygen species production in SK-Hep-1 cells and attenuates acetaminophen-induced liver injury in C57BL/6 mice. *Chem Biol Interact.* 2017;263: 7–17. doi:10.1016/j.cbi.2016.12.011
14. Tang L, Guan H, Ding X, Wang J-S. Modulation of aflatoxin toxicity and biomarkers by lycopene in F344 rats. *Toxicol Appl Pharmacol.* 2007;219: 10–17. doi:10.1016/j.taap.2006.12.001
15. Holzapfel N, Holzapfel B, Champ S, Feldthusen J, Clements J, Hutmacher D. The Potential Role of Lycopene for the Prevention and Therapy of Prostate Cancer: From Molecular Mechanisms to Clinical Evidence. *Int J Mol Sci.* 2013;14: 14620–14646. doi:10.3390/ijms140714620

16. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourão PA, Emonard H, et al. Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. *In Vitro Cell Dev Biol.* 1985;21: 382–90. Available: <http://www.ncbi.nlm.nih.gov/pubmed/4030623>
17. Margis R, Borojevic R. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). *Biochim Biophys Acta - Mol Cell Res.* Elsevier; 1989;1011: 1–5. doi:10.1016/0167-4889(89)90069-4
18. Borojevic R, Guaragna RM, Margis R, Dutra HS. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. *In Vitro Cell Dev Biol.* 1990;26: 361–8. Available: <http://www.ncbi.nlm.nih.gov/pubmed/2188940>
19. SUN K, WANG Q, HUANG X. PPAR gamma inhibits growth of rat hepatic stellate cells and TGF beta-induced connective tissue growth factor expression¹. *Acta Pharmacol Sin.* 2006;27: 715–723. doi:10.1111/j.1745-7254.2006.00299.x
20. Lotersztajn S, Julien B, Teixeira-Clerc F, Grenard P, Mallat A. HEPATIC FIBROSIS: Molecular Mechanisms and Drug Targets. *Annu Rev Pharmacol Toxicol.* 2005;45: 605–628. doi:10.1146/annurev.pharmtox.45.120403.095906
21. Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol.* 2010;7: 425–436. doi:10.1038/nrgastro.2010.97
22. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev.* NIH Public Access; 2008;88: 125–72. doi:10.1152/physrev.00013.2007
23. Hernandez-Gea V, Friedman SL. Pathogenesis of Liver Fibrosis. *Annu Rev Pathol Mech Dis.* 2011;6: 425–456. doi:10.1146/annurev-pathol-011110-130246
24. Sun X, Zhang X-D, Cheng G, Hu Y-H, Wang H-Y. Inhibition of hepatic stellate cell proliferation by heat shock protein 90 inhibitors in vitro. *Mol Cell Biochem.* 2009;330: 181–185.

doi:10.1007/s11010-009-0131-4

25. Son MK, Ryu Y-L, Jung KH, Lee H, Lee HS, Yan HH, et al. HS-173, a Novel PI3K Inhibitor, Attenuates the Activation of Hepatic Stellate Cells in Liver Fibrosis. *Sci Rep. Nature Publishing Group*; 2013;3: 3470. doi:10.1038/srep03470
26. Palozza P, Simone R, Catalano A, Boninsegna A, Böhm V, Fröhlich K, et al. Lycopene prevents 7-ketocholesterol-induced oxidative stress, cell cycle arrest and apoptosis in human macrophages☆. *J Nutr Biochem.* 2010;21: 34–46. doi:10.1016/j.jnutbio.2008.10.002
27. Teodoro AJ, Oliveira FL, Martins NB, Maia G de A, Martucci RB, Borojevic R. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int.* 2012;12: 36. doi:10.1186/1475-2867-12-36
28. Yang C-M, Lu I-H, Chen H-Y, Hu M-L. Lycopene inhibits the proliferation of androgen-dependent human prostate tumor cells through activation of PPAR γ -LXR α -ABCA1 pathway. *J Nutr Biochem.* 2012;23: 8–17. doi:10.1016/j.jnutbio.2010.10.006
29. Soares N da CP, Teodoro AJ, Oliveira FL, Santos CA do N, Takiya CM, Junior OS, et al. Influence of lycopene on cell viability, cell cycle, and apoptosis of human prostate cancer and benign hyperplastic cells. *Nutr Cancer.* 2013;65: 1076–85. doi:10.1080/01635581.2013.812225
30. Hwang E-S, Bowen PE. Cell Cycle Arrest and Induction of Apoptosis by Lycopene in LNCaP Human Prostate Cancer Cells. *J Med Food.* 2004;7: 284–289. doi:10.1089/jmf.2004.7.284
31. Hantz HL, Young LF, Martin KR. Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp Biol Med (Maywood).* 2005;230: 171–9. Available: <http://www.ncbi.nlm.nih.gov/pubmed/15734720>
32. Michael McClain R, Bausch J. Summary of safety studies conducted with synthetic lycopene. *Regul Toxicol Pharmacol.* 2003;37: 274–85. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12726756>

33. Rabus M, Demirbağ R, Sezen Y, Konukoğlu O, Yildiz A, Erel O, et al. Plasma and tissue oxidative stress index in patients with rheumatic and degenerative heart valve disease. *Turk Kardiyol Dern Ars.* 2008;36: 536–40. Available: <http://www.ncbi.nlm.nih.gov/pubmed/19223719>
34. Nahum A, Hirsch K, Danilenko M, Watts CK, Prall OW, Levy J, et al. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27Kip1 in the cyclin E–cdk2 complexes. *Oncogene.* 2001;20: 3428–3436. doi:10.1038/sj.onc.1204452
35. Ivanov NI, Cowell SP, Brown P, Rennie PS, Guns ES, Cox ME. Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines. *Clin Nutr.* 2007;26: 252–263. doi:10.1016/j.clnu.2007.01.002
36. Palozza P, Serini S, Boninsegna A, Bellovino D, Lucarini M, Monastra G, et al. The growth-inhibitory effects of tomatoes digested in vitro in colon adenocarcinoma cells occur through down-regulation of cyclin D1, Bcl-2 and Bcl-xL. *Br J Nutr.* 2007;98: 789–95. doi:10.1017/S0007114507746883
37. Park YO, Hwang E-S, Moon TW. The effect of lycopene on cell growth and oxidative DNA damage of Hep3B human hepatoma cells. *Biofactors.* 2005;23: 129–39. Available: <http://www.ncbi.nlm.nih.gov/pubmed/16410635>
38. Del Giudice R, Raiola A, Tenore GC, Frusciante L, Barone A, Monti DM, et al. Antioxidant bioactive compounds in tomato fruits at different ripening stages and their effects on normal and cancer cells. *J Funct Foods.* Elsevier; 2015;18: 83–94. doi:10.1016/J.JFF.2015.06.060
39. Liu X-J, Yang L, Luo F-M, Wu H-B, Qiang Q. Association of differentially expressed genes with activation of mouse hepatic stellate cells by high-density cDNA microarray. *World J Gastroenterol.* Baishideng Publishing Group Inc; 2004;10: 1600–7. doi:10.3748/WJG.V10.I11.1600

40. Hiramatsu K, Matsuda H, Nemoto T, Nosaka T, Saito Y, Naito T, et al. Identification of novel variants in HLA class II region related to HLA DPB1 expression and disease progression in patients with chronic hepatitis C. *J Med Virol*. 2017;89: 1574–1583. doi:10.1002/jmv.24814
41. Akgöllü E, Bilgin R, Akkız H, Ülger Y, Kaya BY, Karaoğullarından Ü, et al. Association between chronic hepatitis B virus infection and HLA-DP gene polymorphisms in the Turkish population. *Virus Res*. 2017;232: 6–12. doi:10.1016/j.virusres.2017.01.017
42. Twu Y-C, Lee T-S, Lin Y-L, Hsu S-M, Wang Y-H, Liao C-Y, et al. Niemann-Pick Type C2 Protein Mediates Hepatic Stellate Cells Activation by Regulating Free Cholesterol Accumulation. *Int J Mol Sci*. 2016;17: 1122. doi:10.3390/ijms17071122
43. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol*. 2017;14: 397–411. doi:10.1038/nrgastro.2017.38
44. Ben-Dor A, Nahum A, Danilenko M, Giat Y, Stahl W, Martin H-D, et al. Effects of acyclo-Retinoic Acid and Lycopene on Activation of the Retinoic Acid Receptor and Proliferation of Mammary Cancer Cells. *Arch Biochem Biophys*. 2001;391: 295–302. doi:10.1006/abbi.2001.2412
45. Hazra S, Xiong S, Wang J, Rippe RA, Krishna V, Chatterjee K, et al. Peroxisome Proliferator-activated Receptor γ Induces a Phenotypic Switch from Activated to Quiescent Hepatic Stellate Cells. *J Biol Chem*. 2004;279: 11392–11401. doi:10.1074/jbc.M310284200
46. Evans RM, Mangelsdorf DJ. Nuclear Receptors, RXR, and the Big Bang. *Cell*. 2014;157: 255–266. doi:10.1016/j.cell.2014.03.012
47. Sever R, Glass CK. Signaling by nuclear receptors. *Cold Spring Harb Perspect Biol*. Cold Spring Harbor Laboratory Press; 2013;5: a016709. doi:10.1101/cshperspect.a016709
48. Tyagi S, Sharma S, Gupta P, Saini A, Kaushal C. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res*. 2011;2: 236.

doi:10.4103/2231-4040.90879

49. Lenhard JM. PPAR gamma/RXR as a molecular target for diabetes. *Receptors Channels*. 2001;7: 249–58. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11697231>
50. Crowe DL, Chandraratna RA. A retinoid X receptor (RXR)-selective retinoid reveals that RXR- α is potentially a therapeutic target in breast cancer cell lines, and that it potentiates antiproliferative and apoptotic responses to peroxisome proliferator-activated receptor ligands. *Breast Cancer Res*. 2004;6: R546. doi:10.1186/bcr913
51. Yang W, Rachez C, Freedman LP. Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptor coactivators. *Mol Cell Biol*. 2000;20: 8008–17. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11027271>
52. Wei J, Bhattacharyya S, Varga J. Peroxisome proliferator-activated receptor γ : innate protection from excessive fibrogenesis and potential therapeutic target in systemic sclerosis. *Curr Opin Rheumatol*. NIH Public Access; 2010;22: 671–6. doi:10.1097/BOR.0b013e32833de1a7
53. MacDougald OA, Lane MD. Transcriptional Regulation of Gene Expression During Adipocyte Differentiation. *Annu Rev Biochem*. 1995;64: 345–373. doi:10.1146/annurev.bi.64.070195.002021
54. Morrison RF, Farmer SR. Hormonal Signaling and Transcriptional Control of Adipocyte Differentiation. *J Nutr*. 2000;130: 3116S–3121S. doi:10.1093/jn/130.12.3116S

Chapter 3

Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell Biomarkers in Rats

Vanessa Azevedo de Jesus,¹ Monique de Barros Elias Campos,¹ Vanessa Rosse de Souza,¹ Teresa Palmiciano Bede,² Bianca Portugal Tavares de Moraes,³ Adriana Ribeiro Silva,⁴ Cassiano Felipe Gonçalves de Albuquerque,³ Vilma Blondet de Azeredo,² and Anderson Junger Teodoro¹

¹Food and Nutrition Program, Functional Foods Laboratory, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

²Nutrition and Dietetic Department, Fluminense Federal University, Rio de Janeiro, Brazil.

³Immunopharmacology Laboratory, Biomedical Institute, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

⁴Immunopharmacology Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil.

This chapter was published on Journal of Medicinal Food. (2019), 1–8.
DOI: 10.1089/jmf.2019.0014

**LYCOPENE AND TOMATO SAUCE IMPROVES
HEPATIC AND CARDIAC CELLS BIOMARKERS IN RATS**

Vanessa Azevedo de Jesus¹, Monique de Barros Elias Campos¹, Vanessa Rosse de Souza¹, Teresa Palmiciano Bede², Bianca Portugal Tavares de Moraes³, Adriana Ribeiro Silva⁴, Cassiano Felipe Gonçalves de Albuquerque³, Vilma Blondet de Azeredo², Anderson Junger Teodoro¹

¹ Food and Nutrition Program, Functional Foods Laboratory, Universidade Federal do Estado do Rio de Janeiro, RJ, Brazil

² Universidade Federal Fluminense, RJ, Brazil

³ Biomedical Institute, Immunopharmacology Laboratory, Universidade Federal do Estado do Rio de Janeiro, RJ, Brazil

⁴ Oswaldo Cruz Institute, Immunopharmacology Laboratory, Oswaldo Cruz Foundation, RJ, Brazil

***Corresponding Author:**

Anderson Junger Teodoro

Food and Nutrition Program, Functional Foods Laboratory, Universidade Federal do Estado do Rio de Janeiro, RJ, Brazil

E-mail address: atteodoro@gmail.com

ABSTRACT

This study evaluated the effect of tomato sauce and lycopene on hepatic and cardiac cells biomarkers in rats fed with high-fat diet. Animals were split into five groups: control (CG), high-fat (HG), high-fat tomato sauce (TG), high-fat lycopene 2 mg (L2G), and high-fat lycopene 4 mg (L4G). Food and water were offered *ad libitum*, while tomato source and lycopene (2 and 4 mg/day) were offered daily for 60 days. Body weight, heart weight, liver weight, cardiosomatic index, hepatosomatic index, and serum parameters were analyzed in rats. The animals' hearts and liver were processed, and cells were examined by flow cytometry. The groups that receiving tomato sauce and lycopene had lower glycemia. The serum concentration of HDL-cholesterol, hepatic enzymes, and TNF- α did not alter upon the treatments. Tomato sauce and lycopene supplementation avoided the increase in IL-1 β induced by the high-fat diet. Cell cycle analysis of cardiac and liver cells showed a lower percentage of cells in the G₀/G₁ phase and an increase in the G₂/M phase in HG., Both lycopene and tomato sauce reversed this effect. Both treatments avoided cardiac and liver cell death changes by the high-fat diet. Supplementation of tomato sauce and lycopene has a beneficial effect on cardiac and liver cells metabolism. So, we suggest it as a nutritional approach for the prevention and treatment of cardiovascular diseases and non-alcoholic hepatic steatosis.

Keywords: apoptosis; carotenoids; cell cycle; high-fat diet; inflammation.

INTRODUCTION

Obesity is a risk factor for various chronic diseases, the metabolic defects of obesity and type 2 diabetes, characterized by fatty liver disease, insulin resistance, and dyslipidemia, lead to an increased risk of cardiovascular disease and cancer^{1,2}

Although diagnosed worldwide, it has variations in prevalence, reaching about 20 to 30% in western countries. In the United States, a country where $\frac{1}{4}$ of the adult population is obese, the disease affects more than $\frac{2}{3}$ of these individuals. It is estimated that 2-3% of the population presents hepatic steatosis³. The consumption of diets rich in saturated fat is linked to the synthesis of pro-inflammatory cytokines, the increase of reactive oxygen species and the development of oxidative stress, and damages in several biomolecules. It is also a predisposing factor to the development of a variety of chronic diseases such as obesity, cognitive dysfunction, diabetes, and cancer^{4,5,6,7-9}. Thus, a high-fat diet has a central role to the development of oxidative events, as occurs in hepatic steatosis and atherosclerosis. Fatty liver is associated with several atherosclerotic risk factors such as hypertension, diabetes and dyslipidemia^{10,11}. Bioactive compounds supplements are a potential disease-preventing or health-promoting to be taken daily¹². Bioactive compounds are substances discovered from natural sources capable of retarding or inhibiting the oxidation rates and can be produced endogenously or absorbed through the foods in the diet^{13,14,15}. Some authors show an inverse relationship between the consumption of carotenoid-rich foods and the risk of diseases induced by oxidative stress^{6,16,17,18}.

Lycopene protects against many types of diseases. Mainly due to its antioxidative effects, lipid-regulating enzyme activities, adipocyte differentiation and improve the plasma lipid profile in rats fed with a high-fat diet¹². Previous studies have linked the high intake of tomatoes products or lycopene with lower risk for metabolic diseases, protective effect against the high-fat diet, and decreased of hepatic inflammation^{19,20,21,22}. However, there is no consensus in the literature which form of lycopene could

contribute more benefits to these inflammatory diseases, whether it would be from tomato products or from an isolated lycopene supplement.

Considering this body of research, the aim of this study was to investigate the effects of daily ingestion of tomato sauce and lycopene isolated on alterations related to cardiac and hepatic tissue in Wistar rats, hepatic, and cardiac cell cycle.

MATERIALS AND METHODS

Samples

Samples of tomato sauce were obtained from local market and samples of lycopene all-trans WS (water soluble) 10% were supplied by Roche (Rio de Janeiro, Brazil). Samples of tomato sauce were kept refrigerated in a refrigerator (10°C) and samples of lycopene were kept refrigerated in a freezer (-4°C) in appropriate packaging. The total lycopene of Samples of tomato sauce is shown in Fig 1.

Appropriate solutions for each experimental group were prepared daily in the laboratory by dissolving the content in filtered water at 50 ° C and adding 20% refined sugar (to give palatability to the solution).

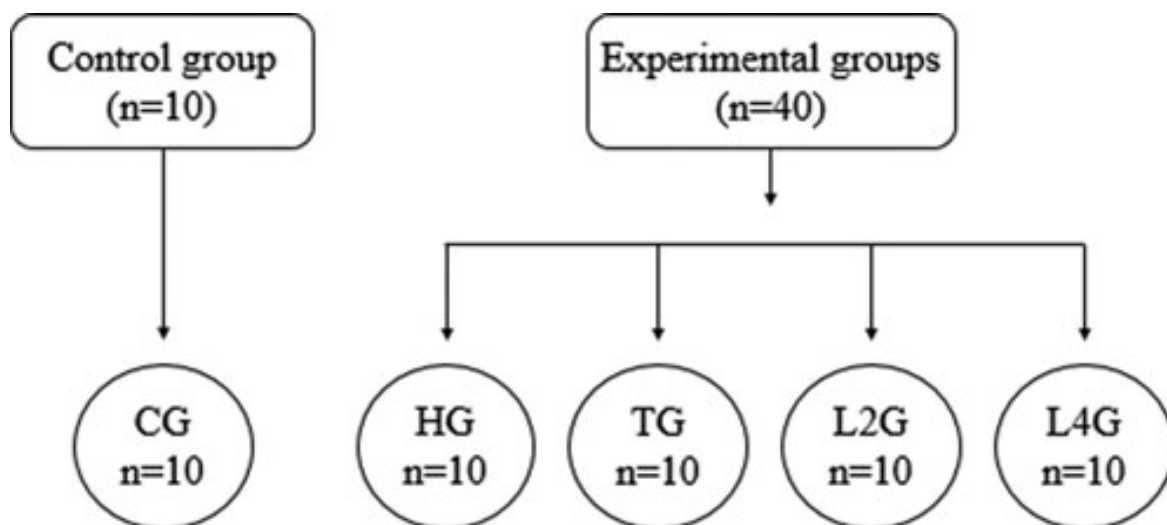


FIG. 1. Dietary protocol. Experimental model: CG: standard diet plus water; experimental groups: HG. high-fat diet plus water; TG. high-fat diet plus solution with tomato sauce and water; L2G. high-fat diet plus solution with 2.0 mg all-trans lycopene and water; L4G. high-fat diet plus solution with 4.0 mg all-trans lycopene and water. Diets and solutions were administered during a period of 60 days. CG, control group; HG, high-fat group; TG, tomato sauce group; L2G, lycopene 2 mg group; L4G, lycopene 4 mg group.

Experimental model

Fifty female, adults, Wistar rats were individually housed and maintained in a 12-h light–12-h dark cycle at 22°C (+/- 2°C). Animal maintenance was in accordance with the ARRIVE guidelines²³. Rat care and experimental protocol were approved by the Institution's Scientific, Academic, and Ethics Board.

Animals were divided in five groups: the control group (CG), which received a standard diet (based casein) *ad libitum*, consisting of protein (minimum) 12.95%, fat (minimum) 4.0%, and water; the high-fat group (HG), which received the high-fat diet *ad libitum*, consisting of protein (minimum) 12.95%, fat (minimum) 20.0%, and water *ad libitum*; the tomato sauce group (TG), which received the high-fat diet *ad libitum*, plus solution with tomato sauce providing 2.0 mg of lycopene per day; the 2.0 mg lycopene group (L2G), which received the high-fat diet *ad libitum*, plus solution with 2.0 mg *all-trans* lycopene (water soluble) 10% dissolved in water per day, and water; the 4.0 mg lycopene group (L4G), which received the high-fat diet *ad libitum*, plus solution with 4.0 mg *all-trans* lycopene (water soluble) 10% dissolved in water per day, and water. Table 2 presents the ingredients for the formulation of the control and high fat rations used in the experiment.

After 60 days of the experiment, all animals were submitted to the vaginal smear procedure to identify the phase in the estrous cycle, so that all were in the same physiological moment and, therefore there was no hormonal interference in the analysis. After the estrous cycle check, the rats that were in the “*estrus*” phase of the cycle were separated and fasted. Body weight was measured, and fasted animals were killed. Serum and organs (heart and liver) were obtained, weighted frozen and kept in -70°C until analysis. Experimental research was done according to the study carried out by Ribeiro *et al.* (2018)¹³.

The heart and liver removed from the animals were weighed to determine the relative weight of the organ denominated cardiosomatic index and hepatosomatic index, which is calculated according to the formula:

$$[(\text{Heart} / \text{Liver Weight (g)} \div \text{Body Weight}) \times 100]$$

Analytical methods

The glucometer measured serum glucose concentration. Serum total cholesterol, high-density lipoprotein (HDL-cholesterol), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), IL-1 β , and TNF- α concentration were measured using BioClin® commercial kits and wavelengths specific to each biochemical indicator, using the colorimetric method with automated spectrophotometer reading (BioClin® BS-120 Chemistry Analyzer®). LDL-C was calculated according to Friedwald's formula²⁴.

Cell cycle

Animal's hearts muscle tissue and liver were processed, and the cardiac and liver cells cycle and apoptosis were measured using flow cytometry. The cells extraction was realized through maceration of the tissue and addition of 0.5 mg/mL collagenase (Sigma®). After centrifugation, the cells were washed twice with phosphate-buffered saline and were resuspended in 500 μ L of ice-cold Vindelov solution containing 0.1% Triton X-100, 0.1% citrate buffer and 0.1 mg/mL RNase, and 50 mg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 min of incubation, cell suspension was analyzed for DNA content by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells with DNA content haploid subG1 (<2n), diploid G₀/G₁ (2n), S phase (>2n and <4n) and G₂/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9. Respectively, the percentage of cell population at a particular phase was estimated with FlowJo software following the acquisition of 30.000 events. The cell dissociation procedure does not affect fluorescence under the experimental conditions that were used in this study or in any others of which we are aware. Nuclei of viable cells were gated according to FL-2W $\times$ FL2-A relation according to the study carried out by Guimaraes *et al.* (2017)²⁵.

Apoptosis assay

For the apoptosis assay, cells were resuspended in 400 μL of binding buffer containing 5 μL of annexin V FITC and 5 μL propidium iodide (Apoptosis Detection Kit II. BD Biosciences) for 15 min at room temperature. Annexin V binding was evaluated by flow cytometry (FACScalibur. BD Biosciences) and after acquisition of 30.000 events. The data were analyzed in CellQuest and FlowJo software.

Data analysis

The main effects of supplementation type were tested using one-way ANOVA followed by Tukey's post hoc test for multiple mean comparison test. Results are expressed as mean–standard deviation and the level of significance was set at $p < 0.05$.

RESULTS

At the end of the experiments, body weights were measured and the final body weight was higher ($p < 0.05$) in the TG ($277.7 \pm 11.48\text{g}$) and lower in the L2G ($197.3 \pm 13.12\text{g}$) and L4G ($188.9 \pm 15.02\text{g}$) compared to the control group (Table 1). Heart weight was higher ($p < 0.05$) in the TG ($1.06 \pm 0.05\text{g}$) and lower in the L4G ($0.72 \pm 0.11\text{g}$) compared with the control group. No differences were observed in the liver weight, hepatosomatic and cardiosomatic index among the groups (Table 1). Heart's images of rats studied are displayed in Fig 2.

TABLE 1. BODY AND TISSUE WEIGHT IN CONTROL AND EXPERIMENTAL GROUPS

Parameters (g)	CG	HG	TG	L2G	L4G
Initial body weight	160.4 – 4.83 ^a	161.4 – 6.23 ^a	163.4 – 5.68 ^a	162.0 – 5.34 ^a	158.6 – 5.94 ^a
Final body weight	261.00 – 31.04 ^a	268.20 – 17.68 ^a	277.70 – 11.48 ^a	197.30 – 13.12 ^b	188.90 – 15.02 ^b
Heart weight	0.94 – 0.21 ^a	0.90 – 0.07 ^a	1.06 – 0.05 ^b	0.80 – 0.16 ^a	0.72 – 0.11 ^c
Liver weight	7.86 – 1.52 ^a	7.10 – 0.57 ^a	8.50 – 1.01 ^a	5.78 – 0.55 ^b	5.82 – 0.37 ^b
Cardiosomatic index	0.36 – 0.09 ^a	0.34 – 0.01 ^a	0.38 – 0.06 ^a	0.40 – 0.06 ^a	0.38 – 0.04 ^a
Hepatosomatic index	2.99 – 0.23 ^a	2.65 – 0.25 ^a	3.06 – 0.43 ^a	2.95 – 0.43 ^a	3.09 – 0.17 ^a

Values are mean - SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

CG, control group; HG, high-fat group; TG, tomato sauce group; L2G, lycopene 2 mg; L4G, lycopene 4 mg; SD, standard deviation; ANOVA, analysis of variance.

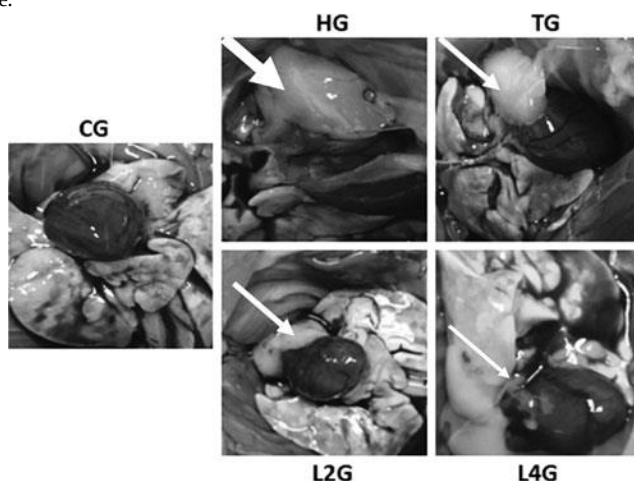


FIG. 2. Heart extracted from sacrificed rats after dietary protocol. *White arrows indicate accumulated fat in tissues.*

Significant differences ($p < 0.05$) in glycemia, cholesterol total and triglycerides were found among the groups analyzed (Table 2). Groups receiving tomato sauce (TG) and lycopene (L2G, and L4G) had lower values of glycemia compared to control. Also, the group receiving high-fat diet had higher levels of triglycerides compared to CG and L4G group had significantly lower triglycerides compared to the HG group. No differences were seen in the concentrations of HDL-cholesterol and liver enzymes (AST and ALT) in serum among the groups (Table 2).

TABLE 2. SERUM PARAMETERS IN RATS FED WITH EXPERIMENTAL DIETS

Parameters (mg/dL)	CG	HG	TG	L2G	L4G
Glycemia	107.00 – 2.65 ^a	101.67 – 4.51 ^{ac}	86.20 – 4.76 ^b	88.60 – 4.56 ^b	88.60 – 6.80 ^b
Total cholesterol	46.00 – 8.60 ^{ab}	45.00 – 3.54 ^b	60.00 – 4.06 ^c	56.80 – 8.14 ^{abc}	53.80 – 4.49 ^{abc}
HDL cholesterol	21.60 – 3.51 ^a	22.40 – 1.34 ^a	25.00 – 2.55 ^a	24.20 – 1.30 ^a	23.40 – .34 ^a
LDL cholesterol	13.62 – 5.01 ^a	26.76 – 2.50 ^b	23.80 – 3.77 ^b	19.53 – 4.80 ^b	21.82 – .01 ^b
Triglycerides	31.60 – 8.17 ^a	47.00 – 7.94 ^b	41.20 – 7.66 ^b	40.00 – 6.20 ^b	33.25 – .68 ^a
AST	195.60 – 52.03 ^a	219.80 – 33.39 ^a	198.20 – 2.77 ^a	177.80 – 41.81 ^a	176.40 – 26.60 ^a
ALT	32.20 – 6.42 ^a	32.00 – 5.39 ^a	29.00 – 4.80 ^a	28.00 – 5.43 ^a	33.20 – .81 ^a

Values are mean - SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

HG showed increase in IL-1 β expression compared to the other groups. Tomato sauce and lycopene, however, avoided this effect with a similar profile. Importantly, TG showed similar results to

CG. There were no differences in the levels of TNF- α among the groups (Fig.3).

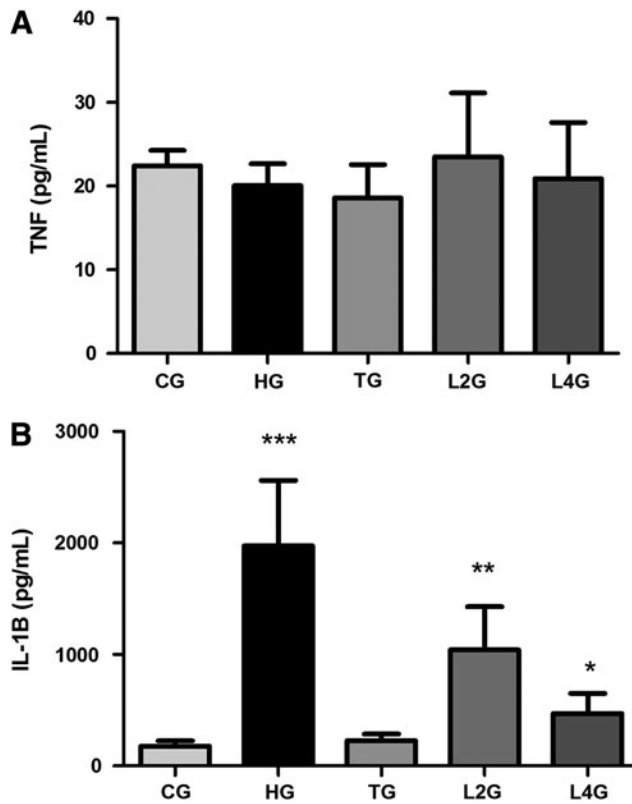


FIG. 3. (A) TNF- α (B) IL-1 β from rats fed a high-fat diet supplemented with lycopene and tomato sauce. * $P < .05$; ** $P < .01$; *** $P < .001$. ANOVA–Tukey’s test. ANOVA, analysis of variance; TNF, tumor necrosis factor; IL, interleukin.

Cell cycle analysis of cardiac cells showed that the HG presented a lower percentage of cells in the G_0/G_1 phase (47.98 ± 6.28), compared to the other groups (Table 3). Besides, the high-fat diet increased the percentage of cells in the G_2/M phase, reversed by the action of both lycopene and tomato sauce ($p < 0.05$).

TABLE 3. EFFECT OF LYCOPENE AND TOMATO SAUCE ON CELL CYCLE PROGRESSION OF CARDIAC CELLS IN RATS FED HIGH-FAT DIET

Groups	Sub-G1	G0/G1	S	G2/M
CG	3.27 – 0.42 ^a	79.80 – 4.34 ^a	8.90 – .44 ^a	7.95 – .50 ^a
HG	6.35 – 1.05 ^b	71.39 – 3.88 ^b	9.76 – .85 ^a	16.03 – 1.82 ^b
TG	1.53 – 1.22 ^a	78.86 – 7.14 ^a	8.53 – .49 ^a	12.66 – 1.62 ^c
L2G	3.44 – 0.29 ^a	81.55 – 7.88 ^a	9.06 – .67 ^a	5.95 – 2.03 ^a
L4G	1.52 – 0.97 ^a	80.17 – 10.35 ^{ab}	10.95 – .73 ^a	8.87 – 1.87 ^a

The results are expressed as the percentages of total cells. Values are mean – SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA, followed by Tukey’s *post hoc* multiple

mean comparison test.

In liver cells, the high-fat diet decreased the number of cells in the G₀/G₁ phase and raised the percentage of cells in the G₂/M phase. We hypothesize that the highest rate of cell death in that group promoted an increase in cellular proliferation as an attempted to surpass cellular loss. Both tomato sauce and lycopene-treated groups increased the number of cells in the G₀/G₁ phase and decreased its numbers in the G₂/M phase induced by the high-fat diet (Table 4).

TABLE 4. EFFECT of LYCOPENE AND TOMATO SAUCE ON CELL CYCLE PROGRESSION of HEPATIC CELLS IN RATS FED HIGH-FAT DIET

<i>Groups</i>	<i>Sub G1</i>	<i>G0/G1</i>	<i>S</i>	<i>G2/M</i>
CG	1.98 – 0.36 ^a	63.49–6.06 ^a	20.20 – 8.47 ^a	14.41 – 3.85 ^a
HG	4.28 – 0.15 ^b	53.67 – 5.79 ^b	28.18 – 3.40 ^b	18.15 – 3.08 ^a
TG	1.22–0.24 ^a	67.50–10.10 ^c	15.66–6.10 ^a	16.84–4.40 ^a
L2G	1.15 – 0.53 ^a	77.96–4.89 ^{cd}	10.57 – 2.05 ^a	11.47 – 2.85 ^a
L4G	1.62 – 0.78 ^a	71.27–7.39 ^d	13.81 – 3.65 ^a	14.92 – 3.93 ^a

The results are expressed as the percentages of total cells. Values are mean – SD.

^{abcd}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

Annexin V and PI biomarkers were used to assess apoptosis. An increase in apoptotic cells was shown in HG compared to CG in liver cells. The lycopene and tomato sauce remarkably diminished the percentage of cells in apoptosis compared to HG (Fig.5) denoting its protector effect against apoptosis induced by a high-fat diet (Fig.4).

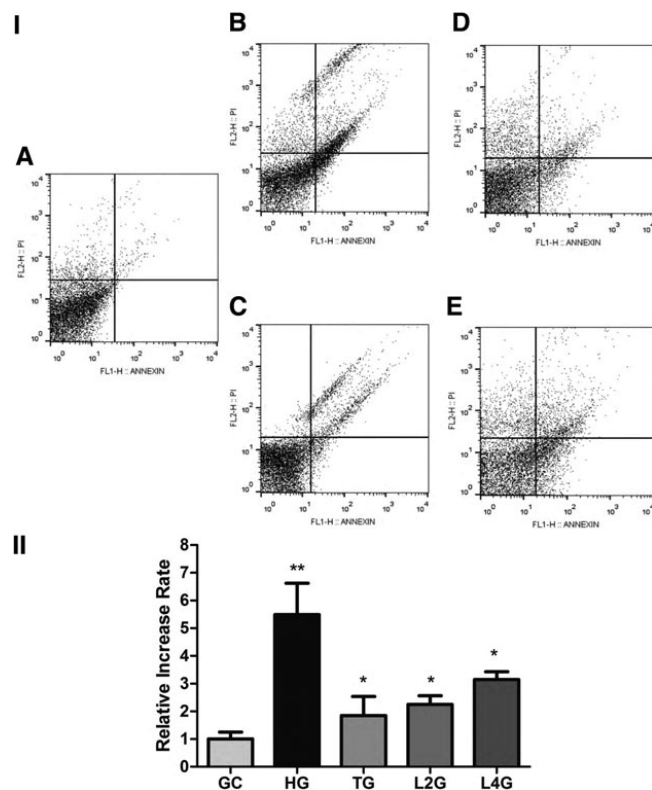


FIG. 4. Monitoring of cell death of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (I) Flow cytometry analysis of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (II) Quantitative effects of lycopene and tomato sauce of cell death of cardiac cells from rats fed a high-fat diet. (A): CG; (B): HG; (C): TG; (D): L2G; (E): L4G. The results are expressed as mean – SD, with significant differences compared by one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. * $P < .05$. ** $P < .01$. SD, standard deviation.

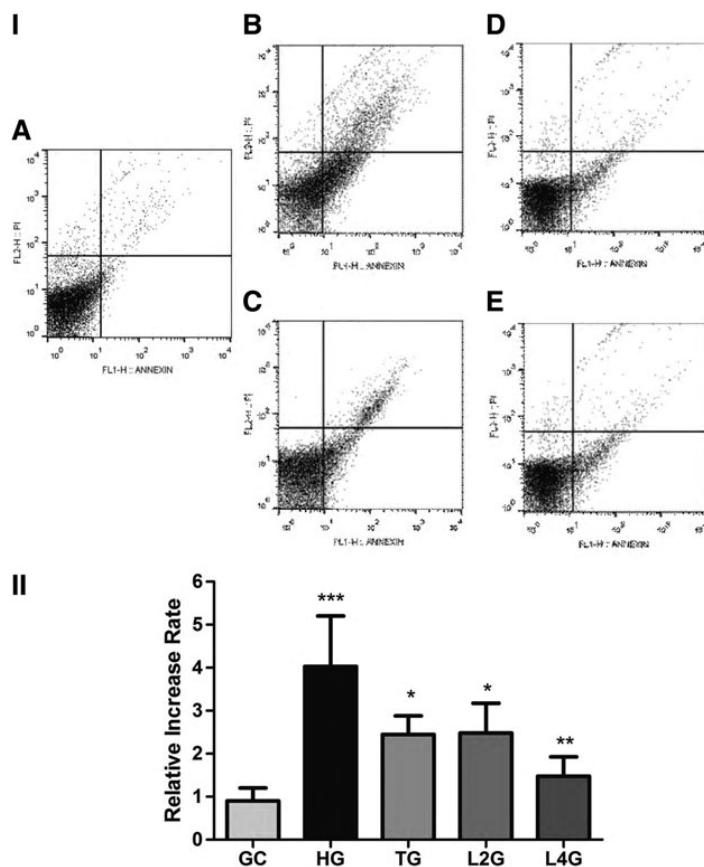


FIG. 5. Monitoring of cell death of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (A) Flow cytometry analysis of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (B) Quantitative effects of lycopene and tomato sauce of cell death of hepatic cells from rats fed a high-fat diet. (A): CG; (B): HG; (C): TG; (D): L2G; (E): L4G. The results are expressed as mean – SD, with significant differences compared by one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. * $P < .05$. ** $P < .01$.

DISCUSSION

Tomato sauce is a rich source of lycopene, which has a potent antioxidant activity. Nevertheless, very little is known about different forms of lycopene supplementation in cardiovascular diseases and non-alcoholic hepatic steatosis^{4,21,26–30}. The present study provided concerning information about the effects of lycopene and lycopene in food matrix supplementation on the cardiac and liver metabolism, cell cycle and apoptosis. Our results agree with studies realized in which quercetin supplementation in rats was able to reduce rats' weight gain even with the ingestion of a high-fat diet and increase the size of the heart when compared to the control¹². Here in, the lycopene supplementation in rats reduced weight gain high-fat diet and was cardioprotective.

Previous studies have shown that the intervention with tomato juice *in vivo* did not affect glucose and lipid profile^{31,32}. Neither changes in the gain weight were observed in accordance with the results found here.

Obesity and dyslipidemia are considered risk factors for this cardiovascular disease and non-alcoholic hepatic steatosis. Also, it has been reported that a high-fat diet and cholesterol can favor metabolic alterations even more³³. Our results revealed that a high-fat diet fostered an increase in triglycerides and cholesterol, and lycopene and tomato sauce supplementation improved rat's lipid profile.

Cellular mechanisms triggered by the consumption of a high-fat diet, includes apoptosis, necrosis, and autophagy^{34–37}. Those effects may be linked to the genesis of cardiovascular diseases and non-alcoholic hepatic steatosis. Therefore, it is vital to identify strategies that may contribute to their prevention and reduction.

Stress factors can induce IL-1 β and TNF- α . Pro-inflammatory cytokine IL-1 β is raised in chronic inflammatory diseases, like obesity, and can be associated with increased cell proliferation, cell cycle arrest, and increased apoptosis in different type cells^{37,38}. We showed that tomato sauce and lycopene supplementation reversed the increase of IL-1 β levels induced by the high-fat diet.

Numerous studies have investigated the effects of individual compounds on vital cellular parameters and apoptosis in an effort to determine the underlying mechanisms of action; however, few studies have investigated the influence of combinations of phytochemicals in this context^{39,40}.

In order to determine the basic mechanisms by which carotenoids present in the food matrix may be more effective in preventing and treating diseases than individual compounds, this study was the approach of compare two versions of lycopene, isolated and in the food matrix, and compare the inhibitory effects of cell growth and apoptosis.

Scarce studies reported the mechanism that fruits and vegetables could prevent or reduce inflammatory diseases, like cardiovascular diseases and non-alcoholic hepatic steatosis. Cardiac cell cycle analysis reported that a high-fat diet promoted a decrease in the G_0/G_1 phase and an increase in the percentage of cardiac cells in the G_2/M phase, showing cell proliferation to compensate higher cell death. Nevertheless, it is important to mention The pronounced cell proliferation may be harmful to the organism.

Lycopene promoted an increase of cell in G_0/G_1 phase and a reduction in the cell percentage on G_2/M phase in different cancer cell lines⁴. Our study showed both tomato sauce and lycopene supplementation increased number of cardiac cell in G_0/G_1 phase and a drop in the number of cardiac cells in G_2/M compared to the high-fat diet. The tomato sauce promoted a reduction of the effect caused by the high-fat diet in the cardiac cells of the animals studied. Lycopene increased the percentage of cardiac cells in the G_0/G_1 phase, perhaps lessening the damage of the high-fat diet. Similar result was observed in the liver cell cycle, where tomato sauce and lycopene supplementation increased the percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in the G_2/M phase.

Consumption of tomato juice and pure lycopene regulates the cell cycle of HepG2 cells. Nonetheless, tomato juice did not promote alteration in apoptosis; only lycopene supplementation was able to induce apoptosis of HepG2 cells^{40,41}.

The present study showed a damaging effect of high-fat diet on cardiac cells inducing apoptosis.

Tomato and lycopene displayed cardioprotector effects. Similar results could be to observe in different cancer cell lines which lycopene promoted an increase of apoptosis^{4,42}. Also, recent studies have demonstrated the protective action of lycopene in cardiomyocytes by the induction of apoptosis *in vitro*^{34,35,43}.

We have shown that tomato sauce and lycopene supplementation has a beneficial effect on cardiac and liver cells metabolism and may be considered as a nutritional approach for the prevention and treatment of cardiovascular diseases and non-alcoholic hepatic steatosis.

ACKNOWLEDGMENT

This research was supported by CNPq and CAPES grants of the Brazilian Federal Government, and FAPERJ grant of the Rio de Janeiro State Government.

AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

REFERENCES

1. World Health Organization. Taking Action on Childhood Obesity. 1–8 (2017).
2. Semenkovich, C.F. Insulin resistance and atherosclerosis. J. Clin. Invest., 116; pp. 1813-1822 (2006).
3. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis.28:155-61(2010).
4. Gloria, N. F. et al. <2014_Kycioebe Abd Beta-Caritebe Induce Cell-Cycle Arrest and Apoptosis in Human Breast Cancer Cell Lines_Gloria.Pdf>. 1386, 1377–1386 (2014).
5. Thies, F. et al. Effect of a tomato-rich diet on markers of cardiovascular disease risk in moderately

overweight, disease-free, middle-aged adults: A randomized controlled trial. *Am. J. Clin. Nutr.* 95, 1013–1022 (2012).

6. McEneny, J. et al. Lycopene intervention reduces inflammation and improves HDL functionality in moderately overweight middle-aged individuals. *J. Nutr. Biochem.* 24, 163–168 (2013).
7. Shiou, Y. L., Huang, I. C., Lin, H. T. & Lee, H. C. High fat diet aggravates atrial and ventricular remodeling of hypertensive heart disease in aging rats. *J. Formos. Med. Assoc.* 117, 621–631 (2018).
8. Zhang, Q. et al. Effects of oral selenium and magnesium co-supplementation on lipid metabolism, antioxidative status, histopathological lesions, and related gene expression in rats fed a high-fat diet. *Lipids Health Dis.* 17, 1–12 (2018).
9. Duavy, S. M. P., Salazar, G. J. T., Leite, G. de O., Ecker, A. & Barbosa, N. V. Effect of dietary supplementation with olive and sunflower oils on lipid profile and liver histology in rats fed high cholesterol diet. *Asian Pac. J. Trop. Med.* 10, 539–543 (2017).
10. Akahoshi M, Amasaki Y, Soda M, Tominaga T, Ichimaru S, Nakashima E, Seto S, Yano K. Correlation between fatty liver and coronary risk factors: a population study of elderly men and women in Nagasaki, Japan. *Hypertens Res.* 24:337–343 (2001).
11. Osono Y, Nakajima K, Hata Y. Hypertriglyceridemia and fatty liver: clinical diagnosis of fatty liver and lipoprotein profiles in hypertriglyceridemic patients with fatty liver. *J Atheroscler Thromb.* 2 Suppl 1:S47–S52 (1995).
12. Ting, Y. et al. Antiobesity Efficacy of Quercetin-Rich Supplement on Diet-Induced Obese Rats: Effects on Body Composition, Serum Lipid Profile, and Gene Expression. *J. Agric. Food Chem.* 66, 70–80 (2018).
13. Ribeiro, V. M. et al. Efectos de dieta con alto contenido de grasa y bebidas ricas en polifenoles en la actividad antioxidante enzimática y no enzimática. *Nutr. Hosp.* 35, 169–175 (2018).

14. Barreiros, A. L. B. S. & David, J. P. Divulgação. *Quim. Nova* 29, 113–123 (2006).
15. Halliwell, B. Biochemistry of oxidative stress: Figure 1. *Biochem. Soc. Trans.* 35, 1147–1150 (2007).
16. Blesso, C. N., Andersen, C. J., Bolling, B. W. & Fernandez, M. L. Egg intake improves carotenoid status by increasing plasma HDL cholesterol in adults with metabolic syndrome. *Food Funct.* 4, 213–221 (2013).
17. Ciccone, M., Cortese, F. & Gesualdo, M. Dietary intake of carotenoids and their antioxidant and anti-inflammatory effects in cardiovascular care. *Mediators Inflamm.* 2013, 1–11 (2013).
18. Karppi, J., Kurl, S., Ronkainen, K., Kauhanen, J. & Laukkanen, J. A. Serum Carotenoids Reduce Progression of Early Atherosclerosis in the Carotid Artery Wall among Eastern Finnish Men. *PLoS One* 8, 1–7 (2013).
19. Melendez-martinez, A. J. et al. Effect of tomato extract supplementation against high-fat diet-induced hepatic lesions. *Orig. Artic.* 2, 198–208 (2013).
20. Fenni, S. et al. Lycopene and tomato powder supplementation similarly inhibit high-fat diet induced obesity, inflammatory response, and associated metabolic disorders. *Mol. Nutr. Food Res.* 61, 1–10 (2017).
21. Kim, J. Y. et al. Effects of lycopene supplementation on oxidative stress and markers of endothelial function in healthy men. *Atherosclerosis* 215, 189–195 (2011).
22. Wang, L., Gaziano, J. M., Norkus, E. P., Buring, J. E. & Sesso, H. D. Associations of plasma carotenoids with risk factors and biomarkers related to cardiovascular disease in middle-aged and older women. *Am. J. Clin. Nutr.* 88, 747–54 (2008).
23. Kilkenney, C., Browne, W., Cuthill, I. C., Emerson, M. & Altman, D. G. Editorial: Animal research: Reporting in vivo experiments-The ARRIVE Guidelines. *J. Cereb. Blood Flow Metab.* 31, 991–993 (2011).

24. Friedewald, W. T., Levy, R. I. & Fredrickson, D. S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. 18, 499–502 (1972).
25. Guimarães, D. D. A. B. et al. Pitaya Extracts Induce Growth Inhibition and Proapoptotic Effects on Human Cell Lines of Breast Cancer via Downregulation of Estrogen Receptor Gene Expression. *Oxid. Med. Cell. Longev.* 2017, (2017).
26. Palozza, P., Catalano, A., Simone, R. E., Mele, M. C. & Cittadini, A. Effect of lycopene and tomato products on cholesterol metabolism. *Ann. Nutr. Metab.* 61, 126–134 (2012).
27. Gajendragadkar, P. R. et al. Effects of oral lycopene supplementation on vascular function in patients with cardiovascular disease and healthy volunteers: A randomised controlled trial. *PLoS One* 9, (2014).
28. Cheng, H. M. et al. Tomato and lycopene supplementation and cardiovascular risk factors: A systematic review and meta-analysis. *Atherosclerosis* 257, 100–108 (2017).
29. Ried, K. & Fakler, P. Protective effect of lycopene on serum cholesterol and blood pressure: Meta-analyses of intervention trials. *Maturitas* 68, 299–310 (2011).
30. Williams, E. J., Baines, K. J., Smart, J. M., Gibson, P. G. & Wood, L. G. Rosuvastatin, lycopene and omega-3 fatty acids: A potential treatment for systemic inflammation in COPD; a pilot study. *J. Nutr. Intermed. Metab.* 5, 86–95 (2016).
31. García-Alonso, F. J., Jorge-Vidal, V., Ros, G. & Periago, M. J. Effect of consumption of tomato juice enriched with N-3 polyunsaturated fatty acids on the lipid profile, antioxidant biomarker status, and cardiovascular disease risk in healthy women. *Eur. J. Nutr.* 51, 415–424 (2012).
32. Tsitsimpikou, C. et al. Dietary supplementation with tomato-juice in patients with metabolic syndrome: A suggestion to alleviate detrimental clinical factors. *Food Chem. Toxicol.* 74, 9–13 (2014).

33. Guzmán-Gerónimo, R. I. et al. Blue Maize Extract Improves Blood Pressure, Lipid Profiles, and Adipose Tissue in High-Sucrose Diet-Induced Metabolic Syndrome in Rats. *J. Med. Food* 20, 110–115 (2017).
34. Estrov, Z. et al. transcription factor NF- κ B , inhibits proliferation , causes S-phase arrest , and induces apoptosis of acute myeloid leukemia cells Resveratrol blocks interleukin-1 α – induced activation of the nuclear transcription factor NF- κ B , inhibits proliferat. 102, 987–995 (2013).
35. Palozza, P., Parrone, N., Simone, R. E. & Catalano, A. Lycopene in atherosclerosis prevention: An integrated scheme of the potential mechanisms of action from cell culture studies. *Arch. Biochem. Biophys.* 504, 26–33 (2010).
36. Viola, J. & Soehnlein, O. Atherosclerosis - A matter of unresolved inflammation. *Semin. Immunol.* 27, 184–193 (2015).
37. Simsa-Maziel, S. & Monsonego-Ornan, E. Interleukin-1 β promotes proliferation and inhibits differentiation of chondrocytes through a mechanism involving down-regulation of FGFR-3 and p21. *Endocrinology* 153, 2296–2310 (2012).
38. Wilcox, T. & Hirshkowitz, A. NIH Public Access. 85, 1–27 (2015).
39. Mesquita, S. D. S., Teixeira, C. M. L. L. & Servulo, E. F. C. Carotenoides: Propriedades, aplicações e mercado. *Rev. Virtual Quim.* 9, 672–688 (2017).
40. Yamagata, K. et al. Mecanismos moleculares de ação anti-inflamatória e antioxidante de polifenóis de uvas e vinho tinto na aterosclerose. *Atherosclerosis* 14, 398–413 (2015).
41. Bojórquez, R. M. C., Gallego, J. G. & Collado, P. S. Propiedades funcionales y beneficios para la salud del licopeno. *Nutr. Hosp.* 28, 6–15 (2013).
42. Teodoro, A. J. et al. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int.* 12, 1 (2012).
43. Gao, J. et al. HHS Public Access. 167, 397–404 (2017).

Chapter 4

Effect of betacarotene and buriti juice on hepatic cells biomarkers in rats fed with high-fat diet

Monique de Barros Elias Campos,¹ Vanessa Azevedo de Jesus,¹ Vanessa Rosse de Souza,¹ Teresa Palmiciano Bede,² Vilma Blondet de Azeredo,² and Anderson Junger Teodoro¹

¹Food and Nutrition Program, Functional Foods Laboratory, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

²Nutrition and Dietetic Department, Fluminense Federal University, Rio de Janeiro, Brazil.

This manuscript was submitted in February 2020.

CONCLUSÕES GERAIS E DESENVOLVIMENTO ADICIONAIS

Os carotenóides se destacam por seus níveis significativos de compostos bioativos e seus benefícios. Muitos autores indicam que a ingestão de licopeno e / ou beta-caroteno e seus metabólitos poderia reduzir a proliferação de células cancerígenas do fígado, efeito decorrente da atividade antioxidante e da modulação do ciclo celular e da taxa de apoptose. No entanto, uma consideração importante em todos os estudos é enfatizar o contexto do composto estudado, uma vez que estudos com um composto isolado podem fornecer resultados muito diferentes dos estudos do composto dentro de uma matriz alimentar.

➤ Com base nos nossos experimentos, demonstramos que o licopeno induziu um fenótipo de lipócitos em células estreladas hepáticas, promoveu a parada do ciclo celular na fase G0 / G1 e diminuiu a viabilidade celular. Durante esse processo, há acúmulo de licopeno na membrana celular, com modificação dos lipídios da membrana. Também demonstramos que a suplementação de licopeno modula as vias moleculares, afetando a expressão de genes relacionados ao ciclo celular, crescimento celular, proliferação e metabolismo lipídico, provavelmente pela ativação da transcrição de PPAR γ e RXR. Tomados em conjunto, os presentes dados sugerem um possível mecanismo de proteção mediado por células estreladas hepáticas de licopeno.

➤ Demonstramos que o molho de tomate e a suplementação de licopeno têm efeitos benéficos no metabolismo hepático e podem ser considerados como uma abordagem nutricional para a prevenção e tratamento de esteatose hepática não alcoólica.

➤ A dieta rica em gordura causou danos ao tecido hepático, alterando o ciclo celular e a histologia hepática. Além disso, a suplementação com beta-caroteno não impediu que o excesso de gordura na dieta reduzisse o conteúdo viável das células e aumentasse o processo de apoptose. Ambos os suplementos aumentaram a morte celular e a necrose das células hepáticas, especialmente a suplementação com 4 mg de beta-caroteno isolado, que agravou o dano

histológico causado pela dieta hiperlipídica, demonstrando um possível efeito tóxico nessas células.

Em conclusão, para a recomendação da suplementação desses compostos, são necessárias mais pesquisas para elucidar o mecanismo pelo qual os carotenóides atuam para modular o câncer e determinar o nível ótimo e seguro de consumo que promove os efeitos esperados.

GENERAL CONCLUSIONS

Carotenoids are highlighted for their selected levels of bioactive compounds and their benefits. Many authors have determined that an intake of lycopene and / or beta-carotene and its metabolites could reduce the proliferation of liver cancer cells, the effect of antioxidant activity and the modulation of the cell cycle and apoptosis rates. However, an important consideration in all studies is to emphasize the context of the compound study, since studies with an isolated compound can offer very different results from the compound studies within a food matrix.

- Based on our experiments, which demonstrate or license a type of lipocyte in hepatic stellate cells, promote a cell cycle arrest in the G0 / G1 phase and decrease cell viability. During this process, there is an increase in lycopene in the cell membrane, with alteration of the membrane lipids. We also demonstrated the supplementation of license modules as molecular pathways, affecting the expression of genes related to the cell cycle, cell growth, proliferation and lipid metabolism, usually by the transcription of PPAR γ and RXR. Taken together, the present data suggest a protective mechanism mediated by liver lycopene star cells.
- We demonstrated that tomato sauce and lycopene supplementation have beneficial effects on liver metabolism and can be considered as a nutritional approach to the prevention and treatment of non-alcoholic liver steatosis.
- The high-fat diet causes damage to liver tissue, altering the cell cycle and liver histology. In addition, beta-carotene supplementation does not prevent excess fat in the reduced diet or viable cell content and increase the apoptosis process. Both supplements increased cell death and necrosis of liver cells, especially supplements with 4 mg of beta-carotene alone, which aggravated or caused histological damage caused by the high-fat diet, demonstrating a possible toxic effect in these diseases.

In conclusion, to recommend supplementation of these compounds, further research is needed to elucidate the mechanism for which the carotenoid activated by modular or cancer is and to determine the optimal and safe level of consumption that promotes the expected effects.

GENERAL REFERENCES

1. Alegria-Ezquerro, E., Vázquez, J. M. C., Barrero, A. A. Obesidad, síndrome metabólico y diabetes: implicaciones cardiovasculares y actuación terapéutica. *Revista Española de Cardiología*, v. 61, n. 7, p. 752-764. 2008.
2. Bucchieri, F., Pubbicomb, S.M., Lordan, J.L., Richter, A., Buchanan, A., Wilson, S.J., Ward, J., Zummo, G., Howarth, P.H., Djukanoviae, R., Holgate, S.T., Davies, D.E. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am. J. Resp. Cell Mol. Biol.*, v.27, n.2, p.179-85, 2002.
3. Charbonneau, A., Unson, C.G., Lavoie, J.M. High-fat diet-induced hepatic steatosis reduces glucagon receptor content in rat hepatocytes: potential interaction with acute exercise. *The Journal of physiology*, v. 579, n. 1, p. 255-267, 2007.
4. Edge, R.; Truscott, T. Singlet oxygen and free radical reactions of retinoids and carotenoids—A review. *Antioxidants*, 7, 5, 2018.
5. Elias, M.B.E., Oliveira, F.L., Fatima Costa Rodrigues Guma, Renata Brum Martucci, Radovan Borojevic and Anderson Junger Teodoro. Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling. *Food & Function*, Issue 4, 2019.
6. Fu S, Watkins SM, Hotamisligil GS. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metab*; 15:623–34, 2012.
7. Geraldo, J.M. & Alfenas, R.C.G. Papel da Dieta na Prevenção e no Controle da Inflamação Crônica – Evidências Atuais, *Arq Bras Endocrinol Metab*, v. 52, n. 6, p. 951-967, 2008.
8. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol*;13:89–102, 2012.
9. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury. *J Gastroenterol Hepatol*, v.15, n.7, p. 718-724, 2000.
10. Jesus, V.A; Campos, M.B.E; Souza, V.R.; Bede, T.P.; Moraes, B.P.T.; Silva, A.R.;

- Albuquerque, C.F.G.; Azeredo, V.B.; Teodoro, A.J. Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell Biomarkers in Rats. *Journal of Medicinal Food* Vol. 22, n 11, 2019.
11. Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol*; 54:795–809, 2011.
 12. Neto LAA. Muito além da transição epidemiológica: doenças crônicas no século XX. *Hist Ciênc, Saúde-Manguinhos* ;26(1):353-5, 2019.
 13. Oliveira M.C. & Schoffen, J.P.F.S. Oxidative Stress Action in Cellular Aging, *Braz. Arch. Biol. Technol.* v.53, n. 6, p. 1333-1342, 2010.
 14. Olthof Mr, Hollman Pch, And Katan Mb: Chlorogenic acid and caffeic acid are absorbed in humans. *American Society for Nutritional Sciences* 131, 66–71, 2000.
 15. Peres, W.A.F.P., Paula, T.P., Silva, R.A.R.N., Coelho, H.S.. A atuação da vitamina A e carotenóides na hepatocarcinogênese *Revista Brasileira de Cancerologia*, 49(2): 113-120, 2003.
 16. Suhett, C.P.A et al. Dieta controle em um estudo sobre dieta com alto teor de gordura em camundongos: comida regular e dieta com baixo teor de gordura purificada têm efeitos semelhantes sobre fenotípicos , resultados metabólicos e comportamentais, *Neurociência nutricional*, 22: 1, 19-28, 2019.
 17. Rohit Sane, Deepali Amin, Vaishali Koli, Rupali Kore, Sneha Paranjpe, Rahul Mandole. Impact of Low-Carbohydrate Diet (LCD) and Obesity Management Program on Obese Patients. *European Journal of Preventive Medicine*. Vol. 7, No. 1,, pp. 1-6, 2019.
 18. Sies, H., Schewe, T., Heiss, C., Kelm, M. Cocoa polyphenols and inflammatory mediators. *Am J Clin Nutr.*, v. 81, n. 1, p.304-312, 2005.
 19. Sherman M, Campbell JAH, Titmuss AS, Kew MC, Kirsch RE. Glutathione S-transferase in human hepatocellular carcinoma. *Hepatology*; 3:170-6, 1983.
 20. Teebor GW, Becker FF. Regression and persistance of hyperplastic hepatic nodules induced by N-2fluorenyacetamide and their relationship to hepatocarcinogenesis. *Cancer*

Res; 31:1-3, 1981.

21. Teodoro, A.J., Perrone, D., Martucci, R.B. et al. Lycopene isomerisation and storage in an in vitro model of murine hepatic stellate cells. *Eur J Nutr* 48, 261–268, 2009.
22. Upadhyaya Kr, Radha Ks, And Madhyastha Hk: Cell cycle regulation and induction of apoptosis by beta-carotene in U937 and HL-60 leukemia cells. *J Biochem Mol Biol* 40, 1009–1015, 2007.
23. Youfa Wang, May A Beydoun, Jungwon Min, Hong Xue, Leonard A Kaminsky, Lawrence J Cheskin, Has the prevalence of overweight, obesity and central obesity levelled off in the United States? Trends, patterns, disparities, and future projections for the obesity epidemic, *International Journal of Epidemiology*, dyy273
24. Yu ChungChooi, CherlynDing, FaidonMagkos. The epidemiology of obesit. *Metabolism*. Volume 92, Pages 6-10March 2019, Pages 6-10, 2019.
25. Zhang, M, Binns CV, Lee AH. Tea consumption and ovarian cancer risk: a case-control study in China. *Cancer Epidemiol Biomarkers Prev.*; 11:713-8, 2002 .

Apêndices - Artigos publicados

Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Monique de Barros Elias^{a*}, Anderson Junger Teodoro^{a*}, Felipe Leite Oliveira^b, Fatima Costa Rodrigues Guma^c, Renata Brum Martucci^b, Radovan Borojevic^d.

Hepatic stellate cells are liver-specific perivascular cells, identified as the major source of collagen in liver fibrosis, following their activation and conversion to myofibroblast-like cells. Lycopene is a carotenoid with biological activities and protective effect described in different pathologies, but little is known about its role in liver protection. We evaluated the influence of lycopene on cell cycle and lipid metabolism and monitored the possible pathways involved in lycopene inhibition of stellate cells activation. Lycopene induced expression of the lipocyte phenotype, with an accumulation of fat droplets in cytoplasm, with high synthesis and turnover of phospholipids and triglycerides. Cell proliferation analysis showed that lycopene reduced the growth of GRX cells. Lycopene induced an arrest in the G0/G1 phase, followed by a decrease of cells in G2/M phase, regardless of the concentration of lycopene used. Lycopene modulated relevant signaling pathways related to cholesterol metabolism, cellular proliferation, and lipid metabolism. Also, lycopene treatment increased the expression of RXR- α , RXR- β , and PPAR γ , important biomarkers of liver regeneration. These results show that lycopene was able to negatively modulate events related to activation of hepatic stellate cells through mechanisms that involve changes in expression of cellular lipid metabolism factors, and suggest that this compound might provide a novel pharmacological approach for prevention and treatment of fibrotic liver diseases.

Introduction

The liver has long been described as the major site of metabolism and accumulation of carotenoids and retinoids in the body. They are mostly found in lipid droplets of the hepatic stellate cells (HSCs), a population of liver nonparenchymal cells, resident quiescent in the perisinusoidal Disse's space. They are specialized in storage, metabolism, and release of vitamins, hormones and endogenous and exogenous lipid mediators. Intracellular retinoids and carotenoids are very high in HSCs, being this an important cellular storage site. After retinol is converted to retinoic acid, it can be released into the blood circulation by the HSCs [1]. The HSCs may be directly or indirectly responsible for the maintenance of the circulating carotenoids homeostasis in periods of a low dietary uptake through the production of the intracellular retinoic acid [2]. HSCs can also uptake, store, isomerize and release into the blood circulation lycopene obtained from the alimentary sources [3].

Besides the storage and redistribution of lipid compounds in the body, HSCs are also the significant players of development and maintenance of hepatic fibrosis. The two activities required induction of HSCs into different and opposed metabolic functions and behaviors. Hepatic fibrosis is a liver tissue response to acute or chronic aggression. This is a response to products released by the injured tissues, mediated by the locally produced or circulating compounds, inflammatory mediators and cytokines [4,5]. Upon tissue injury, HSCs are activated and undergo transdifferentiation to myofibroblasts responsible for overproduction of collagen-rich extracellular matrix [6].

This activation of quiescent HSCs is the major component of hepatic fibrogenesis, including enhancement of proliferation and cell-specific downmodulation of hepatic lipid metabolism. Moreover, activated HSCs also produce pro-inflammatory mediators, which can enhance and further modulate the environment leading to hepatic fibrosis [7,8].

Controlling the degree of HSCs activation may have an antifibrotic protection effect for liver fibrosis. Peroxisome proliferator-activated receptor γ (PPAR γ), one of the PPAR isoforms, is highly expressed in quiescent HSCs of the normal liver, but its expression and activity are dramatically decreased during the HSC activation in vitro and in vivo [9]. Upon binding to cognate ligands, PPAR undergoes a conformational change that releases the co-repressor, allowing its association to its mandatory pair, the X receptor for retinoic acid (RXR). This

^a Address here.

^b Address here.

^c Address here.

[†] Footnotes relating to the title and/or authors should appear here.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See

heterodimer (PPAR:RXR) binds to peroxisome proliferator response elements (PPRE), the specific DNA sequences in target gene promoters, leading to transcriptional activation [10,11]. Being one of the major controllers of the retinoic acid availability, HSCs are one of the major players in these events. However, the RXR can also bind the alternative ligands, including the acyclic carotenoid-derived ones, such as lycopene. This raises the question of the potential consequences of such alternative RXR ligands in HSCs, such as occurs upon lycopene supplementation. This question has been addressed in the present study.

Oxidative stress-related injuries are the potential causes of liver tissue injuries that can lead to hepatic fibrosis. Exogenous antioxidants, both synthetic and natural, have been used in alternative therapeutic approaches for this pathology of liver [12,13]. Epidemiological studies have indicated that food products that contain lycopene, the most potent antioxidant compound among carotenoids, have chemopreventive effects against cancers and other diseases [14]. Lycopene is a bright red carotene, a non-pro-vitamin A carotenoid is a highly unsaturated hydrocarbon containing eleven conjugated and two unconjugated double bonds. Its biological effects in humans have been attributed to mechanisms different from those mediated by the vitamin A, since lycopene lacks the β -ionone ring structure, and it cannot produce the vitamin A in the body [15]. In recent studies, lycopene has shown a protective effect against damage caused by oxidative stress causing liver toxicity, using a model induced by an overdose of acetaminophen. It was observed that lycopene was able to reduce both lipid peroxidation and protein carbonylation [13]. Moreover, it has been suggested that the normalization of liver function markers and lowering of liver damage may be related to the protective effect of lycopene against non-alcoholic liver disease [8]. Lycopene can thus have a double and complementary effect on the activation of HSCs, underlying the evolution of liver fibrosis: lowering their activation level through modulation of the PPAR signaling pathway and protecting the cells of the oxidative stress.

The present study has addressed the question of lycopene effect on metabolism and activation, using an in vitro model of the murine GRX cell line representative of the murine HSCs. These cells are a useful model to study lycopene metabolism since they represent the murine hepatic stellate cells, both in their structural and metabolic properties [16,17,18]. Thus, the present study aimed to investigate the lycopene and their influence on cell cycle, lipid metabolism and the possible pathways involved in lycopene inhibition of activated stellate cells.

Materials and methods

Materials

All-trans lycopene was purchased from Sigma Chemical Company (St. Louis, MO, USA). Water-soluble (WS) lycopene (10%) was provided by Roche (Rio de Janeiro, RJ, Brazil). Dulbecco's cell culture medium and bovine serum albumin were obtained from Sigma, and fetal bovine serum (FBS) from Laborclin (Campinas, SP, Brazil). Tissue culture flasks and cell scrapers were obtained from Nunc (Roskilde, Denmark). All the chemicals were of analytical grade.

Cell Culture and Treatment Protocol

GRX cells were obtained from the Rio de Janeiro Cell Bank (UFRJ, Rio de Janeiro), which certified the cell identity and the absence of microbiological contaminants. GRX cells were plated in 25 cm² tissue culture flasks, 5x10⁶ cells/flask, and maintained routinely in the Dulbecco's medium supplemented with 5% FBS and 2 g/L HEPES buffer, pH 7.4, under 5% CO₂ atmosphere. Under these conditions, they expressed the myofibroblast phenotype. In order to induce the lipocyte phenotype, cells (10⁴ cells/cm²) were incubated for 10 days in the standard culture medium. The culture medium was changed every 5 days and the incubated at different concentrations lycopene WS (1.0–5.0 μ M lycopene water soluble, Roche) dissolved in water at 50°C. All the solutions were prepared every day and studied in the dark to maintain the lycopene stability.

Cell Viability Assay

GRX cells (5 x 10³ cells/seed) were seeded in 96-well plates, and after 24 hours they were incubated with different concentrations of lycopene ranging from 0.625–5.0 μ M lycopene. The supernatant was removed every 2 to 3 days, cells were incubated it in the standard culture medium, and 10 μ L of MTT (5 μ g/mL) were added to each well, leaving the cells in the dark for two hours at 37°C. After incubation, the supernatant was discarded, and the precipitate dissolved in 150 μ L dimethyl-sulfoxide. The plates were read in an enzyme-linked immunosorbent micro-plate reader (BioRad 2550, USA) at 570 nm. Cell viability was determined by MTT assay (Amresco, USA). The lycopene concentrations used in this work are by previous reports.

Cell Cycle Analysis

Cells were rinsed briefly with Ca-Mg free phosphate-buffered saline (PBS) and detached with trypsin at room temperature. After centrifugation, the cells were washed twice with PBS, 1x10⁶ cells were resuspended in 1.0 mL ice-cold VindeLov solution, containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNase and 50 μ g/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 minutes of incubation, the cell suspension was analyzed for DNA content by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells containing the diploid DNA in G0–G1 (2n), S phase (>2n but <4n), and G2/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9, respectively. The percentage of cell population at a particular phase was estimated with EXPO32 V1.2 Analysis software. The cell dissociation procedure does not affect fluorescence under the used experimental conditions.

Incorporation of [14C]-acetate and lipids analysis

All the cells were incubated with 0.25 $\mu\text{Ci/mL}$ [^{14}C]-acetate in 2.5 mL DMEM without serum for 24 hours, on the last days of incubation period with lycopene (5 and 10 days). The radioactive medium was removed, the monolayer washed with BSS-CM. The cells were collected, and centrifugation separated fractioned in fat droplets. The lipids were extracted from the fat droplets and from other cell fractions. Chloroform was evaporated under a nitrogen atmosphere, and the radioactive lipids were analyzed by Thin Layer Chromatography (TLC). Lipids were separated and identified by migration patterns using hexane:ethyl ether:acetic acid (80:20:1, v/v/v) as the mobile phase. The TLC plate was imaged and the incorporation of acetate (%) was quantified by densitometry.

Microarray analyses: cRNA amplification, labeling and hybridization, and cDNA microarray

Cells were incubated with 3,0 μM lycopene and all RNA was extracted from cells using Trizol followed by the RNA extraction kit for its purification (RNeasy Quiagen). The cells were centrifuged to remove the culture medium and subsequently lysed with guanidine isothiocyanate for inactivation of RNAses. After adding 70% ethanol, the sample was applied to the extraction column or silica membrane mini spin RNeasy and subjected to successive centrifugations. The RNA was eluted with 30 or 50 μL of ultra-pure water free of RNAses and stored at -70°C .

The concentration and purity of the extracted RNA were monitored by spectrophotometry of 2 μL sample in the Nanodrop ND-1000. The quality and integrity of the extracted RNA were assessed by electrophoresis in agarose gel marked with ethidium bromide, and by electrophoresis in 2% agarose gel with Tris-Borate (TBE). The quantity of RNA was related to the number of cells. The degree of purity, determined by the ratio of the lengths 260nm/280nm, remained within the threshold between 1.8 and 2.0, indicating the absence of DNA or protein contaminants. The total RNA was characterized by electrophoresis, by the presence of the two bands for ribosomal RNA fractions 28s and 18s.

The cDNA synthesis, sample markup, and hybridization in DNA Microarrays blades (GeneChip[®] 1.0 HumanGene ST) were done according to the standard Affymetrix Protocol WT Analysis. The procedure consists of RNA marking where the total RNA samples were submitted to the reverse transcription reaction for the synthesis of cDNA, adding oligo dT and T7-primers and random primers, followed by an in vitro transcription reaction (IVT), a new cDNA synthesis with random primers, with fragmentation and UDG APE and "labeling" or marking with biotin terminal via TdT (transferase).

The marked cRNA, the hybridization cocktail, probes and controls containing fluorescent marker phycoerythrin were introduced in the DNA chips and, after incubation and washing steps, were read by a suitable scanner, providing the first experimental data defined by the intensity of the detected signals.

Analysis of gene expression

Primary analysis of expression data was performed in the laboratory computers microarrays using GeneChip operating software AFLP[®] Operating Software (GCOS 1.4) and Expression Console[®] 1.1 for correction of noise and quality analysis. The algorithm used in this analysis is known as RMA (Statistical Algorithm) Detection Call (a qualitative measure that indicates whether the transcript is detected, undetected or marginal) for each set. The secondary analyses of the difference of expression (Fold Change) were made with the use of standardization and statistics software Bioconductor Lawson. For each gene, the ratio of signal intensities was calculated and transformed using differentially expressed genes (SDS) common to two different statistical methods (Limma and RankProd, both with $p < 0.05$). The biological pathways identified by the Ingenuity program show the SDRs with red and green colors, with red genes with increased expression and green being reduced. In addition, the colors are presented with different gradients, that is, the redder more expressive, the less red less expressive, the same way for the green color. This software also provides normalization methods and the data of an array were normalized by a global lowess method, based on the local estimation of intensities and a regression calculation weighted toward similar spots. All these normalization steps were represented in Box plots which show averaged ratio of each slide before and after lowess within-array normalization. The t-test clustered significant genes.

Preparation of total RNA and real-time polymerase chain reaction (PCR).

Cells were harvested after 5 and 10 days of treatment with 1, 3 or 5 μM lycopene. GRX cells were washed with BSS CMF, detached with trypsin and counted. To the volume equivalent of 1×10^6 cells, 1 mL Trizol (Gibco) was added and, after homogenization, 200 μL chloroform was added. The RNA was separated following the manufacturer's protocol, and total RNAs was quantified using spectrophotometry. cDNA was synthesized from 3 μg of the total RNA, added 0.5 mg of oligo-(dT) 12-18 and 200 units MLV (reverse transcriptase) (both from Gibco). After 60 min of incubation at 37°C , we raised the temperature to 95°C for 5 minutes and added water (4°C) until the volume of 100 μL .

The PCR reactions were carried with 2.5 μL cDNA, 0.5 U Taq polymerase, 1.5 mM MgCl_2 , 0.5 μL dNTP (10mM) and 0.1 μg of the sense and antisense of each primer (Table 1). The annealing temperature and the number of cycles of amplification reactions varied according to the primer pair were made on the following the manufacturer's specifications. After electrophoresis, the gel was incubated in ethidium bromide solution and analyzed by transillumination (UV), with quantification of the bands by densitometry using the program Image Master VDS, with correction of the bands by the

expression of β -actin in the same sample as described by Sun et al.[19].

Table 1. Primer sequences for the reverse transcription-quantitative polymerase chain reaction.

Primer	Sense	Annealing temperature (°C)	Size PCR product (pb)
Cx 43	5'ATCCAGTGGTACATCTATGG 3' 5'CTGCTGGCTCTGCTGGAAGG 3'	58	593
PPARγ	5'TTTTCAAGGGTGCCAGTTTC 3' 5'TCTGTGACGATCTGCCTGAG 3'	58	501
RARα	GCTTAACCAAGGACGATTCTTGG 3' CGGTGTGCTGTAACCACTGACTGG 3'	52	425
RXRα	5'ACCCAGGTGAACCTCTCGTCCC 3' CGGCTTCCAGAATCTTCTACAGG 3'	52	694
RXRβ	TTCTCTCTCTGGCCACCTCTTACC 3' CCGCAGCAGTATGACCTGATCGTCC 3'	52	796
PPARγ	5'CCTCATGAAGAGCCTTCCAA 3' 5'TTTTGTGGATCCGACAGTTT 3'	55	177
β-actin	5'GTGGGCGCTCTAGGCACCA 3' 5'CTCTTCATGTCACGCACGATTTC 3'	58	560

Statistical Analysis

The presented data are mean values $\pm$ SD of two independent experiments done in triplicate (n=6). Statistical comparisons were carried out by ANOVA and post hoc Tukey's test using Graph Pad Prism 4.0 and Statistical 6.0 program. All $p < 0.05$ values were considered statistically significant.

The data were normalized with the RMA method, available in program R.

We searched for the higher stringency of the analyzes using differentially expressed genes (SDS) common to two different statistical methods (Limma and RankProd), both with p -value < 0.05 ; In this case there are no significant values of p due to the number of samples. We used the 67 SDRs common to the Limma and RankProd methods.

Results

Phenotype conversion of GRX

As described previously [3], under standard culture conditions, GRX cells formed monolayers and had a fibroblastoid morphology corresponding to the activated stellate cells in hepatic tissue (Figure 1A and 1B). Lycopene induced expression of the lipocyte phenotype accumulating fat droplets in their cytoplasm. Similar to other models, induction of lipid storage was not synchronized, and groups of cells with the typical lipocyte phenotype were present with cells that had just begun accumulation of lipid droplets. After 10 days, most of the cells reached the typical lipocyte phenotype (Figure 1C). The lipid nature of cell inclusions could be demonstrated by their affinity for the dye Oil Red O (Figure 1D).

Fig 1. GRX cells in the standard culture medium (A) and incubated with Lycopene 3 (C) for 10 days and after staining with oil-red (B and D respectively). Original magnification x400

Acetate incorporation into GRX cell lipids

GRX cells were supplemented with lycopene for 5 and 10 days and were incubated with labeled acetate in the last days of culture. The cells were then fractionated; lipid droplets and other fractions were separated, and the incorporation of acetate was analyzed by TLC. GRX cells incorporated the labeled acetate into lipids indicating their synthesis and turnover (Table 2). The high total labeling of phospholipids indicated their high synthesis and turnover, particularly in the membranes, where it nearly doubled with the induction of the fat storing phenotype in cells at 10 days. The increased acetate incorporation into triglycerides located in the cytoplasm, followed by the expected segregation into the droplets, indicated that the net syntheses of triglycerides were induced by lycopene to attend this new demand. The presence of labeled free fatty acids in droplets probably indicates that lipid transfer from the cytoplasm to droplets was similar to the formation of fatty droplets in adipocytes, mediated by acyl-transferases.

Table 2. Cell fractionation and composition of lipid droplets of GRX cells incubated with 3 μ M lycopene for 5 and 10 days.

Incubation time (days)	Total		Membrane		Cytoplasm		Nucleus	
	Incorporation (%)							
	5	10	5	10	5	10	5	10
Fosfolipids	62,99	66,84	47,01	85,03	31,30	31,74	64,24	45,77
Monoacylglycerol	0,74	ND	4,01	ND	4,2	ND	2,22	ND
Diacylglycerol	2,54	1,34	2,42	1,16	11,74	ND	1,28	2,51
Triacylglycerol	8,11	17,58	3,62	1,28	22,28	43,62	14,08	17,43
Cholesterol		7,81	2,74	25,80	6,18	6,61	2,60	4,81
Free fatty acid		6,83	3,62	10,81	3,67	19,44	2,19	5,15
							10,23	

Cell Proliferation and Cell Cycle of GRX Cells

Cell proliferation analysis showed that lycopene, from 48 hours on, was able to reduce the growth of GRX cells (Figure 2). Within two days, all cells incubated with different concentrations of lycopene grew 50 to 75% when compared to cells not incubated with lycopene, except the cells incubated with 0.5 μ M of lycopene (Figure 2A). After five days of treatment, all cells incubated with different concentrations of lycopene obtained a reduction of growth by 25%. After seven days of lycopene incubation, the cell growth profile showed no statistically significant differences (Figure 2C), being constant after 10 days of incubation (data not shown).

Fig 2. Growth profile of GRX cells incubated with different concentrations of lycopene (0.625-20 μ M) for 2 days (A), 5 days (B) and 7 days (C). Columns without common letters are statistically different (p <0.05).

GRX cells were incubated with lycopene (1.0-5.0 μ M) for 2 to 7 days after the period of cell cycle analysis was performed. According to the Figure 3A, after 2 days incubation with lycopene, the fraction of GRX cells in G0/G1 phase increased, followed by a decrease of cells in the G2/M phase, regardless of the concentration of lycopene used (p>0.05). This fact showed that there was an increase in intracellular lycopene content at the beginning of the formation of lipocyte phenotype. After 10 days of incubation, the cell cycle was normalized again, with no significant difference between the GRX cells incubated with lycopene and the control ones (p>0.05) (Figure 3B).

Fig 3. Cell Cycle of untreated GRX cells (CT) and treated with lycopene (1-5 μ M) for 2 (A) and 7 days (B). The results are expressed as mean $\pm$ standard deviation and compared by Tukey test (* p <0.05).

Gene expression of lipocyte phenotype

GRX cells were incubated in the presence and absence of lycopene for 10 days and then analyzed for possible expression of genes involved in the formation of lipid droplets.

Cells incubated with different concentrations of lycopene (1.0-5.0 μ M) showed an increased expression of RXR- α , RXR- β , and PPAR γ as compared to controls (Figure 4A-C). No change was detected in the expression of RAR as compared to the untreated groups of cells incubated with lycopene (Figure 4D-E).

Fig 4. Analysis by RT-PCR of untreated GRX cells (CT) and incubated with lycopene (1-5 μ M) and nuclear receptor expression RXR- α (A), RXR- β (B), PPAR- γ (C) and RAR (D). The results are expressed as mean $\pm$ standard deviation (*p <0.05).

Microarray analysis

The differentially expressed genes from the above analyses of Limma e Rank Prod discriminations categorized 67 genes and select 4 genes with a higher statistical difference (Table 3 and Figure 5). All these genes are involved in the metabolism of retinoic acid, but little is known about their activation through lycopene that has not the pro-vitamin A activity. We observed up-regulation of cellular growth and proliferation genes such as HLA-DPB1. Cell cycle-related genes were also modulated by lycopene treatment, with up-regulation of RGL2, C2 and CCHCR1 (Table 3).

Table 3. Top Bio Functions modified by lycopene in GRX cells by microarray analysis.

Gene - DEG	Bio Function	Fold increase
C2	Cholesterol metabolism	8.215
HLA-DPB1	Cellular growth and proliferation	3.907
CCHCR1	Lipid metabolism Small Molecule Biochemistry Endocrine System Development and Function	3.244
RGL2	Cellular growth and proliferation Cell Cycle Genetic Disorder	2.530

Fig 5. Pahways and mechanisms of activation of lycopene in hepatic stellate cells. Red genes are activated by lycopene.

Discussion

This study has described the several prominent sets of information on lycopene in the context of protection and modification of the hepatic stellate cell (HSC) phenotype. Studies suggest that HSCs are the primary source of ECM in liver injury [20,21]. During the progression of liver fibrosis, their proliferation plays a major role in ECM production extremely rich in collagen leads to scar deposition and liver fibrosis. HSCs are activated and transdifferentiated to myofibroblasts that typically express α -smooth muscle actin (α -SMA) and results in overproduction of the extracellular matrix (ECM) [22,23].

HSCs proliferation is a key step during the fibrogenic process [24,25]. Studies suggest having shown that lycopene can modified cell viability of different types of cells [26–29]. In the present study, lycopene was able to reduce the growth of GRX cells and phenotype modification. Depending upon the way and the dose of lycopene administration, it may alter proliferation and production of cell cycle-regulatory proteins. [30,31]. Our results showed no influence of the concentration of lycopene used on cell cycle of GRX cells. The lycopene deposited in plasma, liver, and other tissues had no adverse effects, and no teratogenic effects were observed in rat studies [32,33]. Besides that, previous studies reported that lycopene synthetic was not toxic. Unformulated pure

crystalline lycopene and lycopene as a 10% beadlet formulation are not genotoxic as determined in a comprehensive battery of tests.[34]

We observed that lycopene induced an increase of cells in the G0/G1 phase after 2 days of incubation, followed by a decrease of cells in the G2/M phase. Palozza et al. [26] suggested that lycopene blocked the G1/S phase of the cell cycle, and the possible targets of this block could be the cyclin D1 and inhibitors of the cell cycle. It has been demonstrated that lycopene induced a G1/S cell cycle reduction, expressed by the upregulation of the cyclin A and p27 and downregulation of cyclins E and D1 [30,34,35,36]. Lycopene can induce cell cycle arrest at different stages in different human cancer cells [26,27,30,35–37].

During HSC conversion to the lipocyte phenotype, a reduction of GRX growth and induction of cell cycle arrest was observed, modifying the lipid composition and metabolism. A resting phenotype induction mediated by lycopene can have a protective role in liver pathologies that cause fibrosis. At the same time, induction of the myofibroblasts phenotype with loss of lipid droplets can reduce the storage capacity of the lycopene in hepatic stellate cells.

The overall pattern of lipid accumulation was comparable to that observed by Martucci et al. [2], where triacylglycerols and free fatty acids were the major components of lipid droplets in GRX cells. This accumulation was accompanied by a significant change in lipid content of cell membranes, with an increase of phospholipids and a decrease in membrane cholesterol. These changes turn the membrane more fluid, ensuring greater mobility for rotations, and promoting greater protection against oxidation [38]. In addition, there was a decrease in the percentage of incorporation of mono and diglycerides and free fatty acid, both in total content and in cell fractions, but this was not observed in the nucleus.

Microarray technology provides a genomic approach to explore the markers and molecular mechanisms leading to hepatic fibrosis [39]. Accordingly, we used cDNA microarray analysis to detect the up-regulated genes associated with modification of HSC activation. Such analyses have already revealed roles in selected gene products in cell developmental and physiological functions. The activated pathways often involve retinoic acid, and we decided to test here its functional analog, the lycopene.

Microarray analysis identified many differentially expressed genes that are important in the cell cycle, cellular growth and proliferation and lipid metabolism. To confirm these results and determine the effects of lycopene on pathways other than those specifically involved in HSC, we performed a genome analysis using lycopene on GRX cells. Lycopene treatment modified the expression of 67 DEGS, but only 3 genes with statistical difference. Modification of some of these genes has been reported recently. We observed up-regulation of cellular growth and proliferation genes such as HLA-DPB1 and RGL2. Recent genome-wide studies have demonstrated that HLA-

DPB1 gene may play an essential role on RNA expression and progression of HCV and HBV virus-related liver diseases [40,41]. The C2 protein plays a relevant role in the regulation of intracellular cholesterol homeostasis by binding with free cholesterol, and this protein affects free cholesterol metabolism and regulates the HSCs activation [42].

The mechanisms of HSC activation are crucial for the understanding of liver fibrogenesis. Cytokines promote activation from Kupffer cells and platelets, among which TGF-beta is of great importance. It stimulates the HSC gene expression of ECM molecules, but inhibits proliferation and induces phenotypic transdifferentiation [43]. Previous studies demonstrated that the acyclic retinoic acid equivalent might be a possible metabolite of lycopene that induces apoptosis and regression of cell cycle. Activation of the retinoic acid signaling pathway could be involved in the activation of HSC. Lycopene has not the provitamin A activity, but it can form an acyclic retinoic acid equivalent, able to interact with the nuclear receptor of retinoic acid (RAR) [44].

Depletion of PPAR γ accompanies liver fibrosis and cirrhosis, in which activation of hepatic stellate cells occurs with α -actin expression, loss of the fat droplets, reduction of retinol content, increase in cell proliferation and increase in extracellular matrix production. These processes can be reversed with the restoration of PPAR γ expression [45].

PPARs are transcription factors that belong to the superfamily of nuclear receptors, which regulate expression of a large number of genes involved in biological processes, including energy and lipid metabolism, in response to environmental and dietary changes. PPARs belong to the type II family of nuclear receptors (NR), a group that encompasses non-steroid NRs that form obligate heterodimers with RXR. These heterodimeric receptors have functional domains for DNA and ligand binding. PPARs bind to recognition sequences in the promoter regions of their target genes and act directly to regulate gene transcription. [46,47].

The RXR also has an antifibrogenic protective effect on the liver. PPAR γ /RXR heterodimers regulate transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism, neurodegenerative disorders, cancer and inflammation [48]. PPAR γ activators include prostanoids, fatty acids, thiazolidinediones and N-(2-benzoylphenyl) tyrosine analogs. RXR ligands include naturally occurring retinoic acid and synthetic retinoids. Specific ligands for RXR and PPAR receptors increase metabolic abnormalities associated with type 2 diabetes, obesity, and other cardiovascular risk factors. Although adipose tissue mediated some of the effects of PPAR γ /RXR ligands, other tissues also regulate the effects of these receptors. The activity of the PPAR γ /RXR heterodimer is influenced by posttranslational modifications, receptor turnover, polymorphisms, splice variants, coactivators and corepressors [49].

In human breast cancer cell lines on established in vitro, the RXR-selective ligand potentiates the antiproliferative and

apoptotic responses of PPAR ligands. The molecular mechanisms regulating these effects are currently unclear [50]. PPAR and RXR ligands have been shown to differentially recruit subsets of transcriptional coactivator proteins to the receptor complex [51]. Combinations of these ligands may result in additional coactivator recruitment, leading to enhanced transcriptional activation and cellular effects.

In our study, GRX cells incubated with lycopene augmented their expression of RXR- α , RXR- β , and PPAR γ . PPAR signaling pathway appears to have been activated with the increased expression of PPAR and RXR, forming dimers in cells incubated with lycopene. Yang et al. [28] reported that lycopene increases expression of PPAR γ and RXR α , a direct target gene for the PPAR γ :RXR heterodimer.

Jun Wei [52] examined the regulation of HSC biology through a mechanism involved in the antifibrotic effect of PPAR, including the effect of endogenous PPAR on hepatic fibrosis. An increase in PPAR expression in quiescent primary HSC and PPAR depletion in culture-activated HSC was reported. Quiescent HSCs express adipocyte-specific genes, under the control of adipogenic transcription factor PPAR. Adipogenic transcriptional regulation by PPAR is thus required for the maintenance of the quiescent HSC phenotype [53,54].

Conclusions

Our results indicate that lycopene induced a lipocyte phenotype in hepatic stellate cells, promoted cell cycle arrest in G0/G1 phase and decreased cell viability. During this process, there is an accumulation of lycopene in the cell membrane, with modification of membrane lipids. Also, we demonstrated that lycopene supplementation modulates molecular pathways by affecting the expression of cell cycle-related genes, cellular growth, proliferation and lipid metabolism probably by transcription activation of PPAR γ and RXR. Taken together, the present data suggest possible protective mechanism mediated by lycopene hepatic stellate cells.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

This research was supported by CNPq and CAPES grants of the Brazilian Federal Government, and FAPERJ grant of the Rio de Janeiro State Government.

References

1. Sato M, Suzuki S, Senoo H. Hepatic stellate cells: unique characteristics in cell biology and phenotype. *Cell Struct Funct.* 2003;28: 105–12. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12808230>

2. Martucci RB, Ziulkoski AL, Fortuna VA, Guaragna RM, Guma FCR, Trugo LC, et al. β -Carotene storage, conversion to retinoic acid, and induction of the lipocyte phenotype in hepatic stellate cells. *J Cell Biochem.* 2004;92: 414–423. doi:10.1002/jcb.20073
3. Teodoro AJ, Perrone D, Martucci RB, Borojevic R. Lycopene isomerisation and storage in an in vitro model of murine hepatic stellate cells. *Eur J Nutr.* 2009;48. doi:10.1007/s00394-009-0001-6
4. Krizhanovsky V, Yon M, Dickens RA, Hearn S, Simon J, Miething C, et al. Senescence of Activated Stellate Cells Limits Liver Fibrosis. *Cell.* 2008;134: 657–667. doi:10.1016/j.cell.2008.06.049
5. Kong D, Zhang F, Zhang Z, Lu Y, Zheng S. Clearance of activated stellate cells for hepatic fibrosis regression: Molecular basis and translational potential. *Biomed Pharmacother.* 2013;67: 246–250. doi:10.1016/j.biopha.2012.10.002
6. Lu L, Wang J, Lu H, Zhang G, Liu Y, Wang J, et al. MicroRNA-130a and -130b enhance activation of hepatic stellate cells by suppressing PPAR γ expression: A rat fibrosis model study. *Biochem Biophys Res Commun.* 2015;465: 387–393. doi:10.1016/j.bbrc.2015.08.012
7. Paiva LA, Brand C, Bandeira-Melo C, Bozza PT, El-Cheikh MC, Silva PM, et al. Hepatic myofibroblasts derived from *Schistosoma mansoni*-infected mice are a source of IL-5 and eotaxin: controls of eosinophil populations in vitro. *Parasit Vectors.* 2015;8: 577. doi:10.1186/s13071-015-1197-3
8. Jiang W, Guo M-H, Hai X. Hepatoprotective and antioxidant effects of lycopene on non-alcoholic fatty liver disease in rat. *World J Gastroenterol.* Baishideng Publishing Group Inc; 2016;22: 10180–10188. doi:10.3748/wjg.v22.i46.10180
9. Jin H, Lian N, Zhang F, Chen L, Chen Q, Lu C, et al. Activation of PPAR γ /P53 signaling is required for curcumin to induce hepatic stellate cell senescence. *Cell Death Dis.* 2016;7: e2189–e2189. doi:10.1038/cddis.2016.92
10. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, et al. PPAR α and PPAR γ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* European Molecular Biology Organization; 1996;15: 5336–48. Available: <http://www.ncbi.nlm.nih.gov/pubmed/8895578>
11. Zhang F, Lu Y, Zheng S. Peroxisome proliferator-activated receptor- γ cross-regulation of signaling events implicated in liver fibrogenesis. *Cell Signal.* 2012;24: 596–605. doi:10.1016/j.cellsig.2011.11.008
12. Kilic Suloglu, Ph.D. A, Girgin, Ph.D. APG, Selmanoglu, Ph.D. PG, Balci, MD S, Baydar, Ph.D. PT. Possible Effects of Lycopene and Silymarin on Rat Liver Functions and Oxidative Stress Markers. *Turkish J Biochem.* 2014;39: 344–350. doi:10.5505/tjb.2014.17136
13. Bandeira ACB, da Silva TP, de Araujo GR, Araujo CM, da Silva RC, Lima WG, et al. Lycopene inhibits reactive oxygen species production in SK-Hep-1 cells and attenuates acetaminophen-induced liver injury in C57BL/6 mice. *Chem Biol Interact.* 2017;263: 7–17. doi:10.1016/j.cbi.2016.12.011
14. Tang L, Guan H, Ding X, Wang J-S. Modulation of aflatoxin toxicity and biomarkers by lycopene in F344 rats. *Toxicol Appl Pharmacol.* 2007;219: 10–17. doi:10.1016/j.taap.2006.12.001
15. Holzapfel N, Holzapfel B, Champ S, Feldthausen J, Clements J, Huttmacher D. The Potential Role of Lycopene for the Prevention and Therapy of Prostate Cancer: From Molecular Mechanisms to Clinical Evidence. *Int J Mol Sci.* 2013;14: 14620–14646. doi:10.3390/ijms140714620
16. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourão PA, Emonard H, et al. Establishment of a continuous cell line

- from fibrotic schistosomal granulomas in mice livers. *In Vitro Cell Dev Biol.* 1985;21: 382–90. Available: <http://www.ncbi.nlm.nih.gov/pubmed/4030623>
1. Margis R, Borojevic R. Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). *Biochim Biophys Acta - Mol Cell Res.* Elsevier; 1989;1011: 1–5. doi:10.1016/0167-4889(89)90069-4
 2. Borojevic R, Guaragna RM, Margis R, Dutra HS. In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. *In Vitro Cell Dev Biol.* 1990;26: 361–8. Available: <http://www.ncbi.nlm.nih.gov/pubmed/2188940>
 3. SUN K, WANG Q, HUANG X. PPAR gamma inhibits growth of rat hepatic stellate cells and TGF beta-induced connective tissue growth factor expression1. *Acta Pharmacol Sin.* 2006;27: 715–723. doi:10.1111/j.1745-7254.2006.00299.x
 4. Lotersztajn S, Julien B, Teixeira-Clerc F, Grenard P, Mallat A. HEPATIC FIBROSIS: Molecular Mechanisms and Drug Targets. *Annu Rev Pharmacol Toxicol.* 2005;45: 605–628. doi:10.1146/annurev.pharmtox.45.120403.095906
 5. Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol.* 2010;7: 425–436. doi:10.1038/nrgastro.2010.97
 6. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev.* NIH Public Access; 2008;88: 125–72. doi:10.1152/physrev.00013.2007
 7. Hernandez-Gea V, Friedman SL. Pathogenesis of Liver Fibrosis. *Annu Rev Pathol Mech Dis.* 2011;6: 425–456. doi:10.1146/annurev-pathol-011110-130246
 8. Sun X, Zhang X-D, Cheng G, Hu Y-H, Wang H-Y. Inhibition of hepatic stellate cell proliferation by heat shock protein 90 inhibitors in vitro. *Mol Cell Biochem.* 2009;330: 181–185. doi:10.1007/s11010-009-0131-4
 9. Son MK, Ryu Y-L, Jung KH, Lee H, Lee HS, Yan HH, et al. HS-173, a Novel PI3K Inhibitor, Attenuates the Activation of Hepatic Stellate Cells in Liver Fibrosis. *Sci Rep.* Nature Publishing Group; 2013;3: 3470. doi:10.1038/srep03470
 10. Palozza P, Simone R, Catalano A, Boninsegna A, Böhm V, Fröhlich K, et al. Lycopene prevents 7-ketocholesterol-induced oxidative stress, cell cycle arrest and apoptosis in human macrophages☆. *J Nutr Biochem.* 2010;21: 34–46. doi:10.1016/j.jnutbio.2008.10.002
 11. Teodoro AJ, Oliveira FL, Martins NB, Maia G de A, Martucci RB, Borojevic R. Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int.* 2012;12: 36. doi:10.1186/1475-2867-12-36
 12. Yang C-M, Lu I-H, Chen H-Y, Hu M-L. Lycopene inhibits the proliferation of androgen-dependent human prostate tumor cells through activation of PPARγ-LXRα-ABCA1 pathway. *J Nutr Biochem.* 2012;23: 8–17. doi:10.1016/j.jnutbio.2010.10.006
 13. Soares N da CP, Teodoro AJ, Oliveira FL, Santos CA do N, Takiya CM, Junior OS, et al. Influence of lycopene on cell viability, cell cycle, and apoptosis of human prostate cancer and benign hyperplastic cells. *Nutr Cancer.* 2013;65: 1076–85. doi:10.1080/01635581.2013.812225
 14. Hwang E-S, Bowen PE. Cell Cycle Arrest and Induction of Apoptosis by Lycopene in LNCaP Human Prostate Cancer Cells. *J Med Food.* 2004;7: 284–289. doi:10.1089/jmf.2004.7.284
 15. Hantz HL, Young LF, Martin KR. Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp Biol Med (Maywood).* 2005;230: 171–9. Available: <http://www.ncbi.nlm.nih.gov/pubmed/15734720>
 16. Michael McClain R, Bausch J. Summary of safety studies conducted with synthetic lycopene. *Regul Toxicol Pharmacol.* 2003;37: 274–85. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12726756>
 17. Rabus M, Demirbağ R, Sezen Y, Konukoğlu O, Yildiz A, Erel O, et al. Plasma and tissue oxidative stress index in patients with rheumatic and degenerative heart valve disease. *Türk Kardiyol Dern Ars.* 2008;36: 536–40. Available: <http://www.ncbi.nlm.nih.gov/pubmed/19223719>
 18. McClain M and Bausch J: Summary of safety studies conducted with synthetic lycopene. *Regul Toxicol Pharmacol* 37(2): 274– 285, 2003. doi.org/10.1016/S0273-2300(03)00004-7
 19. Nahum A, Hirsch K, Danilenko M, Watts CK, Prall OW, Levy J, et al. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27Kip1 in the cyclin E–cdk2 complexes. *Oncogene.* 2001;20: 3428–3436. doi:10.1038/sj.onc.1204452
 20. Ivanov NI, Cowell SP, Brown P, Rennie PS, Guns ES, Cox ME. Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines. *Clin Nutr.* 2007;26: 252–263. doi:10.1016/j.clnu.2007.01.002
 37. Palozza P, Serini S, Boninsegna A, Bellovino D, Lucarini M, Monastra G, et al. The growth-inhibitory effects of tomatoes digested in vitro in colon adenocarcinoma cells occur through down-regulation of cyclin D1, Bcl-2 and Bcl-xL. *Br J Nutr.* 2007;98: 789–95. doi:10.1017/S0007114507746883
 38. Park YO, Hwang E-S, Moon TW. The effect of lycopene on cell growth and oxidative DNA damage of Hep3B human hepatoma cells. *Biofactors.* 2005;23: 129–39. Available: <http://www.ncbi.nlm.nih.gov/pubmed/16410635>
 39. Del Giudice R, Raiola A, Tenore GC, Frusciante L, Barone A, Monti DM, et al. Antioxidant bioactive compounds in tomato fruits at different ripening stages and their effects on normal and cancer cells. *J Funct Foods.* Elsevier; 2015;18: 83–94. doi:10.1016/J.JFF.2015.06.060
 40. Liu X-J, Yang L, Luo F-M, Wu H-B, Qiang Q. Association of differentially expressed genes with activation of mouse hepatic stellate cells by high-density cDNA microarray. *World J Gastroenterol.* Baishideng Publishing Group Inc; 2004;10: 1600–7. doi:10.3748/WJG.V10.I11.1600
 41. Hiramatsu K, Matsuda H, Nemoto T, Nosaka T, Saito Y, Naito T, et al. Identification of novel variants in HLA class II region related to HLA DPB1 expression and disease progression in patients with chronic hepatitis C. *J Med Virol.* 2017;89: 1574–1583. doi:10.1002/jmv.24814
 42. Akgöllü E, Bilgin R, Akkız H, Ülger Y, Kaya BY, Karaoğullarından Ü, et al. Association between chronic hepatitis B virus infection and HLA-DP gene polymorphisms in the Turkish population. *Virus Res.* 2017;232: 6–12. doi:10.1016/j.virusres.2017.01.017
 43. Twu Y-C, Lee T-S, Lin Y-L, Hsu S-M, Wang Y-H, Liao C-Y, et al. Niemann-Pick Type C2 Protein Mediates Hepatic Stellate Cells Activation by Regulating Free Cholesterol Accumulation. *Int J Mol Sci.* 2016;17: 1122. doi:10.3390/ijms17071122
 44. Tsuchida T, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol.* 2017;14: 397–411. doi:10.1038/nrgastro.2017.38
 45. Ben-Dor A, Nahum A, Danilenko M, Giat Y, Stahl W, Martin H-D, et al. Effects of acyclo-Retinoic Acid and Lycopene on Activation of the Retinoic Acid Receptor and Proliferation of Mammary Cancer Cells. *Arch Biochem Biophys.* 2001;391: 295–302. doi:10.1006/abbi.2001.2412
 46. Hazra S, Xiong S, Wang J, Rippe RA, Krishna V, Chatterjee K, et al. Peroxisome Proliferator-activated Receptor γ Induces a Phenotypic Switch from Activated to Quiescent Hepatic Stellate Cells. *J Biol Chem.* 2004;279: 11392–11401. doi:10.1074/jbc.M310284200

38. Evans RM, Mangelsdorf DJ. Nuclear Receptors, RXR, and the Big Bang. *Cell*. 2014;157: 255–266. doi:10.1016/j.cell.2014.03.012
39. Sever R, Glass CK. Signaling by nuclear receptors. *Cold Spring Harb Perspect Biol*. Cold Spring Harbor Laboratory Press; 2013;5: a016709. doi:10.1101/cshperspect.a016709
40. Tyagi S, Sharma S, Gupta P, Saini A, Kaushal C. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *J Adv Pharm Technol Res*. 2011;2: 236. doi:10.4103/2231-4040.90879
41. Lenhard JM. PPAR gamma/RXR as a molecular target for diabetes. *Receptors Channels*. 2001;7: 249–58. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11697231>
42. Crowe DL, Chandraratna RA. A retinoid X receptor (RXR)-selective retinoid reveals that RXR- α is potentially a therapeutic target in breast cancer cell lines, and that it potentiates antiproliferative and apoptotic responses to peroxisome proliferator-activated receptor ligands. *Breast Cancer Res*. 2004;6: R546. doi:10.1186/bcr913
43. Yang W, Rachez C, Freedman LP. Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptor coactivators. *Mol Cell Biol*. 2000;20: 8008–17. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11027271>
44. Wei J, Bhattacharyya S, Varga J. Peroxisome proliferator-activated receptor γ : innate protection from excessive fibrogenesis and potential therapeutic target in systemic sclerosis. *Curr Opin Rheumatol*. NIH Public Access; 2010;22: 671–6. doi:10.1097/BOR.0b013e32833de1a7
45. MacDougald OA, Lane MD. Transcriptional Regulation of Gene Expression During Adipocyte Differentiation. *Annu Rev Biochem*. 1995;64: 345–373. doi:10.1146/annurev.bi.64.070195.002021
46. Morrison RF, Farmer SR. Hormonal Signaling and Transcriptional Control of Adipocyte Differentiation. *J Nutr*. 2000;130: 3116S–3121S. doi:10.1093/jn/130.12.3116S

Lycopene and Tomato Sauce Improve Hepatic and Cardiac Cell Biomarkers in Rats

Vanessa Azevedo de Jesus,¹ Monique de Barros Elias Campos,¹ Vanessa Rosse de Souza,¹
Teresa Palmiciano Bede,² Bianca Portugal Tavares de Moraes,³ Adriana Ribeiro Silva,⁴
Cassiano Felipe Gonçalves de Albuquerque,³ Vilma Blondet de Azeredo,² and Anderson Junger Teodoro¹

¹Food and Nutrition Program, Functional Foods Laboratory, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

²Nutrition and Dietetic Department, Fluminense Federal University, Rio de Janeiro, Brazil.

³Immunopharmacology Laboratory, Biomedical Institute, Federal University of the State of Rio de Janeiro, Rio de Janeiro, Brazil.

⁴Immunopharmacology Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil.

ABSTRACT This study evaluated the effects of tomato sauce and lycopene on hepatic and cardiac cell biomarkers in rats fed a high-fat diet. Animals were split into five groups: control group, high-fat group (HG), high-fat tomato sauce group, high-fat lycopene 2 mg, and high-fat lycopene 4 mg. Food and water were offered *ad libitum*, whereas tomato sauce and lycopene (2 and 4 mg/day) were offered daily for 60 days. Body, heart, and liver weights, cardiosomatic and hepatosomatic indices, and serum parameters were also analyzed in rats. The animals' hearts and liver were processed, and cells were examined by flow cytometry. Results showed that the groups receiving tomato sauce and lycopene had lower glycemia. The serum concentration of high-density lipoprotein cholesterol, hepatic enzymes, and tumor necrosis factor- α did not change upon treatment. Tomato sauce and lycopene supplementation did not increase interleukin-1 β in response to a high-fat diet. Cell cycle analysis of cardiac and liver cells showed a lower percentage of cells in the G0/G1 phase and an increase in the G2/M phase in HG. Both lycopene and tomato sauce reversed this effect. Both lycopene and tomato sauce reversed this effect and prevented high-fat diet-stimulated cardiac and liver cell death. Supplementation of tomato sauce and lycopene showed beneficial effects on cardiac and liver cell metabolism; therefore, it is suggested as a nutritional approach for the prevention and treatment of cardiovascular diseases and nonalcoholic hepatic steatosis.

KEYWORDS: apoptosis • carotenoids • cell cycle • high-fat diet • inflammation

INTRODUCTION

OBESITY IS A RISK FACTOR for various chronic diseases, and the metabolic defects of obesity and type 2 diabetes, characterized by fatty liver disease, insulin resistance, and dyslipidemia, lead to an increased risk of cardiovascular disease and cancer.^{1–3} Although diagnosed worldwide, it has variations in prevalence, reaching ~20–30% in western countries. In the United States, a country where 25% of the adult population is obese, the disease affects more than 60% of these individuals. It is estimated that 2–3% of the population has hepatic steatosis.³ The consumption of diets rich in saturated fats is linked to synthesis of proinflammatory cytokines, an increase in reactive oxygen species, development of oxidative stress, and damage to several biomolecules. It is also a predisposing factor in the development of a variety of

chronic diseases, including obesity, cognitive dysfunction, diabetes, and cancer.^{4–9} Thus, a high-fat diet has a central role in the development of oxidative events, as occurs in hepatic steatosis and atherosclerosis. Fatty liver is associated with several atherosclerotic risk factors such as hypertension, diabetes, and dyslipidemia.^{10,11} Bioactive compound supplements are a potential disease-preventing or health-promoting treatment to be taken daily.¹² Bioactive compounds are substances discovered from natural sources, which are capable of retarding or inhibiting oxidation rates and can be produced endogenously or absorbed through foods in the diet.^{13–15} Some authors have demonstrated an inverse relationship between the consumption of carotenoid-rich foods and the risk of diseases induced by oxidative stress.^{6,16–18}

Lycopene is a lipophilic non-provitamin A carotenoid, responsible for the red color in some fruits and vegetables, such as tomatoes. It has a capacity to protect against many diseases, mainly due to its antioxidative effects, lipid-regulating enzyme activities, capacity to induce adipocyte differentiation, and improvement of the plasma lipid profile

Manuscript received 15 January 2019. Revision accepted 25 April 2019.

Address correspondence to: Anderson Junger Teodoro, PhD, Food and Nutrition Program, Functional Foods Laboratory, Universidade Federal do Estado do Rio de Janeiro, Rio de Janeiro 22290-240, Brazil, E-mail: atteodoro@gmail.com

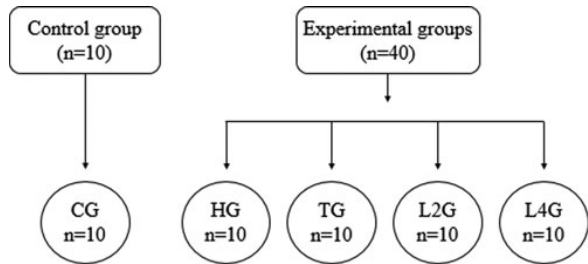


FIG. 1. Dietary protocol. Experimental model: CG: standard diet plus water; experimental groups: HG, high-fat diet plus water; TG, high-fat diet plus solution with tomato sauce and water; L2G, high-fat diet plus solution with 2.0 mg all-trans lycopene and water; L4G, high-fat diet plus solution with 4.0 mg all-trans lycopene and water. Diets and solutions were administered during a period of 60 days. CG, control group; HG, high-fat group; TG, tomato sauce group; L2G, lycopene 2 mg group; L4G, lycopene 4 mg group.

in rats fed with a high-fat diet.¹² Previous studies have linked the high intake of tomato products or lycopene with a lower risk of metabolic diseases, protective effects against high-fat diets, and decreased hepatic inflammation.^{19–22} However, there is no consensus in the literature regarding which form of lycopene (*i.e.*, tomato products or isolated lycopene supplement) is more beneficial to these inflammatory diseases.

The aim of this study was to evaluate the effect of tomato sauce and isolated lycopene on changes related to cardiac and hepatic tissues in Wistar rats, such as glycemia, lipid profile, inflammatory mediators, hepatic, and cardiac cell cycle.

MATERIALS AND METHODS

Samples

Samples of Brazilian tomato sauce (ingredients: tomato [97%], sugar, salt, modified starch, vegetable oil, onion, parsley, marjoram, celery, thickener xanthan gum, aromatizing and potassium sorbate, and sodium benzoate) were obtained from a local market (Rio de Janeiro, RJ, Brazil). Water-soluble lycopene 10% (containing sucrose, corn starch, fish gelatin, lycopene, corn oil, ascorbyl palmitate, and DL-alpha-tocopherol) was provided by Roche (Rio de

Janeiro, RJ, Brazil). Samples of tomato sauce and lycopene were kept in a refrigerator (10°C) and freezer (-18°C), respectively, in appropriate packaging.

Appropriate solutions for each experimental group were prepared daily in the laboratory by dissolving the content in filtered water at 50°C and adding 20% refined sugar to obtain a palatable solution.

Experimental model

Fifty female, adult Wistar rats were individually housed and maintained in a 12-h light–12-h dark cycle at 22°C (±2°C). Animal maintenance was in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.²³ Rat care and experimental protocols were approved by the Institution's Scientific, Academic, and Ethics Board.

Animals were divided into five groups (Fig. 1): control group (CG), which received a standard diet (based casein) *ad libitum*, consisting of protein (minimum) 12.95%, fat (minimum) 4.0%, and water; high-fat group (HG), which received a high-fat diet *ad libitum*, consisting of protein (minimum) 12.95%, fat (minimum) 20.0%, and water; tomato sauce group (TG), which received the high-fat diet *ad libitum*, plus a tomato sauce solution providing 2.0 mg of lycopene per day; 2.0 mg lycopene group (L2G), which received the high-fat diet *ad libitum*, plus a solution containing 2.0 mg all-trans lycopene (water soluble) 10% dissolved in water daily, and water; 4.0 mg lycopene group (L4G), which received the high-fat diet *ad libitum*, plus a solution containing 4.0 mg all-trans lycopene (water soluble) 10% dissolved in water daily, and water. The ingredients for the formulation of the control and high-fat rations used in the experiment are shown in the Supplementary Table S1.

After 60 days of the experiment, the vaginal smear procedure was performed on all animals to identify their phase in the estrous cycle and to establish that all were in the same physiological state without hormonal interference in the analysis. After the estrous cycle check, rats in the “estrus” phase were separated and trapped. Body weight was measured, and trapped animals were sacrificed. Serum and organs (heart and liver) were obtained, weighed, frozen, and kept at -70°C until analysis. Experimental procedures were conducted according to the study of Ribeiro *et al.*¹³

TABLE 1. BODY AND TISSUE WEIGHT IN CONTROL AND EXPERIMENTAL GROUPS

Parameters (g)	CG	HG	TG	L2G	L4G
Initial body weight	160.4 – 4.83 ^a	161.4 – 6.23 ^a	163.4 – 5.68 ^a	162.0 – 5.34 ^a	158.6 – 5.94 ^a
Final body weight	261.00 – 31.04 ^a	268.20 – 17.68 ^a	277.70 – 11.48 ^a	197.30 – 13.12 ^b	188.90 – 15.02 ^b
Heart weight	0.94 – 0.21 ^a	0.90 – 0.07 ^a	1.06 – 0.05 ^b	0.80 – 0.16 ^a	0.72 – 0.11 ^c
Liver weight	7.86 – 1.52 ^a	7.10 – 0.57 ^a	8.50 – 1.01 ^a	5.78 – 0.55 ^b	5.82 – 0.37 ^b
Cardiosomatic index	0.36 – 0.09 ^a	0.34 – 0.01 ^a	0.38 – 0.06 ^a	0.40 – 0.06 ^a	0.38 – 0.04 ^a
Hepatosomatic index	2.99 – 0.23 ^a	2.65 – 0.25 ^a	3.06 – 0.43 ^a	2.95 – 0.43 ^a	3.09 – 0.17 ^a

Values are mean – SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

CG, control group; HG, high-fat group; TG, tomato sauce group; L2G, lycopene 2 mg; L4G, lycopene 4 mg; SD, standard deviation; ANOVA, analysis of variance.

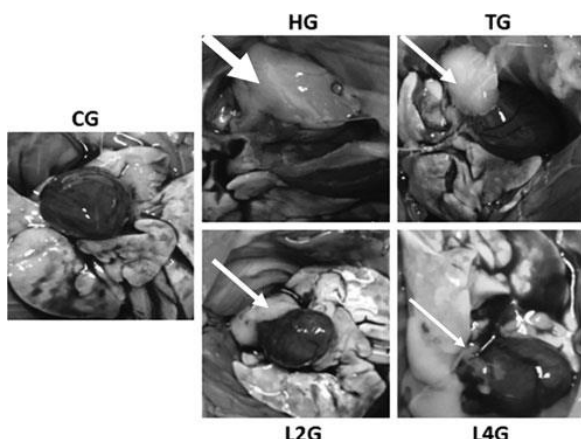


FIG. 2. Heart extracted from sacrificed rats after dietary protocol. White arrows indicate accumulated fat in tissues.

The hearts and livers were weighed to determine the relative weight of the organ denominated cardiosomatic index and hepatosomatic index, which is calculated according to the formula:

$$\% \text{Heart} = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

Analytical methods

A glucometer was used to measure serum glucose concentration. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin (IL)-1 β , and tumor necrosis factor (TNF)- α concentration were measured using the BioClin® Commercial Kits and wavelengths specific to each biochemical indicator, using the colorimetric method with automated spectrophotometer reading (BioClin BS-120 Chemistry Analyzer®). Low-density lipoprotein (LDL) cholesterol was calculated according to Friedwald's formula.²⁴

Cell cycle

Animal heart muscle and liver tissues were processed, and cardiac and liver cell cycles and apoptosis were measured using flow cytometry. Cell extractions were performed

through maceration of the tissue and addition of 0.5 mg/mL collagenase (Sigma®). After centrifugation, the cells were washed twice with phosphate-buffered saline and resuspended in 500 μ L of ice-cold Vindelov solution containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNase, and 50 mg/mL propidium iodide (PI; Sigma Chemical Co., St. Louis, MO, USA). After 15 min of incubation, the cell suspension was analyzed for DNA content by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The relative proportions of cells with DNA content haploid subG1 (<2n), diploid G₀/G₁ (2n), S phase (>2n and <4n), and G₂/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9. Respectively, the percentage of cell population at a particular phase was estimated with FlowJo software following the acquisition of 30,000 events. To our knowledge, the cell dissociation procedure does not affect fluorescence under the experimental conditions used in this study. Nuclei of viable cells were gated according to the FL-2W - FL2-A relationship based on the study conducted by Guimarães *et al.*²⁵

Apoptosis assay

Cells were resuspended in 400 μ L of binding buffer containing 5 μ L of Annexin V fluorescein isothiocyanate and 5 μ L PI (Apoptosis Detection Kit II; BD Biosciences, BD Pharmingen, Mountain View, CA, USA) for 15 min at room temperature (25°C). Annexin V binding was evaluated by flow cytometry (FACSCalibur; BD Biosciences) after acquisition of 30,000 events. The data were analyzed in CellQuest and FlowJo software.

Data analysis

The effects of tomato sauce and isolated lycopene supplementation were analyzed using one-way analysis of variance followed by Tukey's *post-hoc* test for the multiple mean comparison test. Results are expressed as mean $\pm$ standard deviation, and the significance level was set at $P < .05$.

RESULTS

At the end of the experiments, body weights were measured, and the final body weight was higher ($P < .05$) in the

TABLE 2. SERUM PARAMETERS IN RATS FED WITH EXPERIMENTAL DIETS

Parameters (mg/dL)	CG	HG	TG	L2G	L4G
Glycemia	107.00 $\pm$ 2.65 ^a	101.67 $\pm$ 4.51 ^{ac}	86.20 $\pm$ 4.76 ^b	88.60 $\pm$ 4.56 ^b	88.60 $\pm$ 6.80 ^b
Total cholesterol	46.00 $\pm$ 8.60 ^{ab}	45.00 $\pm$ 3.54 ^b	60.00 $\pm$ 4.06 ^c	56.80 $\pm$ 8.14 ^{abc}	53.80 $\pm$ 4.49 ^{abc}
HDL cholesterol	21.60 $\pm$ 3.51 ^a	22.40 $\pm$ 1.34 ^a	25.00 $\pm$ 2.55 ^a	24.20 $\pm$ 1.30 ^a	23.40 $\pm$ 1.34 ^a
LDL cholesterol	13.62 $\pm$ 5.01 ^a	26.76 $\pm$ 2.50 ^b	23.80 $\pm$ 3.77 ^b	19.53 $\pm$ 4.80 ^b	21.82 $\pm$ 4.01 ^b
Triglycerides	31.60 $\pm$ 8.17 ^a	47.00 $\pm$ 7.94 ^b	41.20 $\pm$ 7.66 ^b	40.00 $\pm$ 6.20 ^b	33.25 $\pm$ 7.68 ^a
AST	195.60 $\pm$ 52.03 ^a	219.80 $\pm$ 33.39 ^a	198.20 $\pm$ 52.77 ^a	177.80 $\pm$ 41.81 ^a	176.40 $\pm$ 26.60 ^a
ALT	32.20 $\pm$ 6.42 ^a	32.00 $\pm$ 5.39 ^a	29.00 $\pm$ 4.80 ^a	28.00 $\pm$ 5.43 ^a	33.20 $\pm$ 5.81 ^a

Values are mean $\pm$ SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

TG (277.7 – 11.48 g) and lower in the L2G (197.3 – 13.12 g) and L4G (188.9 – 15.02 g) groups compared with the control group (Table 1). Heart weight was higher ($P < .05$) in the TG (1.06 – 0.05 g) and lower in the L4G (0.72 – 0.11 g) groups compared with the control group. No differences were observed in liver weight or hepatosomatic and cardiosomatic index among the groups (Table 1). Images of rat hearts included in this study are displayed in Figure 2.

Significant differences ($P < .05$) in glycemia, total cholesterol, and triglycerides were found among the groups analyzed (Table 2). Groups receiving tomato sauce (TG) and lycopene (L2G, and L4G) had lower glycemic values compared with the control. Furthermore, the group receiving a high-fat diet had higher triglyceride levels compared with the CG group; however, no differences were observed when compared with the L4G group. In addition, there were no differences in the concentrations of serum HDL cholesterol and liver enzymes (AST and ALT) among the groups (Table 2).

High-fat diet induced an increase in LDL when compared with the control group. However, lycopene and tomato sauce treatment did not promote changes in the LDL concentration caused by the hyperlipidic diet.

HG showed an increase in IL-1 β expression compared with the other groups. Tomato sauce and lycopene, however, did not show this effect and presented a similar profile. Importantly, TG showed similar results to CG. There were no variations in the levels of TNF- α among the groups (Fig. 3).

Cell cycle analysis of cardiac cells showed that the HG group presented a lower percentage of cells in the G₀/G₁ phase (47.98 – 6.28), compared with the other groups (Table 3). Furthermore, a high-fat diet increased the percentage of cells in the G₂/M phase, which was reversed by the action of both lycopene and tomato sauce ($P < .05$). In liver cells, high-fat diet decreased the number of cells in the G₀/G₁ phase and increased the percentage of cells in the G₂/M phase. We hypothesized that the highest rate of cell death in that group promoted an increase in cellular proliferation to reduce cellular loss. Both tomato sauce and lycopene-treated groups increased the number of cells in the G₀/G₁ phase and decreased numbers in the G₂/M phase induced by the high-fat diet (Table 4).

Annexin V and PI biomarkers were used to assess apoptosis. An increase in apoptotic cells was present in liver and heart cells in every group receiving the high-fat diet compared with the CG group. High-fat diet promoted a significant decrease in the population of viable cells and a significant increase of 5.2 and 3.9 times, respectively, in cardiac and hepatic cells compared with controls (Figs. 4 and 5). High-fat diet supplemented with lycopene and tomato sauce reversed the increase in apoptosis caused by the hyperlipidic diet, exhibiting higher values when compared with the control group.

DISCUSSION

Tomato sauce is a rich source of lycopene, which has potent antioxidant activity. Nevertheless, very little is known about

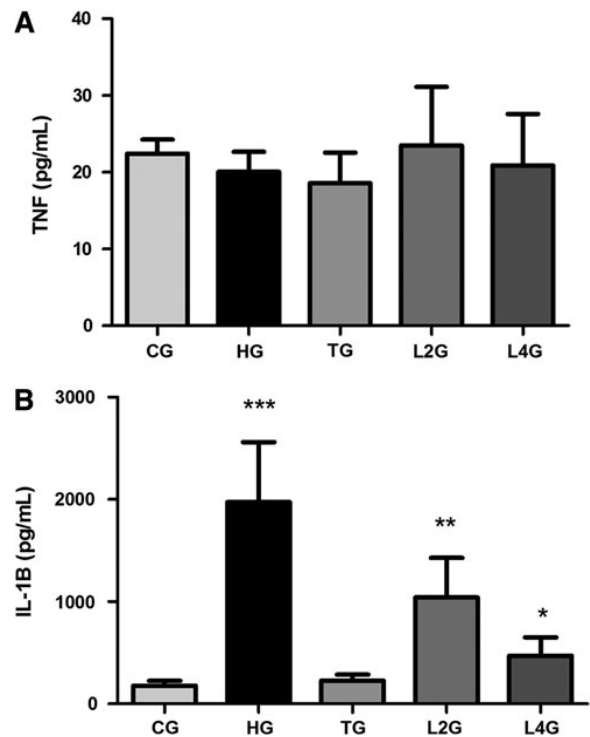


FIG. 3. (A) TNF- α (B) IL-1 β from rats fed a high-fat diet supplemented with lycopene and tomato sauce. * $P < .05$; ** $P < .01$; *** $P < .001$ ANOVA–Tukey’s test. ANOVA, analysis of variance; TNF, tumor necrosis factor; IL, interleukin.

different forms of lycopene supplementation in cardiovascular diseases and nonalcoholic hepatic steatosis.^{4,21,26–30}

Previous study have shown that 762 individuals with hepatic steatosis (76.8%) had at least one atherosclerotic plaque, evidencing a higher prevalence of atherosclerotic plaques in patients with hepatic steatosis. The study observed a direct association between hepatic steatosis and carotid plaques independent of age and sex.¹⁵ For this reason, it is important to evaluate factors that may contribute to their prevention.

TABLE 3. EFFECT OF LYCOPENE AND TOMATO SAUCE ON CELL CYCLE PROGRESSION OF CARDIAC CELLS IN RATS FED HIGH-FAT DIET

Groups	Sub-G1	G0/G1	S	G2/M
CG	3.27 – 0.42 ^a	79.80 – 4.34 ^a	8.90 – 0.44 ^a	7.95 – 1.50 ^a
HG	6.35 – 1.05 ^b	71.39 – 3.88 ^b	9.76 – 0.85 ^a	16.03 – 1.82 ^b
TG	1.53 – 1.22 ^a	78.86 – 7.14 ^a	8.53 – 0.49 ^a	12.66 – 1.62 ^c
L2G	3.44 – 0.29 ^a	81.55 – 7.88 ^a	9.06 – 2.67 ^a	5.95 – 2.03 ^a
L4G	1.52 – 0.97 ^a	80.17 – 10.35 ^{ab}	10.95 – 1.73 ^a	8.87 – 1.87 ^a

The results are expressed as the percentages of total cells. Values are mean – SD.

^{abc}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA, followed by Tukey’s *post hoc* multiple mean comparison test.

TABLE 4. EFFECT OF LYCOPENE AND TOMATO SAUCE ON CELL CYCLE PROGRESSION OF HEPATIC CELLS IN RATS FED HIGH-FAT DIET

Groups	Sub G1	G0/G1	S	G2/M
CG	1.98 – 0.36 ^a	63.49 – 6.06 ^a	20.20 – 8.47 ^a	14.41 – 3.85 ^a
HG	4.28 – 0.15 ^b	53.67 – 5.79 ^b	28.18 – 3.40 ^b	18.15 – 3.08 ^a
TG	1.22 – 0.24 ^a	67.50 – 10.10 ^c	15.66 – 6.10 ^a	16.84 – 4.40 ^a
L2G	1.15 – 0.53 ^a	77.96 – 4.89 ^{cd}	10.57 – 2.05 ^a	11.47 – 2.85 ^a
L4G	1.62 – 0.78 ^a	71.27 – 7.39 ^d	13.81 – 3.65 ^a	14.92 – 3.93 ^a

The results are expressed as the percentages of total cells. Values are mean – SD.

^{abcd}Different letters mean statistical difference between groups. Statistical significance was determined by ANOVA followed by Tukey's *post hoc* multiple mean comparison test.

The present study provided information regarding the effects of lycopene and lycopene in food matrix supplementation on cardiac and liver metabolism, cell cycle, and apoptosis. Our outcomes agree with published results in which quercetin supplementation in rats was able to reduce weight gain and increase heart size even with ingestion of a high-fat diet when compared with the control.¹² Herein,

lycopene supplementation in rats reduced high-fat diet-induced weight gain and was cardioprotective.

Previous studies have shown that a tomato juice intervention *in vivo* did not affect glucose and lipid profiles.^{31,32} Furthermore, no changes in weight gain were observed in accordance with our results.

Obesity and dyslipidemia are considered risk factors of cardiovascular disease and nonalcoholic hepatic steatosis. Furthermore, it has been reported that a high-fat diet and high cholesterol levels can favor metabolic alterations.³³ Our results revealed that a high-fat diet promoted an increase in triglycerides and cholesterol, and lycopene and tomato sauce supplementation improved the rat's lipid profile.

Previous study observed that treatment with a lycopene mix and other bioactive compounds promoted an increase in HDL levels and reduced oxidative stress through prevention of LDL oxidation.¹⁸

Cellular mechanisms triggered by the consumption of a high-fat diet include apoptosis, necrosis, and autophagy.^{34–37} Those effects may be linked to the genesis of cardiovascular diseases and nonalcoholic hepatic steatosis. Therefore, it is vital to identify strategies that may contribute to their prevention and reduction.

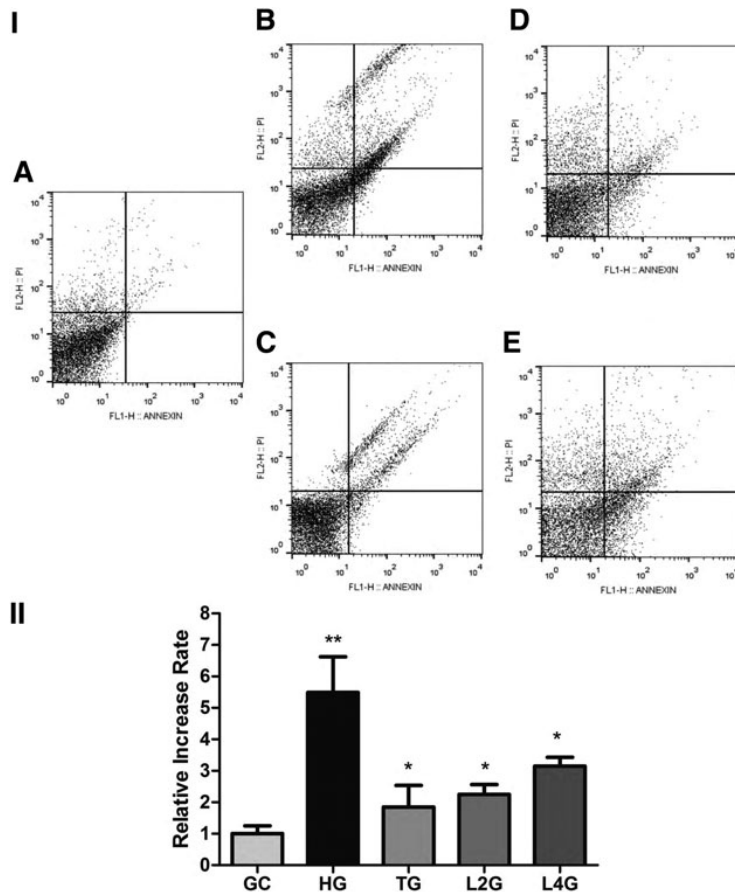


FIG. 4. Monitoring of cell death of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (I) Flow cytometry analysis of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (A): CG; (B): HG; (C): TG; (D): L2G; (E): L4G. The results are expressed as mean – SD, with significant differences compared by one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. **P* < .05. ***P* < .01. SD, standard deviation.

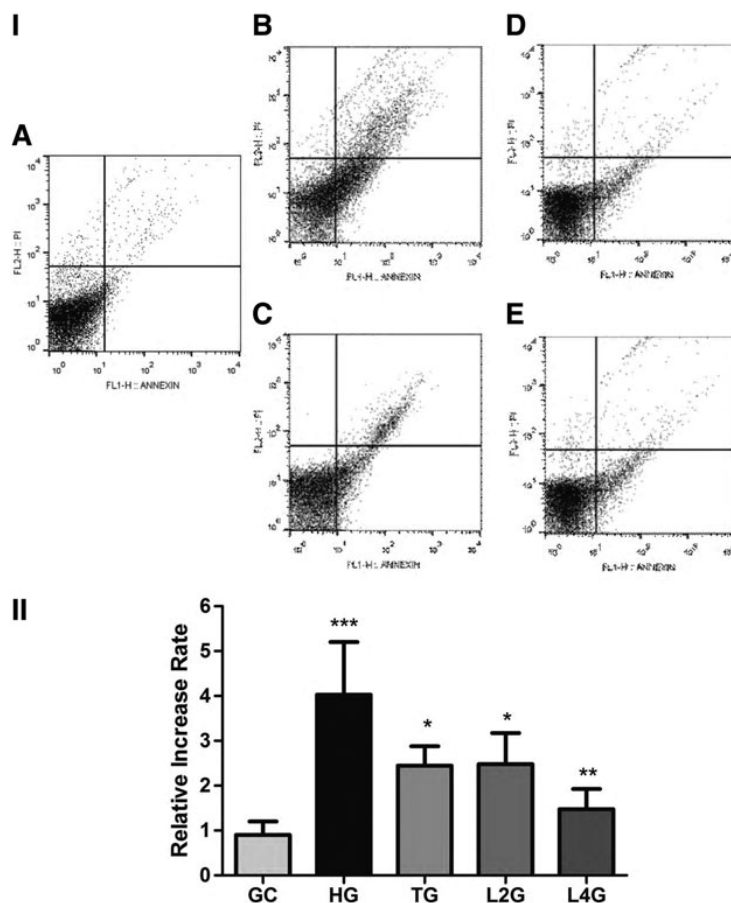


FIG. 5. Monitoring of cell death of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (A) Flow cytometry analysis of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (B) Quantitative effects of lycopene and tomato sauce of cell death of hepatic cells from rats fed a high-fat diet. (A): CG; (B): HG; (C): TG; (D): L2G; (E): L4G. The results are expressed as mean $\pm$ SD, with significant differences compared by one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. * $P < .05$. ** $P < .01$.

Stress factors can induce IL-1 β and TNF- α . Pro-inflammatory cytokine IL-1 β is elevated in chronic inflammatory diseases, such as obesity, and can be associated with increased cell proliferation, cell cycle arrest, and increased apoptosis in different cell types.^{37,38} We showed that tomato sauce and lycopene supplementation reversed the increase in IL-1 β levels induced by high-fat diet.

Numerous studies have investigated the effects of individual compounds on vital cellular parameters and apoptosis to determine the underlying mechanisms of action. However, few studies have investigated the influence of phytochemical combinations in this context.^{39,40}

To determine the basic mechanisms by which carotenoids present in the food matrix may be more effective in preventing and treating diseases than individual compounds, this study compared two versions of lycopene, isolated and food matrix lycopenes, and their inhibitory effects on cell growth and apoptosis.

Few studies have reported the mechanism by which fruits and vegetables could prevent or reduce inflammatory diseases, such as cardiovascular diseases and nonalcoholic hepatic steatosis. Cardiac cell cycle analysis reported that a high-fat diet promoted a decrease in the G₀/G₁ phase and an increase in the percentage of cardiac cells in the G₂/M

phase, demonstrating compensation by cell proliferation for higher levels of cell death. Nevertheless, it is important to mention that the pronounced cell proliferation may be harmful to the organism.

Lycopene promoted an increase of cells in the G₀/G₁ phase and a decrease in the cell percentage in the G₂/M phase in different cancer cell lines.⁴ Our study showed that both tomato sauce and lycopene supplementation increased the number of cardiac cells in the G₀/G₁ phase and a decrease in the number of cardiac cells in the G₂/M compared with the high-fat diet. Tomato sauce reduced the effects caused by high-fat diet in the cardiac cells of the study animals. Lycopene increased the percentage of cardiac cells in the G₀/G₁ phase, perhaps lessening damage caused by the high-fat diet. Similar results were observed in the liver cell cycle, in which tomato sauce and lycopene supplementation increased and decreased the percentage of cells in the G₀/G₁ and G₂/M phase, respectively.

Consumption of tomato juice and pure lycopene regulates the cell cycle of HepG2 cells. Nonetheless, tomato juice did not promote apoptotic changes; only lycopene supplementation was able to induce apoptosis in HepG2 cells.^{40,41}

Apoptosis is characterized by a series of distinct changes in cell morphology, loss of cell attachment, cytoplasmic

contraction, DNA fragmentation, and other biochemical changes, including the activation of caspases through extrinsic and/or intrinsic mitochondrial pathways. Therefore, an inhibitory effect on cell proliferation is very desirable for a compound. It is known that changes in the cell growth process and cell cycle are main features of different pathologies. This study showed a damaging effect of high-fat diet on cardiac cell apoptotic induction. However, tomato sauce and lycopene were not able to reduce high-fat diet-induced apoptosis. Similar results have been observed in different cancer cell lines in which lycopene promoted an increase in apoptosis.^{4,41}

We demonstrated that tomato sauce and lycopene supplementation have beneficial effects on cardiac and liver cell metabolism and may be considered as a nutritional approach for the prevention and treatment of cardiovascular diseases and nonalcoholic hepatic steatosis.

ACKNOWLEDGMENTS

This research was supported by CNPq and CAPES grants of the Brazilian Federal Government and the FAPERJ grant of the Rio de Janeiro State Government.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

SUPPLEMENTARY MATERIAL

Supplementary Table S1

REFERENCES

- World Health Organization: *Taking Action on Childhood Obesity*. World Health Organization, Geneva, Switzerland, 2017, pp. 1–8.
- Semenkovich CF: Insulin resistance and atherosclerosis. *J Clin Invest* 2006;116:1813–1822.
- Bellentani S, Scaglioni F, Marino M, Bedogni G: Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 2010;28:155–161.
- Gloria NF, Soares N, Brand C, Oliveira FL, Borojevic R, Teodoro AJ: Beta-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. *Anticancer Res* 2014;34:1377–1386.
- Thies F, Masson LF, Rudd A, *et al.*: Effect of a tomato-rich diet on markers of cardiovascular disease risk in moderately overweight, disease-free, middle-aged adults: A randomized controlled trial. *Am J Clin Nutr* 2012;95:1013–1022.
- McEneny J, Wade L, Young IS, *et al.*: Lycopene intervention reduces inflammation and improves HDL functionality in moderately overweight middle-aged individuals. *J Nutr Biochem* 2013;24:163–168.
- Shiou YL, Huang IC, Lin HT, Lee HC: High fat diet aggravates atrial and ventricular remodeling of hypertensive heart disease in aging rats. *J Formos Med Assoc* 2018;117:621–631.
- Zhang Q, Qian ZY, Zhou PH, *et al.*: Effects of oral selenium and magnesium co-supplementation on lipid metabolism, antioxidative status, histopathological lesions, and related gene expression in rats fed a high-fat diet. *Lipids Health Dis* 2018;17:1–12.
- Duavy SMP, Salazar GJT, Leite GO, Ecker A, Barbosa NV: Effect of dietary supplementation with olive and sunflower oils on lipid profile and liver histology in rats fed high cholesterol diet. *Asian Pac J Trop Med* 2017;10:539–543.
- Akahoshi M, Amasaki Y, Soda M, *et al.*: Correlation between fatty liver and coronary risk factors: A population study of elderly men and women in Nagasaki, Japan. *Hypertens Res* 2001;24:337–343.
- Osono Y, Nakajima K, Hata Y: Hypertriglyceridemia and fatty liver: Clinical diagnosis of fatty liver and lipoprotein profiles in hypertriglyceridemic patients with fatty liver. *J Atheroscler Thromb* 1995;2(Suppl 1):S47–S52.
- Ting Y, Chang WT, Shiau DK, Chou PH, Wu MF, Hsu CL: Antiobesity efficacy of quercetin-rich supplement on diet-induced obese rats: Effects on body composition, serum lipid profile, and gene expression. *J Agric Food Chem* 2018;66:70–80.
- Ribeiro VM, Bede TP, Rocha GS, Barroso S, Valença S, Azeredo VB: High fat diet and high polyphenols beverages effects in enzymatic and non-enzymatic antioxidant activity. *Nutr Hosp* 2017;35:169–175.
- Barreiros ALBS, David JP, David JM: Oxidative stress: Relations between the formation of reactive species and the organism's defense. *Quim Nova* 2006;29:113–123.
- Halliwell B: Biochemistry of oxidative stress. *Biochem Soc Trans* 2007;35:1147–1150.
- Blesso CN, Andersen CJ, Bolling BW, Fernandez ML: Egg intake improves carotenoid status by increasing plasma HDL cholesterol in adults with metabolic syndrome. *Food Funct* 2013;4:213–221.
- Ciccione M, Cortese F, Gesualdo M, *et al.*: Dietary intake of carotenoids and their antioxidant and anti-inflammatory effects in cardiovascular care. *Mediators Inflamm* 2013;2013:782137.
- Karppi J, Kurl S, Ronkainen K, Kauhanen J, Laukkanen JA: Serum carotenoids reduce progression of early atherosclerosis in the carotid artery wall among Eastern Finnish men. *PLoS One* 2013;8:1–7.
- Melendez-Martinez AJ, Nascimento AF, Wang Y, Liu C, Mao Y, Wang XD: Effect of tomato extract supplementation against high-fat diet-induced hepatic lesions. *Hepatobiliary Surg Nutr* 2013;2:198–208.
- Fenni S, Hammou H, Astier J, *et al.*: Lycopene and tomato powder supplementation similarly inhibit high-fat diet induced obesity, inflammatory response, and associated metabolic disorders. *Mol Nutr Food Res* 2017;61:1–10.
- Kim JY, Paik JK, Kim OY, *et al.*: Effects of lycopene supplementation on oxidative stress and markers of endothelial function in healthy men. *Atherosclerosis* 2011;215:189–195.
- Wang L, Gaziano JM, Norkus EP, Buring JE, Sesso HD: Associations of plasma carotenoids with risk factors and biomarkers related to cardiovascular disease in middle-aged and older women. *Am J Clin Nutr* 2008;88:747–754.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG: Animal research: Reporting in vivo experiments-the ARRIVE guidelines. *Br J Pharmacol* 2010;160:1577–1579.
- Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499–502.
- Guimarães DAB, De Castro DDSB, de Oliveira FL, Nogueira EM, da Silva MAM, Teodoro AJ: Pitaya extracts induce growth inhibition and proapoptotic effects on human cell lines of breast cancer via downregulation of estrogen receptor gene expression. *Oxid Med Cell Longev* 2017;2017:1–13.

1. Palozza P, Catalano A, Simone RE, Mele MC, Cittadini A: Effect of lycopene and tomato products on cholesterol metabolism. *Ann Nutr Metab* 2012;61:126–134.
2. Gajendragadkar PR, Hubsch A, Mäki-Petäjä KM, Serg M, Wilkinson IB, Cheriyan J: Effects of oral lycopene supplementation on vascular function in patients with cardiovascular disease and healthy volunteers: A randomised controlled trial. *PLoS One* 2014;9:e99070.
3. Cheng HM, Koutsidis G, Lodge JK, Ashor A, Siervo M, Lara J: Tomato and lycopene supplementation and cardiovascular risk factors: A systematic review and meta-analysis. *Atherosclerosis* 2017;257:100–108.
4. Ried K, Fakler P: Protective effect of lycopene on serum cholesterol and blood pressure: Meta-analyses of intervention trials. *Maturitas* 2011;68:299–310.
5. Williams EJ, Baines KJ, Smart JM, Gibson PG, Wood LG: Rosuvastatin, lycopene and omega-3 fatty acids: A potential treatment for systemic inflammation in COPD; a pilot study. *J Nutr Intermed Metab* 2016;5:86–95.
6. García-Alonso FJ, Jorge-Vidal V, Ros G, Periago MJ: Effect of consumption of tomato juice enriched with N-3 polyunsaturated fatty acids on the lipid profile, antioxidant biomarker status, and cardiovascular disease risk in healthy women. *Eur J Nutr* 2012; 51:415–424.
7. Tsitsipikou C, Tsarouhas K, Kioukia-Fougia N, *et al.*: Dietary supplementation with tomato-juice in patients with metabolic syndrome: A suggestion to alleviate detrimental clinical factors. *Food Chem Toxicol* 2014;74:9–13.
8. Guzmán-Gerónimo RI, Alarcón-Zavaleta TM, Oliart-Ros RM, Meza-Alvarado JE, Herrera-Meza S, Chávez-Servia JL: Blue maize extract improves blood pressure, lipid profiles, and adipose tissue in high-sucrose diet-induced metabolic syndrome in rats. *J Med Food* 2017;20:110–115.
9. Estrov Z, Shishodia S, Faderl S, *et al.*: Resveratrol blocks interleukin-1 β -induced activation of the nuclear transcription factor NF- κ B, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* 2003;102:987–995.
10. Palozza P, Parrone N, Simone RE, Catalano A: Lycopene in atherosclerosis prevention: An integrated scheme of the potential mechanisms of action from cell culture studies. *Arch Biochem Biophys* 2010;504:26–33.
11. Viola J, Soehnlein O: Atherosclerosis—a matter of unresolved inflammation. *Semin Immunol* 2015;27:184–193.
12. Simsa-Maziel S, Monsonego-Ornan E: Interleukin-1 β promotes proliferation and inhibits differentiation of chondrocytes through a mechanism involving down-regulation of FGFR-3 and p21. *Endocrinology* 2012;153:2296–2310.
13. Gao J, Shi LZ, Zhao H, *et al.*: Loss of IFN- γ pathway genes in tumor cells as a mechanism of resistance to anti-CTLA-4 therapy. *Cell* 2016;167:397–404.e9.
14. Mesquita SDS, Teixeira CMLL, Servulo EFC: Carotenoids: Properties, applications and market. *Rev Virtual Quim* 2017;9: 672–688.
15. Bojórquez RMC, Gallego JG, Collado PS: Functional properties and health benefits of lycopene. *Nutr Hosp* 2013;28:6–15.
16. Teodoro AJ, Oliveira FL, Martins NB, Maia Gde A, Martucci RB, Borojevic R: Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int* 2012;12:36.

Article

Comparative Analysis of Lycopene Content from Different Tomato-Based Food Products on the Cellular Activity of Prostate Cancer Cell Lines

Nathalia da Costa Pereira Soares ¹, Monique de Barros Elias ², Clara Lima Machado ², Bruno Boquimpani Trindade ², Radovan Borojevic ³ and Anderson Junger Teodoro ^{2,*}

¹ Food Science Department, Chemistry Institute, Universidade Federal do Rio de Janeiro (UFRJ), Avenida Athos da Silveira Ramos 149—Cidade Universitária, Rio de Janeiro 21941-909, Brazil; ncpsouares@gmail.com

² Nutritional Biochemistry Core, Laboratory of Functional Foods, Universidade Federal do Estado do Rio de Janeiro (UNIRIO), Avenida Pasteur 296—Urca, Rio de Janeiro 22290-240, Brazil; claramachado55@gmail.com (C.M.); bboquimpani@gmail.com (B.T.); moniquebarros.nutri@gmail.com (M.d.B.E.)

³ Regenerative Medicine Centre, Faculdade de Medicina de Petrópolis (FASE), Avenida Barão do Rio Branco 1003—Petrópolis, Rio de Janeiro 25680-120, Brazil; rrborojevic@gmail.com

* Correspondence: atteodoro@gmail.com; Tel.: +55-21-2542-7785

Received: 25 February 2019; Accepted: 8 May 2019; Published: 10 June 2019

Abstract: Lycopene is more bioavailable in processed tomato products than in raw tomatoes, since arrangement of cis-isomers of lycopene during food processing and storage may increase its biological activity. The aim of the study is evaluate the influence of lycopene content from different tomato-based food products (extract, paste, ketchup and sauce) on cell proliferation, cell cycle, and rate of apoptosis of human prostate cancer cell lines. DU-145 and PC-3 cell lines were treated with lycopene content from different tomato-based food products (500–5000 µg/mL) for 96 h. The data showed a decrease in cell viability in both DU-145 and PC-3 cells after treatment with all lycopene extracts from tomato-based food products. Analysis of cell cycle revealed a decrease in the percentage of prostate cancer cells in G₀/G₁ and G₂/M phases after 96 h of treatment when using lycopene content from tomato paste and tomato extract. However, lycopene extracted from tomato sauce and ketchup promoted a decrease in the percentage of cells in G₀/G₁ phase and an increase in S and G₂/M phases after 96 h of treatment. Lycopene content from all of those tomato-based food products also increased apoptosis in both prostate cancer cell lines. In this regard, lycopene has proved to be a potent inhibitor of cell viability, arrest cell cycle and increase the apoptosis in human prostate cancer cells, suggesting an effect in the balance of human prostate cancer cell lines growth.

Keywords: tomato-based food products; lycopene; prostate cancer; bioactive compounds; chemoprevention

1. Introduction

Prostate malignant growth (PCa) is the most common cancer and the fifth driving reason for death in men, representing 15% of the absolute analyzed tumors in men and 307,000 deaths, speaking to 6.6% of the all-out male disease mortality. [1]. Many factors included diet, lifestyle, environmental, and genetic factors can contribute to enhance the risk factors for PCa [2]. Several studies found inverse relationships between total fruit and vegetable intake [3] or cruciferous vegetable intake and PCa risk [4–8].

Lycopene is commonly found in tomato products. Due to its lipophilic character, the interactions between lycopene and fats enhance its bioavailability [9]. Therefore, cooking processes using oils in the preparation of tomato sauces and paste are very important [10,11]. In most food sources, lycopene exists predominantly in the all-trans conformation. In contrast, the cis-isomer is thought to provide better bioavailability and might be more easily absorbed [12]. However, lycopene solubilization in warm water collapses of the cell walls; this weakens the connection between the lycopene and the tissue matrix. As a result, lycopene is more accessible and isomerization from the all-trans to the cis conformation is increased [13]. Previous studies have suggested that the bioavailability of lycopene is more substantial in tomato paste than fresh tomatoes. Furthermore, lycopene was found in higher quantities in heated, processed tomato juice than in juice that was unprocessed [14,15]. Thus, in light of the epidemiologically defined cancer-preventing properties of carotenoids, the factors affecting their bioavailability should be taken into account [16].

A number of studies described the association between tomatoes and tomato-based food products with PCa, but the conclusions were unclear [2,17]. The antioxidant activity of lycopene, which is abundant in tomatoes, has also been studied specifically for its influence on PCa [12]. Prospective human studies found that higher lycopene consumption or higher lycopene serum levels were associated with lower PCa risk [18,19]. Two short-term preprostatectomy trials using lycopene supplementation or tomato sauce consumption described the lycopene effect in prostate tissue associated with antioxidant and potentially anticancer properties [20,21]. While several clinical trials suggested an inverse relationship between cancer occurrence and lycopene supplementation [22,23], no large trial study has tested the role of lycopene or tomato-based food products on PCa prevention or treatment [24]. In a cohort of 14,000 Seventh-day Adventist men [25], consumption of only tomatoes, beans, lentils, and peas was found to be statistically significantly related to lower prostate cancer risk in a multivariate analysis. β -carotene food sources seems to be unrelated to risk of prostate cancer. In a larger and more comprehensive dietary study, intake of the carotenoids β -carotene, β -carotene, lutein, and β -cryptoxanthin were not associated with the risk of prostate cancer, but high lycopene intake was related to reduction (26%) in risk [26]. High intake of tomatoes and tomato-based food products, which accounted for highest content of lycopene (82%), reduced risk of prostate cancer by 35% and aggressive prostate cancer by 53% in another study [27].

In vitro, lycopene arrests the cell cycle in several PCa cell lines [28–30]. Lycopene-induced delay in progression through the G1 and S phases has also been observed in human cancer cell lines derived from the prostate [31]. Apo-10'-lycopenoic acid and apo-12'-lycopenal, metabolites of lycopene metabolism, [24] can cause cell cycle arrest in cancer cells [32,33]. Cancer cells arrested by serum deprivation in the presence of lycopene cannot return to the cell cycle after serum re-addition [19]. Lycopene and tomato consumption may modify testosterone metabolism and serum concentrations, and may impact gene expression in human prostate cancer cells, normal rat prostate, and prostate cancer xenografts [19,32–34]. In addition to the antioxidant action, lycopene has several biological functions important for human health. Previous studies describes mechanisms in preventing carcinogenesis, including the antiproliferative insulin-like growth factor-1 inhibition, induction of cell differentiation, and apoptosis, connexin, and enhance of gap junctional intercellular communications [35]. For maintaining normal prostate growth and cell renewal, the complex equilibrium between cell growth or differentiation factors and apoptosis inducing factors is critical. Similar to other tissues, prostate is normally composed of cells that divide and replicate in an orderly and controlled manner, however it can likewise contain cells with modified division patterns that give rise to benign or malignant tumors [36].

The treatment of LNCaP cells with physiologically attainable concentrations of lycopene (0.3–3.0 μ M) can significantly reduce the mitochondrial transmembrane potential, induce the release of mitochondrial cytochrome c, and increase annexin V binding, compatible with the induction of apoptosis [37]. Soares et al. [30] showed that lycopene induced apoptosis in prostate cancer cells with an average 1.35-fold increase after 48 h treatment, reaching a maximum 2.25-fold increase after 96 h, at the highest lycopene concentration (10 μ M). It has been demonstrated that lycopene alters the equilibrium of Bcl-2/Bax expression in PCa cells treated with lycopene. Induction of apoptosis is an

important strategy in the tumor suppressive function of p53 [38]. This prompt might be significant for the understanding the effects of lycopene on prostate cancer.

Although the role of lycopene in the prevention of PCa has been studied extensively, human studies examining the role of lycopene in cancer are now being conducted [39]. The majority of studies on the effects of lycopene on cell cycle were performed 48 h after lycopene treatment; this can lead to the underestimation of the effect of this substance.

In the present study, we evaluated the influence of lycopene content from different tomato-based food products (extract, paste, sauce and ketchup) on cell proliferation, cell cycle, and rate of apoptosis of human prostate cancer cell lines (DU-145 and PC-3).

1. Materials and Methods

1.1. Chemicals, Reagents and Materials

Dulbecco's cell culture medium (DMEM) and bovine serum albumin were obtained from Sigma, and fetal bovine serum (FBS) from Laborclin (São Paulo, Brazil). Cell culture flasks and plates were procured from Nunc (Roskilde, Denmark). All chemicals were of analytical grade.

1.2. Samples and Lycopene Extraction

Tomato-based food products (tomato extract, tomato paste, tomato sauce and ketchup) from Brazilian commercial products were purchased in the local supermarket (Rio de Janeiro, Brazil). Lycopene extracts from each tomato-based product were obtained by conventional solvent extraction using organic solvent (absolute ethanol). A 500 g sample was weighed into a 3-L glass tube with a glass filter bottom (50 mm × 1500 mm), and lycopene was extracted for 40 min with 1 L of absolute ethanol in an ultrasonic bath. Subsequently, the solvent was removed using a vacuum evaporator (Marconi®) at 60 °C for 6 h and the material was weighed. After those steps, the lycopene extracts obtained from each tomato-based product were frozen at -78 °C for 24 h. Using a freeze drier (Terroni LD 3000) those extracts described above were submitted to freeze-drying, 20 h, at less than 200 µmHg. The material obtained from this process had a "flour" texture, and it was stored in amber bottles, at -18 °C, until the test procedures with the cell lines [40,41]. The extraction, separation and identification of lycopene were performed using a HPLC (Figure S2).

1.3. High Performance Liquid chromatography (HPLC) Analysis

Profiles of the carotenoids were determined by HPLC (Waters 2695—Alliance Model, Milford, MA, USA) controlled by the Empower software program with the column oven at 33 °C and photodiode array detector (DAD 996-Waters®). Carotenoid separation was obtained in a C30 column (S-3 Carotenoid, 4.6 mm × 250 mm, YCMTM) purchased from Waters. The mobile phase HPLC grade solvents were purchased from Tedia (Rio de Janeiro, RJ, Brazil) and consisted of 8:2 (methanol:t-butyl methyl ether, v:v). The flow rate was 0.8 mL/min and the injection volume samples was 15 µL. Analysis running time was 28 min. All analyses were performed in triplicate. Carotenoids were identified based on their retention times and ultraviolet-visible (UV/Vis) absorption spectra, compared to the retention times of the carotenoid standards. The carotenoid standards were obtained from Sigma-Aldrich. The identification of all-trans lycopene and cis isomers was done by elution order and UV-vis spectra; they were compared to the published data [40,41] (Figure S1). All the solvents and chemicals were purchased from Sigma and Merck. Lycopene standard (90% all-E-lycopene) were obtained from Sigma Aldrich (USA).

1.4. Cell Culture Experiments

Cell lines were obtained from the Rio de Janeiro Cell Bank (BCRJ), which certified their identity and quality (Inmetro, Rio de Janeiro, RJ, Brazil). The BCRJ ensures that all cell cultures undergo microbiological analyzes and tests for mycoplasma detection before being sent to the customers. Prostate cancer cell lines DU-145 (BCRJ code: 0078) and PC-3 (BCRJ code: 0269) were grown in 25 cm²

cell culture flasks at a density of 4.0×10^6 cells/flask and in a 37 °C humidified incubator with 5% carbon dioxide (CO₂) (Thermo Scientific CO₂ Incubator). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4. Cells were handled in safe conditions that meet sterilization procedures standards, using a biosafety cabinet to its manipulation. The cabinet was irradiated for 30 min prior to use and all the surfaces were sterilized using EtOH 70%. Cells were passaged by trypsinization when they reached 70–80% confluence, about twice a week. The experiments have been started after the sixth passage. For each experiment, all cells were plated at a density of 10^4 cells/cm² in 6 and 96-multiwell plates for cell cycle and cell proliferation analyses, respectively. Lycopene extracts were dissolved in water at 50 °C within a range from 500 to 5000 µg/mL and depending on the lycopene concentration of each product, the extract was normalized to in all extracts the concentration of lycopene in the stock solution having the same concentration of lycopene between the different extracts. Lycopene extracts from the different tomato products were then added to the plates. Different plates were used for each lycopene extract and experimental cells were included on the same plate as control cells. Cells were then incubated for 96 h with daily medium replacement.

1.1. Cell Viability Assay

The anticancer activity of extracts on DU-145 and PC-3 cells were determined by the MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used to assess the cytotoxicity. Cells (1×10^4 /well) were plated in 0.2 ml of medium/well in 96-well plates for 24 h. For MTT assay the medium from the wells was removed carefully after incubation. After treatment with lycopene extracts from the different tomato-based food products and incubation for 96 h (six wells for each sample), 20 µL of MTT (5 g/L) were added to each well. After incubation, the medium was removed and 100 µL/well sodium dodecyl sulfate (SDS). Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. The suspension was transferred to enzyme-linked immunosorbent microplate reader (Bio-Rad 2550) at 490 nm. Measurements were performed and cellular viability inhibition rate (CVIR) was calculated using the following formula: $CVIR = (1 - \text{average absorbance value of experimental group} / \text{average absorbance value of control group}) \times 100\%$

1.2. Cell Cycle Analysis

Briefly, cells were washed with calcium and magnesium-free phosphate-buffered saline (PBS) and detached from cell culture flasks with trypsin at 37 °C. The cells were washed twice with PBS, and 1×10^6 cells were resuspended in 1.0 mL of ice-cold VindeLov solution containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNase, and 50 µg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO, USA). Flow cytometry was used to determine the effect of the extracts on the cell cycle using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The relative proportions of cells with diploid DNA content were acquired and analyzed using CellQuest and WinMDI 2.9 software, respectively. Results are expressed as percentage of cells in each phase of cell cycle determined with EXPO32 V1.2 analysis software (Beckman Coulter, Inc., Brea, CA, USA). The cell dissociation procedure did not affect fluorescence under the experimental conditions that were used in this study.

1.3. Apoptosis Assay

DU-145 and PC-3 prostate cancer cell lines were treated with lycopene extracts, at concentrations of 500 and 5000 µg/mL, in 6-well plates. After 96 h of incubation, the non-adherent cells were collected, and adherent cells were quickly washed with PBS and detached with trypsin/EDTA(ethylenediamine tetraacetic acid) 0.125% (Sigma Chemical Co Saint Louis, MO, USA.) at room temperature. Cells were centrifuged to remove the medium, washed with PBS and stained with Annexin V-FITC and PI in binding buffer (BD Pharmingen) according to the manufacturer's instructions. Stained cells were analyzed using a FACSCalibur (BD Bioscience, NJ, USA) and

analyzed using WinMDI 2.9 software. Data were reported as the percentage of apoptosis, obtained by determining the numbers of apoptotic cells versus the total numbers of cells.

1.1. Statistical Analysis

Results were expressed as mean $\pm$ SD for a given number of three independent experiments done in duplicate. Data were analyzed by using one way analysis of variance (ANOVA) followed by the Tukey test using the Graph Pad Prism 5.0 and Statistical 6.0 program (company, city, country (version 5.04, GraphPad Software, San Diego, CA, USA). Statistical differences were considered significant when the value was $p < 0.05$.

2. Results and Discussion

2.1. HPLC Analysis

Table 1 summarizes the data concerning, total carotenoid content, cis-lycopene and all-trans-lycopene isomers from the analyzed tomato-based food products. The mean lycopene content was 96.65% in tomato sauce, 96.48% in ketchup, 95.12% in tomato extract, and 97.78% in tomato paste. However, the lycopene content of tomato sauce was not statistically different from that of ketchup and tomato extract. Among the samples analyzed, ketchup displayed a higher content of cis-lycopene (9.20 $\mu\text{g/g}$). These values are similar to those reported by Barber and Barber [42], as well as Waliszewski and Blasco [43].

Table 1. Total carotenoid content, cis-lycopene, and all-trans-lycopene isomers from the analyzed tomato-based food products.

Sample	Total Carotenoids ($\mu\text{g/g}$)	Cis-Lycopene ($\mu\text{g/g}$)	Trans-Lycopene ($\mu\text{g/g}$)	Lycopene Content (%)
Ketchup	147.81 $\pm$ 8.35 ^a	9.20 $\pm$ 0.89 ^a	133.39 $\pm$ 6.64 ^a	96.48 $\pm$ 0.36 ^a
Tomato Extract	85.60 $\pm$ 1.09 ^b	6.48 $\pm$ 0.88 ^b	74.94 $\pm$ 1.73 ^b	95.12 $\pm$ 0.22 ^b
Tomato Sauce	168.95 $\pm$ 5.36 ^c	7.40 $\pm$ 0.49 ^b	155.94 $\pm$ 7.45 ^c	96.65 $\pm$ 1.63 ^{a,b,c}
Tomato Paste	77.57 $\pm$ 1.81 ^d	5.05 $\pm$ 0.40 ^b	70.80 $\pm$ 2.09 ^b	97.78 $\pm$ 0.10 ^c

Lycopene content (%) was measured through the following formula: (cis-lycopene + trans-lycopene) $\times$ 100/total carotenoids. The data are expressed as mean $\pm$ SD. Variation in the letters between samples indicates significant difference ($p < 0.05$).

Cis-lycopene-rich tomato sauce has higher bioavailability than trans-lycopene-rich tomato sauce in healthy adult subjects. Cis-isomers of lycopene are produced during processing and cooking of tomato products [44,45]. It is conceivable that all-trans-lycopene, a long linear molecule, may be less soluble in bile acid micelles. Lycopene in fresh tomatoes occurs mostly in the trans-form. In contrast, cis-isomers of lycopene may move more efficiently across plasma membranes and preferentially incorporate into chylomicrons [46]. However, is still unclear data for metabolism, biotransformation, distribution, and biological relevance of the cis-isomers of carotenoids in human tissues. Differential absorption, transport, and uptake of specific stereoisomers can also be hypothesized [47,48]. Further endeavors to explain the structures of geometric lycopene isomers and biological mechanisms in the prostate may prompt the advancement of novel chemopreventive agents.

2.2. Effect of Lycopene Extracts on the Number of Viable Cells in Culture

Our study provides evidence that lycopene in tomato products may inhibit the growth of human PCa cells. The human prostate contains lycopene and other dietary carotenoids, supporting the hypothesis that tomato-derived carotenoids may directly impact the prostate. Prostate cancer cell lines (PC-3 and DU-15) were derived from distant metastases of. Accordingly, they have gone through the epithelial-mesenchymal progress and are expected to be different, both from primary prostate cancer and from each other, since each established cancer line passes through extensive

selection both in vivo and in subsequent culture in vitro. DU-145 cells displayed a higher inhibition of proliferation in elevated levels of lycopene compared to the PC-3 cell line.

Both cell lines showed the normal growth characteristics expected under standard in vitro conditions. The plating of cancer cell lines was followed by a 24 h recovery period, and cells were subsequently incubated with 500, 1000, 2500, and 5000 $\mu\text{g/mL}$ of lycopene extracts for 24, 48, 72, and 96 h. Using the MTT assay, we observed a decrease in cell viability in both the cancer cell lines after treatment with all extracts of tomato-based food products. Even after only 24 h of treatment, lycopene promoted an average inhibition of 35% for DU-145 cells, which increased to 55% after 96 h of treatment for all tomato-based food products (Figure 1). Lycopene treatment inhibited viability of PC-3 cells by approximately 40%, after 96 h (Figure 2). A potent inhibitory effect on PC-3 cell viability was observed using lycopene extracted from tomato paste, and the highest reductions in cell viability were achieved even with 24 h of incubation. No statistically significant differences were observed between the effect of lycopene content from tomato-based food products after 96 h of treatment in both the cell lines.

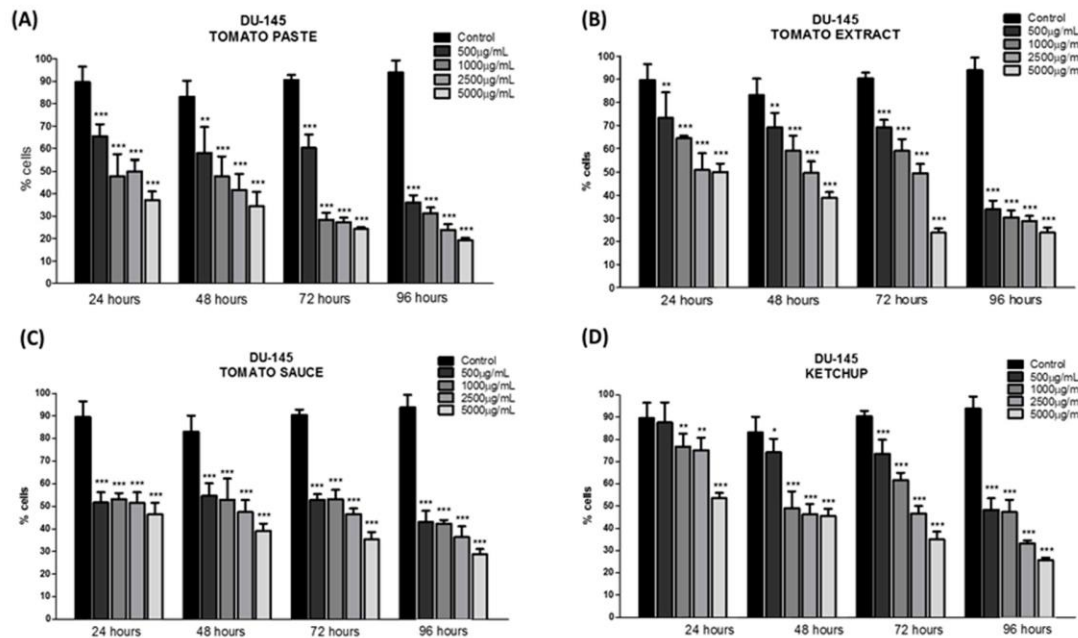


Figure 1. Effect of lycopene obtained of tomato paste (A), tomato extract (B), tomato sauce (C), and ketchup (D) on DU-145 cell viability after 24, 48, 72, and 96 h of exposure, respectively. The results are expressed as mean $\pm$ error standard and significant differences between untreated cells (Control) and those treated with lycopene (500, 1000, 2500, and 5000 $\mu\text{g/mL}$) were compared by one-way ANOVA followed by Tukey's multiple comparison post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

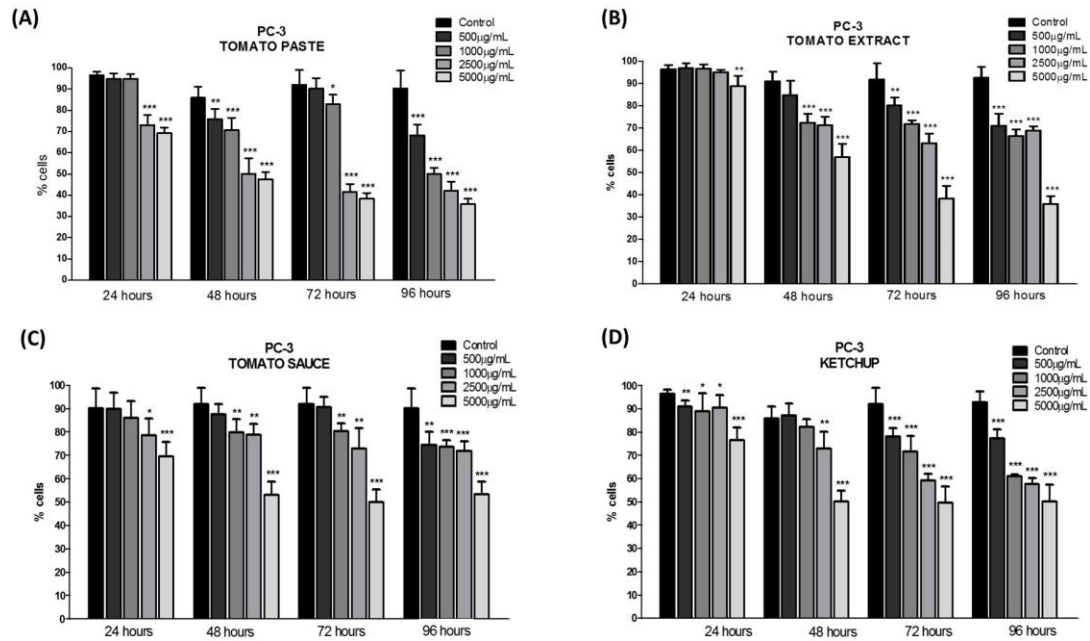


Figure 2. Effect of lycopene obtained of the tomato paste (A), tomato extract (B), tomato sauce (C), and ketchup (D) on DU-145 cell viability after 24, 48, 72, and 96 h of exposure, respectively. The results are expressed as mean $\pm$ error standard and significant differences between untreated cells (Control) and those treated with lycopene (500, 1000, 2500, and 5000 $\mu\text{g/mL}$) were compared by one-way ANOVA followed by Tukey's multiple comparison post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DU-145 cells, which are moderately aggressive, exhibited a greater inhibition of proliferation at high levels of lycopene compared to PC-3 cells, which are highly aggressive. The lycopene effect was found to be time-dependent, because this effect required a relatively long incubation time to achieve improved action.

1.1. Effect of Lycopene Extracts on Cell-Cycle Progression

We treated cells with lycopene for 96 h and quantified the percentage of cells in the different cell-cycle phases to elucidate the mechanism by which lycopene regulated cell growth. Flow cytometry analysis of cell cycle revealed that lycopene extracted from tomato paste (5000 $\mu\text{g/mL}$) decreased the percentage of DU-145 cells in G_0/G_1 and G_2/M phases (Figure 3A). However, lycopene extracted from tomato sauce and tomato extract (Figure 3B,C) decreased the percentage of cells in G_0/G_1 phase and increased in S and G_2/M phases (5000 $\mu\text{g/mL}$) after 96 h of treatment. In addition, lycopene extracted from ketchup (500 $\mu\text{g/mL}$) increased the percentage of cells in G_0/G_1 and G_2/M phases (Figure 3D). No changes were observed when using 5000 $\mu\text{g/mL}$ lycopene extracted from ketchup. In PC-3 cells, a significant reduction in the percentage of cells in the G_0/G_1 and G_2/M phases was achieved with lycopene extracted from tomato paste and tomato extract, when used at higher concentration (Figure 4A,B). The effect of lycopene extracted from tomato sauce and ketchup promoted an accumulation of PC-3 cells in the G_2/M phase (Figure 4C,D).

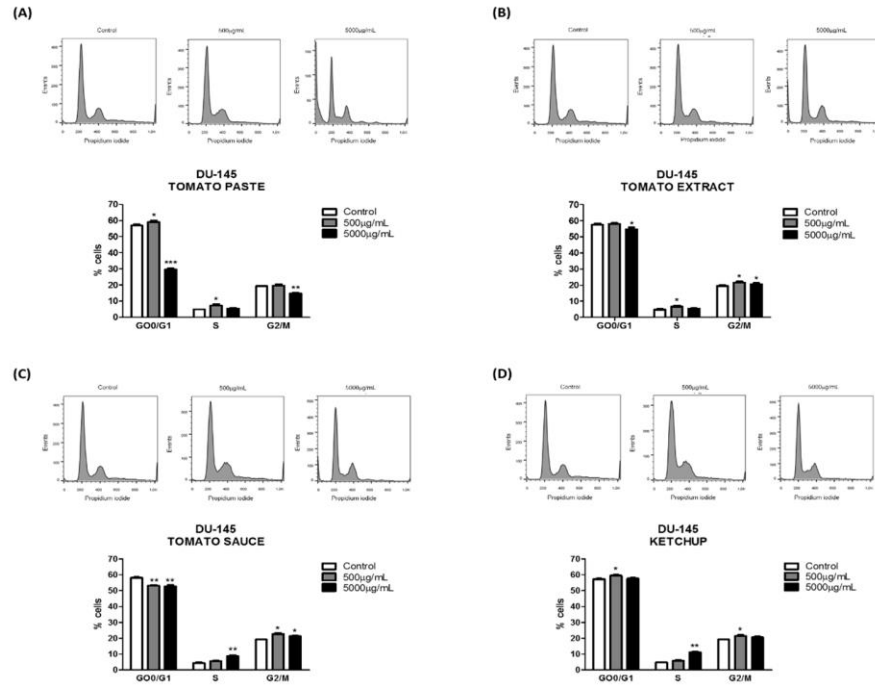


Figure 3. Effect of lycopene obtained from tomato paste (A), tomato extract (B), tomato sauce (C), and ketchup (D) on DU-145 cell cycle after 96 h of exposure, respectively. The results are expressed as mean $\pm$ error standard and significant differences between untreated cells (Control) and those treated with lycopene (500 and 5000 $\mu\text{g/mL}$) were compared by 1-way ANOVA followed by Tukey's multiple comparison post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

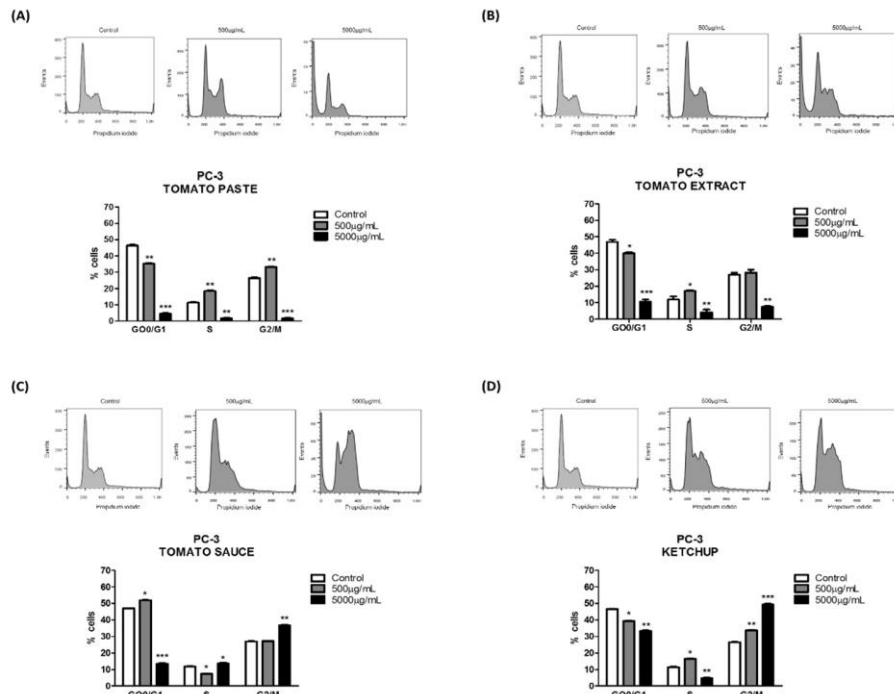


Figure 4. Effect of lycopene obtained of tomato paste (A), tomato extract (B), tomato sauce (C), and ketchup (D) on PC-3 cell cycle after 96 h of exposure. The results are expressed as mean $\pm$ error standard and significant differences between untreated cells (Control) and those treated with lycopene (500 and 5000 $\mu\text{g/mL}$) were compared by 1-way ANOVA followed by Tukey's multiple comparison post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In both cell lines, the cell cycle analysis showed that lycopene reduced the percentage of cells in G₀/G₁ and G₂/M phases after 96 h of treatment in the metastatic PCa cell lines, using lycopene extracted from tomato paste and tomato extract. Although the arrest of cells in G₀/G₁ phase can be reverted, and cells can proceed with proliferation after interruption of the treatment, G₂/M arrest potentially leads to apoptosis. However, lycopene extracted from tomato sauce and ketchup decreased the percentage of cells in G₀/G₁ phase and increased the percentage of cells in S and G₂/M phases after 96 h of treatment. The cell cycle arrest in G₂/M phase is vital, because it leads to apoptosis when cells cannot recover and proceed to cell division.

Furthermore, considering that lycopene extracted from all tomato-based food products interfered with cell cycle and cell viability, it was important to investigate whether these products disturbed apoptosis during the *in vitro* treatment. DU-145 and PC-3 cells displayed a significant increase in apoptosis, suggesting that another mechanism may be involved. According to Renju et al. [49], exposure of PC-3 and DU-145 cell lines to lycopene isolated from *Chlorella marina* at a dose of 20 and 50 µM significantly inhibited cell growth, and apoptosis was strongly induced at 50 µM, demonstrating the anti-proliferative and apoptotic effects of lycopene. Cell-cycle deregulation is an important step in cancer development [49]. Previous studies reported that lycopene induced cell cycle arrest in G₁/S phases, which was mediated by the downregulation of cyclins E and D1, and/or by the upregulation of cyclin A and p27 [50].

1.1. Apoptosis

It has already been elucidated that apoptosis may modulate the malignant phenotype, and studies have uncovered that a high recurrence of apoptosis was seen in unexpectedly relapsing tumors and in tumors treated with cytotoxic anticancer agents [51]. Hence, many studies have been led to demonstrate the impact of tomato-based products on inducing programmed cell death [52,53].

Apoptosis induction was reported by annexin V and PI biomarkers. Altering the balance between proliferation and apoptosis is associated with cancer, and quantification of apoptosis can be a useful measure of cancer cell kinetics. We evaluated the effect of lycopene from all tomato-based products after 96 h of incubation on different stages of the cell death process of DU-145 and PC-3 cells. Table 2 shows the percentage of viable, early apoptotic, late apoptotic, and necrotic cells treated with lycopene extracts from tomato paste, tomato extract, tomato sauce, and ketchup (500 and 5000 µg/mL). Figure 5 shows the influence of lycopene extracts from tomato-based products on the rate of apoptosis.

In DU-145 cells, after 96 h of incubation, tomato paste extract showed an increase upto 34.1 times (5000 µg/mL) in the population of apoptotic cells (early and late apoptosis) compared to control (Figure 5A). Ketchup extract promoted a lower effect from all the tomato-based product extracts analyzed, achieving an increase up to 8.6 times in apoptotic cells, with the highest lycopene concentration (5000 µg/mL). In PC-3 cells (Figure 5B), no statistical difference was observed between lycopene extracts from tomato paste, tomato extract, and tomato sauce at higher treatment concentrations. A potent effect was achieved with a relative increase rate of 58.9 times in the population of apoptotic cells (early and late apoptosis). However, for this cell line, ketchup extract also produced a lower effect compared to all the tomato-based products analyzed, achieving an increase of up to 5.5 times in apoptotic cells.

Increase in early and late apoptotic cells was observed in DU-145 and PC-3 cells treated with extracts from all tomato-based products and both concentrations of lycopene, but the most notable effect was produced by tomato paste extract (Table 2). Despite the unknown comparative bioavailability values for lycopene from various tomato products, lycopene from processed tomato products appears to be more bioavailable than raw tomatoes. The release of lycopene from the food matrix due to processing, the presence of dietary lipids and heat-induced isomerization from all-trans to cis enhance the bioavailability of lycopene [54].

Table 2. Effect of tomato-based products on different cell death stages in human prostate cancer cell lines (DU-145 and PC-3) after 96 h.

Cell Type	Cell death stages	Untreated Cells (Control)	Compounds							
			Tomato Paste		Tomato Extract		Tomato Sauce		Ketchup	
			500 µg/mL	5000 µg/mL	500 µg/mL	5000 µg/mL	500 µg/mL	5000 µg/mL	500 µg/mL	5000 µg/mL
DU-145	Viable cells	93.33 ± 1.23 ^a	89.10 ± 2.71 ^b	64.30 ± 1.56 ^c	89.36 ± 1.48 ^b	70.93 ± 2.02 ^d	88.45 ± 1.48 ^b	78.45 ± 1.20 ^c	91.32 ± 1.24 ^a	89.80 ± 0.32 ^b
	Early apoptosis	0.49 ± 0.12 ^a	2.32 ± 0.53 ^b	13.20 ± 1.70 ^c	2.12 ± 0.29 ^b	8.71 ± 0.40 ^d	3.56 ± 0.56 ^b	6.65 ± 0.35 ^e	1.14 ± 0.28 ^f	2.90 ± 0.30 ^b
	Late apoptosis	0.57 ± 0.14 ^a	4.35 ± 1.09 ^b	21.05 ± 0.64 ^c	4.82 ± 0.54 ^b	19.00 ± 0.85 ^c	3.50 ± 0.34 ^b	14.25 ± 0.64 ^d	3.22 ± 0.46 ^b	5.90 ± 0.25 ^e
	Necrosis	5.61 ± 0.97 ^a	4.23 ± 1.06 ^b	1.45 ± 0.49 ^b	3.70 ± 0.64 ^c	1.36 ± 0.38 ^b	4.49 ± 0.59 ^a	0.65 ± 0.21 ^d	4.32 ± 0.50 ^a	1.40 ± 0.27 ^b
PC-3	Viable cells	97.64 ± 0.08 ^a	90.26 ± 0.23 ^b	37.37 ± 0.04 ^c	87.67 ± 0.89 ^b	40.05 ± 0.49 ^d	93.39 ± 0.04 ^b	42.78 ± 0.37 ^c	93.73 ± 0.37 ^b	94.14 ± 0.18 ^b
	Early apoptosis	0.84 ± 0.07 ^a	1.87 ± 0.15 ^b	21.53 ± 0.33 ^c	3.07 ± 0.22 ^d	16.35 ± 0.35 ^e	1.87 ± 0.20 ^b	18.35 ± 0.35 ^e	1.64 ± 0.28 ^b	2.86 ± 0.29 ^d
	Late apoptosis	0.82 ± 0.07 ^a	5.54 ± 0.35 ^b	39.00 ± 0.57 ^c	8.63 ± 0.48 ^d	42.52 ± 0.35 ^c	2.55 ± 0.49 ^e	38.15 ± 0.64 ^c	3.58 ± 0.49 ^e	2.36 ± 0.32 ^e
	Necrosis	0.7 ± 0.06 ^a	2.33 ± 0.39 ^b	2.10 ± 0.28 ^b	0.63 ± 0.19 ^a	1.08 ± 0.21 ^c	2.19 ± 0.25 ^b	0.72 ± 0.21 ^a	1.05 ± 0.16 ^a	0.64 ± 0.13 ^a

Legend: the cell-cycle phases and quantitative results are illustrated in accordance with the exposure time and carotenoid concentration. The experiment is expressed as mean ± standard error. Small different letters indicate significant differences ($p < 0.05$) within same line among different concentrations versus control group.

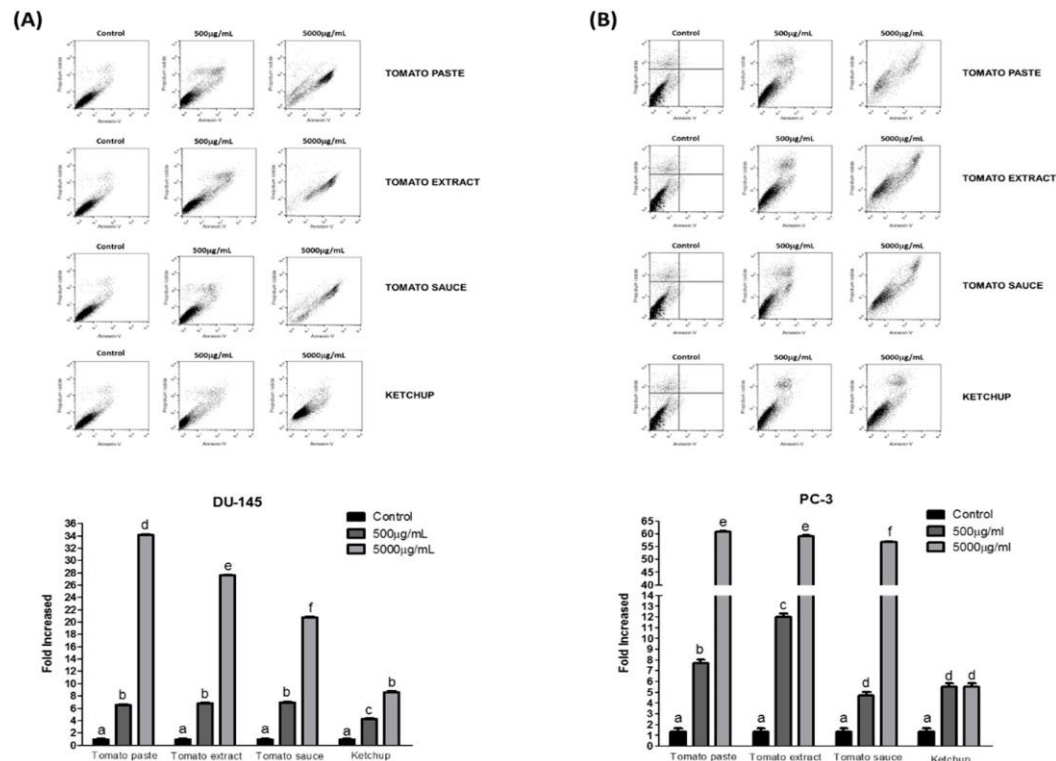


Figure 5. The effect of lycopene obtained from tomato paste, tomato extract, tomato sauce and ketchup in the process of programmed death in DU-145 (A) and PC-3 (B) cells the after 96 h treatment. The flow cytometric analyses are shown according to the exposure time and carotenoid concentration. The quantitative results of lycopene on cell lines are shown after 96 h. The data are expressed as mean $\pm$ standard error. Variation in the letters between samples indicates significance difference ($p < 0.05$).

Recognizing that current evidence of dietary intake and blood concentrations of lycopene reflects consumption of tomatoes and tomato products rather than purified supplements of lycopene is critical. The pharmacokinetic properties of lycopene remain poorly understood. Further research on this potentially important carotenoid's bioavailability, pharmacology, and biology is clearly warranted. Until more definitive information is available on the specific benefits of purified forms of lycopene, current recommendations should highlight the health benefits of diets rich in a variety of fruits and vegetables, including tomatoes and tomato-based products[55,56].

1. Conclusions

Our results may contribute to a better understanding of the potential role of tomato-based products, which are a readily available source of lycopene. The products displayed potent anti-carcinogenic effects against PC-3 and DU-145 cells. Lycopene obtained from tomato paste and extract decreased the percentage of PCa cells in G₀/G₁ and G₂/M phases after 96 h of treatment. However, tomato sauce and ketchup extract decreased the percentage of cells in G₀/G₁ phase and increased percentage of cells in S and G₂/M phases after 96 h of treatment. Lycopene also increased apoptosis in both PCa cell lines. These data show that tomato lycopene inhibits cell proliferation, arrests cell cycle in different phases, and increases apoptosis in human PCa cell lines. Thus, tomato lycopene may constitute the basis of new therapeutic strategies for the treatment of prostate malignancy.

In conclusion, the present study supports the proposal that all-trans-lycopene and lycopene extracted from tomato-based food products may have a protective effect on PCa. These findings add further support to current dietary recommendations to increase consumption of food sources of lycopene to reduce prostate cancer risk.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: HPLC analysis of lycopene standard and UV spectrum of all-trans-lycopene (**A**) and cis-lycopene (**B**); Figure S2. HPLC analysis of tomato extract (**A**), tomato paste (**B**), tomato sauce (**C**), and ketchup (**D**).

Author Contributions: Conceptualization, N.d.C.P.S., C.L.M., A.J.T., and R.B.; methodology, N.d.C.P.S., M.d.B.E., B.B.T., A.J.T., and R.B.; data curation, N.C.P.S., A.J.T., and R.B.; writing—original draft preparation, N.d.C.P.S., C.L.M., B.B.T., A.J.T., and R.B.; writing—review and editing, N.d.C.P.S., M.d.B.E., and A.J.T., R.B.

Funding: This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

Acknowledgments: This research received financial support from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro).

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **2015**, *136*, E359–E386, doi:10.1002/ijc.29210.
2. Lin, P.; Aronson, W. and Freedland, S.J. Nutrition, dietary interventions and prostate cancer: The latest evidence. *BMC Med.* **2015**, *13*, 3.
3. Askari, F.; Parizi, M.K.; Jessri, M.; Rashidkhani, B. Fruit and vegetable intake in relation to prostate cancer in Iranian men: A case-control study. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 5223–5227, doi:10.7314/APJCP.2014.15.13.5223.
4. Liu, B.; Mao, Q.; Cao, M.; Xie, L. Cruciferous vegetables intake and risk of prostate cancer: A meta-analysis. *Int. J. Urol.* **2012**, *19*, 134–141, doi:10.1111/j.1442-2042.2011.02906.x.
5. Richman, E.L.; Carroll, P.R.; Chan, J.M. Vegetable and fruit intake after diagnosis and risk of prostate cancer progression. *Int. J. Cancer* **2012**, *131*, 201–210, doi:10.1002/ijc.26348.
6. Kavanaugh, C.J.; Trumbo, P.R.; Ellwood, K.C. The U.S. Food and Drug Administration's evidence-based review for qualified health claims: Tomatoes, lycopene, and cancer. *J. Natl. Cancer Inst.* **2007**, *99*, 1074–1085, doi:10.1093/jnci/djm037.
7. Heber, D.; Lu, Q.-Y. Overview of mechanism of action of lycopene. *Exp. Biol. Med.* **2002**, *227*, 920–923, doi:10.1177/153537020222701013.
8. Vaishampayan, U.; Hussain, M.; Banerjee, M.; Seren, S.; Sarkar, F.H.; Fontana, J.; Forman, J.D.; Cher, M.L.; Powell, I.; Pontes, J.E.; et al. Lycopene and Soy Isoflavones in the Treatment of Prostate Cancer. *Nutr. Cancer* **2007**, *59*, 1–7, doi:10.1080/01635580701413934.
9. Mozos, I.; Stoian, D.; Caraba, A.; Malainer, C.; Horbańczuk, J.O.; Atanasov, A.G. Lycopene and Vascular Health. *Front. Pharmacol.* **2018**, *9*, 521, doi:10.3389/fphar.2018.00521.
10. Fielding, J.M.; Rowley, K.G.; Cooper, P.; O'Dea, K. Increases in plasma lycopene concentration after consumption of tomatoes cooked with olive oil. *Asia Pac. J. Clin. Nutr.* **2005**, *14*, 131–136.
11. Vallverdú-Queralt, A.; Regueiro, J.; de Alvarenga, J.F.; Torrado, X.; Lamuela-Raventos, R.M. Carotenoid profile of tomato sauces: Effect of cooking time and content of extra virgin olive oil. *Int. J. Mol. Sci.* **2015**, *16*, 9588–9599, doi:10.3390/ijms16059588.
12. Holzapfel, N.P.; Holzapfel, B.M.; Champ, S.; Feldthusen, J.; Clements, J.; Huttmacher, D.W. The Potential Role of Lycopene for the Prevention and Therapy of Prostate Cancer: From Molecular Mechanisms to Clinical Evidence. *Int. J. Mol. Sci.* **2013**, *14*, 14620–14646.
13. Toma, R.B.; Frank, G.C.; Nakayama, K.; Tawfik, E. Lycopene content in raw tomato varieties and tomato products. *J. Foodserv.* **2008**, *19*, 127–132.
14. Olajire, A.A.; Ibrahim, A.O.; Adelowo-Imeokparia, F.E.; Abdul-Hammed, M. and Pak, J. Lycopene in tomato and tomato-based products: Levels and their contribution to dietary lycopene. *Sci. Ind. Res.* **2007**, *50*, 18–21.
15. Tan, H.L.; Thomas-Ahner, J.M.; Grainger, E.M.; Wan, L.; Francis, D.M.; Schwartz, S.J.; Erdman, J.W., Jr.; Clinton, S.K. Tomato-based food products for prostate cancer prevention: What have we learned? *Cancer Metastasis Rev.* **2010**, *29*, 553–568, doi:10.1007/s10555-010-9246-z.

1. Gartner, C.; Stahl, W.; Sies, L. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. *Am. J. Clin. Nutr.* **1997**, *66*, 116–122.
2. Giovannucci, E.; Rimm, E.B.; Liu, Y.; Stampfer, M.J.; Willett, W.C. A prospective study of tomato products, lycopene, and prostate cancer risk. *J. Natl. Cancer Inst.* **2002**, *94*, 391–398.
3. Hori, S.; Butler, E.; McLoughlin, J. Prostate cancer and diet: Food for thought? *BJU Int.* **2011**, *107*, 1348–1359, doi:10.1111/j.1464-410X.2010.09897.x.
4. Chen, P.; Zhang, W.; Wang, X.; Zhao, K.; Singh Negi, D.; Zhuo, L.; Qi, M.; Wang, X.; Zhang, X. Lycopene and Risk of Prostate Cancer. A Systematic Review and Meta-Analysis. *Medicine* **2015**, *94*, E1260, doi:10.1097/MD.0000000000001260.
5. Venier, N.A.; Colquhoun, A.J.; Fleshner, N.E.; Klotz, L.H.; Venkateswaran, V. Lycopene enhances the anti-proliferative and pro-apoptotic effects of capsaicin in prostate cancer in vitro. *J. Cancer Ther. Res.* **2012**, doi:10.7243/2049-7962-1-30.
6. Lian, F.; Smith, D.E.; Ernst, H.; Russell, R.M.; Wang, X.D. Apo-10'-lycopenoic acid inhibits lung cancer cell growth in vitro, and suppresses lung tumorigenesis in the A/J mouse model in vivo. *Carcinogenesis* **2007**, *28*, 1567–1574, doi:10.1093/carcin/bgm076.
7. Ford, N.A.; Elsen, A.C.; Zuniga, K.; Lindshield, B.L.; Erdman, J.W., Jr. Lycopene and apo-12'-lycopenal reduce cell proliferation and alter cell cycle progression in human prostate cancer cells. *Nutr. Cancer* **2011**, *63*, 256–263, doi:10.1080/01635581.2011.523494.
8. Nahum, A.; Hirsch, K.; Danilenko, M.; Watts, C.K.; Prall, O.W.; Levy, J.; Sharoni, Y. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27(Kip1) in the cyclin E-cdk2 complexes. *Oncogene* **2001**, *20*, 3428–3436, doi:10.1038/sj.onc.1204452.
9. Kristal, A.R.; Till, C.; Platz, E.A.; Song, X.; King, I.B.; Neuhauser, M.L.; Ambrosone, C.B.; Thompson IM: Serum lycopene concentration and prostate cancer risk: Results from the Prostate Cancer Prevention Trial. *Cancer Epidemiol. Biomark. Prev.* **2011**, *20*, 638–646.
10. Campbell, J.K.; Stroud, C.K.; Nakamura, M.T.; Lila, M.A.; Erdman, J.W., Jr. Serum testosterone is reduced following short-term phytofluene, lycopene, or tomato powder consumption in F344 rats. *J. Nutr.* **2006**, *136*, 2813–2819, doi:10.1093/jn/136.11.2813.
11. Sharoni, Y.; Linnewiel-Hermoni, K.; Zango, G.; Khanin, M.; Salman, H.; Veprik, A.; Danilenko, M.; Levy, J. The role of lycopene and its derivatives in the regulation of transcription systems: Implications for cancer prevention. *Am. J. Clin. Nutr.* **2011**, *96*, 1173S–1178S, doi:10.3945/ajcn.112.034645.
12. Zhang, J.; Dhakal, I.; Stone, A.; Ning, B.; Greene, G.; Lang, N.P.; Kadlubar, F.F. Plasma carotenoids and prostate cancer: A population-based case-control study in Arkansas. *Nutr. Cancer* **2007**, *59*, 46–53, doi:10.1080/01635580701385900.
13. Zu, K.; Mucci, L.; Rosner, B.A.; Clinton, S.K.; Loda, M.; Stampfer, M.J.; Giovannucci, E. Dietary lycopene, angiogenesis, and prostate cancer: A prospective study in the prostate-specific antigen era. *J. Natl. Cancer Inst.* **2014**, *106*, djt430, doi:10.1093/jnci/djt430.
14. Chen, L.; Stacewicz-Sapuntzakis, M.; Duncan, C.; Sharifi, R.; Ghosh, L.; van Breemen, R.; Ashton, D.; Bowen, P.E. Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. *J. Natl. Cancer Inst.* **2001**, *93*, 1872–1879, doi:10.1093/jnci/93.24.1872.
15. Soares, N.C.P.; Teodoro, A.J.; Oliveira, F.L.; Santos, C.A.; Takiya, C.M.; Junior, O.S.; Bianco, M.; Junior, A.P.; Nasciutti, L.E.; Ferreira, L.B.; et al. Influence of lycopene on cell viability, cell cycle, and apoptosis of human prostate cancer and benign hyperplastic cells. *Nutr. Cancer* **2013**, *65*, 1076–1085, doi:10.1080/01635581.2013.812225.
16. Kucuk, O.; Sarkar, F.H.; Djuric, Z.; Sakr, W.; Pollak, M.N.; Khachik, F.; Banerjee, M.; Bertram, J.S.; Wood, D.P., Jr. Effects of lycopene supplementation in patients with localized prostate cancer. *Exp. Biol. Med.* **2002**, *227*, 881–885, doi:10.1177/153537020222701007.
17. Wan, L.; Tan, H.; Thomas-Ahner, J.M.; Pearl, D.K.; Erdman, J.W., Jr.; Moran, N.E.; Clinton, S.K. Dietary tomato and lycopene impact androgen signaling- and carcinogenesis-related gene expression during early TRAMP prostate carcinogenesis. *Cancer Prev. Res.* **2014**, *7*, 1228–1239, doi:10.1158/1940-6207.CAPR-14-0182.
18. Van Breemen, R.B.; Sharifi, R.; Viana, M.; Pajkovic, N.; Zhu, D.; Yuan, L.; Yang, Y.; Bowen, P.E.; Stacewicz-Sapuntzakis, M. Antioxidant effects of lycopene in African American men with prostate cancer or benign prostate hyperplasia: A randomized, controlled trial. *Cancer Prev. Res.* **2011**, *4*, 711–718, doi:10.1158/1940-6207.CAPR-10-0288.

1. Dini, L.I.; Koff, W.J. Perfil do câncer de próstata no hospital de clínicas de Porto Alegre. *Rev. Assoc. Med. Bras.* **2006**, *52*, 28–31, doi:10.1590/S0104-42302006000100018.
2. Mills, P.K.; Beeson, W.L.; Phillips, R.L.; Fraser, G.E. Cohort study of diet, lifestyle, and prostate cancer in Adventist men. *Cancer* **1989**, *64*, 598–604, doi:10.1002/1097-0142(19890801)64:3<598::AID-CNCR2820640306>3.0.CO;2-6.
3. Giovannucci, E.; Ascherio, A.; Rimm, E.B.; Stampfer, M.J.; Colditz, G.A.; Willett, W.C. Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer Inst.* **1995**, *87*, 1767–1776, doi:10.1093/jnci/87.23.1767.
4. Hantz, H.L.; Young, L.F.; Martin, K.R. Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp. Biol. Med.* **2005**, *230*, 171–179, doi:10.1177/153537020523000303.
5. Schmitt, C.A.; Fridman, J.S.; Yang, M.; Baranov, E.; Hoffman, R.M.; Lowe, S.W. Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell* **2002**, 289–298, doi:10.1016/S1535-6108(02)00047-8.
6. Erhardt, J.G.; Meisner, C.; Bode, J.C.; Bode, C. Lycopene, beta-carotene, and colorectal adenomas. *Am. J. Clin. Nutr.* **2003**, *78*, 1219–1224.
7. Nunes, I.L.; Mercadante, A.Z. Obtenção de cristais de licopeno a partir de descarte de tomate. *Ciênc Tecnol Aliment Campinas* **2004**, *24*, 440–447, doi:10.1590/S0101-20612004000300024.
8. Pacheco, S.; Peixoto, F.; Borguini, R.G.; Nascimento, L.S.M.; Bobeda, C.R.R.; Santiago, M.C.P.A.; Godoy, R.L.O. Microscale extraction method for HPLC carotenoid analysis in vegetable matrices. *Sci. Agric.* **2014**, *71*, 416–419, doi:10.1590/0103-9016-2013-0402.
9. Barber, N.J.; Barber, J. Lycopene and prostate cancer. *Prostate Cancer Prostatic Dis.* **2002**, *5*, 6–12, doi:10.1038/sj.pcan.4500560.
10. Waliszewski, K.N.; Blasco, G. Propriedades nutracéuticas del licopeno. *Salud Publica Mex.* **2010**, *52*, 254–265, doi:10.1590/S0036-36342010000300010.
11. Boileau, A.C.; Merchen, N.R.; Wasson, K.; Atkinson, C.A.; Erdman, J.W., Jr. Cis-lycopene is more bioavailable than trans-lycopene in vitro and in vivo in lymph-cannulated ferrets. *J. Nutr.* **1999**, *129*, 1176–1181, doi:10.1093/jn/129.6.1176.
12. Unlu, N.Z.; Bohn, T.; Francis, D.M.; Nagaraja, H.N.; Clinton, S.K.; Schwartz, S.J. Lycopene from heat-induced cis-isomer-rich tomato sauce is more bioavailable than from all-trans-rich tomato sauce in human subjects. *Br. J. Nutr.* **2007**, *98*, 140–146, doi:10.1017/S0007114507685201.
13. Erdman, J.W., Jr. How do nutritional and hormonal status modify the bioavailability, uptake, and distribution of different isomers of lycopene? *J. Nutr.* **2005**, *135*, 2046S–2047S, doi:10.1093/jn/135.8.2046S.
14. Krinsky, N.I.; Russett, M.D.; Handelman, G.J.; Snodderly, D.M. Structural and geometrical isomers of carotenoids in human plasma. *J. Nutr.* **1990**, *120*, 1654–1662, doi:10.1093/jn/120.12.1654.
15. Von Doering, W.; Sotirious-Leventis, C.; Roth, W.R. Thermal interconversion among 15-cis-, 13-cis-, and all-trans-beta-carotene: Kinetics, Arrhenius parameters, thermochemistry, and potential relevance to anticarcinogenicity of all-trans-beta-carotene. *J. Am. Chem. Soc.* **1995**, *117*, 2747–2757, doi:10.1021/ja00115a010. Renju, G.L.; Muraleedhara, K.G.; Bandugula, V.R.
16. Renju, G.L.; Muraleedhara, K.G.; Bandugula, V.R. Effect of lycopene isolated from *Chlorella marina* on proliferation and apoptosis in human prostate cancer cell line PC-3. *Tumour Biol.* **2014**, *35*, 10747–10758, doi:10.1007/s13277-014-2339-5.
17. Prakash, P.; Russel, R.M.; Krinsky, N.I. In vitro inhibition of proliferation of estrogen-dependent and estrogen-independent human breast cancer cells treated with carotenoids or retinoids. *Nutr. Cancer* **2001**, *131*, 1574–1580, doi:10.1093/jn/131.5.1574.
18. Milani, A.; Basirnejad, M.; Shahbazi, S.; Bolhassani, A. Carotenoids: Biochemistry, pharmacology and treatment. *Br. J. Pharmacol.* **2017**, *174*, 1290–1324, doi:10.1111/bph.13625.
19. Hadley, C.W.; Miller, E.C.; Schwartz, S.J.; Clinton, S.K. Tomatoes, lycopene, and prostate cancer: Progress and promise. *Exp. Biol. Med.* **2002**, *227*, 869–880, doi:10.1177/153537020222701006.
20. Story, E.N.; Kopec, R.E.; Schwartz, S.J.; Harris, G.K. An Update on the Health Effects of Tomato Lycopene. *Annu. Rev. Food Sci. Technol.* **2010**, *1*, 189–210, doi:10.1146/annurev.food.102308.124120.
21. Stacewicz-Sapuntzakis, M.; Bowen, P.E. Role of lycopene and tomato products in prostate health. *Biochim. Biophys. Acta* **2005**, *1740*, 202–205, doi:10.1016/j.bbadis.2005.02.004.
22. Bacanlı, M.; Başaran, N.; Başaran, A.A. Lycopene: Is it Beneficial to Human Health as an Antioxidant? *Turk. J. Pharm. Sci.* **2017**, *14*, 311–318, doi:10.4274/tjps.43043.

1. Moran, N.E.; Erdman, J.W., Jr, Clinton, S.K. Complex interactions between dietary and genetic factors impact lycopene metabolism and distribution. *Arch. Biochem. Biophys.* **2013**, *539*, 171–180, doi:10.1016/j.abb.2013.06.017



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).