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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

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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

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Dedico este trabalho à minha querida mãe.

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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

CHAPTER 2
Lycopene inhibits hepatic stellate cell activation and modulates cellular lipid storage and signaling

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

CHAPTER 4

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

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 estímulam a síntese de citocinas pró-inflamatórias e o aumento das espécies reativas de oxigênio, estresse oxidativo e lesão nas células do tecido (Alegría-Ezquerra, 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

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

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Article

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

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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;

1.Introduction

There is a growing worldwide search for healthy food to use adequate health. It is known that a varied, colorful and balanced diet in terms of quantity and quality, the essential nutrients necessary to maintain and promote good health (Price, 2005; Vicentini,

2016; Migliaccio, 2018);

Scientific evidence has shown that in addition to the essential nutrients present in food, we also have effects of its chemical components and physically active substances that act synergistically for disease prevention and health promotion (Hasler, 2000). These bioactive compounds are represented by natural pigments, vitamins and metabolites, minerals, essential fatty acids, fibers and other substances present in nature (Lampe, 2004; Volp et al, 2009).

In this way, there is a visible increase in interest in studying the beneficial effects of natural dyes as bioactive compounds, and their antioxidant, anti-inflammatory properties, protection against oxidative damage to DNA and cellular components, cancer prevention, chronic diseases, among others (Finley, 2005; Van Gils et al, 2005). It is known that the main causes of death in the western world are cancer and cardiovascular diseases (Alwan et al, 2010). Primary liver cancer is the third leading cause of cancer-related deaths worldwide, and hepatocellular carcinoma (HCC) is responsible for 70% to 85% of cases of primary liver cancer (Siegel et al., 2012; Forner et al, 2012). Thus, there is a growing investigation of chemopreventive strategies to reduce the prevalence of this disease or alternatives to enhance treatments after the occurrence of liver cancer, as HCC has a high mortality rate and poor/bad prognosis (Sporn and Liby, 2013; Singh et al, 2014).

In this context, the objective of this mini review was to describe and discuss the role of two carotenoids of great importance: β-caroteno and lycopene and their effects in relation to hepatocarcinogenesis.

1.1. CAROTENOIDS

Carotenoids are a group of phytochemicals that are responsible for different colors of the foods. They are recognized as playing an important role in the prevention of human diseases and maintaining good health (Arscott E Tanumihardjo, 2010). There is scientific evidence in support of the beneficial role of phytochemicals in the prevention of several chronic diseases. Although the chemistry of carotenoids has been studied extensively, their bioavailability, metabolism and biological functions are only now beginning to be investigated (Finley, 2005).

Carotenoids represent a group of natural pigments with great structural diversity, variety of functions and actions and a wide distribution in animal tissues, algae, bacteria, fungi and yeasts (Capecka et al, 2005). Of all 600 carotenoids identified and characterized,

approximately 20% are found in mammalian plasma and tissues and only 10% are found in the human diet (Young, 2018).

In food, the main sources of carotenoids are fruits and other fresh vegetables, and only 14 types are bioavailable. These carotenoids are released from the food matrix and incorporated into the micelles after gastrointestinal digestion, making them available for intestinal absorption (Martins & Masquio, 2019). Subsequently, carotenoids are transported by low density lipoproteins (LDL) in plasma, and used for various biological activities in the body. In humans, the main circulating carotenoids found are β-carotene, lycopene, lutein, β-cryptoxanthin and alpha carotene. The half-life of plasma carotenoids varies from 12 to 33 days for lycopene, 12 days or less for b-carotene, alpha carotene and cryptoxanthin, and can reach 60 days for zeaxanthin and lutein (Xiang. 2012). Structurally, carotenoids in food are formed by tetraterpenes composed of 40 carbon atoms, formed by the union of 8 isoprenoid units (Silva, 2018). Modifications may occur in the basic skeleton of this molecule, due to hydrogenation, cyclization, dehydrogenation, rearrangements, introduction of groups containing oxygen, among others. Even though they are moderately thermostable, these changes cause color loss, decreased biological activity and formation of volatile compounds that generate undesirable effects (Fu et al, 2017; Galasso et al, 2017).

We can identify 2 groups, regarding the chemical structure. The group of carotenes formed by carotenoid hydrocarbons, which have only carbon and hydrogen atoms, such as beta carotene and lycopene, and the group of xanthophylls, oxygenated derivatives (oxycarotenes), which contain oxygen, carbon and hydrogen atoms, such as beta cryptoxanthin (Silva et al., 2010). They may undergo cyclization at one or both ends of the molecule, presenting in an acyclic (eg lycopene), monocyclic (gamma carotene) or bicyclic (alpha and beta carotene) form (Galasso et al, 2017; Quirós E Costa, 2006).

Carotenoids in trans form are more bioavailable and stable, but some physical and chemical factors such as solar or artificial lighting, heat and pro oxidants can modify this molecule (Rao E Rao, 2013). Carotenoids are pigments, efficient antioxidants scavenging singlet molecular oxygen and peroxyl radicals, protection against oxidation of DNA, modification of the metabolism of carcinogens, alteration in the cell cycle of cancer cells, inhibiting proliferation, among others. In the human organism, carotenoids are part of the antioxidant defense system. They interact synergistically with other antioxidants; mixtures of carotenoids are more effective than single compounds (Britton et al,2004; Yang et al, 2009). It has attracted the attention of researchers in several areas of scientific knowledge, due to its beneficial effect on human health, especially, decreasing the incidence of cancer and acting

predominantly as antioxidants (Lintig and Babino, 2020). Among the most widely studied carotenoids, those with the greatest antioxidant activity are lycopene and β-carotene (Dias et al., 2008; Rao E Rao, 2013; Damodaran et al, 2008). Animals and humans do not have the capacity to synthesize carotenoids and depend exclusively on food sources. The main sources of batacotene and lycopene are shown in Table 1.

TABLE 1. Data on the contents of major carotenoids in fruits and vegetables common in the human diet média (μg)

Food	β-Carotene	Lycopene
Pumpkin	23460	n.d
Celery	4875	n.d
Acerola	3810	n.d
Plum	2080	n.d
Chicory	3700	n.d
Carrot	6150	n.d
Banana	1370	n.d
Potato	21820	n.d
Guava	660	5660
Papaya	65	7957
Cashew Appple	79	4
Peach	137	n.d
Parsley	11382	n.d
Tomato	535	3590
Spinach	2230	n.d
Spinach	2230	n.d
Ketchup	486	10240
Tomato Sauce	1005	19150

Table Brasileira of Foof Composition (TBCA). University of São Paulo (USP). Food Research Center (FoRC). Version 7.0. São Paulo, 2019.

Epidemiological studies have suggested that antioxidant vitamins, including beta-carotene, may play an important role with regard to the primary causes of several diseases, including cardiovascular diseases (Jomova and Valko, 2013). This fact would be associated

with the antioxidant activity of carotenoids, which is a consequence of their unique structure (Ribot et al, 2019). B-carotene can react multiple times with peroxyl radicals to form stable molecules (Chen, et al 2002). Additionally, beta-carotene, a hepato-protective effect in animals with liver fibrosis, has been attributed (Seifert, et al, 1996).

The action of carotenoids on cancer cells has been intensively studied in the last two decades, mainly their metabolism and biotransformation (Teodoro et al., 2009). In different experimental models and in clinical practice, these two carotenoids have been widely studied, but most of these studies have been dedicated to β-carotene and only a few to lycopene (Caris-Veyrat et al, 2017). Lycopene has been described as a significant anti-tumor product in different types of cancer cells, mainly regulating cellular events, such as apoptosis and cell cycle (mitosis), which are widely involved in cancer progression (Teodoro et al., 2012). However, it is poorly understood how lycopene or how the synergy of these two carotenoids would regulate the biology of these cells.

1.2. ROLE OF CAROTENOIDS ON LIVER CANCER

The liver is essential for the metabolism of carbohydrates, proteins and lipids, its main digestive function is the synthesis and secretion of bile, responsible for the emulsification of dietary fats. In addition, the liver acts in the storage of vitamins and minerals, in the degradation and excretion of hormones, in the biotransformation and excretion of drugs and in helping the immune response. (Torales et al, 2019; Charbonneau et al, 2007; Peres et al, 2003).The liver tissue is essential not only for control and homeostasis of the organism, but also for metabolic reactions vital to health that can be deregulated in liver injury processes (Schinoni, 2008). The high energetic activity of the liver generates an increased consumption of oxygen, which leads to a high production of free radicals involved in the pathophysiology of inflammatory diseases and potential damage to proteins, lipids and cellular DNA (Stice et al, 2018; Halliwell & Gutteridgr, 2000; Sies et al., 2005; Jaeschke et al., 2000).

Ingestion of high-fat diets, especially saturated fatty acids, directly affects the integrity and function of liver tissue and can be aggravated by alcohol consumption (Alegría-Ezquerra, 2008). These habits may be associated with the development of oxidative stress, inflammatory state and damage to several biomolecules, including DNA, resulting in single or double breaks and also in the appearance of mutations, favoring the appearance and progression of several chronic diseases (Geraldo et al., 2008; Oliveira & Schoffen 2010; Bucchieri et al, 2002).

Excessive consumption of fats, either by the current dietary pattern of the population or by the appeal of cetogenic diets, lowcarb or hight fat diet (Aragon et al, 2017; Seidelmann et al, 2018), generates an increased flow of lipids to the liver promoting a state of organ lipotoxicity , associated with a high level of oxidative stress and reduction of the body's antioxidant defense (Jian-gao & Qiao, 2009). Diets rich in saturated fat stimulate the synthesis of pro-inflammatory cytokines and the increase in reactive oxygen species, oxidative stress and tissue cell damage (France, 2013; Gentile & Pagliassotti, 2008).

In hepatocytes, oxidative stress and lipid peroxidation promote damage to the plasma membrane, making it vulnerable to apoptosis and favoring the inflammatory response in tissue and the body in general, with increased TGF-B1 secretion and activation of myofibroblasts. responsible for scar formation, where chronic injury can trigger fibrosis of the liver (Cave et al., 2007; França, 2013; Bataller, 2005; Kisseleva, 2008).

In this context, epidemiological studies present evidence that hepatocarcinogenesis is a process that involves multiple stages, preceded by the appearance of hepatic nodules, the incidence in men being twice as high as in women (Gonçalves & Pereira, 1993). Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. There is evidence showing that hepatocarcinogenesis, in rats and humans, is a process preceded by the appearance of foci of altered hepatocytes and hyperplastic liver nodules, which are considered pre-neoplastic lesions (LPN) resulting from clonal expansion from hepatocytes initiates (Sherman et al., 1983; Teebor & Becker, 1981; Peres et al, 2003). Some authors have demonstrated the action of vitamin A and carotenoids in the histopathology of HCC and in the protection of the liver due to their action against oxidative stress (Kaklamani et al., 1991; Yu et al, 2000). Previous studies have shown that obesity and some inappropriate habits such as a high-fat diet or excessive alcohol consumption have promoted liver tumorigenesis by inducing chronic inflammation via Interleukin (IL) - 6) / STAT3, with tumors activated by STAT3 being the most aggressive. in humans (Park et al, 2010; Wang et al, 2009; He et al, 2010). The metabolic surplus of excessive calorie consumption can also increase the synthesis of liver enzymes, which creates an excessive demand in the endoplasmic reticulum (ER). This excess demand in the ER leads to the induction of stress-mediated metabolic responses of the ER, which has been associated with the development of liver cancer (Hetz et al, 2012; Fu et al, 2012; Malhi and Kaufman, 2011).

In this context, we can relate the incidence of cancer to environmental factors, especially food, genetic predisposition and obesity, among other factors (Olthof et al, 2000). Issues related to the role of diet in cancer prevention and treatment are highlighted each year,

including results of studies involving various foods, phytochemicals and nutrients; use of complementary and alternative approaches to prevention and treatment; and ideal diets for those who wish to prevent cancer or its recurrence. Currently, great attention has been paid to preventive strategies and, in this context, the use of bioactive compounds present in food seems to contribute to this process through different mechanisms of action, which are anticancer, antioxidants and anti-inflammatory. (Upadhyaya et al, 2007).

1.3. CAROTENOIDS X ANTIOXIDANTS X OXIDATIVE STRESS

The production of reactive oxygen species (ROS) is an integral part of human metabolism and is observed in several physiological conditions. The main ROSs are the superoxide anion, hydrogen peroxide, hydroxyl radical and peroxide radical, these have an important biological function, as in phagocytosis and apoptosis, a phenomenon that eliminates the aggressive agent and in detoxification reactions promoted by cytochrome P-450 (Rufino et al, 2007).

When its production is exacerbated, the organism has an efficient control system to restore balance through antioxidant compounds, which are substances capable of slowing or inhibiting oxidation rates, which can be produced endogenously or absorbed through food in the diet (Barreiros et al., 2006; HalliwelL & Gutteridge, 2007). Mammals have antioxidant defense systems, divided into enzymatic and non-enzymatic systems (McLean et al., 2005; Halliwell & Gutteridge, 2007). Among the enzymes, we highlight the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR) and glutathione transferase (GST). The non-enzymatic system, for example, consists of bilirubin, glutathione, melatonin, ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), carotenoids (vitamin A, β-carotene, lycopene) and polyphenols (flavonoids) (Halliwell & Gutteridge, 2007; Luz et al., 2011; Laires et al., 2001).

This balance between ROS and antioxidants is necessary for the proper functioning of cells and to maintain physiological homeostasis. When there is an imbalance, as the excess of antioxidant can be harmful, because it decreases the levels of ROS inhibiting apoptosis, an important mechanism in the treatment against cancer cells. (Rufino et al, 2007; Sun et al, 2006).

In addition, it can also generate oxidative stress due to the imbalance between oxidizing and antioxidant compounds, with a predominance of oxidants, with excessive generation of free radicals to the detriment of the lesser capacity for their removal by the body (Halliwell & Gutteridge, 2007).

Oxidative stress is related to the activation of carcinogens in the cell initiation phase, this phase is characterized by alteration of the genetic material, as well as in the promotion phase, when the initiated cell is converted into a pre-malignant cell and in the progression phase, where the pre-malignant cell evolves into a malignant cell, and an uncontrolled cell division can occur (Bertram et al, 1987; Rousseau et al, 1992). According to some authors, the role of carotenoids seems to inhibit the development of the stages of carcinogenesis, through the antioxidant mechanism, which prevents oxidative stress, deactivating reactive singlet oxygen molecules (Edge et al, 2018; Halliwell & Gutteridge, 2007) or by direct antimutagenic action acting in the stages of carcinogenesis, preventing mutation, through any of the following mechanisms: regulation of the communicating junctions, modifying cell-cell communication, potentiating the immune response, potentiating the modulation of the expression of the tumor suppressor gene or inhibiting the growth of tumor cell lines (Zhang et al, 2002), Figure 1.

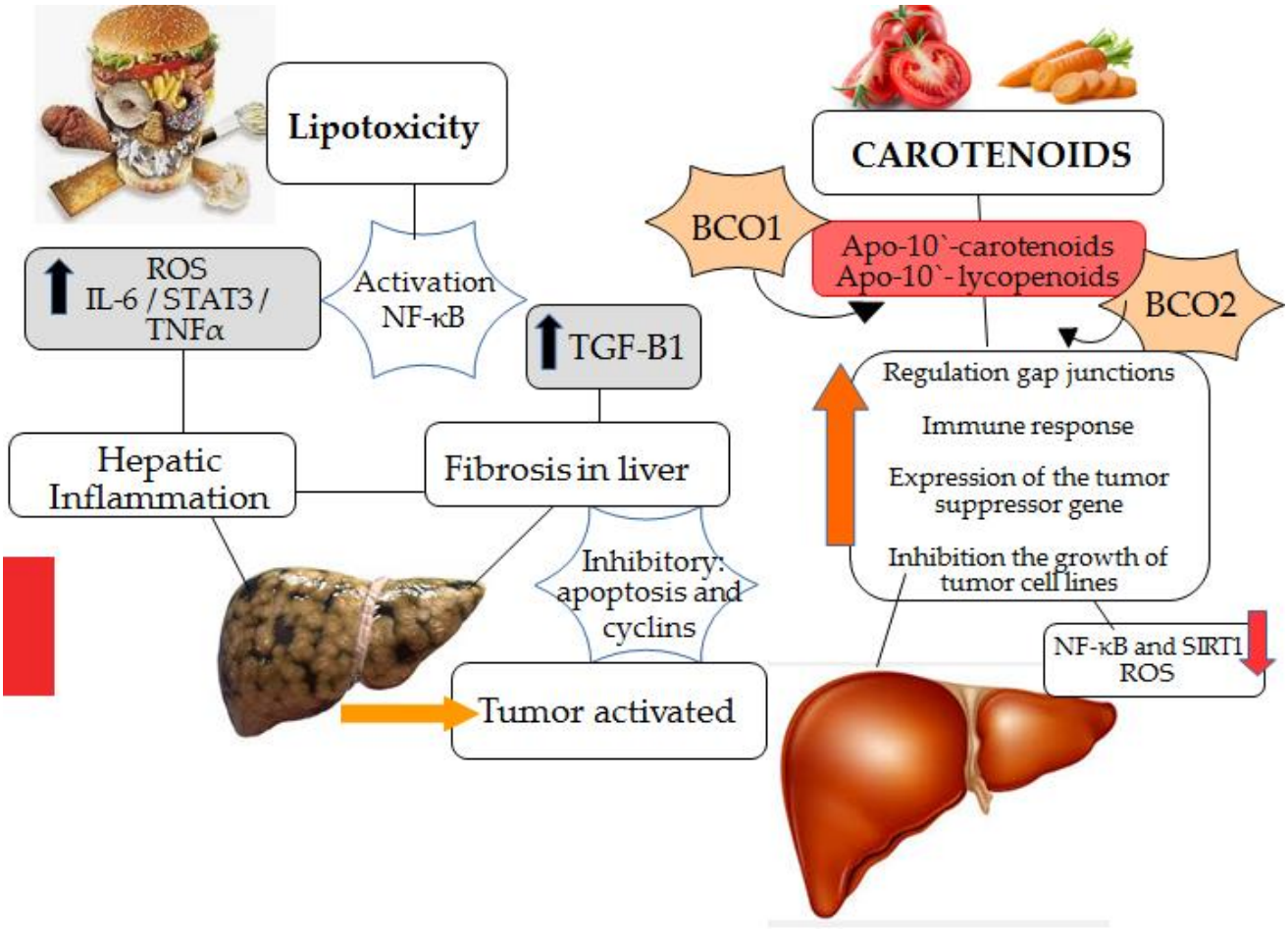

Figure 1. Schematic diagram of potential mechanisms by which carotenoids after cleaved

by the enzymes β-carotene 15,15′-oxygenase (BCO1) and β-carotene 9 ′,10′-oxygenase (BCO2) to form apo-10`-carotenoids and apo-10′-lycopenoids (APO10LA), favoring the reduction of the inflammatory response caused by signaling the nuclear factor-κB (NF-κB) and sirtuin 1 (SIRT1). Apo10LA inhibits the Interleukin (IL) - 6) / signal transducer and activator of transcription 3 (STAT3) pathway, and decreases the secretion of TGF-B1 and activation of myofibroblasts, responsible for the formation of scars, where chronic lesions can trigger liver fibrosis and liver cancer.

In addition, carotenoids, in addition to inhibiting oxidative stress, can protect the integrity and fluidity of the membrane against lipid peroxidation by decreasing cellular changes preventing the aging and appearance of cancer.

1.4. LYCOPENE

Carotenoids are not synthesized by the human body, so they are obtained exclusively through the diet (Scolastici et al, 2007). Lycopene can be found in a limited number of foods; tomatoes and their derivatives are the best dietary contributions. The redder the food, the higher its lycopene concentration. The absorption of lycopene is greater when the food in question is cooked, as the disruption of cell walls facilitates its contact with the intestinal mucosa (Bramley, 2000).

We can find lycopene in plasma and human tissues with great variation in its distribution. Of all the carotenoids, lycopene is one of the most abundant in the human body, and its high concentration is mainly due to the consumption of food (Khachik et al., 2002). Although about 90% of lycopene in food sources is found in the all-trans linear conformation, human tissues contain mainly cis isomers (Boileau et al, 2002), studies show that cis isomers are more available substances, have greater solubility in the micelles of bile acids and can preferably be incorporated into chylomicrons with less tendency for cis isomers to aggregate (Boileau et al, 1999). Lycopene is internalized, stored and isomerized in hepatic stellate cells, which are the main sites of metabolism and storage of retinoids and carotenoids in the body (Teodoro et al, 2009).

Despite not having pro-vitamin A functions, lycopene is considered the carotenoid with the highest antioxidant capacity, at least twice as high as β-carotene, thereby enhancing the protection of molecules against the oxidative process (Boileau et al, 2002 ; Boileau et al, 1999).

Lycopene has been described as a significant anti-tumor product in different types of

cancer cells, mainly regulating cellular events, such as apoptosis and cell cycle (mitosis), which are widely involved in cancer progression (Teodoro et al., 2012). However, it is poorly understood how lycopene regulates the biology of these cells. Murine hematopoiesis can be considered an excellent experimental model to study various events and cellular properties of hepatic adenocarcinoma, including proliferation, differentiation and apoptosis through contact with such carotenoids. Lycopene is a carotenoid whose biological activities and protective effect on prostate and breast cancer have been extensively described (Soares et al, 2019).

Scientific evidence has highlighted some biological actions of lycopene and its derivatives, such as apo-lycopenoids. Through the cleavage of lycopene with a long chain of double bonds conjugated by self-oxidation, oxidation mediated by radicals and singlet oxygen, causing the shortening of the lycopene carbon skeleton, by removing fragments from one or both ends of the chain, with the position the indicated cleavage point, for example apo10`-lycopene from lycopene (Wang, 2012; Mein, 2010; Lindshield et al, 2007).

Thus, lycopene and its metabolites have been relatively consistently associated with a lower risk of a variety of cancers, the evidence is stronger for prostate, lung, stomach, breast and pancreatic cancer and is suggestive for cervical cancer, oral cavity and liver (Boileau et al, 1999).

Ip et al (2013) showed that apo-10′-lycopenoic acid, a lycopene metabolite generated by beta-carotene-9′,10′-oxygenase (BCO2), inhibited carcinogen-initiated, high-fat diet (HFD)–promoted liver inflammation, and hepatic tumorigenesis development.

Previous results from our group showed that lycopene reduced the growth of a lineage of liver stellate cells, the GRX, which actively participate in the process of accumulation of extracellular matrix in hepatic fibrosis damaging liver functions. Lycopene modulated relevant signaling pathways related to cholesterol metabolism, cell proliferation and lipid metabolism, inactivating these cells and increasing the expression of RXR-α, RXR-β and PPARγ, important biomarkers of liver regeneration (Elias et al, 2019). Corroborating these findings, our group conducted a study with rats that received a diet rich in saturated fat and supplemented with tomato sauce and lycopene alone, with beneficial effects on cardiac and hepatic metabolism. The serum concentration of high density lipoprotein cholesterol, liver enzymes and tumor necrosis factor-α did not change and did not increase interleukin-1β in response to a high-fat diet, demonstrating a protective effect of lycopene (Jezus et al, 2019).

Blanche et al. (2014) demonstrad that lycopene supplementation (100 mg/kg diet) for 24

weeks resulted in comparable accumulation of hepatic lycopene could suppress HFD-promoted hepatocellular carcinoma (HCC) progression and multiplicity in mice.

Intriguingly, lycopene chemopreventive effects in wild-type mice were associated with reduced hepatic proinflammatory signaling (phosphorylation of NK-κB p65 and STAT3; IL6 protein) and inflammatory foci. The lycopene suppressed oncogenic signals, provided novel experimental evidence that dietary lycopene can prevent HFD-promoted HCC incidence and multiplicity in mice. Therefore, lycopene metabolites such as APO10LA, may exhibit protective effects against obesity-associated hepatic inflammation and tumorigenesis.

According to Mellert (2002), the supplementation of daily doses of up to 3g / kg of body weight for four weeks did not present toxicity or changes in weight gain and hematological evaluations, urinalysis and analyzed clinical parameters. It is important to note that not all countries have consolidated recommendations for lycopene intake, and these may differ significantly. For example, the recommendation for humans in the United States is 7.8 mg / day of lycopene, while in Denmark the recommendation is 1.4 mg / day (Mortensen, 2004). These doses are easily attainable by eating, since on average red fruits like guava and watermelon for every 100g of them, we have a variation of 5 to 7 mg of lycopene, in fresh tomatoes a variation of 3 to 7 mg / 100g and in tomato by-products such as sauce we can reach an incredible 30 mg of lycopene for every 100 g of the product, issues such as harvest, fruit ripeness, processing and etc. interfere with the concentration of this compound (Shami & Moreira, 2004). A summary of the studies examining the role of lycopene in chronic liver diseases is described in Table 1.

Table 1. Effects of Lycopene in different cell lines and animal models in liver

Cell/Animal model	**Metabolites and derivates**	**Anticarcinogenic activities**	**References**
Rats with hepatitis induced by D-galactosamine/lipopolysaccharide (D-GaIN/LPS)	Lycopene (10 mg/kg body weight for 6 days	Modified metabolism of lipoproteins, ↓ levels of lipid metabolizing enzymes, ↓ levels of cholesterol, triglycerides and free fatty acids, ↓ in the levels of phospholipids in serum and liver.	Shivashangari, et al, 2006. Sheriff, S.A.; Devaki, T, 2012.
Sprague-Dawley rats with hepatocellular carcinoma because a single dose administration of diethylnitrosamine	(15 mg LY/kg BW per day) for 6 weeks	↓ cytochrome P450 2E1, inflammatory foci, and mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-12) were also found.	Wang et al, 2010.

(DEN)			
Mice, induced by a high-fat diet	High-fat diet with or without 0.05% lycopene for 8 weeks	Down regulation of the expression of miRNA-21 (miR-21) / inhibiting the expression of the fatty acid-binding protein 7 (FABP7) and blocking the accumulation of intracellular lipids induced by stearic acid in Hepa 1–6 cells	Ahn et al, 2012.
N-nitrosodiethylamine (NDEA)-induced oxidative stress in rats	Treated with tomato paste, for 28 days in doses which were equivalent to 0.5 - 2.5 mg/kg b.w./day of lycopene	↓ DNA damage induced by NDEA in leukocytes ↓ in plasma concentration of protein carbonyls	Kujawska et al. 2014.
Sprague–Dawley rats with hepatic steatosis induced by a high-fat diet	Tomato juice for 7 weeks	↓ Isoprostanes in urine, , plasma TG and LDL ↑ HDL, lipid hydrolysis, activity of mitochondrial β-oxidation and peroxisomal ↓ steatosis	Martín-Pozuelo et al, 2015.
Mice C57BL/6 and SK-Hep-1 cells	Lycopene -10 or 100 mg/kg by oral gavage	↓ protein carbonylation and areas of necrosis, ameliorating the general appearance of the lesion in mice	Bandeira et al, 2017.
BALB/c mice - N-Nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis.	LycT (5 mg/kg b.w. given orally on alternate days	Modulating cell proliferation, glycolysis, and ultrastructure of liver cells	Gupta et al 2016
Rats - aflatoxin B1 (AFB1)-induced liver injury	LYC (5 mg/kg) and/or AFB1 (0.75 mg/kg) by intragastric administration for 30 days.	↓ AFB1-induced liver injury suppressing oxidative stress and enhancing GST-mediated detoxification.	Xu et al, 2017.
Rats with NAFLD and hypercholesterolemia induced by a high-fat diet	tomato juice of 5 weeks	↓ Regulation of APOB and LPL ↓ Synthesis of fatty acids, triglycerides and cholesterol ↑ Regulation of CD36, FXR, HNF4A and Levels of metabolites related to the antioxidant response	Elvira-Torales et al, 2018.

The benefits of tomatoes and their derivatives are often associated with lycopene. However, it is essential to know the action of other compounds present in these foods that

can interact and add effects on the bioavailability, pharmacology and biology of the effects. As well as, the specific health benefits of purified and / or isolated forms.

1.5. BETACAROTENE

Carotenoids are potent antioxidant and anti-inflammatory micronutrients that have been investigated in the prevention and treatment of various chronic diseases (Rao, 2007).

Beta carotene is a carotenoid present in vegetables and fruits of yellow-orange, with emphasis on carrot, pumpkin, papaya, mango, watermelon as well as in dark green leafy vegetables such as a cabbage. The highest levels of this carotenoid, which presents considerable pro-vitamin A activities have been found in fruits which pulp has orange coloration (Tadmor et al., 2005). It is known that β-carotene is the most potent precursor of retinol, this metabolic conversion of β-carotene to vitamin A is chemically possible due to its molecular structure that contains unsubstituted β-ionone rings, linked to the polyenic side chain (rich in conjugated double bonds). Most of the absorbed β-carotene is converted to retinol (60 to 70%) being metabolized in the enterocyte, reaching the liver through the lymph, carried by remnants of chylomicrons (Parker et al, 1996; Rodriguez- Amaya, 1997). B-carotene is transported in the plasma by lipoproteins and stored mainly in the adipose tissue and liver (Mondul et al, 2013; Middha et al, 2019). In several countries, the average daily consumption of β-carotene is approximately 1.5 to 2.0 mg / day, which can reach 4-6 mg / day in a diet rich in vegetables and fruits, (Sandoval et al, 2019; Bendich, 1994; Block & Langseth, 1994).

The main intervention studies already completed used supplementation with chronic pharmacological doses of β-carotene that varied from 10 to 30mg / day, alone or associated with other micronutrients. Studies indicate that diets rich in this carotenoid are related lower rates of chronic diseases and protection against some cancers (Haider et al, 2017). They can act in the deactivation of reactive species, thus avoiding the initiation of oxidation chains at the cellular level that leads to damage to deoxyribonucleic acid (DNA) and lipid peroxidation (Silva et al., 2010).

Buriti, fruit of the Amazon region, has recently gained great interest in studies because it is the richest natural source of beta carotene known (152,000 μg / 100g in oil) (Aquino et al, 2012). Animal studies have shown extremely high bioavailability, probably due to its oily composition.

Many studies have demonstrated a protective effect of beta carotene against the cell cycle

of some types of cancer cells. These cells have a disturbance in the cell cycle regulation mechanism, causing an uncontrolled cell proliferation, commonly associated with proteins (cyclins) and inhibitory genes (p16, p27, p21, p53) (Martinez, 2018). Cycline-dependent kinases (CDKs), in turn, often present their malignant altered expression and their inhibition can induce apoptosis, being a rational target for cancer therapy. For example, the loss of function of inhibitory genes, such as p16, is associated with melanoma and certain types of tumors. Thus, CDKs could restore checkpoints of the cell cycle and slow growth or induce apoptosis (Folmer et al, 2014). Beta carotene has been associated with the cell cycle arrest of cancer cells, including prostate, breast and liver by means of cyclins and other beneficial effects, such as inhibition of the phase I carcinogen metabolizing enzyme, induction of phase II detoxification enzymes, increased immune system, modulator of gap junctions and modulation of the circulation of hormonal concentrations (Filaire et al, 2013; Dickson and Schwartz, 2009).

In addition, another anti-tumor mechanism would be attacking the tumor-promoting cytokines IL-6 and TNF, which are usually a central mediator in chronic inflammatory diseases, causing liver inflammation and activation of the oncogenic transcription factor STAT3. The chronic inflammatory response caused by obesity and increased production of IL-6 and TNF can also increase the risk of other types of cancer. Obesity promotes liver fat accumulation and fat-induced liver inflammation via TNF and IL-6 cytokines, increasing the predisposition of hepatocellular carcinoma (HCC) in mice. B-carotene supplementation appears to induce the loss of TNF receptor 1 by preventing fat-induced liver damage and ablation of the IL-6 gene, which also prevents the development of obesity-induced HCC (Park et al, 2009; Sueoka et al, 2001). A summary of the studies examining the role of betacarotene in chronic liver diseases is described in Table 2.

Table 2. Effects of Betacarotene in different cell lines and animal models in liver

Cell/Animal model	Metabolites and derivates	Anticarcinogenic activities	References
Rats with monocrotaline-induced steatosis	Diet with or without β-carotene. (52 mg/kg diet)	↓ fat accumulation and liver hemorrhages	Baybutt e Molteni - 1999
Albino rats with carcinogenesis induced by aflatoxin-B1	β-caroteno (120 - 1200 μg 100 g.	↑ levels of vitamin C, glutathione and glutathione related enzymes which act as a free radical scavenger and	Patel e Sail et al, 2006.

		reduced the toxicity effect of AFB1 in rats.	
Ratos Wistar	β-caroteno - 200 mg / kg de peso corporal	↑ lipid peroxide levels, ↓ hepatic reduced glutathione (GSH) and serum protein thiol (PrSHs) along with a nearly twofold increase in hepatic glutathione-S-transferase (GST) activity. The significant increase in GST may be attributed to its being a phase enzyme that predominately participates in the detoxification of the ultimate electrophilic metabolite AFB1-8, 9 epoxide.	Kheir et al, 2008.
Camundongos knockout - fed a diet high in fat	β -caroteno -6 g / 100 g de dieta	↓ plasma cholesterol and atherogenesis index and ↓ of fat accumulation and inflammation was reported in the liver	Harari et al, 2008

2.Conclusions

Carotenoids have stood out for their significant levels of bioactive compounds and their benefits. Many authors indicate that the ingestion of lycopene and / or beta-carotene and its metabolites could reduce the proliferation of liver cancer cells, an effect arising from the antioxidant activity and by the modulation of the cell cycle and the rate of apoptosis. However, an important consideration in all studies is to emphasize the context of the studied compound, since studies with an isolated compound can provide very different results from studies of the compound within a food matrix.

Thus, to recommend supplementation of these compounds, further research is needed to elucidate the mechanism by which carotenoids act to modulate cancer and to determine the optimal and safe level of consumption that promotes the expected effects.

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

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Chapter 2

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

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

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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 G_0/G_1 phase, followed by a decrease of cells in G_2/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 PPARγ, 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

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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 (PPARγ), 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 cm^2 tissue culture flasks, $5x10^6$ 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% CO_2 atmosphere. Under these conditions, they expressed the myofibroblast phenotype. In order to induce the lipocyte phenotype, cells (10^4 cells/cm^2) 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 10^3 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, 1x10^6 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 μCi/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 UGD 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 1x106 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 MgCl2, 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 (ºC)	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´TTTTTGTGGATCCGACAGTTT 3´	55	177
□-actin	5´GTGGGCCGCTCTAGGCACCA 3´ 5´CTCTTTCATGTCACGCACGATTTC 3´	58	560

Statistical Analysis

The presented data are mean values ± 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 indicting 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 3μM lycopene for 5 and 10 days.

	Total		**Membrane**		**Cytoplasm**		**Nucleus**		**Lipid droplets**
	Incorporation (%)								
Incubation time (days)	**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 μM 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 (0.625-20μM) 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 μM) 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 noRlized 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 (1-5μM) for 2 (A) and 7 days (B). The results are expressed as mean ± 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 μM) showed an increased expression of RXR-α, RXR-β, and PPARγ 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 (1-5μM) 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 ± 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 PPARγ 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 PPARγ 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. PPARγ/RXR heterodimers regulate transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism,

neurodegenerative disorders, cancer and inflammation [48]. PPARγ 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 PPARγ/RXR ligands, other tissues also regulate the effects of these receptors. The activity of the PPARγ/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 PPARγ. 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 PPARγ and RXRα, a direct target gene for the PPARγ: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 G_0/G_1 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 PPARγ and RXR. Taken together, the present data suggest possible protective mechanism mediated by lycopene hepatic stellate cells.

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Chapter 3

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

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

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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-1β induced by the high-fat diet. Cell cycle analysis of cardiac and liver cells showed a lower percentage of cells in the G_0/G_1 phase and an increase in the G_2/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 ¼ of the adult population is obese, the disease affects more than ⅔ of these individuals. It is estimated that 2-3% of the population presents hepatic steatosis[3.] 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 daily[12]. 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 diet[12]. 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).

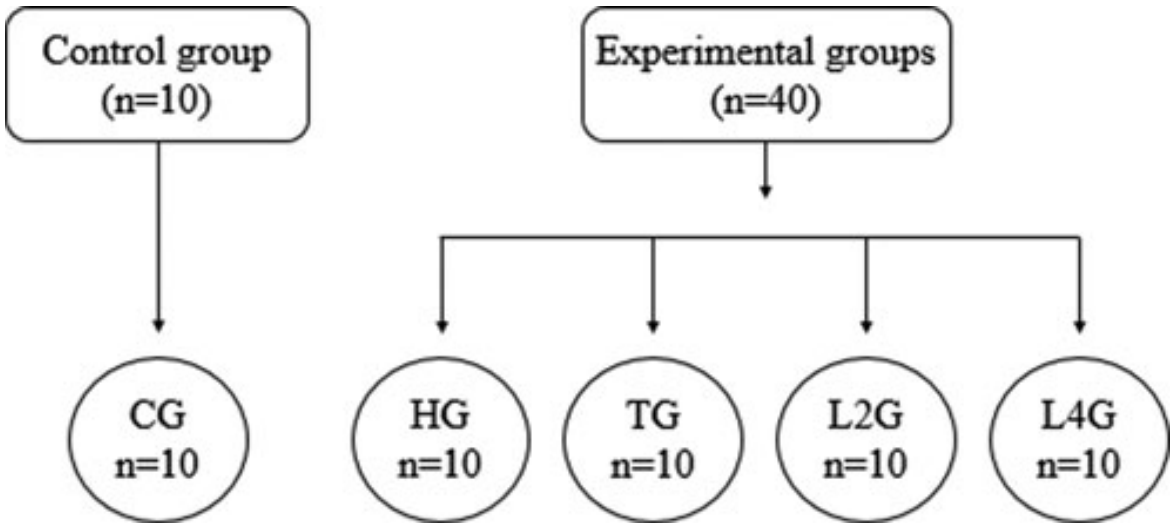

FIG. 1. Dietary protocol. Experimental model: CG: standard diet plus water; experimental groups: HG. high-fat diet plus water; TG. high-fat diet plus solution with tomato sauce and water; L2G. high-fat
diet plus solution with 2.0 mg all-trans lycopene and water; L4G. high-fat diet plus solution with 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 22ºC (+/- 2ºC). Animal maintenance was in accordance with the ARRIVE guidelines[23]. 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:

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

Analytical methods

The glucometer measured serum glucose concentration. Serum total cholesterol, high-density lipoprotein (HDL-cholesterol), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), IL-1β, 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 Analizer®). LDL-C was calculated according to Friedwald's formula[24].

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 μL 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 G_0/G_1 (2n). S phase (>2n and <4n) and G_2/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×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±11.48g) and lower in the L2G (197.3±13.12g) and L4G (188.9±15.02g) compared to the control group (Table 1). Heart weight was higher ($p<0.05$) in the TG (1.06±0.05g) and lower in the L4G (0.72±0.11g) 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.

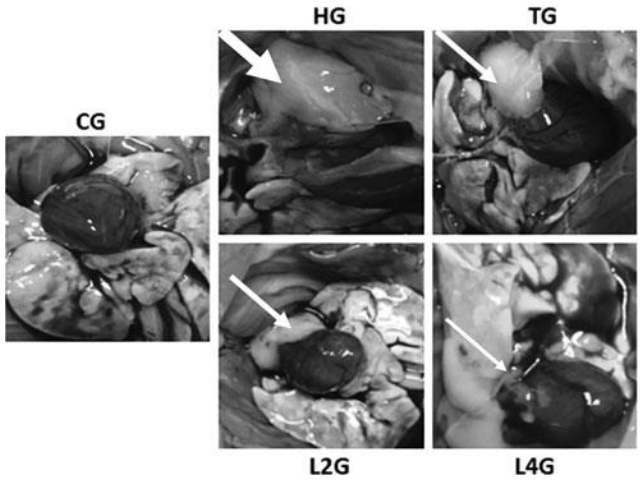

FIG. 2. Heart extracted from sacrificed rats after dietary protocol. *White arrows* indicate accumulated fat in tissues.

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-1β 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).

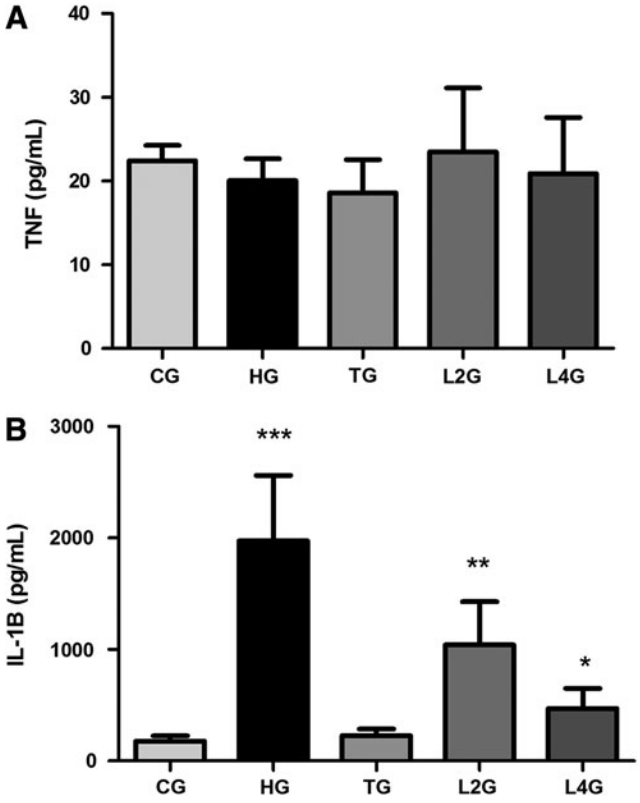

FIG. 3. (A) TNF-*a* (B) IL-1*b* from rats fed a high-fat diet supple- mented with lycopene and tomato sauce. *$P < .05$; **$P < .01$; $P < .001$. ANOVA–Tukey's test. ANOVA, analyis of variance; TNF, tumor necrosis factor; IL, interleukin.

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 G_0/G_1 phase and raised the percentage of cells in the G_2/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 G_0/G_1 phase and decreased its numbers in the G_2/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

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.

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).

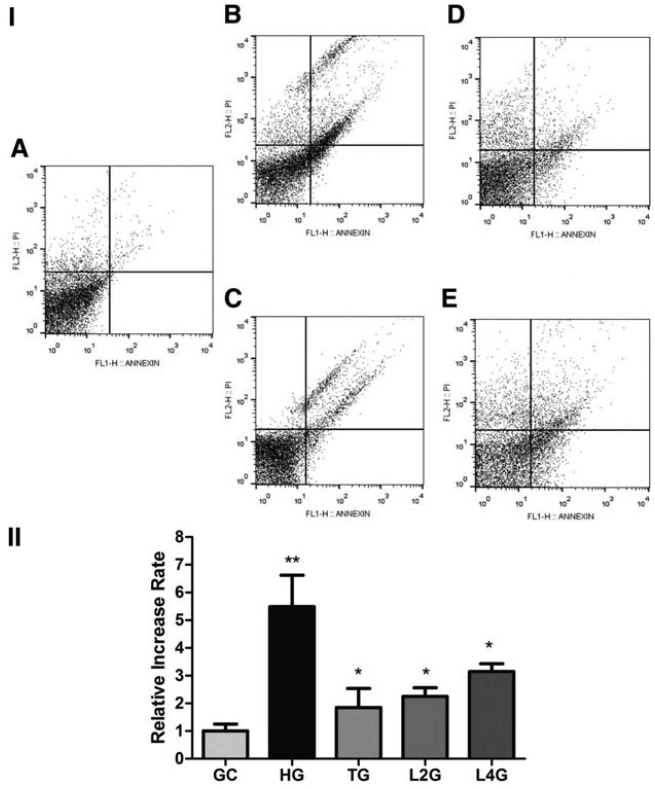

FIG. 4. Monitoring of cell death of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (I) Flow cytometry analysis of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (II) Quantitative effects of lycopene and tomato sauce of 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 com- parison *post hoc* test. **P* < .05. **P* < .01. SD, standard deviation.

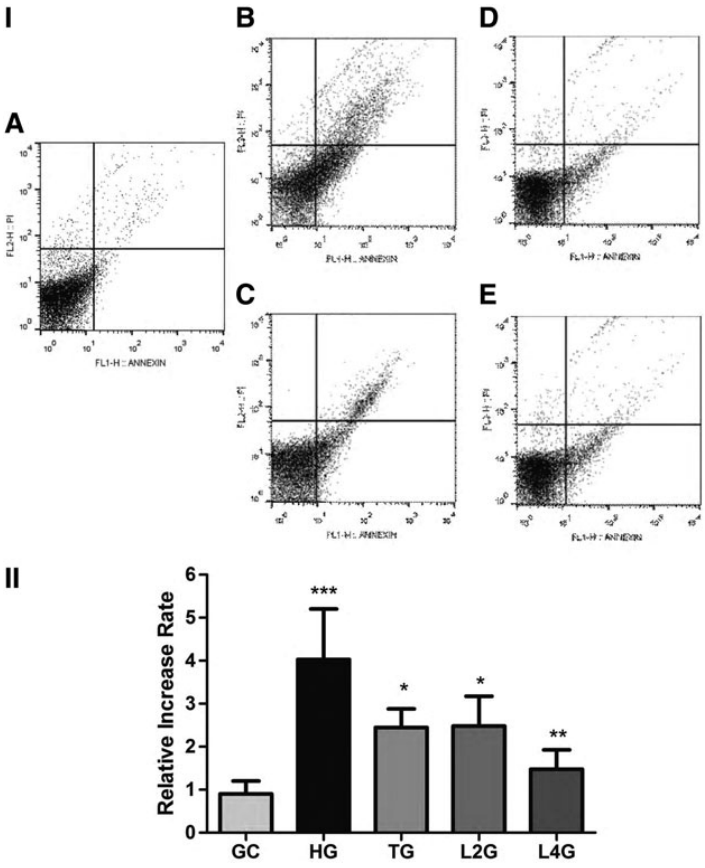

FIG. 5. Monitoring of cell death of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (A) Flow cytometry analysis of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (B) Quantitative effects of lycopene and tomato sauce of 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 com- parison *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 know 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 control[12]. 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 more[33]. 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-1β and TNF-α. Pro-inflammatory cytokine IL-1β 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-1β 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 lines[4]. 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.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

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Chapter 4

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

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

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ABSTRACT

This work evaluated the effect of betacarotene and buriti juice in liver parameters of rats submitted to high-fat diet. Experimental model was conducted with groups of adult females Rattus norvegicus: control (CG); high-fat (HG); buriti juice (BUG); betacarotene - 2mg (B2G) and betacarotene - 4mg (B4G). The high fat diet caused damage to liver tissue, altering the cell cycle and liver histology. In addition, supplementation did not prevent excess dietary fat from reducing viable cell content and increasing the apoptosis process. Both supplements increased cell death and liver cell necrosis, especially the supplementation with 4 mg beta carotene isolated that aggravated the histological damage caused by the high-fat diet, demonstrating a possible toxic effect on these cells.

INTRODUCTION

Liver tissue is essential not only for body control and homeostasis, but also for metabolic and health reactions that can be deregulated in liver injury processes (Schinoni, 2008). The high energetic activity of the liver, associated with a high intake of a high-fat diet, leads to an increase in oxygen consumption, or increases free radical production, causing pathophysiology of inflammatory diseases and damage to substances, lipids and cellular DNA (Halliwell & Gutteridgr, 2000; Sies et al., 2005; Jaeschke et al., 2000), resulting in single or double breaks and also without mutation surgery, favoring or evolving several non-communicable chronic diseases (NCDs) (Braga et al., 2002; Geraldo et al., 2008; Oliveira & Schoffen 2010).

There is a trend of nutritional transition that occurs in this century in many countries and leads to a new dietary model based on a diet rich in saturated fats, sugars and refined foods, and low in complex carbohydrates and fiber, also known as the Western diet (Shively et al, 2019). Excessive fat consumption generates increased lipid flow to the liver promoting a state of lipotoxicity in the organ, associated with a high level of oxidative stress and reduced antioxidant defense of the organism (Bullón-Vela et al, 2018). Diets high in saturated fat stimulate the detection of proinflammatory cytokines and increased reactive oxygen species, oxidative stress, and tissue cell damage (Francis, 2013; Gentile & Pagliassotti, 2008). In hepatocytes, oxidative stress and lipid peroxidation promote plasma membrane damage, become vulnerable to apoptosis, and favor the inflammatory response in the tissue and process in general (Cave et al., 2007; France, 2013).

There are samples demonstrating that hepatocarcinogenesis in rats and humans is a multiple disease process preceded by increased foci of altered hepatocytes and hyperplastic liver nodules, these being preneoplastic lesions caused by hepatocytes initiated (Peres et al., 2003). Studies have shown a carotenoid action on the histopathology of hepatocellular carcinoma and liver protection due to its action against oxidative stress (Kaklamani et al., 1991; Yu et al, 2000). The results of recent investigations suggest that dietary interventions may modulate the expression of some genes and the risk of diseases induced by oxidative stress (Zern & Fernandez, 2005; Silver et al., 2011; Lau et al., 2005; Venturini et al. , 2011, Yilmaz, 2005).

Carotenoids are potent antioxidant and anti-inflammatory micronutrients that have been

investigated in the prevention and treatment of various chronic diseases (Rao, 2007).

Beta carotene is a carotenoid present in vegetables and fruits of yellow-orange, with emphasis on carrot, pumpkin, papaya, mango, watermelon as well as in dark green leafy vegetables such as a cabbage. The highest levels of this carotenoid, which presents considerable pro-vitamin A activities have been found in fruits which pulp has orange coloration (Tadmor et al., 2005). Studies indicate that diets rich in this carotenoid are related lower rates of chronic diseases and protection against some cancers.

Buriti, fruit of the Amazon region, has recently gained great interest in studies because it is the richest natural source of beta carotene known (152,000 μg RAE / 100g in oil) (Santos, 2006). Animal studies have shown extremely high bioavailability, probably due to its oily composition. The effectiveness of buriti in treating and preventing disease has caught the attention of researchers.

Therefore, it is relevant to study liver tissue after treatment with beta-carotene and buriti. However, as most of this human data is still inconclusive, experimental animal models were used to better understand the mechanisms of action of these products. In this sense, the aim of this study is to evaluate the effects of carotenoids on liver function and integrity in in vivo models and to elucidate the mechanisms of action of carotenoids in the hepatic tissue of rats submitted to the high fat diet, in relation to histological, cell cycle and apoptosis parameters.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND SAMPLING

The study was conducted in the Laboratory of Experimental Nutrition at Department of Nutrition and Dietetics of Federal Fluminense University (LabNE-UFF). Samples of Brazilian buriti juice (ingredients: buriti fruit (97%), sugar and water were obtained from Amazônia, Brazil. Water-soluble (WS) betacarotene 10% (containing sucrose, corn starch, fish gelatin, lycopene, corn oil, ascorbyl palmitate, and DL-alphatocopherol) was provided by Roche (Rio de Janeiro, RJ, Brazil). Samples of buriti juice and betacarotene 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. Fifty female, adults Wistar rats were individually housed and maintained in a 12-h light–12-h dark cycle at 22ºC (± 2ºC). Animal maintenance was in accordance with the 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: 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; 2) high-fat group (HG), which received a high-fat diet ad libitum, consisting of protein (minimum) 12.95%, fat (minimum) 20.0%, and water; 3) buriti juice group (BUG), which received the high-fat diet ad libitum, plus a buriti juice solution providing 2.0 mg of betacarotene per day; 4) 2.0 mg betacarotene group (B2G), which received the high-fat diet ad libitum, plus a solution containing 2.0 mg all-trans betacarotene (water soluble) 10% dissolved in water daily, and water; and 5) 4.0 mg betacarotene group (B4G), 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 Materials. 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. (2018).

The hepatic tissue was carefully removed, weighed with a BioPrecisa® precision scale and the relative weight of the organ, denominated liver index, was calculated according to the equation: Liver index = Liver Weight (g) X 100/ Body Weight (g).

After weighing, four fragments of different lobes hepatics were sectioned with a surgical scalpel of each animal and immediately treated for cell cycle analysis, apoptosis assay and histological analysis (Guimarães et al. 2017).

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), IL-1β, 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 formula (Friedewald, 1972).

CELL CYCLE ANALYSIS AND CELL VIABILITY

Flow cytometry analysis was performed to measure cell cycle and cell viability of tissue hepatic. The tissue was macerated and the extractions were made with the addition of 0.5 mg/ml collagenase (Sigma®). The cells were washed twice with phosphate buffered saline and resuspended in 500 μl of ice-cold Vindelov solution containing 0.1% Triton X-100, 0.1% citrate and 0.1 mg/mL of RNase and 50 mg/mL of propidium iodide (Sigma Chemical Co., St. Louis, MO) after centrifugation. Was incubated for 15 minutes of cells and the suspension was analyzed for DNA content by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson. Mountain View, CA). After acquisition of 30.000 events, 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. Considering the experimental conditions that were used in this study, or in any others we were aware of, the fluorescence was not affected by the cell dissociation process. According Guimarães et al. (2017), nuclei of viable cells were gated according to FL- 2W × FL2-A relation.

APOPTOSIS ASSAY

For the apoptosis assay, the cells were subjected to staining with Annexin V conjugated to FITC. The non-adherent cells were collected, and adherent cells were quickly washed with a calcium/magnesium-free buffered saline solution (BSS) and were detached with 0.125% trypsin/EDTA (Sigma Chemical Co., St. Louis, MO, USA) at room temperature. Subsequently, apoptotic and necrotic cells were stained with Annexin V FITC/Propidium

Iodide (PI) (BD Pharmingen, Mountain View, CA, USA) according to the manufacturer's instructions, quantified with a flow cytometer 30.000 events (FACS Calibur, BD Bioscience, Mountain View, CA, USA), and the data were analyzed in CellQuest and FlowJo software.

STATISTICAL ANALYSIS

Data were analyzed using software package was Graphpad Prism for Windows. Differences between the groups were analyzed using the Student's t-test, and the values were considered unpaired and parametric. For means of comparison among the groups, were analyzed using one-way ANOVA followed by Tukey's post-hoc test for the multiple mean comparison test. Results are expressed as mean–standard deviation, and the significance level was set at $p < 0.05$.

RESULTS

During experiment the different dietary treatments did not influence ($p>0.05$) the intake of ration and water of animals which were similar among all groups. However, animals supplemented with beta carotene and buriti juice and with a high-fat diet (B2G, B4G and BUG), showed lower body weight gain ($p <0.05$) (Table 1). Although no difference was observed in the hepato-somatic index of the animals.

The group supplemented with 2mg beta-carotene had higher cholesterol and triglyceride levels when compared to the other groups. The group that received only the hyperlipid diet had the lowest levels of cholesterol and triglycerides. We observed that HG presented higher levels of TGO (219,80 ± 33,39) when compared to the different groups CG (195,60 ± 52,03); B2G (162,80 ± 56,86); B4G (133,80 ± 28,45); BUG (111,60 ±27,52). We found no significant difference in relation to TGP between the groups. The hyperlipid group presented higher levels of IL-1B (60%) than the group supplemented with 4mg of beta-carotene. We did not observe statistical difference in glycemia and TNF analysis in the different groups.

TABLE 1

GROUPS	CG	HG	B2G	B4G	BUG
Peso inicial (g)	207,60 ± 30,33	209,60 ± 17,15	258,00 ± 22,88	253,00 ± 26,54	250,40 ± 10,33
Peso final (g)	261,00 ± 31,04	268,20 ± 17,68	274,00 ± 30,13	262,60 ± 14,50	270,60 ± 13,76
Variação de peso (g)	53,40	58,60	16,00	9,60	20,20
Peso fígado(g)	7,8g ± 1,15	7,1g ± 1,34[6]	7,5g ± 1,85	8,01 ± 0,11	8,25 ± 0,18
Glicemia	100,40 ± 9,24	97,60 ± 6,50	107,20 ± 7,09	107,60 ± 10,71	97,40 ± 9,53
Triglicerides	31,60 ± 8,17	41,25 ± 13,20	73,40 ± 38,71	43,40 ± 11,52	46,00 ± 12,33
Colesterol total	46,00 ± 8,60	45,00 ± 3,54	78,40 ± 22,74	62,80 ± 9,31	65,40 ± 7,54
TGO	195,60 ± 52,03	219,80 ± 33,39	162,80 ± 56,86	133,80 ± 28,45	111,60 ±27,52
TGP	32,20 ± 6,42	32,00 ± 5,39	32,80 ± 11,88	35,00 ± 4,53	22,00 ± 6,56
IL-1	266,38 ± 133,17	1135,38 ± 730,58	571,97 ± 412,78	452,00 ± 208,48	531,60 ± 565,86
TNF-	25,66 ± 7,87	20,05 ± 5,84	35,78 ± 22,24	39,56 ± 14,55	27,42 ± 9,14

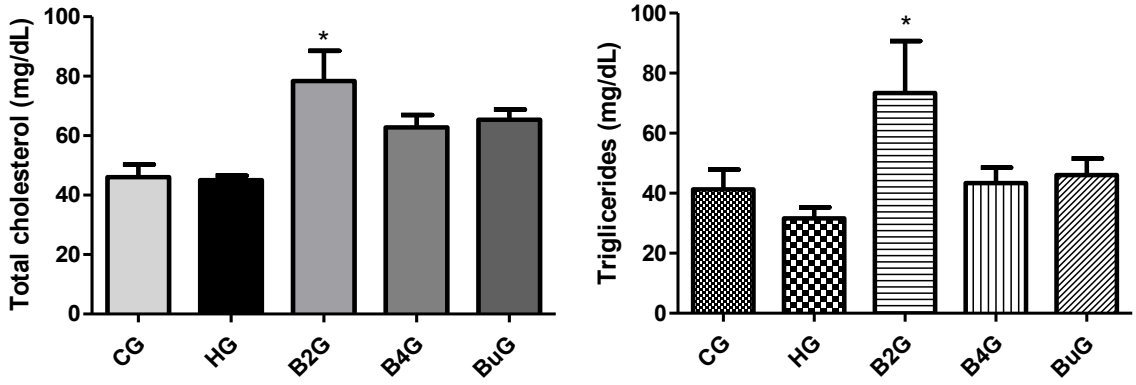

Figure 2. Serum Parameters in Rats Fed with Experimental Diets

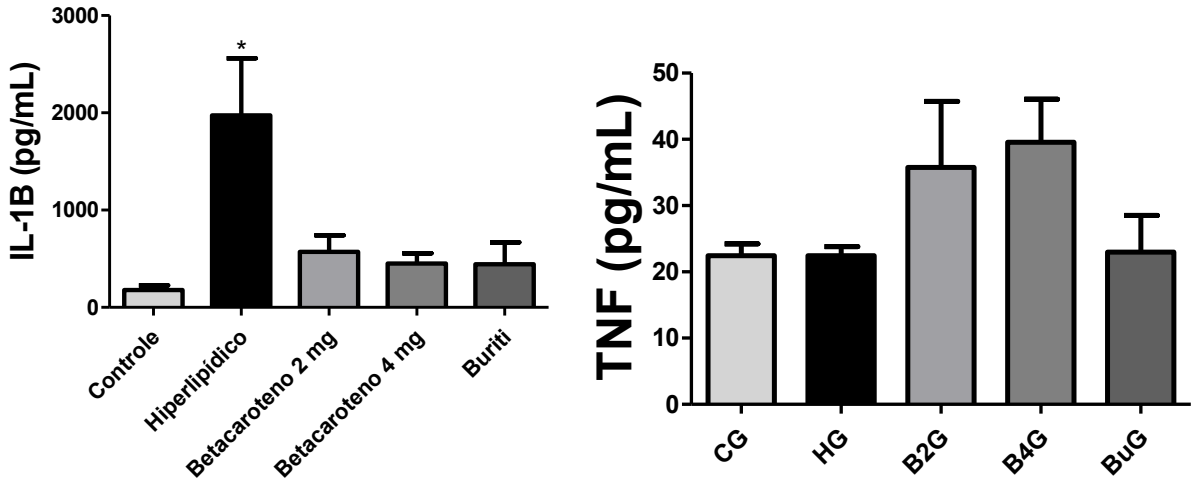

FIG. 3. (A) TNF-*a* (B) IL-1*b* from rats fed a high-fat diet supple- mented with Betacarotene and Buriti juice *$P < .05$; **$P < .01$; $P < .001$
ANOVA–Tukey's test. ANOVA, analyis of variance; TNF, tumor necrosis factor; IL, interleukin.

Our results of cell cycle are described below (Table 2), or GBU presents the lowest percentage of cells in phase G0 / G1 (12.00 ± 2.95), when compared to the groups GC (63.49 ± 6.06), GH (53.67 ± 5.79), GB2 (49.60 ± 29.59) and GB4 (67.50 ± 10.10). In addition, GB2 reached a maximum value of 25.98 ± 16.54 in the G2 / M phase, 55% higher when compared to the CG.

TABLE 2. Effect of Betacarotene and Buriti Juice on Cell Cycle Progression of Hepatic Cells in Rats Fed High-Fat Diet

Grupos	G0/G1	S	G2/M
GC	63,49 ± 6,06 [a]	20,20 ± 8,47 [a]	14,41 ± 3,85[a]
GH	53,67 ± 5,79 [b]	28,18 ± 3,40[b]	18,25 ± 3,08[a]
HP + GBU	12,00 ± 2,95[d]	4,13 ± 2,23[a,c]	9,21 ± 1,40[b]
HP + GB2	49,60 ± 29,59	3,48 ± 0,43[c]	25,98 ± 16,54
HP + GB4	67,50 ± 10,10	12,22 ± 4,95[a,c]	9,21 ± 2,76[b]

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.

The high-fat (HG) diet altered the liver cell cycle of the animals, leading to a smaller number ($p < 0.05$) of viable cells and a higher apoptotic cell rate (Table 3), the supplements do not reversed the effects of high fat diet on apoptosis of liver cells. Beta carotene supplementation, especially B4G showed to be even more aggressive to the liver tissue.

In addition to the alterations already caused by the high-fat diet, beta-carotene treatment caused was an increase in hepatic cells in initial apoptosis in GH (22.98 ± 7.78) when compared to CG (2.18 ± 1.55). In the beta-carotene-treated groups (GBU - 0.04 ± 0.01, GB2 - 0.26 ± 0.27 and GB4 0.75 ± 0.71a), a significant decrease in initial apoptosis was observed when compared to the control group (CG) and hyperlipidic (GH).

However, both groups receiving beta-carotene supplementation (GB2 - 15.27 ± 9.22 and GB4 32.23 ± 20.15) showed a marked increase in the percentage of late apoptosis cells compared to the CG (3.91 ± 1 90), with no significant difference in GBU (5.33 ± 5.82) and

GH (6.59 ± 1.80) in relation to the control group. GBU increased viable cells relative to GH, but also increased necrosis cells compared to GC and CH. The most significant result was GB4, which showed the highest programmed induction of death of these cells, reaching 32.23 ± 20.15 in late apoptosis and 31.68 ± 9.54 in necrosis when compared to the control group (3, 91 ± 1.90; 2.69 ± 1.67) and GH (6.59 ± 1.80; 1.72 ± 0.43), respectively, demonstrating a possible toxic effect on these cells, Table 3.

TABLE 3. Monitoring of cell death of hepatic cells from rats fed a high-fat diet supplemented with Betacarotene and Buriti Juice

Grupos	Células Viaveis (Annexin V- PI-	Apoptose inicial (Annexin V+ PI-)	Apoptose tardia (Annexin V+ PI+)	Non-apoptotic cells (Annexin V- PI+)
GC	89,85 ± 3,96 [a]	2,18 ± 1,55 [a]	3,91 ± 1,90[a]	2,69 ± 1,67[a]
GH	66,58 ± 10,16 [b]	22,98 ± 7,78[b]	6,59 ± 1,80[a]	1,72 ± 0,43[a]
HP + GBU	75,90 ± 12,18[c]	0,04 ± 0,01[c]	5,33 ± 5,82[a]	18,70 ± 6,52[b]
HP + GB2	64,36 ± 12,11[b]	0,26 ± 0,27[c]	15,27 ± 9,22[b]	20,08 ± 6,44[b]
HP + GB4	35,34 ± 12,59[d]	0,75 ± 0,71[a,c]	32,23 ± 20,15[b]	31,68 ± 9,54[b]

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

The consumption of a high energy density, high-fat (HF) diet is thought to be one of the main factors for increased obesity and associated comorbidities. According to Woods (2003), the key point is that when individuals are exposed, on a chronic basis, to a higher mean level of dietary fat, the otherwise incredibly robust negative feedback system that regulates body fat decreases. More fat is stored and the individual moves toward obesity. High fat intake combined with high levels of oxidative stress results in increased liver fat loading in the setting and reduced antioxidant levels resulting in lipotoxicity (Xu, 2010). Obesity is correlate to metabolic syndrome, dyslipidemia, hypertension, intolerance glucose, and insulin resistance. Studies have shown the relationship between metabolic syndrome, especially obesity and dyslipidemia, and the presence of nonalcoholic fatty

liver disease (NAFLD), refers to a wide spectrum of liver damage (Sunik, 2011).

Our outcomes agree with published results in which betacotene supplementation in rats was able to reduce weight gain even with ingestion of a high-fat diet when compared with the control. Our results revealed that a high-fat diet promoted an increase in triglycerides and IL-1B, and betacotene 4mg and buriti juice supplementation improved the rat's lipid profile. Furthermore, the intervention with betacarotene supplementation in vivo did not affect glucose profiles. Stress factors can induce proin- flammatory cytokine IL-1b, associated the chronic inflam- matory diseases, such as obesity, and can be interfere with increased cell proliferation, cell cycle and cell death in different cell types. We showed that betacarotene and buriti juice supplementation reversed the increase in IL-1b levels induced by high-fat diet.

It is suggested that beta-carotene, a carotenoid precursor of vitamin A, an essential nutrient with antioxidant activity, is an important factor in reducing the risk of cancer and chronic diseases. Human cancer risks have been shown to be inversely correlated with high blood levels of retinol or beta-carotene and ingestion of foods containing retinol (Gloria et al, 2014). Thus, many studies investigate the action of bioactive compounds on interferences in cellular parameters and apoptosis of cancer cells. Most studies are directed at isolated compounds and few are concerned with the synergy of compounds in the food matrix.

In this context, our study compared the results of isolated beta-carotene and beta-carotene in the cell matrix and their effects on cell growth and apoptosis in the face of a scenario of tissue inflammation due to the effect of a high-fat diet. . Liver cells cell cycle analysis reported that a high-fat diet promoted a decrease in the G0/G1 phase and an increase in the percentage of hepatic 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. Our results of cell cycle demonstrate GBU presented the lowest percentage of cells in phase G0 / G1, when compared to the all groups In addition, GB2 reached a maximum value in the G2 / M phase, 55% higher when compared to the GC.

Beta-carotene supplementation did not prevent the change caused by the high-fat diet in the cell cycle. In addition, the high-fat diet altered the liver cell cycle of the animals,

leading to a smaller number of viable cells and a higher apoptotic cell rate, the supplements do not reversed the effects of high fat diet on apoptosis of liver cells. Betacarotene supplementation, especially B4G showed to be even more aggressive to the liver tissue. In addition to the alterations already caused by the high-fat diet, beta-carotene treatment caused was an increase in hepatic cells in initial apoptosis in GH when compared to CG. In the beta-carotene-treated groups a significant decrease in initial apoptosis was observed when compared to the control group (CG) and hyperlipidic (GH). However, both groups receiving beta-carotene isoladed supplementation showed a marked increase in the percentage of late apoptosis cells compared to the CG. GBU increased viable cells relative to GH, but also increased necrosis cells compared to GC and CH. The most significant result was GB4, which showed the highest programmed induction of death of these cells, in late apoptosis and in necrosis when compared to the control group and GH, respectively, demonstrating a possible toxic effect on these cells.

Mukherjee (2011), demonstrates even when administered at high doses during long periods of time, beta-carotene does not cause toxicity, whereas high doses of vitamin A and retinoids, as possible prophylactic agents for cancer prevention, can lead to acute hepatotoxicity and cause other adverse effects. On the other hand, contrary to the beneficial effects of beta-carotene, the literature presents cases of hypervitaminosis associated with megadoses, which resulted in hepatic impregnation, causing adverse effects such as injury, fibrosis, portal hypertension and hepatic hydrothorax (Oliveira et al, 2007). There were also reports of experimental and interventionist studies that suggested a possible antioxidant action, which contrasts with its already known antioxidant action.

This study showed a beta-carotene supplementation caused a possible toxic effect on liver cells, increasing the harmful effect of a high-fat diet.

Conclusions

The high fat diet caused damage to liver tissue, altering the cell cycle and liver histology. In addition, supplementation did not prevent excess dietary fat from reducing viable cell content and increasing the apoptosis process. Both supplements increased cell death and liver cell necrosis, especially the supplementation with 4 mg beta carotene isolated that

aggravated the histological damage caused by the hyperlipid diet, demonstrating a possible toxic effect on these cells.

Conflicts of Interest

The authors declare no conflicts of interest

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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 PPARγ 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 PPARγ 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.

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Apêndices -

Artigos publicados

Food and Function

ARTICLE

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

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

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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 PPARγ, 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].

[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

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 (PPARγ), 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 cm^2 tissue culture flasks, $5x10^6$ 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 (10^4 cells/cm^2) 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 10^3 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 [14C]-acetate and lipids analysis

All the cells were incubated with 0.25 µCi/mL [14C]-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 UGD 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 1x106 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 MgCl2, 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 (ºC)	Size PCR product (pb)
Cx 43	5´ATCCAGTGGTACATCTATGG 3´ 5´CTGCTGGCTCTGCTGGAAGG 3´	58	593
PPARγ	5´TTTTCAAGGGTGCCAGTTTC 3´ 5´TCTGTGACGATCTGCCTGAG 3´	58	501
RARα	GCTTAACCAAAGGACGGATTCTTGG 3´ ´CGGTGTGCTGTAACCACTGACTGG 3´	52	425
RXRα	5´ACCCAGGTGAACTCTTCGTCCC 3´ CGGCTTCCAGAATCTTCTCTACAGG 3´	52	694
RXRβ	´TTCTCCTCCTGGCCCACCTCTTACC 3´ CCGCAGCAGTATGACCTGATCGTCC 3´	52	796
PPARγ	5´CCTCATGAAGAGCCTTCCAA 3´ 5´TTTTTGTGGATCCGACAGTTT 3´	55	177
β-actin	5´GTGGGCCGCTCTAGGCACCA 3´ 5´CTCTTTCATGTCACGCACGATTTC 3´	58	560

Statistical Analysis

The presented data are mean values ± 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 indicting 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 3µM lycopene for 5 and 10 days.

	Total		Membrane		Cytoplasm		Nucleus	
	Incorporation (%)							
Incubation time (days)	**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	4,66
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 µM 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 (0.625-20μM) 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 μM) 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 (1-5μM) for 2 (A) and 7 days (B). The results are expressed as mean ± 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 μM) showed an increased expression of RXR-α, RXR-β,and PPARγ 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 (1-5μM) and nuclear receptor expression RXR-α (A), RXR-β (B), PPAR-γ (C) and RAR (D). The results are expressed as mean ± 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 PPARγ 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 PPARγ 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. PPARγ/RXR heterodimers regulate transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism, neurodegenerative disorders, cancer and inflammation [48]. PPARγ 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 PPARγ/RXR ligands, other tissues also regulate the effects of these receptors. The activity of the PPARγ/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 PPARγ. 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 PPARγ and RXRα, a direct target gene for the PPARγ: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 PPARγ 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.

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

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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-*a* did not change upon treatment. Tomato sauce and lycopene supplementation did not increase interleukin-1*b* 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

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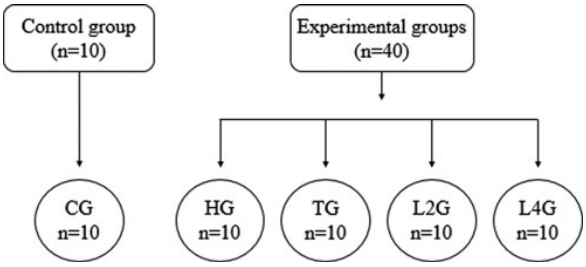

FIG. 1. Dietary protocol. Experimental model: CG: standard diet plus water; experimental groups: HG. high-fat diet plus water; TG. high-fat diet plus solution with tomato sauce and water; L2G. high-fat diet plus solution with 2.0 mg all-trans lycopene and water; L4G. high-fat diet plus solution with 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.

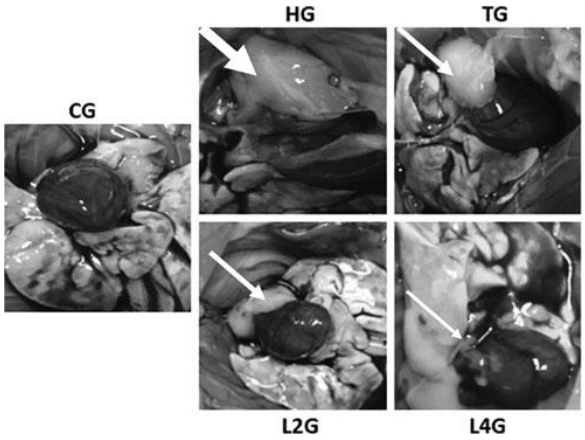

FIG. 2. Heart extracted from sacrificed rats after dietary protocol. *White arrows* indicate accumulated fat in tissues.

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:

$$[(\text{Heart=liver weight (g)}/\text{body weight (g)}) \cdot 100]$$

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*b*, and tumor necrosis factor (TNF)-*a* 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 *l*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 G_0/G_1 (2n), S phase (>2n and <4n), and G_2/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 *l*L of binding buffer containing 5 *l*L of Annexin V fluorescein isothiocyanate and 5 *l*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 – 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 – 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 – 1.34[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 – 4.01[b]
Triglycerides	31.60 – 8.17[a]	47.00 – 7.94[b]	41.20 – 7.66[b]	40.00 – 6.20[b]	33.25 – 7.68[a]
AST	195.60 – 52.03[a]	219.80 – 33.39[a]	198.20 – 52.77[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 – 5.81[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.

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*b* 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-*a* among the groups (Fig. 3).

Cell cycle analysis of cardiac cells showed that the HG group presented a lower percentage of cells in the G_0/G_1 phase (47.98 – 6.28), compared with the other groups (Table 3). Furthermore, a high-fat diet increased the percentage of cells in the G_2/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 G_0/G_1 phase and increased the percentage of cells in the G_2/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 G_0/G_1 phase and decreased numbers in the G_2/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

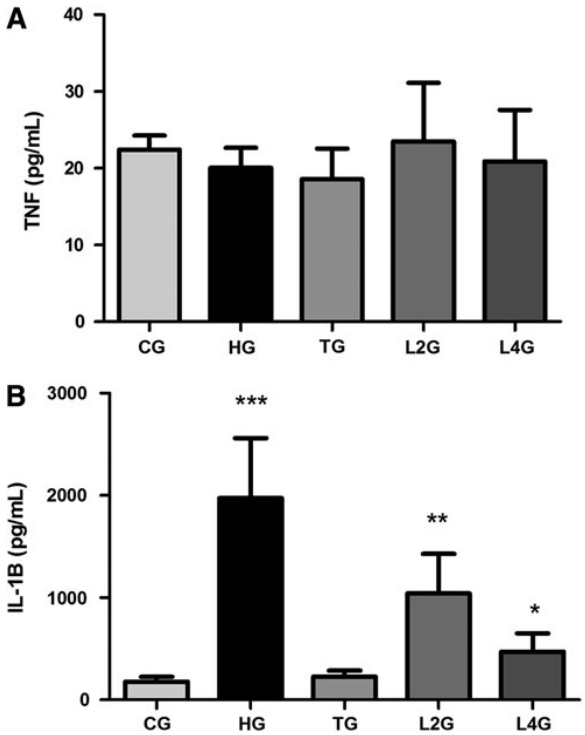

FIG. 3. (A) TNF-*a* (B) IL-1*b* from rats fed a high-fat diet supplemented with lycopene and tomato sauce. *$P < .05$; **$P < .01$; $P < .001$ ANOVA–Tukey's test. ANOVA, analyis of variance; TNF, tumor necrosis factor; IL, interleukin.

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.

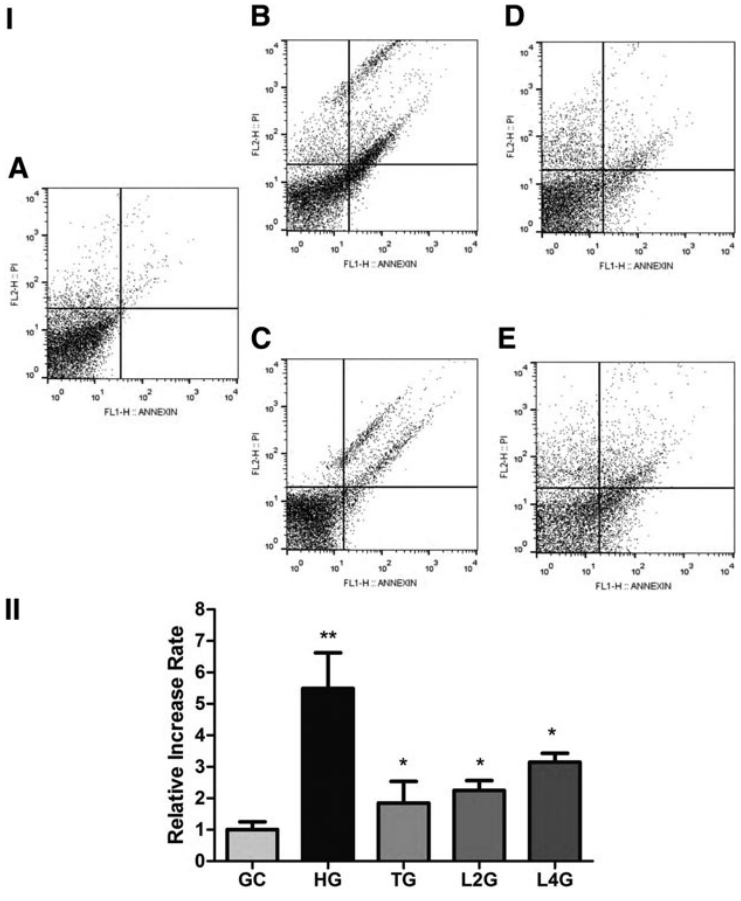

FIG. 4. Monitoring of cell death of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (I) Flow cytometry analysis of cardiac cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (II) Quantitative effects of lycopene and tomato sauce of 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.

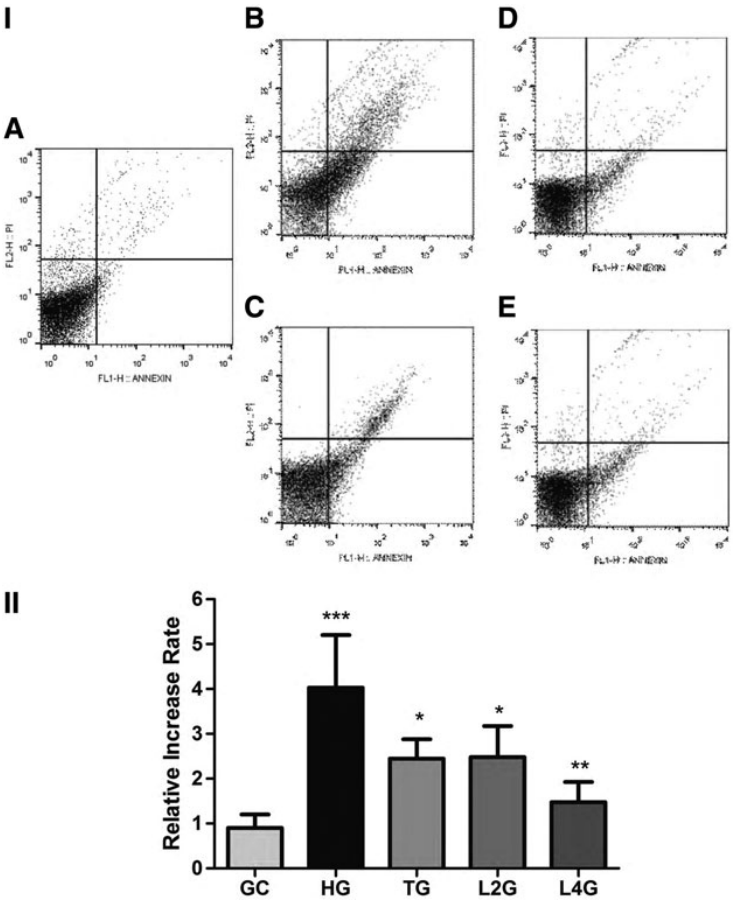

FIG. 5. Monitoring of cell death of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (A) Flow cytometry analysis of hepatic cells from rats fed a high-fat diet supplemented with lycopene and tomato sauce. (B) Quantitative effects of lycopene and tomato sauce of 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. $*P < .05$. $*P < .01$.

Stress factors can induce IL-1*b* and TNF-*a*. Proinflammatory cytokine IL-1*b* 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*b* 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 G_0/G_1 phase and an increase in the percentage of cardiac cells in the G_2/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 G_0/G_1 phase and a decrease in the cell percentage in the G_2/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 G_0/G_1 phase and a decrease in the number of cardiac cells in the G_2/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 G_0/G_1 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 G_0/G_1 and G_2/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.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

SUPPLEMENTARY MATERIAL

Supplementary Table S1

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

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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 G_0/G_1 and G_2/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 G_0/G_1 phase and an increase in S and G_2/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 comsumption 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 μM) 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 μM). 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 cm^2

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 CO_2 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/cm^2 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-2yl)-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 — average absorbance value of experimental group/average absorbance value of control group) × 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 ± 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 μg/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 (μg/g)	*Cis*-Lycopene (μg/g)	*Trans*-Lycopene (μg/g)	Lycopene Content (%)
Ketchup	147.81 ± 8.35 [a]	9.20 ± 0.89 [a]	133.39 ± 6.64 [a]	96.48 ± 0.36 [a]
Tomato Extract	85.60 ± 1.09 [b]	6.48 ± 0.88 [b]	74.94 ± 1.73 [b]	95.12 ± 0.22 [b]
Tomato Sauce	168.95 ± 5.36 [c]	7.40 ± 0.49 [b]	155.94 ± 7.45 [c]	96.65 ± 1.63 [a,b,c]
Tomato Paste	77.57 ± 1.81 [d]	5.05 ± 0.40 [b]	70.80 ± 2.09 [b]	97.78 ± 0.10 [c]

Lycopene content (%) was measured through the following formula: (cis-lycopene + trans-lycopene) × 100/total carotenoids. The data are expressed as mean ± 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 cellline.

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 μg/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.

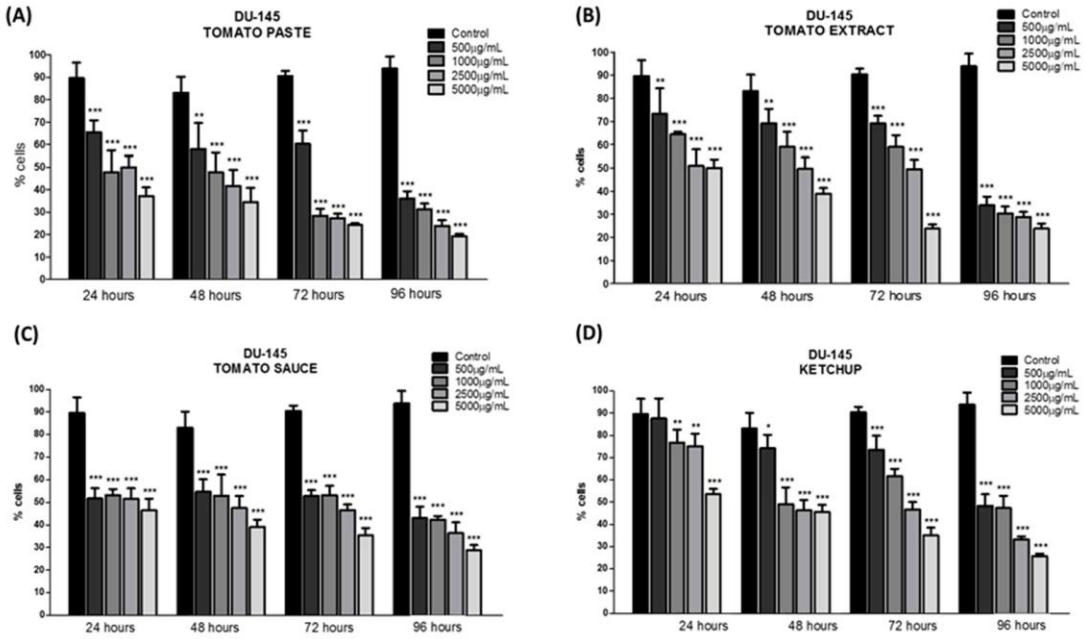

Figure 1. Effect of lycopene obtained of tomato paste (**A**), tomato extract (**B**), tomato sauce (**C**), and ketchup (**D**) on DU-145 cell viability after 24, 48, 72, and 96 h of exposure, respectively. The results are expressed as mean ± error standard and significant differences between untreated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

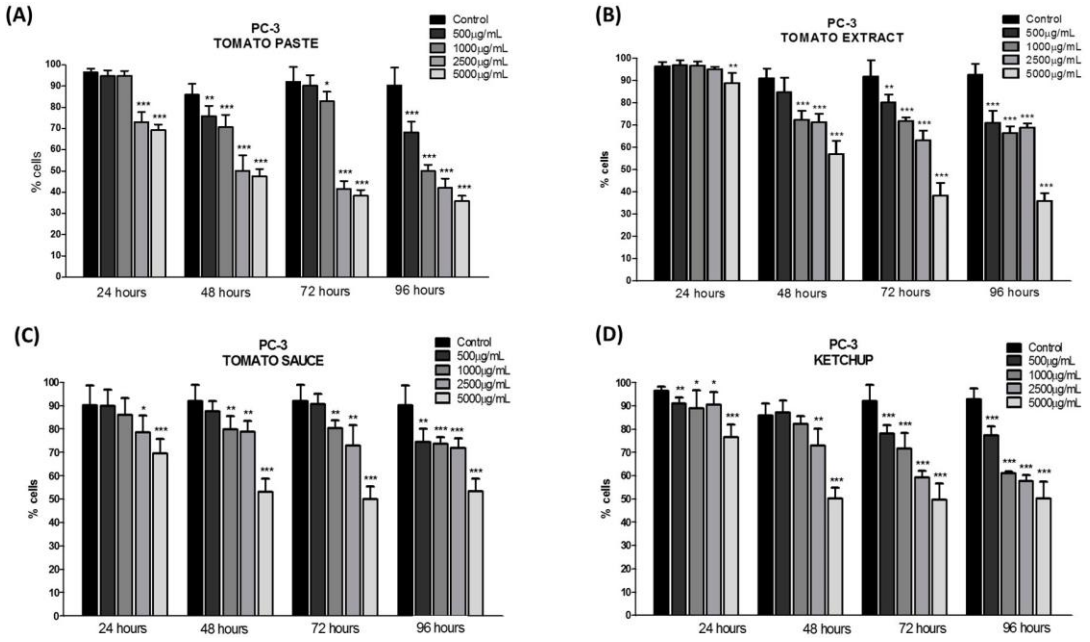

Figure 2. Effect of lycopene obtained of the tomato paste (**A**), tomato extract (**B**), tomato sauce (**C**), and ketchup (**D**) on DU-145 cell viability after 24, 48, 72, and 96 h of exposure, respectively. The results are expressed as mean ± error standard and significant differences between untreated cells (Control) and those treated with lycopene (500, 1000, 2500, and 5000 μg/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 μg/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 μg/mL) after 96 h of treatment. In addition, lycopene extracted from ketchup (500 μg/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 μg/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).

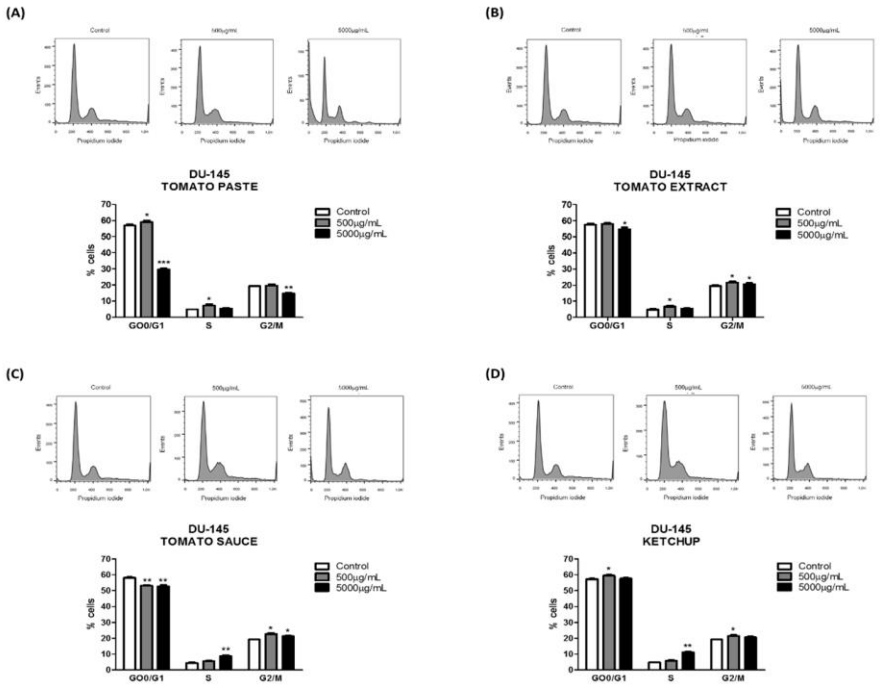

Figure 3. Effect of lycopene obtained from tomato paste (**A**), tomato extract (**B**), tomato sauce (**C**), and ketchup (**D**) on DU-145 cell cycle after 96 h of exposure, respectively. The results are expressed as mean ± error standard and significant differences between untreated cells (Control)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

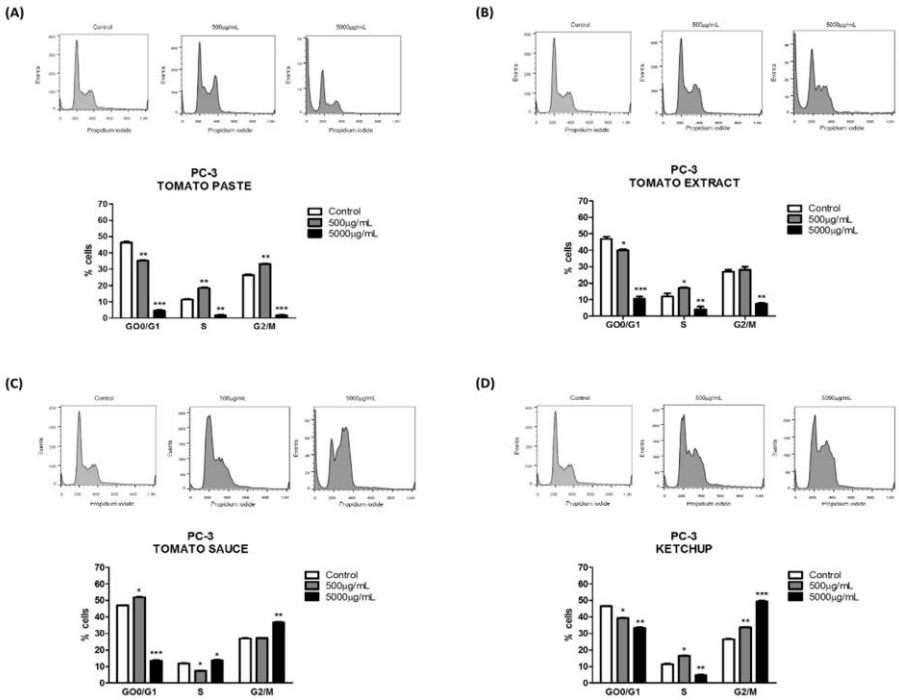

Figure 4. Effect of lycopene obtained of tomato paste (**A**), tomato extract (**B**), tomato sauce (**C**), and ketchup (**D**) on PC-3 cell cycle after 96 h of exposure. The results are expressed as mean ± error standard and significant differences between untreated cells (Control) and those treated with lycopene (500 and 5000 µg/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 G_0/G_1 and G_2/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 G_0/G_1 phase can be reverted, and cells can proceed with proliferation after interruption of the treatment, G_2/M arrest potentially leads to apoptosis. However, lycopene extracted from tomato sauce and ketchup decreased the percentage of cells in G_0/G_1 phase and increased the percentage of cells in S and G_2/M phases after 96 h of treatment. The cell cycle arrest in G_2/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 G_1/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 ^{e}	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.

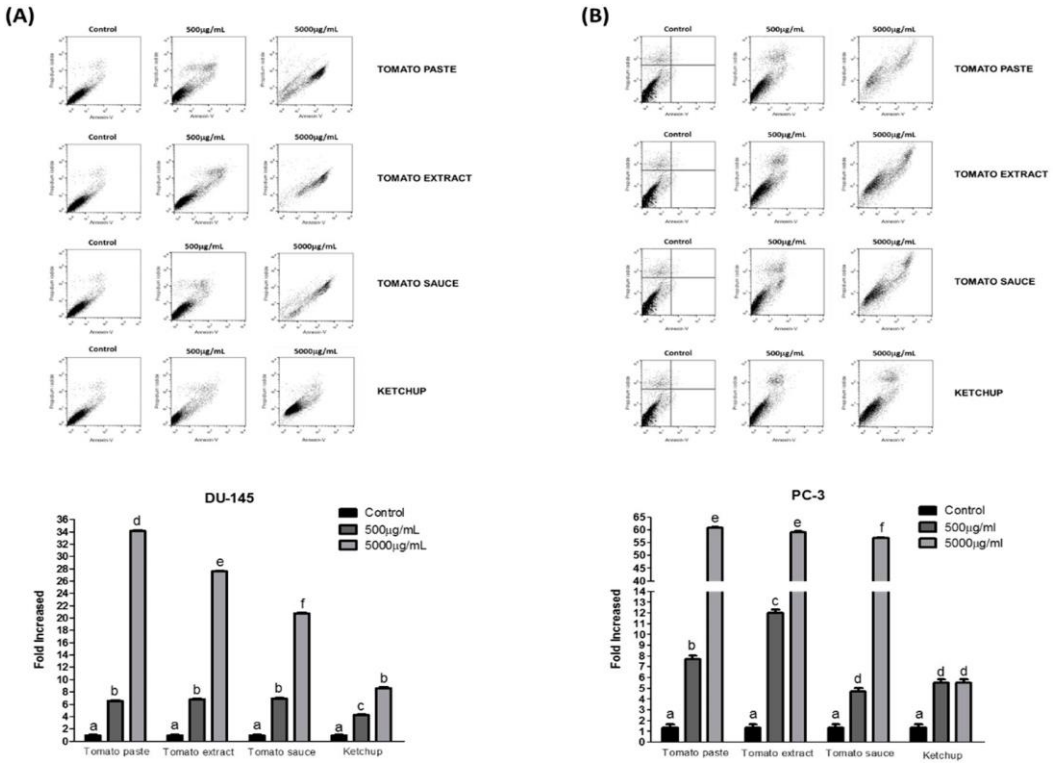

Figure 5. The effect of lycopene obtained from tomato paste, tomato extract, tomato sauce and ketchup in the process of programmed death in DU-145 (**A**) and PC-3 (**B**) cells the after 96 h treatment. The flow cytometric analyses are shown according to the exposure time and carotenoid concentration. The quantitative results of lycopene on cell lines are shown after 96 h. The data are expressed as mean ± 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 G_0/G_1 and G_2/M phases after 96 h of treatment. However, tomato sauce and ketchup extract decreased the percentage of cells in G_0/G_1 phase and increased percentage of cells in S and G_2/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**).

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

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