www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online; ISSN 1308-5301 Print 8/1 (2015) 1-6

Research article/Araştırma makalesi

Effects of α-lipoic acid and resveratrol on rats cancer model induced by 7, 12-DMBA

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Abstract

α-Lipoic acid (LA) has been intensely investigated as a therapeutic agent for several diseases, including hepatic disorder and diabetic polyneuropathy. However, the effects of LA or its reduced form, dihydrolipoic acid (DHLA), on cancer chemoprevention has been slightly reported. In the present study, we examined the effects of DHLA/LA on rat cancer model induced by 7,12-dimethylbenz(a)anthracene (DMBA). Another therapeutic agent; Resveratrol, was found in high concentrations in berry, grape, peanut, pine and wine and act as an antioxidant and antimutagen. In addition, resveratrol and α-lipoic acid inhibited the development of preneoplastic lesions in carcinogen-treated rat cancer model. Datas in this study suggest that resveratrol and α-Lipoic acid, a common constituent of the human diet, merits investigation as a potential cancer chemopreventive agent in humans.

Key words: *α-Lipoic acid, resveratrol, DMBA, fatty acids, vitamins*

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Ratlarda 7, 12-DMBA ile oluşturulan kanser modelinde α-lipoik asit ve resveratrol'un etkileri

Özet

α-Lipoik asit (LA) diyabetik polinöropati ve hepatik hasarları kapsayan çeşitli hastalıklarda terapatik ajan olarak yoğun olarak araştırılmıştır. Bununla birlikte LA ve indirgenmiş formu olan dihidrolipoik asit (DHLA) kanserin, kimyasal yollardan önlenmesi üzerine etkileri rapor edilmiştir. Bu çalışmada DHLA/LA'in 7,12-dimetilbenzentrasen tarafından oluşturulan kanser modeline etkileri araştırılmıştır. Diğer bir terapatik ajan olan resveratrol, antioksidan ve antimutajen olarak dut, üzüm, fıstık, çam ve şarapta yüksek konsatrasyonlarda bulunmuştur. Bunlara ek olarak LA ve resveratrol, ratlarda oluşturulan kanser modelinde kanserojen tedavide preneoplastik lezyonların gelişimini inhibe etmiştir. Bu çalışmada elde edilen veriler, resveratrol ve α-lipoik asitin insanların beslenmelerinde yaygın bir bileşen ve insanlarda kanserin kimyasal olarak önlenmesinde potansiyel bir ajan olduğunu göstermiştir.

Anahtar kelimeler: *α-Lipoik asit, resveratrol, DMBA, yağ asitleri, vitaminler*

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) were identified as carcinogens in 1921 (Bloch, 1921). Since then, studies have been conducted on how the carcinogen functions and; how the mechanism functions between key biological components and PAH (Bloch, 1921). DMBA is widely used as laboratory carcinogen. DMBA has potential mutagenic and carcinogenic characteristics. In vivo and in vitro studies indicate that rat models which are administered with DMBA or are detected with mutagenic responses show a significant increase in number of tumor cells (Khaidakov et al., 2001).

Resveratrol is a phytoalexin found in high concentration in peanuts, grapes and red wine; it exhibits antitumoral, cardioprotective, anti-inflammatory, antioxidant and estrogenic activities (Yang et al., 2013; Park et al., 2012; Bobermin et al., 2012). Resveratrol also presents neuroprotective properties and has been investigated in several

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neurodegenerative models, such as epilepsy, stroke and Alzheimer's and Parkinson's diseases (Bastianetto and Quirion 2010; Fukui et al., 2010; Shetty, 2011).

Lipoic acid is a natural compound widely distributed in plants and animals and functions as a cofactor within mitochondrial enzymes to catalyze the oxidative decarboxylation of alpha-keto acids and glycine cleavage (Ide et al., 2013) however, some researchers have reported protective effect of α-lipoic acid against acetaminophen-hepatotoxicity (Shimaa et al., 2014), miyocardial post ischemia (Magdalena et al., 2014), hypothermia (Sylvia et al., 2014) and liver cirrhosis (Shimaa et al., 2014).

2. Materials and methods

In this study, a total 42-old (16 month aged) male Wistar rats were used after the approval of Firat University local research ethics committee (Official form date and number: 27.07.2006 and 9/3). The animals were housed in cages with air-conditioned room with a 12-h light/12-h dark cycle, and were randomly divided into four groups. The first group was used as a control, the second group DMBA, third group α-LA+DMBA and fourth group R+DMBA. Rats in the DMBA, α-LA+DMBA and R+DMBA groups were injected intrapeitoneally three dose DMBA total 55 mg/kg in sesame oil. After administration of DMBA 6 days, the rats in α-LA+DMBA group was injected α-lipoic acid 25mg/kg and R+DMBA group was injected resveratrol 25 mg/kg on alternate days for 6 weeks. In addition, the rats in control group were injected sesame oil. These treatments were continued for 7 weeks, after which time each experimental rat was decapitate and serum samples were collected and stored in -25ºC prior to biochemical analysis.

2.2. Determination of lipid soluble vitamins

Two hundred microliters of serum samples were homogenized in 3ml acetonitrile/methanol/isopropanol (2/1/1, v/v/v) containing tubes and the samples were vortexed for 30s and centrifuged at 6000 x g for 10 min at 4 ºC. Supernatants were transferred to autosampler vials of the HPLC instrument. For lipophylic vitamins, the mixture of acetonitrile/methanol (3/1, v/v) was used as the mobile phase and the elution was performed at a flow-rate of 1ml/min. The temperature of column was kept at 40 ºC. Supelcosil^{TM} LC 18 DB column (250 mm x 4.6mm, 5µm; Sigma, USA) was used as the HPLC column and detection was performed at 320 nm for retinol (vitamin A) and 215 nm for vitamins D2, D3 and K1, α-tocopherol. Identification of the individual vitamins was performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. Quantification was carried out by external standardization using *Class VP Software*. The results of analysis were expressed as µg/dl.

2.3. Cholesterol analysis

Two hundred microliters of serum was extracted in 3 ml acetonitrile/ isopropanol (70:30, v/v)-containing tubes and the mixture were mixed by vortex for 30 s and centrifuged at 6000 x g for 10 min at 4ºC. Supernatants were transferred to autosampler vials of the HPLC instrument. Acetonitrile/isopropol (70:30) was used as mobile phase at a flow rate of 1ml/min. Supelcosil LC 18^{TM} DB column (250 mm x 4.6 mm, 5µm) was used as the HPLC column. Detection was performed by UV at 202 nm and 40 ºC column oven (Katsanidis and Addis, 1999). Quantification was carried out by external standardization using *Class VP software.* The results were expressed as µmol/dl.

2.4. Vitamin C and MDA

Two hundred microliters of serum sample was homogenized in 2.5 ml 5mM 1-hexane sulphonic acid sodium salt % 0.1 H_3PO_4 containing tubes. Protein were precipitated by the addition 0.5 ml metaphosphoric acid (5 %, w/v) and then samples were centrifuged at 10.000 x g for 5 min. Supernatants were transferred to autosampler vials of the HPLC instrument. Discovery RPAmide C16 (150 mm x 4.6 mm, 5µm) was used as the HPLC column. 5 mM 1-hexane sulphonic acid sodium salt % 0.1 H_3PO_4 and acetonitrile (90% + 10%) were used as the mobile phase and the flow rate was 1ml/min. Detection was performed at UV 244 nm by UV detector and 37 ºC column oven. Quantification was carried out by external standardization using *Class VP software.* The results were expressed as nmol for MDA and µg for VC.

2.5. Extraction of lipids and preperation of fatty acid methyl esters

The lipids of serum samples were extracted by the method of Hara and Radin (1978). 0.5 serum samples were homogenized in 5 ml hexane isopropanol mixture at 3:2 (v/v) for 30 seconds. Samples were centrifuged at 4500 rpm for 10 minutes; supernatant parts were transfered to covered tubes Fatty acids of the lipid extract were converted to methyl esters by using 2 % sulfuric acid (v/v) in methanol (Christie,1992).

Fatty acid methyl ester forms were extracted with n - hexane. Analysis was performed in a Shimadzu GC-17A V3 intstrument gas chromatograph equipped with a flame ionization detector (FID) and a 25 m, 0.25 mm i.d.

Permabond fused-silica capillary column (Machhery –Nagel, Germany). The oven temperature was programmed between 120–220 °C, 5 °C/min. Injector and FID temperatures were 240 and 280 ° C, respectively. The nitrogen carrier gas flow was 1 ml / min. The methyl esters of fatty acids were identified by comparison with authentic external standard mixtures analyzed under the same conditions. *Class GC 10* software version 2.01 was used to process the data. The results were expressed as percent (%).

2.6. Statistical analysis

The experimental results were reported as mean ± SEM. Statistical analysis was performed using SPSS Software. Analysis of variance (ANOVA) and an LSD test were used to compare the experimental groups with the controls.

3. Results and discussion

Serum cholesterol level increased in the DMBA+ α-LA group when compared with the control group, and that it decreased in the DMBA+R group. The high cholesterol level in the DMBA+LA group can be explained by the effect of α-LA. α-LA is a potent antioxidant and also functions as the cofactor (Navari-Izzo et al., 2002) of pyruvate dehydrogenase enzyme (PDH) which is responsible for energy metabolism. Under normal conditions, α-LA is partially synthesized cell. However, injected α-LA increases the activity of the enzyme, in addition to being an antioxidant (Packer, 1998). PDH causes increased formation of citrate because of increased PDH activity and turnover of the tricarboxylic acid cycle. Following the necessary energy provision by the cell, it carries the citrate molecules to the cytoplasm and transforms to cholesterol and fatty acid. The increase of serum cholesterol level in DMBA+ α-LA can be explained by the increase of this mechanism. A study by Mukhopadhyay et al, (2008) reported that cholesterol uptake and content were significantly increased in the tumors.

Table 1. The results of some biochemical parameters in serum

Parameters	Control	DMBA	DMBA+α-LA	DMBA+R
Retinol (µg/dl)	0.261±0.009	**0.181±0.008** [c]	0.270±0.007	**0.215±0.004** [b]
D2 (µg/dl)	0.12±0.008	**0.23±0.015** [c]	**0.51±0.031** [c]	**0.32±0.011** [c]
D3 (µg/dl)	1.32±0.12	**4.00±0.20** [c]	**4.70±0.13** [c]	**3.57±0.11** [c]
α-tocopherol (µg/dl)	4.38±0.15	**3.30±0.18** [c]	**2.02±0.15** [c]	**3.57±0.24** [b]
K_1 (µg/dl)	0.095±0.003	**0.151±0.005** [c]	**0.120±0.003** [b]	**0.116±0.006** [a]
Cholesterol(µmol/dl)	119.76±2.78	131.40±5.91	**145.42±5.80** [c]	**103.82±5.20** [a]
VC (µg/dl)	22.25±0.94	19.58±0.90	19.70±1.23	20.45±1.43
MDA (nmol/dl)	72.38±3.79	**87.42±4.03** [a]	**63.00±2.92** [a]	**54.00±2.34** [b]

a- $p<0.05$ b-$p<0.01$ c-$p<0.001$ d-$p<0.0001$

The amount of MDA, is one of end-product of lipid peroxidation (Senturk et al., 2012), increased in serum samples of the DMBA group but decreased in samples of the DMBA+R group. Free radicals which are formed as endogenous or exogenous agents cause lipid peroxidation by affecting biomolecules in membrane structure because of their high reactivity. Free radicals, which are formed as endogenous or exogenous agents, cause lipid peroxidation by affecting biomolecules in membrane structure because of their high reactivity. The peroxides are easily catabolized to produce secondary products such as MDA. The high level of MDA in the DMBA group indicates that 7, 12-DMBA increased the oxidative stress by having a radical effect or forming reactive radical molecules. The level of MDA in groups which were administered with lipoic acid and resveratrol as well as DMBA was even lower than in the control group. This finding shows that these two antioxidants are effective against lipid peroxidation. The results of the present study are in line with the findings of previous studies within the literature. A study by Arivazhagan et al. (2003) reported that supplementation of lipoic acid in aged rats prevents the elevated levels of lipids.

Tocopherol has antioxidant properties (Kan, 2014). The level of serum α-tocopherol decreased in the DMBA, DMBA+ α-LA and DMBA+R groups ($p<0.01$) when compared with the control group. The amount of α-tocopherol decreased as a result of oxidative harm of 7, 12-DMBA and this damage was partially tolerated with resveratrol and lipoic acid. This reduction is line with the findings of Anbuselvam et al. (2007). The study by Anbuselvam reported that non-enzymic antioxidants like glutathione, ascorbic acid and α-tocopherol were decreased in cancer-bearing animals induced by DMBA when compared to control animals. The significant reduction of α-tocopherol in the group which was administered with lipoic acid can arise from the insufficiency of lipoic acid in activating α-tocopherol radical or usage of α-tocopherol in neutralizing radical molecules (Scholich et al., 1989).

The levels of D2 and D3 vitamins increased in the DMBA, DMBA+ α-LA and DMBA+R groups. A study by Zinser et al. (2003) reported that 1,25-dihydroxy-vitamin-D-3, the active molecule of D-3, prevented cellular development in breast cancer *in vivo* and *in vitro* in their model study with 7,12-DMBA. Active D3 increases

absorbtion of calcium and phosphate from intestinal epithelium. The increases of active D3 in plasma, controls with calcium ion concentration with inverse ratio (Guyton and Hall, 1996).

The increase of D2 and D3 can be a result of the capacity increase due to the resveratrol which was administered intraperitoneally and interoperationally with the antioxidants.

Table 2. The results of fatty acids in serum

Fatty Acids	Control	DMBA	DMBA+LA	DMBA+RES
16:0	19.30±0.30	**16.95±0.71** [b]	**16.68±0.39** [b]	17.72±0.08
16:1n7	1.84±0.23	1.11±0.21	1.34±0.17	**0.97±0.06** [a]
18:0	8.54±0.37	**11.48±0.46** [c]	6.85±0.33	8.81±1.04
18:1 n-9	11.61±0.37	12.74±0.70	11.89±0.86	13.74±1.60
18:1 n-7	2.15±0.11	2.39±0.12	2.23±0.08	2.42±0.28
18:2 n-6	25.00±0.97	**21.56±1.29** [b]	**22.99±0.56** [a]	24.78±1.05
18:3 n-3	0.72±0.11	0.66±0.07	0.50±0.06	0.64±0.39
20:4 n-6	27.00±1.31	**23.02±1.26** [c]	**31.60 ±1.93** [d]	**25.67±1.16** [b]
20:5 n-3	0.57±0.03	0.52±0.12	0.74±0.05	0.31±0.03
22:4 n-6	0.40±0.03	3.77±0.66	0.53±0.03	0.63±0.04
22:5 n-6	0.20±0.01	0.37±0.03	0.82±0.05	0.41±0.05
22:5 n-3	0.54±0.04	**0.87±0.04** [b]	0.83±0.09	0.83±0.07
22:6 n-3	2.13±0.10	**4.60±0.40** [c]	**2.90±0.19** [b]	**3.10±0.36** [b]
∑ Saturated	27.84±0.67	**28.43±1.17** [b]	**23.53±0.72** [b]	**26.53±1.12** [a]
∑ Unsaturated	*72.16*±3.31	***71.61*±4.90** [a]	***76.37*±4.07** [b]	*73.50*±5.09
∑ MUFA	15.60±0.71	**16.24±1.03** [a]	15.46±1.11	**17.13±1.94** [b]
∑ PUFA	56.56±2.60	**55.37±3.87** [b]	**60.91±2.96** [d]	56.37±3.15
∑ n-3	3.96±0.28	**6.65±0.63** [c]	**4.97±0.39** [b]	**4.88±0.85** [b]
∑ n-6	52.60±2.32	**48.72±3.24** [c]	**55.94±2.57** [b]	**51.49±3.30** [b]

a- $p<0.05$ b-$p<0.01$ c-$p<0.001$ d-$p<0.0001$

The fatty acids of serum fatty acid composition such as myristoleic (14:0), palmitic (16:0), palmitoleic (16:1, n–7), stearic (18:0), and oleic (18:1 n–9) are synthesized endogenously in the tissues. 16:0 is the ultimate product of lipogenesis. The fatty acid is synthesized by fatty acid synthase (FAS) and released with different enzymatic reactions. Then, according to cellular needs, it was used in the synthesis of phospholipids, sphingolipids, triglycerides, cholesterol esters, and also the synthesis of palmitoleic acid (16:1, n–7–16:1 n–9) by Δ^9 desaturase (stearoyl CoA) and the synthesis of stearic acid by elongase enzymes (Ntambi, 1999).

Stearoyl CoA desaturase (Δ^9 desaturase) using palmitic (16:0) and stearic acid (18:0) as substrate catalyzes the formation of single double bonds such as 16:1 with 16:0, n9 with n7, 18:1 with 18:0 and n-7 with n-9. 18:0 fatty acid is synthesized by chain extension reaction from 16:0 fatty acid. When the amount of endogenously synthesized fatty acids is taken into consideration, the biochemical and physiological importance of stearoyl CoA desaturase enzyme for organism, tissue and cells can be better understood. This enzyme, which provides a single double bond access between 9-10th C following the –COO carbon atom, has an important function in the viscosity of cellular membranes. The activities of both FAS and Δ^9 desaturase are affected by nutrition, different hormones and substances in diets (Ntambi, 1999).

The level of palmitic acid and palmitoleic acid partially or significantly decreased in the DMBA and antioxidant groups. A study by Çelik and Özkaya et al. (2002) conducted a study on animals which were administered lipoic acid in their muscle tissues. They found that the amount of palmitic acid was lower in their lipoic acid group than the control group; however, the same study reported that lipoic acid in the liver did not affect the amount of palmitic acid.

The level of serum linoleic acid (18:2 n-6) decreased in all groups when compared with the control group. The increase of arachidonic acid in the DMBA+ α-LA group can be a sign of intensive activation of the Δ^6 desaturation pathway. This can result from the metabolic effects of lipoic acid. A previous study assessed that lipoic acid which was administered against the effects of H_2O_2 reduced the level of linoleic acid in brain tissues of subjects and increased the level of arachidonic acid (Çelik and Özkaya, 2002). A study examining the effects of resveratrol on old female rats which were administered with $KBrO_3$ found that linoleic acid decreased in lung, serum, kidney and liver tissues and that the level of arachidonic acid increased (Keser, 2006).

The decrease of both linoleic acid and arachidonic acid in the DMBA group when compared with the control group can be associated with eikosanoid (prostaglandins, leukotrienes an thromboxanes) synthesis. Eicosanoids are synthesized by the lipoxygenase and cyclooxygenase enzymes from the arachidonic acid. The molecules are found in cells at a certain level; however, this can vary in line with physiological, biochemical and toxological conditions. The

decrease of arachidonic acid can be associated with the increase in activities of the lipoxygenase and cyclooxygenase in eikosanoid synthesis.

Docosahexaenoic acid (DHA) (22:6 n–3) is one of the ultimate products of Δ6 desaturation. Studies state that the docosahexaenoic level increases in disease conditions. The enzymes responsible for synthesis can be affected by disease factors (Jelinska et al., 2003; Brener, 2000). In the present study, the level of docosahexaenoic acid was high in the DMBA group. The level also increased in both the disease and DMBA+antioxidant groups. However, these increases were less than the DMBA group. This can be associated with the effects of resveratrol and lipoic acid.

Acknowledgements

I would like to thank FUBAP (Firat University Scientific Research Found) for the financial support it provided to this project (Project Code No: 1312).

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(Received for publication 16 October 2014; The date of publication 15 April 2015)