İstanbul Üniv. Vet. Fak. Derg.
40 (2), 183-190, 2014
Araştırma Makalesi

J. Fac. Vet. Med. Istanbul Univ.
40 (2), 183-190, 2014
Research Article

Effects of Curcumin on Apoptosis in "In Vivo" Solid Ehrlich Ascites Tumor Model in Balb-C Mice[#]

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Geliş Tarihi / Received: 09.07.2013

ABSTRACT

Curcumin is the yellow pigment in turmeric *(Curcuma longa L)* that is widely used as a spice, food coloring (curry) and preservative. Curcumin has a wide range of pharmacological properties including anti-inflammatory, anti-tumor promoter and anti-oxidant effects. In this study, the development and quantity of apoptosis in tumor tissue of solid Ehrlich Ascites Tumor model developed balb-c mouse by applying 100 mg/kg curcumin in different time periods were investigated. For this aim; 54 Balb-c female mice, constituted in vivo tumor by subcutaneous injection of $3x10^6$ EAT cells, were divided into 3 experimental and 3 control groups. Using gavage method, 100 mg/kg curcumin dissolved in 9% alcohol was applied to experimental groups, 9% alcohol was applied to control groups in different time intervals. Long and short diameters measurement, and Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin Nick End Labeling (TUNEL) and hematoxylin-eosin staining were applied to the extracted tumors of all groups and findings were supported with electron microscopic (TEM) evaluations. In long and short diameter measurement of acquired tumors, no significant differences were obtained ($P>0.05$). In microscopic evaluations different results were obtained from cell count of all groups. More apoptotic cells were determined in experimental group 2 ($P<0.001$) and 3 ($P<0.05$) than their control groups. According to statistical analysis, there was no significant difference between experimental group 1 that received longest-term curcumin and control 1 ($P>0.05$). Overall, the long-term 100 mg/kg applications of curcumin do not have any effect on apoptosis.

Key Words: Apoptosis, curcumin, ehrlich ascites tumor, mice

ÖZET

EHRLICH ASCITES TÜMÖRÜ İLE BALB-C FARELERDE OLUŞTURULMUŞ SOLİD TÜMÖR MODELİNDE CURCUMİNİN APOPTOZ ÜZERİNE ETKİLERİ

Curcumin, turmeric (*Curcuma longa L*) de bulunan, yaygın olarak baharat, yiyecek reklendirici ve koruyucu olarak kullanılan sarı renkli pigmentdir. Curcumin anti-inflamatuar, anti-tümöral ve anti-oksidan gibi çeşitli farmakolojik özelliklerinden dolayı yaygın bir kullanıma sahiptir. Bu çalışmada, Balb-c farelerde geliştirilen Ehrlich Ascites Tümör modelinde farklı periyotlarda 100 mg/kg curcumin uygulamasının apoptosis miktarı ve gelişimi üzerine etkisi incelendi. Bu amaçla; 54 adet Balb-c dişi fare, in vivo tumor oluşturmak için $3x10^6$ EAT hücresinin subkutan olarak enjeksiyonunu takiben 3 deney ve 3 kontrol grubuna ayrıldı. Gavaj metodu kullanılarak %9 alkol içerisinde çözündürülen 100 mg/kg curcumin deney gruplarına, %9 alkol kontrol gruplarına farklı zaman aralıklarında uygulandı.

[#] This work is summarized from Corresponding Author's PhD. Thesis.

Tüm gruplardan alınan tümörlerde kısa ve uzun çap ölçümleri, Terminal deoksiribonükleotidil trasferaz-aracılı dUTP-digoksigenin uç işaretleme tekniği (TUNEL) ve hematoksilen-eosin boyama uygulandı. Bulgular ayrıca elektron mikroskobik (TEM) değerlendirmelerle desteklendi. Tümörlerden elde edilen kısa ve uzun çap uzunluklarında herhangi bir farklılık görülmedi (P>0,05). Tüm gruplardan elde edilen hücre sayımlarında mikroskobik değerlendirmelerde farklı sonuçlar gözlendi. Deney Grubu 2 (P<0,001) ve 3 (P<0,05)'de ve Kontrol Gruplarına gore daha fazla apoptotik hücre tespit edildi. İstatistiksel verilere göre, uzun sure curcumin uygulaması yapılan Deney Grubu 1 ve bu gruba ait Kontrol 1 grubu arasında anlamlı bir fark yoktu (P>0,05). Genel olarak, Curcumin'in uzun sure 100 mg/kg uygulamasının apoptoz üzerinde herhangi bir etkisi yoktur.

Anahtar Kelimeler: Apoptoz, curcumin, ehrlich ascites tümör, fare

Introduction

Curcumin is a yellow colored pigment obtained from rhizomes of Curcuma longa. It is used to give color to cheese, butter and cosmetics (Conney et al.,1991; Huang et al., 1997; Limtrakul et al., 1997; Scartezzini and Speroni, 2000). When we look at the result of in vivo and in vitro studies of curcumin which we take with lots of food and drinks naturally, we see that it has anti-oxidant, anti-tumoral, anti-inflammatory and apoptotic effects (Conney et al., 1991; Huang et al., 1997; Ruby et al., 1995). Recently intensive investigations were made especially about the apoptotic effect of curcumin in cancer. Especially its anti-oxidant effect has been investigated greatly and it has been reported that it protects DNA with its free radical holding effect from oxidative damage (Sikora et al., 1993). It is also reported that curcumin decreases NO production in mouse liver by inhibiting INOS gene expression (Chan et al., 1998). It has been shown in histologic sections of kidneys of streptozotocin induced diabetic Wistar albino rats that it has a healing affect on diabetic nephropathy with 0.5% curcumin diet (Babu and Srinivasan, 1997; Babu and Srinivasan, 1998). In studies with rat thymocytes, it has been reported that curcumin has cell contracting, membrane permeability increasing, and mitochondrial membrane potential decreasing effects (Jaruga et al., 1998a). Also in literature (Linke et al., 1997) it has been reported that in tissue cultures it stimulated apoptosis in human hepatoma and leukemia cells and constitute apoptosis in human basal cell carcinoma cells with single dose and time interval doses.

In this study, the effects of applying 100 mg/kg curcumin in different time periods in "in vivo" solid ehrlich ascites tumor (EAT) model developed balb-c mouse on the development and quantity of apoptosis were evaluated with TUNEL and haematoxylin-eosin (HE) staining. All results were supported with electron microscopic evaluations.

Materials and Methods

Animals

Young-adult, female 54 balb-c mice weighting average of 20 gr were used in our study. Out of them 3 groups were formed and each group were divided into 3 experimental (n=10) and 3 control (n=8) groups. All animals were fed ad libitum with pellet feed and tap water. The protocol used in this study was approved by the Istanbul University's Ethic Board with the number 36 and date of 16.11.2001.

EAT Application

In order to develop solid tumor, after diluting with saline (0.9% NaCl) in the way it consists $3x10^6$ EAT cells inside 0.5 ml of it, cells were applied subcutaneous from the nape of the neck of the animals in all groups.

Curcumin Application

100 mg/kg doses of curcumin (Sigma C 1386) were diluted in 9% ethyl alcohol and with gavage method in different time intervals applied to all experimental groups. Control groups received only 9% of alcohol in same time intervals.

Experimental Protocol

Group 1: To the experimental group 20 days before the passages 100 mg/kg curcumin was applied. On 20^{th} day EAT cell passages were carried out. Following the passage

curcumin application continued till sacrification day. Approximately on 13th day after passage sacrifications performed. The same procedures were applied to the experimental group only same amount of ethyl alcohol solution was given to them instead of curcumin.

Group 2: Tumor passages were carried out in the control and experimental groups' animals in this group. 11 days after the tumor passage single dose curcumin to experimental group and single dose ethyl alcohol to control group were applied with gavage method. After 48 h all groups were sacrified under ether anesthesia.

Group 3: Tumor passages were carried out in experimental and control groups. Simultaneously with the tumor passages curcumin to experimental group and ethyl alcohol to control group were applied with gavage method every other day. Application continued till the sacrification on 13th day.

Tissues Samples and Examinations

The long and short diameters of extracted tumors from animals' sacrified under ether anesthesia were measured with calipers compass. Some part of the tumor sections were fixed for light and electron microscope (TEM) examinations and passed through routine histologic procedures for apoptotic cells counts in paraffin sections TUNEL technique and hematoxylin-eosin staining methods were used.

TUNEL technique

Apoptag Plus Peroxidase In situ Apoptosis Detection kit (Intergen 2000 S 7101) method was used. 3'OH ends of 180-200 base-double DNA fragments of apoptotic cells were marked in situ with digoxigenin marked dUTP using Terminal deoxyribonukleotidyl transferase enzyme. To control the specificity of staining 5mg/kg dexamethasone (Sigma D1756) applied adult rat thymus tissue was used, for negative control instead of Tdt enzyme distilled water was used.

Apoptotic Cell Count

Apoptotic cell counts were performed in experimental and control groups with Olympus CH20 light microscope, X40 magnification. In TUNEL technique applied specimens 5 animals in experimental group and 4 animals in control group were studied. In 5 different areas of each slide apoptotic cell count was performed. In haematoxylin-eosin applied tissues average of 9-10 slide in experimental group and 6-8 slides in control group were studied, in 10 different areas of each slide apoptotic cells count performed.

Statistical Analyzes

SPSS for windows was used for statistical analysis. Values of apoptotic cells counts belonging to all groups were evaluated statistically with independent samples *t* test and one-way Anova test.

Results

Tumor Diameter Measurements

Tumors started to become evident on average of 9th day in palpation of EAT passage-performed mouse. No mortality was observed in any group till the day of sacrification. The long and short diameters of extracted tumors were measured with calipers compass and no significant result was obtained among the groups ($P>0.05$) (Table 1).

Apoptotic Cell Count with In Situ DNA End Labeling Technique

In all groups' TUNEL technique applied, apoptotic cell nuclei were brown by the peroxidase activity (Figure 1a, b). Mean apoptotic cell counting results in each group are in Table 2. Mean values in experimental and control groups respectively were 3.04, 3.50 in group 1, 4.28, 1.75 in-group 2, and 4.60, 2.65 in-group 3. In independent samples *t* test statistical evaluation of obtained results while there was no significant difference between experimental group 1 and control 1 ($P>0.05$) apoptotic cell count difference between experimental group 2 and control 2 was highly significant ($P<0.001$), and the difference between experimental group 3 and control 3 was significant ($P<0.05$). Besides apoptotic cells in experimental group 3 were markedly darker than control group 3 (Figure 2). Apoptotic cell counts of all 3 experimental groups with 100 mg/kg curcumin applications

were analyzed with one-way Anova test and no statistical significance obtained (Table 2).

Apoptotic Cell Count with Haematoxylin-Eosin Staining Technique

Mean values of apoptotic cell numbers obtained in experimental and control groups respectively were 3.71, 3.25 in group 1, 3.65, 1.95 in group 2, and 3.72, 1.80 in group 3 (Table 3). In statistical evaluation of obtained results with independent samples *t* test the difference between experimental group 1 which received longest-term curcumin and control 1 was not significant (P>0.05). Similar results were obtained with TUNEL technique as well. In statistical evaluation of experimental group 2 and control 2 the results were highly significant as the results obtained in TUNEL technique (P<0.001) (Figure 3a, b). In group 3 the difference between experimental and control groups were significant (P<0.05) (Figure 4a, b). Parallel to our findings in TUNEL technique, there was no significance in one-way Anova statistical analyzes of apoptotic cell counts obtained with hematoxylin-eosin staining of experimental groups.

Table 1. Mean values of long and short diameter measurement of all groups.
Tablo 1. Tüm grupların kısa ve uzun çap ortalama değerleri.

Groups		Mean Values of Long Diameters	$S_{\bar{x}}$	Mean Values of Short Diameters	$S_{\bar{x}}$
Group 1	**Experimental 1**	2.24 mm	0.16	1.24 mm	0.11
	Control 1	1.88 mm	0.18	1.36 mm	0.20
Group 2	**Experimental 2**	2.36 mm	0.13	1.22 mm	0.12
	Control 2	2.35 mm	0.21	1.25 mm	0.08
Group 3	**Experimental 3**	2.28 mm	0.21	1.24 mm	0.14
	Control 3	2.77 mm	0.15	1.24 mm	0.09

$S_{\bar{x}}$: Standard Error

Table 2. Statistical evaluation of apoptotic cell count values marked using TUNEL technique.
Tablo 2. TUNEL tekniği kullanılarak işaretlenen apoptatik hücrelerin istatistiksel değerlendirilmesi.

	Group 1			**Group 2**			**Group 3**			
	n	$\bar{X}$	$S_{\bar{x}}$	n	$\bar{X}$	$S_{\bar{x}}$	n	$\bar{X}$	$S_{\bar{x}}$	F
Experimental	25	3.04	0.42	25	4.28	0.43	25	4.60	0.69	2.425
Control	20	3.50	0.31	20	1.75	0.49	20	2.65	0.42	
		t=0.838 P>0.05			t= 3.883 P<0.001			t=2.277 P<0.05		

n: counted area number $\bar{X}$ **:** mean value $S_{\bar{x}}$**:** Standard Error F: One-way ANOVA result

Table 3. Statistical evaluation of apoptotic cell count values marked using Hematoxylin-eosin staining.
Tablo 3. Hematoksilen-Eosin ile boyanmış apoptatik hücrelerin istatistiksel değerlendirilmesi.

	Group 1			**Group 2**			**Group 3**			
	n	$\bar{X}$	$S_{\bar{x}}$	n	$\bar{X}$	$S_{\bar{x}}$	n	$\bar{X}$	$S_{\bar{x}}$	F
Experimental	100	3.71	0.30	90	3.65	0.23	100	3.72	0.18	0.019
Control	60	3.25	0.24	80	1.95	0.16	70	1.8	0.20	
		t=1.087 P>0.05			t=5.824 P<0.001			t=6.819 P<0.001		

n: counted area number $\bar{X}$ **:** mean value $S_{\bar{x}}$**:** Standard Error F: One-way ANOVA result

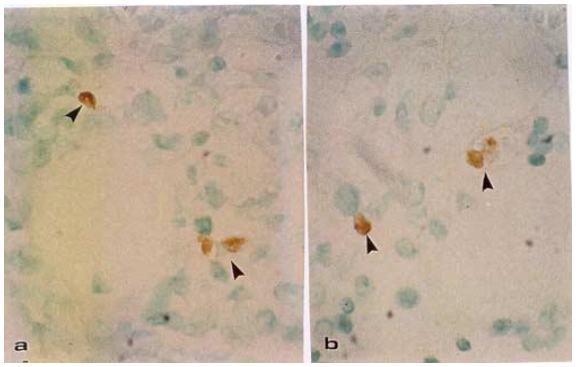

Figure 1. Apoptotic cells marked with TUNEL technique in tumor tissue (arrow head). a) Control 1. Methyl green, 1000X. b) Experiment group 1. Methyl green, 1000X.

Şekil 1. Tümör dokusunda TUNEL tekniği ile işaretlenmiş apoptatik hücreler (ok ucu). a) Kontrol Metil Yeşili, 1000X. b) Deney Grubu 1. Metil Yeşili. 1000X.

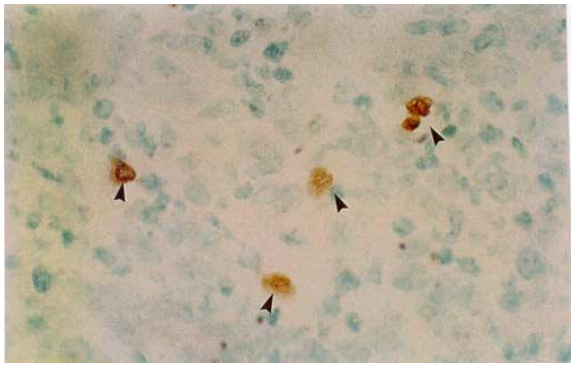

Figure 2. Apoptotic cells darkly marked with TUNEL technique in experimental 3's tumor tissue (arrow head). Methyl green, 1000X.

Şekil 2. Deney Grubu 3'e ait tümör dokusunda TUNEL tekniği ile koyu işaretlenmiş apoptatik hücreler (ok ucu). Metil Yeşili, 1000X.

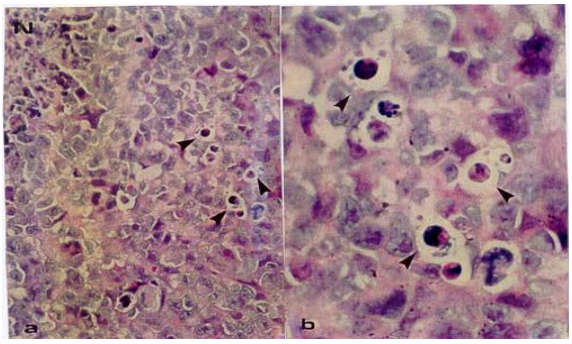

Figure 3. a) Apoptotic (arrow head) and necrotic (N) areas in tumor tissues of short time curcumin received group (Experimental 2) H+E 400X. b) Apoptotic cells in higher magnification (arrow head) H+E, 1000X.

Şekil 3. a) Kısa sureli curcumin uygulanan gruba (Deney Grubu 2) ait tümör dokusunda apoptatik (ok ucu) ve nekrotik (N) bölgeler H+E 400X. b) Yüksek büyütmede apoptotic hücreler (ok ucu) H+E, 1000X.

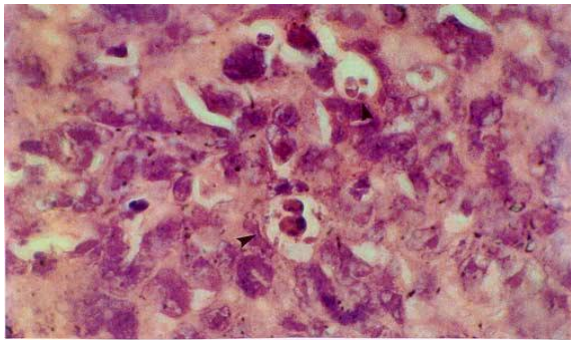

Figure 4. Apoptotic cells in tumor tissue of experimental 3 (arrow head) H+E, 1150X.

Şekil 4. Deney Grubu 3'e ait tümör dokusunda apoptatik hücreler (ok ucu) H+E, 1150X.

Electron Microscopy

In electron microscopic evaluations we performed in order to confirm our apoptotic findings obtained with 'in situ' DNA end labeling and hematoxylin- eosin staining techniques, we established that the EAT cells which have not entered apoptosis showed normal structures of organelles including nucleus and mitochondria, and the EAT cells entered typical apoptosis showed characteristic chromatin thickening and cytoplasmic invaginations in nuclei. It has been proved that the findings we found with other techniques in those EAT cells were typical apoptosis findings (Figure 5 a, b).

Discussion

Jiang et al. (1996) observed the characteristic features of apoptosis of curcumin depending on the concentration and time in mouse sarcoma S180, human colon cancer cell HT-29, human kidney cancer cell 293, human hepatocellular Hep G2 cell cultures. It is also observed that

curcumin administration exposed apoptosis in cell-line COLO205 cells which can be developed in tissue cultures from colorectal carcinomas (Chen et al., 1996), human lung cancer cells in tissue cultures (Ramachandran and You, 1999), AK-5 tumor cells in tissue cultures (Khar et al., 1999) and HL-60 leukemia tissue cultures (Kuo et al., 1996). In our study parallel to those in vitro studies we determined apoptosis development in 'in vivo' solid EAT model cancer cells by administering curcumin.

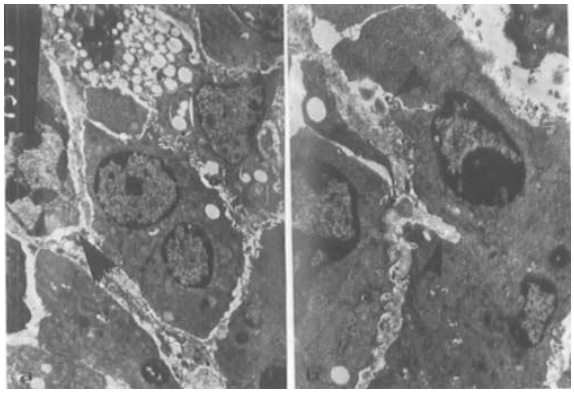

Figure 5. a) Appearance of apoptotic cells (arrow head) in electron microscopy, 3750X. b) Cytoplasmic invaginations.

Şekil 5. a) Elektron mikroskobunda apoptatik hücrelerin görünüşü (ok ucu), 3750X. b) Sitoplazmik invaginasyonlar.

Jaruga et al. (1998b) performed with rat tymocytes in vitro. They also clarified that these events exposed with the effects of curcumin and observed in apoptosis can be sudden and partially reversible. They explicit that the changes observed in cell membrane can be ceased in short time periods and the changes in membrane that curcumin exposed were not dependent to apoptotic schedule. Sikora et al. (1993), in their studies with rat tymocytes and human Jurkat cells, contrary to these studies, showed that curcumin inhibits apoptosis in those cells. Similar to our study, results of counting with TUNEL technique and hematoxylin-eosin applied slides showed that curcumin significantly stimulated apoptosis.

Ruby et. al. (1995) reported that natural curcumins have 53.72-74% of effect on life span of EAT induced rats. In our study in order to obtain apoptotic data, animals were sacrified after specific time periods. Therefore, we could not obtain any results about the effects of curcumin to survival.

Although Ruby et al. (1995) have established regression in tumors by applying curcumin i.p. in their study model of Dalton's lymphoma Ascites Cells (DLA) in rats; they could not stopped tumor related deaths. Also Limtrakul et al. (1997) in their study of skin tumor induced with 7.12-dimethylbenz[α]anthracene and supported with 12-O-tetradecanoylphorbol-13-acetat (TPA) in Swiss albino mouse) they reported that 26 weeks of 2% has no inhibiting effect on tumor development in curcumin diet, however there was significant decrease in tumor sizes. In our study there was no statistical significant difference between curcumin applied groups and control groups' tumor diameter measurements in therapeutic property in the terms of tumor size and development. We think that the contradiction between our results and the results of DLA cells (Limtrakul et al., 1997; Ruby et al., 1995) welded from the differences in tumor type, amount of tumor cells, curcumin dose and application periods.

According to Jang et al. (2002) 230-460mg/dl ethanol application to TM3 mouse leydig cells has apoptotic effect. Consequently apoptotic rate being significantly higher in control 1 than the other control groups in both techniques. Control 1 was received alcohol longer than the other groups. As a result this increases made control 1's results closer to apoptotic results of experimental group 1 and decreased the significance. In contrast to that when mean value of apoptotic cell counts compared in experimental groups, no significant difference determined. Those results showed us that the continuous application of curcumin does not made alterations on apoptotic effect.

Besides when the results obtained from hematoxylin-eosin staining technique compared with TUNEL techniques, there was a similarity between all groups except group 3. In statistical evaluation between experimental group 3 and control 3 results obtained were highly significant in hematoxylin-eosin staining and significant in

TUNEL technique. Piwocka et al. (2001) stated that curcumin causes chromatin thickening, segmentation of DNA with heavy molecular weight and stimulates apoptosis through a non-classic way in Jurkat cells. In our study significance in experimental group 3's results of TUNEL technique similarly can be caused by DNA's with heavy molecular weights exposed by different mechanism of curcumin in apoptosis (Piwocka et al., 2001). Also darker marked apoptotic cells in experimental group 3 with TUNEL technique made us think that 180-200 base-double DNA fragments which expose during apoptosis inside the cell would be higher than control groups.

In electron microscopic evaluation of tumor tissues resembling the basic findings; nuclear apoptotic changes characterized with cytoplasmic invaginations and chromatin thickening in nuclei were determined (Cummings et al., 1997).

The dose of 100 mg/kg curcumin stimulated apoptosis in all groups as aspected in Balb-c mice's EAC tumor. According to the results obtained from TUNEL technique and haematoxylin-eosin staining application of these doses for different time periods did not create any difference in the aspect of apoptosis. But when the results were compared with alcohol given control groups the results obtained from tumor applied single dose curcumin receiving experimental groups were meaningful.

As a result when the apoptotic effect of alcohol is also considered, among those group curcumin has the strongest apoptotic effect. Unexpectedly, no difference in long period curcumin receiving group when compared with control group showed us that this application was not meaningful.

Acknowledgments

"This work was supported by the Research Fund of The University of İstanbul (T-788/0703200)"

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