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Necip İLHAN [1, a]
Oguzhan TATAR [1, b]
Solmaz SUSAM [2, c]
Nevin İLHAN [1, d]

[1] Fırat University, Faculty of Medicine, Medical Biochemistry Department, Elazig, TURKIYE

[2] Adiyaman University, Faculty of Medicine, Medical Biochemistry Department, Adiyaman, TURKIYE

[a] ORCİD: 0000-0001-9997-0418
[b] ORCİD: 0000-0002-3972-1820
[c] ORCİD: 0000-0002-7503-2416
[d] ORCİD: 0000-0002-0208-8929

The Effect of Raloxifene and Fluoxetine Treatment on Oxidant-Antioxidant Status in Breast Cancer Bearing Rats *, **

Objective: 7,12-Dimethylbenz[a]anthracene (DMBA)-induced tumors in mammary tissue closely mimic human breast cancer in morphology and the expression of biochemical and molecular markers. Although significant advances have been seen in the treatment of breast cancer, there is still a great need for alternative approaches. The current experiment was designed to investigate the therapeutic effect of Raloxifene (RAL) and Fluoxetine (FLX) by assessing oxidant and antioxidant parameters in DMBA-induced breast carcinogenesis in rats.

Materials and Methods: Thirty-two female Wistar rats were divided into 4 groups. Group I was treated only DMBA. After tumor induction, cancer bearing rats were orally treated with RAL (Group II), with FLX (Group III), and finally with RAL+FLX (Group IV), respectively. Tumors were induced in the whole groups using DMBA dissolved in olive oil (80 mg/kg body weight). The tumor marker cancer antigen (CA) 15-3 and selected oxidative and antioxidative parameters were analyzed in whole experimental groups by using appropriate methods.

Results: The oral treatment of the therapeutic agents alone or in combination provided a significant decrease in CA 15-3 levels in all therapeutic groups compared to DMBA group. When the level of malondialdehyde was measured, it was noticed a decrease in all treatment groups compared to the DMBA group. Additionally, the activities of enzymatic antioxidants were renovated to better after supplementation with the therapeutic agents, suggesting that this agents exhibiting anticancer effect.

Conclusion: It was proposed that the combination of RAL and FLX was more effective than each of RAL or FLX alone on restoration devastating effects of breast cancer.

Key Words: *Antioxidant enzymes, breast cancer, CA 15-3, DMBA, MDA*

Meme Kanseri Taşıyan Sıçanlarda Raloksifen ve Fluoksetin Tedavisinin Oksidan-Antioksidan Durum Üzerine Etkisi

Amaç: Meme dokusunda 7,12-Dimetilbenz[a]antrasen (DMBA)'nın neden olduğu tümörler, morfolojik açıdan, biyokimyasal ve moleküler belirteçlerin ekspresyonu bakımından insan meme kanserine çok benzemektedir. Meme kanseri tedavisinde önemli ilerlemeler görülmesine rağmen, hala alternatif yaklaşımlara ihtiyaç vardır. Bu çalışma, sıçanlarda DMBA ile indüklenmiş meme karsinogenezinde oksidan ve antioksidan parametreleri değerlendirerek Raloksifen (RAL) ve Fluoksetin (FLX) 'in terapötik etkisini araştırmak amacıyla yapılmıştır.

Gereç ve Yöntem: Otuz iki tane dişi Wistar türü sıçan 4 gruba ayrıldı. Grup I'e sadece DMBA uygulandı. Tümör indüksiyonundan sonra, kanser taşıyan sıçanlar oral olarak RAL (Grup II), FLX (Grup III) ve son olarak RAL+FLX (Grup IV) ile tedavi edildi. Tüm gruplardaki tümörler, zeytinyağı içinde çözündürülmüş DMBA (80 mg/kg vücut ağırlığı) kullanılarak indüklendi. Tüm deney gruplarında tümör markeri olan kanser antijeni (CA) 15-3 ve seçilen oksidatif ve antioksidatif parametreler uygun yöntemler kullanılarak analiz edildi.

Bulgular: Terapötik ajanların tek başına veya kombinasyon halinde oral tedavisi, DMBA grubuna kıyasla tüm terapötik gruplarda CA 15-3 seviyelerinde önemli bir azalma sağladı. Malondialdehit düzeyleri ölçüldüğünde, DMBA grubuna kıyasla tüm tedavi gruplarında bir azalma gözlendi. Bununla birlikte, enzimatik antioksidanların aktiviteleri, terapötik ajanlar uygulandıktan sonra arttı, bu durum bu ajanların antikanser etkisi sergilediğini düşündürmektedir.

Sonuç: Sonuç olarak, RAL ve FLX kombinasyonunun meme kanserinin restoratif yıkıcı etkileri üzerinde tek başına RAL veya FLX'in her birinden daha etkili olduğu düşünülmektedir.

Anahtar Kelimeler: *Antioksidan enzimler, meme kanseri, CA 15-3, DMBA, MDA*

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Correspondence
Yazışma Adresi

Nevin İLHAN
Fırat University,
Faculty of Medicine,
Medical Biochemistry
Department
Elazig - TURKIYE

drnilhan@yahoo.com

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Introduction

Breast cancer is the most widespread cause of malignancy and death in women and constitutes one-tenth of all new cancer diagnoses in the world (1). Free radicals and electrophilic-mediated oxidative stress has a significant function in whole periods of chemical carcinogenesis and tumorigenesis (2). 7,12-dimethylbenz[a]anthracene (DMBA) is an immunosuppressor and a strong organ-specific laboratory carcinogen and commonly used in many research laboratories studying cancer. It is known that the DMBA induce great quantities of free radicals leading to the release of lipid peroxides (2), that's why it was suggested that DMBA-induced breast carcinogenesis is an ideal pattern to study the therapeutic influence of synthetic and natural substances on experimental animals (3).

Various biological tumor indicators are currently being researched to diagnose these early diseases, monitor recurrence or metastasis in treated patients, and predict response or resistance to therapies (4). One of them, cancer antigen 15–3 (CA 15-3) is one of the most widely used markers because it is cheap and easy to use (5). In this study, it was measured tissue CA 15-3 levels instead of serum, because all the other parameters were detected in tissue samples.

Malondialdehyde (MDA), which is one of the parameters showing oxidative damage, is the end product of lipid peroxidation and leads to loss of cell function under oxidative stress (6). It was measured tissue MDA levels in this study because of that MDA is a more precise site of free radical production and has a significant function in assessing the outcome of cancer. Therefore, it was suggested that more accurate result can be acquired compared to plasma values. So, it may be thought that MDA levels have a significant function in assessing the outcome of cancer.

The tissues of animals are continually coping with reactive oxygen species (ROS) produced during many metabolic reactions (7). Oxidative stress develops when ROS generation exceeds the antioxidant capacity of cells. Mitochondria is a major source of intracellular ROS and are particularly vulnerable to oxidative stress, and mitochondrial damage are involved in the pathogenesis of cancer (8). Actually, the cell includes a variety of substances that can remove free radicals and protect them from harmful effects. Antioxidants are molecules that when present, even at very low concentration inhibit or quench free radical reactions and delay or inhibit cellular damage. The protection against free radicals is provided by certain enzymes that which come under a distinctive group, concerned merely with the detoxification of these radicals. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the key enzymatic antioxidants of this defense system by which the oxidative toxic intermediates that are produced during metabolic reactions are removed (9). The function of suppression of antioxidant enzymes in breast cancer is not fully clear; however, it has been suggested that the loss of important antioxidant systems may contribute to the ROS-driven transformation (10).

Currently, breast cancer rates are constantly increasing, with over a million new cases being diagnosed worldwide each year. Therefore, the novel study was focused to assess the effects of Raloxifene and Fluoxetine in combination and alone on oxidant-antioxidant situation in DMBA-induced breast cancer rat model.

Materials and Methods

Research and Publication Ethics: The study procedures were approved by the Ethics Review Committee of Firat University, Elazig, Turkey (Protocol No: 2016/57) and complied with the Animal Welfare Act guidelines.

Animal Design: Female Wistar Albino rats were supplied by the Firat University Experimental Research Center. All animals were maintained on a regular dark-light cycle (12-h light–dark cycle), with free access to pellet diet and water *ad libitum* during the whole experimental duration. All animals were housed in clean polypropylene cages (four rats/cage) under controlled indoor temperature (2±2°C) and humidity (44±5%) conditions.

Experimental Setup: Thirty-*two* healthy female rats were separated into 4 groups. Group I was given a single dose of 80 mg/kg of DMBA dissolved in 1 mL olive oil by gastric gavage on the 15th day of the beginning of experimental period. Breast cancer was allowed for 90 days to monitor the onset of tumorigenesis. Group II, III and IV received DMBA as in Group I and after 90 days, Group II was given 3 mg/kg RAL (dissolved in 1 mL DMSO) by gavage 3 times a week for 6 weeks. Group III rats were given FLX dissolved in 1 mL of distilled water at a dose of 30 mg/kg by oral gavage daily for 6 weeks. Group IV were simultaneously treated by FLX plus RAL at the doses indicated above. Rats were palpated every week to monitor the onset of tumorigenesis.

Biochemical Analysis:

Preparation of Mammary Tissue Homogenate: Homogenate of breast tissues was prepared Phosphate Buffered Saline (PBS) (0,1 M, pH: 7.4) using an automatic tissue homogenizer machine (Ultra TurraxType T25-B, IKA Labortechnic, Germany). Lowry method was preffered to measure total protein contents (11). In an alkaline medium, the copper ion (Cu^{+2}) forms a complex with peptide bonds in proteins and reduced to Cu^{+1}. Reduced copper and the amino acids Tyr, Trp and Cys in the side chain of proteins reduce the Folin-Phenol reagent and cause deep blue color formation. The intensity of the resulting color is measured spectrophotometrically at 660 nm.

Determination of MDA Levels: The levels of MDA were estimated by the thiobarbituric acid test according

to the process described by Ohkawa et al. (12). This method is based on a condensation reaction of two molecules of TBA with one molecule of MDA to give a pink colored compound which can be measured at 532 nm wavelength.

Assay for Mammary Antioxidant Enzymes Activities: Superoxide dismutase enzyme was determined by the method modified by Sun et al. (13). In this method, superoxide radical is produced by xanthine-xanthine oxidase system and the resulting superoxide radical reacts with iodonitrotetrazolium (INT) to form violet colored formazone dye and this color intensity is measured at a wavelength of 505 nm. This reaction is inhibited and % inhibition is calculated due to CuZn-SOD activity in the medium. CuZn-SOD activity is expressed as U/g protein for tissue.

In the GPx activity assay, reduced glutathione (GSH) in the presence of H_2O_2 is oxidized by GPx to oxidized glutathione (GSSG). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is used when oxidized GSSG is converted back into GSH by glutathione reductase enzyme. The amount of NADPH used is monitored at 340 nm wavelength as a decrease in absorbance (14).

CAT activity was measured in tissue homogenate at 25ºC by the method specified by Aebi (15). Here, the decomposition rate of substrate H_2O_2 was monitored spectrophotometrically at 240 nm for 30 seconds.

Assessment of CA 15-3 Levels: The tissue CA 15-3 levels were measured by using Enzyme-Linked Immuno Sorbent Assay (ELISA) for rats (Cat No: YLA0753RA, YL Biont, Shanghai, China, YL Biotech Co, Ltd.) method. A 96 well microplate, pre-coated with an antibody specific for CA 15-3, was provided by the commercial kit. The assay procedure was summarized as follows: standards and samples are pipetted into the wells and then added a Horseradish Peroxidase (HRP) conjugated antibody specific for CA 15-3. To remove any unbound reagent, washing process was carried out. Following a substrate solution was added to the whole wells, the color developed in proportion to the amount of CA 15-3 bound in the initial step was observed. The results were calculated by comparing the absorbance of the samples to the standard curve by ELISA reader.

Statistical Analysis: All data were analyzed using SPSS 22.0 statistical software package (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by *post hoc* Tukey HSD was used to assess difference between the groups. All the results are reported as mean±standard deviation. $P<0.05$ were considered as significant.

Results

The ameliorative effect of Raloxifene and Fluoxetine on mean values of CA 15-3, MDA, CAT, GPx and SOD in control and experimental animals were illustrated in Table 1. It was observed that not only in the combined treatment group but also the drug alone groups were showed significantly decrease in CA 15-3 levels when compared to DMBA group ($P<0.001$, for RAL and for RAL+ FLX; $P<0.05$ for FLX).

The production of MDA, measured as index of lipid peroxidation level, disclosed a significant decrease in whole therapeutic agents (except FLX) applied animals as compared to cancer bearing animals. It was suggested that treatment with these agents reverted the abnormal changes ($P<0.05$, for RAL and RAL+FLX groups).

Upon treatment with therapeutic agents in Group II and Group III no statistically significant changes were depicted on the antioxidant enzyme activities matched to DMBA group ($P>0.05$). However; the combined therapy increased statistically significant antioxidant enzyme activities compared to only DMBA given group ($P<0.05$ for CAT and SOD, $P<0.001$ for GPx activities).

Discussion

Environmental pollutants derived from incomplete combustion of fossil fuels that also exist in tobacco smoke and varied foods have always been known to stimulate different biochemical and physiological changes in the living system. Among the varied pollutants, DMBA is a chemical carcinogen used for the initiation of breast cancer in rat model (16). The administration of DMBA to rats through gavage is widely used to establish a breast cancer model, that is why we chose the using it (17, 18).

CA 15-3 is the tumor-specific marker in the diagnosis and especially the monitoring of breast cancer patients (19). High CA 15-3 and Carcinoembryonic Antigen (CEA) serum levels seem to be associated with a poorer survival (20). In previous studies, the prognostic value of CA15-3 had been confirmed by some researches (21-23). In our study, it was observed that CA 15-3 decreased with the application of treatment agents which may mean that breast cancer responds to treatment.

One of the mechanisms involved in DMBA-induced carcinogenesis is the development of oxidative stress which could be due to the elevated free radical generation and decreased activity of antioxidants (24). In a study conducted by Moselhy et al., on female Sprague Dawley rats, DMBA was used to induce mammary cancer and was evaluated the effects of 2-deoxglucose or malonate individually or in combination on the levels of selected parameters including MDA

Table 1. The mean values of selected parameters in breast tissue

Group	CA15-3 (IU/g protein)	MDA (nmol/g protein)	SOD (U/g protein)	GPx (U/g protein)	CAT (k/g protein)
DMBA	363.78±70.43	258.43±77.62	370.60±100.96	198.47±46.74	0.12±0.06
DMBA+RAL	174.13±45.99***	137.22±70.47*	500.86±91.76	284.62±71.85	0.17±0.09
DMBA+FLX	231.84±56.26*	210.24±92.07	443.57±153.40	271.84±68.80	0.17±0.10
DMBA+RAL+FLX	213.98±60.03***	118.28±60.20**	556.56±156.27*	411.25±141.35***	0.39±0.26**

*P<0.05 compared to DMBA ***P<0.01 compared to DMBA **P<0.01 compared to DMBA

levels. They concluded that MDA levels meaningfully elevated in cancer bearing rats compared the in those controls and it was suggested the combined treatment lead to lowering in MDA levels more potentially compared with individual treatment (25). Again in another study, it was reported that breast cancer bearing rats demonstrated increased levels of mammary lipid peroxidation products including MDA, and berberine administration promoted the reduction of oxidative stress levels and tumorigenicity (26). In our study, results which are compatible with these studies mentioned above were obtained. It was observed that MDA levels decreased in the treatment groups compared to the DMBA group. From the obtained results, it is proposed that the selected therapeutics are capable of restoring the functional degradation and protecting the mammary tissue from oxidative damage.

The members of antioxidant system are crucial in protecting against tumor promoting and they can donate their electrons to stabilize ROS and to inhibit their harmful effects. By scavenging ROS directly or indirectly, they may protect individual against cancer (27). In a study assessing the chemopreventive effect of brucine was reported that antioxidant status in plasma and mammary tissues of DMBA alone injected rats were lower than those in chemopreventive groups (28). In a study conducted Kalaiselvi et al., it was reported that decreased levels of selected enzymatic antioxidants in DMBA induced animals indicates severity of tissue damage. The decreased activities were reverted to near normal by treatment of *Ananus comosus* (*A. comosus*). So, it was suggested that *A. comosus* exhibited therapeutic effect by upregulating antioxidant enzymes in cancer bearing rats (29). Also in our study, the activities of the antioxidant enzymes (SOD, GPx, and CAT) were observed to be lower in the DMBA group. It was observed that the animals 90 days after DMBA treatment, revealed protection against carcinogenesis induced oxidative stress by the supplementation of the therapeutic agents. All treatment agents increased the selected antioxidant activities, but the most effective increase was found in the group in which the combined treatment was applied. This may be due to the therapeutic agents reduces free radical production and mitochondrial oxidative damage during breast cancer formation.

To sum up; the 'fight' between antioxidants and oxidants is an ongoing process and any imbalance leading to increase in the free radical levels may induce cancer formation. It was suggested that DMBA induces oxidative stress in breast in cancer-bearing rats and damages in this tissue could be ameliorated by RAL+FLX treatment.

Conflict of interest: There is no conflict of interest.

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