General Section

Effects of tamoxifen on tissue nitrite/nitrate levels and plasma lipid peroxidation in female rats

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Summary

Purpose of investigation: The effects of tamoxifen on lipid peroxidation and oxidant-antioxidant balance in an animal model were studied. *Methods*: Twelve female adult rats were divided into two groups and DMSO and tamoxifen dissolved in DMSO were administered. Tissues taken from the brain, liver and ovary of rats were dissected. MDA, nitrite, nitrate levels and plasma LDL oxidation in brain, ovary and liver tissues were measured and compared. *Results*: Induced LDL MDA levels were significantly lower in the tamoxifen group ($p = 0.009$). MDA levels in the liver were significantly lower in the tamoxifen group whereas nitrite levels were found significantly higher ($p < 0.05$). Brain and ovarian tissues demonstrated no significant difference with respect to MDA, nitrite and nitrate levels. *Conclusion*: Tamoxifen has no negative effects on lipid peroxidation in an animal model.

Key words: Tamoxifen; MDA; Nitrite; Nitrate.

Introduction

Tamoxifen is a non-steroid estrogenic agent that displays both estrogen agonist and antagonist activity and it is highly effective in the adjuvant therapy of breast cancer [1, 2]. Tamoxifen has mixed estrogenic agonist and antagonist effects and this may cause protective effects regarding cardiovascular diseases [3].

The potential mechanisms of tamoxifen on breast cancer chemoprophylaxis have not yet been completely understood and probably are beyond simple anti-estrogenic activity. In recent years the major aspect of tamoxifen has been the effect on lipid peroxidation and oxidant-antioxidant balance [4]. Breast cancer cells have been demonstrated to proliferate by decreasing lipid peroxidation and the effects of tamoxifen on lipid peroxidation may help us understand its anticancer activity [5]. The focus of this study was to investigate the effects of tamoxifen on lipid peroxidation and oxidant-antioxidant balance in an animal model. Clarification of the effects of tamoxifen on lipid peroxidation may help to reveal the mechanism of its antineoplastic activity. The present study will also clarify the long-term effects of tamoxifen on lipid metabolism and oxidant-antioxidant balance.

Materials and Methods

Animals and treatments

Twelve female adult rats were used in the study after permissions was given by the Animal Ethics Committee of the Faculty of Medicine, Ege University School of Medicine. Rats were approximately nine to ten weeks old and all were fed with a standard diet (pellet feed) under standard daylight (12 hours of light, 12 hours of darkness) and heat conditions (approximately 20°C). Tissue and serum samples were collected in the experimental surgical laboratory and the tissues were analyzed in the laboratory of the Biochemistry Department.

Two groups were formed in accordance with the purpose of the study:

Group 1 (DMSO group) (n = 6): No surgical intervention or medical therapy was performed to female rats included in this group. The rats were administered dimethyl sulphoxide (DMSO) solution which is used as the solvent of tamoxifen.

Group 2 (Tamoxifen group) (n = 6): A dose of 0.2 mg/kg/day tamoxifen dissolved in DMSO was administered intraperitoneally to female rats once daily for 12 days.

After the appropriate anesthesia blood was collected from rats by cardiac injection in both groups two hours after the last dose of treatment. Then laparotomy was performed and tissue samples were collected on ice. The brain, liver and ovary tissues of the decapitated rats were dissected on ice. The tissue samples were kept in a freezer at a temperature of -80°C until the day of biochemical analysis.

Analysis performed on plasma samples

Plasma samples were incubated at room temperature for 30 min with a commercial precipitant reagent (Merck), proposed by Taus *et al*. [6]. After centrifugation at 1,600 g for 10 min, LDL samples were solubilized by 0.15 M sodium hydroxide. LDL oxidation was assesed by basal and induced malondialdehyde (MDA) in samples containing 200 μg of protein.

Measurement of basal malondialdehyde (MDA) was performed by addition of thiobarbituric acid to the tissue homogenate. This solution was boiled at 100°C for 20 min and centrifuged for 10 min at 2000 rpm. The supernatants were analyzed at a wavelength of 532 nm.

LDL-oxidation was induced by addition of 1 mmol of copper sulfate (CuSO4) and was observed over three hours at 10-min intervals at a wavelength of 234 nm. Peak oxidation was detected at 109.2 ± 9.4 min (n: 10). Then at this point (110^{th} min) MDA measurement was repeated for induced LDL-MDA levels. The results were given in nmol/mg LDL protein. Protein measurements were performed according to Lowry's method [7].

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Analysis performed on tissues

All samples were taken out of a -80C° freezer on the study day and then weighed and homogenized in homogenizer tubes with phosphate buffer (0.5 M, pH = 7.4) in a 1/10 (w/v) dilution on ice at 1000 rpm. After centrifugation supernatant was obtained from homogenized samples. All analyses were performed on the obtained supernatant sample. Determination of tissue nitrite was carried out by the colorimetric method based on Griess reaction. Diazonium salt was formed from nitrite via the diazotization of sulfanilamide. Subsequently this salt forms a red compound at 540 nm wavelength by combining with N-ethylenediamine. Nitrate must first be reduced and then measured by the Griess reaction since it is not diazotized with sulfanilamide. After deproteinizing solution (0.5 ml ZnSO4 + 0.5 ml. NaOH) is immersed into tissue homogenate in equal amounts, it is then centrifugated at 3000 rpm for 15 min; 0.5 ml of Griess solution is added to 0.5 ml of the obtained supernatant. After 10 min the developed color is read at the blind sample at a wavelength of 540 nm by a spectrometer. The amount of nitrite is calculated using a standard graph drawn by sodium nitrite (micromole/g wet tissue).

Nitrate was studied by a reductase method. Tissue homogenates must be deproteinized by equal amounts of deproteinization solution before the initiation of the process. The supernatant used for determination of nitrate is collected from samples centrifuged for 15 min at 3000 rpm. After the samples are stored in a dark at room temperature for 90 min, the samples are read at 340 nm against distilled water by a spectrometer. Determination of total nitrite-nitrate (NO) level was done when the tissue nitrite level determined by Griess reaction was added to the measured tissue nitrate level. The sum of nitrite and nitrate levels gives us the total nitrite-nitrate level. Determination of MDA was done by colorimetric measurement that was performed at a wavelength of 532 nm of supernatants after the addition of thiobarbituric acid to the tissue homogenate. This solution was boiled at 100°C for 20 min and centrifuged for 10 min at 2000 rpm.

Statistical Analysis

Statistical analysis was performed using the SPSS 11.0 program. The Mann-Whitney U-test was used to compare groups. The levels are expressed in mean ± standard deviation.

Results

Plasma LDL Oxidation. LDL-MDA levels were assessed in terms of basal and induced levels in both groups. Basal plasma LDL-MDA level was 0.9 ± 0.27 nmol/mg LDL protein (0.55-1.20) in the DMSO group whereas it was 0.74 ± 0.22 nmol/mg LDL protein (0.41 - 0.99) in the tamoxifen group (p = 0.283). The induced level of LDL-MDA was 6.14 ± 1.72 nmol/mg LDL protein (1.78 - 9.14) in the DMSO group. Plasma induced LDL-MDA level was 3.2 ± 1.23 nmol/mg LDL protein (1.50 - 5.23) in the tamoxifen group. The induced levels of LDL-MDA were significantly lower in the tamoxifen group (p = 0.009).

Ovarian tissue. Level of MDA was 0.93 ± 0.13 nm/g wet tissue (0.76 - 1.16) in the DMSO group and 0.79 ± 0.27 nm/g wet tissue (0.49 - 1.21) in the tamoxifen group (p = 0.304). Nitrite level was 7.98 ± 6.63 micromole/g wet tissue (4.65 - 21.40) in the DMSO group and 5.58 ± 1.04 micromole/g wet tissue (4.65 - 7.21) in the tamoxifen group (p = 0.401).

Nitrate level was 245 ± 9.43 micromole/g wet tissue (227.30 - 254.63) in the DMSO group; 258 ± 3.58 micromole/g wet tissue (253.99 - 263.95) in the tamoxifen group and a significant difference was detected between nitrate levels in the ovarian tissue (p = 0.004).

Total nitrite-nitrate (NO) level was 253 ± 12.5 micromole/g wet tissue (232.18 - 270.56) in the DMSO group, but total nitrite-nitrate level measured 263 ± 4.26 micromole/g wet tissue (258.64 - 270.23) in the tamoxifen group (p = 0.093) (Table 1).

Table 1. — *Results of tissue samples*.

	Liver		Brain		Ovary	
	DMSO	Tamoxifen	DMSO	Tamoxifen	DMSO	Tamoxifen
Nitrite	1.49 ± 0.13*	1.74 ± 0.16*	1.59 ± 0.17	1.66 ± 0.2	7.98 ± 6.63	5.58 ± 1.04
Nitrate	205.33 ± 22.28	219.31 ± 21.55	256.93 ± 8.07	257.03 ± 11.85	245.51 ± 9.43†	257.73 ± 3.58†
Total nitrite-nitrate	206.82 ± 22.3	221.06 ± 21.06	258.52 ± 8.11	258.7 ± 12.02	253.5 ± 12.5	263.32 ± 4.26
MDA	0.76 ± 0.81‡	0.63 ± 0.1‡	0.69 ± 0.65	0.66 ± 0.65	0.93 ± 0.13	0.79 ± 0.27

* p = 0.026; ‡ p = 0.043 ; † p = 0.004

Brain tissue. MDA level measured 0.69 ± 0.65 nm/g wet tissue (0.61 - 0.78) in the DMSO group and 0.66 ± 0.65 nm/g wet tissue (0.57 - 0.76) in the tamoxifen group.

Nitrite level was 1.59 ± 0.17 micromole/g wet tissue (1.28 - 1.74) in the DMSO group. It was 1.66 ± 0.2 micromole/g wet tissue (1.28 - 1.86) in the tamoxifen group. Nitrate level was 257 ± 8.07 micromole/g wet tissue (246.27 - 266.52) in the DMSO group and 257 ± 11.85 micromole/g wet tissue (237.27- 269.42) in the tamoxifen group (p = 0.986). Total nitrite-nitrate level measured 258 ± 8.11 micromole/g wet tissue (247.90 - 268.26) in the DMSO group and 259 ± 12.02 micromole/g wet tissue (238.55 - 271.16) in the tamoxifen group (p = 0.976) (Table 1).

Liver tissue. MDA level was 0.76 ± 0.81 nm/g wet tissue (0.38 - 0.88) in the DMSO group, however it was 0.63 ± 0.1 nm/g wet tissue (0.45 - 0.73) in the tamoxifen group. MDA levels in the tamoxifen group was significantly lower (p = 0.043). Nitrite level was 1.49 ± 0.13 micromole/g wet tissue (1.28 - 1.63) in the DMSO group, whereas it was 1.74 ± 0.16 micromole/g wet tissue (1.51 - 1.98) in the tamoxifen group. There was a significant difference in liver tissue nitrite levels between groups (p = 0.026). Nitrate level was 205 ± 22.2 micromole/g wet tissue (187 - 246) in the DMSO group but 219 ± 21.55 micromole/g wet tissue (175 - 231) in the tamoxifen group (p = 0.310).

Total nitrite/nitrate level measured 207 ± 22.3 micromole/g wet tissue (188.62 - 248.22) in the DMSO group and 221 ± 21.61 micromole/g wet tissue (177.17 - 232.70) in the tamoxifen group (Table 1).

Discussion

Breast cancer is the most common type of cancer among women and is the second leading malignancy in mortality following lung cancer [8]. Tamoxifen exhibits high activity in adjuvant hormonal chemotherapy and chemoprophylaxis in breast cancer [9]. The prophylactic use of tamoxifen has been recommended for patients even

without residual disease. The generally accepted approach is 5-year adjuvant use of tamoxifen although it increases the risk of developing endometrial pathologies [10] and carries a risk for venous thromboembolism [11, 12].

The mechanisms of tamoxifen action have not yet been well understood; however, they are probably beyond simple anti-estrogenic activity. Tamoxifen binds to estrogen receptors and modulates transcriptional activities in different ways in different target tissues [13].

In our study significant differences between the DMSO and tamoxifen groups with regard to LDL-MDA levels were not determined by the analysis of plasma LDL oxidation. However, induced LDL-MDA levels were significantly lower in the tamoxifen group. These findings support the studies revealing that tamoxifen reduces or prevents LDL oxidation and reporting that tamoxifen influences lipid peroxidation [14].

One of the compounds developing at the terminal stage of lipid peroxidation is MDA which is used for the determination of lipid peroxide levels. In our study MDA levels in ovary, brain and liver tissues were separately examined and the effect of tamoxifen on lipid peroxidation at the tissue level was investigated. No significant difference with respect to MDA levels in ovary and brain tissues was detected between groups; however, MDA levels were significantly lower in liver tissue. MDA, which is formed as a result of tissue lipid peroxidation, is metabolized at the cellular level. The enzymatic destruction of MDA in the liver is caused by aldehyde dehydrogenases and it is metabolized to CO_2. MDA is known to have mutagenic features and behaves like a chemical carcinogen [15]. Tamoxifen lowers MDA levels in tissue by its effects on lipid peroxidation and this may contribute to the potential useful effects of tamoxifen in long-term use.

Water, ultrafiltrate and plasma nitric oxide oxidize rapidly to nitrite, the stability of which may take hours. However, nitrite is rapidly converted to nitrate in full blood. Nitrites and nitrates lack biological activity but each one is a good indicator to reveal endogenous nitric oxide production. Nitrite and nitrate levels in the tissues were also analyzed in our study. Nitrite levels did not show significant differences between placebo and tamoxifen groups in ovarian and brain tissues; on the other hand, nitrite levels in the liver tissue of the tamoxifen-administered group were significantly higher. No significant difference was determined between groups in terms of nitrate levels in the liver and brain tissues, but nitrate levels were significantly higher in the ovaries of the tamoxifen group.

Higher levels of nitrite in the liver tissue and nitrate in the ovarian tissue in the tamoxifen group is detected with unclear effects of tamoxifen. Nitric oxide is an autocoid which may display both inflammatory and anti-inflammatory activity. Additionally, it may have antioxidant activity. Nitrite and nitrate levels which were indirect criteria of nitric oxide are effected by the tamoxifen activity.

The long duration of antiestrogenic activity of tamoxifen may negatively affect the function of numerous tissues such as endothelium of the blood vessels [16]. Endothelial function may be adversely affected both by the antiestrogenic effects of tamoxifen and its indirect effect on nitric oxide levels.

In conclusion, the study shows that tamoxifen does not have a negative effect on plasma lipid peroxidation; moreover, it reduces LDL oxidation.

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