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The effects of antioxidants on testicular apoptosis and oxidative stress produced by cell phones

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Aim: The aim of this study was to determine the probable effects of cellular phone usage on apoptosis and oxidative/antioxidative balance in testicular tissue, and the probable protective role of vitamin C and E on these processes.

Materials and methods: Forty-two male rats were divided into 6 groups: control, antioxidant, calling, stand-by, calling plus antioxidant, and stand-by plus antioxidant. Oxidant and antioxidant parameters and apoptotic indexes were studied. Two identical cell phones were used in the calling and stand-by groups.

Results: Cell phone usage significantly increased apoptosis in call, but not in the stand-by position, in testicular tissue. Apoptosis produced in the calling mode was significantly more excessive than in the stand-by mode. Oxidative stress was found as high to some extent in both calling and stand-by positions. However, no ameliorating effect of antioxidants could be demonstrated on either apoptosis or oxidative stress.

Conclusion: The effects of cell phones on testicular apoptosis and oxidative stress in the calling and stand-by positions and the effects of antioxidants on both injuries have been investigated for the first time. In addition, increased apoptosis by cell phones has been shown for the first time in this report.

Key words: Cellular phone, apoptosis, oxidative stress, vitamin C, vitamin E

1. Introduction

The current number of mobile phone subscriptions is over 5.3 billion according to the Global System for Mobile Communications (GSM) Association (1). However, new technologies such as mobile phones can cause some health problems while facilitating daily life.

Today, many electronic devices produce a set of electromagnetic waves leading to electromagnetic fields (EMFs). These strongly exposed electromagnetic waves and biological effects of mobile phones have recently become the subject of scientific studies.

Indeed, investigations have revealed that electromagnetic radiation increases oxidative stress and apoptosis on some tissue cells (2,3). However, although there are many studies investigating the effects of electromagnetic waves on testicular cells, there is no study focused on the impacts of a mobile phone on apoptosis or oxidative stress in testicular tissue whether directly in call or in stand-by mode (4–6).

In addition, it is known that antioxidant vitamins, vitamin E, and vitamin C can reduce both oxidative stress and apoptotic effects (2).

In this study, therefore, we investigate whether electromagnetic waves produced by a cell phone in call or in stand-by mode increase the apoptosis and oxidative stress in testicular tissue and whether vitamin C and vitamin E can prevent these adverse events.

2. Materials and methods

2.1. Animals

Forty-two male Wistar rats (200–250 g) were maintained under conditions with temperature of 22 ± 2 °C and

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relative humidity of 65%–70% on a 12-h light–dark cycle and fed ad libitum with commercially available rat chow and water. The animals were housed at 7 animals per cage (50 × 35 × 20 cm) and had free access to water and diet.

2.2. Groups

Rats were divided randomly into 6 groups, each group consisting of 7 rats.

Group I: Control.

Group II: Antioxidant group; rats were given only daily vitamin C (20 mg/kg, intraperitoneally) and vitamin E (50 mg/kg, intramuscularly) as antioxidant substances.

Group III: Call group; rats were exposed to electromagnetic radiation (EMR) emitted by a mobile phone during calling (it was only rung, not switched on) for 10 min every 1 h for 8 h each day, and phones were left in stand-by mode at remaining times of the day over the course of 1 month.

Group IV: Stand-by group; rats in this group were continuously exposed to EMR by a mobile phone in stand-by position for 1 month.

Group V: Call+antioxidant group; in addition to receiving the treatment given to the call group, they were given vitamin C and vitamin E as in group II.

Group VI: Stand-by+antioxidant group; in addition to receiving the treatment given to the stand-by group, they were given vitamin C and vitamin E as in group II.

2.3. Exposure system

Two identical mobile phones (1.00 W/kg SAR value and DCS1800 communication system) were used (7). One cell phone was used for groups III and V and another cell phone was used separately for groups IV and VI.

The groups exposed and not exposed to mobile phones were kept apart in separate rooms (Figure 1). The mean distance of the cell phone from the testis was approximately 22 cm (max: 43.9 cm, min: 0 cm) (Figure 2).

2.4. Surgical procedure

At the end of the study, rats were anesthetized with a cocktail of ketamine (50 mg/kg) and xylazine (10 mg/kg) administered before sacrificing. The testes were then removed and 1 of the testes was put into Bouin solution for histopathological assay; the other was frozen and kept in –80 °C deep-freeze until the biochemical assay.

2.5. Histopathological assay

Testes were fixed in Bouin solution, then included in standard tissue processing, embedded in paraffin, and blocked. After 5-μm-thick sections obtained from paraffin blocks were rehydrated and deparaffinized, 1 sections was stained with the routine hematoxylin and eosin and the other was stained with the TUNEL method to determine germ cell apoptosis. The Takara In Situ Apoptosis Detection Kit (Takara Bio, Tokyo, Japan) was used. Proteinase K (20 μg/mL) was used to deparaffinize and rehydrate, and

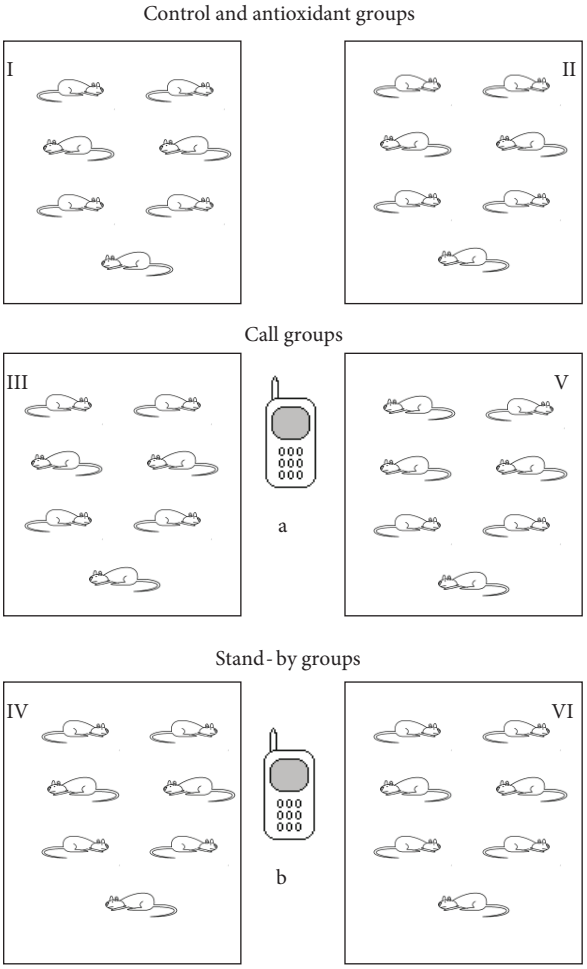

Figure 1. Schematic illustration of the study. Group I, control; group II, antioxidant; group III, call; group IV, stand-by; group V, call+antioxidant; group VI, stand-by+antioxidant; a, cell phone in call + stand-by modes; b: cell phone only in stand-by mode.

sections were kept in distilled water at room temperature for a time period of 15 min. Washed sections in phosphate-buffered saline (PBS) were kept in 3% H_2O_2 solution for the inactivation of endogenous peroxidase. Sections were washed again with PBS, and then incubated with TUNEL reaction mixture (5 μL of terminal deoxynucleotidyl transferase enzyme and 45 μL of reaction solution) in an oven set at 37 °C for 60 min in a moist environment. Sections were washed in PBS solution 3 times separately for 5 min and then they were kept in a composition of anti-FITC HRP at 37 °C for 30 min. Sections were washed in PBS solution 3 times separately for 5 min and then they were colorized with diaminobenzidine at room temperature for 10 min. Counterstained sections with Mayer hematoxylin were evaluated by light microscopy.

Apoptotic cells were counted for at least 500 seminiferous tubules in each tissue. Nuclear staining was

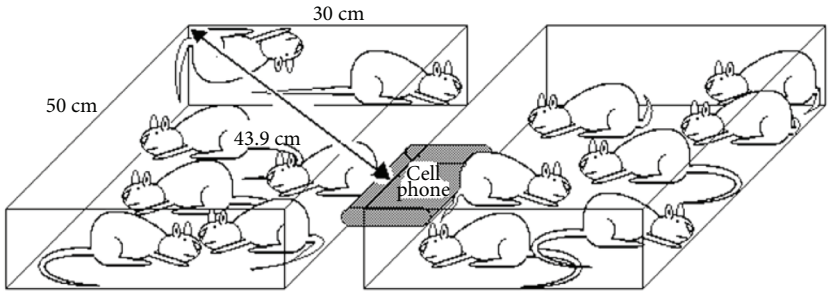

Figure 2. Experimental set-up.

considered as a positive reaction. Two separate apoptotic indexes were used for evaluation. Apoptotic index-1 (AI-1) represents the number of TUNEL-positive apoptotic cells per 100 tubules. Apoptotic index-2 (AI-2) represents the number of tubules containing TUNEL-positive apoptotic cells per 100 tubules.

2.6. Biochemical assay

The total activity of superoxide dismutase (SOD) was measured by the method of Sun et al. (8). First, 100 to 200 mg of testis sample was weighed, diluted at 20% w/v in distilled water, and homogenized. The homogenate was centrifuged at 5000 × *g* for 30 min at 4 °C, and determination was performed in the supernatant fraction. The SOD activity measurements were carried out by inhibiting the SOD activity by nitroblue tetrazolium reduction. Xanthine–xanthine oxidase used as a superoxide generator, and 1 IU was defined as the quantity of SOD required to produce 50% inhibition. The values are expressed as U/mg of protein.

The activity of catalase (CAT) was determined by the Aebi kinetic method (9). A 2.5-mL portion of substrate and a 30% H_2O_2 and 50 mM Tris/HCl buffer of pH 7.4 was mixed with 50 mL of liver homogenate. After 10 s, absorbance was measured at 240 nm, and the kinetic changes of absorbance were marked every 30 s for 2 min. Activity of CAT are expressed in μmol/mg protein.

The tissue malondialdehyde (MDA) levels of the testis tissue were evaluated by the method described by Mihara and Uchiyama (10). The color formed at the end of the reaction of thio-butyric acid (TBA) and MDA was evaluated using this method, and 1.15% KCl, 1% phosphoric acid, 0.6% TBA, and n-butanol reactants were used. Next, 200 mg of tissue homogenized in 1.8 mL of KCl, 3 mL of phosphoric acid, and 1 mL of TBA was added to 0.5 mL of homogenized solvent. This mixture was left in boiling water for 45 min and cooled just after this period. Butanol (4 mL) was then added and centrifuged at 3000 rpm for 5 min. The butanol phase at the upper side was separated and results were defined on a spectrophotometer at 535 and 520 nm. The results were defined as nmol/g tissue after calculations.

The activity of glutathione peroxidase (GSH-Px) was measured by the kinetic method according to Paglia and Valentine (11). Reduced glutathione (GSH) was oxidized by H_2O_2 to GSSG, and then by glutathione reductase was converted back to GSH using NADPH+H+. The rate for GSSG formation was measured by following the decrease in absorbance of the reaction mixture at 340 nm. Activity of GSH-Px was expressed in μmol/mg protein.

The concentration of MDA and the activities of SOD, GSH-Px, and CAT in the testis tissue were recalculated per milligram of protein, the concentration of which was determined according to the method of Lowry et al.(12).

2.7. Data analysis

Analyses were performed using SPSS 13.0. The Kruskal–Wallis test was used in multiple comparisons. In the case of $P < 0.05$ for this test, binary comparisons were performed with the Mann–Whitney U test, and then the limit of significance was considered as $P < 0.003$. The binary comparisons were not performed in the case of detection of $P > 0.05$ by the Kruskal–Wallis test.

3. Results

MDA and CAT levels were not statistically different between groups ($P = 0.243$ and $P = 0.063$, Kruskal–Wallis).

SOD levels between the groups were statistically significant ($P = 0.042$, Kruskal–Wallis). SOD levels were significantly higher in the call group in comparison with the stand-by+antioxidant group in binary comparisons ($P = 0.002$, Mann–Whitney U). Other differences in binary comparisons were not statistically significant ($P > 0.003$, Mann–Whitney U).

GSH-Px levels between the groups were statistically significant ($P = 0.000$, Kruskal–Wallis). GSH-Px levels according to binary comparisons between the control and call groups were statistically significant ($P = 0.001$, Mann–Whitney U). Statistically significant differences were also found between the control and stand-by groups ($P = 0.002$, Mann–Whitney U) and between the antioxidant and call groups ($P = 0.001$, Mann–Whitney U). Other binary comparisons were not statistically significant ($P > 0.003$, Mann–Whitney U).

Statistically significant difference was found among the groups in terms of AI-1 (P = 0.001, Kruskal–Wallis). In binary comparisons, statistically significant differences were found between the antioxidant and call+antioxidant groups (P = 0.002, Mann–Whitney U), between the antioxidant and call groups (P = 0.002, Mann–Whitney U), between the stand-by and call+antioxidant groups (P = 0.002, Mann–Whitney U), and between the stand-by and call groups (P = 0.001, Mann–Whitney U). Other differences in binary comparisons were not statistically significant (P > 0.003, Mann–Whitney U).

The difference among the groups was found as statistically significant in terms of AI-2 (P = 0.001, Kruskal–Wallis). In binary comparisons, the differences were statistically significant between the call and stand-by groups (P = 0.001, Mann–Whitney U), between the call and control groups (P = 0.002, Mann–Whitney U), and between the call and antioxidant groups (P = 0.002, Mann–Whitney U). Other differences in binary comparisons were not statistically significant (P > 0.003, Mann–Whitney U) (Table; Figures 2 and 3a–3c).

4. Discussion

Apoptosis is basically started with 2 pathways: "intrinsic" and "extrinsic". Free radicals and radiation, the possible causes of cell apoptosis, are thought to trigger apoptosis via the intrinsic pathway (13).

Oxidative stress is a state of imbalance between free radicals and antioxidants in favor of the oxidants. Oxygen radicals are the major free radicals formed within biological systems (14).

In our study, radiofrequency (RF) waves emitted by a mobile phone in calling mode significantly increased apoptosis in testicular tissue but did not increase it in stand-by mode. In addition, mobile phones in the calling and stand-by modes also caused a certain degree of oxidative stress. However, antioxidants did not reduce apoptosis or oxidative stress.

There are some studies about the effects of RF waves on apoptosis in various tissues, including testicular, in the range of the wavelength used in the communication systems employed in mobile phones (2,3,15–17).

Dasdag et al. investigated the effects of 900 MHz RF waves emitted by a GSM stimulator on spermatogonia in rat seminiferous tubules. No statistically significant difference was found between the groups and RF waves did not cause apoptosis during spermatogenesis (16). Contrary to the present study, the investigators used a simulator as the RF generator, not a mobile phone, and caspase-3 staining as an apoptotic parameter.

The other relevant study is that by Yilmaz et al. The authors evaluated the effects of 900 MHz waves on apoptosis in the brain and the testis tissues of rats. Contrary to the present study, apoptosis was measured by evaluation

Table. Comparison of parameters according to groups. Group I, control; group II, antioxidant; group III, call; group IV, stand-by; group V, call+antioxidant; group VI, stand-by+antioxidant. The upper values represent means (standard deviation) and the lower values represent medians (minimum–maximum).

	Group I	Group II	Group III	Group IV	Group V	Group VI	P*
MDA	34.62 (10.34) 36.15 (21.54–45.39)	34.95 (5.99) 36.92 (26.92–43.08)	36.81 (6.02) 38.46 (25.39–42.31)	27.69 (7.04) 30.00 (13.85–33.85)	37.95 (9.29) 41.54 (22.31–46.92)	34.29 (5.25) 33.08 (26.15–41.54)	0.243
SOD	33.02 (5.44) 30.76 (27.86–42.19)	36.31 (9.57) 37.38 (25.64–54.79)	32.32 (2.77) [A] 31.64 (28.96–36.34)	32.16 (9.20) 30.91 (17.94–48.81)	29.63 (4.89) 29.20 (24.77–38.44)	25.20 (4.18) [A] 24.49 (20.06–31.19)	0.042
CAT	0.14 (0.03) 0.14 (0.11–0.18)	0.10 (0.02) 0.11 (0.08–0.12)	0.12 (0.03) 0.14 (0.08–0.14)	0.10 (0.02) 0.09 (0.07–0.13)	0.10 (0.02) 0.10 (0.08–0.13)	0.10 (0.01) 0.10 (0.08–0.11)	0.063
GSH-Px	38.28 (18.70) [B, C] 40.56 (16.28–61.92)	52.78 (7.79) [D] 47.39 (46.25–65.34)	91.24 (17.99) [B, D] 93.57 (68.62–121.36)	76.94 (14.43) [C] 81.72 (60.54–98.04)	60.47 (10.41) 59.32 (47.02–77.95)	69.19 (18.95) 67.98 (47.75–102.41)	0.000
AI-1	3.29 (0.88) 3.43 (2.00–4.50)	2.71 (0.49) [E, F] 2.80 (2.00–3.33)	4.77 (0.65) [E, G] 4.79 (4.00–5.50)	2.24 (0.86) [G, H] 2.30 (1.00–3.70)	4.06 (0.41) [F, H] 4.15 (3.45–4.50)	3.40 (1.01) 3.30 (2.20–5.20)	0.001
AI-2	1.80 (0.50) [I] 1.73 (1.10–2.40)	1.71 (0.51) [J] 1.67 (0.20–2.30)	3.04 (0.32) [I, J, K] 3.00 (2.60–3.50)	1.51 (0.50) [K] 1.50 (1.00–2.50)	2.49 (0.33) 2.35 (2.18–3.00)	2.18 (0.47) 2.25 (1.33–2.65)	0.001

*According to the Kruskal–Wallis test. Other P-values according to the Mann–Whitney test at 0.002 for A, 0.001 for B, 0.002 for C, 0.001 for D, 0.002 for E, 0.002 for F, 0.001 for G, 0.002 for H, 0.002 for I, 0.002 for J, and 0.001 for K. P > 0.003 for all comparisons for which P-values are not given.

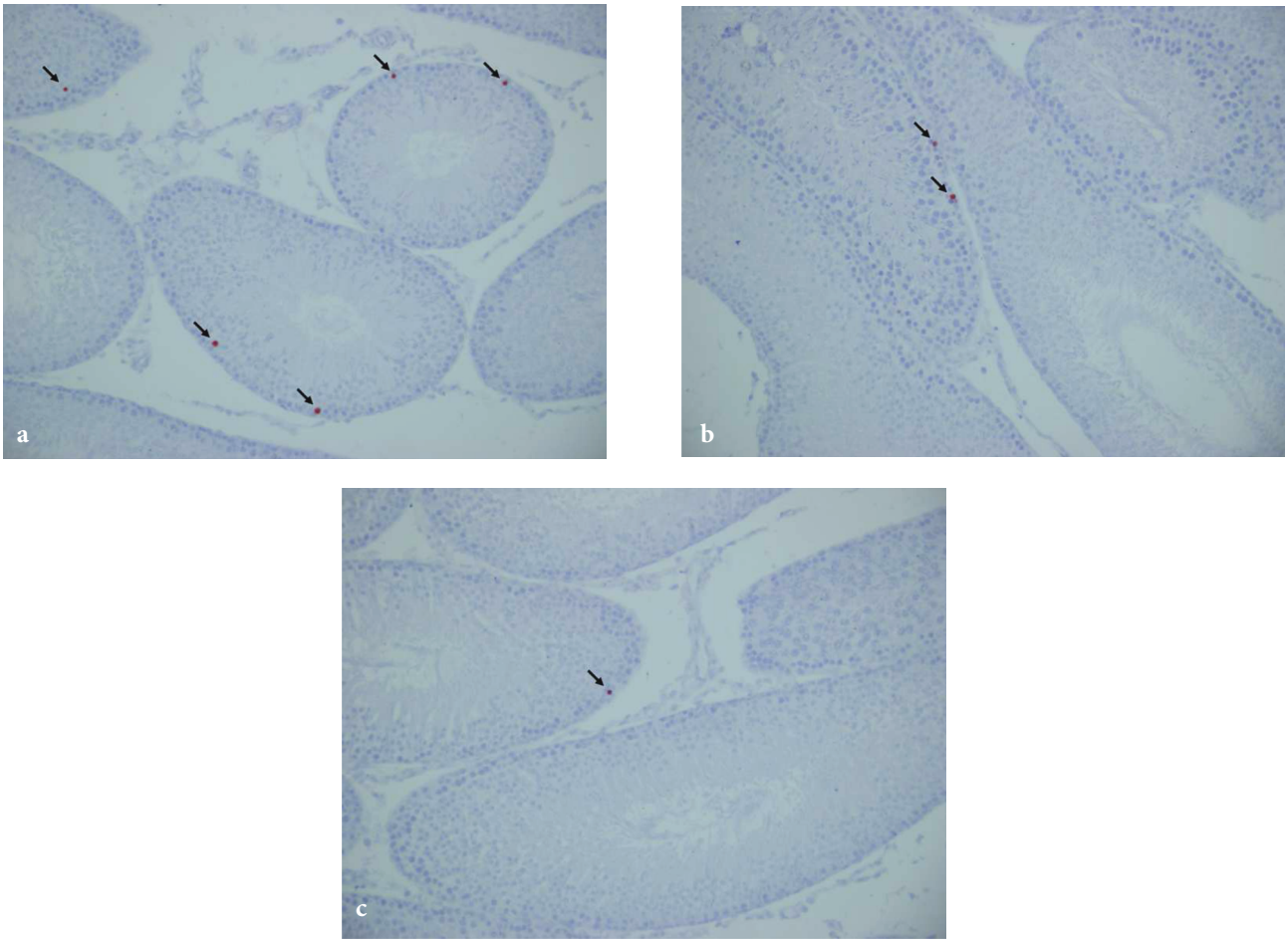

Figure 3. Testicular tissues belonging to: a) rat in the call group (arrows = apoptotic cells, TUNEL, 400×), b) rat in the call+antioxidant groups (arrows = apoptotic cells, TUNEL, 400×), c) rat in the control group (arrows = apoptotic cell, TUNEL, 400×).

of antiapoptotic bcl-2 protein levels. No change in terms of bcl-2 levels was determined between the brain and the testis tissues of rats in the groups (17). A mobile phone was used as the RF generator, like in our study, but the apoptotic effect of stand-by mode was not investigated. In addition, the rats in the EMF-exposed group were exposed to mobile phones in the talking mode for about 600 min during 1 month, but in our study, rats in the call and stand-by groups were exposed to mobile phones in call or stand-by mode for about 2400 min during 1 month. Contrary to the findings of Yilmaz et al., mobile phones caused an apoptotic effect in our study. This effect might be due to the fact that the duration of exposure was 4-fold greater and the mobile phone was used in calling mode, not in talking mode, in our study. As is known, the RF production in the call mode is higher than in the talking mode (18).

To our knowledge, the effects of mobile phones on testicular apoptosis in the call and stand-by modes have been investigated here in a comparative study for the first time. In our study, it was first investigated whether mobile phones in call or stand-by modes can cause apoptosis in testicular tissue, and it was determined that a mobile phone in call mode, but not in stand-by mode, is a significant cause of testicular apoptosis. As noted above, this finding is the first in terms of evaluating the apoptotic effect of mobile phones in stand-by mode on testicular tissue.

However, the apoptotic effect of the mobile phone in stand-by mode on nontesticular tissues has been studied. Indeed, there is only one such study, that by Zhao et al. Those authors investigated whether expression of genes related to cell death pathways was dysregulated in primary cultured neurons and astrocytes by exposure to a working GSM cell phone rated at a frequency of 1900 MHz. They determined that even relatively short-term exposure to cell phone radiofrequency emissions can up-regulate the elements of apoptotic pathways in cells derived from the brain, and that neurons appear to be more sensitive to this effect than astrocytes (15).

Unlike in those studies, the oxidative processes examined in our study have been revealed as being of interest in apoptotic processes, as well as the effects of antioxidants on oxidation and apoptosis. In our study, antioxidants did not significantly reduce apoptosis caused by RF waves produced by cell phones in the call mode. Therefore, to our knowledge, the effect of antioxidants on apoptosis induced with a real cell phone was investigated for the first time here, and it has been shown for the first time that antioxidants did not decrease apoptosis.

Dasdag et al. investigated the testicular effects of cell phones on p53 and MDA parameters, but they found no significant effect (19). If the role of the level of p53 in apoptotic processes is to be considered, this study weakly showed that cell phones do not cause apoptosis in testicular tissue, and in the same way, no peroxidation is constituted because of the relationship between MDA and oxidative stress.

The effects of mobile phones on apoptosis and oxidative stress in nontesticular tissues have been studied by some researchers (2,3). In Oral et al.'s study, the 900 MHz RF waves increased apoptosis and oxidative stress in the endometrial tissues of rats, and vitamin E and C treatments reduced these changes (2).

In our study, the RF waves of the mobile phone both in stand-by and in calling mode significantly increased GSH-Px levels. It was thought that these increased levels of GSH-Px in testicular tissue occurred with increased oxidative stress caused by the RF waves emitted by the mobile phone in both the call and the stand-by mode. However, increased levels of GSH-Px were reduced with antioxidants, but this decrease was not statistically significant. While the mobile phones in the stand-by mode and the call mode caused a significant increase in the levels of GSH-Px in the testicular tissue, interestingly, they did not cause any significant increase in CAT levels. In other research, H_2O_2 was broken down primarily by GSH-Px in low concentrations, but CAT was found to be activated at higher concentrations (20). Therefore, we thought that oxidative stress caused by cell phones in our study did not produce H_2O_2 at a high enough level for the activation of catalase, with oxidation stopped at the level of GSH-Px.

There is only one study about whether the RF waves emitted by mobile phones increase the oxidative stress in testicular tissue. Mailankot et al. evaluated the effects of RF-EMR from mobile phones on free radical metabolism and sperm quality. They found a reduction in the percentage of motile sperm in rats exposed to RF-EMR, as well as significantly elevated lipid peroxidation and decreased GSH content in the testis and epididymis. They used an active mobile phone. However, they did not explain which mode was used as their definition of an "active" mobile phone (21).

In a study by Dasdag et al., it was weakly shown that mobile phones did not increase levels of MDA in testicular tissue or oxidative stress. The other parameters used in the determination of the status of oxidative stress were not studied in that report (19).

There are several studies about whether the RF waves produced by mobile phones increase the oxidative stress on various tissues (22–24). More studies are needed to investigate potential effects of EMFs caused by RF wave-producing devices such as mobile phones in terms of oxidative stress, apoptosis, and protective activities of antioxidants.

In this study, while significant increase of apoptosis was generally found in RF wave-exposed tissues, significant change was not observed in oxidative stress parameters, except for GSH-Px. The effects of antioxidants on the apoptosis and oxidative stress were not significant.

RF waves emitted by mobile phones, especially during the calling mode, led to apoptosis, but did not significantly increase oxidative stress. This situation may point toward some other unknown nonoxidative apoptotic processes. In fact, it was also determined that apoptosis induced by a mobile phone could not be reduced by antioxidants.

As a result, until obtaining more conclusive human studies with larger series, there should be more caution about the biological effects of mobile phones in the stand-by mode, especially in reproductive age groups. In this context, mobile phones should not be kept in the calling mode for long periods and should usually be kept away from the body; communication systems working without RF waves should be preferred; and, finally, more advanced technologies with fewer biological effects should be developed.

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