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Original Research Article

The Effect of Radio Frequency (RF) on Proteomics Pattern of Brain Tissue in Male Wister Rats

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HIGHLIGHTS

- Radio Frequency (RF) waves can affect the protein expression profile of the cell.
- Exposure to nonstandard RF waves changed the expression profile of apoptosis key proteins.
- Changes in the expression of Bcl-2-A1, Bid, Neurofilament and Cytochrome Oxidase under the RF was evident.

ABSTRACT

Radiated waves from mobile phones are in the Radio Frequency (RF) range, so those are unable to cause ionization and electron excitation. RF that are produced and emitted from sender antenna on the surface of mobile phone systems, ranges from 30 up to 300 GHz in electromagnetic wave spectrum. There is no complete information about the effects of RF on protein changes. For this purpose, we have examined the effects of RF waves on the changes in protein expression pattern of the brain. In this study, 10 heads of male Wistar rats, weighing between 200-250 g, were exposed to transmission antenna in 100-180 meter distance. Then, proteome of $RF_{900\ GHz}$ exposed group and control group were extracted. Two-dimensional gel electrophoresis evaluation and proteomics analysis was performed. Results showed altered proteome pattern due to radiation. Tissue exposed to nonstandard waves showed similar pattern of changes in the expression of some proteins, which have key roles in the induction of apoptosis. The expressions of key proteins including Bcl-2-A1, Bid, Neurofilament and Cytochrome Oxidase were decreased. Expression or suppression of apoptosis related proteins such as BCL-2 in rat brain proteome exposed to standard $RF_{900\ MHz}$ (at 180m and beyond), can serve as a biomarker of brain activity, memory and sleep. RF radiated from transmission antenna in urban and standard spaces may not be carcinogenic, but, individuals exposed in nonstandard distances to those antennas (less than 180meter) may be at risk.

Introduction

The theory of electromagnetic (EM) waves of various ranges, which are of practical interest in radio engineering and electronics, including transmission lines, resonators, and radiating systems, has been well presented in the literature. Magnetic waves in vacuum travel at the speed of light and are composed of electric and magnetic fields, which are perpendicular to each other (Hosseinyzadeh et al., 2015). Human beings receive electromagnetic radiation daily and continuously. Although these radiation are low in energy, however, they have risks and harmful

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Table 1. Details of expression profile change; details spots data include: Number spots, molecular weight and isoelectric pH.

#	ANOVA (p)	Fold	PI	MW	Average Normalized Volumes	
					Control	Test
8	5.773e-015	+1.3	5.73	69	1551.590	2006.000
15	6.994e-015	-8.7	9.6	42	3103.331	358.000
20	1.549e-013	-2.9	4.15	66	5.362e+004	1.531e+005
28	1.895e-013	-2.0	5.72	82	3001.268	1517.000
33	2.234e-013	+2.1	3.95	56	3644.273	7737.000
43	2.961e-012	-1.6	3.68	30	1647.051	2616.000
45	3.359e-012	-1.2	6.48	69	3241.324	2785.000
64	6.541e-012	-5.1	5.36	82	4751.843	935.000
66	1.403e-011	-6.7	5.29	80	4827.987	716.000
68	2.464e-011	+2.4	4.01	67	3.464e+004	8.302e+004
70	2.880e-011	-3.5	3.91	77	4450.108	1271.000
72	3.253e-011	+5.7	4.64	44	1831.639	1.045e+004
75	3.355e-011	-2.4	5.17	86	2511.354	1058.000
77	3.707e-011	-2.4	5.05	80	1.633e+004	6719.000
81	4.229e-011	+3.3	5.48	82	3155.665	959.000
87	7.529e-011	-2.7	5.25	94	1.014e+004	3768.000
88	1.082e-010	+2.2	6.07	51	887.328	1931.000
89	1.284e-010	+3.3	5.08	16	5096.712	1.657e+004
90	1.331e-010	-29.3	5.8	25	909.655	131.000
91	1.482e-010	-2.5	5.38	85	6299.076	2510.000
94	1.672e-010	-4.3	6.11	64	2018.518	470.000
92	2.316e-010	-3.9	6.94	82	5458.039	1406.000
93	3.638e-010	-1.9	6.8	26	3396.141	1802.000
96	4.047e-010	+1.8	4.54	53	3223.657	5842.000
102	8.143e-010	-3.4	5.18	80	1.226e+004	3556.000
113	8.977e-010	-7.9	6.85	25	2024.858	255.000
47	9.486e-010	-1.9	6.9	20	4598.640	2431.000
51	1.091e-009	-3.7	7.26	82	5228.023	1401.000
57	1.107e-009	-4.3	6.23	81	1.366e+004	3167.000
61	1.285e-009	3.5-	4.86	81	4192.028	1192.000
53	1.357e-009	-1.7	6.37	50	6540.530	3873.000
58	1.405e-009	2.4+	4.96	40	3837.010	9067.000
97	1.683e-009	-7.2	6.34	83	2701.765	373.000
98	1.923e-009	-3.8	6.4	81	2.037e+004	5426.000
99	2.038e-009	+5.7	5.8	28	376.621	2160.000

Table 1. Continued.

#	ANOVA (p)	Fold	PI	MW	Average Normalized Volumes	
					Control	Test
103	2.060e-009	-1.9	6.31	77	2603.870	1400.000
104	2.182e-009	-4.3	6.9	21	1655.501	381.000
105	2.310e-009	-2.0	5.04	71	5697.809	2849.000
106	2.403e-009	+3.9	4.21	41	477.119	1848.000
107	2.475e-009	-1.5	6.7	69	2590.109	1777.000
108	2.586e-009	-1.8	6.09	69	4313.795	7943.000
109	3.046e-009	-3.1	5.49	95	3780.625	1220.000

*Molecular Weight (MW), Isoelectric pH (PI).

effects. However, the effects of radiation on living organisms had been overshadowed by ionizing radiation for a long time. Studies showed that, the sources of magnetic wave, microwave and RF systems that exist in every environment and to which every individual can be exposed, are non-ionizing (Hossmann and Hermann, 2003). Today, mobile phones are one of the most common devices emitting electromagnetic waves, and are easily available to different ages in almost half of the world population. The urgent need to reconsider exposure limits for low frequency and static magnetic fields, based on combined experimental and epidemiological research regarding the relationship between exposure to non-ionizing radiation and adverse human health effects is felt (Calvente et al., 2010). Mobile technology operates in the microwave frequency range of 300 MHz to 300 GHz. Studies showed that in areas near mobile antenna, the radiation is most effective on cells and tissues of living organisms, through non-thermal effects. Kiliçalp showed that, exposure of guinea pigs to EM fields could cause disturbances in autonomic cardiac regulation (Kilicalp et al., 2009). It has also been shown that, electromagnetic waves can affect the texture of original hematopoietic tissue such as the spleen, liver, yolk sac, and bone marrow (Nylund and Swanson, 1963; Hosseinyzadeh et al., 2015; Pooladi et al., 2018), gonads, and brain (Guney et al., 2007).

On the other hand, proteomics is the study of proteins and their related functions at a large scale. Proteomics studies have the potential to provide perspectives, on molecular dynamics, and trends in pathogenesis. Considerable research has been done to characterize tumor proteins caused by electromagnetic radiation. Proteomics is a powerful tool in identifying multiple proteins that are altered following a neuropharmacological intervention in a disease of the central nervous system (CNS). Protein separation and comparison by two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) can be used to determine the quantity of particular proteins (Pooladi et al., 2013; Hashemi et al., 2014; Khaghani-Razi-Abad et al., 2015).

The International Agency for Research on Cancer (IARC) studies in the radio frequency range, show that 900 MHz mobile phone radiation waves can lead to a significant increase in lipid peroxidation and plasma malondialdehyde as well as superoxide dismutase levels.The radiation also decreases catalase, glutathione reductase and glutathione peroxidase activity in the brain tissue (Hardell and Mild, 2001). Moreover, studies have shown a correlation between cell phone usage and glioma or meningioma (Muscat et al., 2000). Previous studies have reported detrimental effects of the exposure to $RF_{900\ MHz}$ in cell proliferation, apoptosis, and loss of brain neurons.

The underlying mechanisms may be due to increased production of ROS, disruption of mitochondrial functions, intracellular calcium homeostasis, up regulated expression of heat shock proteins, and ultimately specific changes in gene expression profile of the brain (Zhao et al., 2007).

Cell phones often worked with electromagnetic radiation, and it seems that these devices are safe, because of their non-ionizing waves. Although, many manufacturing companies of mobile devices believe that these devices pose no danger to human health, however, the results of many studies suggest some adverse effects on the health of organisms (Calvente et al., 2010; Aboul-Ezz et al., 2013). This study was done to clarify the conflicting data related to the effects of $RF_{900\ GHz}$ on proteome pattern of brain tissues in rats.

In the present study, we evaluated the risk of protein expression of the brain in rats following $RF_{900\ MHz}$ exposure. Here, proteins of test and normal brain tissues

Table 2. The molecular weight and isoelectric pH of the proteins (Bcl-2-A1, Bid, Neurofilament and Cytochrome Oxidase) that were shown suppression in their expression, compared to control independently.

Protein Name	Bcl-2-A1		Bid		Neurofilament		Cytochrome Oxidase	
	MW	PI	MW	PI	MW	PI	MW	PI
Experimental	26	6.3	25	5.8	50	6.37	20	6.9
Fold change	1.88		6.94		1.7		1.89	

were extracted and evaluated by proteomics tools (2D gel electrophoresis).

Materials and Methods

Animals

Two groups of adult male Wistar rats with body weighting 200±25g were purchased and raised in our colony from an original stock of Pasteur institute (Tehran, Iran). The temperature controlled environment was at 23±2 ˚C and animals were kept under a schedule of 12h light and 12h dark cycle (light on at: 08:00 AM). Animals had free access to water and standard laboratory food. Care was taken to examine the animals for general pathological symptoms. Food was withheld for 12-14h before sacrifice. This study was performed according to ethical guidelines of working with laboratory animals (National Research Council, 2011).

$RF_{900\ MHz}$ exposure system

Male Wistar rats were randomly divided into control rats (Group I: in the 180 m range of transmission towers) and experimental rats (Group II: at risk of receiving substandard dose, 100-180 m). Rats were placed in the shuttle box. The test was performed for two hours for each rat and was repeated twice at four day intervals. A spectrum of short wavelength electromagnetic waves ($RF_{900\ MHz}$) was used. For further details about exposure conditions, see references (Sepehrimanesh et al., 2014; Sepehrimanesh et al., 2017).

Tissue preparation and proteins extraction

Whole brains were carefully dissected and lipids extracted according to Folch and collaborators (Folch et al., 1957). In parallel to lipid isolation, the protein fractions were also collected. Typically, brain tissues were frozen in liquid nitrogen and were further homogenized in lysis buffer (Tris-HCl 50 mM, urea 7M, thiourea 2M, CHAPS 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate 2%, C7BzO 0.5%, DTT 20 mM) and 100 µl of a protease inhibitor cocktail (Roche Diagnostics) were added. Then, the homogenates were sonicated for 90s (15% amplitude for 3 cycles of 30s each). All protein extraction steps were performed on ice.

After elimination of debris by centrifugation (10,730 ×g for 20min at 4°C), supernatants were collected and submitted to a 2-D clean-up Kit (GE Healthcare), then, the precipitation step was applies to eliminate interfering compounds.

The protein concentration was subsequently determined using the Bradford method (Bradford, 1976).

In following, 30 µg of protein from rat brain samples were loaded on a 7% stacking polyacrylamide gel and after a short migration and staining, the proteins were cut out from the SDS PAGE gel just after they penetrated into the gel. The revealed gel bands were excised, immersed in a reductive medium containing 5 mM DTT, and cysteines were then irreversibly alkylated in 25 mM iodoacetamide. After extensive washing with water, protein-containing bands were subjected to trypsin digestion (0.5 µg per band, Promega). Several steps of peptide extraction were performed in H_2O/CH_3CN (1:1) solutions acidified with 0.1% TFA and finally, for each sample, peptide extracts were combined and dried in room temperature (Pooladi et al., 2013; Hashemi et al., 2014).

Protein separation

First-dimension separation of samples, based on isoelectric point, was performed by Isoelectric Focusing (IEF). Second-dimension separation based on molecular mass (MW) was performed by vertical large-format SDS-PAGE. First-dimensional gel electrophoresis for protein separation was performed with IPG strips (18 cm) using IEF system. IPG trips were then transformed to SDS-PAGE 12.5% gel and proteins were further separated based on their MW. Followed by a final silver nitrate staining. Gel images were digitally converted to grayscale images and transferred to a UNIX workstation for subsequent analysis of spot densities. The gels were also stained with Sypro Ruby to visualize the total protein in each gel from both samples (Khaghani-Razi-Abad et al., 2015; Kochanek et al., 2006).

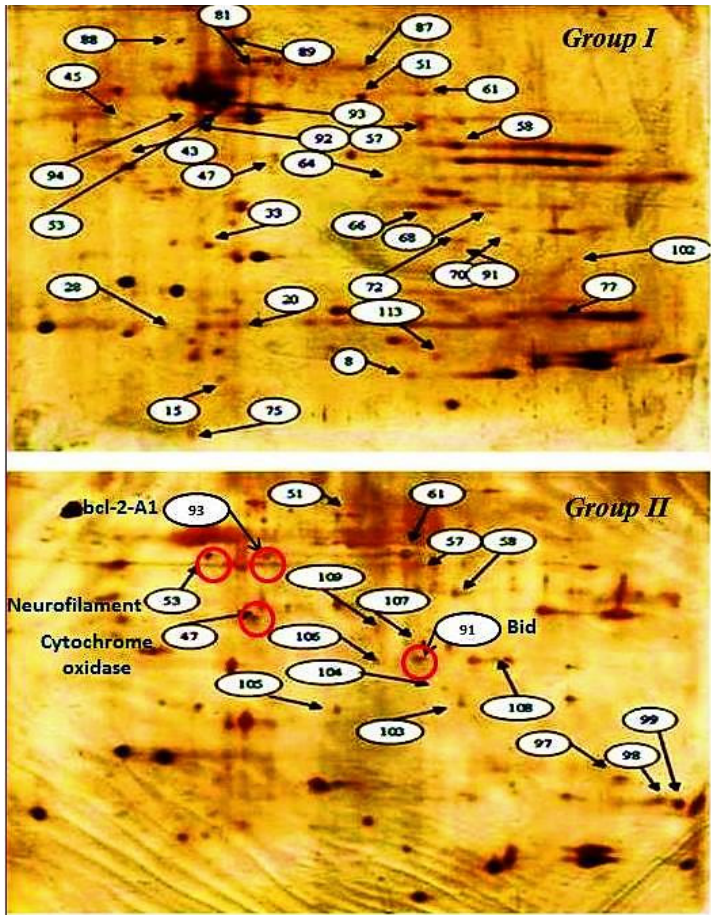

Figure 1. Two-dimensional gel electrophoresis images of control rats (Group I: in the 180 m range of transmission towers) and experimental rats (Group II: at risk of receiving substandard dose, 100-180 m) proteins were separated on the basis of PI and MW.

SDS-PAGE scan and bioinformatics analysis

SDS-PAGE gels were scanned using scanner Densitometer GS-800 (BioRad) scanner at 600 dpi in tagged image file format (TIFF). Image MasterTM 2D platinum v6.0 software was then used to extract and digitize data from graphical images of scanned gels through detecting, normalizing, matching and comparing protein spots according to their volume percent, followed by primary analysis of 2D images by Quantity One® software. The obtained scanned images of SDS-PAGE gels were further analyzed by Non-Linear Dynamics Progenesis Same Spot® Software. After comparing the obtained 2D images with control samples, primary protein detection was performed based on the protein bands. By comparing the results with other published articles, and matching the molecular weight and isoelectric pH values as well as using the webserver of www.ebi.ac.uk/IPI, several protein spots were predicted.

Statistical analysis

All experiments were carried out in triplicate and data are expressed as mean ± SD, unless otherwise specified. One Way Analysis of Variance (One Way ANOVA) was systematically performed to infer statistical significance to the different sets of results. *P*-values < 0.05 was considered significant, using Statistica V5.1 (Statsoft).

Results and Discussion

Statistical comparison of protein dots visualized on gel after 2D electrophoresis in controls and samples tests

The representative set of overlaid spot images is given in Fig. 1. Our results show six spots are common in both the gel sample and the control ($P< 0.05$). Five spots were

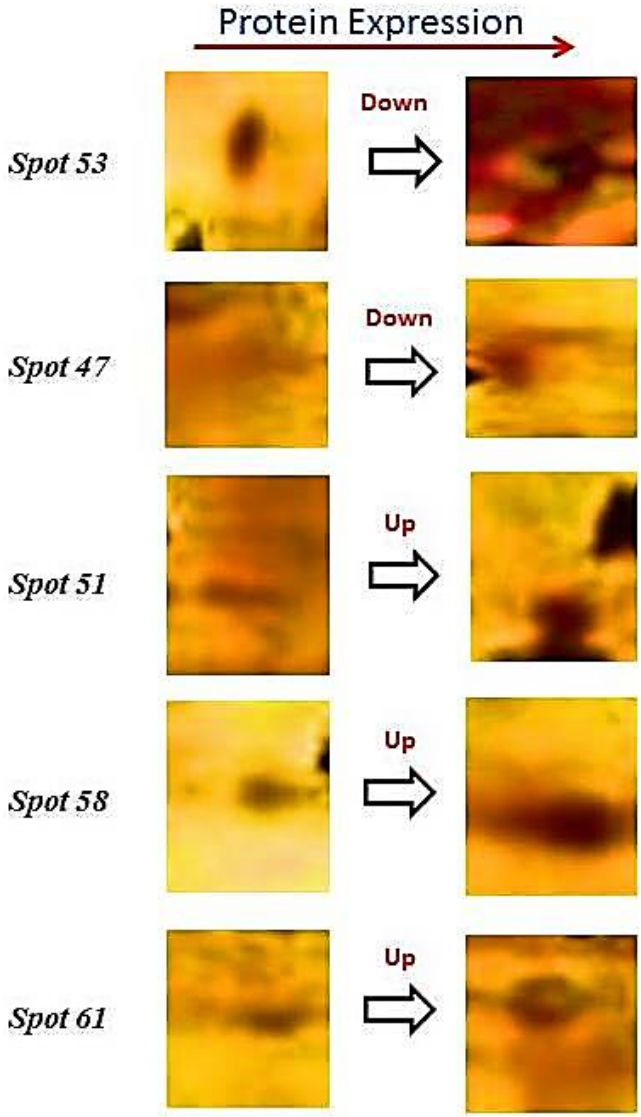

Figure 2. Over and down expression was detected in 5 common spots.

over and down expressed more than two fold. The highest increase is for spot 53 (Fig. 2). The spot has a molecular weight of 50 kDa and isoelectric pH of 6.37. Also, spot 47 in all stains have less than two fold increase (Fig. 2). The spot has a molecular weight of 20 kDa and an isoelectric pH of 6.9.

Analysis of the protein spots

- Spot93: This spot, with a 26 kDa molecular weight and isoelectric pH 6.8, which is likely related to family protein bcl-2-A1.
- Spot91: This spot, with a 25 kDa molecular weight and isoelectric pH 5.8, which is likely related to family protein Bid.
- Spot 53: This spot, with a 50 kDa molecular weight and isoelectric pH 6.37, which is likely related to neurofilament protein family.
- Spot 47 :This spot, with a 20 kDa molecular weight and isoelectric pH 6.9, which is likely related to Cytochrome oxidase protein family.
- Spot 99: This spot, has a molecular weight of 28 kDa and an isoelectric pH of 5.8. It has no relationship with any other protein family and their further characterization requires mass spectroscopy.
- Spot 104 :This spot, with a 21 kDa molecular weight

and isoelectric pH of 6.9. It has no relationship with any other protein family and their further characterization requires mass spectroscopy.

The effects of protein expression changes on brain activity

The findings support the concept that presumably, the high-risk non-ionizing radiofrequency, in distance of 100 to 180 m from the mast can suppress the apoptotic pathways through affecting the expression of cellular proteins, and hence can lead to cancer. It has long been shown that BCL-2 and BCL-XL inhibits the release of cytochrome C and thereby, hinder the cell suicide/ apoptosis response (Yang et al., 1997; Szklarczyk et al., 2015). Other studies had also showed that the exposure of rats to microwave radiation 2450 MHz for 45 minutes, manifested learning loss (Kluck et al., 1997).

Mann and colleagues in 1996 reported that Rapid Eye Movement (REM) sleep in humans decreased upon radiofrequency exposure. The electroencephalogram (EEG) during REM sleep was also altered. REM sleep is essential for learning and memory in the brain. The REM sleep, is required to select and classify new observations and information at the time of awakening and reconnect them with old events (Karimi et al., 2018). It has showed that changes in the expression levels of binding proteins and changes in calcium metabolism cause adverse changes in the hippocampal brain, attention and brain learning systems (Paulraj and Behari, 2006).

The results showed the changes in the expression of proteins, which can serve as biomarkers for the effects of radiofrequency radiation. Our study confirmed that expression of protein in spot 47, a member of cytochrome oxidase family of proteins, with PI ~ 6.9 and a molecular weight of 20 kDa, decreased upon radiation. Its activity levels might be correlated to energy production within brain mitochondria (Ammari et al., 2008; Maskey et al., 2010).

It should be noted that melatonin as a hormone regulates sleep-wake cycle during day and night, showed variable expression levels under the influence of radio waves, and by changing in their expression, its function will alter (Gherardini et al., 2014). The changes in the expression of proteins (Bcl-2-A1, Bid, Neurofilament and Cytochrome Oxidase**)** under the RF could be considered as the evidence for this effect.

Conclusion

Proteins, in the face of a non-standard (unconventional) radio frequency radiation, may be experienced changes in molecular weight and isoelectric pH. Proteomics investigations showed that a large number of apoptosis protein of rat brain was changed after non-standard RF radiation. From the changes were seen in the expression level of key proteins in apoptosis, such as Bcl-2-A1, Bid, Neurofilament and Cytochrome Oxidase, it can be concluded that although non-ionizing radiation emitted from transmission towers, in urban areas may not be carcinogenic per se, when the distances are according to the established guidelines, however, the risk of carcinogenicity increases in the substandard distances.

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Competing Interests

The authors declare that they have no competing interests.

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