



Long term exposure to cell phone frequencies (900 and 1800 MHz) induces apoptosis, mitochondrial oxidative stress and TRPV1 channel activation in the hippocampus and dorsal root ganglion of rats

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Abstract

Mobile phone providers use electromagnetic radiation (EMR) with frequencies ranging from 900 to 1800 MHz. The increasing use of mobile phones has been accompanied by several potentially pathological consequences, such as neurological diseases related to hippocampal (HIPPO) and dorsal root ganglion neuron (DRGN). The TRPV1 channel is activated different stimuli, including CapN, high temperature and oxidative stress. We investigated the contribution TRPV1 to mitochondrial oxidative stress and apoptosis in HIPPO and DRGN following long term exposure to 900 and 1800 MHz in a rat model. Twenty-four adult rats were equally divided into the following groups: (1) control, (2) 900 MHz, and (3) 1800 MHz exposure. Each experimental group was exposed to EMR for 60 min/ 5 days of the week during the one year. The 900 and 1800 MHz EMR exposure induced increases in TRPV1 currents, intracellular free calcium influx (Ca^{2+}), reactive oxygen species (ROS) production, mitochondrial membrane depolarization (JC-1), apoptosis, and caspase 3 and 9 activities in the HIPPO and DRGN. These deleterious processes were further increased in the 1800 MHz experimental group compared to the 900 MHz exposure group. In conclusion, mitochondrial oxidative stress, programmed cell death and Ca^{2+} entry pathway through TRPV1 activation in the HIPPO and DRGN of rats were increased in the rat model following exposure to 900 and 1800 MHz cell frequencies. Our results suggest that exposure to 900 and 1800 MHz EMR may induce a dose-associated, TRPV1-mediated stress response.

Keywords Apoptosis · Dorsal root ganglion · Hippocampus · Mobile phone frequency · Oxidative stress · TRPV1 cation channel

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular free calcium ion
CapN	Capsaicin
CapZ	Capsazepine
DHR	Dihydrorhodamine
DRGN	Dorsal root ganglion neuron
EMR	Electromagnetic radiation
HIPPO	Hippocampal neuron

PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SAR	Specific absorption rate
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1
VGGC	Voltage gated calcium channel
W.C.	Whole cell

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Introduction

The use of mobile phones and Wi-Fi devices that emit electromagnetic radiation (EMR) is increasing worldwide in the twenty-first century at an alarming rate. These devices have improved the quality of life by enhancing data transfer and communication technology. However, powerful energy emitting mobile phones are now commercially available with only limited studies on their hazardous effects on neuronal injury, pain and cancer progression (Nazıroğlu et al. 2012a; Cinar et al. 2013; Ghazizadeh

and Nazıroğlu 2014). The incidence of hippocampal (HIPPO) and dorsal root ganglion neuron (DRGN) related diseases such as epilepsy, migraine and peripheral pain are increasing concurrently with the rate of cell phone usage (Carballo-Quintás et al. 2011; Cinar et al. 2013; Kesari et al. 2013). Individuals of all ages of life are affected by EMR-emitting mobile phone devices. Therefore, understanding the effect of mobile phones frequencies on HIPPO and DRGN is a widespread urgent public safety concern.

The production of reactive oxygen species (ROS) occurs during several physiological processes such as phagocytosis of bacteria and viruses, and the mitochondria respiratory system. Excessive levels of ROS are scavenged by antioxidants. The main targets of ROS are brain and neurons, due to the high rate of oxygen consumption and polyunsaturated fatty acids (PUFAs), and lower antioxidant defense mechanisms (Halliwell 2006). Frequency ranges of 900–1800 MHz EMR are used by second generation mobile phone operators and are defined as non-ionizing EMR. When the 900 and 1800 MHz is absorbed at the molecular level, it may induce thermal effects in heating of macromolecules eukaryotic cells (Bortkiewicz et al. 2012; Nazıroğlu et al. 2013; Yüksel et al. 2016). Therefore, damage to cells due to exposure to harmful EMR is likely due to thermal and oxidative stress mechanisms. Pronociceptive polymodal receptor sensing transient receptor potential (TRP) vanilloid 1 (TRPV1) is a member of the Ca^{2+} permeable TRP super family. The TRPV1 channel is activated different stimuli, including capsaicin (CapN), oxidative stress, high temperature and acidic pH (Caterina et al. 1997; Caterina et al. 1999; Ibi et al. 2008; Pecze et al. 2013; Nazıroğlu et al. 2017). Expression level of TRPV1 is mainly high in neurons such as HIPPO and DRGN (Caterina et al. 1997; Crawford et al. 2009). Therefore, it is also believed to be involved in pain signalling and hippocampal injury (Bodera et al. 2013; Gökçek-Saraç et al. 2017). Activation of TRPV1 through mitochondrial oxidative stress enhanced $[\text{Ca}^{2+}]_i$ accumulation, which is involved in several physiological and pathological processes such as neuronal apoptosis and neuronal recovering signaling (Pecze et al. 2013; Nazıroğlu et al. 2017). Therefore, TRPV1 could act as the final substrate of oxidative stress mediators that operate via distinct intracellular signaling pathways such as NADPH oxidase (Ibi et al. 2008; Nazıroğlu 2017). In acute *in vitro* experiments, we were not able to observe any effect of 900 MHz EMR exposure on TRPV1 activation and heat in HIPPO (Nazıroğlu et al. 2015). However, we recently observed increased high body temperature and oxidative stress effects following long term 900 and 1800 MHz exposure in female rats (Yüksel et al. 2016). Therefore, oxidative stress and heat-dependent activation TRPV1 channels may be involved in the etiology of HIPPO and DRGN injury following exposure to 900 and 1800 MHz EMR in rats.

The mechanism underlying HIPPO and DRGN oxidative injury and programmed cell death through 900 and 1800 MHz EMR exposure remains unclear. To our knowledge, there is no report of the effects of 900 and 1800 MHz EMR exposure on apoptosis, mitochondrial oxidative stress and Ca^{2+} entry in the HIPPO and DRGN of rats. Therefore, the aim of the current study was to examine the molecular mechanisms of 900 and 1800 MHz EMR exposures on apoptosis, oxidative stress and Ca^{2+} entry through TRPV1 activation in the HIPPO and DRGN of rats.

Material and methods

Animals

We used 24 female Wistar rats (aged 12 weeks old with 180 ± 10 g body weight) in the current study. The study was approved by the Local Experimental Animal Ethical Committee of Suleyman Demirel University (SDU) (Protocol number: HADYEK-21438139-604-E-2017). The rats were housed in controlled-temperature (22 ± 2 °C) and humidity (65%) rooms, under a 12:12 h light-dark cycle, with *ad libitum* access to water and food until use.

Study groups and sampling.

The 24 rats were equally divided into three groups as follows: The control group was not exposed to radiofrequencies. In the 900 and 1800 groups, rats were exposed to 900 MHz and 1800 MHz for 60 min/day for one year (5 days/week) as described in a previous study (Yüksel et al. 2016). The 900 and 1800 MHz groups were irradiated for 1 h (between 9:00 AM and 12:00 PM) every day, five days a week (total one year).

Twelve hours after the last exposure to the EMRs, rats were decapitated under propofol inhalation anesthesia in accordance with SDU experimental animal legislation. In the patch-clamp experiment and $[\text{Ca}^{2+}]_i$ concentration assays, the HIPPO and DRGN were further treated with capsaicin (CapN and 10 μM) for activation of TRPV1, and the neurons were blocked using the TRPV1 antagonist, capsazepine (CapZ and 0.1 mM). The effective doses of CapN and CapZ on TRPV1 in several neurons and cells were between 100 nM and 0.1 mM (Rohm et al. 2015; Han et al. 2016). In a recent study (Nazıroğlu 2017), the optimal dose of CapN and CapZ was found to be 10 μM and 0.1 mM in the DRGN, respectively. Therefore, the same optimal doses were used in the current study.

EMR exposure system

The details of the exposure system were previously described in recent studies (Çetin et al. 2014; Yüksel et al. 2016). A generator from Biçer Electronic Co (Sakarya, Turkey)

equipped with a half-wave dipole antenna system was used to irradiate the rats with 900 and 1800 MHz radio frequencies with 217-Hz pulses. The electric field density was set at 20 dB and 11 V/m in order to obtain 0.1 W/kg whole-body average specific absorption rate (SAR). The distance of the antenna from the head, vertebra and lumbar bones of the rats for the mobile phone (900 and 1800 MHz) exposures were selected as 10 cm, because we observed that mobile phone frequencies at 900 and 1800 MHz induced oxidative stress until distances of 10 cm (Çiğ and Nazıroğlu 2015). The rats were placed in plastic cylindrical container with the radio frequency source for 1 h. The exposure system was kept in a specific room, which included plastic furniture for protection from possible radiation reflection. The required electrical field density (0.1 W/kg whole-body average SAR) for 900 and 1800 MHz exposures was continuously recorded every 5 min using a satellite level meter (EXTECH-480836, Extech Instruments Corporation, Nashua, NH, USA) as previously described (Jin et al. 2012).

Dielectric permittivity and conductivity values of the rat tissues at certain frequencies were obtained from a previous study (Jin et al. 2012). The whole-body SAR values varied in the 0.001–1.1 W/kg range, representing a mean SAR value of 0.15 ± 0.10 W/kg for whole-body EMR exposures of 900 and 1800 with a value of 10 V/m at the closest point in the body. The corresponding SAR, in continuous waves, ranged from 8.6 to 93 mW/kg with carrier frequencies of 900 and 1800 MHz (Özorak et al. 2013).

The control rats were placed in a cylindrical container with the radio frequency source switched off during similar times as those used for irradiation as described in a previous study (Özorak et al. 2013). The control animals were kept in their cages without any treatment or restraint of any kind except cage stress.

Preparation of HIPPON and DRGN samples

The DRGN from thoracic and lumbar spinal cord of rats were incubated in 0.25 ml Earle's balanced salts solution (Sigma) and 5 µl collagenase type XI (Worthington) for 15 min at 37 °C (Nazıroğlu et al. 2012b). After adding 0.75 ml cold PBS (without Ca^{2+}), the DRGN was mechanically minced and washed three times and centrifuged (300 g at 4 °C) in 1 ml PBS (three times). After the last centrifugation, DMEM (low (1 g/ml) glucose, Sigma) was added to DRGN and incubated in the medium for 1 h at 37 °C. Neurons were pelleted, suspended in the DMEM medium containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 2 mM glutamine.

Primary cultures of rat HIPPON were prepared as previously described (Nazıroğlu et al. 2015). Hippocampal tissue was dissected and placed in cold Hank's buffered salt solution prior to mechanical dissociation by trituration and incubated

for 30 min with trypsin and mixed every 10 min. It was centrifuged (at 500 g for 5 min) and the supernatant was discarded and replaced by Hanks' solution twice and was then used in assays.

Following isolation and dilution with the DMEM medium, the HIPPON and DRGN were counted using an automatic cell counter system (Casy TT cell counter, Roche Innovatis AG, Reutlingen, Germany). Afterwards, the neurons were diluted into appropriate concentrations in sterile culture flask with the DMEM medium.

Electrophysiology

Whole-cell voltage clamp recording was taken from the DRGN (EPC10, HEKA, Lamprecht, Germany). We used standard extracellular bath and pipette solutions as described in a previous study (Nazıroğlu et al. 2012b). The standard bath solution contained (in mM): 140 NaCl, 1.2 MgCl_2 , 1.2 CaCl_2 , 5 KCl, 10 HEPES, pH 7.4 (NaOH). For Na^+ free solutions, Na^+ was replaced by 150 mM N-methyl-D-glucamine (NMDG) and the titration was performed with HCl. The divalent free bath solution contained (in mM): 150 NaCl, 10 EGTA, 10 HEPES, pH 7.4 (NaOH). The pipette solution contained (in mM) 145 CsCl, 8 NaCl, 2 MgCl_2 , 10 HEPES, pH 7.2 (CsOH) and the Ca^{2+} concentration was adjusted to 100 nM (0.0886 mM Ca^{2+} , 1 mM Cs-EGTA). In some experiments bath solutions were used containing (in mM): 140 NMDG⁺, 10 CaCl_2 and 10 HEPES, pH 7.4 (NaOH). Holding potential of the patch-clamp analyses in the DRGNs was −60 mV. Voltage clamp technique was used in the analyses and current-voltage (I-V) relationships were obtained from voltage ramps from −150 to +150 mV applied over 200 milliseconds. All experiments were performed at room temperature (22 ± 2 °C).

In the experiments, TRPV1 was extracellularly gated by CapN (10 µM), and the channels were extracellularly blocked by CapZ (0.1 mM). The maximal current amplitudes (pA) in a DRGN were divided by the cell capacitance (pF), a measure of the cell surface. Values of current density were expressed as pA/pF in the patch-clamp experiments.

Measurement of $[\text{Ca}^{2+}]_i$ concentration in HIPPON and DRGN

The HIPPON and DGRN samples were separately seeded in sterile cell culture flasks. Then, they were loaded with 4 µM Fura-2/AM in loading buffer for one hour at 37 °C in the dark, washed twice with the phosphate buffer, incubated for an additional 30 min at 37 °C to complete probe de-esterification. The groups were exposed to the stimulations in a water-jacketed cuvette (37 °C) with continuous magnetic stirring. Fluorescence was detected by using a Carry Eclipse Spectrofluorometer (Varian Inc., Sydney, Australia). The

fluorescence at 505 nm was measured at 1 s intervals after excitation at 340 nm and 380 nm, respectively.

For calibration of $[Ca^{2+}]_i$, maximum and minimum fluorescence values were obtained by adding the detergent Triton X-100 (0.1%) and the Ca^{2+} chelator EGTA (10 mM) sequentially at the end of each experiment. Calculation of the $[Ca^{2+}]_i$ concentrations were described in previous studies (Bejarano et al. 2009; Ghazizadeh and Naziroğlu 2014), assuming a K_d of 155 nM. The $[Ca^{2+}]_i$ concentrations in TRPV1 experiments were recorded by using the integral of the rise in $[Ca^{2+}]_i$ for 130 s after addition of CapN (10 μ M), respectively. The $[Ca^{2+}]_i$ concentration is expressed as nanomolar (nM) taking a sample every second as previously described (Bejarano et al. 2009; Ghazizadeh and Naziroğlu 2014).

Measurement of intracellular ROS production and mitochondrial membrane depolarization

Dihydrorhodamine (DHR)123 is an uncharged and non-fluorescent intracellular ROS production indicator and the level of intracellular ROS was assessed by fluorescence microplate reader (Infinite pro200, Tecan Life, Männedorf, Switzerland) with DHR123, which oxidizes to cationic rhodamine 123 in the presence of ROS, as described previously (Bejarano et al. 2009). The resulting data were normalized using the control values.

Mitochondrial permeable lipophilic fluorescent dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, JC-1) was used measurement of mitochondrial membrane potential ($\Delta\Psi_m$) in the HIPPO and DRGN as described in previous studies (Bejarano et al. 2009; Ghazizadeh and Naziroğlu 2014). Reversible formation of JC-1 monomeric form aggregates upon membrane polarization was measured at excitation wavelength (488 nm) with emission of J-aggregate, green (520 nm) and red (596 nm) using the microplate reader (Infinite pro200); the colour of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized (Cossarizza et al. 1993). The ROS and JC-1 values were assessed relative to untreated control cells (fold increase).

Analyses of apoptosis levels, and caspase 3 and 9 activities

Apoptosis is a multistage process during which the activity of caspase enzymes fluctuates, DNA becomes fragmented and phosphatidyl serine is transferred to the outside of the cell membrane. The amount of apoptosis levels were measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) and a commercial kit from Biocolor Ltd. (Northern Ireland) as described in a previous study (Kahya et al. 2014). Briefly, the dye is taken up into the cell via this

phosphatidylserine transmembrane movement as an indicator of apoptosis.

The determinations of caspase 3 and caspase 9 activities in the HIPPO and DRGN neurons were performed in a microplate reader (Infinite pro200) using caspase 3 (N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC), Bachem, Bubendorf, Switzerland) and caspase 9 (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (ACDEVD-AMC), Sigma) substrates. Details of the assays were indicated in recent studies (Ghazizadeh and Naziroğlu 2014; Kahya et al. 2014). The levels of apoptosis, and caspase 3 and 9 activities were expressed as fold-increase after calculating fluorescence units/mg protein.

Statistical analyses

The presence of significance in the three groups was once detected by LSD test. Significance level of p values in the data was analyzed by using Mann-Whitney U test. A P value of < 0.05 was considered significant. All data were reported as means $\pm$ standard deviation (SD). Data were statistically analyzed by using the SPSS statistical program (version 17.0, Chicago, Illinois, USA).

Results

Effects of 900 and 1800 MHz-EMR exposure on $[Ca^{2+}]_i$ concentration through modulation of TRPV1 in HIPPO and DRGNs of rats

For clarifying the role of 900 and 1800 MHz-EMR exposure on TRPV1 activation-induced $[Ca^{2+}]_i$ concentration and influx in the HIPPO and DRGNs, the neurons of 900 and 1800 MHz-EMR exposure rats were further stimulated by CapN (10 μ M) *in vitro*. CapN stimulation caused significant increases in $[Ca^{2+}]_i$ concentration and influx in HIPPO and DRGN of 900 and 1800 MHz exposed rats which was attributed to increased activation of Ca^{2+} -permeable TRPV1 (Fig. 1a and b). We observed high concentration and influx of $[Ca^{2+}]_i$ in the neurons in 900 MHz and 1800 MHz exposed groups. However, the concentration and influx of $[Ca^{2+}]_i$ in the neurons was further increased in 1800 MHz group compared to 900 MHz group ($p \leq 0.001$). The $[Ca^{2+}]_i$ concentration and influx in neurons was decreased by CapZ treatments. The $[Ca^{2+}]_i$ concentration in neurons was significantly ($p \leq 0.001$) decreased in 900 MHz + CapZ and 1800 MHz + CapZ groups compared to 900 MHz and 1800 MHz groups alone. It is likely that the 900 and 1800 MHz EMR exposure induced Ca^{2+} overload through activation of TRPV1 in neurons.

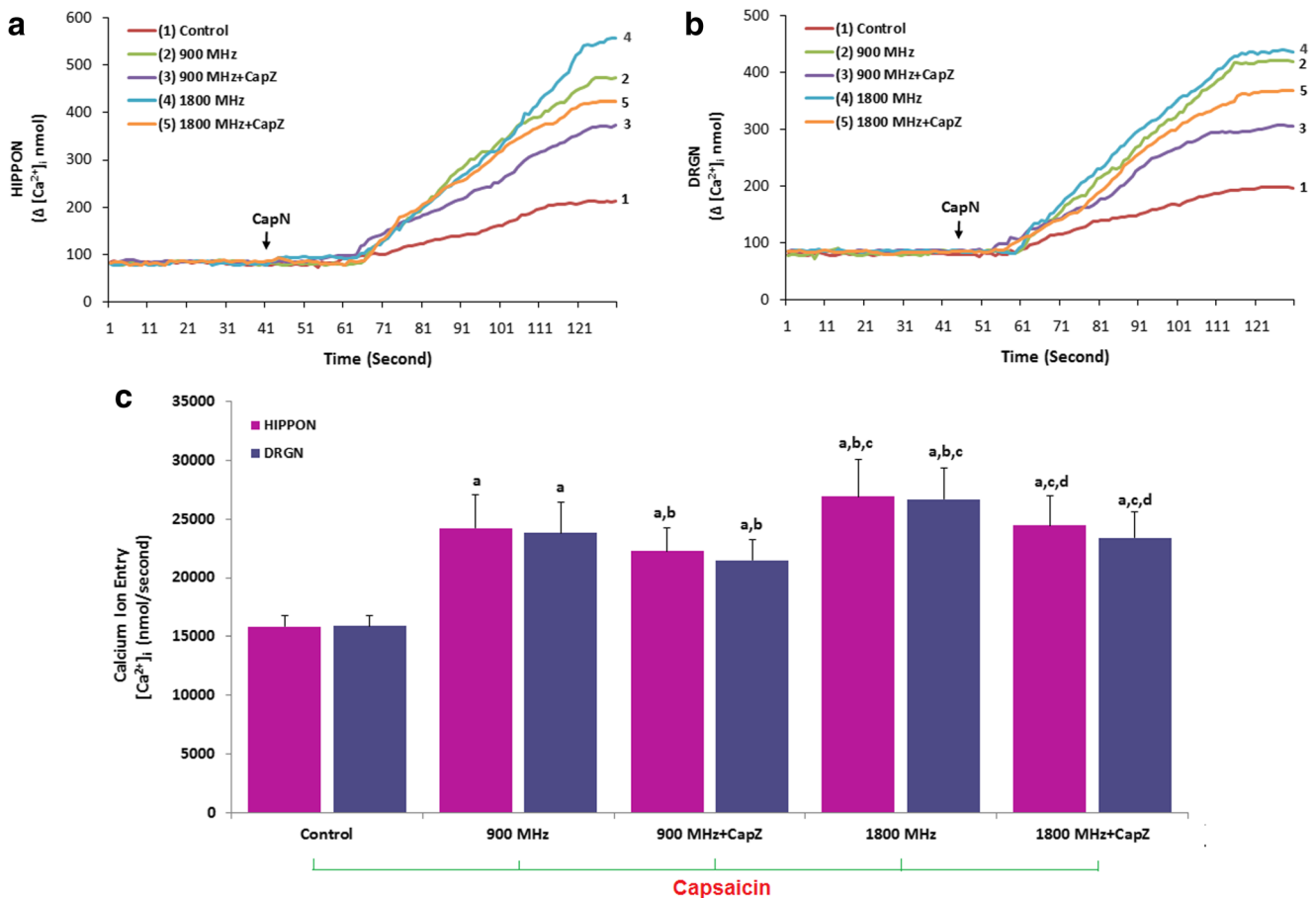


Fig. 1 Effect of 900 and 1800 MHz EMR exposure on $[Ca^{2+}]_i$ concentration through TRPV1 gate in HIPPON (a) and DRGN (b) of rats. ($n = 8$ and mean $\pm$ SD). The animals 900 and 1800 MHz for one year. Then, these dissected neurons of control and FM groups were

further in vitro treated with CapN (10 μ M)) and CapZ (0.1 mM) before loading Fura-2 for 130 s. (^a $p \leq 0.001$ versus control. ^b $p \leq 0.001$ versus 900 EMR group. ^c $p \leq 0.001$ versus 900 MHz + CapZ group. ^d $p \leq 0.001$ versus 1800 MHz group.) (c)

Effects of 900 and 1800 MHz-EMR exposure on TRPV1 activation in DRGNs

Several studies have shown that exposure to extremely low frequency (50 Hz) electromagnetic fields can activate voltage gated calcium channels (VGCC) in neuronal cells (Pall 2013; Sun et al. 2016). Therefore, we investigated the potential role of 900 and 1800 MHz EMR exposure on TRPV1 channel activation in HIPPON and DRGN using whole-cell patch-clamp experiments. The murine TRPV1 channel in the DRGNs of control (2b) was activated by extracellular CapN. The CapN-induced currents were reversibly blocked in the control neurons by extracellular CapZ application and NMDG⁺ (a Na⁺ substitute). There were no currents in the absence of CapN (Fig. 2a). The current densities in neurons was significantly higher in the 900 MHz + CapN group compared to the control and control + CapN groups ($p \leq 0.001$) (Fig. 2c and e). However, the current densities were markedly ($p \leq 0.001$) higher in 1800 MHz + CapN group than in control + CapN and 900 MHz + CapN groups (Fig. 2d and e). The current density was markedly decreased in the 900 MHz + CapN + CapZ and 1800 MHz + CapN + CapZ group following treatment

with the TRPV1 antagonist (CapZ). These results clearly indicate that 900 and 1800 MHz EMR exposure induced overload in Ca^{2+} entry through TRPV1 channel activation.

Effects of 900 and 1800 MHz-EMR exposure on the apoptosis level, caspase 3 and 9 activities in the HIPPON and DRGN of rats

The levels of apoptosis (3a and b), caspase 3 (3c and d) and 9 activities (3e and f) were markedly ($p \leq 0.001$) increased following stimulation of TRPV1 in the HIPPON and DRGN (Fig. 3) by 900 and 1800 MHz exposures. In addition, the level of apoptosis and caspase 9 activities were further increased in 1800 MHz group compared to 900 MHz group ($p \leq 0.001$). However, pre-treatment of neurons with CapZ followed by CapN stimulation reversed CapN-induced programmed cell death (apoptosis) and caspase 3 and 9 activities in control + CapZ, 900 MHz + CapZ and 1800 MHz + CapZ groups. These results imply that 900 and 1800 MHz EMR-induced cytotoxicity and programmed cell death may also be partly due to activation of TRPV1 pathways.

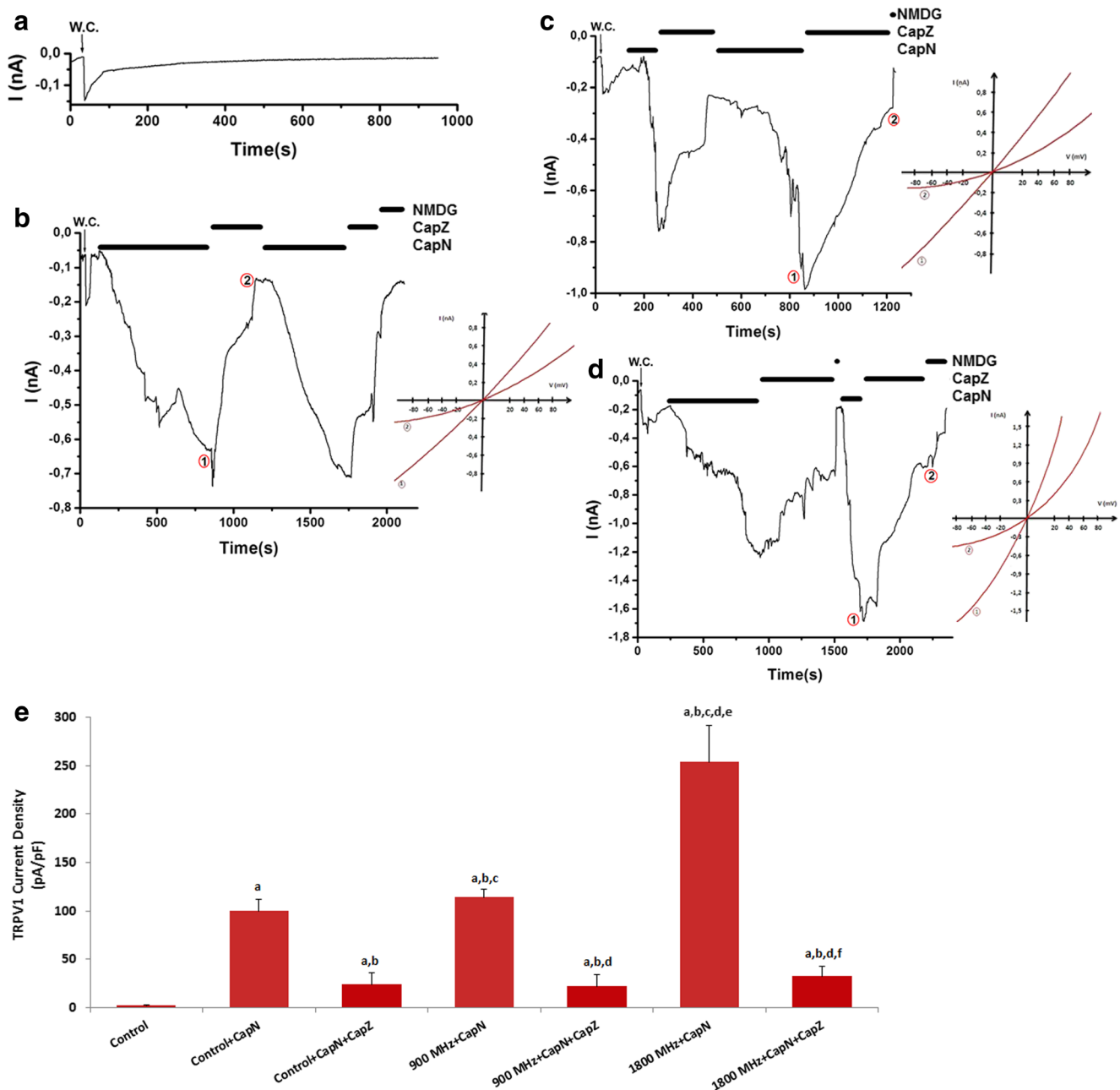


Fig. 2 Effect of 900 and 1800 MHz EMR exposure on TRPV1 channel activation in DRGN of rat (mean $\pm$ SD and $n=3$). The TRPV1 currents in DRGN were extracellularly stimulated by extracellular capsaicin (CapN and 10 μ M) but they were extracellularly blocked by CapZ (0.1 mM) in the patch-chamber. W.C.: Whole cell. A. Control: Original recordings from control neuron. B. Control + CapN group

(without 900 and 1800 EMR exposure). C. 900 MHz exposure group. D. 1800 MHz exposure group. E. TRPV1 channel current densities in the DRGN. (^a $p \leq 0.001$ versus control. ^b $p \leq 0.001$ versus control + CapN group. ^c $p \leq 0.001$ versus control + CapN + CapZ group. ^d $p \leq 0.001$ versus 900 MHz group. ^e $p \leq 0.001$ versus 900 MHz + CapN + CapZ group. ^f $p \leq 0.001$ versus 1800 MHz + CapN group)

Effects of 900 and 1800 MHz-EMR exposure on intracellular ROS production and JC-1 level in the HIPPO and DRGN of rats

In the HIPPO and DRGN, ROS production (Fig. 4a and b) and JC-1 level (Fig. 4c and d) were markedly ($p \leq 0.001$) increased through CapN stimulation by the 900 and 1800 MHz exposures. In addition, ROS production and

JC-1 levels were further increased in the 1800 MHz group compared to the 900 MHz group ($p \leq 0.001$). However, pre-treatment of neurons with CapZ treatment followed by CapN stimulation reversed CapN-induced intracellular oxidative stress (ROS) production and mitochondrial abnormal depolarization (JC-1) in control + CapZ, 900 MHz + CapZ and 1800 MHz + CapZ groups. These results provide further evidence for the involvement of TRPV1 channels in

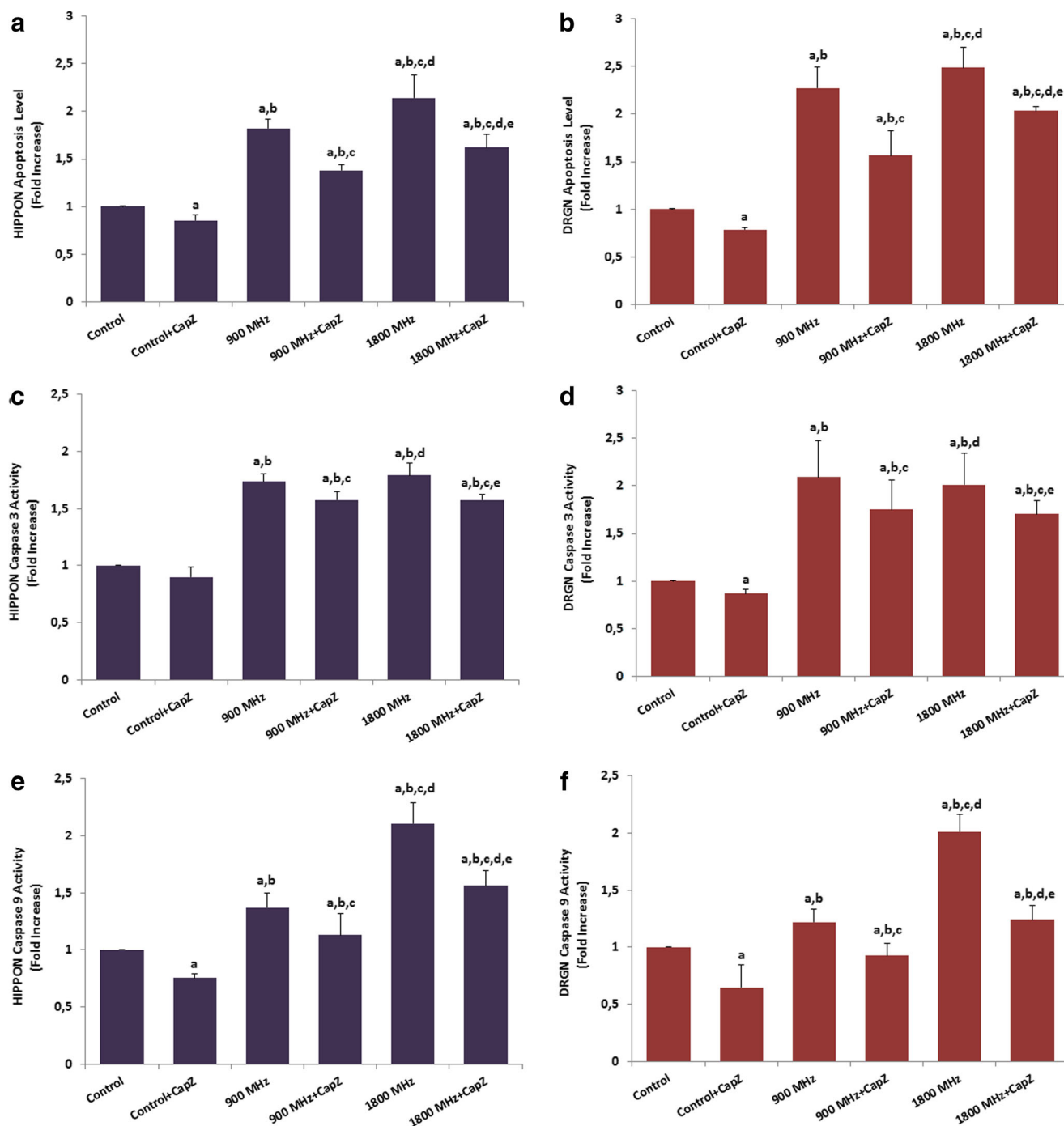


Fig. 3 Effect of 900 and 1800 MHz EMR on the apoptosis level, caspase 3 and 9 activities through TRPV1 channel activations in the HIPPON (a, c and e) and DRGN (b, d and f) of rats (mean $\pm$ SD and $n=3$). These neurons were dissected from exposure and control animals. The TRPV1 in the neurons were opened with capsaicin

(10 μ M), although they were blocked by CapZ (0.1 mM). (^a $p \leq 0.001$ versus control. ^b $p \leq 0.001$ versus control + CapZ group. ^c $p \leq 0.001$ versus 900 MHz group. ^d $p \leq 0.001$ versus 900 MHz + CapZ group. ^e $p \leq 0.001$ versus 1800 MHz group.)

the HIPPON and DRGNs following exposure to 900 and 1800 MHz EMR-induced Ca^{2+} influx and mitochondrial membrane depolarization, which in turn lead to accumulation of intracellular ROS and further TRPV1 channel activation.

Discussion

Accumulating evidence suggests that mobile phone frequency emitted EMR affects neuronal physiology functions and leads to modulation of several molecular pathways (Carballo-

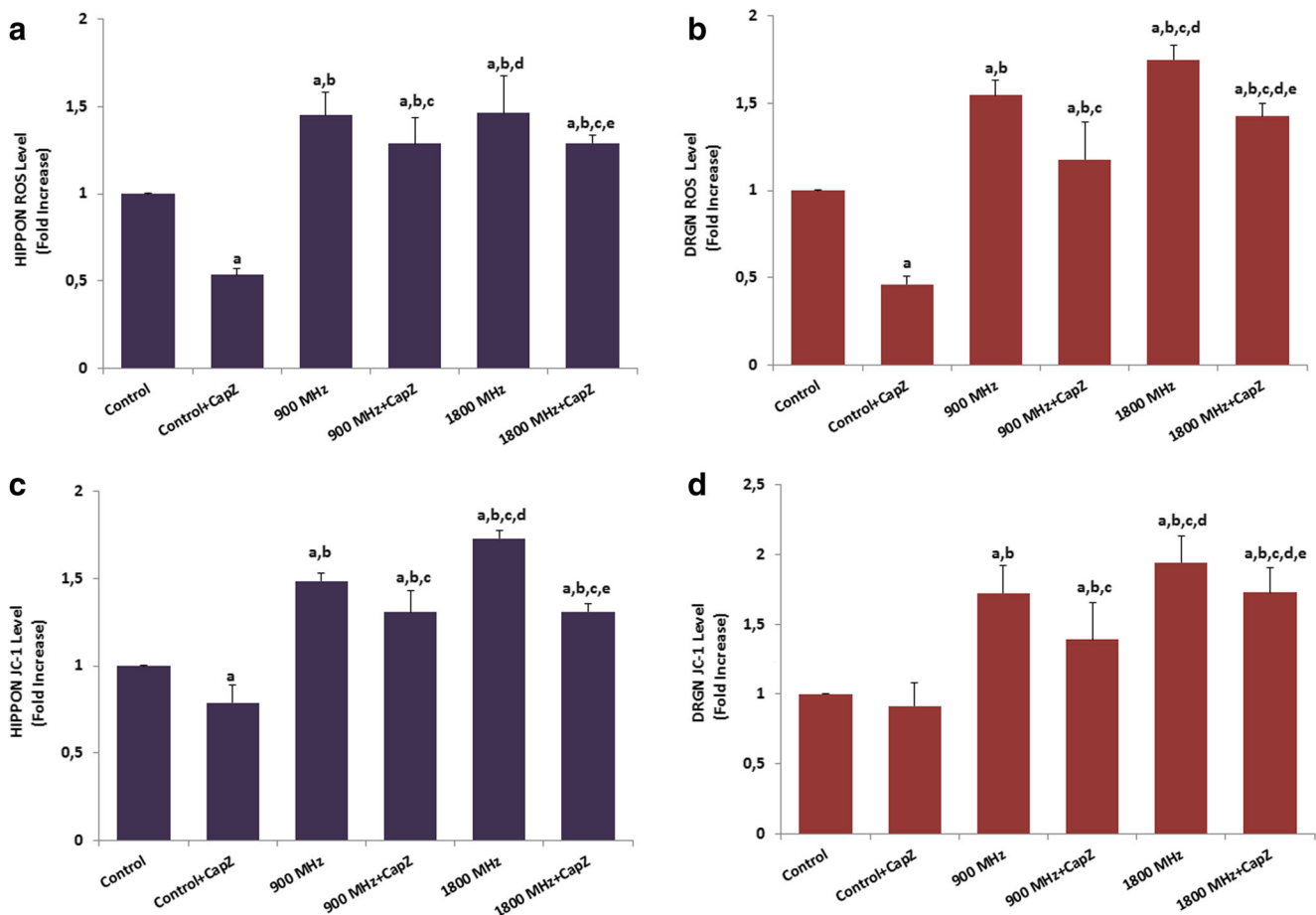


Fig. 4 Effect of 900 and 1800 MHz EMR on the intracellular ROS production and mitochondrial membrane depolarization (JC-1) levels through TRPV1 channel activations in the HIPPO (a and c) and DRGN (b and d) of rats (mean $\pm$ SD and $n = 3$). These neurons were dissected from exposure and control animals. The TRPV1 in the neurons

were opened with capsaicin (10 μ M), although they were blocked by CapZ (0.1 mM). (^a $p \leq 0.001$ versus control. ^b $p \leq 0.001$ versus control + CapZ group. ^c $p \leq 0.001$ versus 900 MHz group. ^d $p \leq 0.001$ versus 900 MHz + CapZ group. ^e $p \leq 0.001$ versus 1800 MHz group.)

Quintás et al. 2011; Hou et al. 2015) that can induce HIPPO injury and pain (de Tommaso et al. 2009; Nazıroğlu et al. 2015). However, the underlying mechanisms through which 900 and 1800 EMR exert their neurotoxic effects responsible for HIPPO and DRGN injury have not been fully understood. It is well known that excessive Ca^{2+} influx and ROS production have important roles for the induction of HIPPO and DRGN injuries (Nazıroğlu et al. 2012a; Kumar et al. 2014). Environmental and nutritional factors such as the hot chili pepper component (CapN), oxidative stress, and high temperature have important roles in overload Ca^{2+} influx through activation of the TRPV1 channel in HIPPO and DRGN (Caterina et al. 1997; Ibi et al. 2008; Pecze et al. 2013; Nazıroğlu et al. 2017). However, the role of TRPV1 channels in 900 and 1800 MHz exposure-induced HIPPO and DRGN injuries has not been clarified yet. The current results implied that exposure to 900 and 1800 MHz may increase $[\text{Ca}^{2+}]_i$ accumulation, mitochondrial ROS and apoptosis levels in the HIPPO by activating TRPV1 channels. To our knowledge, this is the first evidence for the involvement of

TRPV1 channels in pathophysiological process induced by 900 and 1800 MHz exposure in HIPPO and DRGN.

Activation of mitochondrial oxidative stress and heat sensitive cation channels may contribute to peripheral pain and hippocampal injury in humans (Halliwell 2006; Nazıroğlu et al. 2012b). Indeed, increased mitochondrial oxidative stress and body temperature are two common triggers of injuries of non-ionizing EMR such as 900 and 1800 MHz in experimental animals (Bortkiewicz et al. 2012; Yüksel et al. 2016) and human (de Tommaso et al. 2009). Therefore, non-ionizing EMR may be strongly associated with heat and oxidative stress-sensing cation channels. The TRPV1 channel is activated in response to different stimuli, including CapN (Caterina et al. 1997; Caterina et al. 1999; Pecze et al. 2013; Ogawa et al. 2016; Nazıroğlu et al. 2017). In addition, channel activities of TRPV1 are affected by oxidative stress (Ibi et al. 2008; Nazıroğlu 2017) and high temperature ($\geq 43^\circ\text{C}$) in several cell lines (Caterina et al. 1999). Our current results are the first to indicate that activation of TRPV1 in the HIPPO and DRGN is associated with 900 and 1800 MHz exposures. Therefore,

the EMR-induced oxidative stress and heat-dependent activation TRPV1 channels is likely to be involved in the etiology of the HIPPO and DRGN oxidative injuries. Contrary to the present results, *in vitro* studies by our and others were not able previously identify the involvement of short term non-ionizing EMR exposure on TRPV1 and VGCC activation-induced overload Ca^{2+} influx in neurons (Platano et al. 2007; O'Connor et al. 2010; Ghazizadeh and Naziroğlu 2014; Naziroğlu et al. 2015). It is likely that acute exposure to 900 and 1800 MHz EMR has no effect on Ca^{2+} influx *in vitro*. In line with our findings, increases in the mRNA levels of VGCC (P/Q type) has been reported in the presynaptic nerve terminal of mouse brain after extremely low frequency electromagnetic field exposure, which accounts for the increased Ca^{2+} influx upon stimulation (Sun et al. 2016).

The mitochondria play a crucial role in the intrinsic pathway of apoptosis and caspase activation (Shi 2002; Naziroğlu et al. 2013; Carrasco et al. 2015). Increased Ca^{2+} overload and entry enhances mitochondrial membrane depolarization; in turn, excessive ROS production, and caspase 3 and 9 activity occurs in the HIPPO and DRGN (Naziroğlu et al. 2013; Şahin et al. 2015). We observed that 900 and 1800 MHz EMR exposure could increase the $[\text{Ca}^{2+}]_i$ concentration, as well as subsequent apoptosis stimulation, evoked by CapN. It is well known that increased Ca^{2+} entry will depolarize the inner mitochondrial membrane (Carrasco et al. 2015) which increases electron transport chain activity and ROS production. In this respect, results of recent studies indicated that ROS production, caspase activation and apoptosis were effected in different cells except HIPPO and DRGN by 900 and 1800 MHz exposures (Joubert et al. 2008; Xu et al. 2010; Kahya et al. 2014; Türedi et al. 2015) because of depolarization of inner mitochondrial membrane through the overload Ca^{2+} entry. The current study suggests that observed changes in markers of oxidative damage and apoptosis are due to activation of TRPV1 channels in the HIPPO and DRGN. Similarly, exposure of rat primary cortical neuronal cultures to 900 MHz EMR for 24 h induced apoptosis and thermal effect through a caspase-independent pathway (Joubert et al. 2008). Histological analysis from another study further indicated an apoptotic role of 900 MHz exposure (1 h/day for 28 days) in hippocampus of rat (Bas et al. 2009). Results of another study indicated increased number of caspase3-labeled Purkinje neurons and granule cells in the cerebellum in rats following pre- and postnatal exposure to 900-MHz EMF (from prenatal period to 80 day of postnatal period and 24 h/day) (Köktürk et al. 2013), and monthly exposure to 835 MHz induced autophagy in the cortical neurons of mice (Kim et al. 2017).

The hippocampus is a major region of the brain that regulated physiological functions such as behavioural and cognitive functions. DRGN is also important for the modulation of pain processes. The HIPPO and DRGN have been reported

to be vulnerable to non-ionizing radiation wave exposure-induced EMR due to low antioxidant content but high PUFA level and oxygen consumption rate (Köylü et al. 2006; Naziroğlu et al. 2012a; Ghazizadeh and Naziroğlu 2014). In the current study, mitochondrial membrane depolarization and intracellular ROS production were found to be increased in the neurons of rats exposed to frequencies of 900 MHz and 1800 for one year. These results confirm previous reports in HIPPO and DRGN of experimental animals (Köylü et al. 2006; Naziroğlu et al. 2012a; Ghazizadeh and Naziroğlu, 2014).

In conclusion, our data is the first to show that 900 and 1800 MHz EMR exposure may induce mitochondrial ROS production and apoptosis via activation of TRPV1 in HIPPO and DRGN cells. Therefore, TRPV1 may represent an important pharmacological target for the treatment of 900 and 1800 MHz EMR-induced apoptosis and peripheral pain in rat models.

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Author's contribution MN formulated the present hypothesis and was responsible for writing the report. KE, FU and SA were responsible for analyses of spectrofluorometer and plate reader.

Compliance with ethical standards

Disclosures None of the authors have any to disclose. All authors approved the final manuscript.

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References

- Bas O, Odaci E, Kaplan S, Acer N, Uçok K, Colakoglu S (2009) 900 MHz electromagnetic field exposure affects qualitative and quantitative features of hippocampal pyramidal cells in the adult female rat. *Brain Res* 1265:178–185
- Bejarano I, Redondo PC, Espino J, Rosado JA, Paredes SD, Barriga C, Reiter RJ, Pariente JA, Rodríguez AB (2009) Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. *J Pineal Res* 46:392–400
- Bodera P, Stankiewicz W, Zawada K, Antkowiak B, Paluch M, Kieliszek J, Kalicki B, Bartosiński A, Wawer I (2013) Changes in antioxidant capacity of blood due to mutual action of electromagnetic field (1800 MHz) and opioid drug (tramadol) in animal model of persistent inflammatory state. *Pharmacol Rep* 65(2):421–428
- Bortkiewicz A, Gadzicka E, Szymczak W, Zmysłony M (2012) Changes in tympanic temperature during the exposure to electromagnetic fields emitted by mobile phone. *Int J Occup Med Environ Health* 25(2):145–150

- Carballo-Quintás M, Martínez-Silva I, Cadarso-Suárez C, Alvarez-Figueiras M, Ares-Pena FJ, López-Martín E (2011) A study of neurotoxic biomarkers, c-fos and GFAP after acute exposure to GSM radiation at 900 MHz in the picrotoxin model of rat brains. *Neurotoxicology* 32(4):478–494
- Carrasco C, Rodríguez BA, Pariente JA (2015) Melatonin as a stabilizer of mitochondrial function: Role in diseases and aging. *Turk J Biol* 39:822–831
- Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398(6726):436–441
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–824
- Çetin H, Nazıroğlu M, Çelik Ö, Yüksel M, Pastacı N, Özkaya MO (2014) Liver antioxidant stores protect the brain from electromagnetic radiation (900 and 1800 MHz)-induced oxidative stress in rats during pregnancy and the development of offspring. *J Matern Fetal Neonatal Med* 27(18):1915–1921
- Çiğ B, Nazıroğlu M (2015) Investigation of the effects of distance from sources on apoptosis, oxidative stress and cytosolic calcium accumulation via TRPV1 channels induced by mobile phones and Wi-Fi in breast cancer cells. *Biochim Biophys Acta* 1848(10 Pt B):2756–2765
- Cinar N, Sahin S, Erdinc OO (2013) What is the impact of electromagnetic waves on epileptic seizures? *Med Sci Monit Basic Res* 19: 141–145
- Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C (1993) A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochem Biophys Res Commun* 197:40–45
- Crawford DC, Moulder KL, Gereau RW IV, Story GM, Mennerick S (2009) Comparative effects of heterologous TRPV1 and TRPM8 expression in rat hippocampal neurons. *PLoS One* 4(12):e8166
- de Tommaso M, Rossi P, Falsaperla R, Francesco Vde V, Santoro R, Federici A (2009) Mobile phones exposure induces changes of contingent negative variation in humans. *Neurosci Lett* 464(2):79–83
- Ghazizadeh V, Nazıroğlu M (2014) Electromagnetic radiation (Wi-Fi) and epilepsy induce calcium entry and apoptosis through activation of TRPV1 channel in hippocampus and dorsal root ganglion of rats. *Metab Brain Dis* 29(3):787–799
- Gökçek-Saraç Ç, Er H, Kencebay Manas C, Kantar Gok D, Özen Ş, Derin N (2017) Effects of acute and chronic exposure to both 900 MHz and 2100 MHz electromagnetic radiation on glutamate receptor signaling pathway. *Int J Radiat Biol* 93(9):980–989
- Halliwel B (2006) Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 97(6):1634–1658
- Han Q, Kim YH, Wang X, Liu D, Zhang ZJ, Bey AL, Lay M, Chang W, Berta T, Zhang Y, Jiang YH, Ji RR (2016) SHANK3 Deficiency impairs heat hyperalgesia and TRPV1 signaling in primary sensory neurons. *Neuron* 92(6):1279–1293
- Hou Q, Wang M, Wu S, Ma X, An G, Liu H, Xie F (2015) Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells. *Electromagn Biol Med* 34:85–92
- Ibi M, Matsuno K, Shiba D, Katsuyama M, Iwata K, Kakehi T, Nakagawa T, Sango K, Shirai Y, Yokoyama T, Kaneko S, Saito N, Yabe-Nishimura C (2008) Reactive oxygen species derived from NOX1/NADPH oxidase enhance inflammatory pain. *J Neurosci* 28:9486–9494
- Jin Z, Zong C, Jiang B, Zhou Z, Tong J, Cao Y (2012) The effect of combined exposure of 900 MHz radiofrequency fields and doxorubicin in HL-60 cells. *PLoS One* 7(9):e46102
- Joubert V, Bourthoumieu S, Leveque P, Yardin C (2008) Apoptosis is induced by radiofrequency fields through the caspase-independent mitochondrial pathway in cortical neurons. *Radiat Res* 169:38–45
- Kahya MC, Nazıroğlu M, Çiğ B (2014) Selenium reduces mobile phone (900 MHz)-induced oxidative stress, mitochondrial function, and apoptosis in breast cancer cells. *Biol Trace Elem Res* 160(2):285–293
- Kesari KK, Siddiqui MH, Meena R, Verma HN, Kumar S (2013) Cell phone radiation exposure on brain and associated biological systems. *Indian J Exp Biol* 51:187–200
- Kim JH, DH Y, Kim HR (2017) Activation of autophagy at cerebral cortex and apoptosis at brainstem are differential responses to 835 MHz RF-EMF exposure. *Korean J Physiol Pharmacol* 21(2): 179–188
- Köktürk S, Yardimoglu M, Celikozlu SD, Dolanbay EG, Cimbiz A (2013) Effect of *Lycopersicon esculentum* extract on apoptosis in the rat cerebellum, following prenatal and postnatal exposure to an electromagnetic field. *Exp Ther Med* 6(1):52–56
- Köylü H, Mollaoglu H, Ozguner F, Nazıroğlu M, Delibas N (2006) Melatonin modulates 900 Mhz microwave-induced lipid peroxidation changes in rat brain. *Toxicol Ind Health* 22(5):211–216
- Kumar VS, Gopalakrishnan A, Nazıroğlu M, Rajanikant GK (2014) Calcium ion—the key player in cerebral ischemia. *Curr Med Chem* 21:2065–2075
- Nazıroğlu M, Çelik Ö, Özgül C, Çiğ B, Doğan S, Bal R, Gümrall N, Rodríguez AB, Pariente JA (2012b) Melatonin modulates wireless (2.45 GHz)-induced oxidative injury through TRPM2 and voltage gated Ca(2+) channels in brain and dorsal root ganglion in rat. *Physiol Behav* 105:683–692
- Nazıroğlu M, Çiğ B, Blum W, Vizler C, Buhala A, Marton A, Katona R, Jósavay K, Schwaller B, Oláh Z, Pecze L (2017) Targeting breast cancer cells by MRS1477, a positive allosteric modulator of TRPV1 channels. *PLoS One* 12(6):e0179950
- Nazıroğlu M, Özkan FF, Hapil SR, Ghazizadeh V, Çiğ B (2015) Epilepsy but not mobile phone frequency (900 MHz) induces apoptosis and calcium entry in hippocampus of epileptic rat: involvement of TRPV1 channels. *J Membr Biol* 248(1):83–91
- Nazıroğlu M, Tokat S, Demirci S (2012a) Role of melatonin on electromagnetic radiation-induced oxidative stress and Ca2+ signaling molecular pathways in breast cancer. *J Recept Signal Transduct Res* 32(6):290–297
- Nazıroğlu M, Yüksel M, Köse SA, Özkaya MO (2013) Recent reports of Wi-Fi and mobile phone-induced radiation on oxidative stress and reproductive signaling pathways in females and males. *J Membr Biol* 246(12):869–875
- Nazıroğlu M (2017) Activation of TRPM2 and TRPV1 channels in dorsal root ganglion by NADPH oxidase and protein kinase C molecular pathways: a patch clamp study. *J Mol Neurosci* 61:425–435
- O'Connor RP, Madison SD, Leveque P, Roderick HL, Bootman MD (2010) Exposure to GSM RF fields does not affect calcium homeostasis in human endothelial cells, rat pheochromocytoma cells or rat hippocampal neurons. *PLoS One* 5(7):e11828
- Ogawa N, Kurokawa T, Fujiwara K, Polat OK, Badr H, Takahashi N, Mori Y (2016) Functional and structural divergence in human TRPV1 channel subunits by oxidative cysteine modification. *J Biol Chem* 291:4197–4210
- Özorak A, Nazıroğlu M, Çelik Ö, Yüksel M, Özçelik D, Özkaya MO, Çetin H, Kahya MC, Kose SA (2013) Wi-Fi (2.45 GHz)- and mobile phone (900 and 1800 MHz)-induced risks on oxidative stress and elements in kidney and testis of rats during pregnancy and the development of offspring. *Biol Trace Elem Res* 156:221–229
- Pall ML (2013) Electromagnetic fields act via activation of voltage-gated calcium channels to produce beneficial or adverse effects. *J Cell Mol Med* 17(8):958–965
- Pecze L, Blum W, Schwaller B (2013) Mechanism of capsaicin receptor TRPV1-mediated toxicity in pain-sensing neurons focusing on the effects of Na(+)/Ca(2+) fluxes and the Ca(2+)-binding protein calretinin. *Biochim Biophys Acta* 1833:1680–1691
- Platano D, Mesirca P, Paffi A, Pellegrino M, Liberti M, Apollonio F, Bersani F, Aicardi G (2007) Acute exposure to low-level CW and

- GSM-modulated 900 MHz radiofrequency does not affect Ba²⁺ currents through voltage-gated calcium channels in rat cortical neurons. *Bioelectromagnetics* 28(8):599–607
- Rohm B, Riedel A, Ley JP, Widder S, Krammer GE, Somoza V (2015) Capsaicin, nonivamide and trans-pellitorine decrease free fatty acid uptake without TRPV1 activation and increase acetyl-coenzyme A synthetase activity in Caco-2 cells. *Food Funct* 6(1):173–185
- Shahin S, Banerjee S, Singh SP, Chaturvedi CM (2015) 2.45 GHz microwave radiation impairs learning and spatial Memory via oxidative/nitrosative stress induced p53-dependent/independent hippocampal apoptosis: Molecular basis and underlying mechanism. *Toxicol Sci* 148:380–399
- Shi Y (2002) Apoptosome: the cellular engine for the activation of caspase-9. *Structure* 10(3):285–288
- Sun ZC, Ge JL, Guo B, Guo J, Hao M, YC W, Lin YA, La T, Yao PT, Mei YA, Feng Y, Xue L (2016) Extremely low frequency electromagnetic fields facilitate vesicle endocytosis by increasing presynaptic calcium channel expression at a central synapse. *Sci Rep* 6:21774
- Türedi S, Hancı H, Topal Z, Ünal D, Mercantepe T, Bozkurt İ, Kaya H, Odacı E (2015) The effects of prenatal exposure to a 900-MHz electromagnetic field on the 21-day-old male rat heart. *Electromagn Biol Med*. 34:390–397
- Xu S, Zhou Z, Zhang L, Yu Z, Zhang W, Wang Y, Wang X, Li M, Chen Y, Chen C, He M, Zhang G, Zhong M (2010) Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons. *Brain Res* 1311:189–196
- Yüksel M, Nazıroğlu M, Özkaya MO (2016) Long-term exposure to electromagnetic radiation from mobile phones and Wi-Fi devices decreases plasma prolactin, progesterone, and estrogen levels but increases uterine oxidative stress in pregnant rats and their offspring. *Endocrine* 52:352–362