

Pre and postnatal exposure to 900 MHz electromagnetic fields induce inflammation and oxidative stress, and alter renin-angiotensin system components differently in male and female offsprings

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ABSTRACT

Aims: This study was designed to investigate inflammation, oxidative stress and renin-angiotensin system components in brain and kidney tissues of female and male rats prenatally and/or postnatally exposed to 900 MHz electromagnetic field (EMF). It is aimed to evaluate the biological effects of 900 MHz EMF exposure due to the increase in mobile phone use and especially the more widespread use of the GSM 900 system.

Main methods: Male and female Wistar albino offsprings were divided into four groups of control, prenatal, postnatal, and prenatal+postnatal exposed to 900 MHz EMF for 1 h/day (23 days during pregnancy for prenatal period, 40 days for postnatal period). The brain and kidney tissues were collected when they reached puberty.

Key findings: It was found that the total oxidant status, IL-2, IL-6, and TNF- α levels increased ($p < 0.001$) and the total antioxidant status levels decreased ($p < 0.001$) in all three EMF groups comparing to controls in both male and female brain and kidney tissues. The renin-angiotensin system components such as angiotensinogen, renin, angiotensin type 1 and type 2 receptors, and MAS1-like G protein-coupled receptor expression were higher ($p < 0.001$) in all three EMF exposure groups comparing to controls in both male and female brain and kidney tissues. Although there are some differences of the levels of proinflammatory markers, ROS components and RAS components in brain and kidney tissues between males and females, the common result of all groups was increase in oxidative stress, inflammation markers and angiotensin system components with exposure to 900 MHz EMF. **Significance:** In conclusion, our study suggested that the 900 MHz EMF can activate brain and kidney renin-angiotensin system, and this activation is maybe related to inflammation and oxidative stress in both male and female offsprings.

1. Introduction

Mobile phones play an important role in human life nowadays. Especially in the last decade, children's access to mobile phones has become widespread and electromagnetic fields can be more harmful for them as they are in the developmental age. Radio frequency (RF) generated by mobile phones or base stations is in the frequency range 800–2000 MHz [1]. Because the human body has a unique weak electromagnetic field (EMF), the EMFs emitted by cell phones interact with the human organism. The biological effects of EMFs are associated with the stimulation of electric fields in the human body. Strong electric fields, depending on the frequency of interest, can impair cell functions,

leading to degradation in large molecules and imbalance in ionic balance of the cells [2–4]. In addition, 900 MHz EMF disrupts cellular homeostasis by causing the induction of stress proteins, including heat shock proteins (HSPs). HSPs on the other hand, causes oxidative stress, which can cause lipid peroxidation and may be responsible for cell death [5].

Oxidative stress is known to play a role in pathophysiological processes and has important effects on DNA damage process, general and specific gene expression, and cell apoptosis. Exposure to EMF induces the formation of reactive oxygen species (ROS) in animal and human tissues through different pathways. EMF can damage protective enzymatic and non-enzymatic antioxidant systems, and cellular components

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such as proteins, lipids, DNA and resulting in cell damage [6]. In our previous studies, we found that the 900 MHz EMF caused oxidative stress by increasing lipid peroxidation in brain tissues of guinea pigs [7], and increased the blood serotonin level without changing the glutamate level [8]. In addition, EMF can cause inflammation in different tissues and organs. In some studies with male rats, EMF exposure has been shown to cause inflammation in the liver and pancreas tissues [9,10]. There is a reciprocal relationship between oxidative stress and inflammation. “ROS-induced inflammation” has an important role in the progression of pathologies of various diseases. Pathophysiological levels of ROS can trigger signaling cascades that can lead to the initiation and progression of inflammatory diseases by altering the structure of transcription factors and proinflammatory genes [11]. Furthermore, inflammation can be both a cause and a consequence of oxidative stress [5]. Enhanced ROS production by immune cells at the site of inflammation contributes to oxidative stress and tissue damage [11].

The renin angiotensin system (RAS) is a classical endocrine system contributing to body electrolyte homeostasis and blood pressure regulation. In recent years, the presence of a local RAS has been shown in many tissues such as blood vessels, heart, kidney and brain [12]. RAS occurs locally with autocrine/paracrine action on angiotensin mediators in these tissues and is also associated with systemic RAS. There is multiple evidence that RAS components and their specific receptors play a crucial role in cell growth, differentiation, proliferation, apoptosis, formation of reactive oxygen species, tissue inflammation and fibrogenesis in different tissues or organs [13–16]. It is also suggested that the RAS is regulated by sex hormones and contributes to sex differences in cardiovascular and kidney diseases [17]. While exposure to 900 MHz EMF causes oxidative stress and inflammation in brain and kidney tissues, its effects on RAS are still unclear. It is also unclear whether there is a difference between the effect of 900 MHz EMF on female and male individuals. Therefore, the aim of this study was to investigate inflammation, oxidative stress and renin-angiotensin system components in brain and kidney tissues of female and male rats prenatally and/or postnatally exposed to 900 MHz EMF.

2. Methods

2.1. Animals

The Wistar albino rats were obtained from the Bezmialem Vakif University Experimental Animal Centre, and they housed under standard laboratory conditions (12 h light/dark cycles, 22 °C and 60 % humidity) with ad libitum food and water and received humane care according to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Science and published by the National Institutes of Health. The ethic regulations

have been followed in accordance with The National and Institutional guidelines for the protection of animal welfare during experiments. All animal procedures were approved by the Laboratory Animals Ethical Committee of Bezmialem Vakif University (2019/229). All experimental procedures were in accordance with ARRIVE guidelines.

2.2. Experimental groups and study design

Experimental design and groups are illustrated in Fig. 1. Vaginal smear samples of 90 female rats (3-month-old) were examined for determination of their estrus cycles. During the estrus period, a progressive shedding occurs in the superficial mucosal layer and keratinized layers and keratinized epithelial cells without nuclei begin to appear in the vaginal lumen. As the estrus period progresses, the stratum corneum dissolves. As a result, large amounts of cornified epithelial cells begin to shed into the vaginal lumen. The vaginal wash solution taken at each stage was smeared, stained, and evaluated under a light microscope. Cornified squamous epithelial cells, mostly in clusters, were detected in the estrus phase.

Thirty-two females, detected as on their estrus cycles, were mated with 32 males. Each male-female couple was placed in a cage overnight for mating, and the next morning vaginal plug was controlled for the detection of pregnancy. Sixteen females were detected as pregnant, and they were randomly divided into two equal groups ($n = 8$) of prenatally EMF-exposed and prenatally EMF-not exposed. Following the delivery (day 0), the pups were kept in the same cage with their mothers from the birth until the 21st day after birth (suckling period), and the EMF was not applied to prevent the stress that may occur and thus the mother from harming her offspring. Following the suckling period, the pups were divided into four experimental groups.

2.2.1. Control group ($n = 18$)

This group was randomly formed from the female ($n = 9$) and male ($n = 9$) pups of prenatally EMF-not exposed mothers. The pups were also not exposed to EMF until their puberties and handled in the same conditions with other groups.

2.2.2. Prenatal group ($n = 18$)

This group was randomly formed from the female ($n = 9$) and male ($n = 9$) pups of prenatally EMF-exposed mothers. But the pups were not exposed to EMF until their puberties.

2.2.3. Postnatal group ($n = 18$)

This group was randomly formed from the female ($n = 9$) and male ($n = 9$) pups of prenatally EMF-not exposed mothers. But the pups were exposed to EMF until their puberties.

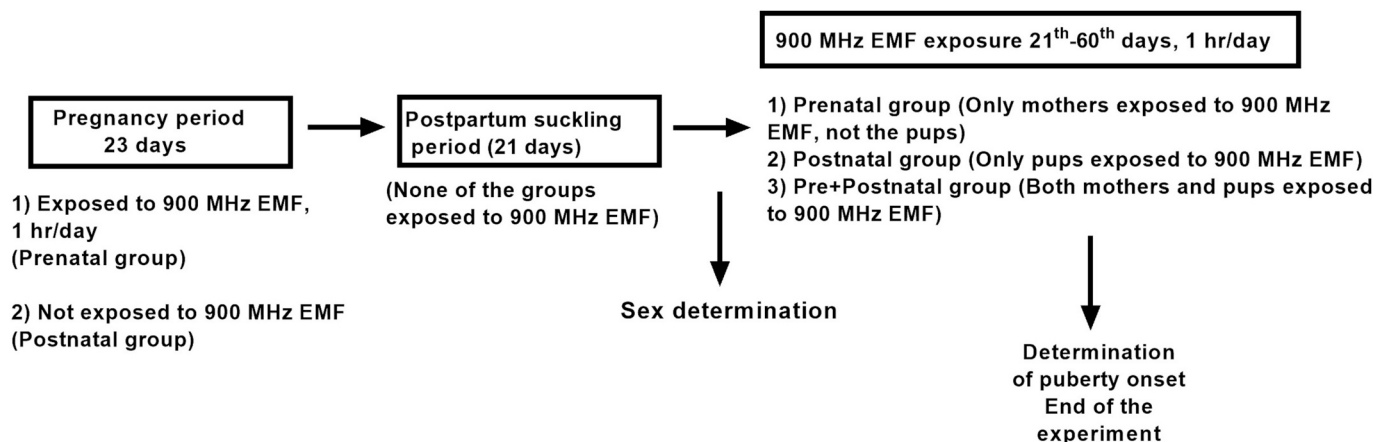


Fig. 1. Study design.

2.2.4. Pre&postnatal group (n = 18)

This group was randomly formed from the female (n = 9) and male (n = 9) pups of prenatally EMF-exposed mothers. The pups were also exposed to EMF until their puberties.

2.3. Determination of puberty onset

Following the 21 days of suckling period, genital examinations were performed by an experienced veterinary surgeon, and complete preputial separation day was referred as the onset of puberty of males [18]. In females, the day of vaginal opening and the first cornified epithelium detection day in vaginal smear specimens was recorded as the onset of puberty [19]. Female rats reached the puberty approximately 47 days while male rats reached at approximately 58 days.

2.4. EMF exposure procedure

2.4.1. During pregnancy

All pregnant females stayed in separate standard cages as they can move and feed freely. Eight pregnant females were exposed to EMF while other 8 females were not. The EMF application started from the 1st day of pregnancy until one day prior to delivery (23 days). The rats in EMF group were exposed to EMF for 1 h every day at the same period of each day. The duration of the EMF exposure has been chosen to mimic prolonged cell phone use in our routine lives. The control group was also handled in the same conditions without EMF exposure. A special cylindrical plexiglass exposure cage with 30 cm diameter was designed to be an antenna of the EMF generator at the top of it (Fig. 2). During the 1 h exposure time, 4 rats were placed in the cage, and could freely move inside it for preventing the stress of the application. After completion of 1 h application to first 4 rats, the second 4 rats were placed into the exposure cage for 1 h EMF application. Every day, the power of the generator was confirmed just prior and following the application, and was reported as approximately 4,5–5,5 V/m.

2.4.2. From 21-day old to puberty

Following the sex determination at the 21st day following the birth

(suckling period), the pups in postnatal and pre + postnatal groups were exposed to EMF 1 h/day in the exposure cage until puberty. During the 1 h exposure time, 5 rats were placed in the cage, and could freely move inside it for preventing the stress of the application.

2.5. EMF exposure device

The rats, kept individually in plexiglass cages (35.60 cm), were exposed to 900 MHz with an average field intensity of 6.0 ± 0.5 V/m (60 min/day, at 09:00 o'clock) every day. A signal source which produces wide band like noise with low power (OEM BG7TBL Wide Band RF Noise Source, China) was attached to a high-power amplifier repeater (OS-B102, Oserjep, China) for Global System for Mobile Communications (GSM) to generate 900 MHz GSM band signal at a range of 935 MHz–960 MHz. Four omnidirectional. GSM antennae (MPN:3A3-1400-N01SJ-050-16, Eightwood Electronics, Shenzhen, China) simultaneously used to irradiate the rats. Each omni-directional antenna was placed central on top of the cages, 45 cm above the rat. The power of the EMF was confirmed by an electromagnetic field meter (TES-593 ElectroSmog Meter, Taiwan), placed under the cage. The electric field strength was 5 ± 0.5 mV/m when the power supply was off and 6.0 ± 0.5 V/m when the power supply was on (Fig. 2).

2.6. Biochemical analysis

At the end of the experiment, rats were anesthetized (ketamine/xylazine, (10 mg/kg, 100 mg/kg)) decapitated, and their whole brains and whole kidneys were removed for molecular and biochemical analysis. The tissues were frozen on ice and stored at -80°C . Tissue samples to use in biochemical assays were homogenized with a steel bead homogenizer (Retsch MM400, Haan, Germany) in 2 ml microtubes in PBS (Phosphate Buffered Saline, pH: 7.4). The homogenates were centrifuged at $15,000 \times g$ for 20 min at 4°C to obtain 2 different fractions, pellet, and supernatant. Firstly, Bradford method was used to determine the total protein amount in homogenates at a 595 nm wavelength (Thermo Scientific Multiskan FC, 2011-06, USA) [20]. After that tissue IL-2 (E0123Ra, BT LAB, Birmingham, UK), IL-6 (E0135Ra, BT LAB,

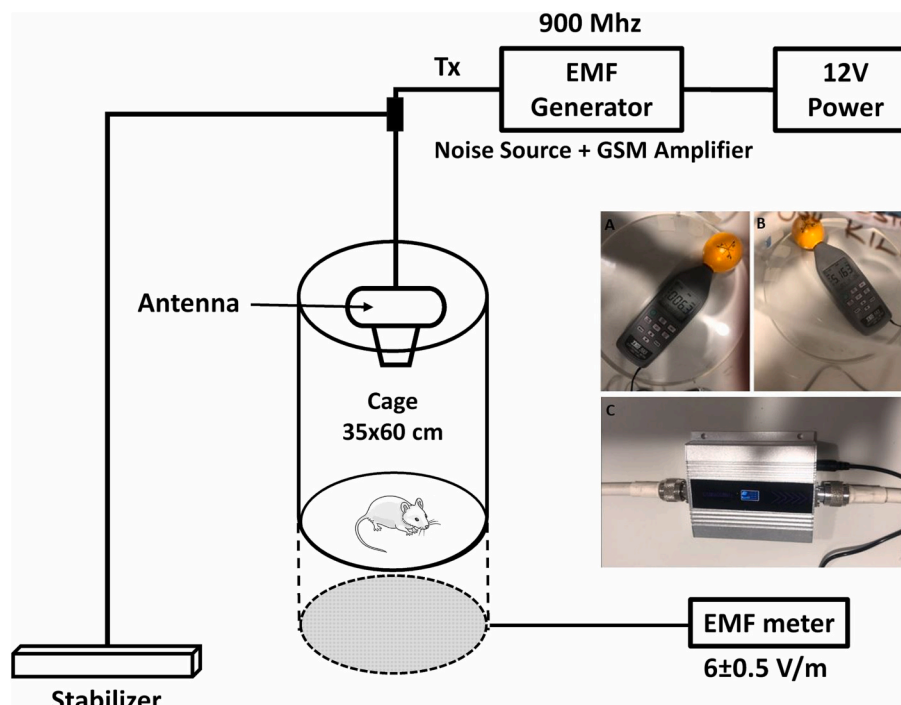


Fig. 2. Design of the EMF exposure.

Birmingham, UK) and TNF- α (E0764Ra, BT LAB, Birmingham, UK) levels were measured with commercial ELISA kits by an expert who was blind to experimental groups.

2.7. Total antioxidant status, total oxidant status determination

TAS and TOS were measured in tissue homogenates at wavelengths of 240 nm and 520 nm, respectively, using a plate reader (Thermo Scientific Multiskan FC, 2011-06, USA). For TAS measurements, Trolox, a water-soluble compound of vitamin E was used as a calibrator and the results were expressed as mmol.Trolox equivalent/L [21]. For TOS measurements, H₂O₂ was used as the standard, and the results were expressed as μ mol-H₂O₂ equivalent/L [22]. Total protein amount in homogenates were measured as above [20]. Oxidative stress index (OSI) values were expressed as Arbitrary Unit according to following formula:

$$\text{OSI} = \left(\frac{\text{TOS } (\mu\text{mol-H}_2\text{O}_2 \text{ equivalent/g protein})}{\text{TAS } (\text{mmol.Trolox equivalent/g protein})} \right) \times 100$$

2.8. Western blotting assay

The brain and kidney tissues were dissected and homogenized (MP FastPrep-24, USA) as mentioned above by using lysis buffer by adding protease and phosphatase inhibitors (ratio was ~50 mg tissue to 1000 μ L of ice-cold lysis buffer) for western blot analyzing. The homogenates were centrifuged at 14,000 x rpm (Beckman Coulter, Krefeld, Germany) for 10 min at 4 °C, and the final supernatant was used as the cytosolic fraction. Total protein concentrations of supernatants were measured using the Bradford method at a 595 nm wavelength (Thermo Scientific Multiskan FC, 2011-06, USA). For each sample, approximately 20 μ g of total protein was loaded into sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It was run at 120 mV for 1 h. After the run, the protein samples in the gel were transferred to polyvinylidene difluoride (PVDF) membrane and incubated at 110 mV for 10 min with a transfer device (Biorad, USA). For each sample, approximately 20 μ g of total protein was loaded into sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It was run at 120 mV for 1 h. After the run, the protein samples in the gel were transferred to polyvinylidene difluoride (PVDF) membrane and incubated at 110 mV for 10 min with a transfer device (Biorad, USA). The membranes were blocked. For blocking, the membranes were incubated for 1 h in an orbital shaker at room temperature in 5 % skim milk and membranes were washed 5 times for 5 min with Tris Buffered Saline with Tween 20 (TBS-T). Primary antibodies Renin (Cat. No: A1585, Abclonal, Woburn, USA), AGT (Cat. No: A15637, Abclonal, Woburn, USA), AT1R (Cat. No: A14201, Abclonal, Woburn, USA), AT2R (Cat. No: A16341, Abclonal, Woburn, USA) and Mas1L (Cat. No: A16162, Abclonal, Woburn, USA) were prepared in 2.5 % skim milk and ratios were 1/1000, 1/1000 1/500, 1/200 and 1/200 respectively. After labeling the membranes with primary antibodies, they were incubated at +4 °C for overnight on a shaker. The membranes were washed with TBS-T 5 times for 5 min, labeled with a 1/1000 secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz Ca USA) and incubated on a shaker for 1 h at room temperature. Stripping method was performed which is a technique used to describe the removal of primary and secondary antibodies from the Western Blot membrane [23]. The bands of antibodies were visualized with chemiluminescence (ECL) Western blot substrate (Pierce, Thermo Scientific, USA) and evaluated on the Vilbert Laurmart Fusion Fx5 device. Beta-actin (ACTB) (Cell signal, USA) was used as a house-keeping reference antibody. The western blot analysis were carried out in triplicate.

2.9. Statistical analysis

The Shapiro–Wilk test was used to test the normality of the data. Data with normal distribution were analyzed by one-way ANOVA test

and data with abnormal distribution were analyzed by Kruskal Wallis tests. Post-hoc comparisons between the groups were performed with Bonferroni and Dunn tests. The sex comparisons of groups were analyzed by two-way ANOVA test followed by Bonferroni post-hoc test. All data were statistically analyzed with GraphPad Prism 8.0.1 (GraphPad Prism Software, San Diego, CA, USA) and presented as mean $\pm$ standard deviation (mean $\pm$ SD). A value of $p < 0.05$ were considered as statistically significant. Correlations between oxidative stress, inflammation and angiotensin system markers were analyzed by Spearman's correlation coefficient using IBM SPSS Statistics 26.

3. Results

3.1. Biochemical results of female brain tissue

The female brain TAS levels were significantly lower in Pre EMF, Post EMF and Pre&post EMF groups ($p < 0.01$, $p < 0.001$, $p < 0.01$; respectively) compared to the control group. The TOS and OSI levels were significantly higher in Pre EMF ($p < 0.05$), Post EMF ($p < 0.001$) and Pre&post EMF ($p < 0.01$) groups than in the control group. The TOS levels were significantly higher ($p < 0.05$) in the Post EMF group compared to the Pre EMF group. In addition, the OSI levels were significantly higher in Post EMF ($p < 0.001$) and Pre&post EMF ($p < 0.001$) groups than in the Pre EMF group.

The IL-2 levels of female brain were significantly elevated in Pre EMF group ($p < 0.01$), Post EMF ($p < 0.001$) and Pre&post EMF groups ($p < 0.001$) compared to the control group. Lastly, the levels of IL-6 and TNF- α were significantly higher in all three EMF groups ($p < 0.001$) than in the control group (Table 1).

3.2. Biochemical results of male brain tissue

The male brain TAS levels were lower in all three EMF groups than in the control group and this decline was statistically significant ($p < 0.001$). The TAS levels were also significantly lower ($p < 0.05$) in the Post EMF group compared to the Pre EMF group. The male brain TOS and OSI levels were significantly higher in Pre EMF group ($p < 0.01$, $p < 0.05$; respectively), Post EMF group ($p < 0.05$, $p < 0.01$; respectively) and Pre&post EMF group ($p < 0.01$, $p < 0.001$; respectively) compared to the control group.

The highest levels of IL-2 in male brain were detected in Pre EMF and Pre&post EMF groups, and this elevation was statistically significant compared to the control group ($p < 0.05$). However, the male brain IL-2 levels were not increasing as dramatic as female brain (Supp. Table 1).

Table 1
Biochemical results of female brain tissue (n = 9).

Female brain				
Parameter	C	Pre EMF	Post EMF	Pre&post EMF
TAS (mmol Trolox Equiv./g protein)	1.14 $\pm$ 0.16	0.91 $\pm$ 0.08**	0.70 $\pm$ 0.13***	0.76 $\pm$ 0.09**
TOS (μ mol H ₂ O ₂ Equiv./g protein)	6.63 $\pm$ 3	10.47 $\pm$ 2.24*	16.77 $\pm$ 1.28*** ⁺	14.70 $\pm$ 3.06**
OSI (Arbitrary Unit)	6.42 $\pm$ 0.7	10.27 $\pm$ 1.64*	24.01 $\pm$ 4.35*** ⁺⁺⁺	22.20 $\pm$ 5.14*** ⁺⁺⁺
IL-2 (ng/g protein)	125.0 $\pm$ 47.79	211.9 $\pm$ 42.06**	337.7 $\pm$ 27.41***	303.1 $\pm$ 90.22***
IL-6 (ng/g protein)	2.27 $\pm$ 1.26	7.48 $\pm$ 1.43***	9.01 $\pm$ 1.38***	7.29 $\pm$ 2.24***
TNF- α (ng/g protein)	152.4 $\pm$ 70.07	381.5 $\pm$ 39.06***	436.8 $\pm$ 67.64***	434.5 $\pm$ 79.46***

Data were presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistical significance compared to the C group. ⁺ $p < 0.05$, ⁺⁺⁺ $p < 0.001$ statistical significance compared to the Pre EMF group. TAS; Total antioxidant capacity, TOS; Total oxidant capacity, OSI; Oxidative stress index, IL-2; Interleukin 2, IL-6; Interleukin 6, TNF- α ; Tumor necrosis factor alpha.

The IL-6 and TNF- α levels of male brain significantly higher in Pre EMF ($p < 0.01$), Post EMF ($p < 0.01$, $p < 0.001$; respectively) and Pre&post EMF ($p < 0.01$, $p < 0.001$; respectively) groups than in the control group (Table 2). However, this increase was more expressive in females than males (Supp. Table 1).

3.3. Biochemical results of female kidney tissue

The TAS levels were significantly lower in Pre EMF ($p < 0.05$), Post EMF ($p < 0.001$) and Pre&post EMF ($p < 0.001$) groups compared to the control group. It was also significantly lower ($p < 0.05$) in the Pre&post EMF group than the Pre EMF group. The TOS levels were significantly higher in all three EMF groups ($p < 0.01$, $p < 0.001$; respectively) than the control group. Levels of OSI were also significantly higher in all three EMF groups ($p < 0.05$, $p < 0.001$; respectively) than the control group. Additionally, it was significantly higher in Pre&post EMF group ($p < 0.001$) than both Pre EMF and Post EMF groups.

IL-2 and TNF- α levels were significantly increased in all three EMF groups ($p < 0.001$) compared to the control group. Suprisingly, levels of IL-6 were significantly increased in just Pre&post EMF group ($p < 0.05$) than the control group. The IL-2 levels of the Pre&post EMF group were significantly higher ($p < 0.001$) than both Pre EMF and Post EMF groups. However, TNF- α levels were lower ($p < 0.01$) in the Pre&post EMF group compared to the Post EMF group (Table 3).

3.4. Biochemical results of male kidney tissue

The TAS levels were significantly lower in all three EMF groups ($p < 0.001$, $p < 0.01$, $p < 0.001$; respectively) than the control group. It was also significantly lower ($p < 0.01$, $p < 0.001$; respectively) in the Pre&post EMF group than both Pre EMF and Post EMF groups. TOS levels were significantly higher in Pre EMF, Post EMF ($p < 0.05$; respectively) and Pre&post EMF ($p < 0.001$) groups compared to the control group. Levels of OSI were also higher in all three EMF groups and this increment was statistically significant ($p < 0.001$). In addition, it was also significantly higher ($p < 0.01$) in the Pre&post EMF group than both Pre EMF and Post EMF groups.

The levels of IL-2 were significantly higher in Pre EMF ($p < 0.01$), Post EMF ($p < 0.05$) and Pre&post EMF ($p < 0.001$) groups than the control group. As opposed to female kidney tissue, levels of IL-6 were significantly increased in Pre EMF ($p < 0.05$), post EMF ($p < 0.05$) and Pre&post EMF ($p < 0.01$) groups compared to the control group. Lastly, TNF- α levels were significantly higher in all three EMF groups ($p < 0.001$) than the control group and the highest levels of TNF- α were

Table 2
Biochemical results of male brain tissue (n = 9).

Male brain				
Parameter	C	Pre EMF	Post EMF	Pre&post EMF
TAS (mmol Trolox Equiv./g protein)	1.45 $\pm$ 0.18	0.93 $\pm$ 0.04***	0.74 $\pm$ 0.02*** ⁺	0.84 $\pm$ 0.05***
TOS (μ mol H ₂ O ₂ Equiv./g protein)	5.04 $\pm$ 1.2	7.36 $\pm$ 1.31**	7.28 $\pm$ 1.82*	8.12 $\pm$ 1.42**
OSI (Arbitrary Unit)	4.12 $\pm$ 1.3	7.88 $\pm$ 1.83*	9.02 $\pm$ 2.59**	10.77 $\pm$ 4.09***
IL-2 (ng/g protein)	110.5 $\pm$ 56.62	165.6 $\pm$ 23.82*	139.1 $\pm$ 32.68	181.7 $\pm$ 71.82*
IL-6 (ng/g protein)	1.9 $\pm$ 1.24	4.23 $\pm$ 1.49**	4.12 $\pm$ 1.04**	4.18 $\pm$ 1.61**
TNF- α (ng/g protein)	205.0 $\pm$ 41.53	298.3 $\pm$ 55.56**	339.1 $\pm$ 42.94***	324.9 $\pm$ 51.97***

Data were presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistical significance compared to the C group. ⁺ $p < 0.05$ statistical significance compared to the Pre EMF group. TAS; Total antioxidant capacity, TOS; Total oxidant capacity, OSI; Oxidative stress index, IL-2; Interleukin 2, IL-6; Interleukin 6, TNF- α ; Tumor necrosis factor alpha.

Table 3
Biochemical results of female kidney tissue (n = 9).

Female kidney				
Parameter	C	Pre EMF	Post EMF	Pre&post EMF
TAS (mmol Trolox Equiv./g protein)	1.22 $\pm$ 0.05	1.01 $\pm$ 0.04*	0.92 $\pm$ 0.02***	0.8 $\pm$ 0.04*** ⁺
TOS (μ mol H ₂ O ₂ Equiv./g protein)	2.21 $\pm$ 0.68	7.6 $\pm$ 0.91**	7.51 $\pm$ 1.06**	10.01 $\pm$ 1.3***
OSI (Arbitrary Unit)	3.02 $\pm$ 0.87	6.79 $\pm$ 0.7*	8.44 $\pm$ 0.75***	12.49 $\pm$ 0.76*** ⁺⁺⁺ ##
IL-2 (ng/g protein)	7.35 $\pm$ 0.55	56.85 $\pm$ 8.63***	60.1 $\pm$ 5.67***	104.4 $\pm$ 7.19*** ⁺⁺⁺ ###
IL-6 (ng/g protein)	1.35 $\pm$ 0.24	1.94 $\pm$ 0.43	2.84 $\pm$ 0.38	4.15 $\pm$ 0.66*
TNF- α (ng/g protein)	105.1 $\pm$ 7.25	229.1 $\pm$ 14.28***	227.3 $\pm$ 6.48***	164.4 $\pm$ 7.17** [#]

Data were presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistical significance compared to the C group. ⁺ $p < 0.05$, ⁺⁺⁺ $p < 0.001$ statistical significance compared to the Pre EMF group. [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ statistical significance compared to the Post EMF group. TAS; Total antioxidant capacity, TOS; Total oxidant capacity, OSI; Oxidative stress index, IL-2; Interleukin 2, IL-6; Interleukin 6, TNF- α ; Tumor necrosis factor alpha.

detected in the Pre&post EMF group and it was statistically significant ($p < 0.05$) compared to the Post EMF group (Table 4).

3.5. Sex comparisons of biochemical analysis

Although there was not any significant change in TAS values of EMF groups in the sex comparisons of the brains, the male brain had higher TAS levels than the female for the control group. Although TOS and OSI are increased in all EMF groups in brain tissue, this increase is attenuated in males compared to females. The inflammatory markers were also significantly decreased in male brains in all three EMF groups compared to the female brains ($p < 0.001$) (Supp. Table 1).

Conversely, in the kidney tissue, TOS and OSI values found to be significantly ($p < 0.01$, $p < 0.001$, respectively) increased in the EMF groups of males compared to females. However, the male kidney had higher TOS levels than the females for the control group.

Surprisingly, there was no change in IL-2 levels between both sexes. Oppositely of the brain results, levels of IL-6 were significantly ($p < 0.001$, $p < 0.05$) increased in male kidney tissue than the females after exposure to EMF. Lastly, TNF- α levels were significantly decreased ($p <$

Table 4
Biochemical results of male kidney tissue (n = 9).

Male kidney				
Parameter	C	Pre EMF	Post EMF	Pre&post EMF
TAS (mmol Trolox Equiv./g protein)	1.16 $\pm$ 0.02	0.88 $\pm$ 0.02**	1.01 $\pm$ 0.02** ⁺⁺	0.73 $\pm$ 0.03*** ⁺⁺ ###
TOS (μ mol H ₂ O ₂ Equiv./g protein)	5.66 $\pm$ 0.11	10.86 $\pm$ 0.35*	11.05 $\pm$ 0.25*	12.06 $\pm$ 0.78***
OSI (Arbitrary Unit)	6.07 $\pm$ 0.23	12.11 $\pm$ 0.87***	11.81 $\pm$ 0.3***	17.28 $\pm$ 1.52*** ⁺⁺ ###
IL-2 (ng/g protein)	30.10 $\pm$ 1.08	76.5 $\pm$ 11.52**	68.23 $\pm$ 2.17*	105.0 $\pm$ 14.59***
IL-6 (ng/g protein)	2.70 $\pm$ 0.21	4.92 $\pm$ 0.44*	4.72 $\pm$ 0.13*	5.24 $\pm$ 0.58**
TNF- α (ng/g protein)	74.59 $\pm$ 11.43	178.8 $\pm$ 11.87***	123.3 $\pm$ 3.61***	216.9 $\pm$ 5.73*** [#]

Data were presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ statistical significance compared to the C group. ⁺⁺ $p < 0.01$ statistical significance compared to the Pre EMF group. [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ statistical significance compared to the Post EMF group. TAS; Total antioxidant capacity, TOS; Total oxidant capacity, OSI; Oxidative stress index, IL-2; Interleukin 2, IL-6; Interleukin 6, TNF- α ; Tumor necrosis factor alpha.

0.01, $p < 0.001$) in the Pre EMF and Post EMF groups and increased ($p < 0.001$) in the Pre&post EMF group of male kidneys compared to the females (Supp. Table 2).

3.6. Western blotting assay

3.6.1. Renin levels

The highest levels of renin expression were detected in Post EMF group compared to the control, Pre EMF and Pre&Post EMF groups and this elevation was statistically significant ($p < 0.001$) in female brain tissue. Levels of renin were also higher ($p < 0.01$) in Pre EMF group compared to the control group (Fig. 3A). Levels of renin expression were significantly higher in Pre EMF ($p < 0.05$) and Pre&post EMF ($p < 0.01$) groups than control groups in male brain tissue (Fig. 3B). Renin expression levels were significantly higher in all three EMF groups ($p < 0.01$, $p < 0.001$; respectively) than control group in female kidney tissue. Renin levels also significantly higher ($p < 0.001$) in Pre&Post EMF group than the Pre EMF and Post EMF groups (Fig. 3C). Similarly, the highest levels of renin expression were detected Pre&Post EMF group than control, Pre EMF and Post EMF groups in male kidney tissue and this increment was statistically significant ($p < 0.001$) (Fig. 3D).

3.6.2. Angiotensinogen levels

Angiotensinogen expression levels were significantly higher in all three EMF groups ($p < 0.001$) than control group in female brain tissue. The levels were also significantly higher in both Post EMF and Pre&post EMF groups ($p < 0.001$) than the Pre EMF group (Fig. 4A). Additionally, levels of angiotensinogen expression were significantly higher ($p < 0.001$) in just both Post EMF and Pre&post EMF groups compared to the control and Pre EMF group ($p < 0.001$) in male brain tissue (Fig. 4B). Similarly, expression levels of angiotensinogen were significantly higher in all three EMF groups ($p < 0.05$, $p < 0.001$; respectively) than control group in female kidney tissue (Fig. 4C). Angiotensinogen expression levels also were higher in both Post EMF and Pre&post EMF groups compared to the control group in male kidney tissue. And this increment was statistically significant ($p < 0.001$, $p < 0.05$; respectively) (Fig. 4D).

3.6.3. AT1R levels

The highest levels of AT1R expression were found in the Post EMF group, and this elevation was statistically significant ($p < 0.001$) compared to the control group in female brain tissue. In addition, levels of AT1R expression in female brain tissue were significantly higher in Pre&post EMF group than the control group. The Post EMF group is greater than the Pre EMF ($p < 0.05$) and Pre&post EMF ($p < 0.01$) groups (Fig. 5A).

Likewise in male brain tissue, levels of AT1R expression were significantly higher in all three EMF groups ($p < 0.01$, $p < 0.001$; respectively) compared to the control group and the levels were also significantly higher in both Post EMF and Pre&post EMF groups ($p < 0.001$) than the Pre EMF group (Fig. 5B). AT1R expression levels were significantly higher in both Post EMF ($p < 0.05$) and Pre&post EMF ($p < 0.001$) groups compared to the controls. Additionally, the Pre&post EMF group had higher ($p < 0.001$) AT1R levels than the Pre EMF group in female kidney tissue (Fig. 5C). AT1R expression were significantly higher in all three EMF groups ($p < 0.05$, $p < 0.001$; respectively) than control group in male kidney tissue (Fig. 5D).

3.6.4. AT2R Levels

Interestingly, levels of AT2R expression in both female (Fig. 6A) and male (Fig. 6B) brain tissues were significantly higher ($p < 0.001$) in all three EMF groups compared to the control group. For brain tissue, levels in the Pre EMF group were higher ($p < 0.05$) than Post EMF group in females, whereas it was higher than both Post EMF ($p < 0.01$) and Pre&Post EMF ($p < 0.05$) groups in males. Likewise in female kidney tissue, levels of AT2R expression were significantly higher in Pre EMF ($p < 0.001$), Post EMF ($p < 0.001$) and Pre&post EMF ($p < 0.05$) groups

than control groups. Levels of AT2R expression were significantly lower ($p < 0.001$) in both Post EMF and Pre&Post EMF groups than in the Pre EMF group (Fig. 6C). In line with female kidney tissue, levels of AT2R expression were significantly higher in all three EMF groups ($p < 0.001$) compared to the control group in male kidney tissue (Fig. 6D).

3.6.5. AT1/AT2 ratios

The ratio for AT1 and AT2 receptors was higher ($p < 0.001$) in male brains compared to the females in all groups including controls. However, the ratio for AT1 and AT2 receptors was lower ($p < 0.01$) in control and Pre&post EMF groups in male kidney tissues than the females. Oppositely, AT1/AT2 ratios of Post EMF group were significantly higher ($p < 0.001$) in male kidneys than the females.

3.6.6. Mas1L levels

Lastly, levels of Mas1L expression were significantly higher ($p < 0.001$) in Pre EMF and Pre&post EMF groups compared to the control group in female brain tissue (Fig. 7A). Levels of Mas1L were lower in both Post EMF and Pre&Post EMF groups ($p < 0.001$) than in the Pre EMF group. On the other hand, levels of Mas1L expression were significantly higher in all three EMF groups ($p < 0.001$, $p < 0.01$; respectively) than the control group in male brains (Fig. 7B). Levels of Mas1L expression in both female (Fig. 7C) and male (Fig. 7D) kidney tissues were significantly higher ($p < 0.001$) in all three EMF groups than the control group. Lastly, levels of Mas1L were lower in both Post EMF and Pre&Post EMF groups ($p < 0.001$) than in the Pre EMF group in female kidney tissue. Mas1L level was also lower ($p < 0.001$) in the Post EMF group than the Pre EMF group in male kidneys.

3.7. Sex comparisons of western blotting assay

Renin, Mas1R and AT2R levels were significantly lower ($p < 0.001$) in the EMF groups of male brain tissue than the female brain tissue. Interestingly, the AGT levels of male brain tissue were significantly lower ($p < 0.001$) in the Post EMF and Pre EMF groups, but significantly higher ($p < 0.001$) in the Pre&Post EMF group compared to the females. Only AT1R levels were significantly elevated ($p < 0.001$) in male brain tissue compared to females in Post EMF and Pre&Post EMF groups (Supp. Table 3).

On the other hand, levels of AT1R, AT2R and Renin were significantly higher ($p < 0.001$) in male kidney tissue than the females after EMF exposure. AGT levels were also significantly higher than the females although this increment statistically significant ($p < 0.05$) only in the Post EMF group. Lastly, levels of MAS1R in males significantly lower ($p < 0.001$) in EMF groups than the females including the control groups in both brain and kidney tissues (Supp. Table 4).

3.8. Spearman's correlation analysis

Considering the correlation analyzes in all groups, there was a positive correlation between angiotensin components and between oxidative stress and inflammation. However, there was a positive correlation between AT1R, AT2R and MasR in some of the EMF exposure groups, while there was a negative correlation in control groups between these components. There was a positive correlation between IL-6 and renin and IL-6- and AT2R in male brains with exposure to EMF. Additionally, there was a positive correlation between OSI and AT2R and OSI and renin in the Post EMF and Pre&Post EMF groups of male brains. Similarly, there was a positive correlation between AGT and OSI and renin and OSI in the Pre EMF group of female kidneys. There was a positive correlation between AT2R and OSI and AGT and TNF- α in the control groups of male kidneys. There was a positive correlation between OSI and IL-2 in Pre EMF group of male kidneys. Lastly, there was a positive correlation between MasR, AT1R and AT2R and IL-6 in Post EMF group of male kidneys (Supp. File 2).

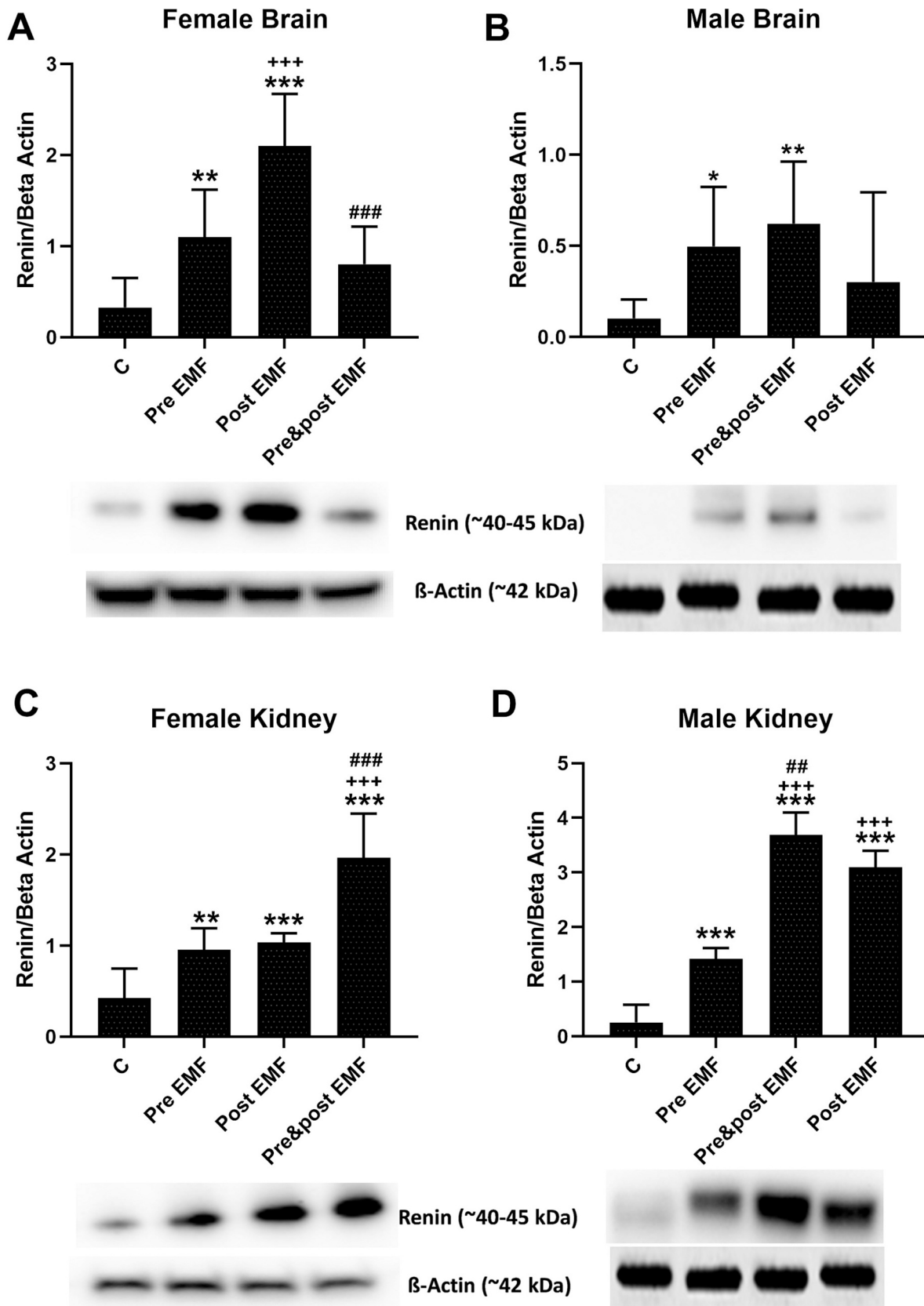


Fig. 3. Western blotting assay results of renin for female brain tissue (n = 9) (A), male brain tissue (n = 9) (B), female kidney tissue (n = 9) (C) and male kidney tissue (n = 9) (D). Data were presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 statistical significance compared to C group. +++p < 0.001 compared to the Pre EMF group. ##p < 0.01, ###p < 0.001 compared to the Post EMF group.

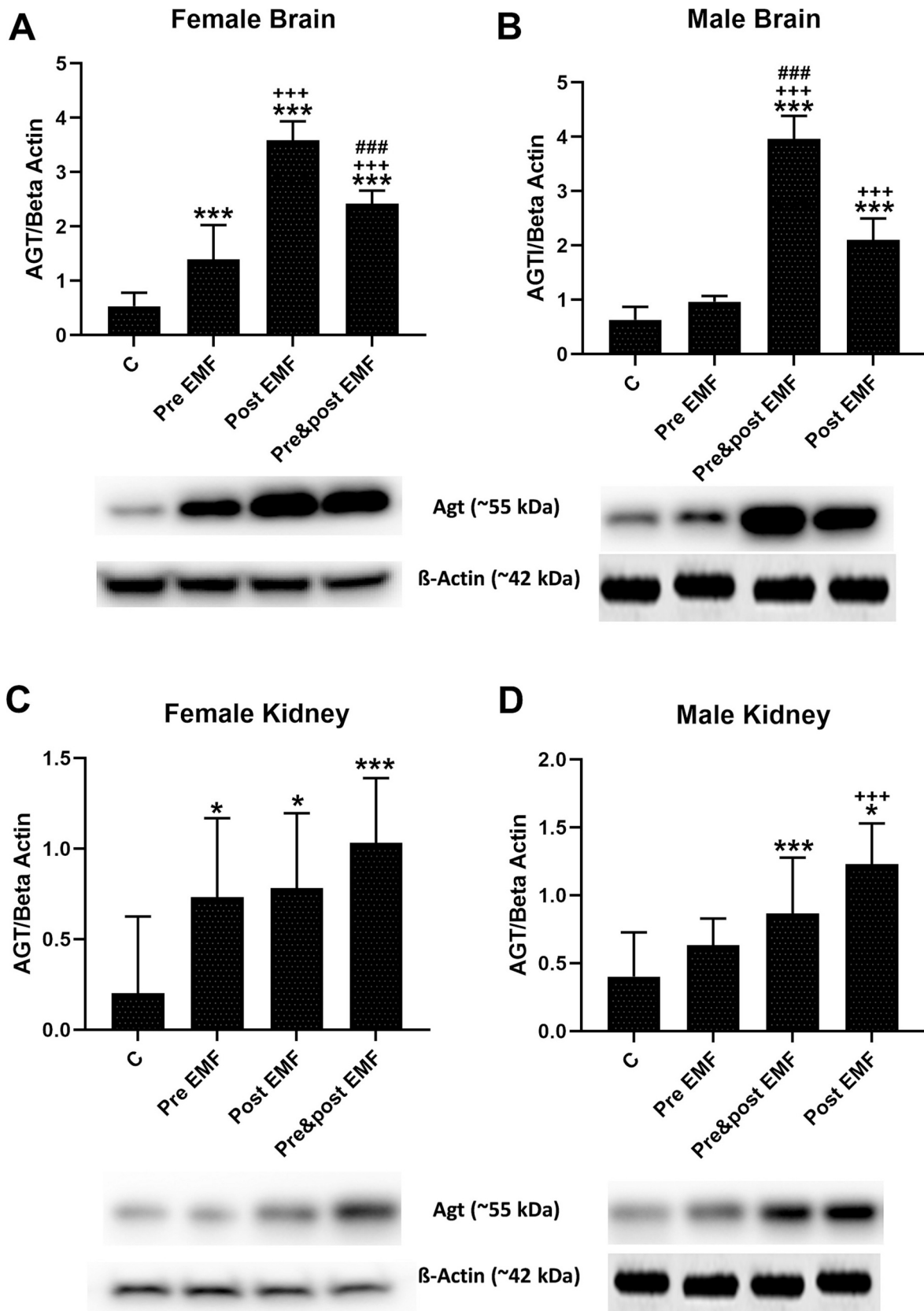


Fig. 4. Western blotting assay results of angiotensinogen for female brain tissue (n = 9) (A), male brain tissue (n = 9) (B), female kidney tissue (n = 9) (C) and male kidney tissue (n = 9) (D). Data were presented as mean ± SD. *p < 0.05, ***p < 0.001 statistical significance compared to C group. +++p < 0.001 compared to the Pre EMF group. ###p < 0.001 compared to the Post EMF group.

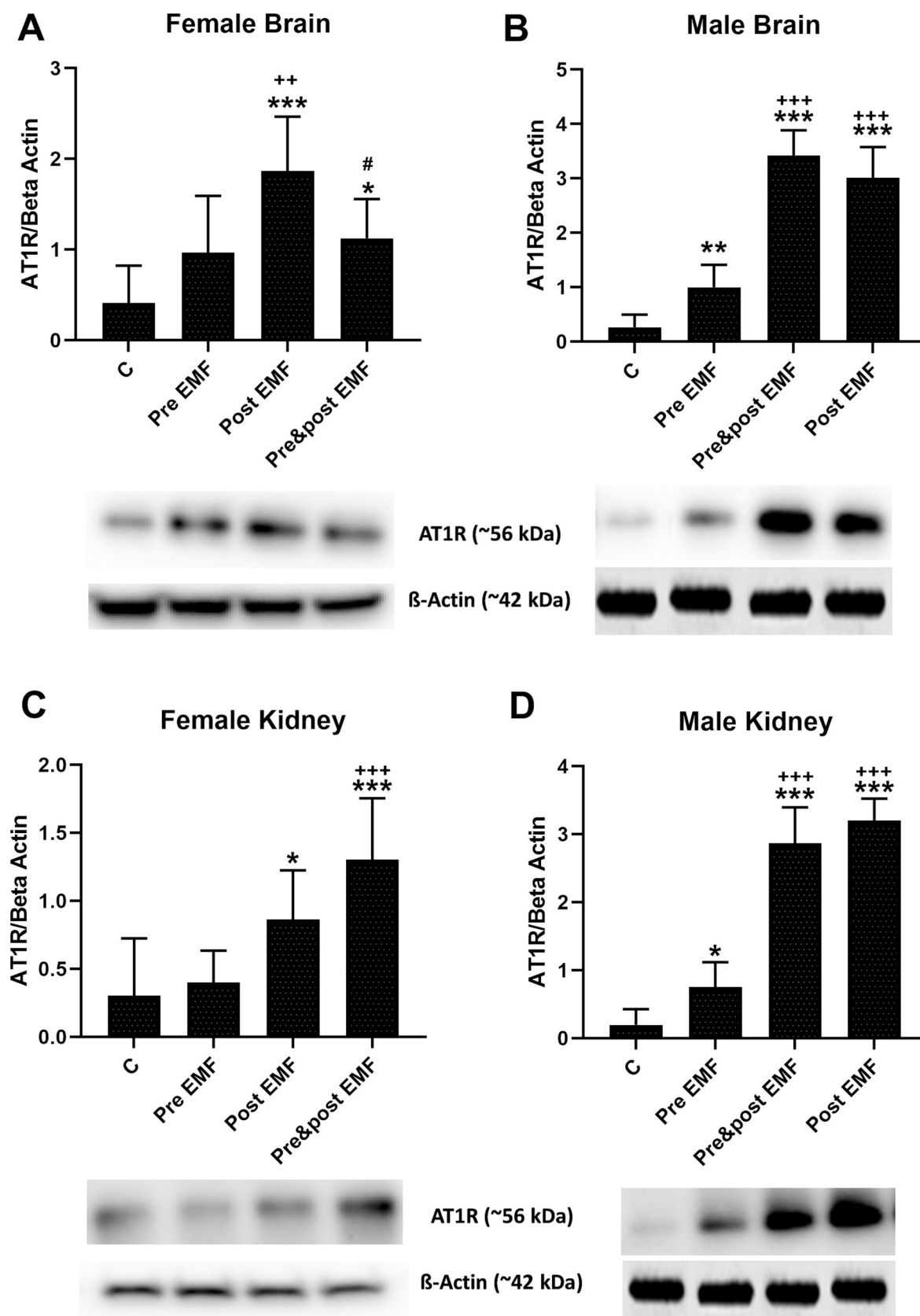


Fig. 5. Western blotting assay results of AT1R for female brain tissue (n = 9) (A), male brain tissue (n = 9) (B), female kidney tissue (n = 9) (C) and male kidney tissue (n = 9) (D). AT1R; Angiotensin II Type 1 Receptor. Data were presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 statistical significance compared to C group. ++p < 0.01, +++p < 0.001 compared to the Pre EMF group.

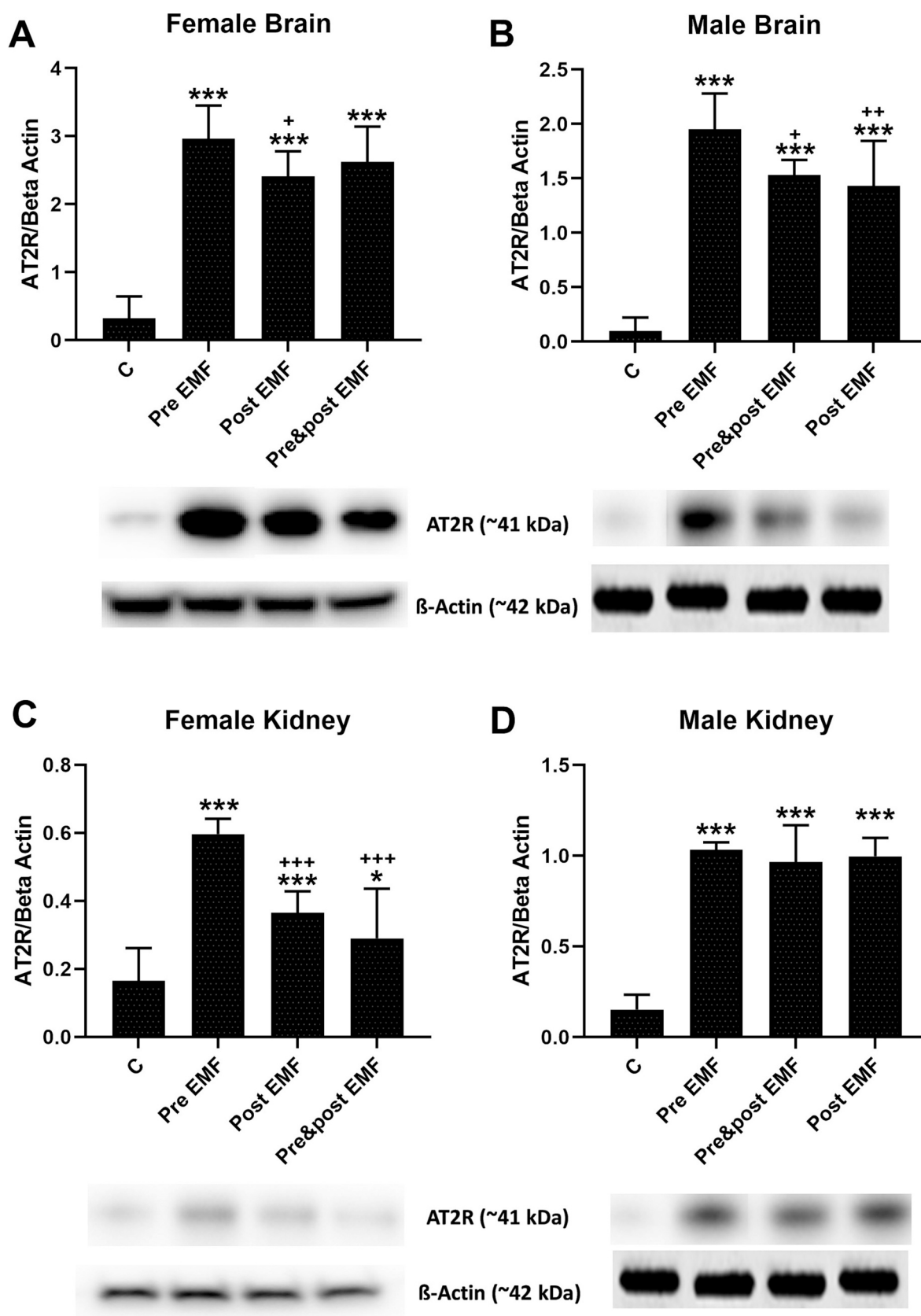


Fig. 6. Western blotting assay results of AT2R for female brain tissue (n = 9) (A), male brain tissue (n = 9) (B), female kidney tissue (n = 9) (C) and male kidney tissue (n = 9) (D). AT2R; Angiotensin II Type 2 Receptor. Data were presented as mean $\pm$ SD. ***P < 0.001 statistical significance compared to C group. +p < 0.05, ++p < 0.01, +++p < 0.001 compared to the Pre EMF group.

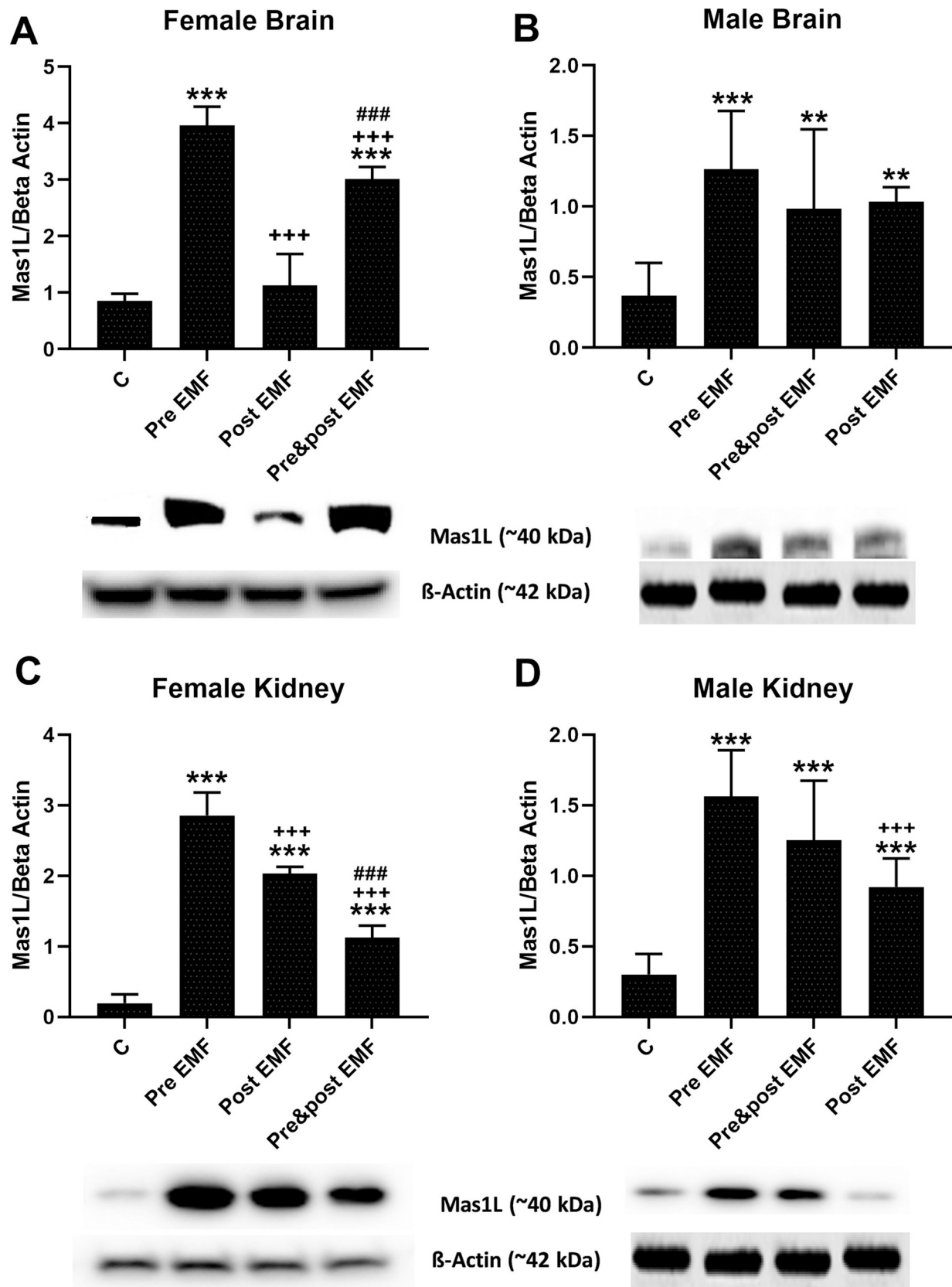


Fig. 7. Western blotting assay results of Mas1L for female brain tissue (n = 9) (A), male brain tissue (n = 9) (B), female kidney tissue (n = 9) (C) and male kidney tissue (n = 9) (D). Mas1L; Mas-related G-protein coupled receptor. Data were presented as mean ± SD. **P < 0.01, ***P < 0.001 statistical significance compared to C group. +++p < 0.001 compared to the Pre EMF group. ###p < 0.001 compared to the Post EMF group.

4. Discussion

Cell phones have become an indispensable part of our routine life over the past two decades. As a result of this widespread use, many controversial effects such as oxidative stress and inflammation occur in living organisms [24,25]. Since the use of mobile phones is close to the brain, the harmful effects of 900 MHz EMF on the brain are a matter of curiosity. The brain is regarded to be particularly indefensible to oxidative damage due to its high oxygen consumption rate, high lipid content and relatively smaller amounts of antioxidant enzymes than other tissues. There are some studies demonstrated that 900 MHz EMF causes oxidative stress [26,27]. In the studies carried out, there is no sex-related difference related to oxidative stress caused by 900 MHz EMF. It was reported that 900 MHz EMF caused oxidative stress in both mothers and offsprings, in a study conducted with rats exposed to 900 MHz EMF during pregnancy [28]. Alkis, et al. [29] suggested that 900 MHz EMF causes oxidative stress and lipid peroxidation in the frontal lobe in male rats. In another study, it has been suggested that 900 MHz EMF leads to significant increment in malondialdehyde (MDA) levels, besides significant decrement in antioxidant defense system [30]. Furthermore in a study of rats exposed to wireless EMF during the prenatal and postnatal periods, it was demonstrated that renal tissue MDA and TOS levels of prenatal group were increased whereas TAS and superoxide dismutase levels of prenatal group were decreased [31]. In addition, Türedi, et al. [32] reported that exposure to 900 MHz EMF increased the levels of kidney MDA and decreased the levels of kidney antioxidant markers, catalase and glutathione. In our study, higher TOS and OSI levels and lower TAS levels in both brain and kidney tissue has been detected in the EMF exposure groups. However, this increment was more marked in female brain tissue. Oppositely, increment of TOS and OSI levels were more marked in male kidney tissues. Also, there was a significantly higher level of TAS in the brain of control males. It may have contributed to greater protection against oxidative stress and inflammation in males. In contrast, a higher level of TOS in the kidneys of males may have contributed to a greater susceptibility of this group to oxidative stress and inflammation. Sex hormones such as estrogens and androgens have protective effects against oxidative stress in several tissues including brain and kidney tissues [33,34]. However, the curative effects of estrogens on oxidative damage are more marked than the androgens [33,35]. The kidney results of females confirm this information. In general, the brains of young women tend to have less oxidative damage compared to men according to other studies [36]. However, some studies have suggested different levels of antioxidant activity in different brain regions of male and female rats. For example, Noschang, et al. [37] demonstrated that the levels of catalase were higher in the striatum of female rats, while it was higher in the prefrontal cortex of male rats. On the other hand, female rats had higher SOD levels in the prefrontal cortex and striatum SOD activity was similar in both sexes. Another study suggested a decrease in antioxidant proteins and an increase in oxidative stress in the hippocampus of C57BL6 female mice compared to males [38]. Since total brain measurements were used in our study, this may be the reason for the difference in females. Moreover, the presence of local RAS in the brain is frequently mentioned in addition to the systemic RAS [39]. Accordingly, one possible reason for the lower oxidative stress in male brains than the females may be that increased estrogen in females leads to enhanced levels of angiotensin II in the brain, further increasing ROS production.

The relationship between oxidative stress and the production of proinflammatory cytokines has been reported in previous studies [40,41]. Oxidative stress causes intracellular signal in proinflammatory cascade via activation of p38 [42]. In the present study, proinflammatory cytokines such as IL-2, IL-6 and TNF- α alterations were consistent with TOS and OSI. While our results of proinflammatory cytokines were lower in male brain than females, the results were opposite in kidney tissue. However, these results are consistent with the biochemical results. It has been reported that the 900 MHz EMF

exposure resulted in increment in levels of IL-2, IL-6 and TNF- α in hippocampi tissue of rats [30]. Similarly Djindjic, et al. [43] demonstrated an increment in serum TNF- α and IL-2 levels of rats exposed to 900 MHz EMF.

In this study, the components of RAS such as renin, angiotensinogen, AT1R, AT2R and MASR were increased with exposure to EMF. Consistent with the inflammatory and oxidative stress results, these components were higher in male kidneys but lower in male brains compared to the females. The higher renin and angiotensinogen levels in the Pre&Post EMF group in both male and female kidneys and brains may be related to the longer EMF exposure time and remain activating the RAS. Ang II and the other RAS components have many effects on different organs and diseases. This complex system also contributes to progression of tissue inflammation and oxidative stress, especially in brain and renal tissue. Ang II contributes to oxidative damage by initiates an inflammatory cascade of reduced nicotinamide-adenine dinucleotide phosphate oxidase, superoxide anions and nuclear factor-kappaB, which mediates transcription and gene expression and increases adhesion molecules and chemokines and exerts a proinflammatory effect via angiotensin type 1 (AT1R) receptor [44]. For instance, there are many studies demonstrated that brain RAS is associated with oxidative stress, inflammation, neurotransmitter secretion and even cognition [45]. Furthermore, activation of the Ang II/AT1R axis in the brain contributes to neuroinflammation and neurodegeneration and is associated with diseases such as Alzheimer's and Parkinson's disease [46]. Ang II/AT1R axis induces inflammation not only in brain but also several tissues especially in kidneys. Dandona, et al. [47] reported that Ang II-mediated inflammation and oxidative stress occurs via interaction between Ang II and AT1R, increasing nicotinamide-adenine dinucleotide phosphate oxidase. Ang II also binds to the angiotensin type 2 (AT2R) receptor, whose expression increases in pathological conditions providing an endogenous protection to inflammatory, oxidative and apoptotic processes and has opposite effects to the actions of the AT1R [48]. Many studies have demonstrated that AT1R blockade reduces oxidative stress and inflammation [49,50]. Tsutamato, et al. [51] reported that AT1R blocker decreases plasma levels of TNF- α and IL-6 in patients with chronic heart failure. Ruiz-Ortega, et al. [52] also suggested that the blockade of Ang II decreases inflammation in animal models of renal injury. Additionally, our previous study has been demonstrated that AT2R blocker PD123319 has more beneficial effects on cardiac ischemia/reperfusion injury than AT1R blocker losartan and played a cardioprotective role via reducing oxidative damage in isolated rat hearts [53]. Based on our results 900 MHz EMF can alter the inflammatory response and activates Ang II/AT1R axis. Tissue angiotensinogen and renin expression in male and female offspring brains and kidneys were detected higher with exposure to EMF.

AT1R and AT2R expression levels were significantly increased in both male and female offspring brains in all three EMF groups, also this increment was more remarkable in AT2R, which can be explained by the rapid increase in AT2R expression in pathological conditions to balance the harmful effects of AT1R. Moreover, AT1/AT2 ratio of male brain tissue was higher in all EMF exposure groups than the females at the same time oxidative stress and inflammation markers were lower in male brains. Consistently, AT1/AT2 ratio of male kidney tissue was lower in Pre&post EMF group and controls compared to the females while oxidative stress and inflammation markers were higher in male kidneys. These results may be related to pre-exposure and post-exposure to EMF. Pre-exposure to EMF increased AT2 expression levels, while post-exposure to EMF increased AT1 expression levels more than the other groups. Our results suggested that the expression levels of AT2 receptor were more marked in the Pre EMF group, while the expression levels of AT1 receptor were more marked in both post-exposure groups unrelated to sex. This may be related to the greater significance of AT2R expression in fetuses than in adults, and this may explain the lowest OSI value in the Pre EMF group in all tissues, again unrelated to sex.

Another cascade of RAS is the ACE2/Ang-(1-7)/Mas axis, which is

the protective arm of RAS and the similar results for AT2R are valid for this axis as well. Angiotensin 1-7 (Ang (1-7)) obtained from Ang II via angiotensin converting enzyme 2 and exerts its effects through the MAS1 oncogene the G-protein coupled receptor (Mas1R). Mas receptor is responsible for the vasodilator, antiproliferative, anti-inflammatory and anti-fibrotic effects of Ang-(1-7) [54]. Many studies have demonstrated that Ang-(1-7) by acting via Mas receptor exerts inhibitory effects on inflammation. A study has suggested the activation of the ACE2/Ang-(1-7)/Mas axis is related to the anti-inflammatory effects of ARBs. It has also been suggested that ARBs cause the reduction of proinflammatory cytokines such as TNF- α , IFN- γ , IL-1 β , IL-6 [55,56]. Additionally, several studies have suggested that treatment with Ang-(1-7) activates an anti-inflammatory response in different pathophysiological conditions [57,58]. In current study, Mas1R expression found significantly higher in both female and male offspring brains and kidneys in all EMF groups. However, in line with the expression of AT2R, pre-exposure to EMF was more potent in raising expression of Mas1L levels. As well as the higher expression of AT1R in fetal life, ACE2/Ang-(1-7)/Mas axis was also found to be higher during the gestation period [59].

5. Conclusion

Although there are some differences of the RAS component alterations in brain and kidney tissues between males and females, the major result of all groups was an increase in all RAS components with exposure to 900 MHz EMF. The increment in renin, angiotensinogen and AT1R is consistent with the increment in ROS and proinflammatory cytokines. Oxidative stress and inflammation were higher in female brains, while these markers were lower for this sex considering the kidneys. However, the two protective arms of RAS, AT2R and Mas1R have also increased but at different levels and this increment was more remarkable in all pre-exposure groups and non-related to sex. This may be related to excess fetal expression of AT2R and higher Mas1R expression during gestation period or may be a compensatory effect of these two components due to the duration of EMF exposure. Further studies are needed for a more comprehensive understanding.

In conclusion, our study suggested that the 900 MHz EMF can activate brain and kidney renin-angiotensin system, and this activation is maybe related to inflammation and oxidative stress in both male and female offsprings.

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CRediT authorship contribution statement

A.K., S.U. and I.M. conceived the experimental design of the study.
A.K. and S.U. performed the animal experiments.
A.K. and H.B. performed the western blotting assay.
A.K., S.U., and H.B. performed the data analysis and interpretation.
A.K. and S.U. wrote most of the paper.
I.M. provided revisions to scientific content of manuscript and grammatical revisions to manuscript.
I.M. provided funding.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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