



1800 MHz radiofrequency fields inhibits testosterone production *via* CaMKI/ROR α pathway

Fenju Qin^{a,*}, Honglong Cao^c, Hongxia Yuan^a, Weiqiang Guo^a, Hailong Pei^d, Yi Cao^b, Jian Tong^{b,*}

^a Department of Biological Science and Technology, Suzhou University of Science and Technology, Suzhou 215009, China

^b School of Public Health, Medical College of Soochow University, Suzhou 215123, China

^c School of Electronic & Information Engineering, Soochow University, Suzhou 215006, China

^d School of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215123, China

ARTICLE INFO

Keywords:

Testosterone
Radiofrequency fields
CaMKI
CaMKI/ROR α
Intracellular Ca²⁺

ABSTRACT

Exposure to radiofrequency fields (RF) has been reported to induce adverse effects on testosterone production and its daily rhythm. However, the mechanisms underneath this effect remain unknown. In this study, male mice were exposed to 1800 MHz radiofrequency fields (RF, 40 μ W/cm² power intensity and 0.0553 W/Kg SAR) 2 h per day for 32 days. The data suggested that RF exposure: (i) significantly reduced testosterone levels, (ii) altered the expression of genes involved in its synthesis (*Star*, *P450scc*, *P450c17* and *β -Hsd*) in testicular tissue, (iii) significantly reduced regulatory protein CaMKI/ROR α . Similar observations were also made in cultured primary Leydig cells exposed *in vitro* to RF. However, all of these observations were blocked by CaMK inhibitor, KN-93, and ionomycin reversed the down-regulation effects on intracellular [Ca²⁺]_i and CaMKI/ROR α expression induced by RF exposure. Thus, the data provided the evidence that RF-induced inhibition of testosterone synthesis might be mediated through CaMKI/ROR α signaling pathway.

Capsule: CaMKI/ROR α signaling pathway was involved in the inhibition of testosterone synthesis induced by RF exposure.

1. Introduction

Exposure to some environmental agents as well as life style has been known to affect male reproduction in humans [1–3]. Among the environmental risk factors, exposure to radiofrequency fields (RF) emitted from mobile phones has become a major concern because of their extensive use during the last few decades [4,5]. RF at frequencies between 400–900 MHz in analog system, and 1800–2200 MHz (digital system) are widely applied for mobile communication devices [6,7]. In humans, male reproductive organ (testis) has been regarded as one of the major organs for RF exposure since usually carry mobile phones in trousers pockets or in waist-bands. The observations made in our earlier studies in rats indicated ‘potentially’ adverse effect of RF exposure on male reproductive functions which included alterations in circadian rhythm and male hormone testosterone [8,9].

Circadian rhythm is regulated by clock genes through a series of clock gene proteins among which BMAL1 is regarded as an essential component of the circadian pacemaker in mammals that regulates the rate-limiting step of steroidogenesis by enhancing the transcription of steroid acute regulatory protein (Star) gene [10]. Other critical

members of the core clock gene machinery are the retinoid-related receptor alpha, ROR α and the clock gene *Bmal1* promoter which contains ROR response element (RORE) [11].

Exposure to various frequencies of RF were reported to evoke a number of alterations at cellular level, including increased Ca²⁺ efflux or Ca²⁺ influx [12,13]. Both Ca²⁺ and cyclic-AMP-dependent pathways are shown to be involved in maximum steroidogenesis induced by Luteinizing hormone (LH) [14,15]. At sub-maximal rates of testosterone production, ionized calcium (Ca²⁺) is the second messenger acting as a mediator for Ca²⁺ sensing protein calmodulin (CaM). When the intracellular Ca²⁺ levels are increased by the release from intracellular storage sites, opening of Ca²⁺-permeable receptors or voltage gated channels, CaM is shown to be activated and stimulated Ca²⁺/CaM-dependent kinases (CaMK) [16]. Among the members of the CaMK family, Ca²⁺/CaM-dependent protein kinase I and IV (CaMK I and IV) activates the transcription of clock gene *Rora* and, Ca²⁺/CaM-independent CaMKII α may inactivate ROR α transcription to repress its constitutive activity [17].

Thus far, there have been no reports on the role played by ROR α associated calcium signaling pathways in response to RF exposure

* Corresponding authors.

E-mail addresses: qinfenju@usts.edu.cn (F. Qin), tongjian@suda.edu.cn (J. Tong).

<https://doi.org/10.1016/j.reprotox.2018.08.014>

Received 17 January 2018; Received in revised form 14 August 2018; Accepted 16 August 2018

Available online 17 August 2018

0890-6238/© 2018 Elsevier Inc. All rights reserved.

emitted from mobile phones. In this study, mice were exposed to RF and its effect was investigated in testis, synthesis of testosterone by Leydig cells and, the role of CaMKI/ROR α signaling pathway to elucidate underlying molecular mechanisms in reproductive health.

2. Materials and methods

2.1. In vivo experiments

2.1.1. Animal handling

Six week old C57BL/6J male mice were obtained from Soochow University's Laboratory Animal Centre, Suzhou, PR China. All experiments were conducted according to the guidelines of IACUC (Institutional Animal Care and Use Committee), Soochow University, China. They were housed under conventional conditions with 12:12 h light (7:00 a.m. to 19:00 p.m.) and dark cycles. The temperature and the relative humidity in the laboratory were kept at $25 \pm 2^\circ\text{C}$ and $50 \pm 5\%$, respectively. The diet and water were provided *ad libitum*. After acclimatization for 4 weeks, mice (26 ± 2 g) and showing circadian rhythmicity in locomotor activity were randomly divided into four groups of six animals each [18]: unexposed controls, 1800 MHz RF exposure at $40 \mu\text{W}/\text{cm}^2$ power intensity for 1 h/day or for 2 h/day for 32 days (starting at 7:00 AM) and sham-exposed (SH, without RF transmission). After the last exposure, all mice were killed by decapitation. From each mouse, testes were collected and stored at -80°C . One testicle was homogenized in normal saline (0.9% sodium chloride), centrifuged and the concentration of testosterone was determined in the supernatant. Another testicular tissue was used to extract RNA for Real-time PCR or Western blotting analyses.

2.1.2. RF exposure and dosimetry

The RF exposure system consisted of a GTEM chamber, signal generator and power amplifier (Fig. 1 a). Continuous wave 1800 MHz RF signal was generated, amplified and transmitted into the GTEM

chamber through an antenna (Southeast University, Nanjing, China). A PMM field strength meter (Cisano sul Neva, Savona, Italy) was used to measure the $40 \mu\text{W}/\text{cm}^2$ power intensity in the GTEM chamber where the animals will be kept for RF or SH exposure. A computer logging system ensured the uniformity of power density by controlling and monitoring the field strength data. The specific absorption rate (SAR) was calculated using a 3D male mouse model and SPEAG Sim4life V3.0 electromagnetic simulation software (IT'IS Foundation, Zürich, Switzerland). At the input power intensity of $0.001327 \text{ W}/\text{m}^2$, the IEEE/IEC62704-1 average specific absorption rate (SAR) distribution on the top view of slice was obtained by the FDTD analysis (Fig. 1b). As a result of the input power differences, the calculation was corrected to re-calculate the real mass-averaged SAR value according to the Eq. (1) as shown following [19].

$$\frac{SAR_E}{SAR_s} = \frac{P_E}{P_s} \times \frac{W_s}{W_E} \quad (1)$$

While SARs is the mass-averaged SAR calculated by simulation in Sim4life, P_s represents the input power density and W_s stands for the weight all for the 3D mouse model. SAR_E shows mouse's mass-averaged SAR value in the experiment, while W_E and P_E represents the mean weight of mouse exposed to RF and input power density, respectively. As calculated by Eq. (1), the approximate SAR_E was $0.0553 \text{ W}/\text{Kg}$.

2.2. In vitro experiments

2.2.1. Isolation, purification and culturing of mouse Leydig cells

The primary Leydig cells were collected from an adult C57BL/6J mouse which showed circadian rhythmicity in locomotor activity. Briefly, the testes were collected, minced and digested with collagenase ($0.5 \text{ mg}/\text{ml}$ in PBS) under 100 times/min agitation for 15 min at 37°C until seminiferous tubules resulted were isolated but not broken. Then, the solution was passed through medium size mesh filters and centrifuged ($800 \times g$) for 10 min at 4°C . The supernatant was discarded and cells were resuspended in DMEM/F12 medium and centrifuged again and centrifuged for 45 min at $25,000 \times g$ at 4°C . The cell pellet containing Leydig cells was purified by separation through 50% Percoll solution (Sigma-Aldrich, Shanghai, China; density: $1.069\text{--}1.074 \text{ g}/\text{ml}$). The enriched Leydig cells washed and counted by a hemocytometer. The purity of Leydig cells in the preparation was determined by the 3 β -hydroxysteroid dehydrogenase (3 β -HSD) staining method [20] and found to be 96%. Separate cultures were set up in petri dishes each having approximately 1×10^6 Leydig cells in 2 ml of DMEM/F-12 medium containing horse serum (5%) and fetal bovine serum (5%). The cells were cultured in an incubator (Thermal Forma, Rockford, USA) maintaining $37 \pm 0.5^\circ\text{C}$, 5% CO_2 and 95% O_2 .

2.2.2. RF exposure and dosimetry

The cells at 45–55% confluency were pre-treated with 20 mIU/ml LH [21] for 30 min, and then exposed to 1800 MHz RF exposure at $40 \mu\text{W}/\text{cm}^2$ power density with or without the CaMK inhibitor, KN-93 ($30 \mu\text{M}$) or without the Ca^{2+} ionophore ionomycin ($1 \mu\text{M}$) for 2 h per day for three days. The calculated averaged SAR distribution inside the culture medium was $0.046 \text{ W}/\text{kg}$ (Fig. 2).

2.2.3. Testosterone measurement

The concentrations of testosterone in the homogenate of testicular tissue as well as in primary Leydig cell culture medium were determined by the Spectra Testosterone Radioimmunoassay (RIA) kit with sensitivity of $0.1 \text{ nmol}/\text{L}$ (Orion Diagnostica, Espoo, Finland) after diethyl ether extraction. The procedure was carried out according to the manufacturer's protocols. Duplicate samples were used for each assay and repeated three times.

2.2.4. Real-time PCR

The total RNA from testis and Leydig cells was extracted using RNA

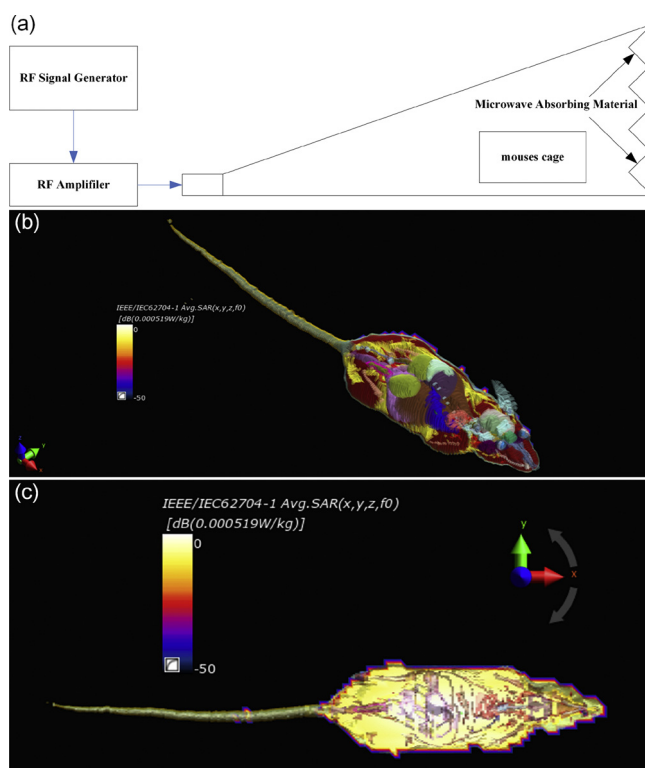


Fig. 1. (a) Schematic diagram of radiofrequency field exposure system; (b) Phantom male mouse and its SAR distribute exposure to 1800 MHz RF in on the top slice.

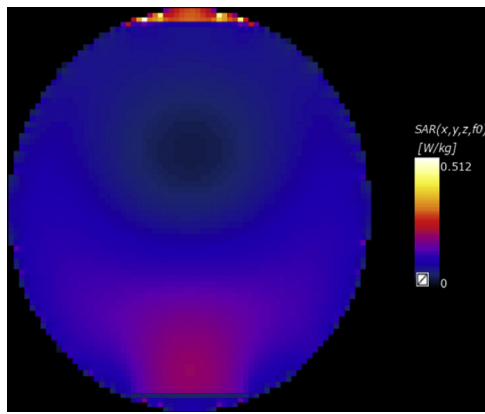


Fig. 2. SAR distribution inside the culture medium hosted in a 35-mm standard Petri dish exposed to 1800 MHz RF.

Table 1
Sequences of primers for Real-time PCR.

Genes	Sequences of primers
Camk1	Sense: 5'- TTGCTGGTCCATAGGAGTCATTG -3' Antisense: 5'- GTCGTCCCAATAAGGAGAGTCAA -3'
Rora	Sense: 5'- TCCCACCTGGAACCTGCCAGT -3' Antisense: 5'- CCACGAGCGATCCGCTGACA -3'
Star	Sense: 5'-AAAGCCAGCAGGAGAACGGGGA-3' Antisense: 5'-GCCTCCATGCGGTCCACAAGTT-3'
P450scc	Sense: 5'- CTGCCTGGGATGTGATTTTCA-3' Antisense: 5'- GTAATGTTGGCCTGGATGTTCT-3'
3β-Hsd	Sense: 5'-GCGGCTGCTGCACAGGAATA-3' Antisense: 5'-GACGCATGCTGCTTCGTGA-3'
P450c17	Sense: 5'- GATCGGTTTATGCCTGAGCG -3' Antisense: 5'- TCCGAAGGGCAAATAACTGG -3'
β-Actin	Sense: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' Antisense: 5'-TAAACGCAGCTCAGTAACAGTCCG-3'

extraction kit (Hoffmann-La Roche Ltd, Basel, Switzerland) and the quality was determined using BioPhotometer (Analytik Jena AG, Jena, Germany). From each sample, 1 µg RNA was reversely transcribed using the Invitrogen reverse transcriptase Kit M-MLV (Life Technologies, Carlsbad, USA). The cDNA sample thus obtained was mixed with FastStart SYBR Green Master (Hoffmann-La Roche Ltd., Basel, Switzerland) to which 10 pmol/l primers were added to prepare the mixture for RT-PCR (7500 Applied Biosystems, Carlsbad, USA). Primers were designed and synthesized by Invitrogen (Life Technologies Corporation, Shanghai, China) and the forward and reverse sequences are presented in Table 1. All values were normalized to β-actin (a mice housekeeping gene). The fold change in mRNA was calculated by the $\Delta\Delta C_t$ method (fold = $2^{\Delta\Delta C_t}$). All samples were tested in duplicates.

2.2.5. Western blotting

Proteins from testicular tissue and primary Leydig cells were extracted utilizing Protein Extraction Kit (Merck KGaA, Darmstadt, Germany) which contained a protease inhibitor [22]. The following primary antibodies were used: monoclonal antibody actin of rabbit (MilliporeSigma, Darmstadt, Germany, Catalog# 1501) (1:1000), monoclonal antibody CamKI of rabbit (Epitomics, Burlingame, USA, Catalog# 2331-1) (dilution, 1:800), polyclonal antibody RARα of rabbit (Santa Cruz Biotech, Santa Cruz, USA, Catalog# sc-551) (dilution, 1:200), monoclonal antibody P450c17 of rabbit (Abcam, Cambridge, UK) (dilution, 1:1000), monoclonal antibody StAR of rabbit (Cell Signaling, Boston, USA, Catalog #8449) (dilution, 1:600), polyclonal P450scc antibody of rabbit (Santa Cruz Biotech, Santa Cruz, USA, Catalog # sc-292456) (dilution, 1:200), or monoclonal antibody 3β-HSD1 of Rabbit (Epitomics, Burlingame, USA, Catalog# 7029-1)

(dilution, 1:800), secondary antibody conjugated with peroxidase (Jackson ImmunoResearch, West Grove, USA, Catalog#115-035-003) (1:2000 goat anti-rabbit IgG). AlphaEase FC software (Cell Biosciences, USA) was used to collect the Western blot scan images, and the target protein expression was normalized and presented as relative gray ratio to the beta-actin. All samples were tested in duplicates.

2.2.6. Cytosolic calcium measurement

The calcium sensitive fluorescent dye, Fluo-8/AM (AAT Bioquest, Sunnyvale, USA) was used to determine the intracellular calcium concentration ([Ca²⁺]_i) in Leydig cells [23]. Cells were harvested after LH/RF or the Ca²⁺ ionophore ionomycin (1 µM) treatment, washed twice with phosphate-buffered saline (PBS), and then incubated with 5 µM Fluo-8/AM in culture medium at 37 °C in the dark. After 45 min, the cells were washed twice to remove excess dye and re suspended in PBS. In each sample, the fluorescence intensity of the dye in cells was measured using a flow cytometer (BD FACSCalibur, Franklin Lakes, USA) at excitation wavelength, 490 nm and emission wavelength, 514 nm. Three independent experiments were conducted.

2.2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to ascertain differences between control/SH-/RF-exposed mice and Leydig /cells. When appropriate, least square significant difference (LSD) test was carried out. A statistical *p* value of < 0.05 between 2 groups was indicative of significant difference. The data presented are mean ± SD.

3. Results

3.1. In vivo experiments

The testosterone levels in controls, Sham- and RF-exposed mice are presented in Fig. 3. The data showed a significant decrease (19.25%) in mice exposed to RF compared with Sham-exposed mice (*p* < 0.05). There was a further reduction (34.79%) in testosterone levels in mice exposed to RF for 2 h (*p* < 0.01). There was no difference between unexposed controls and Sham-exposed mice (*p* > 0.05).

The expressions of *Star*, *P450scc*, *3β-Hsd* and *P450c17* genes involved in testosterone synthesis in control, Sham- and RF-exposed mice from Real-time PCR detection and Western blotting analyses is shown in Fig. 4a–f, respectively. The levels of them were significantly lower in RF-exposed mice (1 h and 2 h) compared with Sham-exposed animals (*p* < 0.01). The decreases were more pronounced after 2 h RF

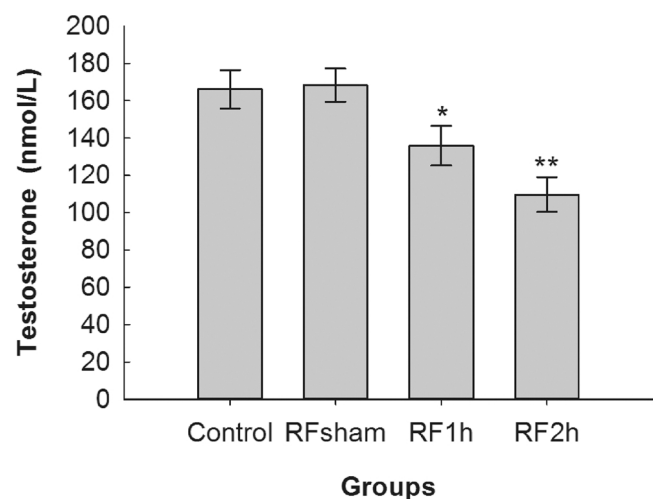


Fig. 3. The levels of testosterone in testis homogenate of mice after 1 h or 2 h RF exposure. The results of RF groups significantly differ with that of the Sham group. Each data point stands for the mean value ± SD of 6 mice for each group. * *p* < 0.05, ** *p* < 0.01.

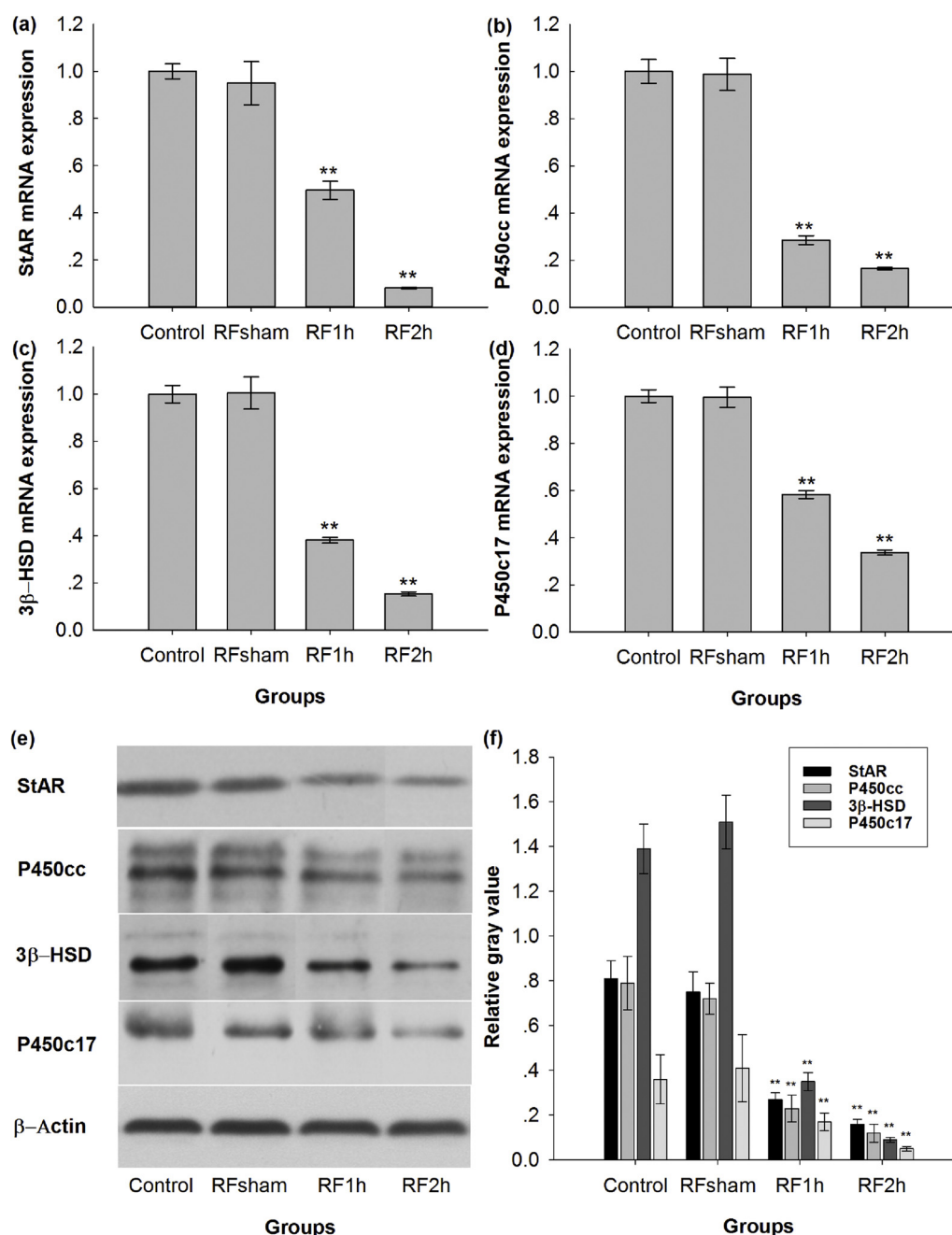


Fig. 4. The expression levels of StAR, P450cc, 3β-HSD, and P450c17 in mice testis after 1 h or 2 h RF exposure as from Real-time PCR (a, b, c, d) and Western blot (e, f). The results of RF groups significantly differ with that of the Sham group. Each data point stands for the mean value $\pm$ SD of 6 mice for each group. ** $p < 0.01$.

exposure (91.43% vs 47.80%; 83.34% vs 71.12%; 84.76 vs 62.11; 66.07 vs 41.40%). There was a positive correlation between mRNA expression levels and protein levels of all these genes.

The mRNA expressions of *CamkI* and *Rora* decreased significantly in RF-exposed mice compared with Sham-exposed animals (Fig. 5 a, b) ($p < 0.01$). There was no difference in mRNA expression between the Sham-exposed and controls. The variations in protein expression are similar to those in gene expression (Fig. 5 c, d).

3.2. In vitro experiments

The observations made in cultured primary Leydig cells exposed to RF were essentially similar to those made *in vivo* exposure experiments in mice.

We explored a potential role for CaMKI-associated changes in the

testosterone synthesis induced by RF exposure using primary Leydig cells. There was a significant decrease in testosterone concentration in Leydig cells exposed to RF for 2 h (Fig. 6) ($p < 0.01$). Treatment with CaMK inhibitor, 30 μ M KN-93, was able to reverse the decreasing effect (KN-93 + RF vs. RF: $p < 0.01$).

The mRNA expression of *CamkI* and *Rora* in cells exposed to RF for 2 h was significantly lower as compared to those in Sham-exposed cells indicating their downregulation ($p < 0.01$) (Fig. 7). Treatment with 30 μ M KN-93 or the Ca^{2+} ionophore ionomycin (1 μ M) reversed the effect and the levels returned to those in Sham-exposed cells (KN-93 or ionomycin + RF2h vs. RF: $p < 0.01$ for *CamkI* and $p < 0.05$ or $p < 0.01$ for *Rora*, respectively). Similar changes were also observed on protein levels of CaMKI and ROR α which were positively correlated with the changes in gene expression.

The $[\text{Ca}^{2+}]_i$ concentration in control and Sham-exposed Leydig

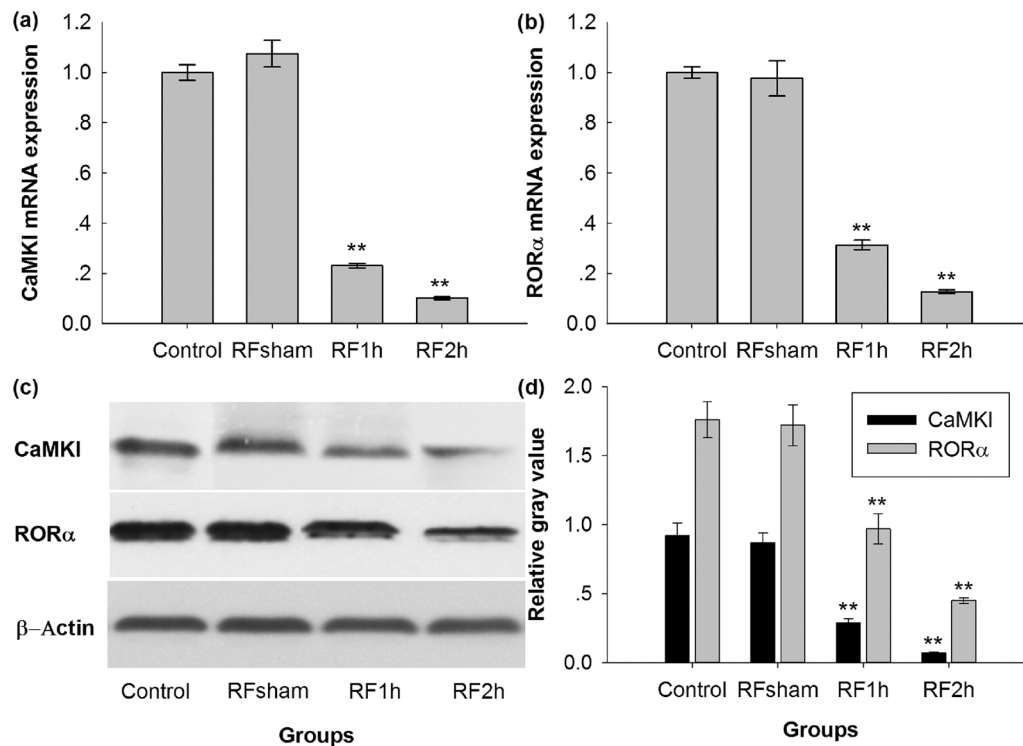


Fig. 5. Expression levels of CaMKI and RORα in the testis of mice exposed to RF 1 h or 2 h as from Real-time PCR (a, b) and Western blot (c, d). The results of RF groups significantly differ with that of the Sham group. Each data point stands for the mean value $\pm$ SD of 6 mice for each group. ** $p < 0.01$.

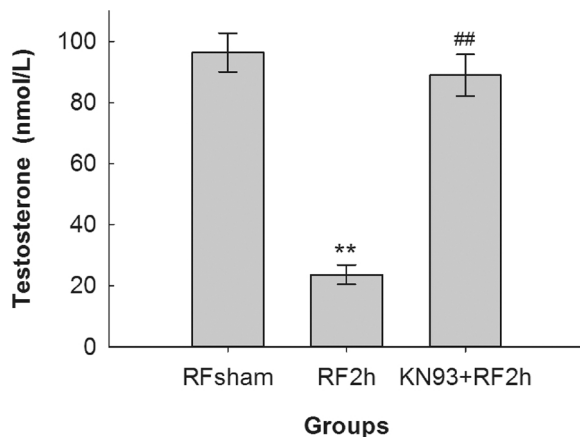


Fig. 6. Testosterone levels in primary Leydig cells medium following 2 h RF exposure, and restoration of levels after treatment with KN-93 (CaMK inhibitor, 30 μ M). Each data point stand for the mean value $\pm$ SD of three alone experiments. The results of RF groups significantly differ with that of the Sham group, ** $p < 0.01$; as was the KN-93 + RF group compared with the RF group, ## $p < 0.01$.

cells were dramatically increased after treatment with LH ($p < 0.01$). Decreased effect was observed even in cells exposed to RF for 2 h and the ionomycin (1 μ M) reversed this change ($p < 0.01$) (Fig. 8).

4. Discussion

Testosterone plays a significant role in maintaining structural and functional integrity of male reproductive organs [24]. As for the effects of RF exposure on male sex hormone testosterone, some studies reported decreased levels in rabbits and rats [25,26] while others claimed an increase in mice and humans [27,28]. The observations made in our previous investigation indicated a decreased daily average level of testosterone as well as alteration in its daily rhythmicity [8].

In mammals, a few clock genes in mouse Leydig cells are reported to be involved in testosterone synthesis. For example, testosterone levels in the serum were found to be decreased in null mice for *Bmal1* or *Per2* or double mutant with *Per1/Per2* [29]. At the cellular level, the regulation of the circadian clock is accomplished by the transcriptional and translational feedback loop. In the feedback loop, *Bmal1* can directly enhance the transcription of the steroidogenic acute regulatory protein (Star) which regulates the pivotal rate-limiting step in testosterone synthesis [30]. Star protein is responsible for the transport of cholesterol from outside to the inside of mitochondrial membrane where the first enzymatic reaction occurs in steroidogenesis [31]. On the other hand, circulating levels of cytochrome P450 (CYP) genes, *P450scc* and *P450c17*, are also reportedly involved in the circadian steroidogenesis [32]. It has been known for many years that biological clock regulates the expression of cytochrome P450 gene family members [33] in which Cyp11 family including *P450scc* is closely correlated with 3 β -HSD protein level [34]. Testosterone levels can be changed by clock gene by regulating the genes in testosterone synthesis, such as *Star*, *P450scc*, 3 β -Hsd and *P450c17*, as observed in the present study.

Inside the core clock machinery, RORα has been reported to regulate the lipid and glucose metabolism. ROR response elements (ROREs) have been identified in several clock genes and these include *Bmal1*, *Clock*, and *Cry1* [35–37]. The *Bmal1* gene contains two ROREs in its promoter region and mutations in ROREs can abolish the circadian rhythmicity of the *Bmal1* promoter activation, indicating that ROREs are essential for rhythmic transcriptional regulation of *Bmal1* [38]. The results obtained in this study suggested that the clock gene *Rora*, together with some steroidogenic genes, viz., *Star*, *P450scc*, 3 β -hsd and *P450c17*, were all down-regulated in mice and cultured primary Leydig cells exposed to 1800 MHz RF exposure. All these data pointed towards the RF-induced inhibitory effect on testosterone biosynthesis mediated by the RORα- Star-P450 Pathway.

Thus far, there have been no reports showing a link between RF exposure and RORα pathway. Phosphorylation is the key in the regulation of nuclear receptor clock protein RORα activity. As an

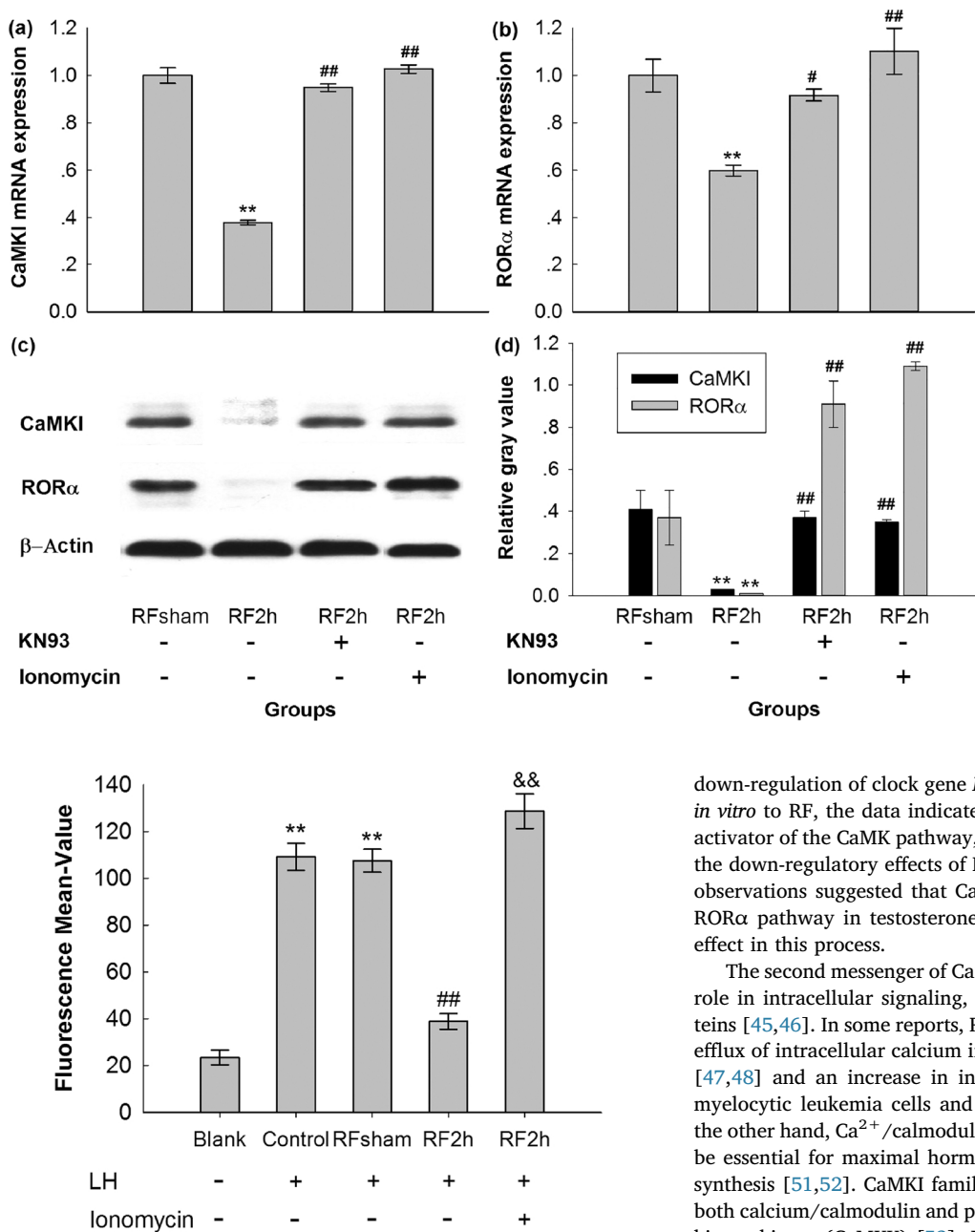


Fig. 8. RF 2 h exposure weakened the increase of Ca^{2+} concentration caused by LH treatment, and ionomycin reversed the effect of RF 2 h on Ca^{2+} concentration. Each data point stands for the Fluorescence of three independent experiments expressed as mean value $\pm$ SD. The Control and Sham groups significantly differ with respect to the Blank group (** $p < 0.01$); the results of RF 2 h group was significantly lower than that of the Sham group (## $p < 0.01$); Ionomycin + RF 2 h group increased compared to the RF 2 h group (&& $p < 0.01$).

important member of CaMK family, CaMKI has been shown to phosphorylate protein kinase and thus, plays a role in the circadian clock as a link between the core master clock and the peripheral circadian rhythm [39]. CaMK family members can also modulate the center clock genes by phosphorylating the circadian clock protein and then, reset the circadian clock [40–42]. The mammalian CaMKI has been implicated in the steroid hormone synthesis which include the transcriptional activation of genes coding for steroidogenic enzymes [43,44]. In this study, the results obtained in RF-exposed mice showed a significant decrease in *CamkI* gene expression and protein with a concomitant

Fig. 7. Reduced levels of CaMKI and RORα gene (a, b) and protein (c, d) expression of primary Leydig cells after RF exposure 2 h, and restoration of levels after treatment with 30 μM KN-93 or 1 μM ionomycin. Each data point stands for three alone experiments expressed as mean value $\pm$ SD. The results of RF groups significantly differ with the Sham group, ** $p < 0.01$; as was the KN-93 + RF or Ionomycin + RF group compared with the RF group, # $p < 0.05$, ## $p < 0.01$.

down-regulation of clock gene *Rora* expression. In Leydig cells exposed *in vitro* to RF, the data indicated that CaMK inhibitor, KN-93, and the activator of the CaMK pathway, ionomycin was able to block or reverse the down-regulatory effects of RORα expression. Taken together, these observations suggested that CaMKI was involved in the regulation of RORα pathway in testosterone biosynthesis and RF exposure had an effect in this process.

The second messenger of Ca^{2+} has been known to play an important role in intracellular signaling, gene expression and regulation of proteins [45,46]. In some reports, RF exposure has been found to induce an efflux of intracellular calcium in avian, feline, rats and human neurons [47,48] and an increase in intracellular calcium ion in human promyelocytic leukemia cells and rat hippocampus neurons [49,50]. On the other hand, Ca^{2+} /calmodulin kinase I (CaMKI) has been reported to be essential for maximal hormone responsiveness in testosterone biosynthesis [51,52]. CaMKI family was reported to be fully activated by both calcium/calmodulin and phosphorylation by the Ca^{2+} /calmodulin kinase kinase (CaMKK) [53]. Increased intracellular Ca^{2+} concentration can activate CaMKK, which in turn leads to activation of CaMKI [54]. In the present study, 1800 MHz RF exposure markedly decreased the intracellular $[\text{Ca}^{2+}]_i$ and down-regulated CaMKI expression in Leydig cells. The Ca^{2+} ionophore ionomycin, the activator of the CaMK pathway can reverse the decrease. This reducing effect was positively correlated that of RORα-StAR-P450 Pathway. Together, these results provided a tentative hypothesis that the RF-induced inhibition of testosterone biosynthesis was brought about via the Ca^{2+} /CaMKI/ RORα pathway, as presented in Fig. 9.

It is important to note that the results from RF-exposed mice *in vivo* and RF-exposed Leydig cell *in vitro* cannot be compared directly since the SAR values were different (*in vivo*, 0.0553 W/Kg; *in vitro*, 0.046 W/Kg). However, both SARs are lower than the exposure limits recommended by the International Commission for Non-Ionizing Radiation Protection ICNIRP (the general public, 0.08 W/kg; occupational exposure, 0.4 W/kg) [55–57]. Therefore, the results obtained in the study can be considered as non-thermal effect of RF exposure. Meanwhile, the results from this study present the public with precaution – no keeping connected wireless devices in close proximity of

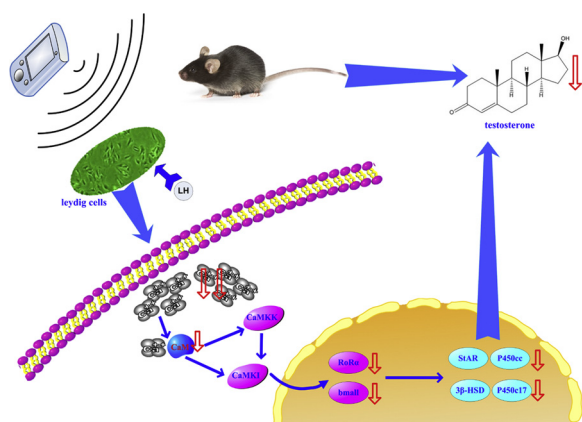


Fig. 9. Proposed hypothesis of CaMKI/ROR α pathway in the regulation of testosterone production by RF exposure.

male gonads.

5. Conclusion

In conclusion, our study provided some evidence that 1800 MHz RF exposure may first disturb Ca^{2+} /CaMKI turnover in Leydig cells and then, inactivate the clock gene ROR α which in turn, down-regulates its target genes involved in testosterone synthesis, including *Star*, *P450sc*, *3β-Hsd* and *P450c17*. All these changes might have resulted in reduced testosterone production.

Declaration of conflict of interest statement

All the authors indicate that there are no potential conflicts of interests.

Acknowledgements

The funding for this study was supported by the National Natural Science Foundation of China (81773463, 81741109), the China Postdoctoral Science Foundation (2016M601883), the Jiangsu Key Lab of senile disease prevention and translational medicine, the Suzhou Science and Technology Development project (SNG2017055), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

References

- [1] M.E. Parsanezhad, S. Mortazavi, T. Doohandeh, B.N. Jahromi, H. Mozdarani, A. Zarei, M. Davari, S. Amjadi, A. Soleimani, M. Haghighi, Exposure to radio-frequency radiation emitted from mobile phone jammers adversely affects the quality of human sperm, *Iran. J. Radiat. Res.* 15 (2017) 63–70.
- [2] N.E. Skakkebaek, A brief review of the link between environment and male reproductive health: lessons from studies of testicular germ cell Cancer, *Horm. Res. Paediatr.* 86 (2016) 240–246.
- [3] A. Agarwal, D. Durairajanayagam, Chapter 13 - Lifestyle Factors and Reproductive Health[M]// Handbook of Fertility, Elsevier Inc, 2015, pp. 145–157.
- [4] International Telecommunication Union, The World in 2016: ICT Facts and Figures, (2016) <http://www.itu.int/en/ITU-D/Statistics/Documents/facts/ICTFactsFigures2016.pdf>.
- [5] G. Zhang, H. Yan, Q. Chen, K. Liu, X. Ling, L. Sun, N. Zhou, Z. Wang, P. Zou, X. Wang, L. Tan, Z. Cui, Z. Zhou, J. Liu, L. Ao, J. Cao, Effects of cell phone use on semen parameters: results from the MARHCS cohort study in Chongqing, China, *Environ. Int.* 91 (2016) 116–121.
- [6] A. Ahlbom, A. Green, L. Kheifets, D. Savitz, A. Swerdlow, Epidemiology of health effects of radiofrequency exposure, *Environ. Health Perspect.* 112 (2004) 1741–1754.
- [7] D. Maskey, J. Pradhan, B. Aryal, C.M. Lee, I.Y. Choi, K.S. Park, S.B. Kim, H.G. Kim, M.J. Kim, Chronic 835-MHz radiofrequency exposure to mice hippocampus alters the distribution of calbindin and GFAP immunoreactivity, *Brain Res.* 1346 (2010) 237–246.
- [8] F.J. Qin, J. Zhang, H. Cao, W. Guo, L. Chen, O. Shen, J. Sun, C. Yi, J. Li, J. Wang, J. Tong, Circadian alterations of reproductive functional markers in male rats exposed to 1800 MHz radiofrequency field, *Chronobiol. Int.* 31 (2014) 123–133.
- [9] F.J. Qin, J. Zhang, H. Cao, C. Yi, J.X. Li, J. Nie, L.L. Chen, J.J. Wang, J. Tong, Effects of 1800-MHz radiofrequency fields on circadian rhythm of plasma melatonin and testosterone in male rats, *J. Toxicol. Environ. Health A* 75 (2012) 1120–1128.
- [10] J.D. Alvarez, A. Hansen, T. Ord, P. Bebas, P.E. Chappell, J.M. Giebultowicz, C. Williams, S. Moss, A. Sehgal, The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice, *J. Biol. Rhythms* 23 (2008) 26–36.
- [11] H.S. Ranchor, Orphan nuclear receptors: current perspectives, *J. Recept. Ligand Channel Res.* 6 (2013) 15–25.
- [12] K.K. Kunjilwar, J. Behari, Effect of amplitude-modulated radio frequency radiation on cholinergic system of developing rats, *Brain Res.* 601 (1993) 321–324.
- [13] M. Naziroglu, B. Cig, S. Dogan, A.C. Uguz, S. Dilek, D. Faouzi, 2.45-Gz wireless devices induce oxidative stress and proliferation through cytosolic Ca^{2+} influx in human leukemia cancer cells, *Int. J. Radiat. Biol.* 88 (2012) 449–456.
- [14] H.S. Abdou, N.M. Robert, J.J. Tremblay, Calcium-dependent Nr4a1 expression in mouse Leydig cells requires distinct AP1/CRE and MEF2 elements, *J. Mol. Endocrinol.* 56 (2016) 151–161.
- [15] M.H. Sullivan, B.A. Cooke, The role of Ca^{2+} in steroidogenesis in Leydig cells. Stimulation of intracellular free Ca^{2+} by lutropin (LH), luteal phase (LHRH) agonist and cyclic AMP, *Biochem. J.* 236 (1986) 45–51.
- [16] S. Sackmann, U. Lichtenauer, I. Shapiro, M. Reincke, F. Beuschlein, Aldosterone producing adrenal adenomas are characterized by activation of calcium/calmodulin-dependent protein kinase (CaMK) dependent pathways, *Horm. Metab. Res.* 43 (2011) 106–111.
- [17] C.D. Kane, A.R. Means, Activation of orphan receptor-mediated transcription by Ca^{2+} /calmodulin-dependent protein kinase IV, *EMBO J.* 19 (2000) 691–701.
- [18] L.L. Chen, F.J. Qin, Y.Z. Chen, J.P. Sun, J. Tong, Chronotoxicity of 1800 MHz microwave radiation on sex hormones and spermatogenesis in male mice, *Wei Sheng Yan Jiu* 43 (2014) 110–115 Chinese.
- [19] H.L. Cao, F.J. Qin, X.G. Liu, J.J. Wang, Y. Cao, J. Tong, H.M. Zhao, Circadian rhythmicity of antioxidant markers in rats exposed to 1.8 GHz radiofrequency fields, *Int. J. Environ. Res. Public Health* 12 (2015) 2071–2087.
- [20] S.O. Abarikwu, E.O. Farombi, M. Kashyap, A.B. Pant, Atrazine induces transcriptional change in steroidogenesis marker genes in primary cultures of rat Leydig cells, *Toxicol. Vitro* 25 (2011) 1588–1595.
- [21] S. Valenti, L. Fazzuolm, G. Giordano, M. Giusti, Changes in binding of iodomelatonin to membranes of Leydig cells and steroidogenesis after prolonged in vitro exposure to melatonin, *Int. J. Androl.* 24 (2001) 80–86.
- [22] F.J. Qin, J. Zhang, L. Zan, W. Guo, J. Wang, L. Chen, Y. Cao, O.X. She, J. Tong, Inhibitory effect of melatonin on testosterone synthesis is mediated by GATA-4/SF-1 transcription factors, *Reprod. Biomed. Online* 11 (2015) 638–646.
- [23] L. Jiang, J. Zhong, X. Dou, C. Cheng, Z. Huang, X. Sun, Effects of ApoE on intracellular calcium levels and apoptosis of neurons after mechanical injury, *Neuroscience* 301 (2015) 375–383.
- [24] J.E. Thistle, B.I. Graubard, M. Braunlin, H. Vesper, B. Trabert, M.B. Cook, K.A. McGlynn, Marijuana use and serum testosterone concentrations among U.S. Males, *Andrology* 5 (2017) 732.
- [25] M.R. Sarookhani, M.A. Rezaei, A. Safari, V. Zaroushani, M. Ziaeiha, The influence of 950 MHz magnetic field (mobile phone radiation) on sex organ and adrenal functions of male rabbits, *Afr. J. Biochem. Res.* 5 (2011) 65–68.
- [26] I. Sehitoglu, L. Tumkaya, Y. Kalkan, R. Bedir, M.C. Cure, O.U. Zorba, E. Cure, S. Yuce, Biochemical and histopathological effects on the rat testis after exposure to electromagnetic field during fetal period, *Arch. Esp. Urol.* 68 (2015) 562–568.
- [27] Z. Forgacs, Z. Somosi, G. Kubinyi, J. Bakos, A. Hudak, A. Surjan, G. Thuroczy, Effect of whole-body 1800MHz GSM-like microwave exposure on testicular steroidogenesis and histology in mice, *Reprod. Toxicol.* 22 (2006) 111–117.
- [28] T. Gutsch, Al-Ali. B. Mohamad, R. Shamloul, K. Pummer, H. Trummer, Impact of cell phone use on men's semen parameters, *Andrologia* 43 (2011) 312–316.
- [29] D.J. Kennaway, M.J. Boden, T.J. Varcoe, Circadian rhythms and fertility, *Mol. Cell. Endocrinol.* 349 (2012) 56–61.
- [30] H. Chen, L. Zhao, M. Kumazawa, N. Yamauchi, Y. Shigeyoshi, S. Hashimoto, M.A. Hattori, Downregulation of core clock gene Bmal1 attenuates expression of progesterone and prostaglandin biosynthesis-related genes in rat luteinizing granulosa cells, *Am. J. Physiol. Cell Physiol.* 304 (2013) C1131–40.
- [31] F. Raucii, A. D'Aniello, M.M. Di Fiore, Stimulation of androgen production by D-aspartate through the enhancement of StAR, P450sc and 3β-HSD mRNA levels in vivo rat testis and in culture of immature rat Leydig cells, *Steroids* 84 (2014) 103–110.
- [32] R. Kofir, U.P. Zmrzljak, T. Bele, J. Acimovic, M. Perse, G. Majdic, C. Prehn, J. Adamski, D. Rozman, Circadian expression of steroidogenic cytochromes P450 in the mouse adrenal gland—Involvement of cAMP-responsive element modulator in epigenetic regulation of Cyp17a1, *FEBS J.* 279 (2012) 1584–1593.
- [33] O. Froy, Cytochrome p450 and the biological clock in mammals, *Curr. Drug Metab.* 10 (2009) 104–115.
- [34] S. Konosu-Fukaya, Y. Nakamura, F. Satoh, S.J. Felizola, T. Maekawa, Y. Ono, R. Morimoto, K. Ise, K. Takeda, K. Katsu, F. Fujishima, A. Kasajima, M. Watanabe, Y. Arai, E.P. Gomez-Sanchez, C.E. Gomez-Sanchez, M. Doi, H. Okamura, H. Sasano, 3β-Hydroxysteroid dehydrogenase isoforms in human aldosterone-producing adenoma, *Mol. Cell. Endocrinol.* 408 (2015) 205–212.
- [35] D.N. Cook, H.S. Kang, A.M. Jetten, Retinoic acid-related orphan receptors (RORs): regulatory functions in immunity, development, circadian rhythm, and metabolism, *Nucl. Recept. Res.* 2 (2015) pii: 101185.
- [36] H. Duez, B. Staels, The nuclear receptors Rev-erbs and RORs integrate circadian rhythms and metabolism, *Diabetes Vasc. Dis. Res.* 5 (2008) 82–88.
- [37] J.S. Takahashi, Molecular components of the circadian clock in mammals, *Diabetes*

- Obes. Metab. 17 (SI) (2015) 6–11.
- [38] F. Guillaumond, H. Dardente, V. Giguère, N. Cermakian, Differential control of *Bmal1* circadian transcription by REV-ERB and ROR nuclear receptors, *J. Biol. Rhythms* 20 (2005) 391–403.
- [39] A. Tovin, S. Alon, Z. Ben-Moshe, P. Mracek, G. Vantine, N.S. Foulkes, J. Jacob-Hirsch, G. Rechavi, R. Toyama, S.L. Coon, D.C. Klein, E. Eisenberg, Y. Gothilf, Systematic identification of rhythmic genes reveals *camk1gb* as a new element in the circadian clockwork, *PLoS Genet.* 8 (2012) e1003116.
- [40] N. Kon, T. Yoshikawa, S. Honma, Y. Yamagata, H. Yoshitane, K. Shimizu, Y. Sugiyama, C. Hara, I. Kameshita, K. Honma, Y. Fukada, *CaMKII* is essential for the cellular clock and coupling between morning and evening behavioral rhythms, *Genes Dev.* 28 (2014) 1101–1110.
- [41] F. Weber, H.C. Hung, C. Maurer, S.A. Kay, Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE-dependent transcription, *J. Neurochem.* 98 (2006) 248–257.
- [42] M. Ermisch, B. Firla, D. Steinhilber, Protein kinase A activates and phosphorylates *ROR α 4* in vitro and takes part in *ROR α* activation by *CaMK-IV*, *Biochem. Biophys. Res. Commun.* 408 (2011) 442–446.
- [43] J.C. Condon, V. Pezzi, B.M. Drummond, S. Yin, W.E. Rainey, Calmodulin -dependent kinase I regulates adrenal cell expression of aldosterone synthase, *Endocrinology* 143 (2002) 3651–3657.
- [44] K. Nanba, A. Chen, K. Nishimoto, W.E. Rainey, Role of *Ca(2+)*/calmodulin-dependent protein kinase kinase in adrenal aldosterone production, *Endocrinology* 156 (2015) 1750–1756.
- [45] H. Plattner, A. Verkhratsky, The ancient roots of calcium signalling evolutionary tree, *Cell Calcium* 57 (2015) 123–132.
- [46] M.C. Pinto, A.H. Kihara, V.A. Goulart, F.M. Tonelli, K.N. Gomes, H. Ulrich, R.R. Resende, Calcium signaling and cell proliferation, *Cell Signal.* 27 (2015) 2139–2149.
- [47] S.K. Dutta, B. Ghosh, C.F. Blackman, Radiofrequency radiation-induced calcium ion efflux enhancement from human and other neuroblastoma cells in culture, *Bioelectromagnetics* 10 (1989) 197–202.
- [48] R. Paulraj, J. Behari, Biochemical changes in rat brain exposed to low intensity 9.9 GHz microwave radiation, *Cell Biochem. Biophys.* 63 (2012) 97–102.
- [49] Z. Jin, C. Zong, B. Jiang, Z. Zhou, J. Tong, Y. Cao, The effect of combined exposure of 900 MHz radiofrequency fields and doxorubicin in HL-60 cells, *PLoS One* 7 (2012) e46102.
- [50] V. Ghazizadeh, 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 (2014) 787–799.
- [51] L.J. Martin, N. Boucher, C. Brousseau, J.J. Tremblay, The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through co-operation with *Ca2+* /calmodulin-dependent protein kinase I, *Mol. Endocrinol.* 22 (2008) 2021–2037.
- [52] J.J. Tremblay, Molecular regulation of steroidogenesis in endocrine Leydig cells, *Steroids* 103 (2015) 3–10.
- [53] S. Takemoto-Kimura, H. Terai, M. Takamoto, S. Ohmae, S. Kikumura, E. Segi, Y. Arakawa, T. Furuyashiki, S. Narumiya, H. Bito, Molecular cloning and characterization of *CLICK-III/CaMKIgamma*, a novel membrane-anchored neuronal *Ca2+* /calmodulin -dependent protein kinase (CaMK), *J. Biol. Chem.* 278 (2003) 18597–18605.
- [54] G.A. Wayman, H. Tokumitsu, M.A. Davare, T.R. Soderling, Analysis of *CaM* -kinase signaling in cells, *Cell Calcium* 50 (2011) 1–8.
- [55] M. Gaestel, Biological monitoring of non-thermal effects of mobile phone radiation: recent approaches and challenges, *Biol. Rev. Camb. Philos. Soc.* 85 (2010) 489–500.
- [56] ICNIRP, Guidelines for limiting exposure to time-varying electric, magnetic, and electromagnetic fields (up to 300 GHz), *Health Phys.* 74 (1998) 494–522.
- [57] A. Lerchl, H. Krüger, M. Niehaus, J.R. Streckert, A.K. Bitz, V. Hansen, Effects of mobile phone electromagnetic fields at nonthermal SAR values on melatonin and body weight of Djungarian hamsters (*Phodopus sungorus*), *J. Pineal Res.* 44 (2008) 267–272.