

ORIGINAL ARTICLE

Alteration of testicular regulatory and functional molecules following long-time exposure to 900 MHz RFW emitted from BTS

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Abstract

The aim of this investigation was to evaluate changes in testosterone and some of the functional and regulatory molecules of testis such as P450scc, steroidogenic acute regulatory protein (StAR), tumour necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β) and nerve growth factor (NGF) following exposure to 900 MHz radio frequency (RF). Thirty adult male Sprague Dawley rats (190 ± 20 g BW) were randomly classified in three equal groups, control (sham, without any exposure), short-time exposure (2 hr) (STE) and long-time exposure (4 hr) (LTE). The exposure was performed for 30 consecutive days. The testosterone level in both exposed groups was significantly less than control ($p < .05$). Level of TNF- α in both exposed groups was significantly greater than control ($p < .05$). IL-1 α and NGF levels in LTE were significantly higher than the STE and control groups ($p < .05$). Level of IL-1 β in LTE was significantly higher than control ($p < .05$). Expression of both P450scc and StAR mRNA was significantly down-regulated in both exposed groups compared to control ($p < .05$). Our results showed that RFW can affect testis and reproductive function through changes in factors, which are important during steroidogenesis, and also through changes in inflammatory factors, which regulate Leydig cell functions.

KEYWORDS

900 MHz radio frequency, cytokine, P450scc, StAR, testosterone

1 | INTRODUCTION

Rapid progression in technology and changes in lifestyle especially life-time exposure to electromagnetic fields increase concerns about their deleterious health effects. Especially, increase in mobile phone usage is the most concerning. The Global System for Mobile Communications (GSM) standard operating in the 900 MHz frequency band is largely used in Europe, Africa, Middle East and Asia (Maaroufi et al., 2014).

Many studies have analysed the biological health effects of exposure to cell phones on general human health. Alternation in electroencephalograph pattern, sleep pattern, neuroendocrine functions, impairment in learning ability and cognitive processes, fatigue, headache, increased resting blood pressure has been observed

with increased cell phone usage (Azimzadeh, Jelodar, Namazi, & Soleimani, 2018; Colak et al., 2012).

The effects of RFW on the brain and testis are the most popular research areas. Moreover, recent studies suggest that RFW emitted from cell phones impairs hormone secretion, such as LH and FSH, due to the deformation of Leydig and Sertoli cells, which may lead to altered cell proliferation and reduce the fertilising potential of men (Desai, Kesari, & Agarwal, 2009; Jelodar, Talebzadeh, & Lari, 2012; Markkanen et al., 2004; Roosli, Michel, Kuehni, & Spoerri, 2007).

Leydig cells are among the most susceptible cells to RFW, and injury to these cells may affect spermatogenesis (Wang et al., 2003). In Leydig cells, the steroid biosynthesis begins by steroidogenic acute regulatory protein (StAR), the first and rate-limiting step of steroidogenesis,

which transfers cholesterol into the mitochondria, and afterwards, the enzyme P450scc cleaves the side chain of cholesterol to create pregnenolone, which finally is transformed into testosterone (Hall, 2016).

In the normal testis, cytokines act as paracrine regulators of spermatogenesis and steroidogenesis (Guazzzone, Jacobo, Theas, & Lustig, 2009). Among these, interleukin-1 (IL-1) and tumour necrosis factor- α (TNF- α) were known to regulate (induce/inhibit) Leydig cell steroidogenesis (Tremellen et al., 2018; Verajankorva, Martikainen, & Pollanen, 2001), which means that under physiological status, they exert a critical role in the normal development and function of testis, but their levels increase during inflammatory conditions (Lysiak, 2004).

It has been shown that IL-1 is an inhibitor of Leydig cell function. It can stimulate steroidogenesis but in general, blocks LH-stimulated testosterone production (Svechnikov, Sultana, & Soder, 2001; Wang, Nagpal, Shimasaki, Ling, & Lin, 1995). The inhibitory effect of TNF- α on Leydig cell function in vitro and in vivo has also been reported (Manna, Chandrala, Jo, & Stocco, 2006; Wu, Zhang, et al., 2012).

Hence, one of the important causes of spermatogenesis and steroidogenesis disorders within the testis may be overproduction of these cytokines, and data reviewed indicate that alterations in the level of cytokines can interrupt steroidogenesis in Leydig cells (Hedger & Meinhardt, 2003).

Nerve growth factor (NGF) is a member of neurotrophins and as an antioxidant has critical roles in the male reproductive system (Li et al., 2010) such as proliferation and differentiation of Leydig cells and testosterone production (Zhang et al., 2013). NGF as a neurotrophin has been suggested to have an autocrine or paracrine function in the regulation of differentiation of immune cells (Otten et al., 2000; Perez-Perez et al., 2003) and also the expression of inflammatory cytokines (Marshall, Gomi, Blennerhassett, & Bienenstock, 1999). In addition, several studies have implicated that NGF is an important regulator of the crosstalk of the signal transduction pathways between the immune and nervous systems (Minnone, Benedetti, & Bracci-Laudiero, 2017; Olgart & Frossard, 2001).

Although reports have demonstrated that exposure to 900 MHz RFW resulted in destructive effects on different organs of the human and animal body, the molecular mechanisms by which RFW induces steroidogenesis or male fertility disorder have not been completely understood.

We investigated the effects of 900 MHz RF on the level of testosterone in the testis tissue and the expression of cytochrome P450scc and StAR protein, as key factors in steroidogenesis process in the Leydig cells. Moreover, we have analysed the effects of RF on the testicular levels of TNF- α , IL-1 α , IL-1 β and NGF as paracrine/autocrine factors, which regulate Leydig cell functions.

2 | MATERIALS AND METHODS

2.1 | Animal experiments

Thirty sexually adult male Sprague Dawley rats (190 $\pm$ 20 g), with free access to food and water, were used. Animal was housed in

polycarbonate cage with dimensions 42 cm $\times$ 26.5 cm $\times$ 15 cm without any metallic interfering materials in the animal room under controlled conditions, 12/12-hr light/dark period (light on at 7 a.m.) and constant temperature (20 $\pm$ 2°C). The irradiation of animals was started at 8 a.m. for 30 consecutive days.

The animals were randomly divided into three groups ($n = 10$): control (sham, without any exposure), short-time exposure (STE) (2 hr) and long-time exposure (LTE) (4 hr).

All of the investigations involving animal use and care were administrated by the Institutional Research Ethics Committee of the School of Veterinary Medicine of Shiraz University.

2.2 | Signal generator

The apparatus and procedure were essentially the same as our previous report (Azimzadeh et al., 2018). Briefly, a simulator with one 12-cm antenna emitting 900 MHz radio frequency wave circularly with average power density of 0.6789 mW/cm² and specific absorption rate (SAR) of testis of 0.3315 W/kg was used. The simulator was centrally placed 1 m away from the cages of exposed groups (STE and LTE) on the floor of the experimental room.

2.3 | Tissue sampling and hormonal assay

In the last day of exposure period, the animals were sacrificed. Right testis in all groups was quickly removed, part of it was homogenised and centrifuged. Then, the supernatant was used to evaluate the concentration of investigating factors including testosterone, TNF- α , IL-1 α , IL-1 β and NGF by ELISA Kit (Shanghai Crystal Day Biotech Co., Ltd.).

2.4 | Protein content of testis

Total protein concentration of testis tissue was determined by the method, described by Bradford (Bradford, 1976).

2.5 | Evaluation of P450scc and StAR expression

Total RNA was extracted by TRIzol reagent (RNX-Plus™ Total RNA Extraction Solution; CinnaGen), as described by the manufacturer's instructions. It should be noted that 260 nm absorbance value was used to determine concentration. The products were treated with DNase I Kit (SINACLON) to remove possible DNA. First-strand cDNA was synthesised from 2 μ g total RNA by using Easy cDNA Reverse Transcription Kit (Pars tous) according to the manufacturer's instructions. Real-time PCR was conducted with LightCycler 96 (Roche) by using SYBR Green/Fluorescein Master Mix 2 $\times$ (Pars tous) following 7.5 μ l Master Mix 2 $\times$ added to 300 nM of each primer set for P450scc or for the StAR. Primer sequences are shown in Table 1. The thermocycling conditions were as follows: preliminary PCR activation step at 95°C for 2 min; 48 cycles of denaturation at 95°C for 20 s, annealing and extension at 59°C for 40 s and melting at 55–97°C. The fold

change for mRNA was determined utilising the $2^{-\Delta\Delta C_t}$ procedure. The amounts of P450scc and StAR were normalised to the β -actin value as an internal control. Moreover, all samples were examined in triplicate.

2.6 | Statistical analysis

All data are presented as mean $\pm$ SEM. The software of SPSS version 21.0 for Windows was used for statistical analysis of data. Analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparisons test was used to compare mean values between groups, and the significance level was set at $p < .05$.

3 | RESULTS

3.1 | The testis tissue levels of testosterone, IL-1 α , IL-1 β , TNF- α and NGF

We found that testosterone level in both exposed groups (LTE and STE) was significantly lower than the control group. No significant difference was observed between the LTE group and the STE group (Figure 1a) ($p < .05$).

IL-1 α level in the LTE group was significantly higher than the STE and control groups ($p < .05$). Although there was a slight increase in IL-1 α level in the STE group compared to the control group, statistically it was not significant (Figure 1c) ($p < .05$).

Level of IL-1 β in the LTE group was significantly higher than the control group ($p < .05$). There was a slight increase in IL-1 β level in the STE group compared to the control group, but statistically, it was not significant (Figure 1c) ($p < .05$).

Level of TNF- α in the STE and LTE groups was significantly higher than control ($p < .05$). There was no significant difference between the LTE and STE groups (Figure 1c) ($p < .05$).

Nerve growth factor level in the LTE group was significantly higher than other groups ($p < .05$). No significant difference was observed between the STE and control groups (Figure 1b) ($p < .05$).

3.2 | mRNA expression levels of P450scc and StAR in testis tissue

mRNA expression levels of P450scc and StAR (Figure 1d) were significantly down-regulated in both exposed groups (LTE & STE)

TABLE 1 The sequences of primers used in real-time PCR for evaluation of expression of P450scc and StAR

Gene	Primer sequence 5' to 3'
P450scc	Forward: AGTCTCCTTGCTCAGCATCACAG Reverse: TTCCTC TTCTTCACCTGCCTCCTG
StAR	Forward: TCAACCACCACCTCCAAGCGAAAC Reverse: AGAAGGAAAGCCAGCAGCAGAACG
β -actin	Forward: GCTCTTTTC CAGCCT TCCTTCTTG Reverse: GTGTTGCATAGAGGTCTTTACGG

compared to control ($p < .05$). No significant difference was noticed between the LTE and STE groups ($p < .05$).

4 | DISCUSSION

Our results indicated that exposure to 900 MHz RFW has potentially deleterious effects on reproductive capacity.

Testosterone as a primary male sex hormone and one of the main products of testis is necessary for development and maintenance of many physiological functions, in which spermatogenesis is the most important one (O'Donnell, Stanton, & Kretser, 2017; Wdowiak, Wdowiak, & Wiktor, 2007). In our study, 900 MHz RF with different time of exposure (STE and LTE) caused a significant decrease in testosterone level in the testis tissue. Although similar results were already reported in serum (Jelodar & Zare, 2008; Meo, Al-Drees, Husain, Khan, & Imran, 2010; Sepehrimanesh et al., 2014), correspondingly, others failed to detect considerable alterations in serum testosterone level (Forgacs et al., 2006; Ribeiro et al., 2007; Salama, Kishimoto, Kanayama, & Kagawa, 2009). However, some discrepancies exist in this context because of differences in the experimental conditions or methodologies such as differences in distance from the source of RFW, exposure periods and frequency of waves used.

StAR and P450scc are two main key elements in the testosterone production in Leydig cells, responsible for the moving of cholesterol into mitochondria and transforming of cholesterol to pregnenolone respectively. Our results revealed a significant reduction in the mRNA levels of the StAR and P450scc in both the STE and LTE groups compared with the control group.

Exposure of Leydig cells (in vitro) to 1,950 MHz RF was also reported to decrease P450scc expression level without changes in StAR (Lin et al., 2017). However, high-dose exposure to cell phone radiation was reported to reduce expression of StAR and testosterone concentration in the testes of mice (Zang et al., 2017). Moreover, exposure to 1,800 MHz RF altered circadian mRNA expression of P450scc and StAR in testis (Qin et al., 2014).

In the normal condition, IL-1 and TNF- α at the physiologically low concentrations may act as a survival factor (Riccioli et al., 2000; Wu, Wang, et al., 2012) and modulate Leydig cell steroidogenesis (Siu, Lee, & Cheng, 2003). However, under the inflammatory and pathologic conditions increase in expression of the above-mentioned cytokines has been reported (Lysiak, 2004; Theas et al., 2008).

Both IL-1 α and IL-1 β are known as inflammatory mediators, which regulate critical physiological functions in the testis (Sahin et al., 2006). IL-1 α is involved in spermatogenesis promotion and steroidogenesis regulation (Guazzone et al., 2009), and IL-1 β has a role in the damage of testicular tissues (Bergh & Soder, 1990). Moreover, it has been proposed that IL-1 α and IL-1 β possibly through cell turnover and tissue repair are physiological and pathophysiological potential growth factors for Sertoli cells (Petersen, Boitani, Froya, & Soder, 2002).

Exposure of Sertoli cells, which are immunocompetent cells, to electromagnetic fields may promote pro-inflammatory actions by

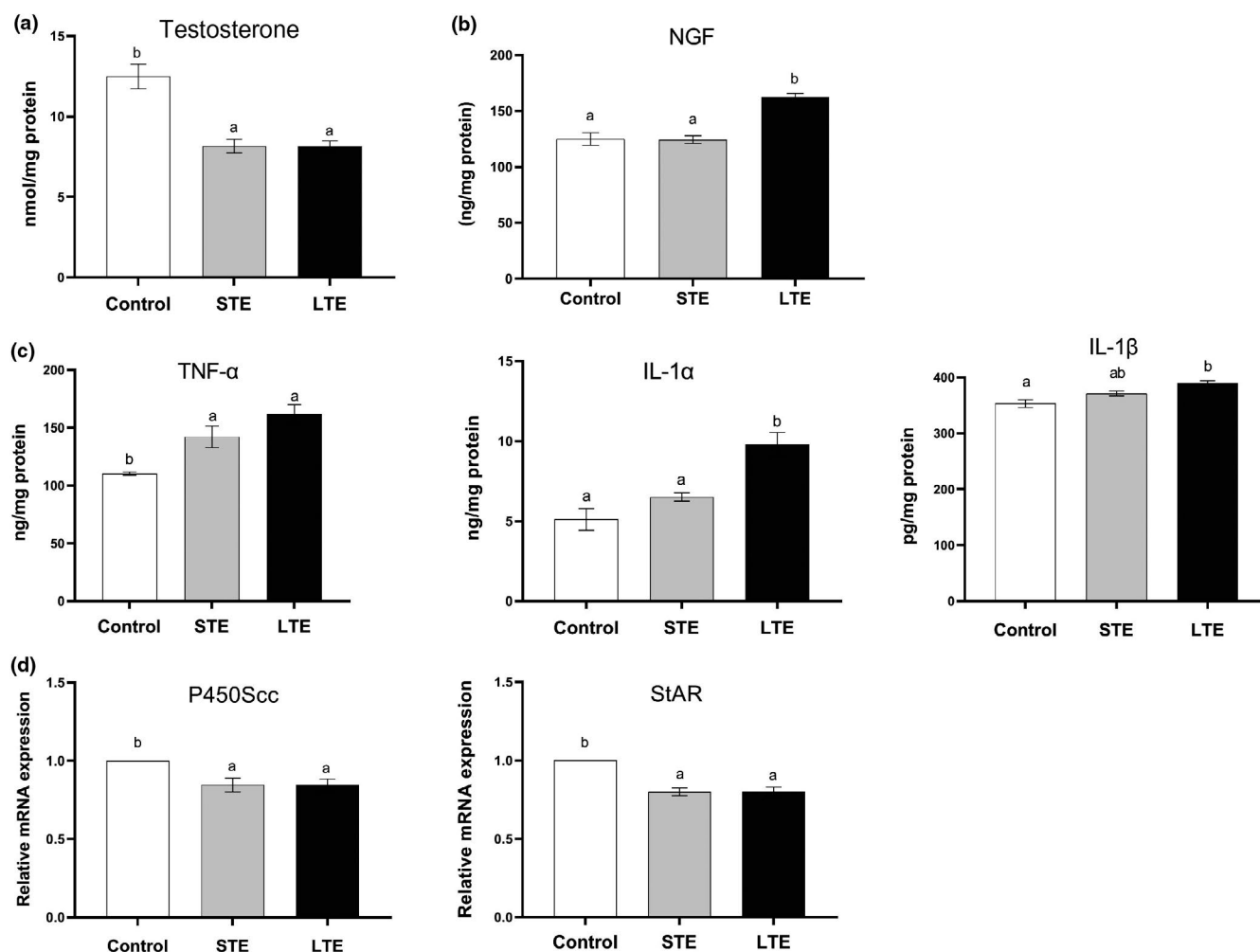


FIGURE 1 (a) Testosterone, (b) NGF and (c) cytokine levels in the testis tissue after 30 consecutive day's radiation. Animals were exposed to 900 MHz radio frequency emitted from BTS in the STE (2 hr) and LTE (4 hr) groups daily. Concentration of all factors in the testis was measured by ELISA. (d) Quantitative analysis for the expression of P450scc and StAR in the testis tissue by real-time RT-PCR. Data are the mean $\pm$ SEM, and different alphabet indicates significant difference between groups ($p < .05$). STE, short-time exposure (2 hr). LTE: long-time exposure (4 hr)

the production of tremendous level of TNF- α , NO, etc. (Yang, Hao, et al., 2010; Yang, He, et al., 2010).

It has been shown that exposure to electromagnetic fields increases both mRNA and protein levels of IL-1 β and TNF- α (Makar, Logani, Bhanushali, Alekseev, & Ziskin, 2006; Wu, Wang, et al., 2012). Moreover, microwave-radiated Sertoli cells produce the high level of cytokines, which could induce oxidative stress and finally interrupted spermatogenesis (Wu, Wang, et al., 2012).

It has been described that IL-1 decrease testosterone formation in Leydig cell, of important this effect, is increased in the presence of TNF- α (Calkins, Guo, Sigel, & Lin, 1990) due to inhibition of both mRNA and protein levels of P450scc and StAR (Mauduit et al., 1998).

However, some reports have demonstrated that IL-1 β can promote testosterone synthesis in Leydig cells (Verhoeven, Cailleau, Damme, & Billiau, 1988; Warren, Pasupuleti, Lu, Platler, & Horton, 1990) although some discrepancies exist in this context which is because of differences in the experimental situations or methodologies.

Collectively, these results emphasise the modulatory effects of cytokines on the steroidogenesis in Leydig cell.

In the present study, IL-1 α level significantly increased in the LTE group compared to the STE group. Given that it has been suggested that IL-1 α , due to its constitutive presence, may act as a repair factor inducing Sertoli cell proliferation after tissue injury in the testis (Petersen et al., 2002), we also suggest that IL-1 α may act as one of innate protective factors against the destructive effects of long-time exposure to RFW in the testis tissue.

Our results showed that the long-time (4 hr) exposure to RF causes a significant increase of NGF level in the testis.

Given that the NGF production due to its dynamic control of innervation is strictly regulated in the body (Sofroniew, Howe, & Mobley, 2001), there are data demonstrating its significant increase in inflamed tissues both in humans and in animals (Aalto, Korhonen, Lahdenne, Pelkonen, & Lindholm, 2002; Fauchais et al., 2009). In addition, NGF levels in pulsed electromagnetic field (PEMF)-treated nerve tissue were significantly decreased and it has been suggested that

nerve regeneration may be promoted by PEMF through amplifying the early decrease in post-injury of NGF activity (Longo et al., 1999).

NGF is a key molecule in the complex network of bidirectional signals between the nervous and immune systems. On the other hand, NGF is part of an endogenous mechanism that, while activating innate immune responses, also activates pathways necessary to dampen inflammatory response and limit tissue damage (Minnone et al., 2017).

Current data suggest that the normally low basal production of NGF has been increased during the inflammatory process at the sites of inflammation and potentiates the release of cytokines and inflammatory mediators (Bayas et al., 2003; Hepburn et al., 2014). Moreover, cytokines such as IL-1 β and TNF- α synergistically stimulate NGF transcription and protein release. Nevertheless, it has been inferred that NGF has a pro-inflammatory activity (Otten et al., 2000).

Other reports have shown that addition of NGF not only inhibits the pro-inflammatory cytokines including IL-1 β and TNF- α generation but also potentiates the release of anti-inflammatory mediators such as IL-10 (Han & Ulevitch, 2005; Liew, Xu, Brint, & O'Neill, 2005).

In our study, NGF level significantly increased in the LTE group compared to the STE group, which can be an evidence for the antioxidant and anti-inflammatory functions of NGF. Therefore, we suggest that NGF could act as another innate protective factor against the destructive effects of long-time exposure to RFW in the testis tissue.

5 | CONCLUSION

Our finding demonstrated that exposure to 900 MHz RF decreases testosterone level in the testis tissue, mainly due to down-regulation of expression of two key factors (P450sc and StAR) which are involved in testosterone production by Leydig cell. Also, exposure to RFW increases the cytokines (IL-1 α , IL-1 β and TNF- α) and NGF levels in the experimental groups. As these factors are involved in inflammatory sites, we suggest that increase in the inflammatory cytokines (IL-1 β and TNF- α) may act as disruptive mediators of exposure to RFW effect. On the other hand, IL-1 α and NGF have a modulatory role in these effects in the testis. Therefore, our data provide a molecular and endocrine evidence through which exposure to 900 MHz RF compromises fertilisation potential.

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