

Down-Regulation of Lipocalin 2 Expression in Mouse Testis after Exposure to Electromagnetic Field

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Abstract

Background: The effects of electromagnetic field (EMF) on reproductive system have been of critical concern for a long time. It has been shown that the EMF can adversely affect testicular cells and tissue and decrease male fertility. The most important determinants of male fertility are sperm development and motility, which are affected by changes in several factors including lipocalin 2 proteins. In the present study, we investigated the effects of exposure to EMF on testis tissue and expression of lipocalin 2 gene.

Methods: Male BALB/c mice (8 weeks old) were exposed to 3 mT EMF for 8 weeks, 4 hours/day. Control group (10 mice) did not receive EMF exposure. After the experimental period, the mice were sacrificed, and their testis tissues were examined by using light microscopy after hematoxylin-eosin staining. Additionally, total RNA and proteins were extracted from testis tissue and used to study the lipocalin 2 expression by real time RT-PCR and Western blot analysis.

Results: The histological changes observed in the testes of experimental group included increased number of spermatocytes and Leydig cells, and increased thickness of basement membrane compared with the control group. The mRNA and protein studies showed that expression of lipocalin 2 gene was down regulated in testes of the mice exposed to EMF.

Conclusion: Our study showed that EMF down regulates the expression of lipocalin 2, a cytoprotective molecule, in testis tissue. This down regulation can be one of the mechanisms that contribute to the decreased fertility observed after exposure to EMF.

Iran J Med Sci 2009; 34(4): 265-270.

Keywords • Electromagnetic fields • testis • fertility • Lcn2 • reactive oxygen species

Introduction

Electromagnetic fields (EMFs) generated by various electrical devices and power transmission lines affect most individuals worldwide.¹ There are several evidences that EMF may adversely affect normal physiologic function of various organs including the reproductive system.² The sensitivity of testis tissue to EMF has been shown before,³ and it was reported that the reactive oxygen species produced as the result of exposure to EMF can lead to pathological

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Received: 3 May 2009

Revised: 4 August 2009

Accepted: 30 August 2009

changes in testis. These changes can lead to sub- or infertility.^{2,4-8}

So far, several reports have shown that some members of the family of lipocalin proteins contribute to the normal function of reproductive system. The main functions of these proteins are transport of small hydrophobic molecules, control of cell metabolism and growth, and synthesis of prostaglandins.^{9,10} However, the functions of several members of this family have not been clearly understood and need to be elucidated, especially with regards to the reproductive system.¹¹ One of the reproduction-related lipocalins is lipocalin 2 (Lcn2), also called neutrophil gelatinase-associated lipocalin (NGAL), which is a 21-kD protein with various biological activities.¹² It has been known that Lcn2 plays a role in renal tubular injury and its level rises rapidly in serum and urine after the injury.^{13,14} Lcn2 mediates delivery of ferric ions to mouse spermatozoa,¹⁵ and enhances sperm motility.¹⁶⁻¹⁸ Successful fertilization requires normal motility of sperm.¹⁹ As EMF decreases sperm motility; we hypothesized that this abnormality could be the result of the effects of EMF on Lcn2 gene expression.

Material and Methods

Mice and Electromagnetic Field Exposure

Seventeen male BALB/c mice aged 8 weeks were used in the present study. The mice were maintained on a 12/12-hours light/dark cycle throughout the experimental period. The mice were divided into two experiment (exposed; 7 mice) and control groups (10 mice). The mice in experiment group were exposed to a 3 mT (50 Hz) magnetic field in the exposure cage for 6 days per week and 4 hours per day from 8:00 AM to 12:00 noon for 8 weeks. After this period the mice were sacrificed by cervical dislocation and testis tissue was removed. Animal experiments were approved by the Ethical Committee of Tabriz University of Medical Sciences.

Histological Study

After removal of testes, the tissues were cut into small pieces and fixed with 10% formalin solution for 24 hours. The fixed tissue was mounted on microscope slides with 5 µm sections, stained with hematoxylin-eosin (H&E) and examined by light microscopy.

mRNA Study

Total RNA from testis tissue was extracted by Trizol reagent (Invitrogen, Carlsbad, Canada) according to the manufacturer's instructions. The quantity and quality of RNA were determined by spectrophotometry (ND-1000; Nanodrop,

Wilmington, Delaware, USA) and electrophoresis, respectively.

Total RNA underwent reverse transcription by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, Canada) followed by DNase I (Invitrogen, Carlsbad, Canada) treatment and heat inactivation.

Semiquantitative PCR was performed using Taq DNA polymerase (Roche, Germany) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA). Primer set for the mouse Lcn2 was forward 5'-CCA GTT CGC CAT GGT ATT TTTC-3' and reverse 5'-CAC ACT CAC CAC CCA TTC AGTT-3'. Expression of the housekeeping gene β -actin was also examined by the following primer set: forward 5'-TTC TAC AAT GAG CTG CGT GTG G -3' and reverse 5'-GTG TTG AAG GTC TCA AAC ATG AT-3'. After initial denaturation (5 min at 94 °C), the Lcn2 PCR reaction was subjected to 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. And the β -actin PCR reaction was subjected to 30 cycles of 95°C for 30 seconds, 59°C for 30 sec and 72°C for 30 seconds. Both reactions were subjected to a final extension at 72°C for 5 min. PCR products were separated in a 2% agarose gel.

Real-time PCR analysis was performed in a Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia) thermocycler. Amplification was conducted using Absolute SYBR Green mix (ABgene, Surrey, UK) according to the manufacturer's instructions. Briefly, 20 µl of total PCR reaction contained 10 µl of 2× SYBR Green mix, 10 pmole forward and reverse primers, and 1 µl of cDNA template. PCR was performed with the same primers mentioned above. The thermal profile was initial denaturation at 94°C for 15 minutes followed by 40 amplification cycles consisting of denaturation at 94°C for 30 seconds, annealing at suitable temperature for 30 seconds and extension at 72°C for 30 seconds. Threshold cycle values were normalized by β -actin expression. Triplicate reactions were set up for each sample and their mean values were used for calculations. Lcn2 values were normalized to β -actin values and their relative expressions were calculated by comparative Ct (threshold cycle) method.

Western Blot Analysis

Total protein was extracted from mice testes by TriPure isolation reagents (Roche, Germany) according to manufacturer's protocol. Protein concentration was determined by Bio-Rad protein assay kit (Bio-RAD, USA) according to manufacturer's instructions. Same amounts of protein from control and exposed samples were

heated in 2× sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95°C for 5 min and run on 12% gel. After Protein samples were separated by SDS-PAGE and subsequently transferred over 1.5 hours to a poly vinyl dene fluoride (PVDF) membrane (Hi-bond Amersham Biosciences, USA; 125 V) using the Mini Trans-Blot Electrophoresis Transfer Cell System from Bio-Rad (Hercules, CA, U.S.A.) in Tris/glycine buffer pH 8.4 containing 20% (v/v) methanol. Then membranes were blocked overnight with a solution containing 5% skimmed milk and 0.1% Tween 20. Blocked membranes were washed with PBS-0.05% Tween 20.

For the detection of Lcn2, membranes were incubated with 1µg/ml of monoclonal anti-mouse LCN2/NGAL (neutrophil gelatinase-associated lipocalin) antibody (Cl 228418, R & D, USA), at room temperature for 1 h. Then, membranes were washed 4 times with phosphate buffered saline (PBS) containing 0.1 % Tween 20 and incubated with 1/5000 dilution of horseradish peroxidase-coupled secondary rabbit polyclonal anti-rat IgG-HRP (ab6734, ABcam, USA) for 1h. Membranes were then washed 4 times with PBS containing 0.1 % Tween 20. Membranes were developed with 3, 3'-diaminobenzidine (DAB) solutions (Sigma, USA).

For the detection of β-actin, membranes were also incubated with 1/5000 dilution of monoclonal anti-mouse β-actin (mAbcam 8226; ABcam, USA) at room temperature for 1 h followed by 4 times washing with PBS containing 0.1% Tween 20. Then, the membranes were incubated with 1/2000 dilution of rabbit polyclonal anti-mouse IgG-HRP (ab6728, ABcam, USA). Membranes were then washed and developed same as described for detection of Lcn2.

Statistical Analysis

The results are expressed as mean ± SD of three independent experiments. Student's t-test was used for comparison of means and a p value of less than 0.05 was considered to be

statistically significant. The data analysis was carried out by INSTAT view software version 3.

Results

Light microscopic examination shows the following changes in the testes tissues of exposed mice compared with the control group: increased number of primary spermatocytes, dense nuclei of primary spermatocytes, increased number of Leydig cells in the interstitial space, and thickening of basement membrane of somniferous tubules (figure 1).

Analysis of Lcn2 mRNA by semi-quantitative RT-PCR has shown that in EMF-exposed mice the mRNA level was decreased in comparison with controls (figure 2). Melt curve analysis has been shown in figure 3. Overlapping of beta-actin and Lcn2 curves indicate that the curve is due to a single band.

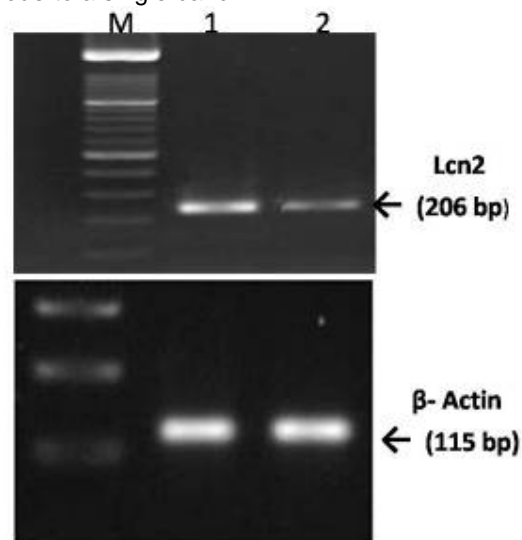


Figure 2: Expression of Lcn2 in mouse testis after exposure to EMF. Eight weeks after exposure to 3mT and 50 Hz of EMF, Lcn2 expression was determined by semi-quantitative RT-PCR. After exposure period, Lcn2 expression was downregulated (lane 2) compared with the control without exposure, (lane 1). M, 100-bp marker. Lower figure indicates the expression of β-actin in both mice groups. M, 100-bp marker.

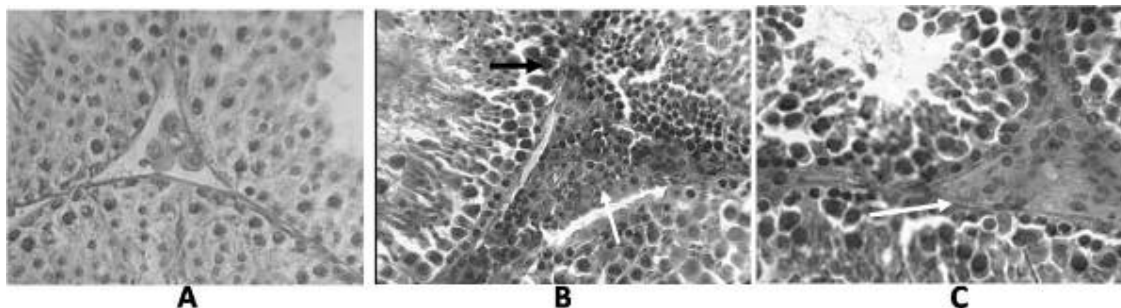


Figure 1: Histological findings in somniferous tubules of testis after 8 weeks of EMF exposure. (A) Unexposed testis tissue (control). (B) Primary spermatocyte with dense nucleus (black arrow) and interstitial cells (white arrow) in exposed testis tissue. (C) Increase of basement membrane thickness has been shown with white arrow in exposed tissue testis.

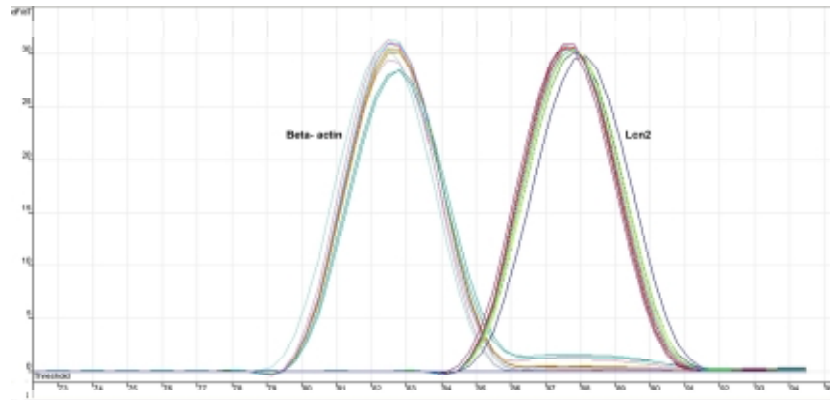


Figure 3: Melt curve analysis. Left: beta-actin, right: Lcn2. Overlapping of beta-actin and Lcn2 curves indicate that the curve is due to single band.

Real time PCR analysis has shown that the Lcn2 mRNA expression level is decreased in exposed mice for about 5-fold (5 ± 2 , $P < 0.001$) in comparison with the controls (figure 4). Consistent with mRNA results, Western blotting shows that the Lcn2 protein level in testis of EMF-exposed mice is lower than the controls (figure 5). These results indicated that electromagnetic field leads to downregulation of lipocalin 2 gene expression in testis tissue.

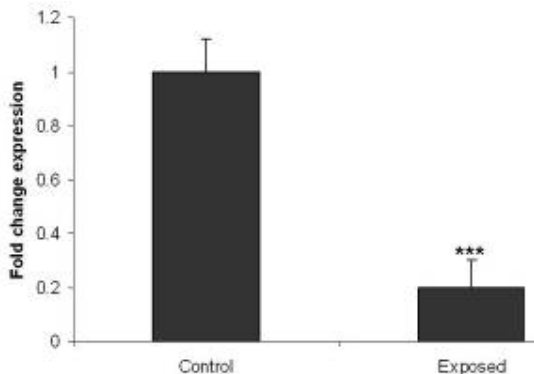


Figure 4: Down- regulation of Lcn2 expression in testes of mice by real time PCR. (Mean \pm SD, ***: $P < 0.001$).

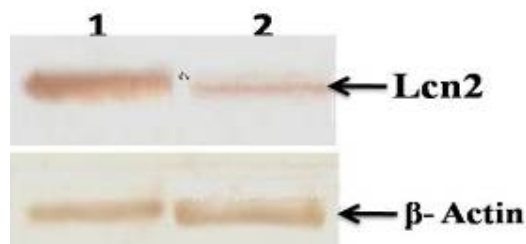


Figure 5: Western blot analysis of Lcn2 expression in mouse testis after exposure to EMF. Lcn2 was downregulated in mouse exposed to EMF (Lane 2) compared with the control without exposure, (Lane 1). Lower figure indicates the expression of β - actin protein in both mice groups.

Discussion

Our results showed that exposure to EMF can lead to morphological changes in somniferous tubules of testis tissue. In an electron microscopic study, Khaki and colleagues showed that exposure of rats to EMF led to increased deposition of fibrin and collagen in boundary tissue of testis.¹ We have also noted thickening of basement membranes and boundary tissue in testes of the experimental group. These features are observed in several testicular pathologies and can interfere with exchange of substances between blood and somniferous epithelium. This can adversely affect normal germ cell development.²⁰ Increased number of Leydig cells in interstitial tissue was another finding, which can be resulted from the cellular proliferative effects of EMF and may share a common mechanism with the cancer-inducing properties of electromagnetic fields.

In an interesting study performed by Chung and co-workers,²¹ pregnant mice were exposed to EMF and F₁ male offspring were reported to have no changes in testis histology and sperm parameters. When compared with our results, it shows that the effects of EMF on reproductive system could be specific to developmental stage. Also, the dosage and duration of exposure could be important. In Chung's study, the mice were exposed to an electromagnetic field of 60 Hz for 10 days (vs 50 Hz for 8 weeks in our study).

It has been previously shown that EMF can induce germ cells apoptosis in somniferous tubules of testis.³ In the current study, presence of dense nuclei in primary spermatocytes might be a marker of early stages of apoptosis. However, further studies are needed to confirm this hypothesis. Recently, the function of Lcn2 in protection of cells from apoptosis has been shown in a number of studies.^{22,23} Our finding

of decreased expression of Lcn2 after EMF exposure may help to explain the mechanism of EMF-induced apoptosis. One of the adverse effects of exposure to EMF is production of reactive oxygen species.⁴⁻⁷ These molecules can lead to several cellular and molecular damages,⁸ and implicated as one of the causes of infertility.²⁴ Reactive oxygen species are considered as a mechanism responsible for pathological changes observed after EMF exposure.¹ We have recently found that Lcn2 acts as a protective factor against H₂O₂ toxicity,²³ and its expression increases after exposure to reactive oxygen species.²⁵ These findings emphasize the role of Lcn2 in reproductive system as is highlighted by its high level of expression in normal testis tissue.²²

Conclusion

Testis is one of the organs in which high level of expression of Lcn2 was detected and its normal physiologic function is affected by exposure to electromagnetic field. We showed that expression of Lcn2 gene is downregulated in testis tissue after exposure to EMF and this may account for some of the observed adverse effects including infertility.

Conflict of Interest: None declared

References

- 1 Khaki AA, Tubbs RS, Shoja MM, et al. The effects of an electromagnetic field on the boundary tissue of the seminiferous tubules of the rat: A light and transmission electron microscope study. *Folia Morphol (Warsz)* 2006; 65, 188-94.
- 2 Ozguner M, Koyu A, Cesur G, et al. Biological and morphological effects on the reproductive organ of rats after exposure to electromagnetic field. *Saudi Med J* 2005; 26: 405-10
- 3 Lee JS, Ahn SS, Jung KC, et al. Effects of 60 Hz electromagnetic field exposure on testicular germ cell apoptosis in mice. *Asian J Androl* 2004; 6: 29-34.
- 4 Kovacic P, Cooksy AL. Unifying mechanism for toxicity and addiction by abused drugs: electron transfer and reactive oxygen species. *Med Hypotheses* 2005; 64: 357-66.
- 5 Lantow M, Lupke M, Frahm J, et al. ROS release and Hsp70 expression after exposure to 1,800 MHz radiofrequency electromagnetic fields in primary human monocytes and lymphocytes. *Radiat Environ Biophys* 2006; 45: 55-62.

- 6 Yao K, Wu W, Yu Y, et al. Effect of superposed electromagnetic noise on DNA damage of lens epithelial cells induced by microwave radiation. *Invest Ophthalmol Vis Sci* 2008; 49: 2009-15.
- 7 Svedenstål BM, Johanson KJ, Mattsson MO, Paulsson LE. DNA damage, cell kinetics and ODC activities studied in CBA mice exposed to electromagnetic fields generated by transmission lines. *In Vivo* 1999; 13: 507-13.
- 8 Di Pietro A, Visalli G, Munaò F, et al. Oxidative damage in human epithelial alveolar cells exposed in vitro to oil fly ash transition metals. *Int J Hyg Environ Health* 2009; 212: 196-208.
- 9 Roudkenar MH, Halabian R, Oodi A, et al. Upregulation of neutrophil gelatinase-associated lipocalin, NGAL/Lcn2, in beta-thalassemia patients. *Arch Med Res* 2008; 39: 402-7.
- 10 Mishra J, Ma Q, Prada A, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; 14: 2534-43.
- 11 Kai MS, Kiyoshi M, Jau YL, Avtandil K. Dual Action of Neutrophil Gelatinase-Associated Lipocalin. *J Am Soc Nephrol* 2007; 18: 407-13.
- 12 Schmidt-Ott KM, Mori K, Li JY, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 2004; 432: 917-21.
- 13 Kuwabara T, Mori K, Mukoyama M, et al. Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. *Kidney Int* 2009 75: 285-94.
- 14 Mishra J, Dent C, Tarabishi R, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet* 2005; 365:1231-8.
- 15 Elangovan N, Lee YC, Tzeng WF, Chu ST. Delivery of ferric ion to mouse spermatozoa is mediated by lipocalin internalization. *Biochem Biophys Res Commun* 2004; 319: 1096-104.
- 16 Fujino RS, Tanaka K, Morimatsu M, et al. Spermatogonial cell-mediated activation of an IκappaBzeta-independent nuclear factor-kappaB pathway in Sertoli cells induces transcription of the lipocalin-2 gene. *Molecular Endocrinol* 2006; 20: 904-15.
- 17 Devireddy LR, Teodoro JG, Richard FA, Green MR. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science*

- 2001; 293: 829-34.
- 18 Lee YC, Liao C Jr, Li PT, et al. Mouse lipocalin as an enhancer of spermatozoa motility. *Mol Biol Rep* 2003; 30: 165-72.
- 19 Das S, Chattopadhyay R, Jana SK, et al. Cut-off value of reactive oxygen species for predicting semen quality and fertilization outcome. *Syst Biol Reprod Med* 2008; 54: 47-54.
- 20 Jarow JP, Budin RE, Dym M, et al. Quantitative pathologic changes in the human testis after vasectomy. A controlled study. *N Engl J Med* 1985; 313: 1252-6.
- 21 Chung MK, Lee SJ, Kim YB, et al. Evaluation of spermatogenesis and fertility in F1 male rats after in utero and neonatal exposure to extremely low frequency electromagnetic fields. *Asian J Androl* 2005; 7: 189-94.
- 22 Tong Z, Wu X, Ovcharenko D, et al. Neutrophil gelatinase-associated lipocalin as a survival factor. *Biochem J* 2005; 391: 441-8.
- 23 Roudkenar MH, Halabian R, Ghasemipour Z, et al. Neutrophil gelatinase-associated lipocalin acts as a protective factor against H₂O₂ toxicity. *Arch Med Res* 2008; 39: 560-6.
- 24 Deepinder F, Cocuzza M, Agarwal A. Should seminal oxidative stress measurement be offered routinely to men presenting for infertility evaluation? *Endocr Pract* 2008; 14: 484-91.
- 25 Roudkenar MH, Kuwahara Y, Baba T, et al. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. *J Radiat Res (Tokyo)* 2007; 48: 39-44.